Combined phylogenetic and chromosomal location studies suggest that the orphan receptor RDC1 is related to CXC chemokine receptors. RDC1 provides a co-receptor function for a restricted number of human immunodeficiency virus (HIV) isolates, in particular for the CXCR4-using HIV-2 ROD strain. Here we show that CXCL12, the only known natural ligand for CXCR4, binds to and signals through RDC1. We demonstrate that RDC1 is expressed in T lymphocytes and that CXCL12-promoted chemotaxis is inhibited by an anti-RDC1 monoclonal antibody. Concomitant blockade of RDC1 and CXCR4 produced additive inhibitory effects in CXCL12-induced T cell migration. Furthermore, we provide evidence that interaction of CXCL12 with RDC1 is specific, saturable, and of high affinity (apparent $K_D = 0.4 \text{ nM}$). In CXCR4-negative cells expressing RDC1, CXCL12 promotes internalization of the receptor and chemotactic signals through RDC1. Collectively, our data indicate that RDC1, which we propose to rename as CXCR7, is a receptor for CXCL12.

In concert with surface adhesion molecules, chemokines are secreted proteins that govern the migration of distinct leukocyte subsets to sites of inflammation and to their specific niches in lymphoid organs (1–3). Two main subfamilies are distinguished according to the position of the first two cysteines, which are separated by one amino acid (CXC chemokines) or are adjacent (CC chemokines) (4, 5). Chemokines mediate their functions by binding to chemokine receptors (CKRs), which belong to the large family of heptahelical G protein-coupled receptors. CKRs share more than 20% sequence identity, and their interactions with chemokines are highly promiscuous and often redundant (6).

The orphan receptor RDC1, which was originally cloned on the basis of its homology with conserved domains of G protein-coupled receptors (7, 8), was primarily believed to act as a receptor for vasostrictent peptide (9), a possibility later dismissed (10, 11). In fact, combined phylogenetic and chromosomal location studies suggested that RDC1 represents a CKR structurally close to CXC receptors (12–14), pointing to CXC chemokines as potential ligands. The RDC1 gene maps to mouse chromosome 1 and human chromosome 2, where the genes encoding CXCR1, CXCR2, and CXCR4 are localized (12,15,16, 48). Like CXCR4, the only known receptor for the chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12, RDC1 can serve as coreceptor for certain genetically divergent human immunodeficiency virus (HIV) and simian immunodeficiency virus strains, in particular for the HIV-2 ROD, an X4-tropic isolate (17). This suggests that both CKRs interact with a conserved domain of the viral envelope glycoprotein gp120, which displays some similarities with chemokines, for binding to CKRs and signaling in T cells (18, 19). Here we provide evidence that RDC1 and CXCR4 also share CXCL12 as a natural ligand.

**MATERIALS AND METHODS**

**Antibodies, Reagents, and Cells**—FITC-conjugated anti-human CD3 (clone SK7, IgG1) and PE-conjugated anti-human CXCR4 (clone 12G5, IgG2a) monoclonal antibodies (mAbs) were from BD Biosciences. The generation of the mouse anti-human RDC1 mAb (clone 9C4, IgG1) is described elsewhere. Briefly, the N terminus of human RDC1 (first 27 amino acids) was fused with carrier proteins, and the recombinant fusion proteins were used for immunization of mice. Positive hybridomas were screened by enzyme-linked immunosorbent assay using a synthetic peptide (DYSEPGNFNSWDSDI) corresponding to amino acids 7–21 of RDC1, designated here as RDC1-(7–21) (see Fig. 2A). The binding of unconjugated anti-RDC1 and anti-CXCR4 (12G5, BD Biosciences) mAbs was revealed using a FITC- or a PE-conjugated goat anti-mouse F(ab’)$_2$, Ab (Dako, Glostrup, Denmark) and analyzed on a FACSCalibur flow cytometer (BD Biosciences) with the CellQuest software. CXCL12, CXCL12-(4–67), and the C-terminal end biotinylated CXCL12 (biot-CXCL12) were kindly provided by Dr. F. Baleux (Institut Pasteur, Paris, France). Interleukin-8/CXCL8, BCA-1/CXCL13 and 6Ckine/CCL21 were from R&D Systems (Minneapolis, MN). PBLs were isolated from heparin-treated blood samples of healthy volunteers as described (20) and cultured overnight in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, penicillin (100 units/ml), streptomycin (100 μg/ml), and streptomycin (100 μg/ml) (complete RPMI medium). Dermal fibroblasts, isolated and expanded from healthy skin biopsies (20), and the HEK 293T (American Type Culture Collection, Manassas, VA) cell line were maintained in complete Dulbecco’s modified Eagle’s medium. The A0.01 T-cell (from Dr. H. T. He, Centre d’Immunologie de Luminy, Marseille, France) and 300.19 murine pre-B cell lines were cultured in complete RPMI medium.

