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Review

Fluorescent approaches for understanding interactions of ligands with G protein coupled receptors ☆

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ABSTRACT

G protein coupled receptors are responsible for a wide variety of signaling responses in diverse cell types. Despite major advances in the determination of structures of this class of receptors, the underlying mechanisms by which binding of different types of ligands specifically elicits particular signaling responses remain unclear. The use of fluorescence spectroscopy can provide important information about the process of ligand binding and ligand dependent conformational changes in receptors, especially kinetic aspects of these processes that can be difficult to extract from X-ray structures. We present an overview of the extensive array of fluorescent ligands that have been used in studies of G protein coupled receptors and describe spectroscopic approaches for assaying binding and probing the environment of receptor-bound ligands with particular attention to examples involving yeast pheromone receptors. In addition, we discuss the use of fluorescence spectroscopy for detecting and characterizing conformational changes in receptors induced by the binding of ligands. Such studies have provided strong evidence for diversity of receptor conformations elicited by different ligands, consistent with the idea that GPCRs are not simple on and off switches. This diversity of states constitutes an underlying mechanistic basis for biased agonism, the observation that different stimuli can produce different responses from a single receptor. It is likely that continued technical advances will allow fluorescence spectroscopy to play an important role in continued probing of structural transitions in G protein coupled receptors. This article is part of a Special Issue entitled: Structural and biophysical characterisation of membrane protein–ligand binding.

Contents

1. Introduction

Transmembrane receptors play critical roles in diverse cell signaling pathways that affect many aspects of cell behavior. Their functions in important physiological processes make them the targets of a large

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fraction of clinically useful drugs and candidate targets for the development of many new drugs [1]. Activation or modulation of downstream signaling pathways by receptors is generally initiated and controlled by interactions of the receptors with different classes of chemical ligands. These include agonists, which lead to activation of downstream pathways, antagonists, which do not activate the downstream pathways, but can inhibit activation by agonists, and inverse agonists, which act directly to inhibit receptor-mediated activation of pathways. Despite the importance of receptor–ligand interactions in controlling cell signaling pathways, the mechanisms by which such interactions elicit downstream responses remain poorly understood.

A major obstacle to understanding the molecular basis underlying receptor–ligand interactions has been the lack of structural information about receptors. Many receptors are transmembrane proteins for which structure determination by X-ray crystallography and NMR is extremely difficult. However, over the past few years, major advances have been made in determining structures of one particularly important class of receptors, the G Protein Coupled Receptors (GPCRs). These constitute a widely-distributed protein superfamily that is responsible for signaling responses to a wide variety of hormones, neurotransmitters, sensory stimuli, metabolites, and ions. GPCRs all consist of membrane proteins with seven transmembrane segments, an N-terminal extracellular portion that can vary considerably in size, and a C-terminal cytoplasmic tail, often involved in downregulation of signaling. Activation of GPCRs generally results in exchange of GTP for GDP bound to the α-subunit of a heterotrimeric G protein, followed by at least partial dissociation of the G protein α-subunit from the β- and γ-subunits. In some cases, activation of GPCRs may also result in signaling via mechanisms that do not involve G proteins, such as through interactions with arrestins [2,3].

To date, structures are available for rhodopsin [4,5] and the β2-adrenergic [6,7], A2a adenosine [8], dopamine D3 [9]; CXCR4 chemokine [10], histamine H1 [11], lyso-phospholipid S1P [12], M2 and M3 muscarinic acetylcholine [13,14], δ-, κ-, and μ-opioid [15–17], neurotensin [18], protease activated [19], serotonin [20], smoothened [21], glucagon [20], and corticotrophin releasing factor [22] receptors. With the exception of rhodopsin, all these structures have been obtained by fusing receptors to stable soluble proteins, or by introducing a variety of stabilizing mutations into the protein to render them stable enough to adopt a single state for crystallization. Such modifications can have significant effects on the functions of receptors [23,24]. However, availability of these structures provides critical information on GPCRs' overall topology, on the nature of their ligand binding sites, and, in some cases, on the nature of the conformational changes associated with receptor activation [25].

Despite the recent structural characterization of GPCRs, the specific ligand–receptor interactions that drive conformational changes of GPCRs that, in turn, result in activation or inhibition of receptor-mediated signaling pathways, are not yet defined. The diverse family of GPCRs apparently share common mechanisms for activating G proteins (for example, many different receptors can activate the same G proteins), but the molecular nature of the ligands that activate GPCRs is astonishingly diverse, ranging from large glycoproteins that interact with large extracellular domains of receptors to small molecules and ions, some of which appear to interact directly with transmembrane regions of the receptors. Classical models of receptor signaling postulated the existence of an active state of a receptor that is stabilized by binding of agonists and an inactive state stabilized by binding of inverse agonists. In this paradigm, ligands that act as antagonists bind with equal affinity to both active and inactive states, providing competition that inhibits activation by agonists, but resulting in no activation of receptors by antagonists added by themselves [26,27]. However, GPCRs appear to be more than simple two-state switches. A particularly intriguing aspect of GPCR signaling is the accumulating evidence for biased agonism, in which different ligands binding to similar sites on a particular receptor are capable of eliciting different downstream signaling responses [28].

Fluorescence-based techniques provide diverse ways of probing the chemical environments and intermolecular interactions that have been extensively applied to understanding receptor-mediated signaling. We focus in this review on applications in which these capabilities are used specifically to probe receptor–ligand interactions and associated conformational changes in GPCRs. Fluorescence has also been extensively used for other types of studies of GPCRs that will not be discussed here, including: 1) cell biological approaches in which fluorescence microscopy is used to characterize the subcellular locations of GPCRs under resting conditions and following stimulation; 2) examination of the dynamic nature of interactions between GPCRs and their cognate G proteins [29–32]; and 3) characterization of the oligomeric state of GPCRs, a complex and controversial topic that is beyond the scope of the present manuscript but has been reviewed in several contexts [29,33–39].

This review will also emphasize the usefulness of fluorescent ligands for studying GPCR signaling in the yeast pheromone response pathway. This signaling system has served as the basis for uncovering several aspects of GPCR signaling that have proved to be broadly relevant to such pathways in mammalian and other systems [40–42]. Haploid cells of the bakers' yeast *Saccharomyces cerevisiae* secrete the mating type-specific peptide pheromones a-factor and α-factor that bind to receptors on cells of the opposite mating type, reporting that a potential mating partner is nearby. Such signaling results in morphological changes, transcriptional reprogramming, and cell cycle arrest that prepare the haploid cell for mating to form a diploid zygote. The receptors for yeast mating pheromones are GPCRs that are, in some cases, functionally interchangeable with mammalian receptors, despite exhibiting very little sequence similarity to their mammalian counterparts [43–46]. In contrast, the sequences of trimeric G proteins in yeast are very similar to those of mammalian G proteins. The genetic approaches possible in yeast, along with the development of robust and diverse readouts for pheromone receptor activation, have resulted in a high level of characterization of this signaling system that has been complemented by the application of quantitative systems-based approaches for detailed analyses of pheromone signaling responses [47–49].

2. Fluorescent ligands

The usefulness of fluorescently labeled ligands for the study of GPCRs has been recognized for several decades [50–54]. Fluorescent GPCR ligands have been used for studies ranging from localization of receptors in tissues and cells (including an early demonstration of the internalization of ligand-bound receptors in cells [53]), to simple binding assays (in many cases, as replacements for radioligands), to sophisticated probing of the geometry and mechanisms of ligand–receptor interactions and receptor–receptor interactions. Several previous reviews have provided compendia of fluorescent ligands for GPCRs that have been reported in the literature [29,55–61]. Table 1 presents an updated list of published fluorescent ligands for GPCRs, including information from these previous reviews.

Since most GPCR ligands are not inherently fluorescent, the use of fluorescent ligands to study GPCRs requires modification of normal ligands to render them fluorescent. A significant problem in the field is the fact that such modifications can alter the ligands' properties, including, importantly, the nature of their interactions with receptors. Alteration of ligand properties is obviously a major issue in creating fluorescent derivatives of small molecule ligands, such as biogenic amines (see [59]), where the native ligands are smaller than any fluorescent moiety to which they can be conjugated. However, the introduction of a fluorophore can also lead to major alterations of the properties of larger ligands, such as peptides [57]. For example, upon testing of fifteen different analogs the yeast peptide mating pheromone, α-factor in which the small NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) fluorophore was attached at 7 out of 13 possible amino acids in the peptide, each of the analogs exhibited at least moderately reduced binding affinity for receptor, and several of the analogs had binding affinities that

Table 1
G protein coupled receptors that have been studied using fluorescent ligands.

Class of receptor	References
α-Adrenergic	[204–208]
β-Adrenergic	[50–52,65,71,87,88,206,209–219]
Adenosine	[69,85,86,220–235]
Angiotensin	[236,237]
Apelin	[238]
Bradykinin	[239]
Cannabinoid	[89,163,240–242]
Chemokine	[72,76,90,91,243–246]
Cholecystokinin/gastrin	[112–114,133,134,247–250]
Dopamine	[251]
Endothelin	[68,73,252,253]
Free fatty acid (GPR40)	[93]
Formyl peptide	[254–262]
Galanin	[92,263]
Glucagon	[67,264,265]
Ghrelin (growth hormone secretagogue)	[117,266]
Histamine	[267–276]
Leukotriene	[277]
Melanocortin	[278,279]
Muscarinic acetylcholine	[78,104–107,194,280–282]
Neuropeptide Y	[94,283–289]
Neurotensin	[290,291]
Opioid	[206,290,292–307]
Oxytocin/vasopressin	[98,111,118,308–312]
Parathyroid hormone	[108,313]
Relaxin	[314]
Secretin	[115,135]
Serotonin	[315–318]
Somatostatin	[95,96,290,319–324]
Tachykinin	[109,110,116,131,132,289,325–328]
Thyrotropin releasing hormone	[329]
Urotensin	[97]
Vasoactive intestinal peptide	[95,289]
Yeast α-mating pheromone	[62–64,82,99,101,172,330–332]

were reduced to undetectable levels [62]. Thus, it is important to note that the diverse ligands included in Table 1 have been characterized to different extents, using a wide variety of different assays. These range from simple microscopic evaluation of patterns of fluorescence localization on cells to full pharmacological characterization of binding and signaling efficacy and potency. In many cases, the actual binding affinities of the modified ligands and their tendencies to bind non-specifically to receptor preparations are not well characterized. Some fluorescent ligands are commercially available from sources such as Abcam Plc., Cell Aura Technologies Ltd., Life Technologies Corp., and Sigma Aldrich Co. However, it is important to note that not all commercially available products have been pharmacologically well-characterized.

