Biochimica et Biophysica Acta 1838 (2014) 3036–3051

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

One motif to bind them: A small-XXX-small motif affects transmembrane domain 1 oligomerization, function, localization, and cross-talk between two yeast GPCRs☆

Antonia Lock [a,1], Rachel Forfar [b,1], Cathryn Weston [b], Leo Bowsher [c], Graham J.G. Upton [d], Christopher A. Reynolds [d], Graham Ladds [b,*,2], Ann M. Dixon [c,**,2]

[a] Department of Genetics, Evolution and Environment, University College London, WC1E 6BT London, UK
[b] Division of Biomedical Cell Biology, Warwick Medical School, Coventry, CV4 7AL, UK.
[c] Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK
[d] School of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK

ARTICLE INFO

Article history:
Received 13 February 2014
Received in revised form 13 August 2014
Accepted 14 August 2014
Available online 23 August 2014

Keywords:
GPCR
TOXCAT
Oligomerization
Trafficking
Signaling
Cross-talk

ABSTRACT

G protein-coupled receptors (GPCRs) are the largest family of cell-surface receptors in mammals and facilitate a range of physiological responses triggered by a variety of ligands. GPCRs were thought to function as monomers, however it is now accepted that GPCR homo- and hetero-oligomers also exist and influence receptor properties. The *Schizosaccharomyces pombe* GPCR Mam2 is a pheromone-sensing receptor involved in mating and has previously been shown to form oligomers in vivo. The first transmembrane domain (TMD) of Mam2 contains a small-*XXX*-small motif, overrepresented in membrane proteins and well-known for promoting helix–helix interactions. An ortholog of Mam2 in *Saccharomyces cerevisiae*, Ste2, contains an analogous small-*XXX*-small motif which has been shown to contribute to receptor homo-oligomerization, localization and function. Here we have used experimental and computational techniques to characterize the role of the small-*XXX*-small motif in function and assembly of Mam2 for the first time. We find that disruption of the motif via mutagenesis leads to reduction of Mam2 TMD1 homo-oligomerization and pheromone-responsive cellular signaling of the full-length protein. It also impairs correct targeting to the plasma membrane. Mutation of the analogous motif in Ste2 yielded similar results, suggesting a conserved mechanism for assembly. Using co-expression of the two fungal receptors in conjunction with computational models, we demonstrate a functional change in G protein specificity and propose that this is brought about through hetero-dimeric interactions of Mam2 with Ste2 via the complementary small-*XXX*-small motifs. This highlights the potential of these motifs to affect a range of properties that can be investigated in other GPCRs.

G protein-coupled receptors (GPCRs) form a large ubiquitous family of transmembrane receptors that share a common topology of seven α-helical transmembrane domains (TMDs). GPCRs are conserved in all eukaryotes, where they act as key regulators of many cellular processes by triggering intracellular signaling cascades in response to environmental stimuli. Although alternative modes of activation exist, transmission of information across the lipid bilayer is commonly achieved by binding of ligand in the GPCR binding pocket, which forces a conformational change in the receptor's transmembrane domains [1,2]. This typically leads to G-protein binding, GDP–GTP exchange on the associated G protein, and propagation of the signal. GPCRs have also been shown to form dimeric or higher order oligomeric complexes, which may be a prerequisite for the correct subcellular targeting and functioning of the receptor [3,4] and may modulate receptor function [5]. Hetero-association of two (or more) GPCRs to form new receptor complexes that participate in "cross-talk" has also been reported, leading to

Abbreviations: GPCR, G protein-coupled receptor; TMD, transmembrane domain; CAT, chloramphenicol acetyltransferase; GpA, Glycophorin A; DMM, defined minimal media; GFP, green fluorescent protein; MBP, maltose binding protein; CHI, CNS searching of helix interactions; RMSD, room mean squared deviation; PVDF, polyvinyl difluoride; ECL, enhanced chemiluminescent substrate; S.E.M., standard error of the mean

☆ This work was funded by an EPSRC studentship through the MOAC Doctoral Training Centre (A.L.), an EPSRC pre-doctoral traineeship (A.L.), BBSRC CASE studentships with Septegen (R.F.), a Warwick Impact Fund (C.W. and G.L.), the University Hospitals of Coventry and Warwickshire NHS Trust (G.L.), the Biotechnology and Biological Sciences Research Council grant number (BB/G01227X/1) (G.L.) and Birmingham Science City Research Alliance (G.L.).

