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Constitutive Agonist-independent CCR5 Oligomerization and Antibody-mediated Clustering Occurring at Physiological Levels of Receptors*[S]

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Hassan Issafras‡, Stéphane Angers§, Sébastien Bulenger, Cédric Blanpain¶, Marc Parmentier¶, Catherine Labbé-Jullié, Michel Bouvier§‖, and Stefano Marullo**

From the Department of Cell Biology, Cochin Institute, INSERM U-567, CNRS UMR-8104 and University René-Descartes, Paris 75014, France, the §Department of Biochemistry, University of Montreal, Montreal, Quebec H3C 3J7, Canada, and the ¶Institut de Recherche Interdisciplinaire en Biologie Humaine et Nucléaire, Université Libre de Bruxelles, B-1070 Bruxelles, Belgium

Although homo-oligomerization has been reported for several G protein-coupled receptors, this phenomenon was not studied at low concentrations of receptors. Furthermore, it is not clear whether homo-oligomerization corresponds to an intrinsic property of nascent receptors or if it is a consequence of receptor activation. Here CCR5 receptor oligomerization was studied by bioluminescence resonance energy transfer (BRET) in cells expressing physiological levels of receptors. A strong energy transfer could be observed, in the absence of ligands, in whole cells and in both endoplasmic reticulum and plasma membrane subfractions, supporting the hypothesis of a constitutive oligomerization that occurs early after biosynthesis. No change in BRET was observed upon agonist binding, indicating that the extent of oligomerization is unrelated to the activation state of the receptor. In contrast, a robust increase of BRET, induced by a monoclonal antibody known to promote receptor clustering, suggests that microaggregation of preformed receptor homo-oligomers can occur. Taken together, our data indicate that constitutive receptor homo-oligomerization has a biologically relevant significance and might be involved in the process of receptor biosynthesis.

G protein-coupled receptors (GPCRs)[1] constitute the largest family of membrane receptors. They are involved in the regulation of most biological functions and represent, collectively, one of the most important targets for therapeutic intervention. A rapidly growing number of studies indicate that GPCRs may be organized as oligomers (1). The biological function of this phenomenon was questioned for some time until it was reported that the hetero-oligomerization of two isoforms of the $GABA_B$ receptor was indispensable for the formation of functional $GABA_B$ binding sites in tissues (2–4). Hetero-oligomerization of other GPCRs has also been proposed as a mean to increase pharmacological diversity and expand signaling modes for this class of receptors (5, 6). However, very little is known about the role that receptor homo-oligomerization could play. The fact that homo-oligomerization has been studied mainly with receptors overexpressed in heterologous systems has even raised concerns about the biological relevance of this phenomenon. Whether oligomeric complexes form at the plasma membrane or in other subcellular compartments and whether or not oligomers result from a dynamic, regulated, and reversible assembly of monomers following receptor activation are two other important and still open questions. The latter question, in particular, was the object of several studies using different experimental approaches that gave different interpretations. For example, it was concluded that agonists could increase homo-oligomerization of β_2-adrenergic (7) and TRH (8) receptors, decrease the homo-dimerization of the δ-opioid receptor (9), or have no effect on the oligomeric state of M3-muscarinic receptors (10).

The issue of receptor oligomerization and its potential regulation by agonists is of particular interest for the chemokine receptor CCR5, one of the two major co-receptors for the human immunodeficiency virus *in vivo*, since it has been suggested that CCR5 dimerization might prevent human immunodeficiency virus infection (11). In one study, CCR5 homo-oligomerization was detected after chemical cross-linking and immunoprecipitation of solubilized receptors but only if cells were previously treated with an agonist or a divalent antibody. The authors concluded that the CCR5 receptor exists exclusively as monomeric entities under basal conditions and that it oligomerizes only upon receptor activation or antibody-mediated reticulation (11). These conclusions are, however, difficult to reconcile with a previous study in which constitutive CCR5 dimers were resolved under low stringent conditions of protein denaturation (12). The fact that two similar studies, both based on immunoprecipitation experiments from cells expressing uncontrolled levels of receptors, reached such divergent conclusions prompted us to develop another experimental approach to study CCR5 homo-oligomerization in living cells at physiolog-

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‡ Recipient of a fellowship from the Association pour la Recherche sur la Polyarthrite Rhumatoïde.

‖ Holder of a Canada Research Chair in Molecular and Cellular Pharmacology.

** To whom correspondence should be addressed: Dépt. de Biologie Cellulaire, Institut Cochin, Bât. G Roussy, 27, rue du Fg. St. Jacques 75014 Paris, France. Tel.: 331-44-41-25-58; Fax: 331-44-41-25-57; E-mail: marullo@cochin.inserm.fr.

[1] The abbreviations used are: GPCR, G protein-coupled receptor; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; GABA, γ-amino butyric acid; hRluc, "humanized" *Renilla* luciferase; MIP-1β, macrophage inflammatory polypeptide 1β; RANTES, regulated on activation normal T cell expressed and secreted; YFP, yellow mutant of the enhanced green fluorescent protein; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting.

ical concentrations of receptor. In the present study, CCR5 receptor oligomerization was investigated in intact HEK-293 cells using bioluminescence resonance energy transfer (BRET). Unambiguous constitutive homo-oligomerization, evidenced by a robust energy transfer in the absence of ligands, could be demonstrated in HEK-293 cells expressing the same density of CCR5 receptors as found in human monocytes and lymphocytes. Furthermore, consistent with the notion of constitutive oligomerization, BRET was detected within the endoplasmic reticulum, indicating that oligomerization occurs early after receptor biosynthesis. Antibodies but not agonists could increase the basal energy transfer, probably as a result of preformed oligomer clustering.

EXPERIMENTAL PROCEDURES

Materials—If not otherwise specified, all chemicals and reagents were from Sigma.

