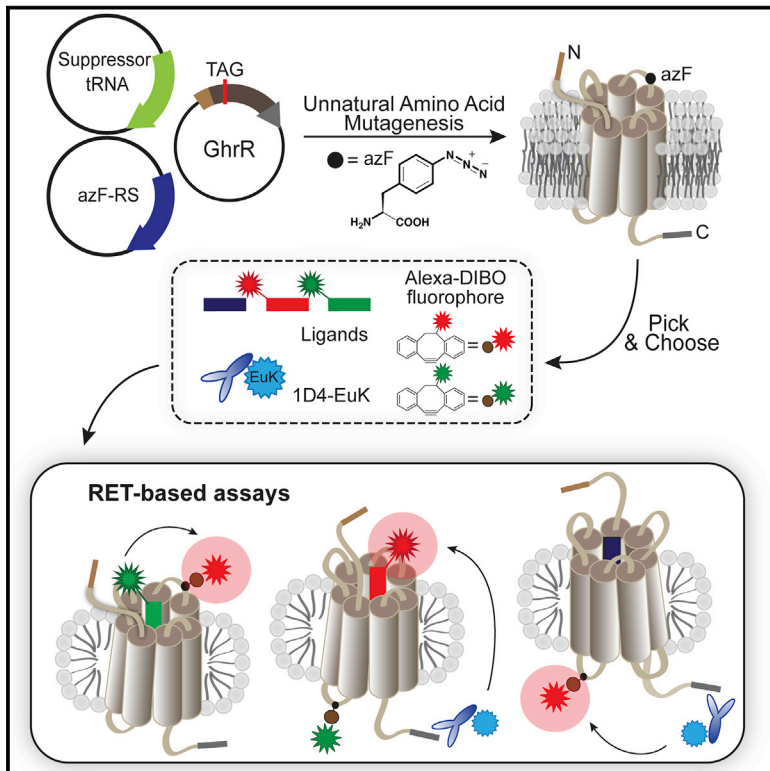# Chemistry & Biology

Bioorthogonal Labeling of Ghrelin Receptor to Facilitate Studies of Ligand-Dependent Conformational Dynamics

Graphical Abstract

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In Brief

Understanding ligand-induced conformations and consequences in ghrelin receptor signaling might assist in drug design. Park et al. use bioorthogonally labeled ghrelin receptors and show that RET-based approaches can reveal distinctive ligand-induced RET signals that are sensitive to inter- and intramolecular conformational changes.

Highlights

- Functional GhrR was site-specifically tagged with the unnatural amino acid, azF

- GhrR azF mutants were labeled bioorthogonally with a fluorophore using SpAAC

- Novel assays were developed to probe binding of fluorescent ligands to labeled GhrR

- Ligand-induced conformational changes could be detected in fluorescent GhrR

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Bioorthogonal Labeling of Ghrelin Receptor to Facilitate Studies of Ligand-Dependent Conformational Dynamics

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SUMMARY

Ghrelin receptor (GhrR) is a promising drug target because of its central role in energy homeostasis. GhrR, known for high constitutive activity, is thought to display multi-state conformations during activation and signaling. We used genetically encoded unnatural amino acids and bioorthogonal labeling reactions to engineer multiple fluorescent donor-acceptor pairs to probe ligand-directed structural changes in GhrR. We demonstrate how conformational dynamics of a G-protein-coupled receptor can be measured in reconstituted systems.

INTRODUCTION

Ghrelin receptor (GhrR), a rhodopsin-like G-protein-coupled receptor (GPCR), is central to various physiological processes, including glucose and lipid metabolism, appetite control, and food intake (Kojima and Kangawa, 2005; Mokrosinski and Holst, 2010). GhrR and its endogenous ligand, ghrelin, have been established as drug targets for metabolic disorders such as obesity and type 2 diabetes mellitus (Cong et al., 2010). However, several ghrelin antagonists have failed to advance as anti-obesity therapeutics due to adverse side effects and lack of efficacy (Chollet et al., 2009). Along with unusually high ligand-independent (constitutive) activity (Holst et al., 2003), GhrR is particularly notable for its propensity for biased signaling through $G\alpha_{q/11}$, $G\alpha_i$, $G\alpha_{12/13}$, and β-arrestin (Sivertsen et al., 2013; Evron et al., 2014). As GPCRs are highly dynamic membrane proteins with different functionally relevant conformational states, biased signaling is thought to arise from the ligand-specific conformations of active receptors (Manglik and Kobilka, 2014; Shukla et al., 2014). GhrR thus provides a useful platform to study the relationships between constitutive activity and ligand-induced stabilization of receptor conformations that are relevant to drug design and discovery.

