Characterization of Gα$_i$ protein interactions with RASA3, 5-HT1A and TNFAIP8

Jeremy Soo, Mireille Daigle, Paul Albert

Department of Cellular and Molecular Medicine (Neuroscience Research Institute) - *University of Ottawa*
451 Smyth Road, Ottawa, Ontario, Canada, K1H 8M5.

INTRODUCTION

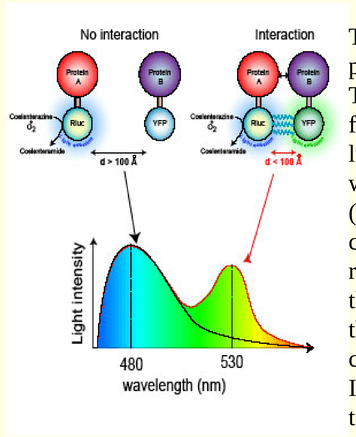


Figure 1: BRET mechanism. (Futura-Santé, 2008)

Bioluminescence Resonance Energy Transfer (BRET) is a method for monitoring protein-protein interactions in living cells. The process involves having one protein fused to an energy donor (Renilla reniformis luciferase, Rluc) and the other protein fused with a fluorescent energy acceptor protein (usually GFP^2). After both proteins are coexpressed, coelenterazine (substrate for rLuc) is added and the BRET is measured. If the BRET signal is above background levels, then the interaction between both proteins is concluded to be in close proximity (< 100 A). In this study, BRETs will be relied on heavily to characterize the interactions between Gα$_i$ proteins with other potential interactors.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are signal transducers that link receptors to the intracellular signal pathway. Though they mediate signaling of a large variety of receptors, the mechanisms and pathways underlying their effects are still unclear. Originally, Gα$_i$ proteins were identified by their ability to inhibit adenylyl cyclase, but even that ability does not explain all of their actions.

Several proteins have been noted to interact with Gα$_i$ proteins, and are thought to interact preferentially with Gα$_i$3. Tumor Necrosis Factor Alpha-Induced Protein 8 (TNFAIP8) was identified as a Gα$_i$-dependent effector by using Gα$_i$3-Q204L as bait in a yeast two-hybrid screen of an NIH-3T3 cell cDNA library (Laliberté et al., 2010). TNFAIP8 is involved in anti-apoptotic and pro-oncogenic signaling in immune and breast cancer (Kumar et *al.* 2000, 2004). It has been noted that Gα$_i$ proteins have been implicated in tumor formation and progression by increasing the survival rate and proliferation and reducing cell death (Dorsam and Gutkind, 2007). Other novel effector proteins include RASA3 (a member of the RasGAPs which mediates D2S receptor signaling via Gα$_i$3 to inhibit thyrotropin-releasing hormone (TRH)-induced ERK 1/2 activation (Nafisi et. *al*, 2008)). We also study Gi coupling of the 5-hydroxytryptamine receptor 1A (5-HT1A, which mediates inhibitory neurotransmission of serotonin). However, the interactions of these proteins were originally characterized were using Gα$_i$1, and not with Gα$_i$3. In this study, the interaction between both Gα$_i$1 and Gα$_i$3 with TNFAIP8, RASA3 and 5-HT1A was investigated, mainly through BRET assays.

METHODS

Yeast Mating/β-galactosidase assay - Gα$_i$3wt and Q204L constructs were subcloned into pAS2-1 vectors, transformed into Y187 yeast strain and selected on –Leu plates while TNFAIP8, TIPE1 and TIPE2 constructs were subcloned into pACT2 vectors, transformed into AH109 yeast strain and selected on -Trp plates. The resultant colonies were mated and selected on SD-Leu, -Trp and -His for 3 days at 30°C. A quantitative β-galactosidase assay was conducted using σ-nitrophenyl β-D-galactopyranoside (Sigma).

BRET constructs: The rLuc gene was inserted into the Gα$_i$1 and Gα$_i$3wt constructs at positions 60, 122, and 91 for the Gα$_i$1 and 91 only for the Gα$_i$3wt. Mutagenesis was conducted to obtain Q204L mutants of the Gα$_i$ constructs. TNFAIP8, 5-HT1A, HCN4 and RASA3 were subcloned into pGFP2-N constructs with GFP2 at the C-terminal.

***Transfections of BRET constructs*:** HEK 293 cells were grown in 10-cm plates in Dulbecco's Modified Eagle Medium (DMEM) + 8% Fetal Bovine Serum (FBS). The following day, cells were transfected by calcium phosphate with 5 µg of the plasmids producing proteins with interactions under investigation. After 48 hours, the cells were analyzed by BRET.