**RDC1 and CXCR4 Expression Vectors and Functional Assays**—The human RDC1, ΔRDC1, and CXCR4 cDNAs were cloned into the pTRIP vector (kindly provided by Dr P. Charneau, Institut Pasteur, Paris, France) and expressed in cells as specified in the legends to Figs. 2–5.
Briefly, transient expression was achieved either by the calcium phosphate-DNA co-precipitation method or by using Nucleofector™ technology (Amaxa Biosystems, Cologne, Germany), whereas stable expression was obtained following a lentiviral-based strategy as described (20) or by electroporation with 20 μg/ml of linearized pcDNA3 containing RDC1 and selection with G418 (Sigma-Aldrich). The RDC1 product was engineered by polymerase chain reaction and lacks the last C-terminal 39 residues (325stop). Competition assays of mAbs with chemokines or RDC1-(7–21) for binding to CKRs were adapted from (21). Briefly, competition of mAb (used at 2 or 5 μg/ml) binding by chemokines or RDC1-(7–21) was performed at 4 °C for 90 min in PBS containing 1% bovine serum albumin, 0.2% Fc, and 0.1% NaN₃. Bound mAbs in treated cells were quantified by flow cytometry as follows: [receptor geometric mean fluorescence intensity (MFI) of treated cells/receptor geometric MFI of untreated cells] × 100. Specific binding of CXCL12 to RDC1 was evaluated using the biot-CXCL12 in A0.01 T cells that lack endogenous RDC1 and CXCR4 at both mRNA (data not shown) and protein (see Figs. 2B and 5A) levels. These cells also lack cell surface heparan sulfates that are known to bind CXCL12. For saturation binding experiments, RDC1-expressing A0.01 cells (2.5 × 10⁶) were maintained at room temperature for 90 min in binding buffer (PBS containing 0.2% bovine serum albumin and 0.1% NaN₃) in the presence of increasing concentrations of biot-CXCL12. Following a washing step with ice-cold binding buffer, specifically bound biot-CXCL12 was subsequently revealed by incubating cells at 4 °C with 1 μg/ml streptavidin (SAv)-PE conjugate (BD Biosciences) in binding buffer for 20 min. Cells were then washed once and fixed in PBS containing 2% paraformaldehyde. Results were analyzed by flow cytometry. Specific binding was estimated by subtracting from the total binding the nonspecific binding determined either in the presence of unlabeled CXCL12 at 10⁻⁶ M (in RDC1-expressing cells) or following the binding of biot-CXCL12 to parental cells. For competition experiments, 7.5 × 10⁻¹⁰ M biot-CXCL12 was used as the tracer in the presence of increasing concentrations of untagged CXCL12. Binding parameters (Kᵣ and IC₅₀) were determined with the Prism software (GraphPad Software Inc., San Diego, CA) using non-linear regressions applied to on-site models. Kᵣ values were deduced according to the Cheng and Prusoff equation Kᵣ = [IC₅₀/(1 + (L/Kᵣ))], where L is the concentration of biot-CXCL12 used.
RDC1 Is a Receptor for CXCL12

