Direct Assessment of CXCR4 Mutant Conformations Reveals Complex Link between Receptor Structure and $G_\alpha_i$ Activation*

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Ligand binding to G protein-coupled receptors (GPCRs) is thought to induce changes in receptor conformation that translate into activation of downstream effectors. The link between receptor conformation and activity is still insufficiently understood, as current models of GPCR activation fail to take an increasing amount of experimental data into account. To elucidate structure-function relationships in GPCR activation, we used bioluminescence resonance energy transfer to directly assess the conformation of mutants of the chemokine receptor CXCR4. We analyzed substitutions in the arginine cage DRY motif and in the conserved asparagine N(3.35)119, which are pivotal molecular switches for receptor conformation and activation. $G_\alpha_i$ activation of the mutants was either similar to wild-type CXCR4 (D133N, Y135A, and N119D) or resulted in loss of activity (R134A and N119K). Mutant N119S was constitutively active but further activated by agonist. Bioluminescence resonance energy transfer analysis suggested no simple correlation between conformational changes in response to ligand binding and activation of $G_\alpha_i$ by the mutants. Different conformations of active receptors were detected (for wild-type CXCR4, D133N, and N119S), suggesting that different receptor conformations are able to trigger $G_\alpha_i$ activity. Several conformations were also found for inactive mutants. These data provide biophysical evidence for different receptor conformations being active with respect to a single readout. They support models of GPCR structure-activity relationships that take this conformational flexibility of active receptors into account.

GPCR2 activation is commonly seen as the consequence of conformational rearrangements in the receptor upon ligand binding. The ternary complex model of GPCR activation and its derivatives postulates a limited number of defined receptor conformations, representing inactive, active, and some intermediate states of activation. However, the link between receptor conformation and activity is not entirely clear, and limitations of this model are beginning to emerge (1).

Receptor mutants have often been used to derive conformational models of receptor activation indirectly from pharmacological and biochemical data. Energy transfer techniques have significantly advanced our capacity to directly assess receptor conformation and conformational responses to ligand binding in live cells (2, 3). However, to date, only few reports directly measured mutant GPCR conformations (4, 5).

CXCR4 is a chemokine receptor involved in human immunodeficiency virus infection, spreading of tumors, and inflammatory diseases. Its sole natural agonist is the chemokine CXCL12. CXCR4/CXCL12 are essential during development, hematopoiesis, and immune system organization. The potential importance of CXCR4 also in the adult organism, where the receptor is expressed on a plethora of different cell types (6), calls for more subtle therapeutic interventions than simple inhibition of the receptor by antagonists. This may be particularly important in long term treatments, such as those required for antiretroviral therapy. Better understanding of the link between CXCR4 conformation and activation is therefore warranted.

We set out to directly measure the conformations of CXCR4 mutants bearing substitutions at positions that potentially affect receptor function. For this purpose, we used a bioluminescence resonance energy transfer (BRET) conformational sensor in live cells based on CXCR4 homodimers. Chemokine receptor homodimers form constitutively and remain associated upon ligand binding, as shown previously by ourselves and others (3, 7–9). Of note, our CXCR4 system uses C-terminal BRET donor (RLuc) and acceptor (yellow fluorescent protein, YFP) fusions of the receptors that preserve receptor activity and do not require receptor overexpression above physiological levels (3).

**MATERIALS AND METHODS**

**Plasmids**—The mutations were introduced into CXCR4 by the Kunkel method and subcloned into CXCR4-YFP and CXCR4-RLuc (described in Ref. 7).

**Reagents**—Recombinant CXCL12 was from R&D systems, and synthetic CXCL12 was a gift from Dr. F. Baleux, Institut Pasteur, Paris, France. AMD3100 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. TC14012, a T140 analogue with similar biological properties, was synthesized as described previously (10).

**Cell Culture and Transfection**—HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with

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2 The abbreviations used are: GPCR, G-protein-coupled receptor; BRET, bioluminescence resonance energy transfer; YFP, yellow fluorescent protein; GTPγS, guanosine 5‘-3-O-(thio)triphosphate.
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FIGURE 1. Mutant expression and G{sub}i activation. A, cell surface expression levels of CXCR4 mutants. B, ΔE, that is, maximal activation versus basal binding of GTP{gamma}S. Results are the percentage of basal binding to membranes from wild-type CXCR4-expressing cells and are representative of 4–5 independent experiments performed in duplicate. C and D, CXCL12-induced [35S]GTP{gamma}S binding to membranes from cells expressing wild-type CXCR4 or receptors mutated in the DRY motif (C) or with substitutions of the Asn-119 residue (D). E, inhibition of cAMP production by CXCR4 mutants. Results are expressed as the percentage of adenylyl cyclase inhibition; background was subtracted from the data. Data are mean ± S.E. from 3 independent experiments.