Various techniques have been used to study the conformational dynamics of GPCRs including nuclear magnetic resonance, electron paramagnetic resonance, and Fourier-transform infrared spectroscopy (Manglik and Kobilka, 2014). Compared with these methods, fluorescence techniques provide superior sensitivity when considering studies of engineered membrane proteins. The application of fluorescence methods, however, has been technically challenging due the lack of general and specific chemistries to label receptors with small organic fluorescent tags, and perturbations caused by the large sizes of fluorescent protein fusion tags (Lohse et al., 2012; Huber and Sakmar, 2014). Using GhrR as a model, we developed a novel fluorescence approach that employs resonance energy transfer (RET) between a site-specifically tagged fluorophore on a minimally engineered GhrR and either a fluorescent ligand or an europium cryptate (EuK)-conjugated monoclonal antibody (mAb), which is sensitive to functional and conformational changes in GhrR.

RESULTS AND DISCUSSION

GhrR Remains Functionally Active after Genetic Encoding of azF

To create a panel of GhrR variants with a site-specific handle for subsequent fluorescent labeling, we introduced an unnatural amino acid, *p*-azido-L-phenylalanine (azF), to various sites in GhrR expressed in mammalian cells using amber codon suppression technology (Ye et al., 2008; Huber and Sakmar, 2014) (Figure 1A). Full-length azF-modified GhrR variants were only expressed when azF was present in the culture medium (Figures 1B and S1). We next assessed the azF-encoded GhrR-mediated functional properties in cell-based assays with readouts like cellular ligand uptake and G_q-mediated inositol phosphate accumulation upon ligand stimulation (Figures 1C and 1D; Table S1). We observed that the potency (EC_{50}) of ghrelin agonism in azF-GhrR mutants mostly remained comparable with that of wild-type (wt)-GhrR. We also found that E_{max} values positively correlated with the cell-surface expression level of receptor variants (Table S1). Immunofluorescence studies of GhrR modified with azF at position 158 revealed that its ligand-independent and ligand-dependent trafficking profiles were similar to those of wt-GhrR (Figure S1).
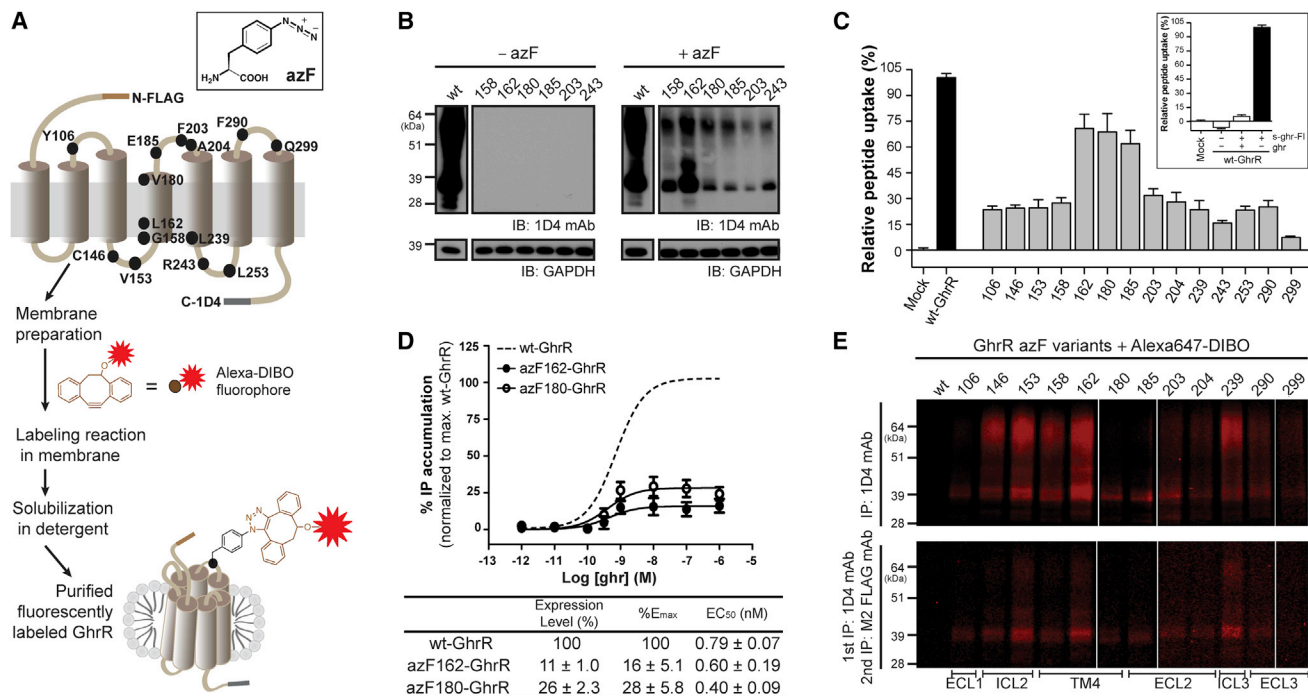
Figure 1. Pharmacological Characterization and Site-Specific Bioorthogonal Labeling on azF-Encoded GhrR Variants Expressed in Mammalian Cells

