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Five-transmembrane domains appear sufficient for a G protein-coupled receptor: Functional five-transmembrane domain chemokine receptors

(signal transduction/desensitization/internalization)

KUN LING*†, PING WANG*†, JIAN ZHAO*†, YA-LAN WU*, ZHI-JIE CHENG*, GUO-XIANG WU*, WEI HU*, LAN MA‡, AND GANG PEI*§¶

*Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, 200031, People's Republic of China; ‡National Laboratory of Medical Neurobiology, Shanghai Medical University, Shanghai, 200032, People's Republic of China; and §Chinese National Human Genome Center, Shanghai, 201203, People's Republic of China

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ABSTRACT **The putative seven-transmembrane (TM) domains have been the structural hallmark for the superfamily of heterotrimeric G protein-coupled receptors (GPCRs) that regulate a variety of cellular functions by mediating a large number of extracellular signals. Five-TM GPCR mutants of chemokine receptor CCR5 and CXCR4, the N-terminal segment of which connected directly to TM3 as a result of a deletion of TM1–2 and the first intracellular and extracellular loops, have been obtained in this study. Laser confocal microscopy and flow cytometry analysis revealed that these five-TM mutant GPCRs were expressed stably on the cell surface after transfection into human embryonic kidney 293 cells. The five-TM CCR5 and CXCR4 functioned as normal chemokine receptors in mediating chemokine-stimulated chemotaxis, Ca^{2+} influx, and activation of pertussis toxin-sensitive G proteins. Like the wild-type GPCRs, the five-TM mutant receptors also underwent agonist-dependent internalization and desensitization and were subjected to regulation by GPCR kinases and arrestins. Our study indicates that five-TM domains, at least in the case of CCR5 and CXCR4, appear to meet the minimum structural requirements for a functional GPCR and suggests possible existence of functional five-TM GPCRs in nature during evolution.**

The heterotrimeric GTP binding protein-coupled receptors (GPCRs) constitute a large and diverse superfamily of signaling molecules. Of about 19,000 ORFs in the genome of *Caenorhabditis elegans*, more than 1,000 are coded for known or unknown GPCRs (1). GPCRs play fundamental roles in regulation of a variety of cell functions by mediating a huge number of extracellular signals such as hormones, neurotransmitters, chemokines, and sensory stimuli (2, 3). Chemokine receptors belong to the GPCRs superfamily and are classified as CC chemokine receptors (CCRs) or CXC chemokine receptors (CXCRs) based on chemokine structures (4, 5). Chemokine receptors mediate many vital functions of chemokines, and it was demonstrated recently that CCR5, CCR2B, CCR3, and CXCR4 are the essential coreceptors on the cell surface for HIV-1 fusion and infection (6, 7).

The GPCR superfamily is evolutionarily conserved and structurally characterized by its possessing putative seven-transmembrane (TM) domains with an extracellular N terminus and a cytoplasmic C terminus (8). The universal adoption of the conserved seven-TM structure by GPCRs, which consequently confers three intracellular and three extracellular loops along with a TM core, generally is speculated as the minimum necessity to achieve their structural stability and functional diversity (3). None of nearly 2,000 GPCRs identified in prokaryotes and eukaryotes to date is known to contain fewer than seven TM domains. Evidence accumulated from studies with "split" GPCRs also indicates that coexpression of receptor fragments with complementary TMs is necessary to reconstitute functional GPCRs in the cases of receptors such as bacteriorhdopsin, rhdopsin, β-adrenergic, M2 and M3 muscarinic receptors, and V2 vasopressin (9). Genetic mutations of chemokine receptors were reported recently. Natural mutants of chemokine receptor CCR5 lacking the last three or five TMs as a result of a deletion or single point mutation in the ORF are nonfunctional (10, 11). The current study, however, clearly demonstrated that chemokine receptors with only five TMs appear to act as functional GPCRs in the aspects of receptor expression, signaling, internalization, and desensitization.

MATERIALS AND METHODS

Materials. Recombinant human RANTES (regulated on activation normal T-cell expressed and secreted), forskolin, 1-methyl-3-isobutylxan-thine, GDP, GTPγS, BSA, pertussis toxin (PTX), and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma. Stromal cell-derived factor 1α (SDF-1α) was from PharMingen. Mouse mAb 12CA5 against the influenza hemagglutinin (HA) epitope was obtained from Boehringer Mannheim. [^{35}S]GTPγS, [^{3}H]cAMP, and $^{45}CaCl_2$ were from Amersham Pharmacia.