Eukaryotic Expression Vectors—The CCR5 and CXCR4 coding sequences without a stop codon were amplified using sense and antisense primers harboring unique *Hin*dIII and *Bam*HI sites. The fragments were then subcloned in frame into the *Hin*dIII/*Bam*HI sites of the pGFP-N1-Topaz, (PerkinElmer Life Sciences) and a humanized form of *Renilla* luciferase (hRluc)-N3, (BioSignal; PerkinElmer Life Sciences) vectors encoding the YFP yellow variant of green fluorescent protein, and of the humanized *Renilla* luciferase. In the resulting constructs, YFP and hRluc were directly fused to the 3′-end of the receptor cDNAs.

Cell Isolation, Culture, and Transfection—Peripheral mononuclear cells were isolated from freshly collected heparinized blood of healthy adult donors by Ficoll density gradient centrifugation (Eurobio Biotechnology, Les Ulis, France) as previously described (13). $CD4^+$ and $CD14^+$ populations were identified by fluorescein isothiocyanate-conjugated monoclonal antibody binding (BD Pharmingen, Heidelberg, Germany). Lymphocytes, separated from monocytes by adherence on plastic, were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, at 37 °C in an atmosphere of 5% CO_2. They were stimulated by 3-day incubation with anti-CD3 antibodies (UCHT-1; Seralab) bound to dynabeads (Dynal) precoated with sheep anti-mouse IgG. Binding to beads was achieved by incubating 0.5 μg of anti-CD3 antibodies with 10^7 beads in PBS, 0.1% bovine serum albumin for 1 h at room temperature. Human embryonic kidney (HEK-293) and HeLa cells, maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and streptomycin (all from Invitrogen), were seeded at a density of 3×10^5 cells in 35-mm plates. Transient transfections were performed the following day using Fugene (Roche Molecular Biochemicals) according to the manufacturer's protocol. Cells were harvested and used 24 h after transfection. Stable cell lines expressing wild-type CCR5 or CCR5 fused to either YFP or hRluc were also selected using 600 μg/ml G418 in the culture medium. The expression level of stable clones was assessed by quantitative FACS analysis as described below.

Cyclic AMP Assay—Cyclic AMP assays were conducted on stable HEK-293 clones expressing wild-type CCR5 or CCR5 fused to either YFP or hRluc. The accumulation of cAMP in intact cells was determined using the Alpha-Screen system (PerkinElmer Life Sciences). This immunoassay is based on the competition between the cAMP contained within the cells to be tested and biotinylated cAMP. Biotinylated cAMP is complexed by streptavidin molecules, which are covalently bound to "donor" beads, whereas an anti-cAMP monoclonal antibody is bound to "acceptor" beads. The interaction between biotinylated cAMP and the antibody bridges donor and acceptor beads. Upon appropriate irradiation (680 nm) of the donor beads, singlet oxygen is released and diffuses to activate the acceptor beads only if donor and acceptor beads are in close proximity. Activated acceptor beads then emit at 520–620 nm. The emission signal, recorded using a polyvalent fluorescence-luminescence detector (Fusion; PerkinElmer Life Sciences) decreased linearly with the increase of competing cellular cAMP concentration. Calibration curves were constructed using known concentrations of added cAMP. Adenylyl cyclase inhibition, promoted by the activation of wild-type CCR5, CCR5-YFP, and CCR5-hRluc, was measured in stable clones incubated with increasing concentrations of MIP-1β (Preprotech Inc., Rocky Hill, NJ), after enzyme prestimulation with 10 μM forskolin.

Receptor Internalization Assay—Receptor internalization was assessed by flow cytometry (FACS). HEK-293 cells, expressing either wild-type CCR5, CCR5-YFP, or CCR5-hRluc were incubated at 37 °C with 100 nM RANTES (R & D Systems Europe, Abingdon, UK) or a 10 μg/ml concentration of the anti-CCR5 MC-1 monoclonal antibody for the indicated time. After washing with ice-cold PBS, cells were incubated for 2 min at 37 °C in an acidic buffer, containing 100 mM NaCl and 50 mM glycine, pH 3, to remove bound RANTES or the MC-1 antibody. Cells were incubated at 4 °C for 1 h with the monoclonal phycoerythrin-conjugated 2D7 antibody, directed against CCR5 (PharMingen, San Diego, CA). Cells were then washed with ice-cold PBS and fixed with 1% formaldehyde. Samples were analyzed with a Becton-Dickinson flow cytometer. Base-line fluorescence, determined from untransfected HEK-293 cells, was subtracted from each sample, and the cell surface expression was defined as the mean fluorescence signal multiplied by the number of positive cells.

BRET Assay—24 h after transfection with the indicated cDNAs encoding BRET donors and acceptors, HEK-293 or HeLa cells were detached with PBS/EDTA and washed in PBS. Aliquots of 1×10^5 cells were distributed in the wells of 96-well microplates (White Optiplate from PerkinElmer Life Sciences) in the presence or absence of CCR5 ligands (100 nM) or antibodies (10 μg/ml). The monoclonal MC-1, MC-5, and MC-6 antibodies, directed against various epitopes of the CCR5 receptor, were generated in the laboratory of Dr. Matthias Mack (University of Munich). Single-chain antibody and Fab fragments from the MC-1 antibody were prepared as described (14). The luciferase substrate, coelenterazine *h* (Molecular Probes Europe, Leiden, The Netherlands), was added at a final concentration of 5 μM, and emitted luminescence and fluorescence were measured simultaneously using the FUSION fluorescence-luminescence detector (PerkinElmer Life Sciences). Cells expressing BRET donors alone (receptors fused to hRluc) were used to determine background. Filter sets were 485 ± 10 nm for luciferase emission and 530 ± 12.5 nm for YFP emission. Mean values from five consecutive measurements were used to determine BRET ratios that were calculated as described (15).