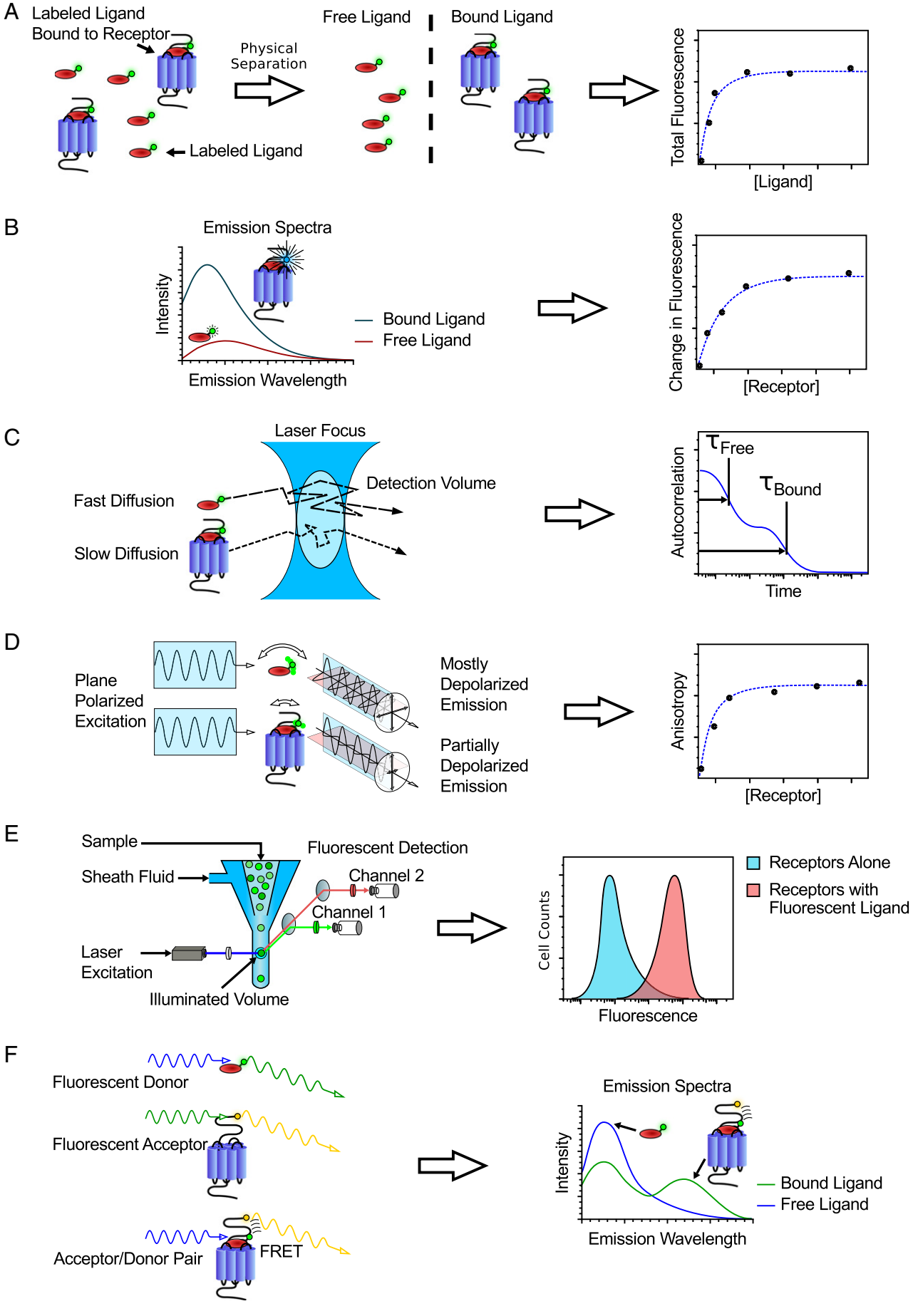
2.1. Binding assays using fluorescent ligands

Initial characterization of many fluorescent ligands has been conducted via competition assays against previously-characterized radiolabeled ligands (in the case of binding assays) and unlabeled ligands (in the case of assays of signaling responses). However, we focus here on assays of ligand binding that specifically make use of the fluorescent properties of ligands (see Fig. 1). As in any binding assay, some of the factors that must be considered relating to fluorescence-based binding assays include the ability to distinguish bound from unbound ligand, the ability to distinguish specific from non-specific binding (or between different classes of binding sites present in the same preparation of receptors), and the ability to distinguish actual reversible binding from internalization of ligand into cells. One aspect of fluorescence-based binding assays that requires additional consideration, compared to radioligand binding assays is the difficulty of quantitating the total concentration of binding sites in a preparation in absolute terms. In fluorescent assays, curve fitting of the data only yields an estimate of relative fluorescence levels at saturation, but no absolute determination of the actual concentrations of receptors, in contrast to radioligand binding assays with tracer of known specific activity. The actual fluorescence levels measured in a binding experiment are strongly affected by the sensitivity and geometry of the detecting instrument. Even if calibrated against a known quantity of a known fluorophore, such as fluorescein, the material used for calibrating must be present in a similar light-emitting geometry to that of the bound ligand, which can be difficult, especially when using flow cytometry. Such a calibration against a standard fluorophore also depends critically on the assumption that the fluorescent properties of the fluorophore used for the binding assay are the same as the standard. This is frequently not the case, since the quantum yield and absorbance of a fluorophore will generally be affected by the details of its chemical environment, such as the mode of conjugation to the ligand and the chemical properties of a receptor's ligand binding site.

Another significant issue in experiments involving ligand binding to whole cells is the problem of distinguishing initial binding to cell surface receptors from effects due to internalization of ligand-bound receptors, which can result in irreversibility of the binding reaction and environmental changes that can affect fluorescence of bound ligand. Internalization can often be prevented by conducting binding experiments at low temperatures or in the presence of metabolic inhibitors, however, this may not completely block endocytosis and may not block initial recruitment to the endocytotic apparatus in cells. Problems associated with receptor internalization generally impose a limit on the length of time over which binding and dissociation experiments can be performed.

Approaches used for assaying the binding of fluorescently-tagged ligands to receptors include:

1) Evaluation of staining patterns by fluorescence microscopy. This is an approach that has been applied for many of the ligands listed in Table 1, dating back to the first uses of fluorescent GPCR ligands [50–53]. It provides useful information about localization in tissues or at the subcellular level, but is of little value for providing insight into the mechanisms of ligand binding or receptor activation. Determining accurate binding affinities by this approach is challenging, since it may be difficult to accurately quantitate cell associated fluorescence and to correct for the contributions from cellular autofluorescence and non-specific binding, and since these measurements can be confounded by significant cell-to-cell variation.
2) Evaluation of bulk binding by separating bound from free ligand. Fluorescent ligands can be used as replacements for radioligands in saturation or competitive ligand binding analyses in which physical means, such as filtration, or centrifugation (for cells or membranes) or affinity or size exclusion chromatography (for purified receptors) are used to separate free from bound ligand. However, quantitation of fluorescent ligands in different fractions may be complicated by the presence of autofluorescent material in receptor preparations, by lower sensitivity of fluorescence detection compared to some radioligands, by differences in ligand fluorescence emission as a function of environment (such as in comparing bound and free ligand), and by the finite off-rate of some ligands, which can allow dissociation during washing steps. As an example, physical separation of free from Ste2p receptor-bound pools of a fluorescent analog of yeast α-factor was reported using affinity chromatography [63]. In some cases, free and bound ligand can be distinguished under equilibrium conditions via equilibrium dialysis, an approach that has also been applied in the yeast pheromone response system [63].
3) Determination of changes in fluorescence emission spectrum or intensity of a bulk solution containing receptors and ligand. In cases where the emission of a fluorescent ligand changes upon binding to receptors binding can be measured using a conventional cuvette-based fluorimeter or a fluorescent plate reader (including real-time PCR instruments) in a bulk solution containing receptor without the need to separate bound from free ligand. This approach relies on the ability to distinguish specific binding-dependent fluorescence changes from fluorescent background contributions arising from unbound ligand,


A
Labeled Ligand
Bound to Receptor
Physical
Separation
Free Ligand
Bound Ligand
Labeled Ligand
Total Fluorescence
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Sample
Sheath Fluid
Fluorescent Detection
Channel 2
Channel 1
Laser
Excitation
Illuminated Volume
Cell Counts
Fluorescence
Receptors Alone
Receptors with
Fluorescent Ligand
F
Fluorescent Donor
Fluorescent Acceptor
Acceptor/Donor Pair
FRET
Emission Spectra
Intensity
Bound Ligand
Free Ligand
Emission Wavelength

cellular autofluorescence, and non-specifically bound ligand. If the receptor is present in cells or membranes, these need to be maintained in a homogeneous suspension. The approach is not widely used, but has been applied to the binding of α-factor to yeast membranes [62,64] based on an observed large increase in fluorescence on binding. Small changes in emission spectrum of the fluorescent antagonist carazolol have been reported upon binding to purified β-adrenergic receptors [65], decreases in fluorescence emission have been reported upon binding of fluorescent derivatives of substance P to membranes containing the neurokinin 1 receptor [66], and an increase in fluorescence intensity has been detected in the binding of a fluorescent glucagon analog to its receptor [67]. An interesting variation of this approach is the spectrofluorimetric determination of the binding of a fluoresceinated N-formyl peptide to its cognate receptor using anti-fluorescein antibodies to selectively quench the fluorescence of unbound ligand [54]. A convenient approach for this type of measurement is to monitor changes in the emission from a constant concentration of fluorescent ligand upon adding increasing concentrations of a receptor-containing preparation. In general, such studies are conducted using fluorescent ligand concentrations that are near or below the dissociation constant for binding, so as to provide the greatest change in overall fluorescence per increment of binding.

