Chapter 7
Optical Control of G Protein-Coupled Receptor Activities in Living Cells

Hideaki Yoshimura and Takeaki Ozawa

Abstract Membrane receptors transmit external signals into cells in response to extracellular stimuli and their activities are controlled spatiotemporally. In recent years, it has become possible to control activity of a target membrane receptor by external light using a photoreceptor protein. It forms oligomers or interacts specifically with its binding partners after light absorption. Controlling receptor activities by external light is now a powerful approach to elucidating the role of receptor activities and its dynamics in various life phenomena. In this review, we describe a new technology of optically controllable receptor using a photoreceptor protein, CRY2, and its application to the interaction of GPCR with β-arrestin in living cells.

7.1 Introduction

Basic knowledge of biochemistry of bioluminescence becomes driving forces behind development of basic tools used for modern life science. A typical example is the discovery of the light source of firefly luciferase; the light source is composed of an enzymatic reaction of luciferase with its substrate, D-luciferin [1]. Since ATP and oxygen molecules are necessary for this reaction, luciferase has been used for highly sensitive luminescence analysis of ATP. Also, the luciferase enzyme is still one of the most versatile proteins as a reporter of gene expression, which is used for measuring transcriptional activities in living cells [2]. Another example is the green fluorescent protein (GFP) discovered in the 1962 [3], which is now indispensable as a tool to visualize target molecules and proteins in living cells and animals. Furthermore, various amino acid mutants of GFP and identification of analogues thereof have advanced the development of fluorescent proteins showing new optical properties [4], which support the current fluorescence imaging technologies. Thus,

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K. Yamanouchi et al. (eds.), *Progress in Photon Science*, Springer Series in Chemical Physics 119, https://doi.org/10.1007/978-3-030-05974-3_7

the technical basis of modern biological science is strongly dependent on the results of photobiological research.

It has been well known that plant cells and algae include many light-absorbing proteins. The proteins contain organic molecules as their chromophore originated from metabolites in the cells. When the chromophore in the proteins absorbs light in a visible region, the proteins undergo dynamical changes in their structure, and translate the light signal into activation of intracellular signaling. Retinal in an ion channel (Fig. 7.1a), for example, absorbs light with a specific wavelength region, and then the channel protein transduces the light signal into the activation of visual cells. Based on the knowledge of light-absorbing ion channel, new technologies called optogenetics have been emerged by artificially expressing such ion channels in neurons [5]; a specific nerve activity in living animals can be controlled by turning on and off of external light. Since the appearance of optogenetics technology, it has spread into various ion channel proteins and their applications [6]. In parallel with the ion channel technologies, various new photoreceptor proteins have also been utilized for directly controlling intracellular enzyme activity and gene expression in living cells and animals [7].

Receptor proteins embedded in the plasma membrane in living cells recognize extracellular ligand molecules and transmit signals into cells, thereby inducing appropriate cellular responses according to environmental differences. One of the important membrane receptor families is G protein-coupled receptors (GPCRs), which are widely distributed in different organs and mediate numerous hormones, neurotransmitters and odor molecules, etc., into intracellular signaling [8]. Although the elucidation of the structure of GPCR is progressing [9], most of the details of the mechanism such as binding with G protein or β-arrestin regulating its function,

