Inhibition of Gαi2 Activation by Gαi3 in CXCR3-mediated Signaling*

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Brian D. Thompson†, Yongzhu Jin‡, Kevin H. Wu†, Richard A. Colvin§, Andrew D. Luster‡, Lutz Birnbaumer†, and Mei X. Wu†‡

From the †Wellman Center for Photomedicine, Department of Dermatology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114, ‡NIEHS, Transmembrane Signaling Group, Laboratory of Signal Transduction, National Institutes of Health, Research Triangle Park, North Carolina 27709, and §Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

G protein-coupled receptors (GPCRs) convey extracellular stimulation into dynamic intracellular action, leading to the regulation of cell migration and differentiation. T lymphocytes express Gαi2 and Gαi3, two members of the Gαi/o protein family, but whether these two Gαi proteins have distinguishable roles guiding T cell migration remains largely unknown because of a lack of member-specific inhibitors. This study details distinct manner. Gαi-mediated blockade of Gαi activation did not result from Gαi activation, but instead resulted from competition or steric hindrance of Gαi interaction with the CXCR3 receptor via the N terminus of the second intracellular loop. A mutation in this domain abrogated not only Gαi2 activation induced by a CXCR3 agonist but also the interaction of Gαi3 to the CXCR3 receptor. These findings reveal for the first time an interplay of Gαi proteins in transmitting G protein-coupled receptor signals. This interplay has heretofore been masked by the use of pertussis toxin, a broad inhibitor of the Gαi/o protein family.

Heterotrimeric G proteins consist of α, β, and γ subunits that couple to seven transmembrane receptors called G protein-coupled receptors (GPCRs).3 The G protein resides attached to the intracellular face of the plasma membrane in an inactive form consisting of the Gα subunit bound to GDP, a structure that is stabilized by interaction with the βγ dimer. Agonist binding to the receptor provokes a conformation change that facilitates the interaction of the Gα subunit to the receptor. Upon interaction with the receptor, Gα protein becomes activated, causing GDP exchange for GTP. This activation to the high energy Gα-GTP results in dissociation of the α and βγ subunits and propagation of downstream signaling by both the Gα-GTP and the βγ subunits. The GTP-binding proteins are classified by the signaling events they instigate, of which there are four major families as follows: Gαq, Gα11, Gα12/13, and Gαi (1, 2). Members of the Gαqα11 family, including Gα11, Gα12, Gα13, Gαo, Gα16, and Gαqγ can all be irreversibly uncoupled from receptors by pertussis toxin (PTX) (3, 4). PTX catalyzes ADP-ribosylation of a specific cysteine residue at position −4 from the C terminus of the α subunits, and thus blocks downstream pathways by preventing receptor interaction (5). The bacterial toxin has proven to be an excellent tool in dissecting the essential role of Gαi proteins in chemotaxis, proliferation, and differentiation of various cells (3, 4, 6, 7). However, it cannot distinguish unique properties of individual Gαi/o protein family members. Investigation of the details of GPCR and G protein interactions has drawn great attention in the past decade, because the interactions dictate the specificity and amplitude of a GPCR-stimulated cellular response, and GPCRs are targets for more than 30% of the current marketed drugs (8).

The primary PTX substrates evident in T lymphocytes are the 40–41-kDa α subunits of the Gαi2 and Gαi3 proteins (9, 10). The importance of PTX-sensitive proteins in lymphocyte trafficking came initially from the unique phenotype of lck-PTX-transgenic mice that expressed the PTX catalytic S1 subunit in thymocytes and peripheral T cells under the control of an lck promoter (11). The transgenic mice had 2–4-fold more mature CD4+ and CD8+ thymocytes in the thymus than did

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3 The abbreviations used are: GPCR, G protein-coupled receptor; Ab, antibody; GTP-γS, guanosine 5′-3-(O-thio)triphosphate; PTX, pertussis toxin; S1P, sphingosine 1-phosphate 1; KO, knock-out; WT, wild type; IFN, interferon; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; ConA, concanavalin A.
control mice and few or no T cells in the peripheral lymphoid tissues because of a defect in thymic emigration (11). Thymocyte emigration appears to be controlled by either Gαi3 or Gαi2, because null mutation of either of them does not affect this process significantly (12, 13). In the periphery, however, their function differs considerably. Gαi2-deficient mice display impairment in lymph node development in several anatomic locations and in Peyer’s patch formation (12, 14). The mice develop inflammatory bowel disease, at least in part, due to a lack of transforming growth factor-β responses in T cells that causes Th1-skewed hyperimmune responses in the colon (12, 15). In contrast, Gαi3-deficient mice develop no overt phenotype (16).