4) Detection of changes in ligand mobility via Fluorescence Correlation Spectroscopy (FCS). FCS, the measurement of time-dependent fluctuations in fluorescence emission from a small illuminated volume provides a way of measuring the diffusion of fluorescently labeled molecules in solution. This can be used to distinguish between ligands that are freely diffusing in solution from those that diffuse more slowly by virtue of being bound to membranes, cells or large particles [60]. Thus, the ability of FCS to provide simultaneous tracking of free and bound populations in the same solution provides a basis for measuring binding in a time-dependent manner under equilibrium conditions, with no need to physically separate bound from unbound ligand. However, one complication is that multiple species of ligand with different characteristic diffusion times are often detected for a given ligand bound to a particular type of receptor [60]. This may reflect different states of homomeric or heteromeric protein–protein association or different subcellular localizations of the particular receptor being studied. Thus, FCS has been used in a number of instances to measure the mobilities of receptors and to provide an estimate of their oligomeric states. It has also been used as an assay for binding of fluorescent ligands to the endothelin [68], adenosine A1 [69], melanin concentrating hormone, gastrin releasing peptide, and β-adrenergic receptors [70,71].
5) Detection of changes in fluorescence anisotropy upon binding. The fact that low molecular weight ligands undergo rapid rotational motion in solution, but are more restricted when bound to large receptors, provides the basis for measuring ligand binding using steady state fluorescence anisotropy or polarization. Molecules that are illuminated with linearly-polarized light will emit with a defined polarization with respect to the incident beam if rigidly held in place, but the orientation of the polarization will be randomized if the molecule rotates significantly during the time span of the excited state of the fluorescent transition. Binding assays based on fluorescence polarization have been used to characterize the ligand binding properties of purified receptors, including chemokine and endothelin receptors [72,73], and are particularly well-suited for use in high-throughput screening [70,74–78]. As with other spectroscopic assays, anisotropy-based binding can be measured in equilibrium experiments, with no need to separate bound from unbound ligand. Measurements can be made using a fluorimeter or a fluorescence plate reader with polarization capabilities. One limitation of such studies stems from the fact that measured steady-state anisotropy of a bulk solution provides only a single number that is averaged over the anisotropies of different species present in the solution, weighted by their fluorescence emission intensities. Thus, in order to significantly change the anisotropy of the population of ligand molecules, a significant proportion of the ligand in solution must become bound to receptor. Meeting this condition requires the presence of a concentration of binding sites that is of the same order as the concentration of ligand, and which must, in a typical binding experiment, also be of the same order as the dissociation constant of the interaction [79]. The use of such high concentrations of binding sites may be difficult to achieve for some receptor preparations, and may lead to high backgrounds if the receptor preparation is associated with significant autofluorescence. To circumvent some of these problems, fluorescence polarization assays are often performed as competitions, under conditions where the fluorescent ligand is predominantly bound to receptors until displaced by unlabeled ligand. Binding experiments are also often performed as titrations of increasing amounts of receptor preparation into a constant concentration of fluorescent ligand. Polarization changes of the fluorescent ligand carazolol upon binding to β-adrenergic receptors have also been monitored by chromatographic separation of free ligand receptor–ligand complexes [65].
6) Flow cytometry. Sklar and coworkers originally demonstrated that flow cytometry can provide unique advantages for assays of ligand binding to receptors (see [54,80,81]). Flow cytometers measure fluorescence only when a cell (or other particle) passes through the narrow fluid path of a flow cytometer, collecting emitted light only from a small volume surrounding the particle. This allows determination of the amount of fluorescent ligand associated with cells in a homogeneous suspension with no need to physically separate free from bound ligand. The approach can be used even when binding of fluorescent ligand to receptors does not result in any change in fluorescence emission. However, its sensitivity is enhanced in cases where fluorescence of the bound ligand increases or alters in wavelength upon binding, as observed for binding of fluorescent derivatives of yeast α-factor to the α-factor receptor [82]. The fact that flow cytometry can detect binding under equilibrium conditions raises the possibility of examining relatively low affinity interactions for which problems of ligand dissociation could be severe in assays

Fig. 1. Schematic representations of ligand-binding assays using fluorescent ligands. In each case, the fluorescent ligand is indicated by a red oval with an attached green fluorophore and the receptor is represented as purple hepta-helical bundle. A) Determination of ligand binding using physical separation of bound and free ligand. The concentrations of free and bound ligand in separate fractions are determined through measurement of total fluorescence emission intensities in each fraction. B) Determination of ligand binding based on changes in fluorescence emission spectrum or intensity of a bulk solution containing receptors and ligand. Relative changes in fluorescence intensity or wavelength reflecting binding to receptors are quantitated as a function of increasing ligand or receptor concentration. C) Determination of ligand binding through detection of changes in ligand mobility via Fluorescence Correlation Spectroscopy (FCS). Rapid fluctuations in fluorescence emission of ligand in a small illuminated volume reflect the diffusion time of the labeled entity. Quantitation of binding is accomplished by distinguishing the population of rapidly diffusing free ligand from that of slower diffusing bound ligand. Analysis of the fluctuations in emission allows assignment of a diffusion time (related to τ, the time scale of the observed fluctuations) and an estimate of the fraction of the population associated with each diffusion time. D) Determination of ligand binding based on changes in fluorescence anisotropy upon binding. When free ligand undergoing rapid rotational diffusion is illuminated with linearly polarized light, the emitted fluorescence is depolarized because the emitting dipole rotates during the time interval between fluorescence excitation and emission. Receptor-bound ligand undergoes much slower rotation, leading to fluorescence emission that maintains linear polarization. The extent of polarization of a solution of fluorescent ligand and receptors is a weighted average of the emissions from the free and bound populations of ligand. E) Determination of ligand binding using flow cytometry. Illumination of a small volume containing receptors expressed on cells or bound to beads allows determination of the amount of bound fluorescent ligand. Data is presented as a histogram showing the percentage of cells or beads exhibiting a given fluorescence emission intensity. Often a control sample containing cells or beads without receptors or a sample incubated with fluorescent ligand in the presence of a large excess of non-fluorescent competitor is used as a control for autofluorescence and non-specific binding. F) Determination of fluorescent ligand binding based on FRET between a fluorescent ligand and a fluorescently labeled receptor. Binding of ligand to receptor leads to transfer of fluorescence from donor to acceptor that can be detected via a decrease in donor emission or an increase in acceptor emission. Although the illustration shows the donor conjugated to ligand and the acceptor conjugated to receptor, the opposite configuration is also used.

involving separation of free ligand and the requisite washing steps. However, a practical lower limit on the affinities of interactions that can be studied in this way arises from the fluorescence of high concentrations of unbound ligand in the illuminated volume of the flow cytometer when working at high ligand concentrations needed to study weak binding. On the other hand, assays can be conducted at very low cell densities, resulting in a low overall concentration of receptors, as needed for assaying interactions with low dissociation constants without complications introduced by ligand depletion. Ligand binding can be quantitated in either saturation- or competition-type assays. A practical lower limit of detection of about 1000 fluorescein molecules per cell or particle arises from difficulty of distinguishing the ligand emission from the background of autofluorescence of the cells or particles (see [80]). This limit is usually well above any limitations from the fluorescence detection sensitivity of current instruments. The capability to time-stamp data from each cell passing through the cytometer makes it possible to follow the kinetics of ligand–receptor interactions. The variety of excitation lasers and detection channels available on modern flow cytometers allows the use of a wide range of fluorophores, either singly or in multiplexed configurations. Although flow cytometry-based assays of ligand binding are generally performed with cells, the benefits of the approach have been realized in assays of binding of components to purified receptors attached to synthetic beads, including a reconstitution of ligand interactions with formyl peptide receptors [83] and β_2-adrenergic receptors with trimeric G proteins [84] on the surface of beads.

Many of the early studies establishing flow cytometry as a ligand binding assay were performed with formyl peptides serving as ligands for chemotactic receptors. However, the approach has also been used for ligands for adenosine [85,86], adrenergic [87,88], cannabinoid [89], chemokine [90,91], galanin [92], long chain fatty acid [93], neuropeptide Y [94], somatostatin [95,96], urotensin [97], vasopressin [98] and yeast α-factor [82,99–102] receptors.

7) Ligand–Receptor Fluorescence Resonance Energy Transfer. The binding of a fluorescent or dye-labeled ligand to a receptor can be monitored via Fluorescence Resonance Energy Transfer (FRET) between the ligand and a fluorescent group attached to the receptor. FRET is the non-radiative transfer of energy between two fluorophores or chromophores where the emission from one overlaps the excitation of the other. The strong ($1/r^6$) distance dependence of the interaction makes it useful for measuring distances or determining whether the labels are in close proximity on the scale of 1–10 nm. The presence of FRET can be detected either via enhanced emission of the longer wavelength (acceptor) fluorophore, or via loss of emission from the shorter wavelength (donor), even if the acceptor is not fluorescent. In many cases, binding can be measured based on FRET changes without any requirement to separate bound from unbound ligand, allowing determination of binding kinetics on a rapid time scale. If fluorescent backgrounds are low and there is minimal bleedthrough of donor emission into the acceptor channel and minimal direct excitation of the acceptor by donor excitation, FRET-based approaches can be sufficiently sensitive to detect relatively low occupancies of ligand bound to receptors.