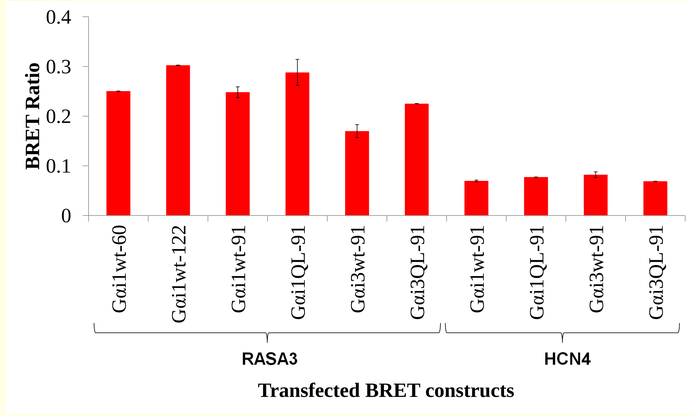
BRET: Transfected cells were washed twice with D-PBS, detached with HBBS-EDTA and then centrifuged at 700 x g. The pellet was resuspended in BRET buffer (2 µg/mL aprotinin in D-PBS). Cells were placed in duplicates in a 96 well opaque plate, and incubated in the dark for 30 min. Agonists (4 µM 5-HT, 10 ng/µL TNF) were added to the appropriate samples prior to reading. The plates were read using a Victor V3 multi-label counter (Perkin Elmer). The transfection efficiencies were verified by examining the GFP levels before adding 20 µM of DeepBlueC and measuring the signals at λ = 480 nm and at λ = 530 nm. The BRET ratio was calculated by dividing the GFP2 signal by the rLuc signal.

RESULTS

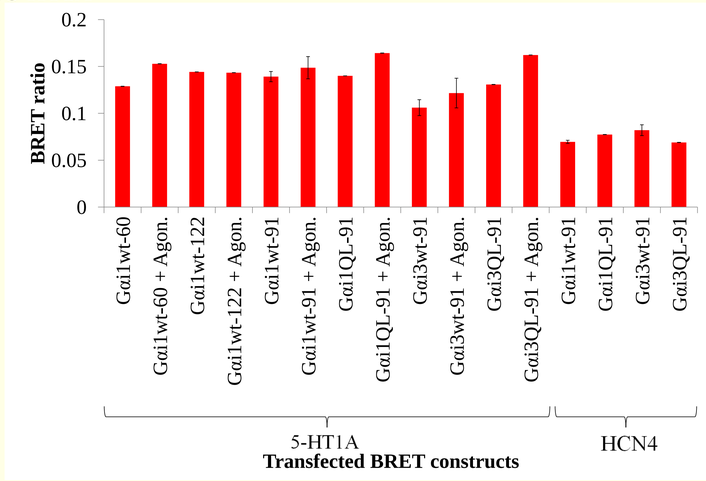
***Gα$_i$1 and 3* proteins interacts with RASA3, 5-HT1A and TNFAIP8**

To address the interactions between G$_α$i 1 and 3 proteins with the novel proteins in living cells, BRETs were conducted (Figure 2). Based on the BRET ratios, RASA3, 5-HT1A and TNFAIP8 appears to be significantly greater than the negative control potassium channel HCN4-GFP2 which has been documented as having no interaction with G-proteins (Whitaker and Accili, 2008). In comparison to 5-HT1A and RASA3, a strong interaction between TNFAIP8-GFP2 and the Gαi proteins were seen. The optimal position (largest BRET ratio) of the rLuc in the Gα$_i$ proteins with RASA 3 and TNFAIP8 seemed to be at positions 122 and 91 respectively. No significant difference in the BRET ratios was seen in the different positions of the rLuc for the 5-HT1A –Gα$_i$ interaction.

A) RASA3



B) 5-HT1A



C) TNFAIP8

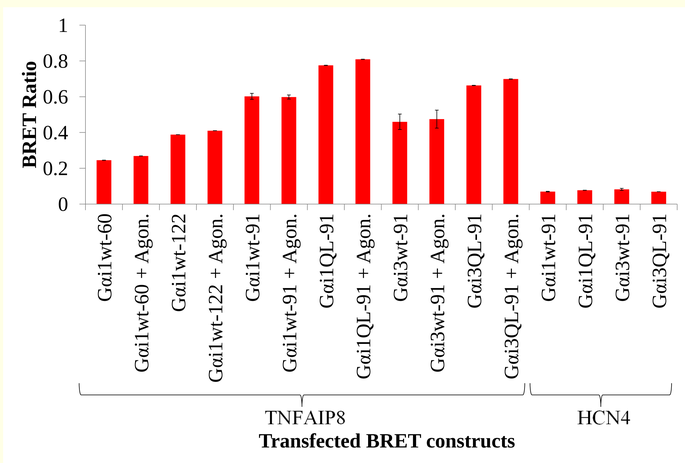


Figure 2: BRET ratios of Gα$_i$1 and Gα$_i$3 wt and QL constructs with A) RASA 3, B) 5-HT1A, C) TNFAIP8. BRET ratio is calculated using the corrected GFP signal (at λ = 480 nm) divided by the corrected luciferase signal (at λ = 530 nm). A GFP^2-HCN4 plasmid was used as a control. In B) and C), the agonist used was 4 µM 5-HT, 10 ng/µL TNF respectively. The position of the rLuc gene is indicated with the Gα$_i$ constructs. HCN4 was used as a negative control to obtain background, since it does not interact with G-proteins.

