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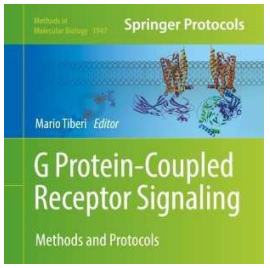
A Combined Cell-Free Protein Synthesis and Fluorescence-Based Approach to Investigate GPCR Binding Properties



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We use this protocol and it's working

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Abstract

Fluorescent labeling of de novo synthesized proteins is in particular a valuable tool for functional and structural studies of membrane proteins. In this context, we present two methods for the site-specific fluorescent labeling of difficult-to-express membrane proteins in combination with cell-free protein synthesis. The cell-free protein synthesis system is based on Chinese Hamster Ovary Cells (CHO) since this system contains endogenous membrane structures derived from the endoplasmic reticulum. These so-called microsomes enable a direct integration of membrane proteins into a biological membrane. In this protocol the first part describes the fluorescent labeling by using a precharged tRNA, loaded with a fluorescent amino acid. The second part describes the preparation of a modified aminoacyl-tRNA-synthetase and a suppressor tRNA that are applied to the CHO cell-free system to enable the incorporation of a non-canonical amino acid. The reactive group of the non-canonical amino acid is further coupled to a fluorescent dye. Both methods utilize the amber stop codon suppression technology. The successful fluorescent labeling of the model G protein-coupled receptor adenosine A2A (Adora2a) is analyzed by in-gel-fluorescence, a reporter protein assay, and confocal laser scanning microscopy (CLSM). Moreover, a ligand-dependent conformational change of the fluorescently labeled Adora2a was analyzed by bioluminescence resonance energy transfer (BRET).

Attachments

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Guidelines

1 Introduction

G protein-coupled receptors (GPCRs) are involved in vital processes including the regulation of signaling pathways and the subsequent triggering of essential physiological responses. Up to date more than 50% of pharmaceutical drugs are targeting GPCRs and GPCR interaction partners [1]. Therefore, the development of novel production processes of GPCRs for structural and functional characterization remains essential. In a natural environment, membrane proteins are only present in small concentrations [2]. The overexpression of membrane proteins is usually performed in heterologous systems involving a complex vector design. Nevertheless, overexpression obstacles such as low solubility, cytotoxicity, and low stability can occur [3]. In addition, detergents used to solubilize membrane proteins often induce altered ligand binding properties [4]. Cell-free protein synthesis based on translationally active lysates represents an approved alternative for membrane protein synthesis. Eukaryotic cell lysates based on insect, CHO, and human cell lines are predestined for membrane protein synthesis due to the presence of endogenous membrane structures [5]. These microsomes enable an integration of membrane proteins into a biological membrane. In addition, posttranslational modifications such as disulfide bridging, phosphorylation, and partly glycosylation are possible [6]. Furthermore, the CHO cell-free system is characterized by a high productivity of complex proteins [7].

In the context of protein modification and fluorescent labeling by using genetic code expansion [8], cell-free systems are of special interest due to their open reaction mode [9]. Exogenously prepared components can be easily and directly added to the cellfree protein synthesis reaction to incorporate a defined non-canonical amino acid site-specifically at the position of a redefined codon within the gene sequence [10]. In a first step, addressing of a defined amber stop codon can be verified by the addition of a precharged tRNA to the cell-free protein synthesis reaction. The tRNAs in this report are aminoacylated to a lysine residue that is coupled to a Bodipy TMR dye (BP). The tRNA recognizes either the amber stop codon or the phenylalanine codon. The lysine residue with the coupled fluorescent dye is subsequently sitespecifically (BP-CUA) or statistically (BP-GAA) incorporated. The lack of a re-aminoacylating mechanism of the tRNA leads to the consumption of large amounts of precharged tRNA. Therefore, we describe an alternative system that is inspired by the natural aminoacylation of tRNAs in vivo.