(A) Schematic of GhrR with sites for azF tagging (black circles) and a labeling workflow with Alexa fluorophore (red star) conjugated to DIBO (brown).

(B) Immunoblot analysis for azF-GhrR mutants. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a loading control.

(C) GhrR-mediated peptide ligand uptake. After the incubation with 1 μM s-ghr-Fl for 30 min, cells were lysed and the relative fluorescence intensity of internalized s-ghr-Fl in cell lysate was determined as a measure of receptor functionality. The specificity was confirmed by the complete reduction in fluorescence intensity when wt-GhrR was simultaneously treated with s-ghr-Fl and a saturating concentration of unlabeled ghrelin (ghr) (see inset graph). Each data point represents mean ± SEM (n = 3).

(D) Ghrelin-induced inositol phosphate (IP) accumulation. %E_{max} and EC_{50} values of representative azF-encoded receptors, azF162- and azF180-GhrR, were expressed as mean ± SEM (n = 3).

(E) In-gel fluorescence images of labeled GhrR variants. Alexa 647-labeled GhrR receptors were enriched by 1D4 mAb (C-terminal 1D4 epitope; upper panel) and further by M2 FLAG mAb (N-terminal FLAG epitope; lower panel).

See also Figures S1–S3 and Table S1.

AzF-GhrR Variants Were Fluorophore-Tagged via Bioorthogonal SpAAC Reaction

Selected functional azF-GhrR variants were then labeled with alkyne-bearing Alexa-dibenzocyclooctyne (DIBO) fluorophore (Figure S2) using strain-promoted [3 + 2] alkyne-azide cycloaddition (SpAAC), which allows high specificity (Debets et al., 2011; Huber et al., 2013; Tian et al., 2014). The labeling reactions were carried out in crude plasma membrane preparations to target properly folded cell-surface-expressed receptors and provide a native-like environment over the course of reaction. By doing so, the harsh denaturing/renaturing procedures used in previous biophysical studies for GhrR (Damian et al., 2012, 2015; Mary et al., 2012) can be avoided. Using optimized conditions (Figure S2), azF-GhrR variants were labeled with Alexa 647-DIBO. The labeled receptors were solubilized and then enriched using 1D4 mAb against the C-terminal 1D4 epitope (Figure 1E, upper panel) and further using M2 FLAG mAb against the N-terminal FLAG tag (Figure 1E, lower panel). These in-gel fluorescence images showed distinct fluorescent bands corresponding to the purified Alexa 647-labeled receptors. The relative ratios of label to receptor (Alexa 647-fluorescent band/1D4 band) indi-

cated that azF tags on GhrR variants could be bioorthogonally labeled with suitable fluorophores while wt-GhrR exhibited minimal background labeling (Figure S2).