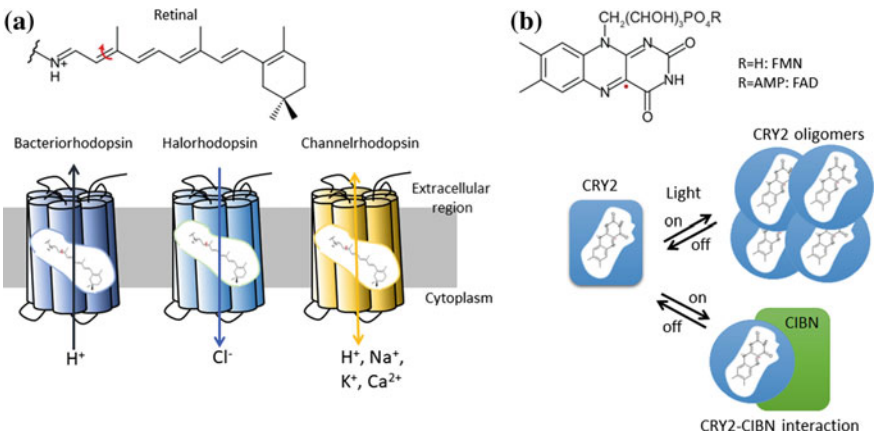

Fig. 7.1 Different types of optogenetics tools. **a** Structure of retinal and different types of ion channels. Arrows in the bottom figure indicate direction of each ion flow. **b** Structure of FMN and FAD and character of CRY2. Upon blue light absorption, CRY2 forms oligomers or interacts with its specific binding protein, CIBN

its accompanying intracellular signals, and dynamic process of the translocation to endosomes. Many studies using specific ligands and inhibitors for GPCRs have been carried out so far, it is difficult to manipulate these compounds in the spatiotemporal way. Therefore, it is desirable to develop new technologies for simultaneous analysis of GPCR and its related proteins in living cells. We herein focus on a new methodology of the analysis of GPCR and β-arrestin interactions using a photoreceptor proteins, CRY2 and new insight into the mechanism of intracellular trafficking of GPCRs.

7.2 Optogenetics Tool of CRY2

Many photoreceptor proteins undergo their structural changes upon light absorption by their chromophores. In optogenetics, the property of photoreceptor proteins is used for controlling activity of specific proteins of interest in living systems. In particular, photoreceptor proteins that form dimers and oligomers by light absorption are widely used as a module that induces protein-protein interactions and localization changes of a target protein in living cells.

Photoreceptor proteins responsive to blue light are often used as optogenetics tools. Of various photoreceptor proteins absorbing blue light, the light-oxygen-voltage sensing (LOV) domain is a representative: Aureochrome 1a derived from *Vaucheria frigida* which is a kind of yellow-green alga has a LOV domain (AU1) enveloping flavin mononucleotide (FMN) as a chromophore (Fig. 7.1b). Blue light irradiation induces the protein dimer formation reversibly, which was applied for manipulating gene expression by external blue light [10].

On the other hand, Cryptochrome is a family of photoreceptor proteins having flavin adenine dinucleotide (FAD) as a chromophore. Among them, Cryptochrome 2 (CRY2) derived from *Arabidopsis thaliana* is widely used as a light control module in response to blue light. CRY2 exhibits two important characteristics with blue light absorption; one is a reaction in which CRY2 forms an oligomer (Fig. 7.1b). Particularly in plant cells, it forms agglomerates in a microscopic observable sizes called Photobody in the nucleus. Until now, the CRY2 protein has been used as an optogenetics module to control activities of tyrosine kinase and membrane receptors such as Trk and DCC receptors [11, 12]. The other is a character of interaction between CRY2 and CIB1 protein, which is a target protein of CRY2. Because CRY2 interacts with the N-terminal domain of CIB1, the truncated domain (called CIBN) is used as a tool for light manipulation. The interaction of CRY2 with CIBN is reversible and their complex dissociates promptly upon turning off the light (Fig. 7.1b). Taking these advantages of CRY2-CIB1 interactions, some researchers have demonstrated their application to controlling kinase activities such as intracellular kinases Akt and Ras [13, 14].