Quantitative FACS Analysis—Cell surface expression of CCR5 and CXCR4 was quantified by flow cytometry using the phycoerythrin-conjugated 2D7 and 12G5 monoclonal antibodies (PharMingen), respectively. The number of PE molecules bound on the surface of lymphocytes, monocytes, and transfected HEK-293 cells was determined in parallel, using the Quanti-BRITE PE Fluorescence Quantitation kit (Becton Dickinson Immunocytometry Systems, San Jose, CA) and the software provided by the manufacturer. For each experiment, fluorescence calibration curves were established with beads coated with known amounts of epitopes. Receptor density was expressed as the number of epitopes recognized by the antibodies per mg of total proteins, measured using the BCA protein assay (Pierce). To determine the proportion of receptors at the cell surface, comparative FACS analyses were conducted on intact cells and cells permeabilized with 0.05% saponin for 1 h at 4 °C.

BRET Analysis after Subcellular Fractionation—HEK-293 cells grown in 10-cm dishes were transiently transfected with CCR5-hRluc and CCR5-YFP plasmids. 48 h after transfection, the cells were washed three times with ice-cold PBS, scraped off the dish, and lysed with 1 ml of ice-cold hypotonic lysis buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 2 mM EGTA, 6 mM magnesium chloride, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, 1 mM benzamidine). The cell lysate was homogenized with 50 strokes of a Dounce homogenizer. Cellular debris and unlysed cells were removed by centrifuging at $1000 \times g$ for 5 min at 4 °C. The supernatant was collected, and sucrose was added to obtain a final concentration of 0.2 M sucrose. The discontinuous sucrose step gradient was made using the above hypotonic lysis buffer with the addition of sucrose at the following final molar concentrations: 0.5, 0.9, 1.2, 1.35, 1.5, and 2.0. Each step in the gradient had a total volume of 5 ml. The samples were centrifuged for 16 h at 27,000 rpm in a Beckman SW28 rotor. A total of 31 fractions of 1 ml each were collected from the top of the tube. BRET was measured on 100 μl of each fraction. The efficiency of the sucrose gradient to resolve the plasma membrane from the endoplasmic reticulum was verified by immunoblotting. Each fraction was probed with a mouse monoclonal antibody that specifically recognizes the α subunit of the Na/K-ATPase pump (Sigma A-276) (plasma membrane marker) at a dilution of 1:250 and a polyclonal anti-calnexin antibody (Stressgen Biotechnologies SPA-860) (endoplasmic reticulum marker) at a dilution of 1:8000.

Overexpression of Wild-type and Dominant Negative K44A Dynamin—HeLa cells expressing either wild-type dynamin or the K44A dominant negative mutant of dynamin, both under the control of a regulated tetracyclin promoter (Tet-off system), were generated in the laboratory of Dr. Sandra Schmid (Scripps Clinic, San Diego, CA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml each penicillin and streptomycin, 400 μg/ml G418, 200 ng/ml puromycin, and 1 μg/ml doxycyclin.

Overexpression of wild-type and dominant negative K44A dynamin was induced by removing doxycyclin from the medium 12 h after the transfection with the cDNAs encoding CCR5-hRluc and CCR5-YFP. BRET analysis and the endocytosis assay were conducted in parallel 48 h after transfection. Under these conditions, a ~20-fold overexpression of dynamin could be obtained, as determined by Western blots carried on whole-cell lysates with a monoclonal anti-dynamin antibody from the laboratory of Dr. Sandra Schmidt.

Immunofluorescence—For immunofluorescence studies, HeLa cells were seeded on coverslips in six-well plates, transfected with a plasmid encoding CCR5-YFP, and used for immunofluorescence 2 days post-transfection. After the indicated treatments, cells were fixed and processed for fluorescence microscopy as described previously (16). Samples were examined under an epifluorescence microscope (Leica DM-IRB) attached to a cooled CCD camera (Micromax 1300YHS; Princeton Instruments). Images were processed using the Metamorph™ software.

RESULTS AND DISCUSSION

Previous studies, based on immunoprecipitation assays after cell solubilization, indicated that CCR5 receptors might form homo- and hetero-oligomers (12, 11, 17), as reported for a growing number of GPCRs (reviewed in Ref. 18). However, these studies were discrepant on important issues such as the constitutive or induced nature of CCR5 oligomerization. In an effort to better characterize CCR5 oligomerization in living cells, we took advantage of a biophysical assay based on BRET, which was initially developed to monitor homodimerization of cyanobacteria clock proteins (19) and more recently applied to the study of GPCRs oligomerization (see Ref. 18 for a review).

Based on the Förster equation (20), it was estimated that the distance allowing energy transfer between BRET pairs is on the order of 25–100 Å. Therefore, the detection of a specific BRET between two receptors demonstrates a physical proximity that can be explained best by the formation of receptor dimers or oligomers (15). For this purpose, fusion proteins were constructed between CCR5 and either hRluc or YFP. FACS analysis of intact and permeabilized HEK-293 cells, transiently expressing each chimeric receptor, revealed a subcellular distribution similar to that of control wild-type CCR5 receptor: about 70% of both native or chimeric receptors was found at the plasma membrane 24 h after transfection (data not shown). Fusion receptors maintained their functional properties, as indicated by the similar potencies of MIP-1β to inhibit forskolin-promoted cAMP production via wild-type CCR5, CCR5-hRluc, and CCR5-YFP (IC_{50} of 1.61 ± 0.08, 1.5 ± 0.07, and 0.83 ± 0.03 nM, respectively; Fig. 1*A*). Also, agonist binding induced a similar extent and rate of receptor internalization of each receptor construct (Fig. 1*B*).

The cDNAs encoding CCR5-hRluc and CCR5-YFP were transiently co-expressed in HEK-293 cells, and the energy transfer was measured after the addition of coelenterazine *h*, the cell-permeable substrate of hRluc. As shown in Fig. 2*A*, a significant ($p < 0.01$) energy transfer was observed between CCR5-hRluc and CCR5-YFP upon coelenterazine *h* addition in the absence of any CCR5 ligand. No such signal was observed in cells expressing CCR5-hRLuc alone or when CCR5-YFP was replaced by a closely related chemokine receptor, fused to YFP (CXCR4-YFP). The CXCR4 receptor was preferred to the CCR2 as a negative control for BRET, as it was reported that the latter receptor might form functional hetero-oligomers with CCR5 (17). The lack of BRET between CCR5-hRluc and CXCR4-YFP did not result from the inability of CXCR4 to serve as a BRET acceptor, since a particularly strong signal was observed upon co-expression of CXCR4-YFP and CXCR4-hRluc. The stronger signal observed with CXCR4 does not necessarily reflect a higher proportion of receptors engaged in constitutive oligomers, since it could result from differences in orientation and/or distance between BRET partners. The selectivity of the interaction was also demonstrated by BRET competition experiments where the BRET signal was studied in the presence of increasing concentrations of native receptors. Overexpression of CCR5 but not of CXCR4 inhibited the transfer of energy between CCR5-hRluc and CCR5-YFP (Fig. 2*B*).