FRET Signal Serves as a Measure of the Functionality of Labeled GhrR

We measured fluorescence resonance energy transfer (FRET) between Alexa 647-labeled GhrR and s-ghr-Fl, a stabilized ghrelin analog with fluorescein at Lys-20 (Figure S3), to monitor the formation of receptor-ligand complex in detergent micelles. In pilot experiments, six azF-GhrR variants with Alexa 647 fluorophore at different sites on the extracellular loops were evaluated for FRET efficiency (Figure 2A). The extent of FRET was calculated as a ratio of fluorescence intensity of Alexa 647 at 665 nm (F665, acceptor) normalized to the fluorescence intensity at 525 nm (F525, donor) after excitation of the fluorescein donor at 488 nm. Labeled GhrR variants showed an increased FRET response of up to 61% of that of wt-GhrR (Figure 2B). By comparing relative FRET ratios among the six mutants in the presence and absence of excess unlabeled ghrelin, we identified position 180 as the most suitable and non-perturbing Alexa 647
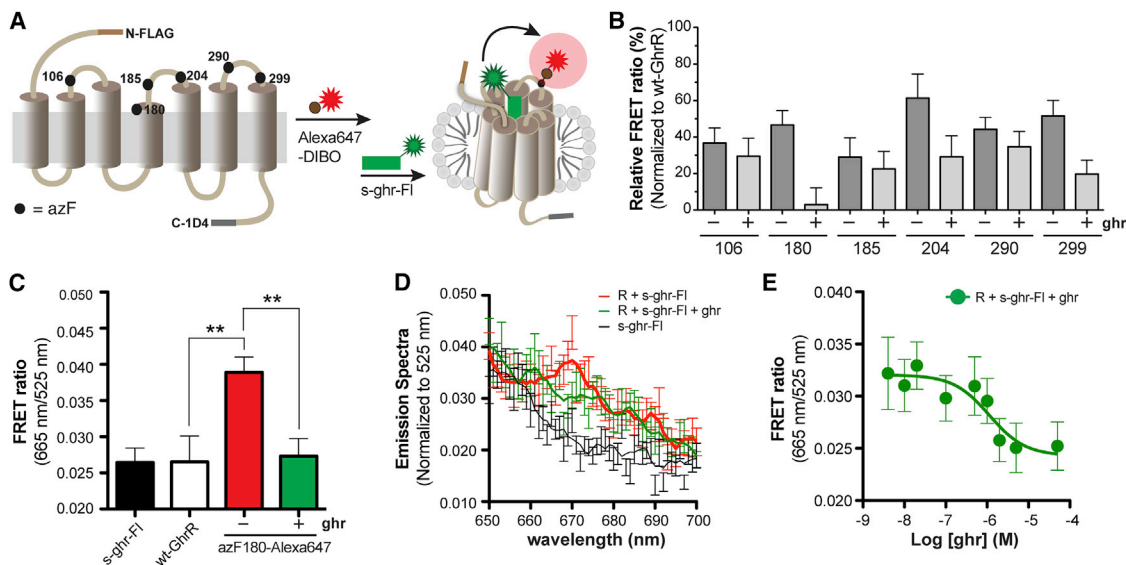
Figure 2. FRET Readouts as a Measure of Ligand-Receptor Complex Formation

(A) The principle of FRET between a donor (fluorescein of s-ghr-Fl) and an acceptor (Alexa 647 tagged on GhrR).

(B) Relative FRET ratio change (%) of Alexa 647-GhrR variants was calculated in the presence and absence of excess unlabeled ghrelin (ghr).

(C) Specificity and reproducibility of FRET signal for azF180-Alexa647-GhrR. Competition with 100-fold excess ghr abolished the FRET response. The statistical significance was evaluated by t-test, and the corresponding p values (**p < 0.01) relative to wt-GhrR were obtained using GraphPad Prism 5.0.

(D) Representative normalized emission spectra of azF180-Alexa647-GhrR (denoted as R). Emission spectra of azF180-Alexa647-GhrR were obtained when alone, in the presence of s-ghr-Fl, or in presence of both s-ghr-Fl and the excessive unlabeled ghrelin.

(E) Dose-dependent FRET reduction of azF180-Alexa647-GhrR with unlabeled ghrelin (ghr). Each data point represents mean ± SEM performed in duplicate (n = 3) and is fitted by non-linear curve fitting using "log (inhibitor) versus response (three parameter)" in GraphPad Prism 5.0.

See also Figures S2 and S3, and Table S2.

tagging site to monitor ligand binding. Using azF180-Alexa647-GhrR as a model system, we validated the reproducibility and specificity of FRET reads (Figures 2C and 2D). We further confirmed the specificity of FRET response by demonstrating that unlabeled ghrelin displaced the fluorescent donor, s-ghr-Fl, with pK_i of 8.2 (Figure 2E). This value is in a good agreement with pK_i reported in the previous literature and also with pK_i derived from a radioligand competition assay in this study (7.8–8.1 and 8.4, respectively) (Table S2). These experiments demonstrate the proof of concept that a bioorthogonally labeled GhrR expressed in mammalian cells in culture can be used for RET-based ligand binding assays.