7.3 Signal Transduction of GPCR and Downstream Molecules

GPCRs represent the largest protein family and mediate large number of external stimuli such as hormones, and neurotransmitters, which are therefore implicated in myriad of physiological functions and diseases in our body [15, 16]. Even though the GPCR family consists of nearly 1000 members, their structural features and the signal transduction pathways through them are highly conserved throughout the members. GPCRs consist of single polypeptide chain that spans 7 times across the cell membrane. The GPCRs activated with a specific agonist lead to interaction with heterotrimeric G proteins inside of the cells and stimulate signal transduction through G proteins. The activated GPCRs are then C-terminally phosphorylated by GPCR kinases (GRK), and then β-arrestin is recruited through interaction to the phosphorylated site on the GPCRs. The β-arrestin recruitment induces stimulation of the MAP/ERK signaling pathway, receptor desensitization, and clathrin-dependent endocytosis.

Hetero trimeric G proteins are typical downstream proteins of activated GPCRs, consisting of three subunits, Gα, Gβ, and Gγ. Upon an agonist binds to GPCR, the Gα subunit in heterotrimeric G protein, which are tethered to the inner leaflet of the plasma membrane are associated with the active GPCRs. Then, the signal is transduced from the GPCR to the heterotrimeric G protein, leading activation and dissociation of the heterotrimeric G protein into a Gα and a Gβγ dimer.

GPCRs in an active form undergo the function of G protein-coupled receptor kinase (GRK) and are phosphorylated at the C-terminal region [17]. Then, β-arrestin, another downstream molecule of GPCRs, interacts with the phosphorylation site and forms a complex with the GPCRs. β-arrestin attaching to a GPCR has different functions. First, β-arrestin inhibits the activity of GPCRs to transduce signal to G proteins by preventing GPCR-G protein interaction (desensitization) [18]. Second, upon attaching to GPCRs, β-arrestin transduce signal to its downstream molecules such as ERK1/2. In addition, β-arrestin provides a scaffold to concentrate clathrin and its related proteins, and induces endocytosis of the GPCRs [19]. Beside the importance of the G-protein signaling pathway, the roles of β-arrestin are thus also critical to regulate GPCR functions.

7.4 Light Regulation of GPCR-β-Arrestin Interactions

The interaction between GPCRs and β-arrestin is crucial for GPCR signaling, regulation of GPCR activity, and GPCR trafficking in the cells. Artificial control of GPCR-β-arrestin interaction is a promising approach to control the function of GPCRs comprehensively. We have established a method to manipulate GPCR-β-arrestin interaction through photo irradiation in living cells. We introduced briefly the work of photo regulation of GPCR-β-arrestin interaction in living cells [20].

β2-adrenergic receptor (ADRB2), which is one of the most investigated GPCRs, was selected as a target GPCR. ADRB2 was fused with CIBN whereas β-arrestin was connected to CRY2. Upon blue light irradiation, photo-induced interaction between the CRY and CIBN leaded association between ADRB2 and β-arrestin (Fig. 7.2) [21, 22]. In addition, a SNAP tag, which is a tag protein to conjugate an organic fluorescent dye, and a red fluorescent protein mCherry were attached to ADRB2-CIB and β-arrestin-CRY, respectively, to monitor their localization in cells. The SNAP tag was conjugated with SNAP-Cell 647-SiR, which is a far red fluorescent dye, in microscope observation, allowing simultaneous localization analysis of ADRB2-CIB and β-arrestin-CRY in the same living cells.

Before blue light irradiation, ADRB2-CIB and β-arrestin-CRY were localized on the plasma membrane and in the cytosol, respectively. This localization property was consistent with those of ADRB2 and β-arrestin in the resting state without stimulation. Upon blue light irradiation, interaction of CRY2 and CIBN led to β-arrestin recruitment to the plasma membrane within 2 min, then particle-like structures that include ADRB2 and β-arrestin appeared in the cytoplasm 15 min after the light irradiation (Fig. 7.3). Treatment of the sample cells with a dynamin inhibitor, Dingo4a, before the blue light irradiation hampered the generation of the particle-like structures in the cytoplasm, indicating that the particle-like structures were endosomes that form through clathrin-dependent endocytosis of ADRB2-β-arrestin complexes.