The addition of non-canonical amino acids in vivo that usually do not permeate the cell wall can be realized in cell-free systems due to the missing cell wall. Even high concentrations of non-canonical amino acids can be added to the reaction. Moreover, the suppressor tRNA and the modified aminoacyl-tRNA-synthetase can be applied to the cell-free reaction in defined concentrations enabling an identification of optimal concentrations for highest suppression efficiency. Here, the described experimental setup is based on an evolved aminoacyl tRNA-synthetase (aaRS) derived from *E.coli* [11, 12] and a modified suppressor tRNA recognizing the UAG amber stop codon [13]. The combination of the aaRS, tRNA, and a phenylalanine-derivate (p-propargyloxy-L-phenylalanine, pPa) with an alkyne group in para-position resulted in an efficient incorporation of the non-canonical amino acid into the model protein adenosine A_{2A} receptor (Adora2a). The human Adora2a is expressed in almost every tissue of the body. Primarily, the membrane protein can be detected in the brain, cells of the immune system, leucocytes, and platelets. In peripheral tissues, the Adora2a is involved in inflammation reactions and affects the control of the cardiovascular system [14]. The interaction of the Adora2a with different neurotransmitters in the brain is of highest interest since the receptor displays an important role in the regulation of dopamine and

glutamate release thereby making it a promising therapeutic target for the treatment of depression, insomnia, and Parkinson's disease [15]. Consequently, a better understanding of the detailed interactions between membrane proteins and their specific ligands is mandatory. Information on the structural and functional relationship of a defined GPCR might be gained by a visualization of the target membrane protein in a nature-like lipid environment [14]. A highly preferred method to determine GPCR interaction is based on the spatial proximity of two fluorescent moieties. Interaction partners can be determined by an intermolecular fluorescence resonance energy transfer (FRET) and specific conformation changes by an intramolecular FRET. For the Adora2a, intermolecular as well as intramolecular FRETs were established to analyze the receptors' behavior [16, 17]. In this way, the conformational rearrangement of the helices III and VI induced by agonist binding was elucidated [18]. A coupled fluorescent dye in the region of amino acid 215 will be excited by a C-terminally fused NanoLuciferase (Nluc) resulting in a detectable bioluminescence resonance energy transfer (BRET). A signal change of the BRET value after the addition of a ligand indicates a conformational change of the Adora2a. The main objective of this chapter is to describe a cell-free protein synthesis-based alternative methodology to the standard protein visualization methods typically used in vivo. Our cell-free procedure is based on the site-specific and statistical incorporation of a precharged tRNA and a site-specific incorporation of a non-canonical amino acid into the target protein. The cell-free synthesized protein is subsequently labeled by using a chemoselective reaction. Moreover, we adapted an intramolecular BRET for the Adora2a to show a ligand-dependent conformational change. In addition, we present the preparation of the evolved aminoacyl-tRNA-synthetase and the suppressor tRNA.

4 Notes

1. Preparation of CHO lysate: The cultivation was carried out in a Biostat B-DCU II bioreactor (Sartorius Stedium Biotech GmbH) at 37 °C with a chemical defined and serum-free media. Cells were harvested at a density of 3.5–5 × 10^6 cells/ml by centrifugation at 200 × g for 5 min. The cell pellet was resuspended in 40 mM HEPES-KOH, pH 7.5, 100 mM NaOAc and 4 mM DTT. The cell suspension was passed through a 20-gauge needle to mechanically disrupt the cell membrane. Nuclei and cell debris were removed by a centrifugation step at 10,000 × g for 10 min. Raw lysate is applied to equilibrated Sephadex G-25 columns. Elution fractions with the highest measured RNA concentration were pooled and treated with micrococcal S7 nuclease to digest endogenous mRNA. The inactivation of the calcium-dependent nuclease was performed by adding 6.7 mM EGTA to complex the calcium ions. The lysate was further supplemented with creatine kinase (f.c. 100 µg/ml) to ensure the regeneration of ATP out of creatine phosphate. The prepared lysate was shock frozen in liquid nitrogen and stored at –80 °C.
2. The cell-free reaction in a dialysis mode is performed in a two-chamber device. The reaction chamber (1.1 ml, red lid) and the feeding chamber (11 ml, colorless lid) are separated by a semipermeable membrane with a molecular weight cut-off of 10 kDa. Whereas inhibitory byproducts such as accumulating phosphates are removed, amino acids and energy components are delivered to the reaction chamber.
3. It is recommended to collect samples of the translation mix, supernatant, purification steps including flow through, washing fractions and elution fractions as well as buffer exchange procedure and concentration. The aliquots can be diluted in SDS-PAGE sample buffer and loaded to the SDS-PAGE in order to monitor the purity of the aminoacyl-tRNA-synthetase during the preparation.