Cloning and Plasmid Construction. The full-length cDNA encoding the wild-type CXCR4 was cloned by reverse transcription–PCR (RT-PCR) from THP-1 cells using primers designed based on the published sequences (GenBank accession no. X71635), and the wild-type human CCR5 was cloned as described (12). A minor PCR product of approximately 800 bp, in addition to the expected product of 1,020 bp, was generated in PCRs to amplify CCR5 by using human leukocyte cDNAs as a template. The origin of the minor PCR product was not known; it was probably a result of the suboptimal conditions of the PCR. However, after being cloned and sequenced, the shorter PCR product was found to encode a CCR5 mutant lacking 72 aa residues (Leu-37–Gly-107). Subsequently, a similar mutation of CXCR4 with a deletion of 72

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Abbreviations: TM, transmembrane; GPCR, G protein-coupled receptor; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; HA, hemagglutinin; RANTES, regulated on activation normal T cell expressed and secreted; SDF-1α, stromal cell-derived factor 1α; GRK, G protein-coupled receptor kinase; HEK, human embryonal kidney; PTX, pertussis toxin; PMA, phorbol-12-myristate-13-acetate.

†K.L., P.W., and J.Z. contributed equally to this work.

¶To whom correspondence should be addressed at: Shanghai Institute of Cell Biology, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai, 200031, People's Republic of China. e-mail: gangpei@sunm.shcnc.ac.cn.

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aa (Ile-43–Val-114) corresponding to those in CCR5 was purposely designed and the mutant receptor cDNA was prepared by PCR using the wild-type construct as a template. The amplified human chemokine receptor cDNA fragments were cloned into a modified pcDNA3 vector (Invitrogen) with the sequence of HA epitope tag at the 5′ end. Goα1 and β-arrestin 2 were amplified by RT-PCR using human brain mRNA as a template and cloned into pcDNA3. The authenticity of the DNA sequences was confirmed by sequencing. Expression plasmids for human β-arrestin 1 (13), human Giα2 (14), and bovine G protein-coupled receptor kinase (GRK) 2 and GRK5 (15) were as described.

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were cultured in MEM (GIBCO/BRL) supplemented with 10% heat-inactivated FBS. HEK293 cells (1×10^6) plated in a 60-mm tissue culture dish were transfected with 5 μg DNA by using the calcium phosphate-DNA coprecipitation method and used 48 hr after transfection unless indicated otherwise. For chemotaxis assay, the transfected HEK293 cells were placed in medium containing 1 mg/ml Geneticin (GIBCO/BRL) 48 hr after transfection, and the transfectants were maintained in the same medium to select Geneticin-resistant cells stably expressing chemokine receptors. The expression of transfected receptors on the Geneticin-resistant cells was evaluated by fluorescence-activated cell sorting using 12CA5.

Chemotaxis Assay. The assays were performed in blind-well chambers (Neuroprobe, Gaithersburg, MD) as described (16). The agonist was added to the lower wells, and the polycarbonate membrane (pore size 8 μm, Poretics) in the upper chamber was coated with collagen. The HEK293 cells were resuspended at a density of 5×10^6 per ml of MEM containing 0.1% BSA, and 200 μl of cell suspension was added to the upper chamber. After incubation at 37°C for 6 hr, the cell suspension in the upper chamber was removed, and the membrane was fixed and stained. For each agonist concentration tested, cells migrated through to the underside of the membrane were counted in eight high-power fields, in a blinded fashion. The migration index for each experiment was calculated as the mean number of cells that migrated toward medium containing agonist divided by mean number of cells that migrated toward medium containing BSA only.

Ca^{2+} Influx Determination. As described (17), the cells were challenged with agonists at 37°C for 10 min in 20 mM Hepes (pH 7.4), 130 mM NaCl, 5 mM KCl, 6 mM glucose, 200 μM $CaCl_2$, 100 μM $LaCl_3$ containing 0.1 μCi $^{45}Ca^{2+}$, washed three times with 7% sorbitol solution containing 1 mM $LaCl_3$ on ice, and lysed in 5% SDS. The amount of $^{45}Ca^{2+}$ contained in the homogenized cell lysate was determined in a liquid scintillation spectrophotometer, and the protein content was determined according to Lowry's method. The Ca^{2+} influx across the plasma membrane in each sample was calculated as: $([Ca^{2+}]_{total}/protein_{sample}) \times (cpm_{sample}/cpm_{total})$.

[^{35}S]GTPγS Binding Assay. The assay was carried out as described (12). Cells were lysed in 5 mM Tris·HCl, pH 7.5/5 mM EDTA/5 mM EGTA at 4°C. The membrane pellet resulted from a 30,000 × *g* centrifugation was resuspended, and aliquots containing 10 μg protein were incubated at 30°C for 1 hr in 50 mM Tris·HCl, pH 7.5/1 mM EDTA/5 mM $MgCl_2$/100 mM NaCl/40 μM GDP/0.5 nM [^{35}S]GTPγS (1,200 Ci/mmol, Amersham Pharmacia) in the presence or absence of the agonists in a total volume of 100 μl. The reaction was terminated by adding cold PBS and filtering through GF/C filters, which were counted in a liquid scintillation spectrophotometer. Data were means of duplicate samples. Basal binding was determined in the absence of agonists, and nonspecific binding was obtained in the presence of 10 μM GTPγS (Sigma). The percentage of stimulated [^{35}S]GTPγS binding was calculated as $100 \times (cpm_{sample} - cpm_{nonspecific})/(cpm_{basal} - cpm_{nonspecific})$.