In endocytosis on various receptor molecules including ADRB2, some cytosolic proteins were recruited to endosomes to initiate, compose, and/or maintain the endosomes. To assess the recruitment of the endocytosis-related proteins to the photo-induced endosomes of ADRB2-β-arrestin complexes, localization of one of such proteins, Mdm2 [23], was monitored during the photo-induced ADRB2 endocytosis under a fluorescence microscope. Upon blue light irradiation of the sample cells, Mdm2 was recruited to the photo-induced endosomes. After the blue light irradiation stopped, Mdm2 relocated to the cytosol homogenously as the endosomes were decomposed. This result showed that the photo-induced interaction between ADRB2 and β-arrestin induced formation of endosomes of the same properties as those generated upon physiological stimulations.

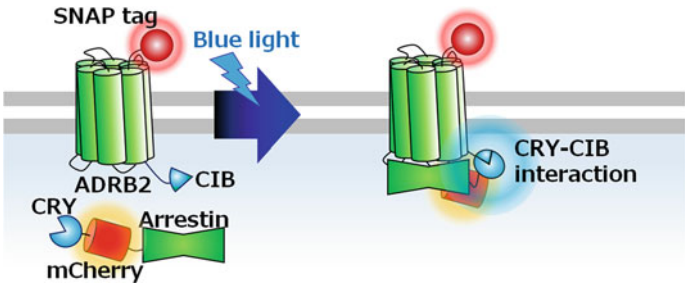

Fig. 7.2 Schematic of the optogenetic tool to control ADRB2-β-arrestin interaction

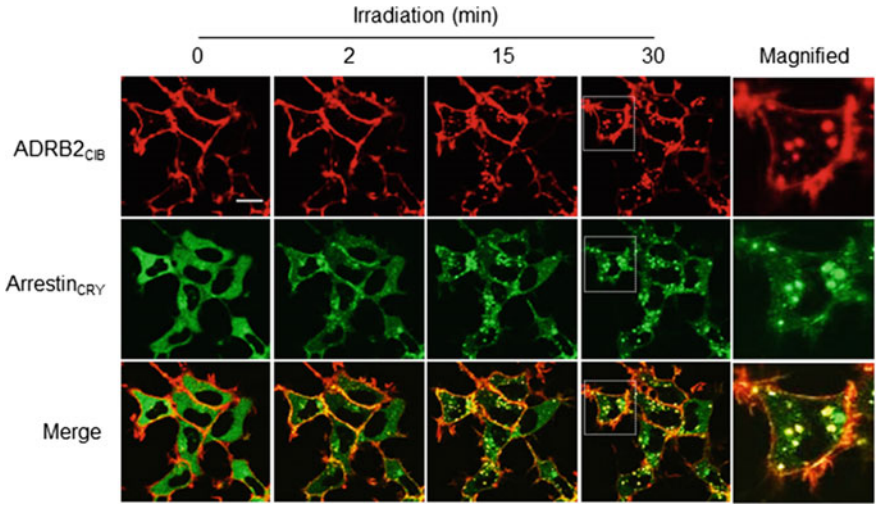

Fig. 7.3 Light-induced endocytosis of ADRB2-CIB. Scale bar, 20 μm. Blue light was irradiated for 30 min to the cells expressing ADRB2-CB and Arrestin-CRY under a confocal fluorescence microscope

Considering the reversibility of CRY-CIB interaction, dissociation of photo-induced ADRB2-β-arrestin complex after stopping light irradiation was tested. The cells expressing ADRB2-CIB and β-arrestin-CRY were irradiated with blue light for 30 min, which was sufficient to form endosomes in the cytosol. Then the light irradiation was quitted and the cells were kept in dark. In the dark condition, the endosomes disappeared in 20 min, and ADRB2-CIB and β-arrestin-CRY relocated to the plasma membrane and the cytosol, respectively (Fig. 7.4). The same endosome disappearing and relocation of ADRB2 and β-arrestin occurred in case of stimulation with an agonist, isoproterenol (ISO). ISO treatment for 60 min led to reduction of ADRB2 on the plasma membrane, whereas ADRB2 recovered on the plasma membrane over time after washing ISO out. Thus the photo-regulation system on ADRB2-β-arrestin interaction reproduces the trafficking dynamics of ADRB2 and β-arrestin upon agonist stimulation.