Labeling of GPCRs for this type of assay can be accomplished by fusing fluorescent proteins to the extracellular N-terminal region of the receptors, by specifically attaching small fluorescent tags to the N-terminal of the receptor, or through binding of a fluorescently labeled antibody to the native N-terminal of a receptor or an epitope fused at this position. In some cases, FRET can be measured in a time-resolved mode, using a lanthanide-containing donor with a long-lived excited state and a large Stokes shift (between excitation and emission) to remove background contributions from autofluorescence, direct excitation of acceptor, and bleedthrough of donor emission (see [103]). It is also sometimes possible to detect binding in circumstance where one member of a FRET pair (either donor or acceptor) is specifically labeled and the other member is not, since the FRET-dependent change in the specific label should only occur when it interacts with the non-specifically labeled partner. This, for example, raises the possibility of measuring binding of a specifically fluorescently labeled ligand to a cell in which surface proteins have been non-specifically labeled with a fluorophore that can serve as a FRET partner to the ligand.

FRET between labeled ligands and GPCRs fused to fluorescent proteins has been used to detect ligand binding M1 muscarinic acetylcholine receptors [104–107], parathyroid hormone receptors [108], and neurokinin NK2 receptors [109,110]. FRET between labeled ligand and fluorescent antibody bound to vasopressin receptors has also been reported [111]. FRET with fluorescent ligands has also been used in combination with small fluorophores covalently attached to introduced cysteine residues in cholecystokinin (CCK) receptors [112–114] and secretin receptors [115] and via biosynthetic incorporation of unnatural amino acids into the neurokinin-2 receptor [116]. Several studies have also taken advantage of recently-developed enzyme-based approaches for tagging receptors with small molecule fluorophores for ligand–receptor FRET studies. These include studies of ligand binding to ghrelin receptors [117], V2 vasopressin receptors [118], and numerous additional types of receptors [119].

2.2. Stabilization of GPCRs for structure determination based on preservation of fluorescent ligand binding by mutant receptors

Recent crystal structures of GPCRs have highlighted the importance of protein stability in the generation of protein crystals. The structures of the β_1-adrenergic, the A2a adenosine, and neurotensin receptors were solved using constructs that incorporated several stabilizing mutations [18,120,121], while several others have been solved by replacing a flexible intracellular loop with T4 lysozyme or other stable soluble proteins, which is thought to decrease the conformational flexibility of the protein while also providing extra surfaces for crystal contacts (see [122]). Other GPCR structures have been solved using antibodies to stabilize the protein [123–125]. Thus, a major bottleneck in the determination of GPCR crystal structures appears to be the identification of ways to improve the stability of solubilized receptors. Recently, fluorescent GPCR ligands have been used in conjunction with Fluorescence Activated Cell Sorting (FACS) of cells expressing libraries of randomly mutated receptors to screen for receptor mutations that improve the stability of GPCRs.

One such directed evolution-based approach focusing on receptors expressed in *Escherichia coli* led to the identification of mutations in the gene for the neurotensin receptor 1 that improved GPCR expression ~10-fold [126]. When tested for thermal stability, a high-expressing mutant clone was also found to exhibit improved stability. In a more comprehensive study the laboratory of Andreas Plückthun generated libraries of mutants in which all 64 possible codons were substituted at every codon position of the same neurotensin receptor [127,128]. FACS based on fluorescent ligand binding was used to select for high expressing clones which were then subjected to high throughput sequencing to determine the frequency of each codon at each position. By combining several of the mutations, a clone was identified that improved expression even further compared to the clone identified in the prior study. When similar mutagenic and fluorescent screening techniques were applied to three other GPCRs, the tachykinin receptor NK1, the α_{1a}-adrenergic receptor, and the α_{1b}-adrenergic receptor, clones with improved expression and thermal stability were identified for each receptor, indicating that the procedure can be generally applied to proteins for which fluorescent ligands are available [129].

A similar approach has recently been applied for the identification of stabilizing mutations from randomly mutagenized libraries of yeast α-factor receptors expressed in yeast cells (Zuber and Dumont, *unpublished results*). Binding of NBD-labeled α-factor was also used to identify α-factor receptors retaining ligand binding activity from a

library of mutated receptors with T4 lysozyme inserted at random positions in the vicinity of the third intracellular loop. A further selection for T4 lysozyme-containing receptors that retain signaling function was also conducted, allowing recovery of lysozyme-inserted variants that retain full ability to bind ligand and activate the pheromone response pathway [102]. Flow cytometric measurements of binding of a fluorescent alprenolol derivative have also been used in evaluation of the ligand-binding capabilities of potentially stabilizing site-directed mutations in the β_2-adrenergic receptor [88].

Some of the attempts to achieve mutagenic stabilization of GPCRs assayed by fluorescent ligand binding have been based on the assumption that there will be a correlation between receptor expression in cells and receptor stability in detergent. In these studies, receptors were first optimized through directed evolution for expression in a cell system, and then tested individually for improved stability in the solubilized state. A new approach by Scott and Plückthun, in contrast, directly screens for protein stability in detergent using a technique referred to as CHESS (Cellular High-throughput Encapsulation, Solubilization, and Screening) [130]. Prior to receptor solubilization, bacterial cells are encapsulated within a polymer coating that is impermeant to large molecules, while allowing small molecules such as fluorescent ligands and detergents to pass through. This confines solubilized receptors within a matrix that can be sorted using flow cytometry while maintaining an association between each mutated receptor and its genetic coding information, as is required to identify the relevant mutations in the stabilized variants. This method has been used to identify stabilizing mutations in neurotensin and α_{1a}-adrenergic receptors.

2.3. Use of fluorescent ligands to probe the geometry and environment of GPCR ligand binding sites

2.3.1. FRET-based mapping of ligand binding sites

The strong dependence of FRET efficiency on the spatial separation between donor and acceptor has served as a basis for using ligand–receptor FRET to map the distances between different labeled groups on fluorophores and labeled groups on receptors. Such measurements can be much more precise when the FRET is measured between small fluorophores than when the donor or acceptor is a large fluorescent protein, where the actual location of the absorbing and emitting dipoles may be poorly defined and close apposition of donor and acceptor is hindered by steric constraints. A model of the interactions of CCK peptides with CCK receptors has been derived from triangulation of distances determined by FRET between small fluorophores attached at different positions to peptide ligands and small fluorophores specifically attached to receptors in tissue culture cells at cysteine residues introduced by site-directed mutagenesis [112,114]. Although many proteins at the cell surface are labeled with acceptor dye by this approach, the specificity of the observed FRET signal is provided by the binding specificity of the labeled ligands. The same group also took a similar approach for modeling the geometry of secretin binding to its receptor [115].

2.3.2. Probing of ligand binding sites based on fluorescent properties of labeled ligands

In addition to providing an assay for whether or not ligand binds to a particular GPCR, the use of fluorescent ligands makes it possible to probe the environment and geometry of the ligand binding site, as well as kinetic aspects of ligand binding to receptors. For example, some environmentally-sensitive fluorophores change their quantum yields or emission spectra in response to changes in their surroundings, particularly the polarity of their immediate environment. Such changes are readily observable upon examination of the fluorescent properties of the fluorophore or the fluorescently tagged ligand in the presence of solvents with different polarities. A useful measure of the accessibility of bound ligand to aqueous solution is also provided by the degree of quenching observed upon addition of quenchers such as iodide ions to a suspension or solution containing ligand–receptor complexes. In such experiments, it is important to control for the effects of the changing ionic strength of the solution at different iodide concentrations [64].

Examples of GPCRs with fluorescent ligands that have been used to probe the environment of ligand binding site include: 1) the galanin receptor. Analysis of quenching was used to determine that the receptor-bound ligand is sequestered in a hydrophobic binding site [92]; 2) the neurokinin NK1 receptor. The bound state of a dansylated non-peptide antagonist exhibited an increase in emission intensity, a blue-shift of emission, and a reduced susceptibility to collisional quenching, compared to the same ligand in the unbound state. No such fluorescent changes were seen for any dansyl groups attached to each of four different positions on the NK1 peptide agonist substance P, underscoring the existence of significant differences in binding between peptide and small molecule ligands [131]; 3) the neurokinin NK2 receptor. Variations in the length of the spacer arm connecting an environmentally sensitive NBD fluorophore to the N-termini of peptide antagonist ligands resulted in drastic changes in fluorescence emission and in susceptibility to quenching. This led to the inference that the labeled region of these antagonists is buried to a depth of only 5–10 Å in a hydrophobic binding pocket. On the other hand, similar fluorophores attached to N-termini of agonist peptide ligands did not show these hallmarks of inaccessibility to solvent [132]. A fluorescent non-peptide antagonist exhibited even higher fluorescence emission and anisotropy than labels attached to the peptide antagonists, implying that it resides in a site that is even more sequestered from solvent and restricted in motion than peptide ligands [131]; and 4) β-adrenergic receptors. The fluorescent antagonist carazolol, when bound to receptors, exhibited changes in emission spectrum and susceptibility to quenching on binding, indicative of residing in a hydrophobic binding site [65].

