Cloning and Functional Expression of mCCR2, a Murine Receptor for the C-C Chemokines JE and FIC*

(Received for publication, February 23, 1996, and in revised form, March 26, 1996)

Takao Kurihara and Rodrigo Bravo‡
From the Department of Oncology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000

The C-C chemokines human monocyte chemoattractant protein-1 and -3 (MCP-1 and MCP-3) and mouse JE and FIC are potent activators of monocytes. Several receptors for MCP-1 and MCP-3 have been cloned from human monocytes. Several receptors for MCP-1 and MCP-3 have been cloned from human monocytes. Several receptors for MCP-1 and MCP-3 have been cloned from human monocytes.

**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.**

The nucleotide sequence(s) reported in this paper has been submitted to GenBank™/EMBL Data Bank with accession number(s) U51717.

‡To whom correspondence should be addressed: Dept. of Oncology, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543-4000. Tel.: 609-252-5744; Fax: 609-252-3307.

The abbreviations used are: MCP, monocyte chemoattractant protein; RANTES, regulated on activation normal T cell expressed; kb, kilobase(s); PCR, polymerase chain reaction; MIP, macrophage inflammatory protein.

MARC are structurally related (50–72% identity) C-C chemokines that are potent in vitro chemoattractants and activators of monocytes (4–14). In vivo, MCP-1 has been implicated in monocyte infiltration of tissues during several inflammatory diseases including atherosclerosis (15, 16) and rheumatoid arthritis (17), while JE has been implicated in macrophage-mediated tumor growth suppression in mice (18). Recent transgenic mouse models have suggested a role for JE in monocyte/macrophage recruitment and in host responses to intracellular pathogens (19, 20). To further understand the biological functions of MCP-1, MCP-3, JE, FIC, and related chemokines, it will be important to identify and characterize the receptors that bind and mediate their responses.

Three cDNAs, ccr1, ccr2A, and ccr2B, that encode receptors for MCP-1 and/or MCP-3, have been cloned from human monocyte cell lines (21–23). All three cDNAs encode seven-transmembrane G protein-coupled receptors, and the alternatively spliced ccr2A and ccr2B receptors differ only in their intracellular carboxyl-terminal tails. When expressed in human embryonic kidney 293 cells, mCCCR2 specifically bound 125I-JE with high affinity. FIC was less potent than JE in competing 125I-JE binding to mCCCR2-expressing cells, while three other mouse chemokines, MIP-1α, C10, and N51/KC, did not compete. mccr2 RNA expression was detected in elicited peritoneal macrophages as well as in several mouse organs. The cloning of mCCCR2 provides an important tool to investigate monocyte/macrophage responses to JE and FIC, to identify other targets for their action, and potentially to study models of CCR2 function in the mouse.

Chemokines are small secreted molecules that chemotact and activate specific leukocyte subpopulations in vitro and are thought to be important for leukocyte trafficking in vivo (1–3). The chemokine superfamily has traditionally been divided into two subgroups, C-X-C or C-C, based upon the presence or absence of an amino acid between the first two cysteine residues of a conserved four-cysteine motif. In general, C-X-C chemokines attract neutrophils, while C-C chemokines attract mononuclear cells. Human monocyte chemoattractant protein-1 (MCP-1), 1 MCP-2, MCP-3, mouse JE, and mouse FIC/...
Four positive cDNA inserts contained all or part of the mCCR2 coding region by DNA sequencing.

Northern Blotting—Total RNA was prepared from mouse organs and cultured cells using RNAzol (Cinna/BioTecx). 10 μg of total RNA was electrophoresed through a 1% agarose, 2 mM formaldehyde gel, the electrophoresed RNA was stained with ethidium bromide to check for equivalent loading, and the RNA transferred in 10 × SSC to GeneScreen Plus (DuPont NEN) membranes. Northern blots were hybridized and washed in an identical manner to colony hybridization except for the addition of 100 μg/ml sonicated salmon sperm DNA to the hybridization. To generate the mCCR2 probe, a 1.1-kb mCCR2 cDNA region fragment was PCR-amplified from cDNA with the primer pair: sense, 5′-GCGGAACTTGAGAAGACATATGTTACCT-3′, and antisense, 5′-GCGTGCTTATAGTCAACCCAGGACCTTG-3′. The 0.6-kb PCR-amplified β-actin probe (31) was used as a control.