RESULTS AND DISCUSSION

We constructed mutants D133N, R134A, and Y135A in the conserved DRY motif (residues 3.49, 3.50, and 3.51) following the assignment of Ballesteros and Weinstein (14), a pivotal conformational switch for ligand-induced rearrangements leading to receptor activation (15). In addition, mutants N119D, N119K, and N119S were introduced in position asparagine 119 (Asn-119, or 3.35), a key residue in which replacements change receptor activity as a function of the size of their lateral chain (16–18). Surface expression of mutants D133N and R134A was somewhat reduced, whereas the remaining substitutions showed similar surface expression as wild-type CXCR4 (Fig. 1A).

To analyze activation properties of the mutants, we measured G protein activation using a GTP{gamma}S binding assay (Fig. 1, B–D). D133N and Y135A displayed no apparent reduction of agonist-induced G protein activation, whereas R134A activity was strongly reduced. Replacement of the Asn-119 (N3.35) residue by lysine, serine, or aspartic acid resulted in the phenotypes previously reported by Zhang et al. (16). The N119D substitution resembled wild-type CXCR4, and N119K leads to loss of activity, whereas constitutive activity is seen with the N119S mutant. GTP{gamma}S binding of the N119S mutant is further increased in the presence of agonist to maximal activity identi-

10% fetal bovine serum (Wisent), 100 units/ml penicillin and streptomycin, 2 mM L-glutamine (Invitrogen). Transfections were performed in 6-well dishes using the polyethylenimine method (11). Receptor expression was tested by flow cytometry using the anti-CXCR4 monoclonal antibody 12G5 as described (3).

GTP{gamma}S Binding—[35S]GTP{gamma}S binding experiments were carried out on crude membrane preparations from transfected cells, as described previously (12). 10 μg of membrane proteins were incubated in 96-well microplates for 15 min at 30 °C in assay buffer (20 mM Hepes, pH 7.4, containing 100 mM NaCl, 10 μg/ml saponin, 3 mM MgCl{sub}2, 1 μM GDP) in the presence of the indicated concentrations of CXCL12. [35S]GTP{gamma}S (Amersham Biosciences) at 0.1 μM was added, and membranes were further incubated for 30 min at 30 °C. After centrifugation at 4 °C and removal of supernatants, the plates were counted in a Wallac 1450 MicroBeta Trilux.

Adenylyl Cyclase Activity—cAMP was determined by radioimmunoassay using the Amersham Biosciences cAMP assays system following the manufacturer’s instructions.

BRET Measurements—Usually 0.01–0.2 μg of RLuc constructs were cotransfected with increasing quantities of the corresponding YFP-tagged construct, completed to 2 μg with empty vector. Transfected cells were seeded in 96-well white clear bottom plates treated with poly-D-lysine and left in culture for 24 h. Cells were then washed once with phosphate-buffered saline and coelenterazine H (Nanolight Technology) added to a final concentration of 5 μM in phosphate-buffered saline. Readings were collected using a Mithras LB 940 reader (Berthold) by sequential integration of the signals detected in the 480 ± 20 (for luciferase) and 530 ± 20 nm (for YFP) windows. The BRET signal is the ratio of the receptor-YFP over the receptor-RLuc emission. The values were corrected to net BRET by subtracting the background BRET detected in cells expressing the RLuc constructs alone. Ligands were incubated in the presence of 0.1% bovine serum albumin (Sigma) with the cells at 37 °C for 5 min before the addition of coelenterazine H and BRET reading. In BRET titration experiments, net BRET ratios were expressed as a function of the [acceptor]/[donor] ratio (13). Total fluorescence (excitation filter at 485 nm and emission filter at 535 nm) and luminescence (measured 10 min after the addition of coelenterazine in the absence of emission filter) were used as a relative measure of total acceptor and donor protein expression. Ligand-induced BRET changes were monitored at maximal BRET (BRET{sub}max), and we ensured that BRET{sub}max did not depend on the quantity of transfected RLuc or YFP fusion plasmids.