In this study, we investigate unique properties of Gαi2 and Gαi3 in chemotaxis of activated T cells induced by CXCR3 ligands, because expression of the CXCR3 receptor has been associated with pathophysiology of Th1-type autoimmune diseases in both humans and experimental models (17–19). The receptor has the following three known ligands: monokine-induced IFN-γ (MIG)/CXCL9, IFN-γ-inducible 10-kDa protein (IP-10)/CXCL10, and IFN-γ-inducible T cell α-chemotactant/CXCL11. These three chemokines exhibited discernible biological effects in vivo and in vitro (20–24), but they all activated exclusively Gαi2 through the CXCR3 receptor, as indicated by a lack of CXCR3-mediated responses in Gαi2−/− T cells, regardless of the agonist employed. Interestingly, we found that Gαi3 was negatively involved in the CXCR3-mediated signaling. This translated to cellular responses, where a lack of Gαi3 significantly enhanced T cell migration toward CXCR3 agonists. Gαi3 appears to hinder Gαi2 activation via the N terminus of the second intracellular loop. A mutation in this region abolished both activation of Gαi2 and the binding of Gαi3 to the receptor. This work begins to unravel an interplay between individual Gαi proteins that has previously been masked by the use of PTX.

**MATERIALS AND METHODS**

**Animals**—Gαi2-knock-out (KO), Gαi3-KO, and wild type (WT) control mice on the mixed 129Sv/C57BL/6 background were generated by gene targeting and backcrossed with C57BL/6 (B6) mice for six times as described (12, 25). Both female and male mice were used at 4–6 weeks of age unless otherwise indicated. The mice were housed in conventional cages at the animal facilities of the Massachusetts General Hospital in accordance with institutional guidelines.

**T Cell Preparation**—Single-cell suspensions prepared from lymph nodes and spleens were treated with a mixture of rat anti-mouse monoclonal antibodies (Abs) against CD19, CD32, and CD16 followed by depletion of Ab-bound cells with BioMag goat anti-rat IgG (Polysciences Inc., Warrington, PA) per the manufacturer’s instructions. This preparation isolated an ~90% pure population of T cells. CXCR3 expression on these T cells was induced by stimulation for 2 days at 37 °C and 5% CO2 with 2.0 μg/ml concanavalin A (ConA) in a complete RPMI 1640 medium (10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol). The cells were continuously cultured for 2 more days in the medium with replenishment of 15% ConA supernatant, which gave rise to a maximal level of CXCR3 expression.

**Cell Membrane Preparation**—Cell membrane was prepared as described with some modifications (26). Briefly, T cells stimulated with or without ConA as above were collected, suspended in ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 3 mM EGTA, 1× protease inhibitor mixture, and 1 mM PMSF), and Dounce-homogenized. In some cases, the cells were treated with 10 ng/ml PTX for 1 h at 37 °C before suspension in hypotonic buffer and homogenization. Disrupted cells were centrifuged at 500 × g at 4 °C for 10 min to remove nuclei and unbroken cells. The remaining supernatant was spun at 32,000 × g for 25 min at 4 °C to pellet the cell membrane that was then suspended in membrane solubilization buffer (20 mM glycerine, pH 7.2, 1 mM MgCl2, 250 mM sucrose, 1× protease inhibitor mixture, and 1 mM PMSF). The suspended cell membrane was left on ice for at least 2 h with periodic vortexing and then frozen until use.

**GTPγS Assay**—Cell membrane equivalents of 20 μg of protein were first incubated with the indicated concentrations of chemokines on ice for 1 h to prime Gαi activation by the receptor (27). G protein activation was initiated by placing samples at room temperature for 2 h in the presence of 0.3 nM [35S]GTPγS (1250 Ci/mmol; PerkinElmer Life Sciences) in a GTP-binding buffer (50 mM Tris/NaCl, 1 mM EGTA, and 5 mM MgCl2) supplemented with fresh 0.1% bovine serum albumin, 1 mM dithiothreitol, and 2 mM GDP (28). Activation of Gαi proteins at room temperature instead of 37 °C minimizes background radioactive incorporation (28). The concentrations used for each CXCR3 ligand were empirically determined based on the most robust migratory response and our preliminary tests. All chemokines were obtained from PeproTech Inc. (Rocky Hill, NJ). Where indicated, a myristoylated Gαi2 protein or control Gαi protein (Calbiochem) at varying concentrations was preincubated with the cell membrane for 1 h on ice to allow the added Gα protein to interact with the βγ subunit in the membrane prior to chemokine stimulation (27). To determine the Gαi protein in association with the receptor, 20 μg/ml monoclonal Ab specific for Gαi2 (IgG2b, Chemicon, Temecula, CA) or an isotype-matched control Ab was added to the samples, along with the chemokine to block Gαi2 protein activation (29). The samples were then transferred to a 96-well plate and suctioned through a glass fiber filter by a TomTeC cell harvester (model MACH 3 M, Hamden, CT). The filters were washed five times with a cold GTP washing buffer (20 mM Tris, pH 8.0, 100 mM NaCl, and 25 mM MgCl2) and dried, and radioactive count was determined on a 1450 MicroBeta scintillation counter (Wallac, Turku, Finland). Percentages of specific GTPγS incorporation in activated T cell membranes were calculated as

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((\text{counts} / \text{min of sample}) - (\text{counts} / \text{min of background control})) / (\text{counts} / \text{min control without a stimulus}) \times 100
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Migration Assays—The Transwell system with 3-μm pore size polycarbonate membrane (Corning Glass, Corning, NY) was used for all transmigration assays, because T cells are relatively small in size. This pore size significantly reduced the basal.
levels of T cell migration in our study. T cells at $3 \times 10^6$ cells/ml in Aim-V medium (Invitrogen) were added to the upper chamber at 0.3 ml per well, with the indicated concentrations of chemokines in 0.6 ml of Aim-V medium in the lower chamber. Freshly isolated T cells or T cells activated by ConA for 4 days were allowed to migrate for 4–6 h at 37 °C and 5% CO$_2$. At the end of the migration, the cells in the lower chamber were collected and counted on a hemocytometer.