HTRF Signal Reflects the Structural Integrity of Labeled GhrR

Next, we established a complementary assay based on homogeneous time-resolved fluorescence (HTRF) technology to evaluate the structural integrity of labeled GhrR variants regardless of the fluorophore location. We adapted our previously reported HTRF assay (Knepp et al., 2011), which utilized the dual-wavelength detection of donor (615 nm) and acceptor (665 nm) fluorescence after excitation of EuK at 320 nm. We used EuK-labeled 1D4 mAb (1D4-EuK; donor) and a fluorescent ligand, substance P analog labeled with Atto647N fluorophore (Atto647N-SPA; acceptor, Figure S3) as a RET pair (Figure 3A). The simultaneous binding of 1D4-EuK and Atto647N-SPA to GhrR ensures that the HTRF signals are only obtained from full-length functional receptors. By demonstrating the dynamic

range of HTRF changes over increasing concentration of receptor, we first showed that Atto647N-SPA binds to wt-GhrR in a saturable and specific manner (Figure 3B). We then chose position 162 at the end of transmembrane (TM) 4 for Alexa 488 tagging, because it was tolerant to azF incorporation and faces away from the RET pair, thus preventing any potential interference with HTRF signals. We subsequently demonstrated that the HTRF response from azF162-Alexa488-GhrR was comparable with that of wt-GhrR (Figure 3C) with only a 3-fold difference in K_D of Atto647N-SPA (Table S3).

We validated the specificity of HTRF readouts by competition experiments using a nonapeptide (C9) that competes for binding of 1D4-EuK to the engineered GhrR (Figure 3D). The RET signals of wt-GhrR and selected Alexa 488-labeled variants representing each topological domain in GhrR (positions 162, 180, 203, and 243) decreased as the concentration of C9 increased (Figures 3E and 3F). Noticeably, the C9 peptide exhibited a wide range of IC_{50} values (0.8–6.3 μM), which we attribute to the varied cell-surface expression level of azF-GhrR variants (i.e., the amount of receptor tested in each experiment). Nevertheless, these results show that labeled GhrR variants have normal functional activity with respect to ligand binding.

HTRF Signal Features Ligand-Directed Structural Changes in Labeled GhrR

We next reconfigured the HTRF assay for monitoring ligand-specific conformational changes in GhrR. To do so, we directly anchored the acceptor fluorophore (Alexa 647) to GhrR instead
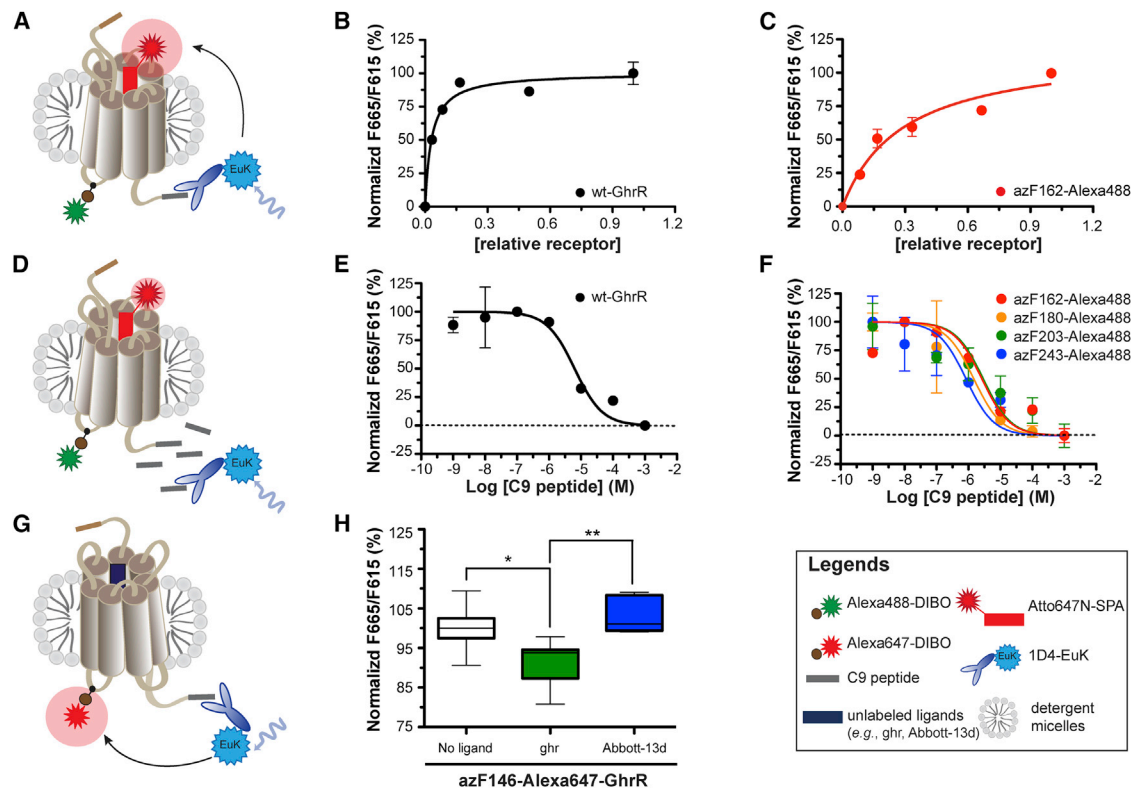
Figure 3. HTRF Assay of GhrR Function