FIGURE 2. CXCL12 competes for binding of the 9C4 mAb to RDC1. A, alignment of the amino acid sequences of human RDC1 and CXCR4. Amino acid residues with identity (*), high similarity (.), and moderate similarity (.) are marked. Transmembrane domains are unlabeled, whereas intracellular and extracellular domains are highlighted in blue and red, respectively. A synthetic peptide (framed residues, RDC1-(7–21)) was used to screen hybridomas producing anti-RDC1 mAbs. B, RDC1-(7–21) prevents binding of the 9C4 mAb to RDC1. Left section, cell surface expression level of CXCR4 in A0.01 T-cells expressing the receptor after transduction was determined by flow cytometry using the 12G5 anti-CXCR4 mAb (12G5, red histogram). No labeling was detected using the corresponding isotype-matched control Ab (CTRL; gray histogram) or the anti-RDC1 9C4 mAb (green histogram). Right section, binding of the 9C4 mAb to RDC1 in A0.01 T-cells expressing the receptor after transduction is shown in the absence (9C4 untreated; red histogram) or presence of 10−5 M (dotted histogram) or 10−3 M (blue histogram) of RDC1-(7–21). No labeling was detected using the corresponding isotype-matched control Ab (CTRL; gray histogram) or the anti-CXCR4 12G5 mAb (data not shown), and binding of the 9C4 mAb was not affected by pre-incubating cells with an excess of RDC1-(7–21) (data not shown). C, HEK 293T cells expressing CXCR4 or RDC1 were generated by the calcium phosphate-DNA co-precipitation method and were assessed for the binding of the 9C4 or 12G5 mAb in the absence (untreated; red histogram) or presence (blue histogram) of 10−5 M CXCL12. Nonspecific binding was assessed after incubation of cells with the corresponding isotype-matched control Ab (gray histograms) or in the presence of the anti-RDC1 9C4 (in CXCR4-expressing cells; left section, green histogram) or anti-CXCR4 12G5 (in RDC1-expressing cells; right section, green histogram) mAb. Representative experiments of three independent determinations are shown. D, different concentrations of the chemokines (CA) CXCL12 and CXCL12-(4–67) were tested for their ability to compete with the binding of the 12G5 anti-CXCR4 (left section) or 9C4 anti-RDC1 (right section) mAb. Values are normalized for binding in the absence of CA (arbitrarily set at 100%; open bars) and represent means ± S.E. of three independent determinations.

In competition binding experiments. Receptor internalization and chemotaxis assays were performed as described (20). Statistical analyses consisted of unpaired two-tailed Student’s t tests and were conducted with the Prism software.

**Immunofluorescence**—PBLs (2 × 10⁵) were plated in RPMI medium supplemented with 1% bovine serum albumin and 20 mM HEPES onto polylysine-coated glass coverslips for 3 h at 37 °C and fixed in PBS with 4% paraformaldehyde. Cells were stained at room temperature with the anti-CXCR4 or anti-RDC1 mAb (10 μg/ml) for 30 min followed by FITC- or Texas Red-conjugated horse antimouse Ab (Vector Laboratories, Burlingame, CA). After three washes with PBS, cells were incubated with the FITC-conjugated anti-human CD3 mAb and finally mounted in Vectashield medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories). For competitive experiments, cells were incubated at 4 °C with a mAb to CXCR4 or RDC1 (2 μg/ml) in the presence of 10 μM CXCL12. Fluorescence was imaged on a Zeiss microscope (Oberkochen, Germany) using a Plan Apochromat 100×, 1.4 oil immersion objective. Images were collected with a cooled charge-coupled device camera (Axiocam MRm), piloted by Axiovision imaging software (Zeiss). Optical sectioning was performed according to the structured illumination principle using the ApoTome system (Zeiss).