Western Blot Analysis—To examine Ga$_{12}$ expression in T cell membranes, T cells with or without stimulation were collected and lysed in cold nondenaturing lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1× protease inhibitor mixture), followed by centrifugation at 500 × g for 10 min to remove nuclear fragments and unbroken cells. Crude cell membrane was pelleted from the supernatant by centrifugation at 32,000 × g for 25 min. Protein samples of ~100 µg were separated on a 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted with anti-Ga$_{12}$ monoclonal Ab (Chemicon, Temecula, CA). Ga$_{12}$ protein was visualized by incubation of the membrane with sheep anti-mouse Ab conjugated with horseradish peroxidase (Amersham Biosciences) followed by reaction with SuperSignal West Pico ECL reagents (Pierce) and exposure to Kodak film (Eastman Kodak Co.). For assessment of Ga$_{13}$ protein expression, Ga$_{13}$ protein was first depleted by anti-Ga$_{13}$-specific Ab because of cross-reaction of anti-Ga$_{12}$ Ab with Ga$_{12}$ protein and similar molecular mass of these two G proteins. Briefly, T cell membranes prepared above were incubated for 1 h on ice with anti-Ga$_{12}$ Ab-conjugated protein G-agarose beads that had been treated previously with 1 mg/ml bovine serum albumin to block nonspecific binding. The beads were removed by centrifugation, and the depletion was repeated three times. The resultant Ga$_{12}$-depleted T cell membranes were subjected to Western blotting analysis using anti-Ga$_{13}$ Ab (BIOMOL International LP) as detailed above. The same membranes were re-probed, after stripping, with anti-G protein pan-β subunit Ab (Upstate Biotechnology, Inc., Lake Placid, NY) for equal protein loading controls.

Flow Cytometry—To determine chemokine receptor expression, T cells with or without stimulation with ConA or transfected COS-7 cells were incubated with specific Abs followed by analysis on a FACSCalibur cytometer equipped with a CellQuest software (BD Biosciences). A murine CXCR3-specific Ab (clone 4c4) was a generous gift from Dr. Dominic Picarella (Millenium Pharmaceuticals) (30). Fluorescence-conjugated rat anti-mouse Abs specific forCCR7 or CXCR4 and mouse anti-human CXCR3 Ab were purchased from Biologend (San Diego, CA), R&D Systems (Minneapolis, MN), or Pharmingen, respectively.

In Vitro Transcription and Translation—Ga$_{12}$ and Ga$_{13}$ proteins were synthesized and 35S-labeled by the TnT® Quick-Coupled in Vitro Transcription/Translation System per the manufacturer’s instructions (Promega, Madison, WI). In brief, 1 µg of a Ga$_{12}$ or Ga$_{13}$ plasmid was mixed with the rabbit reticulocyte lysate provided, 100 µM myristic acid, and [35S]methionine (1000 Ci/mmol) for 75 min at 30 °C. The resulting product was verified by SDS-PAGE on the basis of the size and compared in the presence or absence of the specific plasmid.

Immunoprecipitation—To study CXCR3-associated Ga$_{i}$ proteins, COS-7 cells were transfected with pcDNA plasmids containing either WT or mutated CXCR3 receptors or a control HA-tag-S1P$_1$ (sphingosine 1-phosphate 1) receptor using Lipofectamine according to the manufacturer’s instructions (Invitrogen). Three mutants were as follows: 1) an R149N-CXCR3 that had asparagine (Asn) substitution for arginine (Arg) at position 149; 2) LIL-CXCR3 generated by replacement of alanines for leucines (Leu) at positions 332–334 in the C terminus; and ST(–)-CXCR3 constructed by substitution of all serines (Ser) and threonines (Thr) in the C terminus with alanines (24, 31, 32). The transfected cells were lysed, and the cell membrane was prepared and suspended in a GPCR solubilization buffer (1 mM MgCl$_2$, 250 mM sucrose, 1× protease inhibitor mixture, and 1 mM PMSF in phosphate-buffered saline) as described (24, 31, 32). Cell membranes with equivalent of 60 µg of protein were incubated with 3 µl of in vitro synthesized 35S-labeled Ga$_{12}$ or Ga$_{13}$ for 1 h on ice to allow the added Ga$_{i}$ protein to interact with the βγ subunit in the membrane, after which the cell membrane was either left unstimulated or stimulated for 2 h on ice by indicated chemokines and activated at room temperature as described (27). The stimulated membranes were then cross-linked for 2 h on ice with 20 µM dithio-bis-sulfosuccinimidyl propionate (Pierce). The cross-linker was quenched by the addition of 50 µM Tris, pH 7.5, for 15 min on ice. To the chemokine-stimulated membranes, anti-CXCR3 Ab or control Ab was added, and the Ab-bound receptor was pulled down by pre-cleaned protein G-agarose beads (Invitrogen). For HA-S1P$_1$-transfected cells, immunoprecipitation using anti-HA Ab was carried out in place of anti-CXCR3 Ab. The resultant beads were washed extensively with a cold GPCR washing buffer (1 mM EGTA, 5 mM MgCl$_2$, and 0.1% bovine serum albumin in phosphate-buffered saline). Radioactivity of the resultant beads was quantified by scintillation counting on the 1450 MicroBeta scintillation counter to determine the amount of 35S-Ga$_{12}$ or 35S-Ga$_{13}$ in association with the CXCR3 receptor.