Macrophage Preparation—Mice were injected intraperitoneally with 3 ml of 3% brewer’s thioglycollate (Difco) in phosphate-buffered saline, and 4 days post-injection, peritoneal cells were lavaged with Dulbecco’s modified Eagle’s medium, 5% fetal bovine serum. Cells were collected by centrifugation, resuspended at 1 × 10⁶ cells/ml, and plated in tissue culture dishes for 1 h at 37°C. Nonadherent cells were rinsed by three changes of complete medium.

Binding Assays—A 3.8-kb cDNA fragment containing the entire mCCR2 coding region was excised with PvuI and Xhol restriction enzymes and cloned into the EcoRV-Xhol sites of pcDNA3 (Invitrogen). 10–20 μg of pcDNA3 mCCR2 was transfected into HEK293 cells, and individual G418-resistant clones expressing mCCR2 (293/mCCR2) were identified by Northern blotting. 293/mCCR2 cells were seeded into 10-cm dishes for 1 h at 37°C. Nonadherent cells were rinsed by three changes of complete medium and 4 days post-infection, peritoneal cells were lavaged with Dulbecco’s modified Eagle’s medium, 5% fetal bovine serum. Cells were collected by centrifugation, resuspended at 1 × 10⁶ cells/ml, and plated in tissue culture dishes for 1 h at 37°C. Nonadherent cells were rinsed by three changes of complete medium.

Degenerate Cloning of a Murine cDNA Homologous to ccr2B—Receptors for the murine C-C chemokine FIC were previously identified on human monocytes, and cross-desensitization studies indicated that FIC, J E, and MCP-1 can signal through common receptors (13). These results suggested that murine receptors for FIC and J E might be structurally similar to CCR1, CCR2A, and CCR2B. Since specific FIC binding sites had been identified on the mouse monocyte cell line WEHI265.1 (13), we utilized a PCR strategy using WEHI265.1 cDNA template and degenerate primers based on conserved sequences in ccr1 and ccr2B to clone potential murine receptors for FIC and J E.

Degenerate primers encoding the conserved sequences NLAISDLL in transmembrane domain 2 and FLFWTPY in transmembrane domain 6 amplified a PCR product from WEHI265.1 cDNA that shared sequence similarity with ccr1, ccr2A, and ccr2B. The subcloned PCR product was used as a probe to isolate four cDNA clones from a WEHI265.1 cDNA library. The longest 3.8-kb cDNA clone (pCA-4A) contained an open reading frame of 373 amino acids that is highly homologous to CCR2B (80% identity; Fig. 1) and to CCR2A (71% identity). The 373 residue sequence was also similar but less homologous to CCR1 (56% identity), murine MIP-1α/MmCCR1 (55% identity) (28, 29), and murine MIP-1αR/mCCR3 (55% identity) (28, 29). Because of the highest sequence homology to the two CCR2 receptors, we designated this potential murine receptor mCCR2.

Similar to other cloned chemokine receptors, the mCCR2 sequence contains seven hydrophobic regions suggesting a G-protein-coupled receptor. Several other hallmarksof C-C chemokine receptors are also contained in the mCCR2 sequence, including conserved extracellular cysteine residues, the conserved sequence IFFILLTLIDRYAILHAVFAL from the middle of transmembrane domain 3 to intracellular loop region 2, an extremely basic intracellular loop region 3, and a serine/threonine-rich COOH terminus (23, 34). The predicted NH2-terminal extracellular region of mCCR2, like that of mCCR1 and mCCR3, contains no predicted N-glycosylation sites, unlike human CCR1, CCR2A, and CCR2B.