* Corresponding author. Tel.: +44 2476 150220; fax: +44 2476 523701.
** Corresponding author. Tel.: +44 2476 150037; fax: +44 2476 524112.
E-mail addresses: graham.ladds@warwick.ac.uk (G. Ladds), ann.dixon@warwick.ac.uk (A.M. Dixon).

[1] These authors contributed equally to the data shown in the manuscript.
[2] These authors contributed equally to the project.

http://dx.doi.org/10.1016/j.bbamem.2014.08.019
0005-2736/

modified G protein coupling and signaling [6]. Despite these and other excellent studies highlighting the importance of GPCR homo- and hetero-oligomerization, the specific interactions mediating complex formation are poorly understood due to the significant technical challenges involved in structural characterization of membrane proteins.

Lower eukaryotes are attractive model organisms in which to study GPCRs as they are amenable to genetic manipulation and exhibit less cross-talk between signaling pathways [7]. Several studies of the GPCR Ste2, the pheromone receptor from the budding yeast *Saccharomyces cerevisiae* which mediates the mating response, agree that oligomerization of this protein is facilitated (at least in part) by interactions involving transmembrane domain 1 (TMD 1) [8–11]. These interactions were further traced to a small-*XXX*-small motif (specifically a G_{56}-*XXX*-G_{60} motif) in TMD 1 [12]. Small-*XXX*-small motifs are motifs of two residues (typically glycine, but also alanine or serine) separated by three amino acids in the polypeptide chain, thus physically placing them on the same face of an α-helix. Co-location of these two small residues results in a "groove" which allows two helices to interlock via many favorable van der Waals contacts, thereby promoting helix–helix interactions. While these motifs have been frequently found at the oligomeric interface of helical membrane proteins [13–16], they have also been shown to be highly dependent upon the surrounding amino acid sequence [17,18], and are not always effective in driving protein interactions. Therefore, simple identification of these motifs in the primary sequence of a transmembrane domain is not a silver bullet to understanding protein interactions, but their presence can be used to design targeted mutagenesis strategies to verify their role (or lack thereof) experimentally. In this way, mutation of the G-*XXX*-G motif in Ste2 TMD 1 was shown in one study to interfere with oligomerization and localization (but not ligand binding) of the receptor [12]. However, other studies before and since have reported that Ste2 TMD 1 is not sufficient to drive oligomerization on its own, and that other domains in the receptor are also involved in complex formation including the N-terminus and TMD 2, TMD 4, and TMD 7 [9–11]. Evidence also exists that suggests the sites of contact between receptors change upon ligand-binding [9]. Moreover, it should be noted that, while small residues may mediate TMD packing, this is not always essential and indeed some GPCRs have been known to include more bulky residues such as isoleucine and leucine. In short, this growing body of work has not yet yielded a consensus model for Ste2 oligomerization, apart from the agreement that TMD 1 participates in some way.

The *Schizosaccharomyces pombe* P-factor receptor Mam2 [19] is orthologous to the Ste2 receptor, and immunoblotting (performed under non-denaturing conditions) has suggested the existence of oligomeric complexes [20] although a direct interaction has not been determined. Unfortunately, classical approaches to confirm dimerization of Mam2 have proven unsuccessful. In the past we have been able to immunoprecipitate heterologously expressed mammalian receptors from *S. pombe* cells [20,21], but somewhat surprisingly this has not been possible for Mam2 despite the production of two in-house antibodies. Likewise, we have been unable to utilize fluorescent-based techniques such as Förster resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) due to the large size of the C-terminal tail of Mam2 (45 amino acids). Unlike Ste2, where it has been possible to delete the C-terminal tail and still produce a functional receptor [12], Mam2 has an absolute requirement of its C-terminal tail to facilitate efficient downstream signal transduction [22]. The tail of Mam2 is absolutely required for an interaction with the regulator of G protein signaling 1 (Rgs1) protein that both negatively and positively modulates signal transduction dependent upon the dose of stimulating pheromone [22,23]. Deletion of the Mam2 C-terminal tail generates a receptor with severely attenuated signaling abilities. Consequently, a novel approach that attempts to understand the Mam2 dimerisation interface has been required and we described here our use of the bacterial expression system TOXCAT [24] to investigate the Mam2 dimerization interface.

