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G-Protein-Coupled Receptor Interaction with Small GTPases

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Abstract

In addition to heterotrimeric G-proteins, Ras-like small GTPases are also involved in regulating physiological functions of the G-protein-coupled receptor (GPCR) superfamily. In particular, Rab and ARF GTPases function either as “traffic cops” to coordinate receptor targeting to specific locations or as “signal transducers” to directly mediate receptor signal propagation. As revealed in protein–protein interaction assays, GPCRs may use specific motifs to physically interact with small GTPases, providing important insights into the underlying molecular mechanisms. In this chapter, we describe coimmunoprecipitation and GST fusion protein pull-down approaches to study the GPCR-small GTPase interaction, by focusing on the interaction of α_{2B} - and β_2 -adrenergic receptors with the small GTPases Rab8 and ARF1.

1. INTRODUCTION

The multiphysiological functions of G-protein-coupled receptors (GPCRs) are largely mediated by activating heterotrimeric G-proteins and are regulated by transport of both the receptors and the G-proteins to specific locations (Qin, Dong, Wu, & Lambert, 2011; Sorkin & von Zastrow, 2009; Wu, Benovic, Hildebrandt, & Lanier, 1998). In addition to heterotrimeric G-proteins, the Ras-like Rab and ADP-ribosylation factor (ARF) small GTPases, which have been well described to function as “traffic cops” to control cargo transport between various intracellular compartments (Takai, Sasaki, & Matozaki, 2001), are also involved in the regulation of GPCR systems. Rab GTPases are involved in almost every step of vesicle-mediated transport including the targeting, tethering, and fusion of transport vesicles with the appropriate acceptor membrane (Grosshans, Ortiz, & Novick, 2006), whereas ARFs function as crucial regulators in the formation of transport vesicles from distinct intracellular compartments (D’Souza-Schorey & Chavrier, 2006). Among the five ARF GTPases identified in humans, ARF1 is the best-studied and well-understood member. ARF1 plays a crucial role in both anterograde and retrograde trafficking, and its traffic function is mediated through regulating the formation of COPI- and clathrin-coated vesicles. Consistent with their well-established traffic functions, several Rabs and ARFs have been demonstrated to modulate the cell surface transport of GPCRs (Charette, Holland, Frazer, Allen, & Dupre, 2011; Dong, Li, & Wu, 2011; Dong & Wu, 2007; Dong, Yang, et al., 2010; Dong, Zhang, et al., 2010; Dupre et al., 2006; Filipeanu, Zhou, Claycomb, & Wu, 2004; Filipeanu, Zhou, Fugetta, & Wu, 2006; Luo, Wang, & Reiser, 2007; Satoh, O’Tousa, Ozaki, & Ready, 2005; Wang & Wu, 2012; Wu, Zhao, & He, 2003; Zhang et al., 2009). Furthermore, ARF1 has also been shown to modulate the activation of phospholipase D and

extracellular signal-regulated kinases 1/2 (ERK1/2) by GPCRs (Dong et al., 2011; Mitchell et al., 1998, 2003; Shome, Nie, & Romero, 1998).