RNA Expression of mCCR2—To characterize mCCR2 RNA expression, Northern blots were performed on total RNA isolated from WEHI265.1 cells and stable HEK293 clones (293/mCCR2) transfected with an expression plasmid containing a 3.8-kb cDNA fragment from pCA-4A (Fig. 2A and data not shown). In both WEHI265.1 cells and 293/mCCR2 cells, a predominant mRNA of ~3.8 kb was detected, indicating that the 3.8-kb...
cDNA insert of pCA-4A represents approximately a full-length cDNA clone. A smaller mRNA species was also detected in both WEHI265.1 and 293/mCCR2 cells. Since the smaller mRNA was not detected in control or vector-transfected 293 cells (data not shown), it is likely to be an alternatively spliced version of the 3.8-kb mCCR2 mRNA rather than a cross-hybridizing mRNA.

To determine the distribution of mCCR2 expression in the mouse, Northern blot analyses were performed on total RNA extracted from multiple organs (Fig. 2A). Out of 12 organs analyzed by Northern blotting, mCCR2 expression was detected in the kidney, lung, spleen, and thymus, although the mRNA levels were significantly lower than in WEHI265.1 cells. The low levelsof mCCR2 mRNA in the organs may represent expression in a specific subset of cells within the particular organs or in contaminating leukocytes such as monocytes/macrophages. Since mCCR2 was cloned from the monocyto cell line WEHI265.1, we determined if mCCR2 mRNA was also expressed in mouse mononuclear cells. When Northern blot analysis was performed on total RNA from thioglycollate-elicited peritoneal macrophages, mCCR2 mRNA was detected at lower levels than in WEHI265.1 cells (Fig. 2B). Unlike the WEHI265.1 cells, the predominant mCCR2 mRNA form in the elicited peritoneal macrophages is the smaller 2.8-kb species. The preferential expression of larger or smaller mRNAs in the WEHI265.1 cells and peritoneal macrophages suggests differential transcriptional regulation of mCCR2 in different cell types.

Chemokine Binding to 293/mCCR2 Cells—To investigate chemokine binding to mCCR2, several stable HEK293 lines expressing high levels of mCCR2 mRNA were cloned (Fig. 2A). Since mCCR2 is most homologous to the MCP-1/MCP-3 receptor CCR2B, 293/mCCR2 cells were tested for their ability to bind 125I-JE. In two independent 293/mCCR2 lines, 75–80% specific binding of 1.0 nM 125I-JE was detected that reached equilibrium within 15–20 min at 37°C, while no specific binding was detected to control HEK293 cells (data not shown). Equilibrium binding of increasing concentrations of 125I-JE to 293/mCCR2 cells was saturable and approached maximal binding at 5 nM (Fig. 3A). Scatchard transformation of the binding data revealed a single class of 125I-JE binding sites with a Kd of 2.1 nM (Fig. 3B). This Kd value is representative of high affinity chemokine binding to chemokine receptors expressed in HEK293 cells.

To determine the ligand binding specificity of mCCR2, competition binding analyses were performed on 293/mCCR2 cells with 0.5 nM 125I-JE and increasing concentrations of unlabeled chemokines (Fig. 4). Both JE and FIC competed for 125I-JE binding to mCCR2, with JE competing approximately ten times more effectively than FIC. Two other mouse C-C chemokines, MIP-1α (35) and C10 (36), and the mouse C-X-C chemokine N51/KC (37, 38) did not effectively compete for 125I-JE binding to mCCR2. These results suggest that mCCR2 preferentially binds JE and FIC among the mouse chemokines tested so far and that mCCR2 is yet another chemokine receptor that binds more than one ligand.

The sequence and binding specificity of mCCR2 make it the most likely mouse species analog of CCR2B. mCCR2 binds JE with high affinity and FIC with lower affinity, while CCR2B binds MCP-1 with high affinity and MCP-3 with lower affinity. Interestingly, it has been proposed by sequence similarities that JE is the mouse MCP-1 analog and FIC the mouse MCP-3 analog. 

**FIG. 3.** Binding of 125I-JE to 293/mCCR2 cells. A, 0.2–7.5 nM 125I-JE was bound to 293/mCCR2 cells in the presence or absence of unlabeled JE as detailed under “Experimental Procedures.” The difference between total and nonspecific binding is the specific binding. Each data point is the mean of duplicate samples, and the experiment shown is representative of three independent experiments. Error bars depict the standard deviation for the mean values of total and nonspecific binding data. B, Scatchard transformation of the binding data.