cAMP Assay. Cells were challenged with agonist in the presence of 10 μM forskolin and 500 μM 1-methyl-3-isobutylxanthine at 37°C for 10 min. The reaction was terminated with 1 N perchloric acid, which then was neutralized with 2 M K_2CO_3. The cAMP level of each sample was determined by using RIA as described (18). Data were averages of duplicate samples and presented as a percentage of control, being calculated as $100 \times [cAMP_{(forskolin + agonist)} - cAMP_{(basal)}]/[cAMP_{(forskolin)} - cAMP_{(basal)}]$. $cAMP_{(forskolin + agonist)}$ is cAMP level in the presence of forskolin and agonist, $cAMP_{(basal)}$ is cAMP level in the absence of forskolin and agonist, and $cAMP_{(forskolin)}$ is cAMP level in the presence of forskolin alone.

Flow Cytometry. Cells were incubated with 12CA5 (5 μg/ml) in PBS containing 2% BSA at 4°C for 1 hr. The presence of HA-tagged chemokine receptors on the cell surface was detected by incubation with FITC-conjugated goat anti-mouse IgG (Tago). The cells were analyzed on a FACSCalibur flow cytometer. Basal cell fluorescence intensity was determined with cells stained with the secondary antibody alone.

Immunofluorescence Microscopy. Immunofluorescence microscopy was done as described (19). After stimulation with the agonists at 37°C for 30 min, the cells grown on coverslips were fixed in 1% polyformaldehyde for 20 min and incubated with methanol at −20°C for 20 min. Then the cells were treated with 12CA5, and the presence of HA-tagged chemokine receptors was detected with FITC-conjugated goat anti-mouse IgG. The control experiments were done with mock-transfection cells. Images were recorded by using a Leica TCS NT laser confocal scanning microscope.

Immunoprecipitation and Western Blotting. The experiment was performed as described (20). Briefly, cells were lysed in 20 mM Tris·HCl, pH 7.4/150 mM NaCl/0.4% digitonin on ice for 45 min. The lysate was centrifuged at 12,000 × *g* for 30 min, and the supernatant was incubated with 0.5 μg 12CA5 and Protein A-Sepharose (Amersham Pharmacia) on ice for 4 hr. After washing, the immunocomplexes absorbed onto Protein A-Sepharose were eluted in 50 mM Tris·HCl (pH 7.4), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue and subjected to Western blot analysis using 12CA5 as described (13).

RESULTS

Molecular Cloning and Cell Surface Expression of the Five-TM Receptors. A chemokine receptor CCR5 mutant (mCCR5) bearing a 216-bp deletion in the ORF and encoded for a receptor lacking 72 aa residues was unexpectedly obtained in our effort to clone chemokine receptor CCR5 by PCR. Interestingly, analysis of the deduced amino acid sequence revealed that the mutant receptor lacks the region (Leu-36–Gly-107) coding for the first and second putative TM and the first intracellular and extracellular loops of GPCR (Fig. 1*A*). Similar mutation of another chemokine receptor CXCR4 with deletion of 72 aa residues from Ile-43 to Val-114 (mCXCR4) then was purposely designed and constructed (Fig. 1*A*). As shown in Fig. 1*B*, both mutant chemokine receptors possess only the last five TMs, according to the two-dimensional models of CCR5 and CXCR4 (21), with the intact N terminus directly connected to the third TM.

Certain mutations in GPCR impair stability of the receptor on the membrane; therefore, these mutant receptors are unable to translocate to cell surface. It has been reported that CCR5-Δ32 mutant encoding for a truncated receptor and even CCR5 with certain point mutations express poorly on the cell surface (10, 22). The expression of the five-TM chemokine receptor mutants therefore were examined in HEK293 cells. First, immuoprecipitation experiments confirmed expression of the wild-type and the truncated chemokine receptors in HEK293 cells (Fig. 2*A*). Surface expression of the wild-type