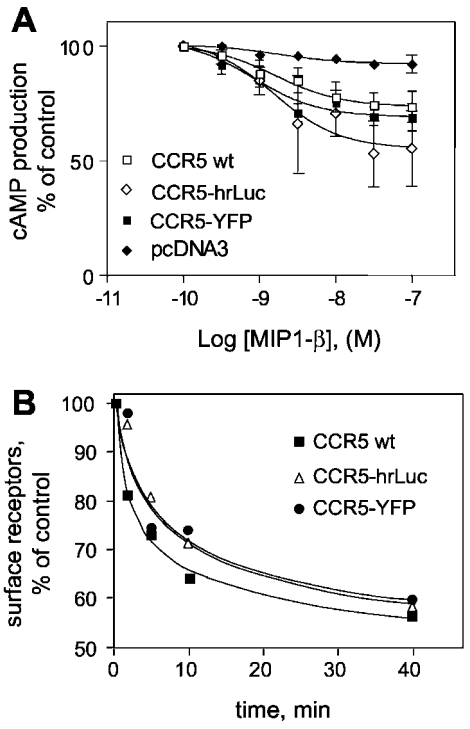

FIG. 1. **Functional characterization of wild-type, hRluc-fused, and YFP-fused CCR5 receptors.** *A*, receptor-mediated cAMP inhibition. HEK-293 cells expressing wild-type CCR5 or the receptor fused at its carboxyl terminus to either hRluc (CCR5-hRluc) or YFP (CCR5-YFP) were stimulated with increasing concentrations of the CCR5 agonist MIP-1β in the presence of 10 μM forskolin. Cellular cAMP levels were measured as described under "Experimental Procedures." Results are expressed as percentage of the forskolin-stimulated cAMP levels and represent the mean ± S.E. of three independent experiments. *B*, receptor internalization. HEK-293 cells expressing wild-type CCR5, CCR5-hRluc, or CCR5-YFP were incubated for the indicated times with 100 nM RANTES. Cell surface expression of wild-type and fused receptors was assessed by FACS using an antibody (2D7) directed against an extracellular epitope of the CCR5 receptor. Results, expressed as percentage of surface receptor levels in the absence of RANTES, are representative of three independent experiments.

BRET data, which support the existence of constitutive CCR5 oligomerization, are in agreement with a previous report in which CCR5 oligomers could be immunoprecipitated from cell lysates of untreated cells and visualized by Western blot (12). However, in other reports, CCR5 oligomers became apparent only when intact cells were preincubated with agonists or bivalent antibodies before chemical cross-linking, solubilization and immunoprecipitation (11, 17). By comparing the experimental protocols used in these studies, we noticed that the authors, who could not detect the presence of constitutive dimers, used more stringent denaturing conditions. We therefore conducted a series of immunoprecipitation studies on solubilized CCR5, in which we varied the composition of the sample buffer, and confirmed that the relative proportion of oligomeric receptors markedly decreased as the denaturing conditions became more stringent. CCR5 oligomers completely disappeared after resuspension of immunoprecipitated material in a classical Laemmli buffer, whereas, under low stringent conditions, oligomers were visible even in the absence of agonist treatment (see supplementary material). These data suggest a different interpretation of the actual role that agonists

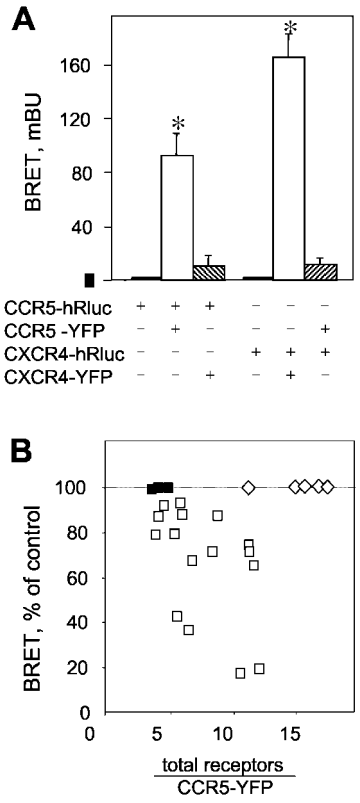

FIG. 2. **Constitutive BRET between CCR5-hRluc and CCR5-YFP.** *A*, HEK-293 cells were transfected with cDNAs encoding the CCR5 and CXCR4 receptors fused at their carboxyl termini to hRluc or YFP as indicated. Energy transfer was initiated by the addition of the cell-permeable luciferase substrate coelenterazine *h*, and the signals were measured in a polyvalent luminescence-fluorescence detector. The BRET signal was determined by calculating the ratio of the light emitted by the receptor-YFP (518–548 nm) over the light emitted by the receptor-hRluc (480–490 nm). The values were corrected by subtracting the background signal detected when either CXCR4-hRluc or CCR5-hRluc was expressed alone and expressed as the BRET ratio × 1000 (*mBU*). Data represent the mean ± S.E. of 4–6 independent experiments. *B*, HEK-293 cells were transfected with a constant amount of CCR5-hRluc and CCR5-YFP cDNAs in the presence of increasing concentrations of plasmid encoding wild-type CCR5 (*open squares*) or CXCR4 (*open diamonds*). The *closed squares* represent the BRET values obtained in the absence of competing receptor. The data are expressed as percentage of the BRET detected in the absence of competitor. The amount of receptor competitor is expressed as a ratio of the total number of receptors over the amount of CCR5-YFP expressed in each case. The amount of CCR5-hRluc, as assessed by the luminescence signal, remained stable (within 20% of variation) for all transfections.

and antibodies play in the visualization of CCR5 oligomers following covalent cross-linking (11, 17); the chemical bridging of adjacent CCR5 molecules, which maintains the complexes in denaturing conditions, may only occur when receptors adopt a specific conformation promoted by the agonist or when receptors are preclustered by antibodies.