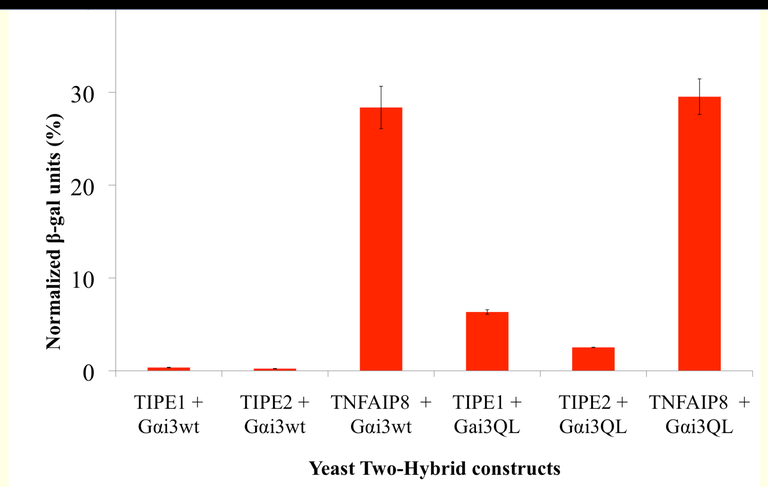


Figure 3:β-galactosidase assay of TIPE1 and 2, and TNFAIP8 with Gα$_i$3 wt and QL. Yeast strains were transformed with pAS2-1-Gα$_i$3 wt or QL and pACT2-TNFAIP8, TIPE1 or TIPE2, and then assessed by β-galactosidase assay. The β-gal activities are normalized to the positive control vector pCL1-1, and presented as mean ± SE (N = 3).

***Characterization of the interaction between TNFAIP8 with Gα$_i$3* proteins using Yeast-two hybrid and β-galactosidase assay**

In order to further investigate the specificity of the TNFAIP8-Gα$_i$3 interaction, a yeast two-hybrid was conducted. Selection upon –Leu, -His, -Trp revealed growth on TNFAIP8 with Gα$_i$3wt and QL, and TIPE 1 and TIPE2 with Gα$_i$3QL. To quantify the strength of the interaction, a β-galactosidase assay was conducted (Figure 2). TIPE 1 and TIPE 2, both of which are TNFAIP8-related proteins found in mice displayed minimal β-gal activity with both Gα$_i$3wt and QL, while TNFAIP was observed to have a high β-gal activity.

DISCUSSION

***Gα$_i$1 and 3* proteins interacts with RASA3, 5-HT1A and TNFAIP8**

Since all the BRET ratios were quite significantly different from the control (HCN4-GFP^2), BRET results (Figure 2) demonstrate that Gα$_i$1 and Gα$_i$3 both interact with RASA3, 5-HT1A, and very much so with TNFAIP8. There was no preference for Gα$_i$3, which may be due to the over-expression of both proteins required to detect BRET signals. The optimal positioning of the rLuc in the Gαi proteins is most likely 122 for RASA3, and 91 for TNFAIP8 since the highest BRET ratio was obtained at those positions. Meanwhile, the position of rLuc did not affect the interaction between Gαi proteins and 5-HT1A.

The specificity of the TNFAIP8-Gα$_i$ interaction was investigated further using a yeast two-hybrid system followed by a β-gal assay. In the Yeast-two hybrid, the conjugated protein system allows for the production of His, and thus allowing yeast containing both proteins that interact with each other to survive. The β-gal assay further reinforces the idea that TNFAIP8 is specific for Gα$_i$3 proteins. TIPE1 and TIPE2 proteins that are TNFAIP8 related proteins in mice show virtually no β-galactosidase activity and thus, minimal interactions between proteins.

The next step is to move the position of rLuc in the Gα$_i$3 construct since only position 91 was examined for the Gα$_i$3. The optimal position of rLuc for investigation of the RASA3-Gα$_i$3 interaction may be 122 as seen with Gα$_i$1.

REFERENCES

Dorsam RT, Gutkind JS. (2007). G-protein-coupled receptors and cancer. Nat Rev Cancer 7:79–94.

Kumar D, Gokhale P, Broustas C, Chakravarty D, Ahmad I, Kasid U. (2004). Expression of SCC-S2, an antiapoptotic molecule, correlates with enhanced proliferation and tumorigenicity of MDA-MB 435 cells. Oncogene 23:612–616.

Laliberté, B., Wilson, A.M, Nafisi, H., Mao, H., Zhou, Y.Y., Daigle, M., Albert, P.R. (2010). TNFAIP8: A New Effector for Galpha(i) coupling to reduce Cell death and induce cell transformation. J. Cell. Physiol. 225(3): 865-874.

Nafisi, H., Banlhasheml, B., Daigle, M., Albert, P.R. (2008). GAP(IP4BP)/RASA3 mediates Galphai-induced inhibition of mitogen-activated protein kinase. J. Biol. Chem. 283(51): 35908-17.

Whitaker GM, Accili EA. (2008). Using bioluminescence resonance energy transfer to measure ion channel assembly. Methods Mol Biol 491:189–197.