**FIG. 4.** Competition of 125I-JE binding to 293/mCCR2 cells. 0.5 nM 125I-JE was bound to 293/mCCR2 cells in the presence of increasing concentrations of the indicated unlabeled chemokines as detailed under “Experimental Procedures.” Each data point is the mean of duplicate samples, and the experiment shown is representative of three independent experiments. Error bars depict the standard deviation for the mean values of each data point.
analog (39, 40). Since mCCR2 does not bind MIP-1α, while murine MIP-1αR/mCCR1 and murine MIP-1αRL2/mCCR3 have not been reported to bind J E or FIC, mCCR2 most likely mediates distinct functions from the other two doned murine C-C receptors. mCCR2 expression was detected in both the WEHI265.1 monocytic cell line and in elicited peritoneal macrophages, suggesting that mCCR2 will be important for mediating mononuclear cell responses to JE and FIC. The generation of mice with a targeted deletion of the m ccr2 gene will be useful for testing the role of mCCR2 in J E-mediated monocyte/macrophage recruitment and host defense and may be useful for developing mouse models for MCP-1/CCR2 functions.

Acknowledgments—We thank Naomi Thomson and Tom Nelson for assistance with DNA sequencing, Ed O’Rourke and John Stevens for recombinant protein expression and purification, Daniel Carrasco and Anne Lewin for mouse work, Maria Webb for advice in binding assays, and Heather MacDonald-Bravo for comments on the manuscript.

