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GPCR-Gα protein precoupling: Interaction between Ste2p, a yeast GPCR, and Gpa1p, its Gα protein, is formed before ligand binding *via* the Ste2p C-terminal domain and the Gpa1p N-terminal domain

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ABSTRACT

G protein coupled receptors bind ligands that initiate intracellular signaling cascades *via* heterotrimeric G proteins. In this study, involvement of the N-terminal residues of yeast G-alpha (Gpa1p) with the C-terminal residues of a full-length or C-terminally truncated Ste2p were investigated using bioluminescence resonance energy transfer (BRET), a non-radiative energy transfer phenomenon where protein-protein interactions can be quantified between a donor bioluminescent molecule and a suitable acceptor fluorophore. Constitutive and position-dependent BRET signal was observed in the absence of agonist (α-factor). Upon the activation of the receptors with α-factor, no significant change in BRET signal was observed. The location of Ste2p–Gpa1p heterodimer was investigated using confocal fluorescence microscopy and bimolecular fluorescence complementation (BiFC) assay, a technique where two non-fluorescent fragments of a fluorescent protein reassemble *in vivo* to restore fluorescence property thereby directly reporting a protein-protein interaction. BiFC experiments resulted in a dimerization signal intracellularly during biosynthesis on the endoplasmic reticulum (ER) and on the plasma membrane (PM). The constitutive BRET and BiFC signals observed on ER between Ste2p and Gpa1p in their quiescent and activated states are indicative of pre-coupling between these two proteins.

This study is the first to show that the extreme N-terminus of yeast G protein alpha subunit is in close proximity to its receptor. The data suggests a pre-coupled heterodimer prior to receptor activation. The images presented in this study are the first direct *in vivo* evidence showing the localization of receptor - G protein heterodimers during biosynthesis and before reaching the plasma membrane.

1. Introduction

G protein-coupled receptors (GPCRs) belong to the largest cell surface receptor family in eukaryotic cells. Considering their role in sensing extracellular signals, the GPCR family plays very important roles in sensory systems and detect a large assortment of chemicals (hormones, neurotransmitters, chemo attractants and ions) and sensory signals (light, odorants and taste molecules) [1]. Activated GPCRs often transmit these extracellular signals to intracellular signaling cascades by heterotrimeric G proteins [2], which consist of three subunits, Gα, Gβ and Gγ. Mutations and functional problems of these receptors are linked to many human diseases such as diabetes, cardiovascular diseases, hypertension, cancer, hypothyroidism, retinitis pigmentosa and many psychotic disorders [3]. This is why GPCRs are the targets of ~25% of pharmaceuticals on the market thus being a major focus of molecular pharmacology research [4, 5]. However, the action mechanism of the GPCRs and the G proteins still has lots of unresolved discussions. Understanding the interactions between the GPCRs and their G proteins is pivotal to explain the plasticity of the signal transduction pathways.

GPCR structure and function has been studied in many model systems to avoid the complications arise from complex mammalian systems. In contrast to the human genome, which encodes nearly 1000 GPCRs [6], the budding yeast *Saccharomyces cerevisiae* encodes only

Abbreviations: BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; C-EGFP, C-terminal domain of enhanced green protein; CFP, cyan fluorescent protein; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; GPCR, G-protein coupled receptors; Gα, G protein alpha subunit; Gβ, G protein beta subunit; Gγ, G protein gamma subunit; MAPK, mitogen activated protein kinase; MLT, medium lacking tryptophan; MLTU, medium lacking tryptophan and uracil; MLU, medium lacking uracil; N-EGFP, N-terminal domain of enhanced green protein; PM, plasma membrane; RET, resonance energy transfer; RGS, regulators of G-protein; RLuc, *Renilla luciferase*; SDS, sodium dodecyl sulfate; TM, transmembrane; Tα, transducin; YFP, yellow fluorescent protein

