Selective G Protein Coupling by C-C Chemokine Receptors*

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The C-C chemokines are major mediators of chemotaxis of monocytes and some T cells in inflammatory reactions. The pathways by which the C-C chemokine receptors activate phospholipase C (PLC) were investigated in cotransfected COS-7 cells. The C-C chemokine receptor-1 (CRK-1), the MCP-1 receptor-A (MCP-1Ra), and MCP-1Rb can reconstitute ligand-induced accumulation of inositol phosphates with PLC β2 in a pertussis toxin-sensitive manner, presumably through Gβγ released from the Gα proteins. However, these three receptors demonstrated different specificity in coupling to the α subunits of the Gα family. While none of the receptors can couple to Gαq or Gα16, MCP-1Rb can couple to both Gα14 and Gα16, but its splicing variant, MCP-1Rb, cannot. Since MCP-1Ra and -b differ only in their C-terminal intracellular domains, the C-terminal ends of MCP-1R determine G protein coupling specificity. CRK-1 can couple to Gα14 but not to Gα16, suggesting some of the C-C chemokine receptors, unlike the C-X-C chemokine receptors, discriminate against Gα16, a hematopoietic-specific Gα subunit. The intriguing specificity in coupling of the Gα family of G proteins implies that the chemokines may be involved in some distinct functions in vivo. The commonality of the chemokine receptors in coupling to the Gα-Gβγ-PLC β2 pathway provides a potential target for developing broad spectrum anti-inflammatory drugs.

Chemokines are a large family of small (8–10 kDa), inducible, secreted, proinflammatory cytokines, which are produced by various cell types. Members of the chemokine family share 20–90% homology in their amino acid sequences. The sequences usually have four conserved cysteine residues except lymphotactin. On the basis of the positions of the cysteine residues, the chemokine family can be divided into three subfamilies: the C-X-C or chemokine family, the C-C or chemokine family, and the C or γ family. The α family includes IL-8, GRO (growth-related oncogene), NAP-2, ENA-78, platelet factor 4, IP-10, and GCP-2, while the β family includes macrophage chemotactic protein (MCP)-1, -2, and -3, RANTES (regulated upon activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α and -1β, I309, and V10 (for reviews see Refs. 1–3). The newly discovered γ family has only one member, lymphotactin. Lymphotactin, unlike other chemokines, has only two conserved cysteine residues (4). The exact physiological and pathophysiological functions of these factors are not yet clearly defined; however, it is generally believed that their main function is recruitment and activation of leukocytes at the site of inflammation.

Two receptors, IL-8RA and IL-8RB, have been cloned for the C-X-C chemokine family (5). We have characterized the G protein-coupled pathways for these two receptors by using the cotransfection assay (6). Recently, three receptors for the C-C chemokines were also cloned: CRK-1 (7, 8), MCP-1Ra, and MCP-1Rb (9). CRK-1 binds to MIP-1α, RANTES, MIP-1β, and MCP-1 with varying affinities. However, only MIP-1α and RANTES can induce biological effects at physiological concentrations (7). MCP-1Ra and -b are two alternative splicing variants, and they differ only in their C-terminal intracellular domains (9). MCP-1Rb binds to MCP-1 and MCP-3 but not to MIPs, RANTES, or MCP-2 (9, 10). The C-C chemokine receptors share about 50% sequence homology among themselves and less than 30% homology with the C-X-C chemokine receptors (2).

CRK-1 and MCP receptors have typical structural characteristics of G protein-coupled receptors, and they induce cytosolic Ca2+ efflux (7, 9), presumably through activation of phospholipase C (PLC). Five cDNAs that encode the α subunits of the Gα class have been characterized, Gαq, Gα11, Gα14, Gα15, and Gα16 (11), all of which can activate isoforms of PLC β, PLC β1–4, to stimulate the release of inositol phosphates (IPs) (12–17). COS-7 cells contain Gαq and Gα11 but not Gα14, Gα15, or Gα16 (15). The expression of Gα15 and Gα16 was detected only in hematopoietic cells (Gα15 may be the mouse counterpart of human Gα16) (18–20), while Gα14 is expressed in some lineage of hematopoietic cells as well as other cell types (19). Many receptors, including the IL-8 receptors, were found to couple to some of the α subunits of the Gα family to activate PLC. Recently, the Gβγ subunits of G proteins were also found to activate specific isoforms of PLC β. The Gβγ-linked pathway may account for the PTX-sensitive activation of PLC mediated by the IL-8 receptors in mature leukocytes (6).