BRET experiments, indicating that both CCR5 and CXCR4 receptors form constitutive homo-oligomers but not CCR5-CXCR4 hetero-oligomers, are also consistent with recent electron microscopy observations indicating that chemokine receptors, including CCR5, CXCR4, and CCR2, are found within closely apposed but distinct clusters of 50–100 nm at the plasma membrane of macrophages, T cells, and fibroblasts (21).

One of the major concerns when studying receptor oligomerization is the level of expression necessary to monitor oligomer formation, since a high concentration of receptors might cause random interactions between overexpressed receptor molecules. A recent analysis of the theory for FRET indicated a way to distinguish between FRET due to specific "clustering" of donors and acceptors and FRET due to high concentrations of donors and acceptors that are randomly distributed in the membrane. If donor and acceptor fluorophores are clustered, FRET is independent of absolute donor and acceptor surface densities but is dependent on the donor/acceptor ratio. In contrast, if donor and acceptor fluorophores are randomly distributed, FRET varies with acceptor density, and it is insensitive to the donor/acceptor ratio (22, 23). Since FRET and BRET are based on the same physical principle, the same rules determined for membrane protein clustering in FRET experiments should apply for BRET. Therefore, BRET studies were conducted in HEK-293 cells transfected with either increasing amounts of a fixed ratio of donor/acceptor cDNAs or with decreasing donor/acceptor ratios (Fig. 3). In the first set of experiments, the energy transfer remained stable for surface receptor levels ranging from 40 to 100 fmol of receptor/mg of membrane proteins (Fig. 3*A*). In contrast, the BRET signal increased linearly over the same range of total surface receptors, when a fixed amount of donor DNA was coexpressed with increasing concentrations of the acceptor DNA (Fig. 3*B*). These data indicate that the constitutive energy transfer between CCR5-hRluc and CCR5-YFP reflects a nonrandom association between the two receptor species. In addition, to determine whether specific BRET signals could be detected at physiologically relevant expression levels, precise assessments of cell surface receptors were carried out by quantitative FACS analysis. The receptor densities in transfected HEK-293 cells were then compared with those existing in human hematopoietic cells prepared from healthy donors. Specific BRET was detected in cells expressing as little as 40 fmol of receptor per mg of membrane proteins, corresponding to $8–10 \times 10^3$ surface receptors/cell (Fig. 3*A*). These receptor concentrations fall well within the range measured in human activated lymphocytes and monocytes (Fig. 3*C*).

Another important issue in studies on GPCR oligomerization has been to determine whether ligands can modulate the amount of oligomers. We found that incubation with RANTES or MIP-1β for up to 10 min had no effect on the energy transfer between CCR5-hRluc and CCR5-YFP (Fig. 4*A*), indicating that CCR5 agonists cannot modulate the constitutive oligomerization of CCR5. The incapacity of CCR5 agonists to modulate BRET cannot be attributed to signal saturation, since weak BRET values observed for higher donor/acceptor ratios remained insensitive to the ligands (Fig. 4*B*). Among previous energy transfer-based studies on GPCR oligomerization (using either FRET or BRET), a similar insensitivity to the ligand was observed for the yeast α-factor receptor (24) and for the human δ-opioid receptor (25). In contrast, an agonist-dependent enhancement of energy transfer was found for somatostatin, β_2-adrenergic, gonadotropin-releasing hormone, and thyrotropin-releasing hormone receptors (6, 8, 15, 26), and an agonist-promoted decrease of energy transfer was recently reported for the cholecystokinin receptor (27). In some instances, these results were interpreted as increase, lack of change, or decrease in the amount of oligomers. However, other parameters may explain the observed changes in energy transfer. Both BRET and FRET efficacies vary with the sixth power of the distance between the energy donor and acceptor ($E = Ro^6/Ro^6 + R^6$, where E is the energy transfer efficacy, R represents the distance between the donor and the acceptor, and Ro is the distance allowing 50% of maximal energy transfer) and is also sensitive to their dipole orientations. It follows that all changes that would influence the distance or the relative orientation between the donor and the acceptor are also susceptible to induce changes in energy transfer. Receptor activation by agonists is associated with conformational changes