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three known G protein coupled receptors and has one of the best-studied heterotrimeric G protein signaling pathways [7]. Basic principles of G protein signaling and regulation were first elucidated by genetic and biochemical assays analyzing the response of yeast to its mating pheromone peptide. Many of the important mechanisms of G protein and mitogen-activated protein (MAP) kinase signaling, the three-tiered structure of MAP kinase module [8] and the existence of kinase scaffold proteins [9] were first shown in *S. cerevisiae*. The first demonstration of mono-ubiquitination as a signal for receptor endocytosis [10, 11], the positive signaling role of Gβγ subunits [12] were discovered in yeast. The desensitization of signaling by regulators of G protein signaling (RGS) proteins were shown for the first time using the genetic disruption of *SST2* in yeast [13]. Homologs of Sst2 also exist in higher eukaryotes, named RGS proteins, were shown to accelerate G protein GTPase activity, thus leading to subunit re-association [7]. Furthermore, most of the GPCR mediated signaling pathway and its elements in mammalian cells are structurally and functionally similar to yeast pheromone signaling pathway [14]; the G protein and kinase components share extensive sequence similarity with their mammalian counterparts [15]. Thus, in this study we used Ste2p and Gpa1p to investigate GPCR Gα interactions.

The most studied contact site between Ste2p, the yeast mating pheromone α-factor GPCR and its cognate Gα is the C-terminal domain of Gα with the third intracellular loop of Ste2p. It was also shown that the N-terminus, the αN–β1 loop, the α4–β6 region and the α5 helix of Gα are involved in the interactions between Gα and GPCRs [16–23]. In rhodopsin transduction (Tα) complex, it was shown that both the N and the C termini are in close vicinity to the third cytoplasmic loop of rhodopsin Tα [24] but in most x-ray studies the extreme N- and C-termini of Gα cannot be depicted since they are disordered [25].

The mechanism of the dynamics of GPCR–Gα interaction is still under debate. Two different models were proposed: the “collision model” assuming that random collisions occur between receptors and G proteins in their inactive state without activating the signaling pathway that upon the stimulation of the receptors with their ligand, conformational changes on the receptor activates the G protein [26, 27], and a second model that presumes receptors and G proteins reside as a “pre-coupled” complex and stimulation of the receptors leads to immediate activation of the pre-coupled G protein [28–31]. One way to investigate these two models is to use resonance energy transfer (RET) assays in live cells.

Ability of tagging proteins with a fluorescent tag allows researchers to use these tagged proteins in order to study interactions *in-vivo*, in real-time [32]. Bioluminescence resonance energy transfer (BRET), a quantitative assay preferred in this study, is an electrodynamic phenomenon that occurs when an excited state “donor” molecule such as *Renilla luciferase*, transfers its energy to a ground state “acceptor” molecule, a fluorescent protein, in a non-radiative process through long range dipole-dipole interactions. Förster distance, which is defined as the distance at which RET efficiency is 50% [33] ranges from 2 to 9 nm. Such distances are far below the spatial resolution of a optical microscopy and are comparable to the size of biomolecules and/or the distance between sites on multi-subunit proteins. Therefore, in physiological conditions, RET can only be observed when two proteins are interacting since the average molecular distance is in the order of the Förster distance. The second technique used in this study is bimolecular fluorescence complementation (BiFC) assay, which allows to visualize protein interactions in live cells, and based on the facilitated association of complementary fluorescent protein fragments fused to interaction partners. Complex formation by the interaction partners brings the fluorescent protein fragments into close proximity thereby facilitating their complementation [34, 35]. The BiFC assay enables sensitive visualization of protein complexes with high spatial resolution, however the temporal resolution may be limited by the time required for fluorophore formation, as well as the stabilization of complexes by association of the fluorescent protein fragments [32, 36].