**RESULTS AND DISCUSSION**

**CXCL12-induced Chemotaxis of T lymphocytes Is Inhibited by an Anti-RDC1 mAb**—Like CXCR4, the RDC1 gene is ubiquitously expressed in non-hematopoietic and hematopoietic tissues, and its mRNA is found in brain, heart, kidney, spleen, and PBLs (7, 17, 22). We investigated by immunofluorescence whether RDC1 is expressed in leukocytes using a mAb raised against human RDC1 (9C4). Leukocytes including CD3⁺ T lymphocytes (Fig. 1A) were stained either with the 9C4 anti-RDC1 mAb or 12G5 mAb, which recognizes an epitope in the second extracellular loop of CXCR4 (23–25). As expected, the concomitant addition of 10 μM CXCL12 abolished staining of CXCR4 with mAb 12G5. Interestingly, a molar excess of CXCL12 also prevented the 9C4 mAb from binding to T lymphocytes (Fig. 1A, lower sections). As cell migration features chemokine responses, we asked whether the 9C4
mAb could interfere with the CXCL12-stimulated chemotaxis of T lymphocytes. The chemotactic effect of 30 nM CXCL12 was inhibited by 40–50% in the presence of the 9C4 anti-RDC1 mAb or the 12G5 anti-CXCR4 mAb (10 μg/ml). When added together, both mAbs produced an additive inhibitory effect that extends up to 70% of the CXCL12-induced chemotaxis (Fig. 1B). By contrast, migration of lymphocytes in response to CCL21 (Fig. 1B) or CXCL13 (data not shown) was not affected by the addition of anti-RDC1 and anti-CXCR4 mAbs. These findings suggest that RDC1 is expressed at the surface of T lymphocytes and contributes to cell migration toward the CXCL12 gradient. However, the physiological role of RDC1 in T cell migration remains to be determined. Interestingly, CXCL12 displaces the binding of the anti-RDC1 mAb, which reacts with the N terminus of RDC1. Sequence alignment reveals that the N termini of CXCR4 and RDC1 display amino acid similarities and encompass acidic and aromatic residues (Fig. 2A) that are known to be critical for CXCR4 to bind human immunodeficiency virus gp120 and CXCL12 (26, 27). These findings suggest that CXCL12 interacts with the N terminus of RDC1.

CXCL12 Binds to RDC1—To confirm that the 9C4 mAb specifically recognizes RDC1, we first assessed whether the synthetic peptide RDC1-(7–21), which encompasses the epitope recognized by the 9C4 mAb, prevents binding of the mAb to the receptor. In A0.01 cells that lack endogenous expression of RDC1 and CXCR4, ectopic expression of RDC1 restored binding of the 9C4 mAb (Fig. 2B, right section, red histogram and Fig. 5A, right section) but not that of the anti-CXCR4 12G5 mAb (data not shown and Fig. 5A). Conversely, ectopic expression of CXCR4 promoted binding of the 12G5 mAb but not that of the anti-RDC1 9C4 mAb (Fig. 2B, left section, and see Fig. 5A, left section). When
pre-incubated with RDC1-(7–21), the 9C4 mAb (5 μg/ml) was found to be impaired in its ability to recognize RDC1 in a dose-dependent manner (Fig. 2B, right section). We further evidenced that the 9C4 anti-RDC1 mAb bound to HEK 293T cells expressing the receptor (Fig. 2C, right section, red histogram) but not to the parental cells (data not shown) or to CXCR4-expressing (Fig. 2C, left section, green histogram) or CCR5-expressing (data not shown) cells. Conversely, the anti-CXCR4 12G5 (Fig. 2C, right section) or anti-CCR5 2D7 (data not shown) mAb did not label cells that expressed RDC1 alone. These findings indicate that the 9C4 mAb specifically recognizes RDC1. We then investigated the interaction between CXCL12 and RDC1 by assessing the ability of the chemokine to compete with the binding of the 9C4 mAb to RDC1. CXCL12 displaced the 9C4 as well as the 12G5 mAbs (Fig. 3, B–D). When added at increasing concentrations, truncated CXCL12 displaced binding of 7.5 × 10⁻¹⁰ M biot-CXCL12 to RDC1-expressing A0.01 cells in a dose-dependent manner with IC₅₀ = 5 ± 1.8 nM (Fig. 3, C and D). Overall, this demonstrates that RDC1 is a high affinity receptor for CXCL12.