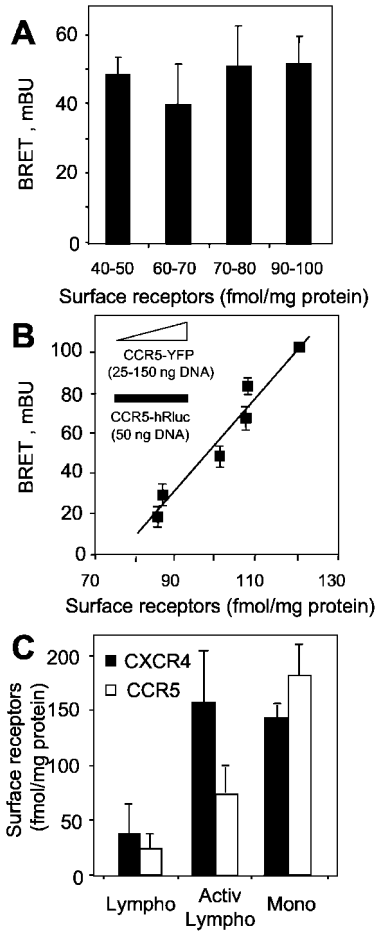

FIG. 3. **Effects of receptor density on the constitutive BRET signal between CCR5-hRluc and CCR5-YFP.** *A*, HEK-293 cells were transfected with increasing concentrations of plasmids encoding CCR5-hRluc and CCR5-YFP while maintaining an identical ratio (1:1) of the two constructs. BRET was measured as described in the legend to Fig. 2*A*, and receptor number was determined by quantitative FACS analysis as described under "Experimental Procedures." BRET values were averaged for group of experiments in which receptor densities were within intervals of 10 fmol/mg. The data presented for each interval is the mean ± S.E. of three independent transfections. *B*, HEK-293 cells were transfected with a fixed concentration of CCR5-hRluc cDNA (50 ng) and increasing concentrations of the CCR5-YFP construct (from 25 to 150 ng) for ratios varying form 2:1 to 1:3. BRET and the total receptor number were measured for each individual transfection. The BRET values represent the mean ± S.E. of triplicate transfections. *C*, CCR5 and CXCR4 receptor densities were determined in circulating mononuclear cells by quantitative FACS analysis. Lymphocytes (*Lympho*) and monocytes (*Mono*) were obtained from human blood as described under "Experimental Procedures." The lymphocytes were used either directly (*Lympho*) or following activation with an anti-CD3 antibody (*Activ. Lympho*). The data represent the mean ± S.E. of 4–6 independent determinations.

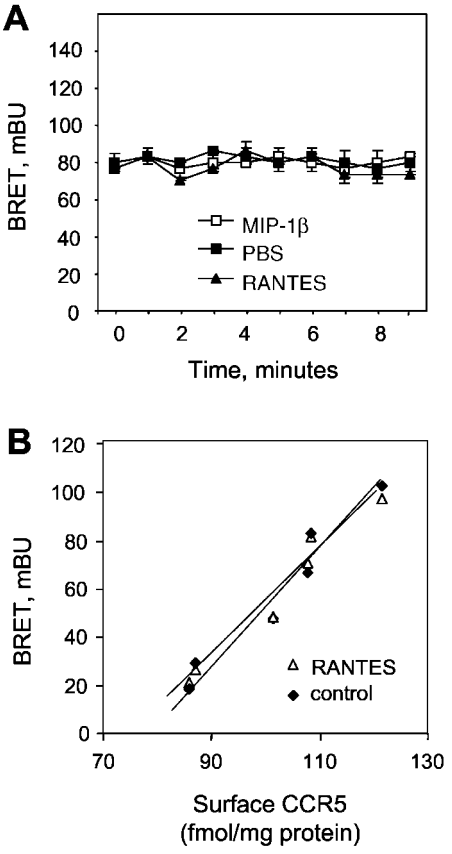

FIG. 4. **Effects of receptor activation on the constitutive BRET signal between CCR5-hRluc and CCR5-YFP.** *A*, HEK-293 cells, co-transfected with CCR5-hRluc and CCR5-YFP plasmids at a 1:3 ratio, were incubated with the CCR5 agonists, MIP-1β (100 nM; *open squares*), RANTES (100 nM; *closed triangles*), or vehicle (PBS; *closed squares*) for the indicated time. The transfer of energy was then initiated by the addition of coelenterazine *h*, and the BRET was measured as in Fig. 2*A*. The data shown represent the mean ± S.E. of triplicates in an experiment representative of six independent experiments. *B*, HEK-293 cells, transfected with a fixed concentration of CCR5-hRluc cDNA and increasing concentrations of the CCR5-YFP construct, were stimulated (*open triangle*) or not (*closed diamonds*) with RANTES (100 nM) for 10 min before the addition of coelenterazine *h* and BRET measurements. Receptor density was measured for each individual transfection. *mBU*, BRET ratio × 1000.

within the transmembrane core of GPCRs (28), coupling to G proteins, receptor phosphorylation by specific kinases, and arrestin translocation. The relative distance and the orientation of the energy donor and acceptor may or may not be affected by these events, depending on their position on the receptor and on the specific structural features of each receptor. Therefore, the agonist-promoted modulation of the energy transfer reported for some GPCRs cannot be readily interpreted as a change in receptor oligomerization. The experimental confirmation of the hypotheses above is provided by a recent study on melatonin receptors showing that ligand-promoted BRET enhancement represents specific ligand-induced conformational changes of preexisting receptor oligomers rather then increased oligomerization linked to the activation state of the receptors (29).

The observation that the constitutive oligomerization of the CCR5 receptor cannot be modulated by agonists may be explained by the fact that, at physiological concentrations, CCR5 receptors exist only as constitutive oligomers in intact cells. Such a model was recently proposed for the dopamine D2 receptors to explain complex binding patterns of some ligands (30). Alternatively, receptor monomers and oligomers may coexist, but the equilibrium between the two species cannot be dynamically regulated by receptor activation. The fact that in both hypotheses receptors exist at the plasma membrane as stable dimers raises the question of whether receptor dimers form only after reaching the plasma membrane or if they may assemble earlier during the biosynthetic maturation process. To address this issue, BRET was monitored after subcellular fractionation. Comparable BRET signals were detected in both cell surface and endoplasmic reticulum membranes resolved on a sucrose gradient, indicating that receptors exist as oligomers in both compartments (Fig. 5). Oligomeric assembly within the endoplasmic reticulum is a general feature of the export system that controls the quality of several plasma membrane proteins.

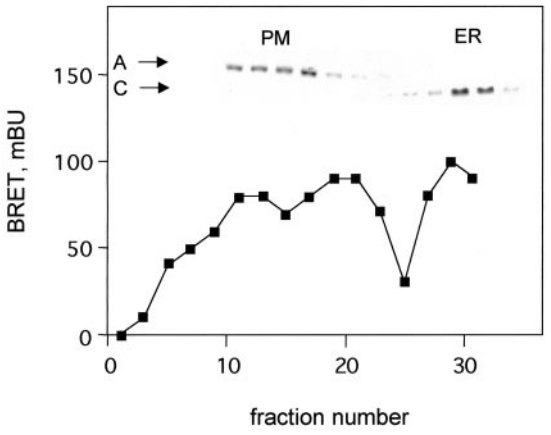

FIG. 5. **Constitutive BRET signals between CCR5-hRluc and CCR5-YFP in plasma membrane and endoplasmic reticulum subfractions.** HEK-293 cells, co-transfected with CCR5-hRluc and CCR5-YFP plasmids at a 1:3 ratio, were lysed, and subcellular fractions were resolved on a sucrose gradient as described under "Experimental Procedures." BRET was then measured in each fraction as described in the legend to Fig. 2*A*. Individual fractions were also resolved by SDS-PAGE. The presence of the plasma membrane and endoplasmic reticulum markers (the Na/K-ATPase (*A*) and calnexin (*C*), respectively), were then probed by immunoblotting using the appropriate antibodies. *Fraction 0* indicates the top of the gradient. The data shown are representative of two independent experiments.