Since the C-C chemokines play important roles in chemotaxis of monocytes and some T cells, we characterized the G protein-coupled signal transduction pathways for the three C-C chemokine receptors by the cotransfection assay system in COS-7 cells. We found that the C-C chemokine receptors showed different specificity in coupling to the Gα subunit of the Gα family, while the receptors can all couple to the Gβγ subunits of the Gα family.
was determined as described in Ref. 6. In brief, cells were lysed in 10% perchloric acid and neutralized with KOH. IPs were retained by AG1-X8 ion exchange resin and eluted with formic acid. Portions of eluted samples were counted in a scintillation counter. The basal IP level in COS-7 cells is about 2000 dpm. Transfection or cotransfection with cDNAs encoding the C-C chemokine receptors, G\(\alpha\)14, PLC \(\beta_1\), Lac Z, did not alter the basal level. Cells transfected with the G\(\alpha\)16 and PLC-\(\beta_2\) cDNA increased the basal levels to 2500 and 3500 dpm, respectively. MCP-1 and MIP-1\(\alpha\) were purchased from R&D Systems. All the assays were repeated at least three times. The representative ones were shown.

cDNA Cloning—The cDNAs encoding CKR-1, MCP-1Ra, and MCP-1Rb were cloned from human THP-1 monocyte cells by polymerase chain reaction using primers based on the published sequences (7, 9). The sequences were verified by DNA sequencing. The cloned receptors were ligated into pcDNA/AMP (Invitrogen).

Ligand Binding Assay—Cos-7 transfectants were incubated with \(^{125}\text{I}\)-labeled ligands (Amersham Corp., 2000 Ci/mmol) in Dulbecco’s modified Eagle’s medium containing 0.25% bovine serum albumin three times. Finally the cells were solubilized in 0.1 N NaOH, and aliquots were counted by a \(\gamma\)-counter. The maximum binding sites and affinities were determined by Scatchard analyses.

RESULTS AND DISCUSSION

We tested in cotransfected COS-7 cells whether the newly cloned C-C chemokine receptors, including the MCP-1Ra, MCP-1Rb, and CKR-1, can couple to the \(\alpha\) subunits of the \(G\alpha\) class of G proteins. We have previously shown that receptors that can couple to G\(\alpha\)16 or G\(\alpha\)11 gave ligand-induced accumulation of IPs in COS-7 cells transfected with the receptor cDNAs (21). Thus, to test whether these C-C chemokine receptors can couple to G\(\alpha\)16 or G\(\alpha\)11, we transfected the cDNAs corresponding to each of the C-C chemokine receptors into COS-7 cells and determined ligand-induced accumulation of IPs. There was little MIP-1\(\alpha\)-induced accumulation of IPs in cells expressing CKR-1, and neither was there MCP-1-induced accumulation of IPs in cells expressing MCP-1Ra or MCP-1Rb (Fig. 1A). These results indicate that these receptors cannot couple to G\(\alpha\)16 or G\(\alpha\)11. To test whether the receptors can couple to other members of the \(G\alpha\) class, we cotransfected COS-7 cells with each of the receptor cDNAs and the G\(\alpha\)1A,-B,-and-C (21), \(G\alpha\)z or \(G\alpha\)a proteins were detected with anti-\(G\alpha\) bodies specific to G\(\alpha\)14 or G\(\alpha\)16. We and others have previously found that all the receptors tested in the cotransfection assay, including \(G\alpha\)1A, \(G\alpha\)B, and \(G\alpha\)D (21), \(\beta_2\)-adrenergic receptors (22), the \(\beta_2\)-muscarnic receptor, D1-dopamine receptor, V2, \(V_1\)a-vasopressin receptor, \(A_2\)a-adenosine receptor, \(\mu\)-opioid receptor, \(\delta\)-opioid receptors, and thrombin receptor (17) can couple to G\(\alpha\)16. However, neither CKR-1 nor MCP-1Ra can couple to G\(\alpha\)16, since cells coexpressing G\(\alpha\)16 and CKR-1 or MCP-1Ra showed little ligand-induced accumulation of IPs (Fig. 1A). Interestingly, MCP-1Rb, the alternative splicing variant of MCP-1Ra, gave ligand-dependent release of IPs when coexpressed with G\(\alpha\)16 (Fig. 1A), suggesting that MCP-1Rb can couple to G\(\alpha\)16. The activation of G\(\alpha\)16 by MCP-1Rb was insensitive to PTX (Fig. 1B). Furthermore, these C-C chemokine receptors demonstrated different selectivity in coupling to G\(\alpha\)14; CKR-1 and MCP-1Rb can couple to G\(\alpha\)14, while MCP-1Ra cannot (Fig. 1A). The concentration-dependent responses to ligand indicate a mean effective concentration (EC\(_{50}\)) for MCP-1Rb-mediated activation of G\(\alpha\)16 and G\(\alpha\)14 of about 7 nm.