Data Analysis—Data were analyzed using GraphPad Prism 4.0 software. GTP{gamma}S binding was analyzed using nonlinear regressions applied to a sigmoidal dose-response model (variable slope). The statistical significance of the net BRET differences between the different mutants and conditions was calculated using one-way analysis of variance with Tukey’s post-test for p value less than 0.05.
Energy acceptor over donor titrations demonstrated that most mutants retained BRET$_{50}$ values (the YFP/RLuc ratio at which half-maximal BRET is obtained) in the same range as wild-type CXCR4 (Fig. 2A), and the observed differences are not statistically significant. BRET$_{50}$ has been interpreted as the propensity of a receptor to dimerize (13, 19). The maximal BRET varied between wild-type and mutant CXCR4 with the exception of N119D. These BRET$_{max}$ differences were significant, as were the differences between several mutants (Fig. 2B). Lower BRET results from the greater distance between the energy donor and acceptor or from altered orientation, which is likewise indicative of conformational change. This conclusion is in line with evidence presented by Zhang et al. (16) as a result of receptor modeling, which suggested conformational differences between wild-type CXCR4 and the N119S mutant. Of note, it is improbable that the mutations directly modify the orientation of the fused fluorophores since they are in TM3 and adjacent residues of the receptor. We therefore conclude that the observed differences in BRET$_{max}$ are due to overall conformational changes within the receptor.

We next measured BRET in the presence of the natural CXCR4 agonist CXCL12, the weak agonist AMD3100, or the inverse agonist TC14012. We have previously shown that these ligands induce distinct conformational changes in CXCR4 homodimers without affecting BRET$_{50}$ values (3). Acceptor over donor titration analyses performed with the constitutively active mutant N119S and the inactive mutant N119K in the absence and presence of ligands revealed no significant changes of BRET$_{50}$ suggesting that ligand binding also to mutant CXCR4 does not change their propensity to dimerize (N119S at basal condition, 4.01 ± 0.13; in the presence of CXCL12, 7.93 ± 0.32; in the presence of TC14012, 5.99 ± 0.24; and N119K at basal condition, 4.85 ± 0.93; in the presence of CXCL12, 3.43 ± 0.93; in the presence of TC14012, 5.57 ± 2.46, n = 4). Fig. 3A shows the observed ligand-induced BRET$_{max}$ changes of the CXCR4 mutants, expressed as the percentage of change relative to the respective BRET$_{max}$ in the absence of ligand. The relative responses to the agonist CXCL12 of the mutants were different. Although the differences between wild-type CXCR4 and the mutants did not reach statistical significance, the differences between mutant Y135A and mutants D133N, R134A, N119K, and N119S were significant (p < 0.05). The relative BRET
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FIGURE 3. Ligand-induced BRET changes. A, relative BRET changes induced by the CXCR4 agonist CXCL12 (200 nM) (n = 4), the weak agonist AMD3100 (1 μg/ml) (n = 4), and the inverse agonist TC14012 (1 μg/ml) (n = 2), with respect to basal BRET of each mutant. Statistical significance: p < 0.05 for CXCL12 responses of D133N, R134A, N119K, and N119S with respect to Y135A; p < 0.05 for AMD3100 responses between CXCR4 and mutants N119K and N119S, D133A and N119K, R134A and Y135A, N119D, N119K, and N119S, and N119D and N119K; p < 0.05 for TC14012 responses between wild-type CXCR4 and D133N and D133N and mutants Y135A and N119S, B, the same values expressed on a scale relative to wild-type CXCR4 BRET in the absence of ligands (set as 100%). The error bars represent the mean ± S.E. of 4 independent experiments (except for TC14012, where 2 independent experiments were performed). One-way analysis of variance with Tukey’s multiple comparison test of all data found for 115 out of 378 comparisons shows significant differences (p < 0.05), the most appealing ones being between inactive wild-type CXCR4/basal and between active wild-type CXCR4/CXCL12 and N119S/basal, D133N/CXCL12 and N119S/CXCL12, and between active N119D/CXCR4 and N119D/CXCL12. Comparison of selected inactive receptors found significant differences between R134A/basal and N119K/basal and between R134A/basal and wild-type CXCR4/CXCL12 is shown.

