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Quaternary structure of the yeast pheromone receptor Ste2 in living cells☆

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ABSTRACT

Transmembrane proteins known as G protein-coupled receptors (GPCRs) have been shown to form functional homo- or hetero-oligomeric complexes, although agreement has been slow to emerge on whether homo-oligomerization plays functional roles. Here we introduce a platform to determine the identity and abundance of differing quaternary structures formed by GPCRs in living cells following changes in environmental conditions, such as changes in concentrations. The method capitalizes on the intrinsic capability of FRET spectrometry to extract oligomer geometrical information from distributions of FRET efficiencies (or FRET spectrograms) determined from pixel-level imaging of cells, combined with the ability of the statistical ensemble approaches to FRET to probe the proportion of different quaternary structures (such as dimers, rhombus or parallelogram shaped tetramers, etc.) from averages over entire cells. Our approach revealed that the yeast pheromone receptor Ste2 forms predominantly tetramers at average expression levels of 2 to 25 molecules per pixel ($2.8 \cdot 10^{-6}$ to $3.5 \cdot 10^{-5}$ molecules/nm^2), and a mixture of tetramers and octamers at expression levels of 25–100 molecules per pixel ($3.5 \cdot 10^{-5}$ to $1.4 \cdot 10^{-4}$ molecules/nm^2). Ste2 is a class D GPCR found in the yeast *Saccharomyces cerevisiae* of the mating type **a**, and binds the pheromone α-factor secreted by cells of the mating type **α**. Such investigations may inform development of antifungal therapies targeting oligomers of pheromone receptors. The proposed FRET imaging platform may be used to determine the quaternary structure sub-states and stoichiometry of any GPCR and, indeed, any membrane protein in living cells. This article is part of a Special Issue entitled: Interactions between membrane receptors in cellular membranes edited by Kalina Hristova.

1. Introduction

G protein-coupled receptors (GPCRs) recognize and respond to a variety of stimuli ranging from light to molecular ligands such as odorants, hormones, and neurotransmitters [1–5]. While several experiments have indicated that GPCRs form functional homo- or hetero-oligomeric complexes in vivo as well as in vitro [6–13], there have been more than occasional suggestions that not all GPCRs are multimeric or that homo-oligomerization is not essential for function [14–16]. Whether this uncertainty stems from data over-interpretation or built-in structural and functional versatility of these receptors is yet to be clarified [12, 17]. In this work, we utilized a combination of fluorescent tag-based approaches introduced recently to investigate the oligomerization properties of the yeast pheromone receptor Ste2 [18], which is often used as a general model for fungal pheromone receptors [19].

Protein interactions in living cells may be probed via detection of light from fluorescent tags attached to the proteins of interest. If an "acceptor" (A) fluorescent molecule lies within less than 10 nm of an optically excited "donor" molecule (D), it can extract energy from the donor through non-radiative Förster Resonance Energy Transfer (FRET) [20] and re-emit it at longer wavelengths. Using FRET, it has been possible to determine intramolecular distances, probe association of molecules into oligomeric complexes, determine the spatial distribution of such complexes in living cells, and assess the effects of ligand binding [7, 20–25].

Previously, a strategy was proposed for determination, at every pixel in a fluorescence image [8], of the *apparent FRET efficiency* (E_{app}), i.e., the average fraction of energy transferred within a population of donor-tagged and acceptor-tagged molecules, some of which may be interacting. The method relies on acquisition of full spectral information from sample voxels containing donors and acceptors, and their unmixing using known donor and acceptor spectra. The resulting

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pixel-level E_{app} values in an image are used to generate an E_{app} distribution (i.e., a *histogram*), rather than an average over a region of interest. Such distributions are then interpreted using models of molecular complexes (or oligomers) with certain *quaternary structures* (i.e., *sizes* and *geometries*) [8,9]. Each quaternary structure entails multiple configurations of donors and acceptors, each of which generates a specific peak in the E_{app} histograms. Taken collectively, the peaks represent a unique FRET fingerprint corresponding to a certain oligomeric structure, while the E_{app} histograms constitute veritable *FRET spectrograms*. The underlying method therefore is called *FRET spectrometry* [26].

Using this FRET spectrometry approach, it has been shown that the α-factor receptor Ste2 forms complexes as large as tetramers [8]. Ste2 is a class D GPCR found in the yeast *Saccharomyces cerevisiae* [27–30] of the mating type **a**, and binds the pheromone α-factor secreted by cells of the mating type **α**. Previous work has not been able to demonstrate whether the Ste2 tetramers were stable structures or formed by reversible association of dimers, and whether structures larger than tetramers formed at higher expression level of the receptors.

The novel FRET imaging platform introduced herein expands on the previous methods to allow determination of the proportion of different quaternary structures of the α-factor receptor in living cells for two different ranges of concentrations. We used the FRET spectrometry method to determine the dominant quaternary structure of Ste2 from FRET spectrograms. The method for determination of E_{app} histograms relied on two photon excitation of the donor at a wavelength (800 nm) at which the acceptor is virtually unexcited, concomitant with spectrally-resolved detection of fluorescence which is spectrally unmixed (i.e., deconvoluted) into donor and acceptor components for each pixel in an image. A second excitation at a wavelength (960 nm) which directly excited both the donors and the acceptors allowed determination of average values for donor and acceptor concentrations as well as E_{app} for each cell. This in turn allowed us to probe the partition of receptor populations among different quaternary structures, using the classical statistical ensemble approach [26,31,32] supplemented with geometrical information from FRET spectrometry. Our results suggested that Ste2 assumes tetrameric and octameric forms, with tetramers dominating at low concentrations.

