Visualizing G protein signaling in living cells
Adjobo-Hermans, M.J.W.

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Outline

The importance of both spatial and temporal cues in the activation of signaling pathways has recently become evident. Standard biochemical assays like cell fractionation do not allow for the study of events in single cells and therefore such spatial information cannot be obtained. Likewise, asynchronous (oscillatory) processes in living cells cannot be assessed by these techniques. The discovery of the genetically-encoded probe GFP has greatly facilitated the study of the spatiotemporal dynamics of signaling events in single living cells by means of fluorescence microscopy. The development of a varied palette of spectral variants permits co-imaging of several sensors and proteins of interest simultaneously (multi-parameter imaging). Making use of these probes we studied the mechanisms of heterotrimeric G protein signaling.

Even though the resolved crystal structures of heterotrimeric G proteins have been instrumental in the understanding of many aspects of signaling, they inherently cannot provide information as to the dynamic changes that occur during receptor-stimulated G protein activation. Therefore, we opted for the use of GFP-tagged G protein subunits that do supply this information and, moreover, do so in their natural environment: at the plasma membrane. The major part of this thesis addresses the activation and regulation of the heterotrimeric G protein Gq and its effector PLCβ in living mammalian cells. However, we started out investigating heterotrimeric G protein signaling in plant cells. Plant heterotrimeric G proteins are proposed to play roles in cell division, auxin, gibberellin and abscisic acid signaling, light responses, ion channel regulation and Nod-factor signaling and thus make interesting subjects of investigation. In plants, heterotrimeric G protein signaling seems to deviate significantly from the mechanisms reported in mammalian cells, but remains poorly defined. As described in Chapter 1, Section 3.2, the process of lipidation has been studied in detail for both mammalian and yeast heterotrimeric G proteins. However, lipidation of the plant heterotrimer had not been described when the research for this thesis started. The lipidation of the plant heterotrimer is examined in Chapter 2. The exact location of heterotrimerization remains controversial as is evident from the literature described in Chapter 1, Section 3.3.3. In Chapter 2, the location of assembly of the plant heterotrimer is addressed.

The longstanding dogma states that the heterotrimer dissociates upon activation, as described in Sections 3.1 and 3.3.1. The first study on G protein activation by means of FRET was published in 2001 by Devreotes and co-workers. FRET ratio-metric imaging was applied and the decrease in FRET upon addition of an agonist was interpreted as a dissociation of the subunits. However, in 2003, Bunemann et al. recorded an increase of FRET upon stimulation of Gq and were able to prove the occurrence of a change in
conformation instead of dissociation of the subunits. We studied the effect of activation on the integrity of the plant heterotrimer, as there was no information available on this effect. The results of this study are presented in Chapter 2.

Studying $\alpha_q$ is imperative, since $\alpha_q$ is implicated in the development of myocardial hypertrophy upon mechanical stress of the heart. This is one of the triggers of cardiac failure, a leading cause of death in the Western world and therefore drugs to downregulate $\alpha_q$ are much sought after. Proteins belonging to the Gq class are also involved in synaptic transmission, cell growth, platelet aggregation, glucose secretion, actin cytoskeletal rearrangements, hematopoietic cell differentiation, leukocyte activation and contraction of smooth muscle, indicating yet again their importance in human physiology.

In general, receptor activation of heterotrimeric G proteins is sampled by measuring the relationship between ligand concentration and the binding of $^{35}$S-GTP$_\gamma$S (a GTP analogue resistant to hydrolysis) by the G protein. However, $\xi_\alpha$ subunits are the predominant species in most cell types and their basal $k_{\text{off}}$ (i.e. spontaneous GDP release) is considerably higher ($\sim$3-fold) as compared to other $\alpha_\xi$ subunits (Milligan, 2003). The $\alpha_q$ subunit does not share the favorable characteristics of $\xi_\alpha$ subunits and therefore its activation cannot be ascertained by means of this assay. In addition, $\alpha_q$ is refractive to pertussis toxin as stated in Section 3.10.2. For these reasons we started the development of a sensor in order to directly measure Gq activation in living cells. In Chapter 3 the quest for a functional GFP-tagged $\alpha_q$ subunit is described and a FRET pair is presented that enables the real-time visualization of Gq activation in living cells and the influence accessory proteins have on this activation. Using ratio-metric FRET imaging and FLIM we addressed the dissociation dogma with respect to the Gq heterotrimer. Additionally, the effects of lipidation on the localization of $\alpha_q$ are described. As evident from Section 3.2.1, a lot of controversy exists as to the effect of activation on the localization of $\alpha_\xi$ subunits in general. In Chapter 3, we tried to elucidate the conflicting data published for $\alpha_q$, which have been described in Section 3.11.

The Gq-effector PLC$_\beta$ is the subject of Chapters 4 and 5. The activation mechanism of PLC$_\beta$ isozymes by $\alpha_q$ is insufficiently understood. Basal knowledge as to e.g. their subcellular location is also scant, as is apparent from the available literature discussed in Section 4.3. PLC$_\beta$ isozymes have been implicated in biological/patho-biological processes ranging from fear condition, opioid sensitivity and cancer to the development of the cerebellum, visual processing and taste recognition, which makes research on their action at the molecular level highly relevant.
Chapter 4 deals with the localization of the full-length PLCβ isoforms and their separated domains. The C-terminal module termed the CT domain is elaborately studied with respect to its ability to act as a dominant negative in Gq-signaling. As described in Section 4.4, most of the research on Gαq-PLCβ interaction has been done in vitro and involves only one isoform: PLCβ1. Since the expression pattern of the isoforms varies significantly between tissues/cell types (Section 4.1), the signal output upon stimulation with an identical concentration of agonist may differ considerably depending on the expressed PLCβ isoform(s). Hence, we investigated the differences between the isoforms. In addition, the effect of the CT domain on the localization of active Gαq is described, as well as the kinetics of the CT domain, as measured by means of FLIP. The localization of PLCβ3 and several mutant forms is examined in living cells upon stimulation and in the presence of a PDZ domain-containing protein that has been described to interact with this PLCβ isoform (Section 4.7).

It was hypothesized that PLCβ isozymes would move to the plasma membrane upon activation of Gαq. However, this process had not been visualized in living cells. In Chapter 5, the real-time visualization of PLCβ1a is presented and a novel mechanism of regulation of PLCβ1a signaling is described.

In general, microscopy is crucial in the study of signal transduction in living cells. Processes occurring at the plasma membrane are especially difficult to study and require the application of advanced microscopy techniques. In Chapter 6, the TIRF technique, first presented in Chapter 5, and FLIM are used in combination, enabling the study of annexin A4 interactions at the plasma membrane.

The implications of the results obtained in this thesis are summarized and discussed in Chapter 7 with respect to the current understanding of heterotrimeric G protein signaling. In addition, future experiments are proposed to further our knowledge in this exciting field of research.