Various HTRF assay formats were designed to evaluate receptor function and conformational changes upon ligand addition.

(A–C) Binding of Atto647N-SPA to serially diluted wt-GhrR and azF162-Alexa488-GhrR. HTRF signals only occur when Atto647N-SPA (acceptor) binds to functioning receptors with full length recognized by 1D4-EuK (donor). Data were fitted by non-linear regression analysis, assuming a single binding site.

(D–F) Validation of specificity of HTRF response using C9. The C9 peptide competes against the interaction between 1D4-EuK and receptor.

(G and H) An HTRF format for monitoring ligand-induced conformational changes in GhrR. Receptors were treated with 5 μM ligands and HTRF changes were monitored. At least three independent experiments in duplicate were carried out, and data are expressed as mean ± SEM. Non-linear regression analysis for curve fitting and statistical analysis using the t-test with corresponding p values (*$p < 0.05$, **$p < 0.01$) were performed in GraphPad Prism 5.0.

Bottom right panel shows color-coded symbols for (A), (D), (G), and (H). See also Figures S2 and S3, and Table S3.

of the ligand (Figure 3G). We first tested position 146 in intracellular loop (ICL) 2, because the ICLs are known to undergo structural change during receptor interaction with G proteins (Venkatakrishnan et al., 2013). Also, position 146 has been subjected to bimane labeling with intact functionality (Mary et al., 2012). The RET responses of azF146-Alexa647-GhrR were measured upon addition of the agonist ghrelin and a small-molecule inverse agonist, Abbott-13d (Figure S3). When ghrelin was added, a significantly lower RET signal was observed compared with the signal for ligand-free receptor (8.2% decrease). On the other hand, the addition of Abbott-13d caused little change in the RET signal (Figure 3H). The RET responses of three additional variants labeled at ICLs supported that distinct HTRF signals reflect specific conformations of GhrR induced by ligands (Figure S2). Since the RET acceptor tag is in ICLs and the donor 1D4-EuK binds to the C-terminal 1D4 epitope, the agonist-specific RET signal suggests structural changes involving either ICLs or the C-terminal tail. Previously, the C terminus of β₂ adrenergic receptor (β₂AR) in a purified form was shown to experience ligand-specific conformational changes, possibly by moving away from the core of the receptor (Granier et al., 2007). Furthermore, it has been established that

TM domains and the cytosolic surface of the agonist-occupied β₂AR rearrange conformationally upon receptor activation, creating a binding interface for Gα$_s$ protein (Venkatakrishnan et al., 2013). Regulatory functions of the C terminus of GhrR have been also suggested during basal or agonist-induced internalization (Holliday et al., 2007). Our data from HTRF assays are in agreement with the previous studies, providing evidence that the C-terminal tail of GhrR may undergo ligand-directed structural changes with its potential role in cellular signaling.

SIGNIFICANCE

We present a novel strategy to introduce a site-specific fluorescent tag to GhrR in mammalian cell membranes, which involves amber codon suppression to genetically incorporate azF in GhrR followed by bioorthogonal labeling with a fluorophore using SpAAC. The labeling method is site specific but general, since in principle any location on the GhrR can be labeled. The most ideal site for a fluorescent probe, however, will be where the probe is informative while not perturbing underlying receptor function. Using labeled GhrR in combination with a panel of fluorescent peptide ligands, we

developed a robust FRET-based ligand binding assay and HTRF assays that can be used to monitor ligand binding and ligand-dependent conformational changes. Our strategy complements previous biophysical methodologies used to study GhrR, because the labeling reaction is carried out under gentle conditions in a native membrane and also because the modified receptor can be studied in cell-based assays in parallel. Some promising applications of the present approach include: (1) monitoring dynamic intra- and intermolecular interactions of GhrR with other GPCRs or signalosome components (e.g., G proteins, G-protein-coupled receptor kinases, β-arrestin) in a ligand-independent and -dependent manner; (2) screening drug candidates including allosteric modulators (e.g., manipulating the orthosteric site for endogenous ligands) and functionally biased ligands (e.g., monitoring RET responses from a GPCR coupled to a certain G protein); and (3) identifying and preparing uniformly labeled GhrR suitable for single-molecule fluorescence studies. The approach described here should be transferable to any expressed GPCR that is amenable to amber codon suppression.