We have demonstrated in many of our reports (6, 15, 21, 23) that coexpression of one protein does not significantly affect the expression of others. Nonetheless, we determined the expression of G\(\alpha\)16 and G\(\alpha\)14 in cells cotransfected with cDNA encoding CKR-1, MCP-1Ra, or MCP-1Rb to eliminate the possibility that the inabilities of CKR-1 and MCP-1Rb to couple to G\(\alpha\)16 or G\(\alpha\)14 were the results of lower expression levels of the proteins. As shown by Fig. 1, C and D, the expression levels of

**Fig. 1.** Coupling of the C-C chemokine receptors to the \(\alpha\) subunits of the \(G\alpha\) class. A, COS-7 cells were cotransfected with the cDNA (0.25 \(\mu\)g) encoding the C-C chemokine receptors and the cDNA (0.25 \(\mu\)g) corresponding to G\(\alpha\)14, G\(\alpha\)16, or Lac Z (\(\beta\)-galactosidase, as a control) as indicated in the figure. Forty-eight hours after transfection, MIP-1\(\alpha\)-induced (7 nm) accumulation of IPs in cells expressing CKR-1 and MCP-1-induced (20 nm) accumulation of IPs in cells expressing MCP-1Ra or -B were determined 30 min after addition of ligands. B, concentration-dependent accumulation of IPs to MCP-1 was determined in cells coexpressing MCP-1Rb and G\(\alpha\)14 or G\(\alpha\)16 in the presence (closed symbols) or absence (open symbols) of PTX. PTX (500 nm/ml) was added 4 h before the PLC assay. C and D, COS-7 cells were cotransfected with the G\(\alpha\)16 cDNA (0.25 \(\mu\)g) and cDNA (0.25 \(\mu\)g) encoding one of the C-C chemokine receptors. The cells were lysed in SDS sample buffer 48 h after transfection. The proteins were separated on 12% SDS-polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane. The G\(\alpha\)16 (C) and G\(\alpha\)14 (D) proteins were detected with antibodies specific to G\(\alpha\)16 and G\(\alpha\)14, respectively.

G\(\alpha\)16 and G\(\alpha\)14 were similar regardless of the nature of the coexpressed receptors. We also determine the receptor levels by using \(^{125}\text{I}\)-labeled MCP-1 or MIP-1\(\alpha\). The cells transfected with CKR-1, MCP-1Ra, or MCP-1Rb all show about 525–650 fmol of ligand-binding sites/1 \(\times\) 10\(^5\) cells, and the affinities are around 4.5 nm for CKR-1, 2.2 nm for MCP-1Ra, and 1.7 nm for MCP-
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The responses to the C-C chemokines (including MCP-1, MIP-1α, and RANTES) in monocyte phagocytes were found to be mostly PTX-sensitive (1-3), yet the signal transduction pathways mediated by the α subunits of the Gα class are PTX-resistant (11). PTX is a bacterial toxin, which modifies the C-terminal Cys residues of the Gαi and Gαq subunits. The modification prevents interactions between receptors and G proteins. Recently, we proposed a novel pathway to explain the PTX sensitivity; receptors interact with PTX-sensitive G proteins to release the Gβγ subunits, which then activate PLCβ2 (6, 23). Since there are abundant Gαi proteins (predominantly Gαs, Gαi1, and Gαi3) (24, 25) and the PLCβ2 pathway is likely to occur in vivo, to test whether the C-C chemokine receptors can couple to endogenous PTX-sensitive Gαi proteins of COS-7 cells to activate PLCβ2, we transfected COS-7 cells with cDNA encoding PLCβ2 and cDNA encoding each of the C-C chemokine receptors. COS-7 cells contain endogenous Gαi2 but not Gαqα16 proteins, and they contain endogenous PLCβ1 but not PLCβ2 as determined by specific antibodies (14). The accumulation of IPs in response to varying concentrations of MCP-1 or MIP-1α was determined. All three receptors can induce activation of PLCβ2 with EC50 of 4 × 10−11 M for CRK-1 and 3 × 10−10 M for MCP-1Ra and MCP-1Rb (Fig. 2). In addition, we found that the ligand-induced responses in cells coexpressing the receptors and PLCβ2 were PTX-sensitive (Fig. 2). Thus, we conclude that all three C-C chemokine receptors can couple to endogenous PTX-sensitive Gαi proteins, presumably the Gα2 protein, to activate PLCβ2 via Gβγ (the Gαqα16 subunits cannot directly activate PLCβ) (14). The finding that MCP receptors can inhibit adenylyl cyclase activity in A293 human kidney cells expressing the receptor confirms our notion that the receptor can couple to the Gαi proteins (27). The inability of the chemokine receptors to activate endogenous PLCβ (Fig. 1A) or recombinate PLCβ1 (data not shown) is consistent with our previous observation that Gβγ could not activate PLCβ1 in the cotransfection system (6, 21). In addition, we found that MCP-1 could not activate PLC in cells expressing CRK-1 and that MIP-1α could not induce IP formation in cells expressing MCP receptors.