Statistical Analysis—The Student’s two-tailed t test was used to analyze the significance of experimental groups compared with relevant controls.

RESULTS

Altered Migration toward CXCR3 Ligands of Ga$_{12}$- or Ga$_{13}$-deficient T Cells—CXCR3 agonists induce chemotactic responses of activated T cells in vitro with a potency of CXCL11 > CXCL10 > CXCL9 in a PTX-sensitive manner (24, 34). These three chemokines also mediate distinct biological effects in vivo (20, 21, 23, 35). In an attempt to understand whether the varying responses evoked by these agonists resulted from activation of different Ga$_{i}$ proteins, we tested the role of Ga$_{12}$ and Ga$_{13}$ in the migration of activated T cells toward CXCL9, CXCL10, or CXCL11 (36). Stimulation of WT T cells with increasing concentration gradients of CXCR3 ligands elicited a weak chemotactic response to CXCL9 (data not shown) but vigorous responses to CXCL10 or CXCL11 in a dose-dependent manner (Fig. 1, A, B, and E), mirroring previous investigations (37). However, migration toward these three individual chemokines was all reduced to control levels in cells

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**Figure 1**

- **A**: Chemotaxis of WT T cells were assayed toward CXCL9, CXCL10, or CXCL11 in a dose-dependent manner (Fig. 1, A, B, and E).
- **B**: The chemotactic response to CXCL9 was all reduced to control levels in cells.
**Goα3 Antagonizing Goα2 Activation**

**FIGURE 1.** Chemotactic responses in the presence or absence of Goα2 or Goα3. A–E, Goα2 and Goα3 have opposing effects on CXCR3-stimulated chemotaxis. Activated T cells prepared from WT, Goα3−/−, and Goα2−/− mice were either left untreated (A, B, and E) or treated with 100 ng/ml PTX overnight (C and D) and then added to the upper chamber. CXCR3 agonists at indicated concentrations were added to the lower chamber in triplicate. After 4 h of incubation, the migrated cells in the lower chamber were collected and counted. Data are presented as mean percentages ± S.D. of cell migration relative to cell input. One representative experiment in A–D and cumulative data from at least five independent experiments (n = 15) in E are shown. Chemokine concentrations used in E were the optimal concentrations for stimulating maximal migration toward CXCL9 (100 ng/ml), CXCL10 (10 ng/ml), and CXCL11 (100 ng/ml). Medium controls are designated as 100%, and mean percentages ± S.D. of cell migration relative to the control are shown. * and **, statistical significance (p < 0.05 or p < 0.01, respectively) in the presence versus absence of a specific Goα protein. F–H, varying effects of Goα2 and Goα3 on T cell chemotaxis induced by different chemokines. The migration was assayed as above, except that nonstimulated T cells were used in place of activated T cells in chemotactic responses to CXCL12 and CCL19 in F and G. Data are presented as mean percentages ± S.D. of cell migration relative to cell input as A and B. One representative result of three independent experiments with each performed in triplicate.

lacking Goα2 over a wide range of agonist concentrations tested (Fig. 1, A, B, and E). The lack of chemotactic responses toward CXCR3 agonists was not due to aberrant cell viability or migration of these cells, because they migrated similarly as WT counterparts in response to CCL19 or CCL21 (data not shown), two ligands for the CCR7 receptor (Fig. 1F). These results indicate that CXCR3-mediated migration depends predominantly on Goα2, irrespective of the ligands.

Unexpectedly, the cell migration toward CXCL9 and CXCL11 was not attenuated but instead drastically increased in the absence compared with presence of Goα3 (Fig. 1, A and E). The migration toward CXCL10 was also increased, albeit to a lesser extent (Fig. 1, B and E). However, such increased chemotaxis could not be induced by CCL21 (data not shown) or CCL19 in the cells, regardless of whether or not the cells were activated (Fig. 1, G and H). We also observed no augmented chemotactic response in Goα3−/− cells stimulated with CXCL12, a ligand for another Goα-dependent receptor CXCR4. In fact, CXCL12-provoked chemotaxis was diminished in a lack of either Goα2 or Goα3 (Fig. 1F). The inflammatory chemokines MCP-1 and MIP-1α were unable to elicit a significant migratory response given our T cell activation conditions (data not shown). Together, these data stress that the increased migration toward CXCR3 agonists in the absence of Goα3 is receptor-specific.