Along with the suggestion that Mam2 forms oligomers [20], Mam2 further resembles Ste2 in that it also contains two consecutive small-*XXX*-small motifs (G_{49}-*XXX*-S_{53}, S_{53}-*XXX*-A_{57}) in TMD 1. Given the lack of a consensus as to the precise mechanisms governing oligomerization of fungal GPCRs, and guided by previous studies of Ste2 as well as the presence of well-known helix interaction motifs, we describe here for the first time the effects of targeted mutation of the small-*XXX*-small motifs on oligomerization, function, and localization of Mam2. Equivalent experiments were performed on Ste2 for comparison to Mam2 and the wider literature. Our results demonstrate that the TMD 1 small-*XXX*-small motifs play a critical part in the correct localization and function of full-length Mam2, and this behavior is mirrored in parallel experiments performed on Ste2. The small-*XXX*-small motifs also promote strong self-association of Mam2 TMD 1 in isolation (a result which is not observed in parallel experiments on Ste2 TMD 1). Using a series of chimeric G protein expressing strains [21], we also demonstrate that Mam2 can form functional heterodimers with Ste2, and our mutagenesis data suggest that this occurs via complementary small-*XXX*-small motifs in the first TMD of each receptor. Molecular models of the relevant helix–helix interactions are used to support the experimental results and illustrate plausible interaction modes. The data presented here highlight the importance of this well-known motif and offer novel insight into the mechanisms used by Mam2 to functionally assemble into homo- and hetero-oligomeric complexes.

1. Materials and methods

1.1. TOXCAT assay and construction of chimera

The self-association of the first transmembrane domains of *S. cerevisiae* Ste2 (V_{49}–W_{70}) and *S. pombe* Mam2 (L_{46}–C_{67}) were studied using the TOXCAT assay, which has been described previously [24]. Briefly, the DNA sequence encoding the transmembrane domain of interest was cloned into the pccKAN vector between the dimerization-dependent DNA binding domain of ToxR at the N-terminus, and maltose binding protein (MBP) at the C-terminus. The resulting fusion protein was expressed in *Escherichia coli* NT326 cells lacking endogenous MBP. TM domain-driven oligomerization of the fusion protein leads to ToxR mediated activation of the reporter gene chloramphenicol acetyltransferase (CAT), with CAT expression levels indicating the strength of TM self-association. Before performing assays, correct insertion and orientation of the TOXCAT constructs in the *E. coli* inner membrane was confirmed using the protease sensitivity in spheroplast assay [24]. Expression levels for all constructs were determined via western blots against maltose binding protein (MBP). The chloramphenicol acetyltransferase (CAT) reporter gene was quantified using the FAST CAT kit (Invitrogen). CAT activity was normalized to the expression level of each construct using the ImageJ tool [25] to quantify band intensities on a western blot, and all CAT activities are reported relative to the value obtained for the positive control, the strongly-dimerizing TMD of Glycophorin A (GpA). A point mutant of GpA, G_{83}I, which impairs TMD association, was used as a negative control. Values given are the means (±S.D.) for three or more independent measurements and a student's T-test was performed to determine the significance of changes in CAT activity.

1.2. Yeast strains, plasmid construction and culture conditions

The yeast strains used in this study are listed in Table S1. All yeast strains were derived from JY546 which contains the *sxa2* > *lacZ* construct for quantification of pheromone-dependent transcription. To facilitate the expression of heterologous GPCRs in *S. pombe*, we have previously described the generation of a series of Gα-transplants, integrated at the *gpa1* locus, in which the C-terminal five amino acids of Gpa1 were replaced with the corresponding residues from mammalian Gα-subunits [21]. General yeast procedures are as described previously