important conformational changes or that these changes do not translate into important BRET changes. In turn, the higher BRET responses of most mutants to AMD3100 suggest that the conformational changes that these mutants undergo upon AMD3100 binding are dissimilar from those of the wild-type receptor. This, in principle, might result from their different basal conformations. To directly compare the ligand-bound conformations of the mutants, BRETmax values of the mutants in the presence of ligands were plotted as the percentage of ligand-bound wild-type CXCR4 BRETmax under basal conditions (Fig. 3B, and for a summary, Table 1). This direct comparison reveals that the mutants also have altered conformations in complex with ligand. In the presence of CXCL12, only mutants Y135A and N119D have BRETmax values similar to the wild type, indicating that agonist-bound receptor conformations of the other mutants are different, including mutants D133N and N119S (p < 0.05), which nevertheless stimulate Gαi activity in the presence of CXCL12. Differences in inactive conformations are suggested by the differences of R134A, N119K, and CXCR4 in the presence of inverse agonist (p < 0.05). Interestingly, despite the obvious functional differences between mutants N119K and N119S, almost identical BRET in the presence and absence of ligands was measured. This suggests that despite the recorded differences among many of the mutants, the technical limits of our method may not permit appreciation of subtle but functionally paramount conformational differences.

BRETmax values are average values of a receptor population; it can therefore not be categorically ruled out that different BRETmax values represent different states of equilibrium between only two distinct conformations (for example, one inactive and one active conformation). However, we find no linear link between BRETmax and G protein activation (for example, R134A with CXCL12 has lower BRETmax and higher activity than N119K with or without CXCL12, p < 0.05). Moreover, inactive and active receptors can have similar BRETmax (such as mutants N119K and N119S in the presence of CXCL12). We therefore conclude that different BRETmax corresponds to genuinely different receptor conformations rather than to changes in equilibrium between active and inactive receptors.

We have shown that mutations of the CXCR4 sequence can alter both the basal conformation and the conformational rearrangements induced by ligand binding. Confor-
mational rearrangements of a given mutant in response to agonist are not predictive of G protein activation (for example, mutant N119K conformationally responds to CXCL12 yet fails to activate G proteins). It remains to be investigated whether conformational responses could be predictive for receptor activities other than G protein activation; we do, however, not believe that conformational response per se must be followed by functional responses (i.e. the inverse agonist TC14012 induces conformational response but not activity). In turn, conformational differences between mutants in the presence of CXCL12 do not necessarily correlate with gross differences in G protein activation. For instance, the N119S mutant and wild-type CXCR4 have different conformations in the presence of CXCL12, yet both activate G proteins. Although it remains to be seen whether such mutants are functionally different with respect to other readouts, such differences would not affect our conclusion that distinct receptor conformations can mediate the same activity. Taken together, we detect important differences on both the functional and the conformational level in our mutant panel, but the link between function and conformation eludes simplification.

Theoretical models that describe GPCR activation have been repeatedly amended to accommodate new experimental data (20). The dominant ternary complex model and its derivatives assume the existence of a limited number of receptor conformations that derive from one another by intermediates between the extremes of one active and one inactive conformation. This assumption is increasingly difficult to reconcile with accumulating pharmacological evidence that different receptor ligands can stabilize different receptor conformations, as deduced from different functional consequences, and further revision of current models may be warranted (1). In this context, direct measurement of receptor conformation offers an additional perspective, contributing independent evidence to the elucidation of structure-function relationships in GPCRs. To date, few studies using these methods have employed mutant receptors in live cells (4, 5).

Our data derived from a set of CXCR4 mutants provide biophysical evidence that active receptor conformations can have a degree of conformational diversity that was previously unappreciated and that is consistent with multiple receptor conformations being able to perform the same activation step. This fits models that take conformational heterogeneity of receptors into account. Such models were initially forwarded to better describe allostery and suppose that collections of receptor conformations make up functionally similar receptor subpopulations (21–23). Although it might be rightly argued that recombinant systems expressing receptor mutants in cell lines do not reflect what wild-type receptors actually perform in vivo, such artificial test systems highlight aspects of the conformational plasticity of the active receptor, as they have previously permitted the accumulation of functional information.