Detailed analyses of changes in fluorescence emission, quenching, anisotropy and lifetime on ligand binding to CCK and secretin receptors have been conducted by Miller and co-workers. Binding of fluorescent ligands to preparations containing these receptors results in small changes in emission intensity and small spectral shifts. In the case of the CCK receptor, differences were observed between the binding-associated fluorescence changes of related agonist and antagonist peptides that were similarly labeled at their amino termini. The differences were indicative of greater exposure of N-termini of agonist ligands to aqueous solution than for similarly labeled antagonists. Treatment of the receptor preparations with non-hydrolyzable GTP analogs resulted in a shift in the fluorescence of bound agonist toward a more antagonist-like state [133]. Surprisingly, in comparisons of the related type A and B CCK receptors, significant differences were seen in the apparent solvent accessibility of the bound state of the same fluorescently labeled peptide ligand, despite the fact that this peptide binds both receptor types with the same affinity and exhibits similar potencies toward activation of the two types [134]. In the case of the secretin receptor, quenching of the fluorescence of Alexa Fluor 488-labeled secretin was enhanced by the presence of a non-hydrolyzable GTP analog when the label was attached at two internal amino acids of the peptide sequence, suggesting that these regions become more solvent-exposed when the secretin receptor is in the active state. Corresponding changes in anisotropy and fluorescence lifetime were also observed for labels at these two positions [135].

Fluorescent analogs of the yeast mating pheromone α-factor have been extensively used to probe the environment of the ligand binding site of the α-factor receptor Ste2p. Naider and co-workers found that, in contrast to other positions on the 13-residue peptide ligand, Lys^7, could be modified by attachment of the small environmentally-sensitive fluorophore NBD with minimal effects on signaling responses [62,64,82]. Binding of the Lys^7 derivative, [K^7(NBD),Nle^{12}] α-factor, to Ste2p results in blue-shifting of the fluorescent emission, an increase in the intensity of emission, and enhanced resistance to collisional quenching by iodide, compared to the fluorescent properties of this same ligand in aqueous solution. These changes indicate that binding

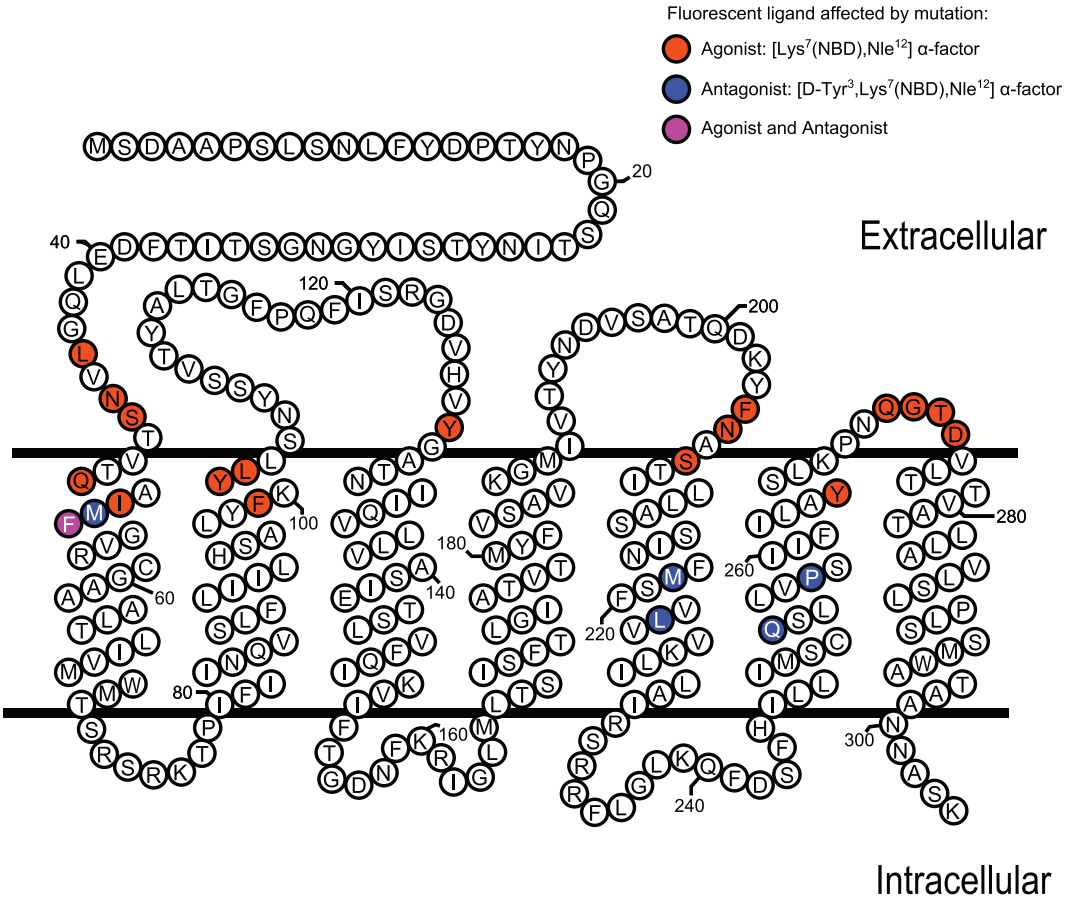

Fig. 2. Predicted topology of a C-terminally truncated form of the α-factor receptor Ste2p indicating the positions of residues identified as affecting the fluorescence emission of labeled agonist and antagonist [100]. The residues indicated in red or purple are the sites of mutations that red-shift the emission wavelength of receptor-bound agonist [K^7(NBD),Nle^{12}] α-factor. These sites are postulated to interact directly with the ligand, rendering the environment of the fluorophore more polar. The residues indicated in blue or purple are sites at which mutations lead to blue-shifting of the receptor-bound antagonist [DTyr^3,Lys^7(NBD),Nle^{12}]α-factor, indicative of transfer to a less polar environment. Mutations at these sites are postulated to act indirectly on the fluorescent ligand by shifting the overall receptor conformation to a state resembling that of the activated receptor, since they lead to constitutive signaling activity and enhanced affinity for antagonist without, in most cases, affecting the spectrum of bound agonist.

to receptor results in a reduction in the accessibility to solvent of the side chain of this Lys^7. However, this reduced accessibility does not result in deep burial of the Lys^7 side chain, since changing the Lys spacer arm by even a single carbon atom results in significant alteration of emission properties and susceptibility to quenching [64].

Our laboratory [100] used a genetic screen in conjunction with fluorescent ligand binding to identify the regions of the receptor that are differentially involved in interactions with the normal agonist, α-factor, and with a series of known peptide antagonists for this receptor [136–138]. The results are summarized in Fig. 2. This approach was based on the establishment of flow cytometric methods for monitoring ligand binding by Sklar and co-workers [58,80], and on the application of flow cytometry to genetic screening of large mutational libraries in yeast developed primarily by Wittrup and co-workers [139,140]. It was also based on the discovery of binding-dependent fluorescence changes in the intensity and wavelength of fluorescence emission of the ligand [K^7(NBD),Nle^{12}] α-factor and other related α-factor analogs [62,64,100].

To identify residues in the receptor that are close enough to the NBD fluorophore of the bound ligand to affect its fluorescence emission, we conducted a screen for amino acid substitutions in receptors that alter the emission spectrum of the NBD on bound ligand, while retaining normal receptor conformation, expression levels, and targeting to the plasma membrane (based on fluorescent ligand binding). This was accomplished by creating a library of yeast cells expressing randomly mutagenized *STE2* genes encoding α-factor receptors, then using fluorescence activated cell sorting of cells pre-incubated with NBD-labeled ligand to collect cells that exhibit an altered emission spectrum, based on detection of changes in the ratio of emission intensities in two different wavelength channels of the cell sorter. By performing the cell sorting in the presence of a relatively low concentration of ligand, and sorting for cells that exhibit high overall levels of bound fluorescence, we were able to directly screen for cells expressing mutant receptors that retain high affinity for ligand (indicating that they retain a native-like overall fold) and that are present at the cell surface at nearly normal levels. A set of 18 amino acid substitutions in the α-factor receptor that cause red-shifting of the bound ligand analog was identified. A spectral shift in this direction is suggestive of mutation-induced changes that increase exposure of the labeled region of α-factor to the aqueous solvent. No substitutions causing blue-shifting were recovered. Despite the fact that the mutagenized regions extended over all the predicted loops and transmembrane segments of the receptor, the 18 mutations that were recovered were all located in a region predicted by topological models to reside at the interface between the transmembrane helices and the external loops. Several of the identified mutations occurred at residues that had already been implicated in ligand contact or recognition, however some mutations were also found at sites that had not been previously implicated in interactions with ligand. None of the recovered mutations resulted in any significant alteration of the fluorescence emission of an α-factor analog labeled with NBD at a position that corresponds to His^3 of the normal pheromone. This analog binds the α-factor receptor with somewhat lower affinity than the Lys^7

derivative. Furthermore, in accordance with the conditions of the screen, none of the recovered mutations significantly altered the affinity of the receptor for α-factor [100].

Based on fluorescent analyses, interactions between the α-factor receptor and fluorescent antagonists were found to differ drastically from those involving the agonist [100]. The characterized antagonists for the α-factor receptor are peptides that differ from the normal agonist, α-factor, in that they contain truncations or alterations in the first few amino-terminal amino acids. They can be labeled with the fluorophore NBD at a position corresponding to Lys^7 of α-factor, the same site used to label the normal agonist. When bound to normal α-factor receptors, these labeled antagonists exhibit emission spectra that are significantly red-shifted compared to the spectra of receptor-bound NBD-labeled α-factor, indicating that the labeled moiety of the antagonist resides in a more polar environment than is the case for similarly labeled agonist. A mutational screen similar to that conducted for labeled agonist was performed for the labeled antagonist [DTyr^3,Lys^7(NBD), Nle^{12}]α-factor. Surprisingly, the mutations recovered from this screen turned out to be completely different from those found in the screen using the labeled agonist in the following respects: 1) They resulted in blue-shifting of the emission spectrum, indicating that they make the environment of the ligand-attached fluorophore more hydrophobic. 2) They occurred at positions with diverse predicted topologies in the receptor, suggesting that the observed changes in emission spectrum were not the result of changes in residues in direct contact with ligand. 3) All the mutated variant receptors recovered in the antagonist screen were capable of initiating signaling responses upon binding ligands that act as antagonist toward normal α-factor receptors and most of them resulted in enhanced constitutive signaling. 4) Most of the recovered mutations resulted in enhanced binding affinity for ligands that act as antagonists for normal receptors. The changes in fluorescence emission of ligand bound to these mutant receptors, together with the changes in binding affinity and signaling function, indicate that the mutations recovered from the antagonist-based screen cause a global switch in the receptor conformation toward a state that is easier to activate. This altered receptor conformation also leads to decreased solvent accessibility of ligands that act as antagonists toward normal receptors. These results are consistent with a model in which interactions of the C-terminal portions of α factor-related peptides with one set of surfaces on the receptor are responsible for the overall high binding affinity for receptor, whereas interactions of the N-terminal regions of these peptides with a second set of surfaces on the receptor mediate activation of signaling responses [100].