Although the C-C chemokine receptors can couple to the GαiGβγPLCβ2 pathway, these receptors demonstrate interesting specificity in coupling to the α subunits of the Gα class. While none of the three receptors couples to Gαq, MCP-1Rα can couple to both Gα16 and Gα14, but its splicing variant MCP-1Ra cannot couple to either Gα14 or Gα16. CRK-1 couples to Gα14 but not to Gα16. The differences between MCP-1Rα and MCP-1Rβ in G protein coupling indicate that the C-terminal intracellular domains are critical in determining the G protein coupling specificity, since these two receptors differ only in the C-terminal ends (9). Moreover, the finding further supports our previous notion, drawn from our study of the α1B-adrenergic receptor, that different receptor sequences are required for activation of different Gα subunits of the Gα class (28). The study of the α1B-adrenergic receptor indicates that the α1B-adrenergic sequences required for activation of Gα14 are located in the third intracellular loop, whereas the sequences required for activation of Gα16 do not appear to be localized within the third inner loop. In this report, however, points out that the sequences in the C-terminal intracellular domain are critical for activation of both Gα14 and Gα16. We interpret the apparent discrepancy to suggest that G protein-interacting sequences on different receptors may be located at different sites or that there exist multiple G protein-interacting sites on a receptor so that alteration of any one of them abolishes the ability of the receptor to couple to the G protein. In this report we did not account for the influences of different Gβγ subunits on the coupling of these receptors to different Gα subunits because there were no significant differences observed for different Gβγ subunits in interaction with Gα14 or in regulation of PLCβ2 (15) or of adenylyl cyclase activities (29). Furthermore, the same system (COS-7 cells) was used in the studies; thus, the differences in the coupling of these chemokine receptors to different Gα subunits cannot be attributed to Gβγ.

The physiological relevance of the pathways mediated by Gα14, Gα16, and Gα12 is not clear. All these Gα subunits were found in various hematopoietic cells. Although more systematic studies of the expression of these Gα subunits are needed, previous studies suggest that there are very abundant Gα14 subunits with the majority of Gα12 and some Gα16 in leukocytes (24, 25) and that the levels of the Gα subunits increase along with differentiation (18). Gα16 and PLCβ2 was detected only in hematopoietic cells. Gα16 was detected in neutrophils, monocytes, lymphocytes, and erythrocytes as well as various hematopoietic progenitor cells, and its expression was also observed in HL-60 promyeloid cells decreases by 90% after differentiation (18). These results, in addition to the findings that responses to chemokines in mature leukocytes were mostly PTX-sensitive, suggest that the Gα16-linked pathway may be the predominant one in chemokine-mediated effects in mature leukocytes, such as chemotaxis and activation of leukocytes. If this hypothesis is correct, the activation of PLCβ2 by Gβγ would be an excellent target for developing broad spectrum anti-inflammatory drugs, because all the known chemokine receptors, including the C-C chemokine receptors, can couple to the Gα16Gβγ-PLCβ2 pathway. The fact that PLCβ2 is expressed only in hematopoietic cells may limit potential side effects.

Recently, some evidence indicates that chemokines may be directly or indirectly involved in the regulation of hematopoesis; MIP-1α inhibits proliferation of the hematopoietic stem cells (30), and the IL-8 receptor-null mice have expanded populations of neutrophils and B cells, in addition to their reduced abilities to respond to inflammatory stimuli (31). The Gα14-linked pathway may play a role in hematopoiesis as well as in other hematopoietic functions, although there is a lack of evidence. Nevertheless, regulation of expression levels by differentiation, specificity in interactions between receptors and G proteins and between G proteins and effectors, and diversity of molecular nature of receptors, G proteins, and effectors in leukocytes underlie the molecular basis for the complex func-

![Fig. 2. Activation of PLC β2 by the C-C chemokine receptor in transfected COS-7 cells.](http://www.jbc.org/)

The figure shows the activation of PLC β2 by the C-C chemokine receptor in transfected COS-7 cells. COS-7 cells were cotransfected with the PLC β2 cDNA (0.25 μg) and cDNA (0.25 μg) corresponding to CRK-1 (A), MCP-1Ra (B, squares), and MCP-1Rb (B, triangles). Ligand-induced accumulation of IPs was determined 30 min after addition of ligands (MIP-1α in A, MCP-1 in B) in the presence (closed symbols) or absence (open symbols) of PTX. PTX (50 ng/ml) was added 4 h before the PLC assay.
tion of signal transduction networks in the hematopoietic system. Alternative splicing further expands the signal-processing capabilities of eukaryotic cells.

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