EXPERIMENTAL PROCEDURES

azF Tagging on GhrR

GhrR azF mutants were expressed in mammalian cells as previously reported (Ye et al., 2008) with a slight modification. In brief, the amber stop codon (TAG) was introduced into selected sites of GhrR using site-directed mutagenesis. The ratio of DNA in micrograms was optimized at 1:1:1 = pcDNA.GhrR-amb/pSVB.Yam/pcDNA.AzRS to obtain high expression. For wt-GhrR, 10-fold less pcDNA.GhrR-wt was transfected, but the total amount of DNA was maintained at the same level using an empty expression vector, pcDNA-3.1(+). See Supplemental Methods for detailed procedure.

Labeling on azF-Encoded GhrR Variants

50 μM Alexa-DIBO fluorophore of choice was added to each membrane extract and the labeling mixture was incubated at 4°C overnight on a nutator. On the following day, the labeling reaction was stopped and unreacted dye was removed by ultracentrifugation at $200,000 \times g$ for 25 min at 4°C. After two washes with buffer A (see Supplemental Methods for its composition), the labeled membranes were resuspended, aliquoted, and stored at −20°C prior to use.

Plate-Based FRET Measurements

For FRET measurements, fluorescence emission spectra from 500 to 700 nm with 1-nm increments were recorded after excitation at 488 nm (fluorescein) using a BioTek Synergy *Neo*. Samples in black-bottom 384-well microplates were uniformly mixed at room temperature before measurements. For the competitive inhibition FRET setup, s-ghr-Fl and unlabeled ghrelin with 100-fold higher concentration were added simultaneously. The FRET ratio was obtained by calculating ratio of emission of Alexa 647 (665 nm) to fluorescein (525 nm). The FRET ratio was converted to relative FRET ratio (%) by normalizing it to that of wt-GhrR using the following equation: relative FRET ratio (%) = $(F665_{sample}/F525_{sample} - F665_{wt-GhrR}/F525_{wt-GhrR})/(F665_{wt-GhrR}/F525_{wt-GhrR}) \times 100$. See Supplemental Methods for detailed procedures of sample preparation.

Plate-Based HTRF Assay Design

Preparation of 1D4-EuK, assay setup, and fluorescence measurements were carried out as previously reported (Knepp et al., 2011) with a slight modification. Dual-channel fluorescence of samples in black opaque-bottom 384-well microplates was measured using an Envision (PerkinElmer) with excitation at 320 nm (5,000 flashes per measurement per well) and emission collection in ten 200-μs windows (emission filters at 615 and 665 nm). Normalized F665/F615 was defined as $(F665_{sample}/F615_{sample} - F665_{negative}/F615_{negative})/(F665_{negative}/F615_{negative})$, where negative control values were obtained from the mixed labeled components in the absence of receptor and then expressed as a percentage. See Supplemental Methods for detailed sample preparations.

REFERENCES

Chollet, C., Meyer, K., and Beck-Sickinger, A.G. (2009). Ghrelin—a novel generation of anti-obesity drug: design, pharmacomodulation and biological activity of ghrelin analogues. J. Pept. Sci. *15*, 711–730.

Cong, W.N., Golden, E., Pantaleo, N., White, C.M., Maudsley, S., and Martin, B. (2010). Ghrelin receptor signaling: a promising therapeutic target for metabolic syndrome and cognitive dysfunction. CNS Neurol. Disord. Drug Targets *9*, 557–563.

Damian, M., Marie, J., Leyris, J.P., Fehrentz, J.A., Verdie, P., Martinez, J., Baneres, J.L., and Mary, S. (2012). High constitutive activity is an intrinsic feature of ghrelin receptor protein: a study with a functional monomeric GHS-R1a receptor reconstituted in lipid discs. J. Biol. Chem. *287*, 3630–3641.

Damian, M., Mary, S., Maingot, M., M'Kadmi, C., Gagne, D., Leyris, J.P., Denoyelle, S., Gaibelet, G., Gavara, L., Garcia de Souza Costa, M., et al. (2015). Ghrelin receptor conformational dynamics regulate the transition from a preassembled to an active receptor:Gq complex. Proc. Natl. Acad. Sci. USA *112*, 1601–1606.