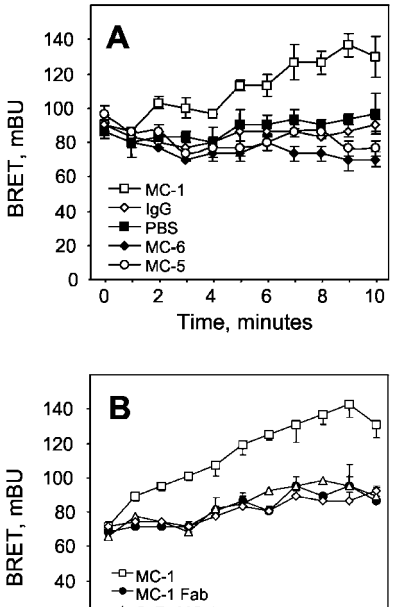

FIG. 6. **Effects of antibody treatment on the constitutive BRET signal between CCR5-hRluc and CCR5-YFP.** HEK-293 cells, co-transfected with CCR5-hRluc and CCR5-YFP plasmids at a 1:3 ratio, were incubated with a 10 μg/ml concentration of the monoclonal MC-1, MC-5, and MC-6 antibodies (*A*), the Fab and ScFv fragments of MC-1, preimmune IgG, and PBS (*B*) for the indicated periods of time. The transfer of energy was then initiated by the addition of coelenterazine *h*, and the BRET was measured as in Fig. 2*A*. The data shown represent the mean ± S.E. of triplicates obtained in an experiment representative of three independent experiments.

For instance, oligomerization of ion channel subunits was shown to occur in the endoplasmic reticulum (31). Only fully assembled and correctly folded oligomers could leave this compartment and be processed through the secretory pathway. The concept that early oligomerization of GPCRs in the maturation

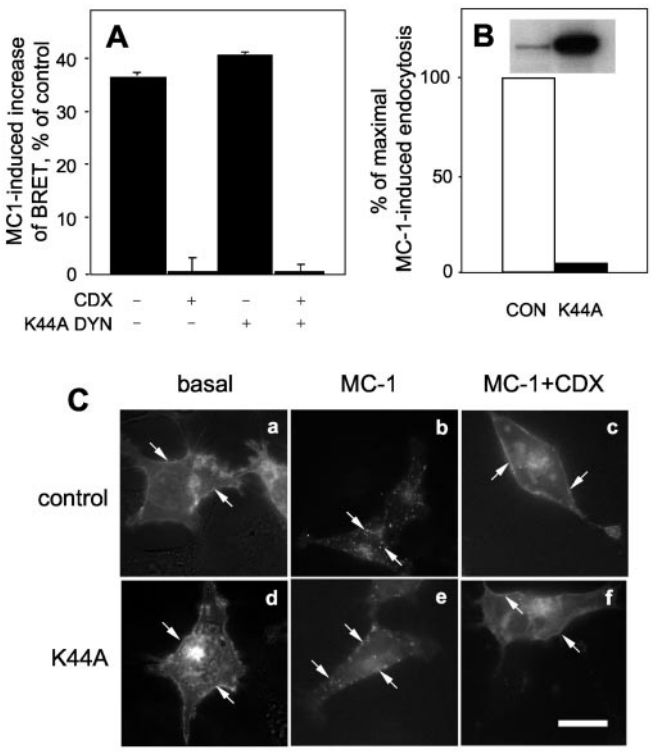

FIG. 7. **Effects of endocytosis inhibition and cholesterol depletion on BRET enhancement promoted by the MC-1 antibody.** *A*, HeLa cells stably expressing the wild-type or the dominant negative K44A mutant of dynamin under the control of the "tet-off" promoter were co-transfected with CCR5-hRluc and CCR5-YFP plasmids at a 1:3 ratio. BRET was then measured as in Fig. 2*A* following a 10-min treatment with 10 μg/ml of the MC-1 antibody or the vehicle alone in the presence or absence of methyl-β-cyclodextrin (*CDX*) and in cells overexpressing or not the K44A dominant negative mutant of dynamin (*K44A DYN*). BRET values obtained in the presence of overexpressed wild type dynamin were similar to those measured in the presence of K44A DYN (not shown). The data are expressed as percentage increase in BRET promoted by the MC-1 treatment and represent the mean ± S.E. of three independent experiments. *B*, MC-1-promoted internalization was measured in HeLa cells overexpressing (*K44A*) or not (*CON*) the dominant negative mutant of dynamin. Cell surface expression of the CCR5 receptor was assessed by quantitative FACS analysis following a 10-min treatment with a 10 μg/ml concentration of the MC-1 antibody or the vehicle alone. The *inset* is a Western blot showing the level of dynamin K44A overexpression after doxycyclin removal, compared with control cells expressing wild type endogenous dynamin. *C*, control HeLa cells (*a*, *b*, and *c*) and HeLa cells expressing K44A dynamin (*d*, *e*, and *f*) were transfected with the CCR5-YFP plasmid. 48 h after transfection, the cells were incubated (*c* and *f*) or not (*a*, *b*, *d*, and *e*) with methyl-β-cyclodextrin (*CDX*) for 45 min at 37 °C and then incubated (*b*, *c*, *e*, and *f*) or not (*a* and *d*) with the MC-1 antibody for 10 min at 37 °C. After fixation, cells were processed for immunofluorescence as described under "Experimental Procedures." The *arrows* indicate CCR5-YFP. In the presence of K44A dynamin, CCR5 clusters induced by the antibody were mostly located at the plasma membrane (compare *panels b* and *e*). CDX inhibited receptor clustering in both control cells and cells expressing K44A dynamin (compare *b* and *e* with *c* and *f*) and endocytosis in control cells (compare *b* and *c*). Note that in many cells expressing K44A dynamin, a strong accumulation of CCR5-YFP was visible in the Golgi area, which probably corresponds to the inhibition of vesicle budding from the Golgi apparatus.