Next, the dependency of endosome trafficking on blue light irradiation time was assessed [20]. The cells expressing ADRB2-CIB and β-arrestin-CRY were irradiated blue light for 120 min and colocalization of generated endosomes with lysosome marker protein LAMP1 were monitored. In the fluorescence microscope images of LAMP1 and ADRB2-CIB, ADRB2-CIB predominantly localized on lysosomes, which were represented as particles including LAMP1. This result suggests that prolonged ADRB2-β-arrestin interaction leads the endosomes to lysosomal degradation pathway.

Finally, biochemical activity of ADRB2-CIB as a GPCR upon blue light irradiation was estimated. In the assay of ADRB2 activity to transduce the signal to G protein pathways, nanobody 80 (Nb80), which selectively bind to the active state

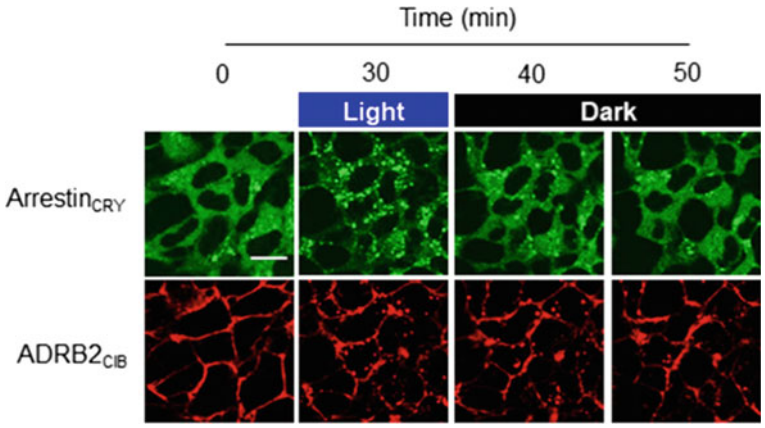

Fig. 7.4 Recycling of ADRB2-CIB after quitting light irradiation. Scale bar, 20 μm. The cells expressing ADRB2-CIB and β-arrestin-CRY was illuminated with blue light for 30 min and kept in dark for further 20 min under a confocal fluorescence microscope

ADRB2 interacting to G proteins [24], was used. The gene of GFP-fused Nb80 (GFP-NB80) was introduced and expressed in the cells stably expressing ADRB2-CIB and β-arrestin-CRY, and the localization of GFP-Nb80 was monitored upon blue light or ISO stimulation under a fluorescence microscope. Whereas 30 min ISO stimulation led to formation of endosomes that contained GFP-Nb80, the endosomes formed after blue light irradiation did not include GFP-Nb80. In addition, the signal transduction activity through the β-arrestin pathway was assessed by Western blotting analysis to detect ERK1/2 phosphorylation, which is a downstream of the β-arrestin signal pathway. Different from ISO stimulation, blue light irradiation of ADRB2-CIB and β-arrestin-CRY-expressing cells failed to induce ERK1/2 phosphorylation. Phosphorylation on the C-terminal region of ADRB2, which occurred upon ISO stimulation, was also assessed by Western Blotting analysis. Even upon blue light irradiation, the C-terminal region did not phosphorylated. These results indicates that the photo activation system of ADRB2-CIB and β-arrestin-CRY induces endocytosis of ADRB2-β-arrestin complex, even though such biochemical activities of ADRB2 as signal transduction through G-protein pathway, ERK1/2 pathway stimulation, and phosphorylation of the ADRB2 C-terminal region were not introduced.