CXCL12 Induces Down-regulation of RDC1—Receptor internalization is a widely described process that occurs upon chemokine stimulation (29). We thus investigated CXCL12-induced internalization of RDC1. Treatment of pre-B 300.19 cells expressing human RDC1 with increasing concentrations of CXCL12 induced a dose-dependent internalization of RDC1 (Fig. 4, A and B). Similarly, 2 × 10⁻⁷ M CXCL12 induced down-regulation of RDC1 expressed on dermal fibroblasts (Fig. 4, C and D) or on Chinese hamster ovary K1 or A0.01 cells (data not shown). By contrast, RDC1 was not internalized in response to other CXC chemokines, CXCL8 and CXCL13 (data not shown). Agonist-induced CKR internalization generally relies on the phosphorylation of C-terminal Ser/Thr residues that, in turn, promotes binding of β-arrestins for targeting to clathrin-coated pits (30). The C-terminal sequence of RDC1 contains multiple Ser/Thr residues (Fig. 2A) that could serve as structural determinants for receptor internalization.
CXCL12 Promotes Cell Migration through RDC1—We tested the ability of CXCL12 to trigger chemotaxis of A0.01 T-cells expressing either RDC1 or CXCR4 (Fig. 5A). Addition of the chemokine resulted in a dose-dependent migration of RDC1-positive cells (Fig. 5B). The magnitude of this process was comparable with that of CXCR4-positive cells, with a maximum migration obtained at 10−8 M CXCL12. CXCL12 also triggers chemotactic signal through the truncated ΔRDC1 receptor (data not shown), indicating that an intact receptor C terminus is not required for chemotaxis. This finding highlights that the structural determinants required for RDC1-mediated chemotaxis may differ from those involved in receptor internalization, as reported for other CKRs (34, 35). The 9C4 mAb strongly inhibited migration of RDC1-expressing cells but did not modify that of cells expressing CXCR4 (Fig. 5C). Conversely, the anti-CXCR4 12G5 mAb abolished chemotaxis of cells expressing CXCR4 but had no effect on CXCL12-promoted migration of cells expressing RDC1 alone. These results confirm that CXCL12 triggers chemotactic signals directly through RDC1. CCL21, CXCL8, CXCL13 (Fig. 5D), CCL4, RANTES (regulated on activation of normal T cell expressed and secreted)/CCL5 (data not shown), or CXCL12-(4–67) (Fig. 5D) did not promote chemotaxis of RDC1-expressing cells.

Taken together, our data indicate that, in cells ectopically expressing RDC1, CXCL12 binds to and signals via RDC1 and further suggest that activation of RDC1 by CXCL12 requires the intact N terminus of the chemokine.

Conclusions—Collectively, our data demonstrate that the orphan receptor RDC1 is a high affinity receptor for CXCL12. Currently, six CXC receptors have been characterized and, according to the nomenclature for CKR families (6), we propose that RDC1 be renamed as CXCR7. The highly conserved sequences of RDC1 and CXCL12 among species suggest that CXCL12/RDC1 interactions may have conserved functional significance. However, the responsiveness of RDC1 to CXCL12 is intriguing because of the nearly identical phenotypes of CXCR4- and CXCL12-deficient mice. The notion that both CXCR4 and CXCL12 knock-out mice die perinatally and display profound defects in hematopoiesis and cerebellar development was generally interpreted as a monogamous relationship between CXCR4 and CXCL12 (36, 37). The interaction between CXCL12 and RDC1 opens questions about the role of this receptor during embryonic life. RDC1 may function in organogenesis and hematopoiesis in concert with CXCR4. Alternatively, but not exclusively, RDC1 may exert distinct activities to those of CXCR4. To this end, it would be important to understand the spatio-temporal regulation and distribution of RDC1 in embryonic tissues.

In postnatal life, as shown for CXCR4, our results are also consistent with a possible role of RDC1 in CXCL12-mediated regulation of CD34+ stem cell homing in the bone marrow and leukocyte trafficking (38–41). Accumulating evidence shows involvement of the CXCL12/CXCR4 axis in inflammatory diseases, tumor metastasis, and angiogenesis (42–45). The reported expression of RDC1 in tumor endothelial
cells of brain and peripheral vasculature (46) or in Kaposi’s sarcoma-associated herpesvirus dermal microvascular endothelial cells (47) also supports the view that pathological processes might implicate the CXCL12/RDC1 axis.

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