**Goα2 and CXCR3 Expression in T Cells**—The opposing responses of Goα2 and Goα3-deficient T cells to CXCR3 ligands argue for Goα3 antagonizing the function of Goα2. To conclude this, we must rule out that the increased migration toward CXCR3 agonists in Goα3−/− cells resulted from increased expression of Goα2 that could potentially provide a compensatory mechanism to offset the lack of Goα3 (38). Fig. 2A clearly showed that Goα2 expression was not increased in activated Goα3−/− T cells compared with WT T cells (lanes 3 versus 1, lower panel), as was the case for Goα4 expression in activated Goα4−/− T cells (lanes 5 versus 1, lower panel). Neither Goα2 nor Goα3 expression was increased in freshly isolated KO T cells compared with WT T cells (Fig. 2A, top panel). We also detected comparable levels of the CXCR3 receptor on these three groups of T cells (Fig. 2B). The expression of other receptors, including CCR7 on stimulated and unstimulated T cells and CXCR4 on resting T cells, was also comparable in these cells, despite some variations (Fig. 2B). To exclude another possibility that the increased chemotactic response was ascribed to coupling of the CXCR3 receptor to other G protein family members in the absence of Goα3, the cells were treated with 100 ng/ml PTX overnight before they were assayed for chemotaxis. The pretreatment ablated CXCR3-mediated chemotactic
responses in both WT and Goi3-deficient T cells (Fig. 1, C and D), confirming involvement of PTX-sensitive Goi2 protein only in the increased migratory response in the absence of Goi3. Our investigation thus concludes that Goi3 participates as an inhibitor in CXCR3-stimulated signaling, and null mutation of Goi3 directly accounts for the increased migration of Goi3(−/−) T cells toward CXCR3 ligands.

Altered GTPyS Incorporation in the Absence of Goi2 or Goi3—
Our migration data show involvement of both Goi2 and Goi3 in CXCR3-mediated signaling, presumably with Goi2 activating downstream effectors to drive T cell migration. Yet Goi3-mediated inhibition of Goi2 activity in a receptor-specific fashion has never been explored. So in the subsequent studies, we set out to determine whether Goi3 directly blocked Goi2 activation or diverted signals downstream of Goi2. For downstream signaling disruption, we would expect Goi3 activation to be an indispensable receptor-stimulated event in the G protein-signaling cascade, which can be readily measured by a GTPyS incorporation assay. All three CXCR3 chemokines at the concentrations optimal for cell migration were able to stimulate significant GTPyS incorporation in WT T cell membranes as reported previously (Fig. 3A) (39). In contrast, T cells from Goi2(−/−) mice failed to incorporate GTPyS significantly above controls following stimulation with CXCL9 or CXCL11 under similar conditions (Fig. 3A). A small increase in GTPyS incorporation was seen in the cells stimulated with CXCL10, consistent with lesser Goi3 inhibition in response to this chemokine (Fig. 3A and Fig. 1B). The lack of GTPyS incorporation in Goi2(−/−) T cell membranes was not a failure of these cells to incorporate the GTP analog, because adenosine elicited comparable GTPyS incorporation in T cells isolated from all these mice (Fig. 3A) (40). Instead, the result argues strongly that Goi3 cannot be activated by CXCR3 agonists, despite its ability to inhibit Goi2 function. Moreover, GTPyS incorporation in these T cells proportionally correlated with their migratory responses stimulated by CXCR3 agonists, increasing G protein activation in Goi3(−/−) T cells and lacking such activation in Goi2(−/−) T cells as compared with WT T cells. The increased GTPyS incorporation in Goi3(−/−) cells was solely contributed by Goi2 activation, as demonstrated by complete abrogation of the response following PTX treatment (Fig. 3B). The findings not only indicate that Goi2 is the predominant G protein activated by the CXCR3 receptor, but also suggest that CXCR3 receptor activation cannot elicit exchange of GDP for GTP in Goi3 protein, arguing that Goi3 interferes with Goi2 signaling without being activated.

Inhibition of CXCR3-mediated Goi2 Activation by Goi3—
The consistent and significant increases in GTPyS incorporation in Goi3(−/−) T cell membranes and in the migration of these T cells argue strongly for inhibition of Goi2 activation by Goi3. Moreover, inhibition of Goi2 activation without a concomitant rise in Goi3 activation raises an intriguing possibility that Goi3 may interact with the receptor after ligand stimulation but remain inactive and continue its association with the receptor, by which it blocks Goi2 interaction with the receptor. To address this, we first used an Ab directed against the C terminus of the Goi2 protein to block the receptor-G protein interaction in the GTPyS incorporation assay. As shown in Fig. 3C, pre-treatment with Goi2-specific Ab but not control Ab completely abolished CXCL10- and CXCL11-stimulated GTPyS incorporation in Goi3(−/−) T cell membranes. A specific and complete blockade of G protein activation by anti-Goi2 Ab further rules
**Goα13 Antagonizing Goα12 Activation**

**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)

**D**

![Graph D](image)