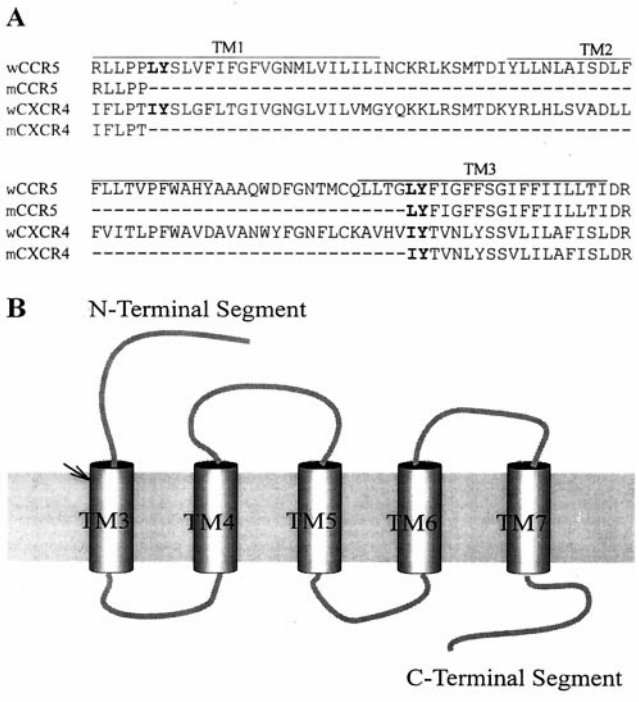

FIG. 1. (*A*) Amino acid sequence alignment of the putative TM1–3 region of the wild-type and the five-TM chemokine receptors CCR5 (wCCR5 and mCCR5) and CXCR4 (wCXCR4 and mCXCR4). The deleted residues in the mutants are presented by dashes. (*B*) Schematic two-dimensional model of the five-TM mutant chemokine receptors. The arrow indicates the connecting point of the N terminus to TM3.

and the mutant chemokine receptors CCR5 and CXCR4 tagged with HA at their N termini (23, 24) was detected by laser confocal microscopy and flow cytometry after immunofluorescence staining with 12CA5. The results show that both five-TM receptor mCCR5 and mCXCR4 were expressed stably on the plasma membranes at a level comparable to their wild-type seven-TM counterparts (Fig. 2 *B–F*).

Agonist-Stimulated Receptor Internalization. Once stimulated with receptor agonist, most GPCRs undergo rapid endocytosis, termed as internalization (25). As detected with confocal immunofluoresence microscopy and fluorescence-activated cell sorting, the wild-type and the mutant chemokine receptors CCR5 or CXCR4 expressed on HEK293 cell surface quickly internalized in the response to the stimulation by RANTES (agonist of CCR5) or SDF-1α (agonist of CXCR4) (Fig. 2 *B–F*). Chemokine treatment of 30 min caused approximately 20% reduction in cell surface fluorescence in cells expressing wCCR5 or mCCR5 and more than 40% reduction in cells expressing wCXCR4 or mCXCR4 (Fig. 2*F*). The above results indicate that the five-TM mutant chemokine receptors also undergo agonist-dependent internalization as their wild-type counterparts do and that TM1 and TM2 as well as the first intracellular and extracellular loops are not required for internalization of CCR5 and CXCR4.

Receptor-Mediated Cellular Responses to Chemokines. GPCR-mediated cellular responses critically depend on the functional interaction of receptor and its agonist and the subsequent G protein activation induced. The potencies and efficacies of the receptor agonist RANTES and SDF-1α to stimulate the mutant chemokine receptors were not significantly different from those for wCCR5 and wCXCR4 as measured with GTPγS binding and cAMP assays (Fig. 3). Similar results also were obtained by using MIP-1β, another agonist of CCR5 (data not shown). Other receptor-mediated cellular responses to chemokines also were examined. As shown in Fig. 3, the cells expressing mCCR5 and mCXCR4