As revealed in different protein–protein interaction assays, the functions of these small GTPases in regulating the trafficking and signaling of GPCRs may be mediated through their physical and specific association with the receptors (Table 6.1) (Barclay et al., 2011; Deretic et al., 2005; Dong, Yang, et al., 2010; Dong, Zhang, et al., 2010; Dong et al., 2011; Esseltine, Dale, & Ferguson, 2011; Hamelin, Theriault, Laroche, & Parent, 2005; Madziva & Birnbaumer, 2006; Mazelova et al., 2009; Mitchell et al., 1998, 2003; Parent, Hamelin, Germain, & Parent, 2009; Robertson et al., 2003; Satoh et al., 2005; Seachrist et al., 2002; Tai, Chuang, Bode, Wolfrum, & Sung, 1999; Wikstrom et al., 2008). We have demonstrated that α_{2B} -adrenergic receptor (α_{2B} -AR) and β_2 -AR use different motifs in the C-termini to interact with Rab8, and these interactions likely dictate their post-Golgi trafficking (Dong, Yang, et al., 2010). We have also demonstrated that α_{2B} -AR uses the unique double Trp (diW) motif in the third intracellular loop to interact with ARF1 in an agonistdependent manner, and it is this interaction that modulates the activation of ERK1/2 by α_{2B} -AR (Dong et al., 2011). Therefore, studying the interaction between GPCRs and small GTPases may provide important insights into the molecular mechanisms underlying the regulation of GPCRs by small GTPases. Many protein–protein interaction assays have been successfully developed to measure the interaction between GPCRs and small GTPases. In this chapter, we focus on co-immunoprecipitation (co-IP) and GST fusion protein pull-down assays, which have been employed in many laboratories to study the GPCR-small GTPase interaction.

2. ASSAYS FOR GPCR INTERACTION WITH SMALL GTPases

In general, it is very difficult to immunoprecipitate GPCRs by using antibodies against the receptors themselves due to the poor quality of the antibodies and the extremely hydrophobic property of the receptors. To overcome this issue, one could label the receptors with an epitope, such as HA, FLAG, or GFP, at their terminal tails. The epitope-tagged receptors can be easily immunoprecipitated by high-affinity antibodies against the epitope. It has been well demonstrated that epitope-tagging does not significantly influence the properties of the receptors, including intracellular trafficking, ligand binding, and G-protein coupling. Our laboratory has used both HA and GFP to label GPCRs, resulting in the receptors with similar characteristics to their wild-type counterparts (Duvernay et al., 2009, 2011; Wu et al., 2003). We have also studied the interaction of HA- and GFP-tagged receptors with small GTPases by co-IP using antibodies against the epitopes (Dong, Yang, et al., 2010; Dong, Zhang, et al., 2010; Dong et al., 2011). In contrast to the co-IP assay to detect the interaction of full-length receptors with small GTPases, GST fusion protein pull-down assay is particularly useful in defining the intracellular subdomains or searching for the specific motifs that mediate GPCR interaction with small GTPases. The following sections describe the co-IP and GST fusion protein pull-down assays which were used in our laboratory to study the interaction of α_{2B} -AR and α_2 -AR with the small GTPases Rab8 and ARF1.

2.1. Co-IP assay

- 2.1.1. HEK293 cells are cultured on 100-mm dishes and transiently transfected with 2 μ g of GFP-tagged α_{2B} -AR in the pEGFP-N1 vector together with 2 μ g of ARF1 for 36 h. The cells transfected with the pEGFP-N1 vector and ARF1 are used as a control. Generation of α_{2B} -AR tagged with GFP at its C-terminus is described elsewhere (Wu et al., 2003).