The study described above shows a potential of the optogenetics tool based on CRY and CIB to induce a particular physiological event. In the case of ADRB2 and β-arrestin, CRY-CIB interaction upon blue light irradiation induces ADRB2-β-arrestin complexes formation, resulting in endocytosis of the complexes (Fig. 7.5). It is noteworthy that the light irradiation triggers complex formation of ADRB2 and β-arrestin and following endocytosis, a downstream event of ADRB2—β-arrestin complex formation. On the other hand, upstream phenomena of the endocytosis, such as G-protein pathway signaling, the C-terminal phosphorylation, or ERK1/2 pathway signaling, were not induced. These results imply two aspects of GPCR endocytosis.

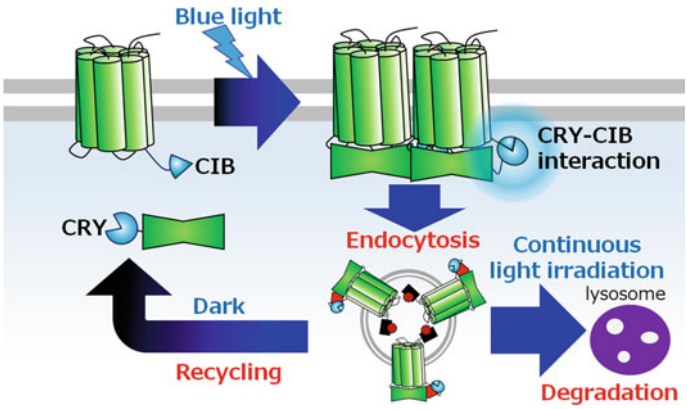

Fig. 7.5 Schematic on trafficking of ADRB2-CIB and β-arrestin-CRY upon photo irradiation

First, formation of endosomes is induced upon GPCR-β-arrestin complex formation, which would provide a scaffold to recruit various endocytosis related proteins to the endosomes. Second, endocytosis of GPCR does not require other upstream biological activation, such as signaling activity through G-proteins and ERK1/2, and phosphorylation of the GPCR. These finding are newly provided as a GPCR functioning mechanism, and would not be revealed without a photo-activation system based on CRY and CIB.

7.5 Perspective

Upon an extracellular signal is induced into a cell, the signal is transmitted through many molecules and also branched multiple pathways. In addition, temporal pattern of signal input causes a variety of signal outputs. Analyses of the function and mechanism of each signaling molecule are still difficult in conventional methods. Recent development of optogenetic tools allows for spatiotemporally-specific signal input, as well as stimulation of a particular target molecule in the middle of a signaling pathway. As shown in the case of optogenetic control of ADRB2-β-arrestin interaction, different duration time of molecular-molecular interaction results in various outputs; ADRB2 was recycled after β-arrestin dissociation whereas continuous ADRB2-β-arrestin interaction for 60 min or longer led them to a degradation pathway in lysosomes. Activation of ADRB2-β-arrestin interaction, which is a mid-point in whole signaling cascades starting from ADRB2, revealed that upstream signaling events such as activation of G-protein signaling and ERK1/2 signaling are not required to initiate and control ADRB2 trafficking. Such analyses were realized by artificial manipulation of ADRB2-β-arrestin interaction by the CRY-CIB-based optogenetic tool.

Further development of optogenetic tools and their practical application will provide much suggestive information in biological studies. Variation of spatial and temporal patterns in signal input generated by an optogenetic technique and quantitative analysis of signal output will provide clues to establish a mathematical model on a target signaling cascade, which will highly contribute to works on systems biology. Optogenetic signal input to a particular cell in living organ will also be useful to investigate cell-cell communication and intercellular signal transduction. Thus, optogenetic techniques will provide new aspects in investigation on signal transduction and mechanisms in biological phenomena of living animals.

Acknowledgements This work was supported by CREST (JPMJCR1752 to T.O.) from Japan Science and Technology (JST) and the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grants-in-Aid for Scientific Research (S) 26220805 to T.O.).

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