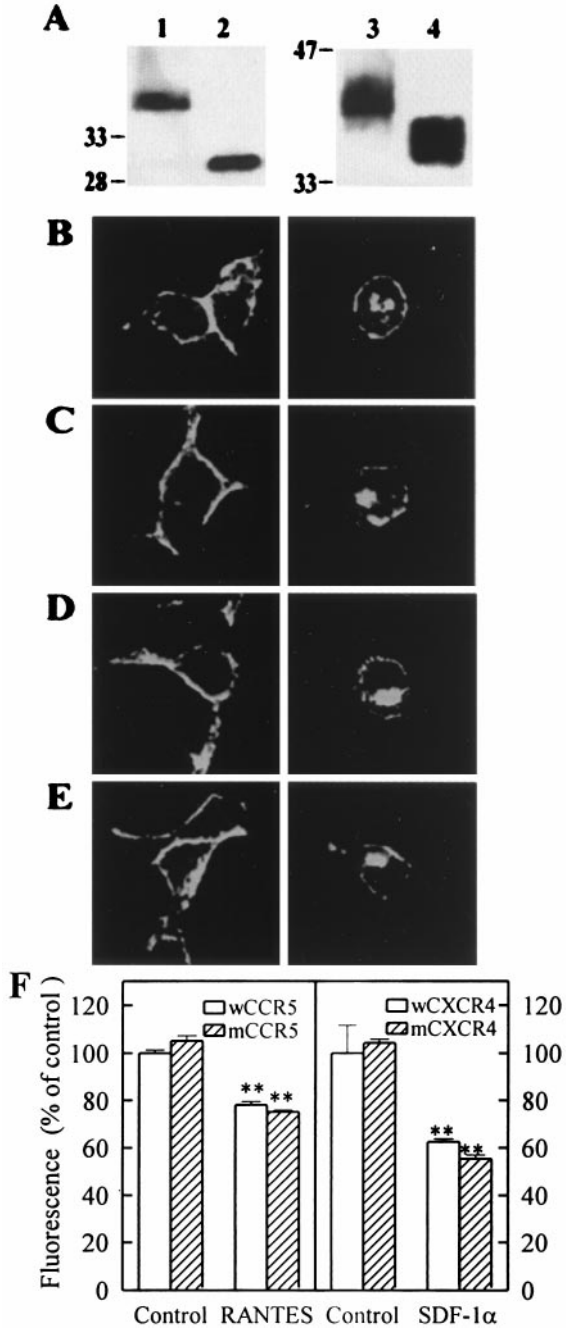

FIG. 2. Expression and internalization of the five-TM chemokine receptors. (*A*) The expression of wCCR5 (lane 1), mCCR5 (lane 2), wCXCR4 (lane 3), and mCXCR4 (lane 4) in transiently transfected HEK293 cells was detected by immunoprecipitation and Western blotting with 12CA5. (*B–E*) Cells transiently transfected with wCCR5 (*B*), mCCR5 (*C*), wCXCR4 (*D*), and mCXCR4 (*E*) were incubated without (*Left*) or with (*Right*) 10 nM agonist (RANTES for CCR5 and SDF-1 for CXCR4) for 30 min, and internalization of receptors from the cell surface was analyzed by laser confocal fluorescence microscopy using 12CA5 and FITC-conjugated anti-mouse IgG. (*F*) Similarly, internalization of the wild-type and five-TM mutant CCR5 or CXCR also was determined by using flow cytometry after incubation with or without (control) 10 nM chemokine. Untransfected cells or mock-transfected cells showed negative staining under the same conditions (not shown). Pictures shown in *A–E* are representative of two separate experiments. The data in *F* indicate averages and error ranges of two independent determinations in duplicate. **, $P < 0.01$ compared with unpretreated controls.

showed increased migration (*C*) and extracellular calcium influx (*D*) in response to chemokine stimulation at levels comparable to the wild-type controls. These data indicate that