4. Concentrate the synthetase to a concentration of 5 g/l to ensure a minimal final concentration of 100 µM. If necessary repeat the concentration step.
5. The PCR product is purified with the QIAquick PCR Purification Kit and the concentration is determined by using a Nano-Drop 2000c. For further analysis prepare a 1% (w/v) agarose gel and load 1 µl of the PCR product. The expected band size is 123 bps.
6. The prepared RNA can be analyzed by gel-electrophoresis run in 1× TBE buffer (**Protocol 2.2** "Preparation of Suppressor tRNA", item 11 of Subheading "Generation of PCR Product"). Therefore, prepare a 2% (w/v) agarose gel. Mix 2 µl of the RNA with 6 µl MOPS sample buffer (**Protocol 2.2** "Preparation of Suppressor tRNA", item 6 of Subheading "Generation of RNA Transcript") and load the sample to the agarose gel. Use a RNA ladder. The expected band size is around 200 bps.
7. After centrifugation three phases are present: on top the aqueous phase with approximately 50% of the total volume, containing the RNA; a middle interphase that is nearly invisible and below the red phenol/chloroform phase. Try to isolate only the aqueous phase.
8. It is important that the components are dissolved completely to ensure the correct concentration. Vortex the components and store them in aliquots to avoid repeated thaw and freeze cycles.
9. The plasmid concentration can be varied and can be dependent on the chosen vector backbone. It is recommended to apply different plasmid concentrations in the range of 20–100 nM to the cell-free protein synthesis reaction.
10. The fluorescent dye is susceptible to light. An illumination will decrease the fluorescence intensity of the dye. Use colored tubes or wrap the tube with aluminum foil. In addition, keep in mind that the precharged tRNACUA will address the amber stop codon whereas the precharged tRNAGAA will address statistically phenylalanine codons.
11. The optimal temperature for cell-free protein synthesis reaction in a CHO lysate is 30 °C. However, a temperature series is recommended to determine the optimal conditions for an individual protein in terms of folding and activity. The Adora2a-Nluc construct showed the highest Nluc activity at 27 °C.
12. It is recommended to analyze the incorporation efficiency of the non-canonical amino acid. With a low efficiency the subsequent labeling reaction will yield as well in a low amount of labeled protein. Therefore, two different methods can be utilized. (a) Adding a reporter protein downstream of the amber stop codon. The reporter protein will only be translated if the amber stop codon is addressed by the tRNA and the non-canonical amino acid is transferred to the polypeptide chain. The intensity of the reporter protein signal is directly correlated to the amount of full-length protein and in conclusion to the incorporation efficiency. A comparison to a DNA construct without amber stop codon is possible. (b) Determine the amount of full-length protein with autoradiography. If the termination product has an adequate amount of incorporated ^{14}C-leucine during the cell-free protein synthesis reaction and a detectable size, an additional band should be visible in the autoradiography. The ratio of full-length product and termination product can be calculated.
13. It is recommended to evaluate different agonist concentrations because at a defined concentration the binding sites of the cell-free synthesized Adora2a should be completely occupied resulting in a saturation effect in the BRET signal. The saturation effect supports the specific conformational rearrangement after ligand binding.
14. The non-canonical amino acid is solubilized in 0.5 M NaOH. The alkaline pH of NaOH will shift the pH of the lysate that might result in an inactivation of enzymes involved in protein synthesis. Therefore, dilute the ncAA in translation mix, water and components that are not affected by an alkaline pH.

15. Reactive groups of ncAA are often instable and susceptible to illumination. If the reactive group is inert a following coupling to a fluorescent dye is not possible. Therefore, keep the ncAA in the dark by using colored or wrapped tubes.
16. Copper has a toxic effect on proteins due to oxidative damage, but it is necessary as catalyst for the copper(I)-catalyzed alkyneazide cycloaddition (CuAAC). It is recommended to use different copper concentrations for the labeling reaction. For the Adora2a_amb construct we have seen a highest labeling efficiency with 200 µM copper. The lowest copper concentration resulting in a detectable fluorescent band was 50 µM. Keep in mind that the concentration of THPTA has to be adjusted as well (threefold concentration of THPTA to $CuSO_4$). In addition, it is recommended to adjust the incubation time of the labeling reaction. In general, CuAAC is a fast and efficient click reaction. A decrease in incubation time might enhance proteins activity.
17. The same microscopic settings that are chosen for the fluorescently labeled sample should be applied to the negative control to exclude an unspecific labeling of microsomal structures. Fluorescent dyes are usually highly hydrophobic and tend to stick unspecific to lipid membranes. It is recommended to evaluate the unspecific binding of different dyes to figure out the most suitable dye for certain applications. For our images a pinhole of 1 Airy unit (118 µm), laser intensities of 22% (bright field) and 8% (fluorescence) resulted in the optimal recording of fluorescently labeled Adora2a.

Acknowledgments

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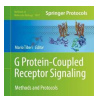

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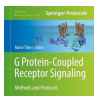

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