- 2.1.2.** The cells are washed twice with phosphate saline buffer and harvested by centrifugation at $500\times g$ for 5 min at 4 °C.
- 2.1.3.** The cells are lysed with 500 μ l per dish of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, and Complete Mini protease inhibitor cocktail (Roche Diagnostics). After gentle rotation for 1 h, samples are centrifuged at $14,000\times g$ for 15 min at 4 °C.
- 2.1.4.** The supernatant is incubated with 50 μ l of protein G Sepharose 4B beads for 1 h at 4 °C, and the beads are discarded after centrifugation. This step will remove the proteins that may nonspecifically bind to the beads during incubation with antibodies.
- 2.1.5.** The supernatant is incubated with 3 μ g of GFP antibodies (Santa Cruz Biotechnology) overnight at 4 °C with gentle rotation followed by incubation with 50 μ l of protein G Sepharose 4B beads for 5 h.
- 2.1.6.** The Sepharose beads are collected by centrifugation and washed three times each with 500 μ l of lysis buffer without SDS.
- 2.1.7.** α_{2B} -AR and ARF1 bound to the Sepharose beads are eluted with 100 μ l of 1 $\times$ SDS gel-loading buffer. Thirty microliters from each sample is then separated by SDS-PAGE to probe ARF1 in the immunoprecipitates by immunoblotting using ARF1 antibodies.
- 2.1.8.** To determine the amount of the receptors in the immunoprecipitates, each sample is further diluted five times with 1 $\times$ SDS gel-loading buffer, separated by SDS-PAGE and probed with GFP antibodies. Based on our experience, further dilution of the immunoprecipitates with SDS gel-loading buffer is an important step in order to clearly detect the receptors by immunoblotting.
- 2.1.9.** Similar to GPCRs, small GTPases can also be tagged with epitopes and coexpressed with the receptors. If so, small GTPases in the immunoprecipitates can be verified by immunoblotting using epitope antibodies. We have studied the interaction of HA-tagged α_{2B} -AR with GFP-tagged ARF1 and Rab8 by co-IP (Dong, Yang, et al., 2010; Dong et al., 2011).
- 2.1.10.** To measure agonist activation-dependent interaction of α_{2B} -AR with ARF1, HEK293 cells are transfected with HA-tagged α_{2B} AR together with ARF1 as described above. After starvation for at least 3 h, the cells are stimulated with UK14,304, an α_2 -AR agonist, at a concentration of 1 μ M for different time periods. α_{2B} -AR is immunoprecipitated with anti-HA mouse monoclonal antibodies 12CA5 (Roche Diagnostics). After elution of the bound proteins, HA-tagged α_{2B} -AR and ARF1 in the immunoprecipitates are detected by immunoblotting using HA and ARF1 antibodies, respectively. Our studies have shown that α_{2B} -AR interaction with ARF1 was markedly augmented by UK14,304 stimulation (Fig. 6.1) (Dong et al., 2011).
- 2.1.11.** To determine if the GPCR-GTPase interaction depends on the activation of GTPase, HEK293 cells are transfected with the receptor together with either inactive GDP-bound mutant or active GTP-bound mutant of the GTPase. After immunoprecipitation as described above, the amounts of the mutated small GTPase in the immunoprecipitates are compared. Our studies have shown that both α_{2B} -AR and β_2 -AR preferentially interacted with GDP-bound form of Rab8 (Dong, Yang, et al., 2010).

2.2. GST fusion protein pull-down assay

- 2.2.1. HEK293 cells (with or without ARF1 transfection) or brain tissues are homogenized in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40, and total lysates are prepared as described previously (Wu, Bernard, & Lanier, 2002).
- 2.2.2. GST and GST fusion proteins encoding the intracellular domains of α_{2B} -AR are expressed in bacteria and purified using glutathione Sepharose 4B beads as described previously (Wu et al., 2002). The purity of purified GST fusion proteins is analyzed by Coomassie Brilliant Blue staining following SDS-PAGE before the experiments. GST fusion proteins tethered to the glutathione beads are either used immediately or stored at 4 °C for no longer than 3 days.
- 2.2.3. The GST fusion proteins (about 5 μ g) immobilized on the glutathione resin are incubated with 1 mg of cell or tissue lysates in a total volume of 500 μ l binding buffer containing 20 mM Tris-HCl, pH 7.5, 2% NP-40, and 70 mM NaCl. Concentrations of both NaCl and NP-40 in the binding buffer should be optimized. We have used NaCl from 70 to 150 mM and NP-40 from 0.5% to 4% in the assay. The incubation may be carried out at 4 °C overnight or at room temperature for 3 h.
- 2.2.4. The resin is washed four times with 1 ml of binding buffer. Bound ARF1 is eluted with 1 $\times$ SDS gel-loading buffer, separated by SD-SPAGE, and detected by immunoblotting using ARF1 antibodies.
- 2.2.5. To determine if the interaction between GPCRs and small GTPases is direct, small GTPases are purified and then incubated with GST fusion proteins encoding receptor domains. We have used the following protocol to show the direct interaction between the C-terminus and the third intracellular loop of α_{2B} -AR and ARF1 (Fig. 6.2A). ARF1 was tagged with the epitope His at its N-terminus, and His-ARF1 was purified by using His SpinTrap kit from GE Healthcare. One microgram of purified His-ARF1 was incubated with GST fusion proteins at 4 °C for 2 h. The resin was washed four times with 1 ml of binding buffer, and the retained proteins were solubilized in 1 $\times$ SDS gel-loading buffer and separated by SDS-PAGE. Bound His-ARF1 was detected by immunoblotting using His or ARF1 antibodies (Dong, Zhang, et al., 2010).
- 2.2.6. This method can also be used to determine if the interaction between GPCRs and small GTPases depends on the activation of the small GTPases. For this purpose, inactive GDP- or active GTP-bound mutants of the GTPase are expressed in cells, and the cell lysates are incubated with GST fusion proteins encoding the receptor domains. The amounts of the mutated small GTPase bound to the receptor domains are compared (Dong, Yang, et al., 2010).