2.4. Use of fluorescent ligands to monitor the kinetics of ligand binding to GPCRs

Measurement of the kinetics of ligand interactions with GPCRs and comparison of these kinetics with the time scales of ligand-dependent conformational changes in receptors and of receptor interactions with downstream components such as G proteins, kinases, and arrestins are of great importance for understanding receptor function. The ability to monitor the progress of receptor–GPCR interactions in real time has been recognized as an important capability of fluorescent approaches since the early applications of fluorescent ligands [53,54] and extends today into the possibility of measuring the protein–ligand interactions at the single molecule level on very short time scales. A significant advantage of fluorescent approaches for measuring binding kinetics is the capability for following the progress of a binding reaction in homogeneous solutions without the need to separate free ligand from bound and perform time-consuming washing steps. Kinetic measurements may be conducted by monitoring time-dependent changes in fluorescence emission, such as changes in quantum yield or emission wavelengths, or by using microscopy or flow cytometry to simply quantitate the amount of fluorescent ligand associated with cells as a function of time. The time stamp function provided by most flow cytometers makes it possible to readily display time-dependent fluorescence changes in a population of cells. For situations where it is necessary to measure fluorescence changes at time scales shorter than what can be conducted in conventional fluorimeters, microscopes, or flow cytometers, fluorescence can be measured in stop-flow instruments or by using specially designed rapid mixing flow cytometers [141–144].

In an early study using flow cytometry to monitor the kinetics of binding of fluorescein-labeled formyl peptide to chemotactic receptors on human neutrophils, Sklar et al. [54] were able to recapitulate previously reported experiments that measured binding kinetics using radiolabeled ligand. They determined a single bimolecular association rate of 10^8–10^9 M^{-1} min^{-1}, consistent with binding via a diffusion controlled reaction to a site with partially restricted accessibility. They also measured dissociation rates, finding that multiple processes with different rate constants were required to fit the data. This may reflect a multiplicity of fates for ligand-bound receptors, including internalization and sequestration into specialized regions of membrane.

More complex kinetics of association between GPCRs and ligands have been observed in a number of cases. Based on FRET measurements, the time-dependence of binding of Texas Red-labeled neurokinin A, an agonist for the neurokinin NK2 receptor, to EGFP-tagged receptors can be described by two kinetic components, a rapid rate that depends on the concentration of ligand (2×10^6 M^{-1} s^{-1}) and a slower rate (~0.05 s^{-1}) that was not linearly dependent on ligand concentration [110]. The data could be fit by assuming that association proceeds via an initial bimolecular association that is followed by an isomerization of the complex. The time course of the rapid binding step correlated well with that of the calcium response triggered by the receptor, whereas cyclic AMP (cAMP) responses correlated better with the slower step. Thus, the initial binding event may result in transition of the receptor to an activated state for calcium signaling followed by a slower conversion to the activated state for cAMP signaling. A natural N terminally-truncated variant of neurokinin A that elicits only the calcium response exhibited only the rapid phase of binding, suggesting that interaction of the receptor with the N-terminal region is responsible for the slower cAMP response. These approaches were subsequently extended to the analysis of additional ligands and mutant NK2 receptors [109].

A two-stage mechanism has also been used to describe the kinetics of association of tetramethyl rhodamine-labeled parathyroid hormone (PTH) with GFP-tagged PTH receptor [108]. As was observed for the neurokinin receptor, binding could be described by a fast phase that depended linearly on the concentration of ligand (apparent rate constant of ~10^6 M^{-1} s^{-1}) and a concentration-independent slower phase (rate constant ~1 s^{-1}) that could be modeled as an isomerization following binding. Competition with a truncated PTH derivative known to bind only to the transmembrane regions or loops of the PTH receptor, but not to its large extracellular N-terminal region, resulted in elimination of the slower phase of PTH binding. The time course of receptor activation, monitored via a fluorescent assay for cAMP production and via FRET changes in a doubly-labeled PTH receptor [145], corresponded to the slower phase of the changes in receptor–ligand FRET. This led to the interpretation that the rapid phase of binding represents an interaction of the full-length agonist with the N-terminal domain of the receptor, which is then followed by a conformational rearrangement to a signaling-competent state involving interaction of ligands with the transmembrane region of the receptor.

The kinetics of binding of the yeast mating pheromone α-factor to the α-factor receptor could also be conveniently studied using α-factor peptide tagged with the environmentally sensitive fluorophore NBD, ([K^7(NBD),Nle^{12}] α-factor). Global kinetic analysis of binding kinetics at different concentrations could not be effectively fit by a reaction scheme based on a single bimolecular association but, instead, was consistent with a sequential pathway involving an initial association with receptor, followed by a rearrangement or redistribution of the bound ligand. Independent support for the existence of two steps in the association kinetics came from examination of changes in the

emission wavelength of the [K^7(NBD),Nle^{12}] α-factor as a function of time during binding, based on detection of differential changes in different wavelength channels of the flow cytometer. Although the NBD fluorophore attached to α-factor undergoes a large overall blue-shift over the course of binding to receptors, this change actually takes place in two phases, an initial large blue shift followed by a smaller shift back toward longer wavelengths. Taken together, these results suggest that α-factor initially binds to its receptor in a hydrophobic environment, but that during a subsequent rearrangement it shifts so as to move the tagged lysine side chain to an environment with increased accessibility to solvent [82].

3. Fluorescence-based approaches for detecting ligand-dependent conformational changes in GPCRs

3.1. Spectroscopic approaches for detection of changes in the environment of labeled sites in receptors

Ligand binding to a G-protein coupled receptor must be associated with conformational changes in the receptor that result in activation of downstream signaling. However, the nature of such changes, the mode of their coupling to ligand binding, and the mechanisms by which they result in altered interactions with G proteins or arrestins remain unclear. There is a growing body of evidence from biophysical studies indicating that GPCRs may be capable of adopting many more than the two (active and inactive) states that served as the basis for early models [26,27]. In addition, there are numerous examples of systems in which different activated states of GPCRs resulting from the binding of different ligands lead to biased agonism, the differential activation of distinct downstream signaling pathways [146].

Diverse biophysical, biochemical, and molecular biological approaches have been applied to understanding ligand-induced conformational changes in GPCRs [25,147,148]. The recent determination of the X-ray structure of the β_2-adrenergic receptor in complex with a heterotrimeric G protein has been particularly informative in this regard [149]. However, among the biophysical approaches, fluorescence-based assays of conformational change have some significant advantages. 1) The high time-resolution of fluorescence measurements makes it possible to follow, in real time, transitions from one state to another, where the static states are defined by X-ray crystallography. 2) The sensitivity of fluorescence measurements allows monitoring of conformational changes in single molecules and small numbers of molecules, allowing analysis of conformational trajectories at a level of detail that is not possible with large numbers of molecules in bulk solution experiments. 3) Fluorescence approaches allow probing of particular regions of receptor structure into which fluorescent reporters are introduced. 4) Some fluorescent labeling approaches allow examination of receptors in their native environments in cells or membranes.

Detection of changes in the fluorescence emission of intrinsic or introduced fluorophores at different positions in GPCRs has provided important information about the nature of conformational changes in receptors (see Fig. 3). Despite the fact that intrinsic tryptophan absorbance spectroscopy provided initial evidence for conformational change upon activation of rhodopsin [150], only a few measurements of the intrinsic tryptophan fluorescence of GPCRs have been conducted, presumably because of the complexity of the spectra in proteins containing multiple tryptophan residues. Tryptophan emission changes in the leukotriene B4 receptor [151], the metabotropic glutamate receptor subtype 1 [152], and the thromboxane A2 receptor [153] have all provided indications of ligand-specific conformational changes.

Multiple labeling strategies have been used to introduce fluorescent reporters at different sites in GPCRs. One of the first studies of this kind, conducted by Kobilka and co-workers, introduced the small, membrane-permeable, environmentally-sensitive probe, IANBD (N,N′-dimethyl-N-(iodoacetyl)-N′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine) for labeling of cysteine residues in the β_2-adrenergic receptor [154]. Purified IANBD-labeled β_2-adrenergic receptors exhibited a decrease in fluorescence intensity in response to agonist binding that was reversed by addition of antagonist. A remarkable linear correlation was observed between the magnitude of the decrease and the efficacy of the different ligands, consistent with the hypothesis that the change in fluorescence reflected conformational changes associated with receptor activation. In agreement with this hypothesis, this group also showed that inverse agonists produced small, but reproducible, increase in IANBD fluorescence. A subsequent study [155] localized the relevant IANBD labeling sites in the receptor to Cys125 and Cys285, in the third and sixth transmembrane segments, respectively, implicating these two helical segments in ligand-dependent conformational changes in agreement with contemporaneous results from electron paramagnetic resonance data [156].