Debets, M.F., van Berkel, S.S., Dommerholt, J., Dirks, A.T., Rutjes, F.P., and van Delft, F.L. (2011). Bioconjugation with strained alkenes and alkynes. Acc. Chem. Res. *44*, 805–815.

Evron, T., Peterson, S.M., Urs, N.M., Bai, Y., Rochelle, L.K., Caron, M.G., and Barak, L.S. (2014). G Protein and beta-arrestin signaling bias at the ghrelin receptor. J. Biol. Chem. *289*, 33442–33455.

Granier, S., Kim, S., Shafer, A.M., Ratnala, V.R., Fung, J.J., Zare, R.N., and Kobilka, B. (2007). Structure and conformational changes in the C-terminal domain of the beta2-adrenoceptor: insights from fluorescence resonance energy transfer studies. J. Biol. Chem. *282*, 13895–13905.

Holliday, N.D., Holst, B., Rodionova, E.A., Schwartz, T.W., and Cox, H.M. (2007). Importance of constitutive activity and arrestin-independent mechanisms for intracellular trafficking of the ghrelin receptor. Mol. Endocrinol. *21*, 3100–3112.

Holst, B., Cygankiewicz, A., Jensen, T.H., Ankersen, M., and Schwartz, T.W. (2003). High constitutive signaling of the ghrelin receptor—identification of a potent inverse agonist. Mol. Endocrinol. *17*, 2201–2210.

Huber, T., and Sakmar, T.P. (2014). Chemical biology methods for investigating G Protein-coupled receptor signaling. Chem. Biol. *21*, 1224–1237.

Huber, T., Naganathan, S., Tian, H., Ye, S., and Sakmar, T.P. (2013). Unnatural amino acid mutagenesis of GPCRs using amber codon suppression and bioorthogonal labeling. Methods Enzymol. *520*, 281–305.

Knepp, A.M., Grunbeck, A., Banerjee, S., Sakmar, T.P., and Huber, T. (2011). Direct measurement of thermal stability of expressed CCR5 and stabilization by small molecule ligands. Biochemistry *50*, 502–511.

Kojima, M., and Kangawa, K. (2005). Ghrelin: structure and function. Physiol. Rev. *85*, 495–522.

Lohse, M.J., Nuber, S., and Hoffmann, C. (2012). Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. Pharmacol. Rev. *64*, 299–336.

Manglik, A., and Kobilka, B. (2014). The role of protein dynamics in GPCR function: insights from the beta2AR and rhodopsin. Curr. Opin. Cell. Biol. *27*, 136–143.

Mary, S., Damian, M., Louet, M., Floquet, N., Fehrentz, J.A., Marie, J., Martinez, J., and Baneres, J.L. (2012). Ligands and signaling proteins govern the conformational landscape explored by a G protein-coupled receptor. Proc. Natl. Acad. Sci. USA *109*, 8304–8309.

Mokrosinski, J., and Holst, B. (2010). Modulation of the constitutive activity of the ghrelin receptor by use of pharmacological tools and mutagenesis. Methods Enzymol. *484*, 53–73.

Shukla, A.K., Singh, G., and Ghosh, E. (2014). Emerging structural insights into biased GPCR signaling. Trends. Biochem. Sci. *39*, 594–602.

Sivertsen, B., Holliday, N., Madsen, A.N., and Holst, B. (2013). Functionally biased signalling properties of 7TM receptors—opportunities for drug development for the ghrelin receptor. Br. J. Pharmacol. *170*, 1349–1362.

Tian, H., Naganathan, S., Kazmi, M.A., Schwartz, T.W., Sakmar, T.P., and Huber, T. (2014). Bioorthogonal fluorescent labeling of functional g-protein-coupled receptors. Chembiochem *15*, 1820–1829.

Venkatakrishnan, A.J., Deupi, X., Lebon, G., Tate, C.G., Schertler, G.F., and Babu, M.M. (2013). Molecular signatures of G-protein-coupled receptors. Nature *494*, 185–194.

Ye, S., Kohrer, C., Huber, T., Kazmi, M., Sachdev, P., Yan, E.C., Bhagat, A., RajBhandary, U.L., and Sakmar, T.P. (2008). Site-specific incorporation of keto amino acids into functional G protein-coupled receptors using unnatural amino acid mutagenesis. J. Biol. Chem. *283*, 1525–1533.