In conclusion, we find that heterogeneous CXCR4 conformations can lead to similar G protein activation, implying a flexibility of active receptors that is unaccounted for by the ternary complex model and its derivatives. Our findings might also help meet the conceptual challenges inherent to therapeutic targeting of CXCR4. The use of competitive inhibitors of CXCL12 binding to CXCR4 results in side effects that make the drug unsuitable for long term antiretroviral treatment in human immunodeficiency virus infection. Our data suggest that noncompetitive allosteric compounds, which modulate the receptor conformation rather than blocking ligand binding and thereby preserve some of the functions of the receptor, may be compatible with the maintenance of vital CXCR4 signaling but impinging on other properties such as the mediation of viral entry.

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REFERENCES

1. Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobylka, B. K., Weinstein, H., Javitch, J. A., Roth, B. L., Christopoulos, A., Sexton, P., Miller, K., Speeding, M., and Mailman, R. B. (2006) J. Pharmacol. Exp. Ther. 320, 1–13
2. Ayoub, M. A., Couturier, C., Lucas-Meunier, E., Angers, S., Fossier, P., Bouvier, M., and Jockers, R. (2002) J. Biol. Chem. 277, 21522–21528
3. Percherancier, Y., Berchiche, Y. A., Slight, I., Volkmer-Engert, R., Tama-mura, H., Fuji, N., Bouvier, M., and Heveker, N. (2005) J. Biol. Chem. 280, 9895–9903
4. Tateyama, M., Abe, H., Nakata, R., Saito, O., and Kubo, Y. (2004) Nat. Struct. Mol. Biol. 11, 657–642
5. Vilardaga, J. P., Steinmeyer, R., Harms, G. S., and Lohse, M. J. (2005) Nat. Chem. Biol. 1, 25–28
6. Murdoch, C. (2000) Immunol. Rev. 177, 175–184
7. Issasfras, H., Angers, S., Bulenger, S., Blainpan, C., Parmentier, M., Labbe-Jullie, C., Bouvier, M., and Marullo, S. (2002) J. Biol. Chem. 277, 34666–34673
8. El-Asmar, L., Springael, J. Y., Ballet, S., Andrieu, E. U., Vassart, G., and Parmentier, M. (2005) Mol. Pharmacol. 67, 460–469
9. Springael, J. Y., Le Minh, P. N., Urizar, E., Costagliola, S., Vassart, G., and Parmentier, M. (2006) Mol. Pharmacol. 69, 1652–1661
10. Tamamura, H., Omagari, A., Hiramatsu, K., Gotoh, K., Kanamoto, T., Xu, Y., Kodama, E., Matsuoka, M., Hattori, T., Yamamoto, N., Nakashima, H., Otaka, A., and Fujii, N. (2001) Bioorg. Med. Chem. Lett. 11, 1897–1902
11. Boussif, O., Lezoualh, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7297–7301
12. Lagane, B., Ballet, S., Planchenault, T., Balabanian, K., Le Poul, E., Blanpain, C., Percherancier, Y., Staropoli, I., Vassart, G., Oppermann, M., Parmentier, M., and Bachelerie, F. (2005) Mol. Pharmacol. 67, 1966–1976
13. Mercier, J. F., Salaghour, A., Angers, S., Breit, A., and Bouvier, M. (2002) J. Biol. Chem. 277, 44925–44931
14. Gutierrez, N., and Hug, R. (2005) Methods Neuosci. 25, 366–428
15. Coteccchia, S., Fanelli, F., and Costa, T. (2003) Assay Drug Dev. Technol. 1, 311–316
16. Zhang, W. B., Navenot, J. M., Haribabu, B., Tamamura, H., Hiramatsu, K., Omagari, A., Pei, G., Manfredi, J. P., Fujii, N., Broach, J. R., and Peiper, S. C. (2002) J. Biol. Chem. 277, 24515–24521
17. Hunyady, L., Vauquelin, G., and Vanderheyden, P. (2003) Trends Pharmacol. Sci. 24, 81–86
18. Feng, Y. H., Miura, S., Husain, A., and Karnik, S. S. (1998) Biochemistry 37, 15791–15798
19. Ramsay, D., Kellett, E., McVey, M., Rees, S., and Milligan, G. (2002) Biochem. J. 365, 429–440
20. Kenakin, T. (2002) Nat. Rev. Drug Discov. 1, 103–110
21. Onaran, H. O., and Costa, T. (1997) Ann. N. Y. Acad. Sci. 812, 98–115
22. Kenakin, T., and Onaran, O. (2002) Trends Pharmacol. Sci. 23, 275–280
23. Kenakin, T. (2004) Trends Pharmacol. Sci. 25, 186–192