The Kobilka group was also able to achieve site-specific attachment of labels to Cys265 in the third intracellular loop of purified β_2-adrenergic receptors by reacting with either fluorescein maleimide or tetramethylrhodamine-maleimide (TMR) [157–159]. Binding of agonists to the receptor labeled with fluorescein at this position resulted in a decrease in fluorescence emission and in susceptibility to iodide quenching, consistent with the ligand-dependent motion of this loop, which is near the site of interaction with the G protein [158]. Receptors labeled with fluorescein at Cys265 were also studied using fluorescence lifetime measurements, to assay conformational heterogeneity of the protein [157]. Measurement of the lifetimes associated with decay of fluorescent excited states provides much of the same information that can be obtained from steady state measurements of emission, but fitting these decays to multiple exponential functions introduces the important additional capacity for distinguishing properties of different subpopulations in samples where not all molecules are behaving identically. Fluorescein attached to unliganded receptor exhibited a single broad distribution of fluorescent lifetimes, indicative of a single, but flexible, conformational state. This distribution narrowed in the presence of antagonist, suggesting that antagonist binding causes a reduction in flexibility. Binding of the full agonist isoproterenol led to the appearance of an additional class of short fluorescence lifetimes mixed in with a population of fluorescence decays with lifetimes similar to those of unliganded receptor. These results led to the important idea that, while agonist binding may lead to the appearance of a distinct activated state of receptors, at any given time, not all ligand-bound receptors adopt this state. Fluorescence lifetime experiments were also used to show that the states resulting from binding of partial agonists to the β_2-adrenergic receptor were different from the states induced by binding of the full agonist, implying that different types of agonists may cause receptors to adopt a diversity of receptor conformations not restricted to simple inactive and active states, perhaps providing a structural basis for biased agonism.

Fluorescence-based approaches were also used to specifically detect conformational changes around proposed “ionic lock” interactions between the third and sixth transmembrane segments of GPCRs [160]. Starting with a variant β_2-adrenergic receptor from which all reactive cysteine residues had been removed, a cysteine was introduced in place of Ala271 in the sixth transmembrane segment and a tryptophan was substituted for Ile135 in the third transmembrane segment. This allowed the specific introduction of a fluorescent bimane group at position 271 that could be quenched if, as seen in crystal structures, disruption of the ionic lock results in alteration of a helical twist in the sixth transmembrane, bringing the bimane into close proximity to the introduced tryptophan at position 135. Minimal quenching was observed in the absence of antagonist, but binding of different agonists led to reductions in bimane emission that could be correlated with the particular ligand structures. Ligand-dependent changes in fluorescence of a bimane group attached to Cys265 were also used to demonstrate retention of native-like responses to ligands by a modified β_2-adrenergic receptor used for X-ray structure determination [23].

The kinetics of conformational changes in the β_2-adrenergic receptor upon binding to different ligands were analyzed by Kobilka and coworkers using β_2-adrenergic receptors labeled with TMR at Cys265. This allowed the development of a detailed model in which sequential interactions of different chemical groups on ligands lead to different stages of receptor activation and internalization [161,162]. A disadvantage of the use of fluorescent reporters attached to purified receptors is the relatively slow rates of change of fluorescence emission (compared with physiological responses and changes detected by FRET) upon addition of ligands. This is most likely the result of solubilization and purification of receptors, and of conducting the analysis in detergent micelles in the absence of other cell components [160,163].

Covalently attached environmentally sensitive fluorescent reporters have also been used to monitor conformational changes in GPCRs other than the β_2-adrenergic receptor. Bimane groups attached to cysteine residues introduced into rhodopsin at different positions were used to monitor conformational changes upon light-dependent conversion to the Meta II state [164]. Quenching of similarly introduced bimane groups resulting from proximity to tryptophan residues in peptide analogs of the C-terminal of the G protein transducin was used to map the geometry of the rhodopsin–transducin interaction [165]. The mobility of fluorescent probes attached to rhodopsin was analyzed in different states of activation using time resolved anisotropy measurements [166]. Bimanes introduced at specific positions in the ghrelin receptor reconstituted into lipid nanodisks exhibited ligand-dependent changes in steady state emission and lifetime distributions [167]. In addition, changes in emission from this labeled receptor were observed upon the addition of G proteins and arrestin, providing evidence for a complex landscape of ligand- and effector-induced conformational states. Bimane labeling has also been used to study the effects of an allosteric ligand of the cannabinoid CB1 receptor, providing evidence that the ligand stabilizes an agonist-bound state of the receptor that is, surprisingly, not capable of efficient G protein activation [163].

3.2. Studies of ligand-dependent conformational changes in GPCR studies using intramolecular FRET

Changes in emission of a single fluorescent label, while providing information about changes in the environment of the specific reporter group, can be difficult to interpret in terms of actual structural changes in receptors. Furthermore, most studies involving conjugation of small environmentally-sensitive fluorophores to receptors require prior solubilization and purification of receptors, removing them from their native environments. FRET-based approaches make it possible to detect changes in distances between fluorescent groups at specific locations in receptors in the presence and absence of different ligands (see [168]). When FRET is measured between different fluorescent proteins fused to receptors, distance changes can be detected while the receptors are present in the membranes of living cells. A significant concern is the problem of distinguishing intramolecular FRET from intermolecular FRET, since FRET-based approaches are also commonly used to study the nearly ubiquitous oligomerization of GPCRs. In a number of cases, the absence of intermolecular contributions to FRET has been demonstrated by the absence of FRET observed when molecules that are separately singly labeled at the two relevant positions are mixed together or co-expressed [145,169].

Although it is convenient to conduct FRET using genetically encoded fluorescent proteins, such proteins have disadvantages compared to small fluorophores: 1) They are large, sometimes of greater mass than the proteins to which they are fused. This can lead to alterations in localization, stability, or functional properties of the proteins being studied. For example, in some cases, introduction of large fluorescent proteins [170], and even small tagging sequences [171] in the third intracellular loop of a receptor can interfere with activation of G proteins by receptors. The overall size of fluorescent proteins makes it difficult to obtain distance information for groups that are expected to be in close proximity. 2) The actual locations of the exciting and emitting dipoles in fluorescent proteins are not well-defined, and fluorophores are generally attached to proteins via flexible linkages, making it difficult to interpret FRET efficiencies in terms of structurally relevant distances. 3) Fluorescent protein tags can be subject to proteolytic cleavage separating them from the proteins to which they are supposed to be attached [172]. Thus it is important to check that FRET signals are not being interpreted based on cleaved fluorescent proteins with irrelevant locations. 4) Fluorescent proteins do not have optimal photochemical and photophysical behaviors. They are not as bright or photostable as some commonly used small molecule fluorophores, making them, for example, generally unsuitable for single molecule studies.

To avoid some of the problems with the use of fluorescent proteins, various approaches have been used for incorporating genetically encoded sequences that allow attachment of small fluorophores at specific positions to GPCRs without the need for purification of receptors. One of the most commonly used approaches is the use of small membrane permeant arsenical compounds, known as FlAsH reagents [173], that bind with high affinity to sequences with a particular configuration of four cysteine residues. Refinements in labeling conditions and acceptor sequences have rendered these reagents fairly specific for target sequences, with minimal background and toxicity to cells (see [169]). Other, less frequently used approaches for introducing small molecule labels at defined positions in receptors include the use of fluorophore-conjugated metal chelating compounds expected to bind to histidine repeats [174], enzyme tags that catalyze self-labeling reactions [175], and short sequences that can be recognized by exogenous enzymes as acceptors for labeling reactions (see [176]). However, the metal chelating compounds are relatively low affinity reagents that bind reversibly and the enzyme tags result in fusion proteins that are almost as massive as the fluorescent proteins. To date, the potential usefulness of short enzyme target sequences for labeling GPCRs does not seem to have been fully exploited.

FRET-based approaches have been applied by several groups to detecting conformational changes in β-adrenergic receptors. Initial studies used purified protein with a FlAsH reagent in the C-terminal tail and an Alexa Fluor 568 covalently attached to Cys265 (see above) [177]. The distance from the C-terminal to the third intracellular loop, calculated based on FRET efficiency was ~60 Å, indicating that the tail may adopt an extended conformation. All tested ligands produced an increase in FRET efficiency when the FlAsH reagent was positioned at the proximal end of the C-terminal tail. Results were less consistent when the FlAsH was at the extreme distal end of the tail. Different changes in FRET efficiency of labels at different sites in the C-terminal region were detected based on different types of ligand bound to the receptors. FRET between a FlAsH label in the third intracellular loop of β_2-adrenergic receptors and cyan fluorescent protein fused at the C-terminal has also been detected in living cells [178]. This study also detected an increase in FRET upon binding of ligand. The fact that the time scale of the increase was shorter than that of fluorescence changes that have been reported for studies with purified detergent-solubilized β_2-adrenergic receptors suggests that the particular structure of the surrounding amphiphilic molecules can significantly affect receptor conformational transitions, a conclusion that is also supported by NMR studies of β_2 in different detergents [179]. A subsequent study examined FRET between cyan fluorescent protein fused at the third intracellular loop of the β_2-adrenergic receptor and yellow fluorescent protein inserted into the C-terminal tail. As found in other studies, differences in the FRET efficiencies and the kinetics of FRET changes indicated that the receptor can adopt a range of conformations, consistent with the idea of biased agonism [180].

The α_{2A}-adrenergic receptor has also been the target of a number of analyses using intramolecular FRET. In initial studies of receptors containing combinations of CFP and YFP inserted into the third intracellular loop and at the C-terminal tail of receptors expressed in live cells, a decrease in FRET was detected upon addition of agonist [145,181].