3. FUNCTION OF GPCR–SMALL GTPase INTERACTION

3.1. Role in GPCR trafficking

Over the past several years, great progress has been made in defining the role of small GTPases in intracellular trafficking of GPCRs, including cell surface export, internalization, and recycling. The fact that GPCRs may use highly specific motifs to directly interact with small GTPases to coordinate their trafficking between various intracellular organelles provides an excellent example indicating that the cargo GPCRs may physically associate with components of transport machinery to control their transport. For example, the dileucine (LL) motif, which is highly conserved in the membrane-proximal C-termini of the family A GPCRs (Duvernay, Zhou, & Wu, 2004), mediates β_2 -AR interaction with Rab8

(Fig. 6.2B), and the interaction is likely involved in the post-Golgi traffic of the receptor (Dong, Yang, et al., 2010). As the LL motif has also been demonstrated to be important in the export of newly synthesized GPCRs from the endoplasmic reticulum (Carrel, Hamon, & Darmon, 2006; Duvernay et al., 2004; Lachance et al., 2011; Sawyer, Ehlert, & Shults, 2010; Schulein et al., 1998), it may function at multiple intracellular compartments. Another excellent example is that rhodopsin uses its C-terminal VxPx motif to interact with ARF4 GTPase to modulate its movement from the TGN to the plasma membrane in a polarized environment (Deretic et al., 2005; Mazelova et al., 2009). As the mutation of the VxPx motif has been well described to contribute to the pathogenesis of neurodegenerative diseases, particularly the severe forms such as autosomal dominant retinitis pigmentosa, rhodopsin interaction with ARF4 may play a crucial role in performing its physiological function.

3.2. Role in GPCR signaling

In addition to coordinating receptor trafficking, the interaction of GPCRs with small GTPases may directly modulate receptor-mediated signal propagation. We have recently used GST fusion protein pull-down assay in combination with site-directed mutagenesis to identify the diW motif located in the third intracellular loop of α_{2B} -AR as an ARF1-binding site (Fig. 6.2C). Surprisingly, mutation of the diW motif to disrupt α_{2B} -AR interaction with ARF1 did not alter the cell surface transport and endocytosis of α_{2B} -AR, but significantly attenuated ERK1/2 activation by α_{2B} -AR (Dong et al., 2011). These data suggest that diW motif-mediated α_{2B} -AR interaction with ARF1 may play a role in regulating α_{2B} -AR function. These data, together with the other reports that demonstrated a crucial role of ARF1 in the activation of phospholipase D and phosphatidylinositol 4-phosphate 5-kinases (Brown, Gutowski, Moomaw, Slaughter, & Sternweis, 1993; Cockcroft et al., 1994; Jones et al., 2000; Mitchell et al., 1998, 2003; Shome et al., 1998), indicate that ARF1 may function as a signaling molecule mediating GPCR signal propagation.