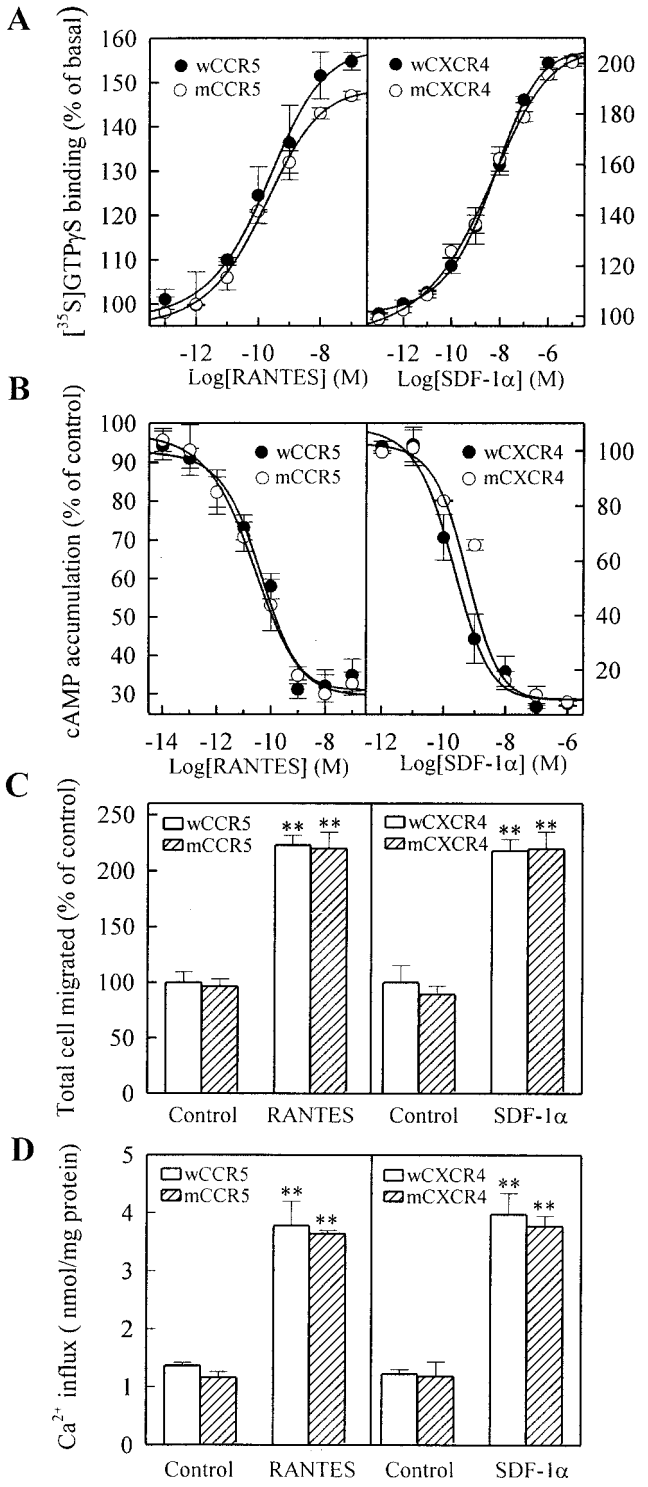

FIG. 3. The five-TM chemokine receptor-mediated cellular responses. HEK293 cells transiently (*A*, *B*, and *D*) or stably (*C*) expressing the wild-type or mutant chemokine receptors were treated with indicated amounts (*A* and *B*), 1 nM (*C*), or 10 nM (*D*) of chemokines. The chemokine-stimulated G protein activation (*A*), adenylyl cyclase inhibition (*B*), cell migration (*C*), and Ca^{2+} influx (*D*) were measured. The EC_{50} values of the chemokine-induced GTPγS binding were estimated: wCCR5, 0.15 nM; mCCR5, 0.18 nM; wCXCR4, 2.6 nM; and mCXCR4, 4.6 nM. The basal GTPγS binding values were in the range of 1.11 ± 0.04 nmol/mg protein. The EC_{50} values for cyclase inactivation were 30 pM for wCCR5, 22 pM for mCCR5, 200 pM for wCXCR4, and 550 pM for mCXCR4. The untreated forskolin-stimulated cAMP levels were in the range of 129 ± 11 pmol/mg protein. The migration number of the untreated cells was 12.6 ± 0.3. All data are means ± SE of three independent experiments performed in duplicate.

functional interaction of chemokine with CCR5 and CXCR4 is not significantly altered by the deletion of TM1–2 and that the five-TM chemokine receptors are able to transduce various signals across membrane.

Coupling of Receptor to PTX-Sensitive Inhibitory G Proteins. Our previous research has demonstrated that chemokine receptors couple to PTX-sensitive G proteins (12). Stimulation of chemokine receptors activate inhibitory G proteins (12), which induce chemotaxis (16). As shown in Fig. 4, pretreatment of PTX abolished the agonist-stimulated G protein activation in the HEK293 cells expressing either wild-type or mutant receptors, showing coupling of both types of receptors to PTX-sensitive G proteins. Further experiments using the

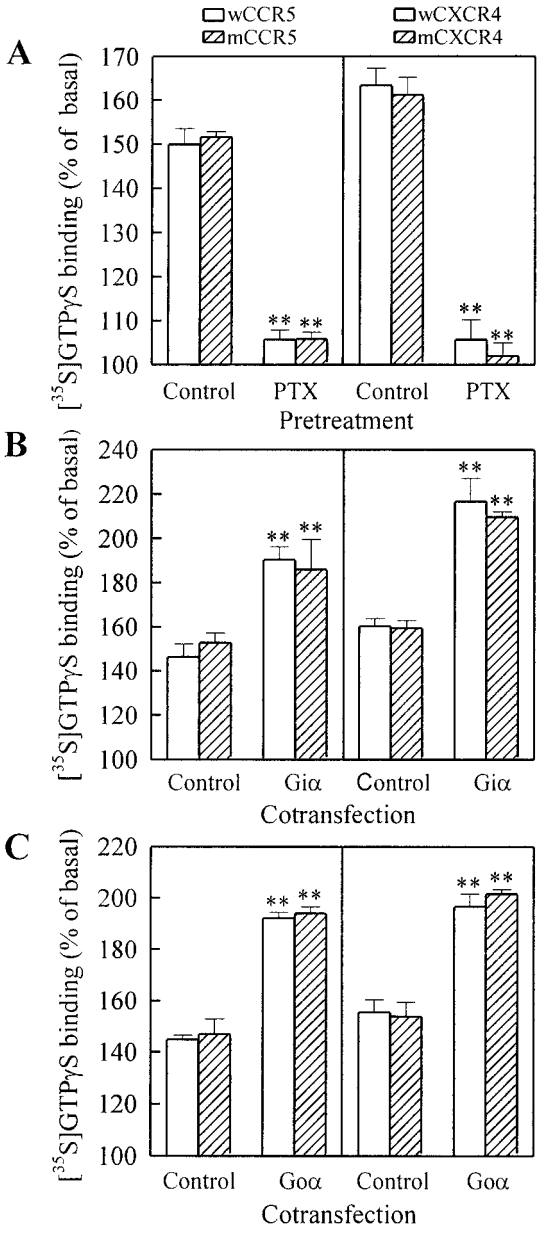

FIG. 4. Functional coupling of the five-TM chemokine receptors to PTX-sensitive G proteins. HEK293 cells were transiently transfected the wild-type or mutant chemokine receptors and pretreated with or without (control) 100 ng/ml PTX for 24 hr (*A*). The cells were transfected with the indicated chemokine receptor alone (control) or cotransfected with Giα2 (*B*) or Goα1 (*C*). The basal GTPγS binding was 1.08 ± 0.05 nmol/mg protein. Data presented are averages and error ranges of two separate experiments performed in duplicate. **, $P < 0.01$ compared with controls.