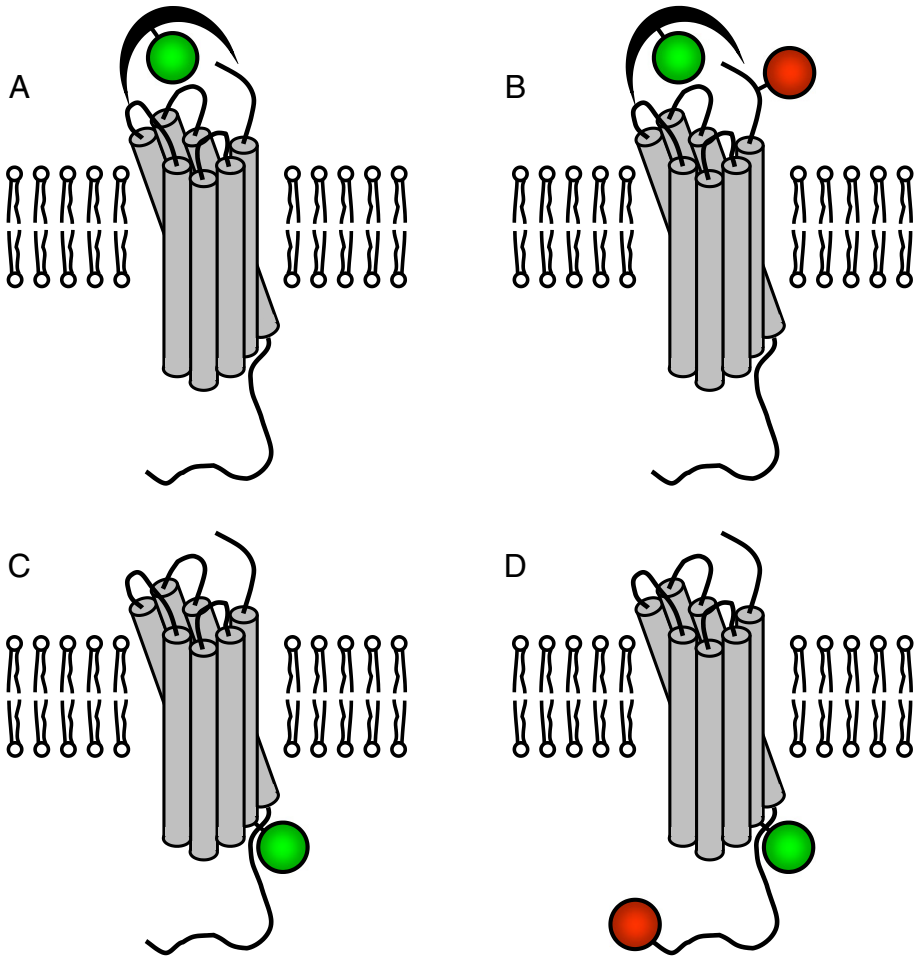

Fig. 3. Schematic diagram of four types of fluorescent experiments used to examine ligand–receptor interactions and ligand-dependent conformational changes in G protein coupled receptors. Fluorophores used as reporters are shown as colored disks. A) Use of a fluorescently tagged ligand with an untagged receptor for measuring ligand binding, determination of the environment of the bound ligand, and the kinetics of ligand binding. The ligand is represented as a black crescent. B) Use of intermolecular ligand–receptor Fluorescence Resonance Energy Transfer (FRET) to measure ligand binding, map the geometry of ligand–receptor interactions, and measure the kinetics of ligand binding. C) Use of a single fluorescent label attached to receptor (most often at the cytoplasmic end of the sixth transmembrane helix) to measure changes in conformation induced by ligand binding. D) Use of intramolecular FRET between two labels on a receptor (most often the cytoplasmic end of the sixth transmembrane helix and the C-terminal tail of the receptor) to measure distance changes induced by ligand binding. Note that, for simplicity, receptors are shown as monomers, despite a large body of evidence that they exist as oligomers in membranes. Also, the figure is not drawn to scale and does not reflect that fluorophores consisting of fluorescent proteins can be of approximately the same size as the entire receptor.

Again, the time course of the observed changes (<40 ms) was much more rapid than had been observed with previous studies of conformational change in purified detergent solubilized receptors [145]. Binding of inverse agonist results in a FRET change that is opposite to that induced by agonist binding [182]. Similar conformational changes in the α_{2A}-adrenergic receptor, as well as a correlation between agonist efficacy and the half-time of receptor activation were detected using constructs containing FlAsH bound to the third intracellular loop and CFP fused at the C-terminal [183]. The short time resolution of the system was also exploited in order to measure the dependence of the kinetic parameters of the receptor response on ligand concentration, providing a view of the pharmacology of the system that may be more relevant to what happens in cells than typical long-term assays where the components are all allowed to reach an equilibrium state. The change in receptor conformation reported by FRET changes occurred on a faster time scale than activation of G protein, assayed using a separate FRET reporter system [184].

Intramolecular FRET has also been used to monitor activation of muscarinic acetylcholine receptors. An M2 receptor construct with a FlAsH tag in the third intracellular loop and CFP at the C-terminus, expressed in HEK cells, exhibited a decrease in FRET in response to the agonists acetylcholine and carbachol, though at levels of agonist that were higher than are typically required to elicit responses in other assays [185]. Allosteric negative modulators of signaling, tested by themselves, had little or no effect on FRET, but inhibited the responses of orthosteric agonists. Similar FRET reporter systems based on FlAsH and CFP have been constructed for M1, M3, and M5 muscarinic acetylcholine receptors and a reporter based on CFP and YFP has been used to study the M1 receptor [170,171,186,187]. Each of these exhibits decreased FRET upon binding to agonists. A FRET construct based on the M3 receptor was used to examine the kinetics of activation and deactivation of a constitutively active M3 variant. The mutation's effects could be explained by a decrease in the rate of deactivation rate better than by an increase in activation rate, a conclusion that would be difficult to draw without the time resolution that is possible with FRET measurements [188].

Among other receptors studied by intramolecular FRET, the parathyroid hormone receptor stands out for its slow kinetics of activation (~1 s time constant, compared with <40 ms for the α_{2A}-adrenergic receptor measured in parallel) [145]. Conformational changes have also been detected based on FRET changes in the A_{2a}-adenosine receptor [169] and the bradykinin B_2 receptors [189]. FRET measurements have also been used as evidence for changes in the conformation of the bradykinin receptor in response to fluid shear stress.

4. Single molecule fluorescent studies of GPCRs

Major technological advances allowing detection and fluorescence-based imaging of single molecules have, to date, had relatively little impact on understanding mechanisms of ligand dependent signaling by GPCRs, although such approaches have found important applications in characterizing the oligomeric states of receptors and their dynamics in cell membranes [190–194]. A commonly used approach for single molecule fluorescence is to tether the molecule of interest to a solid surface in order to keep it in the field of view for the longest possible time. Such tethering has been reported for single CCR5 chemokine receptors [195]. However, it is not clear to what extent tethering could affect the dynamics of receptors being studied (see [196]). Analysis of photon bursts from freely diffusing single solubilized β_2-adrenergic receptors specifically labeled with fluorescein at Cys265 led to the conclusion that the attached fluorophore experiences more than one environment, even under native, unliganded conditions. Binding of the agonist isoproterenol significantly reduced the burst size (equivalent to a reduction in emission in bulk experiments), while retaining heterogeneity of burst sizes, indicative of heterogeneity of environments of the label. Such heterogeneity could be the result of rapid binding and unbinding of ligand [197]. A subsequent study overcame limitations on the length of time over which it is possible to observe freely diffusing molecules through the use of an "Anti-Brownian ELectrokinetic" (ABEL) trap to retain individual β_2-adrenergic receptors labeled at Cys265 with tetramethylrhodamine in the field of view for up to many hundreds of milliseconds. This approach allowed monitoring of the intensities and lifetimes of fluorescence emission of individual molecules for long enough to obtain well-defined measurements and even observe discrete transitions of individual receptors between different states. The transitions occurred over time scales ranging from milliseconds to seconds. A 2D "map" of intensity and lifetime generated from the single molecule data showed differences in the occupancy of different intensity and lifetime states between the ligand-free receptor and agonist-bound receptor. However, both appeared to sample a wide variety of different environments [198].

5. Outlook

The contributions made by fluorescence-based approaches for studying ligand-dependent signaling by GPCRs are likely to continue. It is to be hoped that a greater proportion of the wide variety of fluorescent ligands reported in literature will become available commercially, or by inter-laboratory exchange. These ligands will continue to be useful for high-throughput screening for new GPCR ligands, for understanding the mechanisms underlying the relationship between the binding of different ligands and receptor activation, for the development of thermostable receptors for structural studies, and as a replacement for radioligands in routine binding assays. It can also be anticipated that the application of new and improved technologies to fluorescent reporters and FRET pairs coupled to receptor proteins will be valuable in extrapolating from the rapidly growing wealth of structural information about GPCRs to an understanding of the mechanisms and dynamic aspects of receptor signaling and regulation. Technologies that are likely to be critical for progress in this area include: 1) improved tagging procedures for specifically introducing small fluorophores at specific positions in receptors in living cells; 2) improved approaches for immobilizing and trapping receptors for single molecule spectroscopy; 3) introduction of new technologies that could greatly enhance detection of weak fluorescence emission, such as enhancement by metals [199] and optical antennas [200]; 4) procedures for simultaneously collecting and integrating intensity, anisotropy, and lifetime data in single molecule experiments; 5) continuing optimization of the fluorescent, biochemical, and pharmacological properties of fluorescent ligands for particular receptors; 6) integration of fluorescence spectroscopy with newly-developed technologies for super-resolution imaging [201]; and 7) application of quantum dots to molecular spectroscopy for understanding receptor function. Although they provide bright fluorescent sources with useful spectral properties, the relatively large dimensions of quantum have prevented them from being useful as environmental probes of particular regions of proteins [202,203].

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