**FIGURE 3.** CXCR3 agonist-stimulated G protein activation in the presence or absence of Goα12 or Goα13. A, CXCR3 agonist-stimulated GTPγS incorporation in activated T cells. Cell membranes equivalent to 20 μg of protein prepared from activated T cells with or without Goα12 or Goα13 were stimulated in triplicate with 100 ng/ml CXCL9, 10 ng/ml CXCL10, 100 ng/ml CXCL11, or 10 μM adenosine in the presence of 0.3 mM [35S]GTPγS as described under "Materials and Methods." Percentages ± S.D. of specific [35S]GTPγS incorporation relative to background controls are shown with unstimulated cell membranes designed as 100%. The result represents four separate experiments. * and ** are statistical significance (p < 0.05) or high significance (p < 0.01), respectively, in the presence versus absence of a specific Goα protein. B, inhibition of G protein activation by PTX treatment. Activated T cells prepared from activated T cells treated with 10 ng/ml PTX for 1 h before the cell membranes were prepared and assayed for GTPγS incorporation induced by either 10 ng/ml CXCL10 or 100 ng/ml CXCL11 at A. Data represent three separate experiments with each sample in triplicate as A. C, blockade of G protein activation in Goα13−/− T cell membrane by anti-Goα12 Ab. G protein activation in cell membrane prepared from Goα13−/− T cells was assayed in the presence of either 3 μg of anti-Goα12 monoclonal Ab or isotype-matched control Ab (Control Ab) during chemokine stimulation as in A. The data represent three independent experiments with each sample in triplicate as A. **statistical significance (p < 0.01) in the presence versus absence of a specific anti-Goα12 Ab. D, inhibition of Goα12 activation in Goα13−/− T cell membranes by exogenous Goα12 protein. Cell membranes prepared from Goα13−/− T cells were preincubated with indicated concentrations of in vitro synthesized Goα12 or Goα13 protein before stimulation of the membrane by 100 ng/ml CXCL11 and assaying G protein activation as in A. The data represent three separate experiments with each sample in triplicate. * and **, statistical significance (p < 0.05 or p < 0.01, respectively), in the presence of Goα12 versus Goα3 protein.
**Gα<sub>i3</sub> Antagonizing Gα<sub>i2</sub> Activation**

Furthermore, significant GTPγS incorporation induced by CXCL11 was seen in the cell membranes prepared from COS-7 cells transfected with WT CXCR3 receptor and all variants, except for the R149N variant that was totally ineffective (Fig. 4C). The inability of the R149N mutant to activate Gα<sub>i3</sub> and to interact with Gα<sub>i3</sub> suggests that Gα<sub>i3</sub> may compete for or sterically interfere with Gα<sub>i2</sub> binding to the CXCR3 receptor directly or indirectly via the DRY domain, by which it controls the amplitude of a migratory response elicited by the receptor.

**DISCUSSION**

Our results clearly show that a deletion of Gα<sub>i3</sub> augments Gα<sub>i2</sub>-mediated migratory responses toward CXCR3 ligands, whereas deletion of Gα<sub>i2</sub> ablates the response. The Gα<sub>i3</sub>-mediated inhibition of CXCR3 signaling is not reliant on the exchange of GDP for GTP by Gα<sub>i3</sub>, but it does require ligand-stimulated interaction between Gα<sub>i3</sub> and the CXCR3 receptor. Presumably, a conformational change of the receptor caused by ligand binding provides conditions that are optimal for Gα<sub>i3</sub> binding to the receptor. However, the receptor-bound Gα<sub>i3</sub> appears unable to undergo a conformational change required for dissociation of GDP and/or binding to GTP, thus preventing it from full activation and leaving the receptor. By occupancy of the receptor, Gα<sub>i3</sub> blocks Gα<sub>i2</sub> activation. It has been shown that the DRY motif at the N terminus of the second intracellular loop is key for proper G protein recognition and interaction (31). Our data suggest that this motif may be also essential for the blockade induced by Gα<sub>i3</sub>, which requires further investigation. Substitution of Arg<sup>149</sup> amino residue to Asn ablates not only activation of Gα<sub>i2</sub> but also binding of Gα<sub>i3</sub> to the receptor. Under similar conditions, receptors carrying mutations at the C terminus can activate Gα<sub>i3</sub> and preserve Gα<sub>i3</sub> interaction similarly to the WT CXCR3 receptor (Fig. 4B). Lack of Gα<sub>i3</sub> binding to and Gα<sub>i3</sub> activation by the R149N mutant cannot be ascribed to the levels of receptor expression on the cells. Although the R149N-CXCR3 mutant was expressed on the cells at levels slightly lower than the WT kine receptors (24, 41). Therefore, “incomplete activated” Gα<sub>i3</sub> may prevent Gα<sub>i2</sub>-mediated signaling via the DRY site.