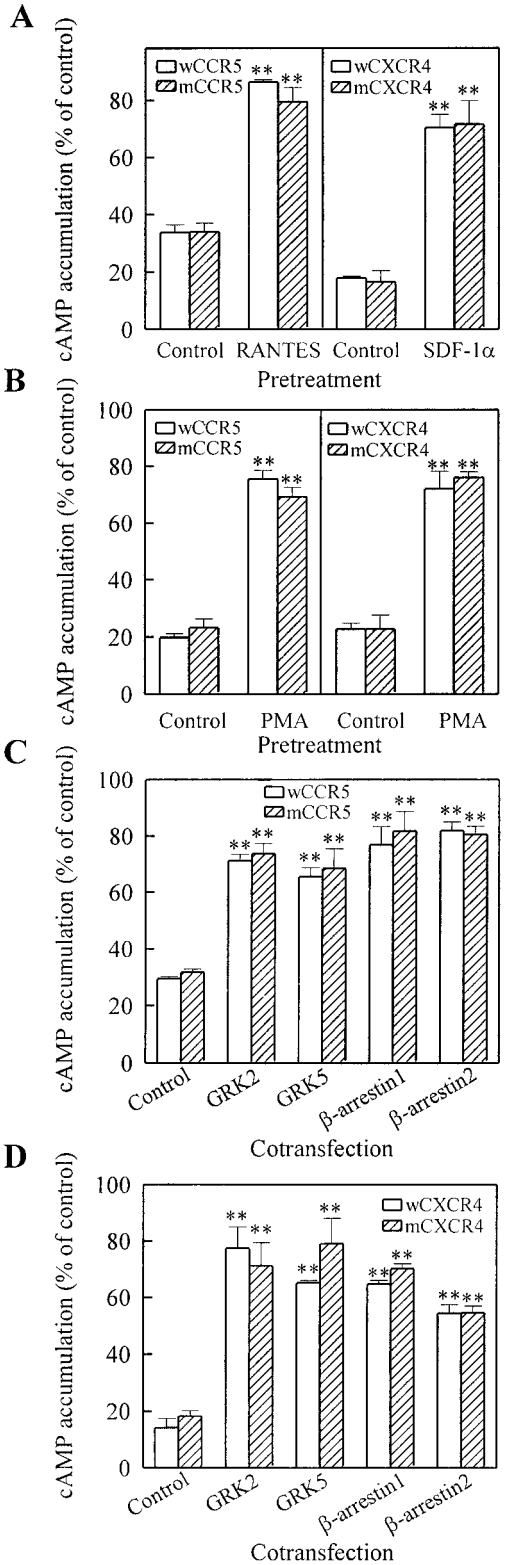

FIG. 5. Desensitization and functional interaction of the five-TM chemokine receptor with receptor kinases and arrestins. HEK293 cells transiently expressing the wild-type or mutant chemokine receptors were pretreated with PBS (as control), 10 nM indicated chemokine (*A*), or 1 μM PMA (*B*) for 15 min at 37°C. After rinsing with PBS, the cells were challenged with 10 nM chemokine (RANTES for CCR5 and SDF-1α for CXCR4) and forskolin-stimulated cAMP formation was determined. The cells were transiently transfected with the wild-type or mutant CCR5 (*C*) or CXCR4 receptors (*D*) alone (control) or cotransfected with GRK2, GRK5, β-arrestin 1, or β-arrestin 2 as indicated. The inhibition of the cAMP formulation induced by 10 nM chemokines (RANTES for CCR5 and SDF-1α for CXCR4) was determined. (*A*) The untreated forskolin-stimulated cAMP levels were in the range of 123 ± 12 pmol/mg protein. Data presented are averages and error ranges of two separate experiments performed in duplicate. **, $P < 0.01$ compared with controls.

cells cotransfected with receptors and α subunits of either Gi or Go demonstrate that the mutant chemokine receptors couple to these two PTX-sensitive G proteins just as well as their wild-type counterparts (Fig. 4 *B* and *C*). Our data suggest that receptor-G protein coupling is not affected by the truncation and imply that the first intracellular loop of CCR5 and CXCR4 is not involved in the coupling of receptor to these G proteins.