pathway is a prerequisite for proper targeting to the plasma membrane, was validated experimentally in the case of the GBR1 and GBR2 isoforms of metabotropic $GABA_B$ receptor. The GABA-binding GBR1 isoform contains an endoplasmic reticulum retention signal, within its carboxyl-terminal tail (32) that prevents its transport to the cell surface (2–4). GBR1-GBR2 oligomerization, which involves a coiled-coil interaction between the two carboxyl tails, hides the GBR1 retention signal, allowing the functional hetero-oligomer to be exported to

the plasma membrane (32). Although not directly demonstrated yet, the hypothesis that receptor homo-oligomerization plays a similar role during CCR5 maturation is plausible and consistent with our data. If true, it would be of particular physiopathological interest, since previous studies showed that the infection by human immunodeficiency virus is directly related to the density of its co-receptors at the cell surface (33–35). Defining the CCR5 oligomerization interface and its molecular dynamics could offer new pharmacological targets to decrease the number of human immunodeficiency virus binding sites. Consistent with this notion is the suggestion that CCR5 mutants that are retained in the endoplasmic reticulum might act as inhibitors of wild-type receptor maturation and targeting to the cell surface (12, 36, 37).

Although our results suggest that receptors may be synthesized as oligomers, they do not exclude the possibility that oligomers may assemble to form larger structures such as clusters of oligomers. Such a possibility is supported by recent FRET studies demonstrating microaggregation of the GnRH receptor (26). Because antibodies can bridge adjacent molecules, they are known to cause capping or patching of membrane antigens, and it was reported recently that an anti-CCR5 antibody could allow the detection of CCR5 oligomeric structures in immunoblot experiments (11). Therefore, we investigated the effect of anti-CCR5 monoclonal antibodies on constitutive BRET. Several antibodies, directed against the extracellular domains of the receptor, were recently characterized in terms of epitope recognition, competition with chemokine binding, receptor activation, and trafficking (14). The MC-5 antibody, which mapped to the amino-terminal domain, inhibited MIP-1β binding. The MC-1 antibody, which binds to the second extracellular loop, promoted a strong β-arrestin- and clathrin-independent receptor endocytosis (probably through caveolae), and the MC-6 antibody, which mapped to a conformational epitope covering multiple extracellular domains, induced receptor signaling. As shown in Fig. 6*A*, the MC-1 antibody, not the MC-5 nor the MC-6 or control IgG, markedly enhanced the BRET in a time-dependent manner, reaching a plateau after 7–10 min of incubation with the antibody at 37 °C. Similarly to what was reported for the MC-1-promoted CCR5 endocytosis (14), only the native, divalent, antibody could induce the BRET enhancement, and neither a single-chain fragment of MC-1 (ScFv-MC-1) nor a purified Fab fragment of MC-1 affected BRET (Fig. 6*B*). However, receptor endocytosis was not involved in the enhancement of BRET promoted by the MC-1-antibody. Indeed, it was still observed after the overexpression of the K44A dominant negative mutant of dynamin (Fig. 7, *A* and *C*, *panel e*), a mutant known to inhibit both clathrin-dependent and -independent endocytosis (38, 39), which effectively blocked receptor endocytosis triggered by the MC-1 antibody (Fig. 7*B*). The fact that only MC-1 and not MC-5 or MC-6 antibodies induced a BRET increase, whereas all three antibodies bound the receptor, suggests a level of epitope-driven specificity. Interestingly, only the MC-1 antibody led to receptor clustering even in the presence of the K44A dynamin (see Ref. 14 and Fig. 7*C*, *panels b* and *e*), thus suggesting that the BRET increase results from this epitope-specific clustering. Further supporting this hypothesis is the observation that cholesterol depletion induced by methyl-β-cyclodextrin simultaneously blocked both the BRET increase promoted by the antibody (Fig. 7*A*; not the constitutive BRET, not shown) and receptor clustering (Fig. 7*C*, compare *panels b* and *c* and *panels e* and *f*). The precise mechanism by which receptor clustering promotes BRET enhancement remains speculative. The simple narrowing of preconstituted oligomers may increase the local concentration of BRET acceptors or determine an optimal orientation between acceptors and donors. Alternatively, the same changes could be promoted by the recruitment of cytoplasmic components to clustered receptors (*e.g.* proteins of the endocytic machinery). The possibility that constitutive BRET could also result from clustering of the receptors within finite microdomains rather than direct protein-protein interactions cannot be formally excluded. Recent FRET analysis indeed suggested that targeting of energy donor and acceptors to lipid rafts could result in efficient energy transfer (40). However, our observation that the constitutive BRET was insensitive to methyl-β-cyclodextrin treatment, which disrupts raft and was previously shown to inhibit FRET resulting from clustering, makes it an unlikely hypothesis.

The present study shows that, at physiological concentrations, CCR5 receptors exist as constitutive oligomers that form early after biosynthesis, in the endoplasmic reticulum. These observations suggest that, as demonstrated in the case of the $GABA_B$ receptor heterodimerization, homo-oligomerization might play a role in endoplasmic reticulum export quality control. The fact that CCR5 agonists did not modulate the constitutive BRET indicates that activation is not accompanied by a change in the receptor oligomerization state. Our data also invite the reinterpretation of previous studies, which concluded that antibodies promoted oligomer formation by cross-linking receptor monomers. Indeed, we show here that antibodies most likely cause the clustering of preexisting oligomers.

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