2. Materials and methods

2.1. Sample preparation

Yeast cells (*Saccharomyces cerevisiae*) were engineered to express the sterile 2 α-factor receptor protein fused to one of two different types of fluorescent tags, i.e. either GFP_2 [33] or YFP [34], at position 304 in the Ste2 amino acid sequence. All but eight amino acids of the Ste2 cytoplasmic tail were removed for increased FRET efficiency [35]. This removed tail contains a sequence of amino acids that is required for receptor internalization and desensitization [18,36,37]. Therefore, the resulting Ste2Δtail-GFP_2 and Ste2Δtail-YFP are endocytosis defective, a property that we used to our advantage in this work. The tagged Ste2Δtail proteins do remain biologically active (see Fig. S7 in Supporting materials). Both GFP_2 and YFP are variants of the green fluorescent protein (GFP) and contain the following amino acid substitutions: F64L, A206K (GFP_2), and S65G, S72A, T203Y, A206K (YFP). This choice of fluorescent tags offers the advantage of a high spectral overlap between the donor emission and acceptor excitation spectra, as well as a low direct excitation of the acceptor, because GFP_2 has a blue-shifted excitation maximum (398 nm), compared to the commonly used eGFP. The A206K mutation was incorporated into both fluorescent tags to eliminate their natural propensity to dimerize at high concentrations [38].

The DNA constructs, Ste2Δtail-GFP_2 and Ste2Δtail-YFP, were expressed (either singly or together) from high-copy (*2 μ*) plasmids in the yeast strain KBY58 (*MATa leu2-3,112 ura3-52 his3-Δ1 trp1 sst1-Δ5 ste2Δ*) lacking a functional copy of the chromosomal *STE2* gene [39]. These haploid cells of mating type ***a*** carry a mutation (*ho*) that prevents them from switching their mating type from type ***a*** to type **α**. In this way, any homo-oligomerization of Ste2 detected in the experiments performed in the absence of the ligand α-factor is not affected by endogenous ligand from cells with switched mating type.

Yeast cells carrying one or two of the above plasmids were grown at 30 °C on synthetic complete medium lacking uracil and/or tryptophan, to provide plasmid selection. Cells gently scraped from the solid medium were suspended in 800 μL of 100 mM KCl buffer; 200 μL of cell solution was then pipetted onto 35-mm gridded glass-bottom dishes (Grid 50; Ibidi, Martinsried, Germany). Prior to adding the cell suspension, the dishes were coated with concanavalin A (Sigma Aldrich, St. Louis, MO) to prevent the cells from being moved when additional solutions were introduced to the medium. To this end, 100 μL of a 0.5 mg/mL solution of concanavalin A (in deionized water) was placed onto a gridded dish, and the dish was then covered for 30 min to allow the deposition to occur. The remaining solution was then removed and the dishes were allowed to air-dry for a minimum of 24 h. The yeast cell suspension was pipetted onto the dish prepared as above, and after allowing the cells to fully adhere to the dish for 10 min, the cells were imaged using a two-photon optical micro-spectroscope we have developed (see next).

2.2. Two-photon fluorescence micro-spectroscopy

The two-photon optical micro-spectroscope was comprised of a Nikon Eclipse Ti™ (Nikon Instruments Inc., Melville, NY) inverted microscope stand and a modified OptiMiS scanning/detection head from Aurora Spectral Technologies (Grafton, WI). The scanning/detection head was modified to incorporate a line-scan protocol and a module for automatically changing the excitation wavelength while maintaining the excitation power. The line-scan protocol leads to signals two orders of magnitude higher than those achievable with a point-scan-based system for the same line dwell time, which reduces the sample photobleaching and thereby improves the accuracy of the method [40]. A mode-locked laser (MaiTai™; Spectra Physics, Santa Clara, CA), which generates 100 fs pulses with center wavelengths tunable between 690 nm and 1040 nm and a full-width half maximum of ~7 nm, was used for fluorescence excitation. The excitation beam was focused to a line in the plane of the sample using an infinity-corrected, plan apochromat, oil immersion objective (100×, NA = 1.45; Nikon Instruments Inc.). The OptiMiS detection head employed a non-descanned detection scheme, in which the emitted fluorescence was projected through a transmission grating onto a cooled electron-multiplying CCD (EMCCD) camera (iXon X3 897, Andor Technologies, Belfast, UK). The spectral components of the fluorescence emission are separated when passing through the transmission grating, and strike the CCD camera in the form of a line perpendicular to the excitation line. The different wavelengths of light comprising the emitted fluorescence are separated as a function of pixel position on the CCD array. Therefore, the micro-spectroscopic data set captured by the OptiMiS detection system contains three-dimensional information; two of the dimensions are spatial dimensions (440 × 300 pixels), and the third dimension is wavelength, i.e. each pixel in the 2D image is sampled at 200 different wavelength channels. The spectral bandwidth of the wavelength channels ranges from 415 nm to 615 nm with a spectral resolution of ~1 nm. The optical scanning head (for laser beam scanning) and EMCCD camera used for image acquisition were controlled by the same computer using in-house custom software written in C++.

Using the OptiMiS detection system, spectral information was obtained from each sample voxel on a time scale much shorter than that which would correspond to molecular diffusion of the fluorescently labelled receptors [8]. Furthermore, by exciting the donor with a wavelength which leaves the acceptor virtually unexcited (800 nm in this work), any signal detected from the acceptor could be safely attributed to FRET. This sample scan, which we term "the FRET scan," allowed determination of both the FRET efficiency and donor concentration at pixel

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