Receptor Desensitization and Interaction with Receptor Kinases and Arrestins. Agonist-dependent desensitization of GPCR has been under extensive investigation. Some of the underlying mechanisms have been clearly depicted, and GRKs and arrestins have been shown to play important roles (25). Previous research by our laboratory (12) and Aramori *et al.* (23) showed that chemokine receptors desensitize after agonist pretreatment, and this process involves GRKs and β-arrestins. Chemokine-induced desensitization of the wild-type and the mutant receptors was tested in this study. As shown in Fig. 5*A*, the responsiveness of both the wild-type and the five-TM mutant receptors reduced greatly after preincubation with the agonist of the corresponding receptor, suggesting the deletion of 72 aa residues apparently does not affect the feedback regulation of CCR5 and CXCR4. Chemokine or PMA treatment causes phorsphorylation and desensitization of chemokine receptors (24). As shown in Fig. 5*B*, treatment with protein kinase C (PKC) activator PMA mimicked the agonist pretreatment whereas it had no significant effect on basal adenylyl cyclase activity (Fig. 5*B*), indicating recognition and regulation of the wild-type seven-TM and the mutant five-TM chemokine receptors by PKC in these cells (24). Furthermore, coexpression of the chemokine receptors with either GRKs (2 or 5) or β-arrestins (1 or 2) significantly attenuated the receptor-mediated inhibition of cAMP, and no significant difference between the wild-type and mutant chemokine receptors was observed (Fig. 5 *C* and *D*). These results thus indicate that the interaction of the receptor with GRKs or arrestins is not altered by the truncation, and the deleted TM1–2 region is not critical for regulation of the receptor responsiveness by GRKs and arrestins.

DISCUSSION

The structure and function relationship of GPCRs has attracted a great deal of attention because of the importance of GPCRs in regulation of numerous cellular functions. The putative seven-TM structure is considered as the molecular fingerprint shared by all known GPCRs and therefore has been widely used to identify novel GPCRs even with unknown functions. No GPCR with fewer than seven TMs has been reported to date, which has led to a reasonable speculation that five TMs may be insufficient whereas nine TMs would be more than enough to form a stable, yet flexible, TM core (3). The current study, however, clearly demonstrates that five-TM chemokine receptors CCR5 and CXCR4 function in many aspects tested indistinguishably from their seven-TM counterparts and indicate that the five-TM structure is feasible within the chemokine receptor family. Thus, our study suggests that the five-TM core structure appears sufficient for a functional GPCR with enough stability at least in the case of chemokine receptors.

Several distinct modes of GPCR-ligand binding have been observed, which depend on whether ligand binds to the TM core, to both core and extracellular loops, to extracellular

loops and N terminus, or exclusively to N terminus of GPCRs (3). It has been shown that functional interaction of chemokine receptors with their ligands or HIV involves both the N-terminal segment and second extracellular loop of the receptor (26). The current study further demonstrates that the first and second TMs and first extracellular loop of chemokine receptors are not essential for chemokine binding and subsequent receptor activation. This model apparently is limited to certain GPCR subtype(s) because TMs 1–4 are required for the ligand binding in the case of adenosine A_1 receptor (27), and the first extracellular loop and TM2 consist of parts of the ligand binding sites in formyl peptide receptors (3).

Agonist-induced conformational changes in GPCRs lead to activation of G proteins selectively coupled to these receptors and thus transduce the extracellular signals into specific cellular responses. Numerous studies have indicated that in most cases the second and third intracellular loops of GPCRs are the major sites for receptor to interact with G proteins, and the amino acids residues in these regions are the primary determinants of the selectivity of G protein coupling (2). The current study provides additional evidence that the first intracellular loop is not critically involved in the coupling of chemokine receptors to PTX-sensitive G proteins. Furthermore, the selectivity of coupling of the chemokine receptors to either Gi or Go proteins seems unaffected by the deletion of TM1–2, at least under the conditions of overexpression of G proteins. It is also of great interest to notice that the feedback regulation of the five-TM mutant chemokine receptors, namely agonist-dependent internalization and desensitization of receptors, seems indistinguishable from their seven-TM counterparts and that their functional interactions with other signal molecules such as GPCR kinases, arrestins, or protein kinase C apparently were not altered. Taken together, data from this study suggest that the five-TM domain structure appears to be sufficient for a functional GPCR, at least in the cases of CCR5 and CXCR4.

On the extracellular surface, the seven-TM domains in GPCRs are proposed to be arranged counterclockwise as a cluster, in which TM1 and TM2 are somewhat apart from the rest of the five TMs (28). This arrangement may render the structural basis for the separation of TM1–2 from TM3–7 as an independent folding unit in the "split" GPCRs (9) or as a functional dispensable part in the current study of chemokine receptors. However, the question still remains unanswered: why has the seven-TM core been universally adopted by all GPCRs identified to date, and in other words, why were five-TM GPCRs unable to evolve naturally even if the seven-TM receptors emerged first in evolution? The current study suggests the possible existence of functional GPCRs with five-TM domains in nature that have not been discovered yet because of the limits of our knowledge and methodology. Otherwise, there has to be some unknown restraint to prevent natural mutations of five-TM GPCRs from occurring and propagating. The lack of a sufficient size and versatility for the five-TM GPCRs to offer a great number of specificities in response to so many diverse signals (3) could be one such reason.

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