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Figure 6.1.

Agonist activation-dependent association of α_{2B} -AR with ARF1 as measured in co-immunoprecipitation assay. HEK293 cells were transfected with HA- α_{2B} -AR and ARF1 and stimulated with 1 μ M UK14,304 for different time periods (0–40 min). HA- α_{2B} -AR was immunoprecipitated (IP) with HA antibodies. HA- α_{2B} -AR and ARF1 in the immunoprecipitates were detected by immunoblotting (IB) using HA and ARF1 antibodies, respectively (Dong et al., 2011).

Figure 6.2.

Interaction of α_{2B} -AR and β_2 -AR with Rab8 and ARF1 as determined by GST fusion protein pull-down assay. (A) Interaction of the third intracellular loop (ICL3) and the C-terminus (CT) of α_{2B} -AR with purified ARF1. ARF1 was tagged with 6 $\times$ His and purified. The ICL3 and the CT of α_{2B} -AR were generated as GST fusion proteins and incubated with purified His-tagged ARF1. Bound His-ARF1 was detected by immunoblotting using ARF1 antibodies (Dong, Zhang, et al., 2010). (B) Interaction of the β_2 -AR CT with Rab8 and the role of the LL motif. The β_2 -AR CT and its mutant in which LL was mutated to AA were generated as GST fusion proteins. Rab8 tagged with GFP was expressed in HEK293 cells, and total cell homogenates were incubated with the GST- β_2 -AR CT fusion proteins. Rab8 bound to the β_2 -AR CT was revealed by immunoblotting using GFP antibodies (Dong, Yang, et al., 2010). (C) The diW motif in the ICL3 mediates α_{2B} -AR interaction with

ARF1. Each residue in the ICL3 fragment W359-E369 was mutated to alanine and generated as GST fusion proteins. Their interaction with ARF1 was determined by GST fusion protein pull-down assay (Dong et al., 2011).

Table 6.1

Interaction of GPCRs with small GTPases

GPCRs	Domains/motifs	GTPases	Assays	Functions
AT1R	Unknown	ARF1,3	Co-IP	PLD activation
AT1R	CT: Pro-Cys	Rab4,5,7,11	Co-IP, GST, Y2H	Trafficking
α_{2B} -AR	CT	Rab8	Co-IP, GST	Golgi-to-PM
α_{2B} -AR	CT	ARF1	Co-IP, GST	PM transport
α_2 B-AR	ICL3: di-Trp	ARF1	Co-IP, GST	ERK1/2 activation
β_2 -AR	CT: di-Leu	Rab8	Co-IP, GST	Golgi-to-PM
β_2 -AR	CT	Rab11	Co-IP, GST	Recycling
M3-MR	ICL3	ARF1,6	Co-IP, GST	PLD activation
5-HT _{2A} R	CT, ICL3	ARF1	Co-IP, GST	PLD activation
TP β	CT, ICL1	Rab11	Co-IP, GST	Recycling
IP	CT	Rab11	Co-IP, Y2H	Recycling
Rhodopsin	CT: Val-X-Pro-X	ARF4	GST, cross-link	TGN-to-PM
V2R	Unknown	ARF6	Co-IP	ER-to-PM

AT1R, angiotensin II type 1 receptor; AR, adrenergic receptor; MR, muscarinic receptor; 5-HT_{2A}R, 5-hydroxytryptamine 2A receptor; TBbeta, thromboxane A₂receptor; IP, prostacyclin receptor; V2R, vasopressin V2 receptor; CT, C-terminus; ICL1, the first intracellular loop; ICL3, the third intracellular loop; Co-IP, co-immunoprecipitation assay; GST, GST fusion protein pull-down assay; Y2H, yeast two hybrid screening assay; PLD, phospholipase D; PM, plasma membrane; ERK1/2, extracellular signal-regulated kinases 1/2; TGN, trans-Golgi network; ER, endoplasmic reticulum.