In parallel experiments, immunoprecipitation of the CXCR3 receptor did not pull down Gα<sub>i2</sub> as expected, presumably because of its rapid dissociation from the CXCR3 receptor upon activation. The 35S-Gα<sub>i3</sub> could interact well with the S1P<sub>1</sub> receptor, another PTX-sensitive GPCR (Fig. 4B, right panel), ruling out that undetectable 35S-Gα<sub>i3</sub> in CXCR3 immunoprecipitates resulted from a lack of binding function of the protein.
CXCR3 receptor, its expression was comparable with the LLI-CXCR3 mutant (Fig. 4A) (24). The latter could activate G\(\alpha_{i2}\) and bind with G\(\alpha_{i3}\) as the WT CXCR3 receptor (Fig. 4, B and C). Murine G\(\alpha_{i2}\) and G\(\alpha_{i3}\) are 83% identical in amino residue sequence. This structural similarity may be the basis for their competition for the same binding domain of the CXCR3 receptor or for G\(\alpha_{i1}\) interference with G\(\alpha_{i2}\) interaction of the receptor. A subtle difference in the receptor conformation induced by a specific agonist is likely to determine whether the activated receptor can interact and activate a given G\(\alpha\) protein or interact with a G\(\alpha\) protein without inducing its full activation. Therefore, function of a given G\(\alpha\) protein as an activator or inhibitor is receptor-specific, and the interplay between these two G proteins may be essential in the control of the amplitude of a GPCR-induced signal. To the best of our knowledge, this is the first description of a G protein interacting with a receptor after ligand binding for the sole purpose of diminishing the other G protein interaction with the receptor. This finding gives novel insight into regulation of GPCR signaling by heterotrimeric G proteins.

GPCRs initiate a signaling cascade through activation of heterotrimeric G proteins following stimulation by an extracellular agonist (43). To meet the complexity and versatility of cell migration in the body, many chemokine receptors have evolved to recognize more than one chemokine, such as the CXCR3 and CCR7 receptors, or one chemokine receptor can be activated by multiple chemokines like CCL5 (regulated on activation normal T cell expressed and secreted), CCL3 (MIP-1\(\alpha\)), MCP-2, and MCP-3 (44). Our finding that a migratory response is tied proportionally to the amplitude of G protein activation indicates that stoichiometry of G protein activation is directly linked to the propensity of a cell to migrate. The precise activation level of the G\(\alpha_{i2}\) protein stimulated by ligation of the CXCR3 receptor is determined by the affinity of the ligand as well as the interplay of G\(\alpha_{i2}\) and G\(\alpha_{i3}\) with the receptor. In accordance with CXCR3 signaling dependent on G\(\alpha_{i3}\) and inhibition of the signaling by G\(\alpha_{i9}\), our in vivo study showed that similar to T cells from mice lacking the CXCR3 receptor, G\(\alpha_{i2}\)-deficient T cells failed to elicit an acute graft-versus-host defense reaction after being transfused into full major histocompatibility complex-mismatched Balb/c SB-17 severe combined immunodeficiency mice (30,45). In contrast, transfer of G\(\alpha_{i3}\)-deficient T cells into the severe combined immunodeficiency mice stimulated an aggravating graft-versus-host defense response compared with WT T cells. Thus, an interplay among different heterotrimeric G proteins is likely to have a role to play in vivo, introducing another level of complexity in the control of cell migration systemically. The complex regulation of GPCR signaling at multiple levels is crucial in temporal and spatial regulation of cell migration in the body.

Determination of receptors linked to G\(\alpha\) proteins has been greatly aided by the use of PTX. PTX has also allowed insight into downstream effectors that are either partially or fully involved in propagating a signal by G\(\alpha\) proteins. Although PTX has provided extensive and important details about the agonist and signal transduction mechanisms, the specifics about individual G\(\alpha_{i\alpha}\) family members has gone largely unresolved because PTX blocks receptor interaction by several G\(\alpha_{i\alpha}\) family members. With the advent of the genetic manipulation of mice, we can tease out the specifics of G\(\alpha_{i1}\), G\(\alpha_{i2}\), and G\(\alpha_{i3}\) in a migratory response stimulated by a specific receptor. Studies of B cell migration in G\(\alpha_{i2}\)-/- mice suggest an irreplaceable role of G\(\alpha_{i3}\) in chemotactic responses to chemokines CXCL12 and CXCL13 (14). On the other hand, G\(\alpha_{i2}\) and G\(\alpha_{i3}\) are redundant in thymic egress as indicated by no significant defects in thymic export in G\(\alpha_{i2}\)- and G\(\alpha_{i3}\)-deficient mice (12, 13). Although the proportions and the numbers of CD4- and CD8+ single positive thymocytes were increased in G\(\alpha_{i2}\)-/- mice as lck-PTX-transgenic mice, the increases were not caused by a defect in thymic egress, rather by an accelerated transition from the double positive to single positive thymocytes as shown by our previous investigations (12, 46). In support, nearly normal numbers and percentages of CD4+ and CD8+ T cells were seen in the spleen of G\(\alpha_{i2}\)-/- mice (11–13). Likewise, mature thymocytes in G\(\alpha_{i3}\)-/- mice populated lymphoid tissues normally in the periphery, and there was no aberrant accumulation of single positive thymocytes in the mice (13). The redundancy of G\(\alpha_{i2}\) and G\(\alpha_{i3}\) in thymic egress was also consistent with our recent study showing that G\(\alpha_{i2}\)-KO and G\(\alpha_{i3}\)-KO T cells migrated indistinguishably from WT T cells in response to an increasing concentration of sphingosine 1-phosphate (S1P), a lipid mediator controlling T cell egress (33).