The heterotrimeric G protein cycle was quantitatively characterized recently in yeast measuring the FRET between CFP tagged Gpa1p (Gα) and YFP tagged Ste18p (Gγ) [37]. In another study, Gillen et al. reported that the first 67–36- or 9-amino acids of Gpa1p are sufficient to direct GST to the plasma membrane in yeast [38]. Gpa1p was shown to be myristoylated at the 2nd amino acid residue [39] and palmitoylated at the 3rd amino acid residue [40], and these post-translational modifications are required for plasma membrane targeting and membrane anchorage. Harashima et al. tagged Gpa2p (Gα of yeast glucose receptor) with GFP after position 10 without altering the protein function and showed that Gpa2p has lipid modifications, a myristoylation site on the Gly2 and possible palmitoylation sites at cysteine residue near this myristoylation site of its N-terminus, which is required for membrane localization similar to Gpa1p [41].

In this study, the interactions of the N-terminal residues of Gpa1p (Gα) with the C-terminal residues of its cognate receptor, Ste2p was investigated. For this purpose, Gpa1p was tagged with EGFP at various positions on the N-terminus, while a full-length Ste2p and a C-terminally truncated receptor (Ste2p-Δ305) were tagged with RLuc at position 304. GPCR–Gα interaction was quantified *in vivo* with BRET. Whereas the location of Ste2p–Gpa1p heterodimer, was assayed by BiFC and colocalization of reconstructed EGFP signal with organelle markers. This study is the first to show that the extreme N-terminus of yeast G protein alpha subunit is in close proximity to its receptor and both proteins exist in a pre-coupled heterodimer prior to receptor activation. The *in vivo* visualization data presented in this study also suggest that the heterodimerization occurs at the endoplasmic reticulum during the biosynthesis, before reaching the plasma membrane.

2. Methods

2.1. Yeast strains, plasmids and media

DK102 (MAT***a***, ura3-52 lys2–801^{am} ade2-101^{oc} trp1-Δ63 his3-Δ200 leu2-Δ1 ste2::HIS3 sst1-Δ5) [42], BJS21 (MAT***a***, prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 ste2::Kan^{R}) [43] and TM5117 (MAT***a***, trp1-Δ63, ade2-101^{oc}, bar1, his3-Δ200 leu2-Δ1 ste2::HIS3, FUS1-lacZ::URA3, gpa1Δ, far1Δ) [44] *Saccharomyces cerevisiae* strains were used. TM5117 was used as a Gpa1p knock-out strain to investigate the Ste2p–Gpa1p interaction. DK102 was used for alpha-factor induced growth arrest assay and the protease deficient BJS21 strain was used in Western blot analysis due to the decreased protein degradation.

For constitutive expression of Ste2p and Gpa1p, pSP-G1 and pST-G1 bidirectional double promoter plasmids [45], with 2-μm based shuttle vector, a *TEF1* promoter-*ADH1* terminator region and *PGK1* promoter–*CYC1* terminator region were used. pSP-G1 carrying a *URA* marker and pST-G1 carrying a *TRP* marker for selection in yeast.

The pBS-35S-RLUC plasmid, *Renilla Luciferase* cDNA vector, was a generous gift from Dr. Albrecht von Arnim (University of Tennessee Knoxville, USA). pEGFP-N2, enhanced green fluorescence cDNA vector was obtained from Prof. Dr. Henry Lester's laboratory (California Institute of Technology, USA) [46]. ER marker plasmid, YIplac204/TKC-DsRed-HDEL was a generous gift from Dr. Benjamin S. Glick (University of Chicago, USA).

Yeast strains were grown in YEPD (yeast extract-peptone-dextrose) broth at 30 °C and were maintained on agar plates at 4°C for short-term storage. For selection of successful yeast transformants with constructed plasmids, media lacking tryptophan (MLT, Supplementary materials), media lacking uracil (MLU, Supplementary materials) and media lacking both Tryptophan and Uracil (MLTU, Supplementary materials), were used respectively.

2.2. Chemical reagents and other materials

Phusion Hot Start II High-Fidelity DNA Polymerase was purchased from Thermo-Fisher Scientific (MA, USA). All restriction enzymes were