REFERENCES
1. Oppenheim, J. J., Zachariae, C. O. C., Mukaida, N., and Matsushima, K. (1991) Annu. Rev. Immunol. 9, 617–648
2. Miller, M. D., and Krangel, M. S. (1992) Crit. Rev. Immunol. 12, 17–46
3. Baggiolini, M., Dewald, B., and Moser, B. (1994) Adv. Immunol. 55, 97–179
4. Furutani, Y., Nomura, H., Notake, M., Oyamada, Y., Fukui, T., Yamada, M., Larsen, C. G., Oppenheim, J. J., and Matsushima, K. (1989) Biochem. Biophys. Res. Commun. 159, 249–255
5. Yoshinura, T., Yuki, N., Moore, S. K., Appella, E., Lerman, M. I., and Leonard, E. J. (1989) FEBS Lett. 244, 487–493
6. Chang, H. C., Hsu, F., Freeman, G. J., Griffin, J. D., and Reinherz, E. L. (1989) Int. Immunol. 1, 388–397
7. Van Damme, J., Proost, P., Lenaerts, J.-P., and Opdenakker, G. (1992) J. Exp. Med. 176, 59–65
8. Minty, A., Chalon, P., Guillenet, J.-C., Kaghod, M., Liauzun, P., Magazin, M., Miloux, B., Minty, C., Ramond, P., Vita, N., Lupker, J., Shire, D., Ferrara, P., and Caput, D. (1993) Eur. Cytokine Netw. 4, 99–110
9. Opdenakker, G., Froyen, G., Fiter, P., Proost, P., and Van Damme, J. (1993) Biochem. Biophys. Res. Commun. 191, 535–542
10. Cochran, B. H., Reffel, A. C., and Stiles, C. D. (1983) Cell 33, 939–947
11. Rollins, B. J., Morrison, E. D., and Stiles, C. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3738–3742
12. Kulmberg, P. A., Huber, N. E., Scheer, B. J., Wran, M., and Baumraker, T. (1992) J. Exp. Med. 176, 1773–1778
13. Heinrich, J. A., Ryseck, R. P., MacDonald-Bravo, H., and Bravo, R. (1993) Mol. Cytol. Biol. 13, 2020–2030
14. Sozzani, S., Locati, M., Zhou, D., Rieppi, M., Luini, W., Lamorte, G., Bianchi, G., Pontarutti, N., Allavena, P., and Mantovani, A. (1995) J. Leukoc. Biol. 57, 788–794
15. Nishi, N. A., Coughlin, S. R., Gordon, D., and Wilcox, J. N. (1991) J. Clin. Invest. 88, 1121–1127
16. Yla-Herttuala, S., Lipton, B. A., Rosenfeld, M. E., Sarkia, T., Yoshimura, T., Leonard, E. J., Witzum, J. L., and Steinberg, D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5252–5256
17. Koch, A. E., Kunkel, S. L., Harlow, L. A., Johnson, B., Evanoff, H. L., Haines, G. K., Burdick, M. D., Pope, R. M., and Stieter, R. M. (1992) J. Clin. Invest. 90, 772–779
18. Rollins, B. J., and Sunday, M. E. (1991) Mol. Cell. Biol. 11, 3125–3131
19. Fuentes, M. E., Durahan, S., Swerd, M. R., Lewin, A. C., Barton, D. S., Megli, J. R., Bravo, R., and Lira, S. A. (1995) J. Immunol. 155, 5769–5776
20. Rutledge, B. J., Rayburn, H., Rosenberg, R., North, R. J., Gladue, R. P., Corless, C. L., and Rollins, B. J. (1995) J. Immunol. 155, 4838–4843
21. Nede, K., DiGregorio, D., Mak, J. Y., Horuk, R., and Schall, T. J. (1993) Cell 72, 415–425
22. Gao, J.-L., Kuhns, D. B., Tiffany, H. L., McDermott, D., Li, X., Francke, U., and Murphy, P. M. (1993) J. Exp. Med. 177, 1421–1427
23. Charo, I. F., Myers, S. J., Hernandez, A., Franch, C., Connolly, A. J., and Coughlin, S. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2752–2756
24. Myers, S. J., Wong, L. M., and Charo, I. F. (1995) J. Biol. Chem. 270, 5786–5792
25. Frand, C., Wong, L. M., Van Damme, J., Proost, P., and Charo, I. F. (1995) J. Immunol. 154, 6511–6517
26. Contadiere, C., Ahuja, S. K., and Murphy, P. M. (1995) J. Biol. Chem. 270, 16491–16494
27. Ben-Baruch, A., Xu, L., Young, P. R., Bengali, K., Oppenheim, J. J., and Wang, J. M. (1995) J. Biol. Chem. 270, 22123–22128
28. Galu, J. A., and Murphy, P. M. (1995) J. Biol. Chem. 270, 17494–17501
29. Post, T. W., Bozic, C. R., Rothenberg, M. E., Luster, A. D., Gerard, N., and Gerard, C. (1995) J. Immunol. 155, 5199–5305
30. Van der Eb, A. J., and Graham, F. L. (1980) J. Virol. 36, 581–587
31. Carrasco, D., Weh, F., and Bravo, R. (1994) Development 120, 2491–2501
32. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
33. Heinrich, J. N., O’Rourke, E. C., Chen, L., Gray, H., Dorfman, K. S., and Bravo, R. (1994) Mol. Cell. Biol. 14, 2849–2861
34. Murphy, P. M. (1994) Annu. Rev. Immunol. 12, 593–633
35. Davatelas, G., Tekamp-Olson, P., Wolpe, S. D., Hennsen, K., Luedcke, C., Gallegos, C., Col, D., Merryweather, J., and Cerami, A. (1988) J. Exp. Med. 167, 1399–1444
36. Orlodsky, A., Berger, M. S., and Prystowsky, M. B. (1991) Cell Regul. 2, 403–412
37. Ryseck, R. R., MacDonald-Bravo, H., Mattei, M.-G., and Bravo, R. (1989) Exp. Cell Res. 180, 266–275
38. Oquendo, P., Albert, J., Wen, D., Graycar, J. L., Derynck, R., and Stiles, D. (1990) J. Biol. Chem. 265, 4137–4147
39. Rollins, B. J., Stier, P., Ernst, T. E., and Wong, G. G. (1989) Mol. Biol. Cell. Biol. 9, 4687–4695
40. Thiriot, S., Nys, G., Fiten, P., Masure, S., Van Damme, J., and Opdenakker, G. (1994) Biochemistry. Biophys. Res. Commun. 201, 493–499