How the redundancy works mechanistically is not fully understood at present. Association with the same subunit of G\(\beta\gamma\) may be one of the common mechanisms whereby G\(\alpha_{i2}\) and G\(\alpha_{i3}\) can compensate for the absence of one to another. Hwang et al. (42) have shown that functional silence of either G\(\alpha_{i2}\) or G\(\alpha_{i3}\) by specific short interfering RNA has little effect on the migration of macrophages toward an increasing concentration gradient of C5a or C3a. Deletion of G\(\beta 2\), however, ablated C5a- or C3a-provoked migration, suggesting that both G\(\alpha_{i2}\) and G\(\alpha_{i3}\) can transduce the migration signal as long as the G\(\beta 2\) subunit is presented (42). On the contrary, the CXCR4 receptor requires both G\(\alpha_{i2}\) and G\(\alpha_{i3}\) for a full response. Absence of either gene impaired T cell migration induced by CXCL12 (Fig. 1F), although decreased expression of the CXCR4 receptor on these cells may also be partially involved. We should point out that a slight increase or decrease (10–15%) in the G\(\alpha_{i3}\) expression levels in G\(\alpha_{i3}\)-/- T cells or G\(\alpha_{i2}\) in G\(\alpha_{i2}\)-/- T cells was observed in some mice (Fig. 2A). However, the levels of G\(\alpha\) protein expression were not correlated with migratory changes for any of the studied chemokines. In particular, the slight decrease in G\(\alpha_{i2}\) expression in G\(\alpha_{i3}\)-KO T cells was discordant with the increased activity for the CXCR3 receptor. Similarly, although the levels of G\(\alpha_{i2}\) and G\(\alpha_{i3}\) expression were slightly decreased in activated as compared with nonactivated T cells, the decrease affects little their chemotactic response to CCL19 stimulation (Fig. 1, G and H). This is probably attributed to the following two reasons. First, G\(\alpha_{i2}\) or G\(\alpha_{i3}\) is a lot more abundant than any individual chemokine receptor.

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4 Y. Jin, K. H. Wu, Z. Zhou, and M. X. Wu, manuscript in preparation.

5 B. D. Thompson, Y. Jin, W. Hu, L. Charles, L. Birnbaumer, and M. X. Wu, unpublished data.
in a cell. Second, constant recycling of $\alpha_i$ proteins from an active to inactive status further increases the size of a $\alpha_i$ protein pool that is already far in excess of any individual chemo-
line kinase receptor. Therefore, distinct function of $\alpha_{i2}$ and $\alpha_{i3}$ as described in this study is unlikely ascribed to a slightly altered level of $\alpha_{i2}$ or $\alpha_{i3}$ protein. Nevertheless, this does not exclude that the absolute levels of $\alpha_{i2}$ and $\alpha_{i3}$ can affect activation of a given GPCR, when the relative levels of $\alpha_{i2}$ and $\alpha_{i3}$ differ drastically. Our data thus argue that $\alpha_{i2}$ and $\alpha_{i3}$ proteins may play overlapping, distinct, antagonizing, and additive roles depending on a specific receptor. The $\alpha_{i2}$- and $\alpha_{i3}$-null mutation mice provide a unique system to explore fundamental signaling differences of a specific GPCR coupling to $\alpha_{i2}$, $\alpha_{i3}$, or both and potential interplay between these $\alpha_i$ proteins.

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REFERENCES

1. Wilkie, T. M., Gilbert, D. J., Olsen, A. S., Chen, X. N., Amatruda, T. T., Korenberg, J. R., Trask, B. J., de Jong, P., Reed, R. R., Simon, M. I., Jenkins, N., and Copeland, N. (1992) Nat. Genet. 1, 85–91
2. Fields, T. A., and Casey, P. J. (1997) Biochem. J. 321, 561–571
3. Kaslow, H. R., and Burns, D. L. (1992) Eur. J. Immunol. 22, 143–150
4. Kehrl, J. H. (1998) J. Immunol. 160, 1515–1520
5. Zhang, Y., Finegold, M. J., Jin, Y., and Wu, M. X. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 878–883
6. Alnadjim, Z., Haddad, W., and Kaptanoglu, L. (2004) J. Biol. Chem. 279, 6122–6128
7. Droz, P., Leung, B. Y., and Kehrl, J. H. (2005) J. Exp. Med. 201, 192, 1515–1520
8. Nakano, S., Jakubzick, C. N., Zaitsev, A., and Luster, A. D. (2002) J. Immunol. 168, 3205–3212
9. Kehrl, J. H. (1998) J. Immunol. 160, 1515–1520
10. Boyce, S. W., and Boyce, B. J. (1997) Immunol. Rev. 158, 192, 1515–1520
11. Wu, Z. Y., Jin, Y., Edwards, R. A., Zhang, Y., Finegold, M. J., and Wu, M. X. (2005) J. Immunol. 174, 6122–6128
12. Huang, T. T., Zong, Y., Dalwadi, H., Chung, C., Miceli, M. C., Spicher, K., Birnbaumer, L., Braun, J., and Aranda, R. (2003) Int. Immunol. 15, 1359–1367