Effective Solubilization and Stabilization of Functional G Protein-Coupled Receptors

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PURPOSE

The requirement for stabilized G protein-coupled receptors (GPCRs) in their native, functional form presents significant challenges for in vitro functional and structural characterization. Efficient solubilization of the receptor most often requires stringent extraction that compromises structural integrity and activity. In addition, most activity assays must be performed immediately following solubilization of the receptor, with activity rapidly degrading over time. Our aim was to develop a simple method to efficiently solubilize active GPCRs that are stable outside of their native membrane environment.

METHODS

Protein Extraction

Membrane Protein Extraction: $1x10^7$ cultured cells or 50-100mg of tissue were washed in PBS, homogenized in 1mL hypotonic buffer, followed by an incubation at 4ºC for 15 minutes. Samples were then centrifuged at 16,000xg for 20 minutes. The supernatant was removed, and the pellet was resuspended in extraction reagent for 30-60 minutes at 4ºC to solubilize membrane proteins. Insoluble material was removed by centrifugation at 16,000xg for 20 minutes and the supernatant containing the membrane proteins was collected.

Whole Cell Protein Extraction: $1x10^7$ cultured cells or 50-100mg of tissue were washed in PBS, lysed in 1mL extraction reagent, and incubated for 30-60 minutes at 4ºC. After incubation samples were centrifuged at 16,000xg for 20 minutes. The supernatant containing the solubilized protein was collected.

Generation of styrene maleic acid co-polymer lipid particles (SMALP): 50-100mg of tissue were washed in PBS, resuspended in 1mL of DPMC, and incubated for 30 minutes at 37ºC in a sonicating water bath. After incubation 1mL of 2.5% polymer (SMA) was added to sample and equilibrated at room temperature for 1 hour. Samples were centrifuged at 40,000xg for 30 minutes. The supernatant containing the solubilized protein was collected.

Any extractions using commercially available reagents were performed according to provided product manual. For all above sample preparations, the remaining pellet was resuspended in 1 mL of RIPA Lysis Reagent and sonicated for 10 seconds at 50% Amps. Protein in each fraction was determined using the BCA Assay Kit.

Western Blot

Normalized samples were separated on a 4-20% Tris Glycine gel and transferred to nitrocellulose membrane via the Pierce G2 Fast Blotter. Membranes were then blocked, incubated in primary antibody for one hour at room temperature or overnight at 4ºC, washed, incubated in secondary antibody for 30 minutes at room temperature, washed, and incubated for 5 minutes in Thermo Scientific SuperSignal™ West Dura Extended Duration Substrate. Blots were then visualized on the Thermo Scientific iBright™ Imaging System.

Purification

Frozen cell pellets containing $1x 10^7$ Expi293 cells expressing ADORA2A-GFP-6xHis were thawed and lysed with the Pierce™ GPCR Extraction & Stabilization Reagent. Extracts (500mg) were then purified with Thermo Scientific Pierce Ni-NTA Magnetic Agarose according to the user manual, with the modification of making equilibration, wash, and elution in the presence of one tenth strength GPCR Extraction & Stabilization Reagent.

Radioligand Binding Assay

M3 lysates were prepared from a GeneBLAzer expression cell line (Thermo Fisher Scientific). For the binding assay, extracts (400ug) were incubated with H^3-4-DAMP (PerkinElmer) alone or with Ipratropium Bromide and H^3-4-DAMP (competitive binding assay) for 75 minutes at room temperature. Free radioligand was then removed and bound radioligand was then quantified using a TRI-CARB 2000 TR scintillation counter.

Tissue lysates were prepared from frozen mouse brain (Pel-Freeze Biologicals). For the binding assay, extracts (600ug) were incubated with H^3-Adenosine (PerkinElmer) alone or with Adenosine (Sigma) and H^3-Adenosine (competitive binding assay) for 75 minutes at room temperature. Free radioligand was then removed and samples were then analyzed on a TRI-CARB 2000 TR scintillation counter.

ADORA2A-GFP-6xHis lysates were prepared from a Expi293 expression cell line (Thermo Fisher Scientific). For the binding assay, extracts were incubated with H^3-Adenosine (PerkinElmer) alone or with unlabeled and H^3-Adenosine (competitive binding assay) for 75 minutes at room temperature. Free radioligand was then removed and bound radioligand was then quantified using a TRI-CARB 2000 TR scintillation counter.

RESULTS

When comparing different methods for extraction and stabilization of GPCRs the following trends were observed:

- Improved solubilization enhances western blot results
- Receptor stabilization at the time of extraction is required to preserve >75% GPCR Activity
- Solubilization and stabilization of active receptor can be achieved using a whole cell lysis method.
- Receptor can be purified in its functional form

Figure 1: Improved western blot results. *G protein-coupled receptors Free Fatty Acid Receptor 4 (GPR120), Serotonin Receptor (5HT1A) and Thrombin Receptor (TR) were extracted from HeLa and HEK293 cells using both fractionation using a competitor kit (A&B) and whole cell methods (C & D). The Pierce™ GPCR Extraction & Stabilization Reagent (C) shows improved banding and yield in the soluble fraction (S) when compared to the insoluble pellet (P) and other methods tested. RIPA buffer (D) is the harshest condition and expected to give maximal solubility of membrane proteins.*

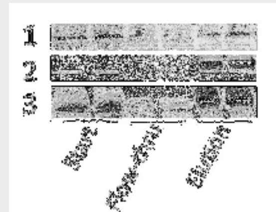

Figure 2: Purification of His-tagged adenosine receptor type 2A. *Expi293 cells expressing ADORA2A-GFP-6xHis lysed using the GPCR Extraction and Stabilization Reagent. Extracts were then purified using Pierce™ Ni-NTA Magnetic Agarose. Samples were eluted with 0.3M imidazole, pH 8.0 and analyzed by western blot using three different primary antibodies for detection (1= anti-ADORA2A, 2 = anti-6x His, 3 = anti-GFP). Start, flow-thru, and elution fractions, shown in duplicate in all three blots, show effective capture and elution of the 6xHis tagged adenosine receptor type 2A.*

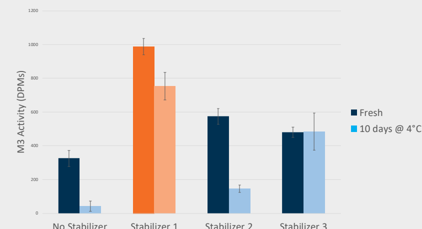

Figure 3: Improved stability of active receptor. *Muscarinic acetylcholine receptor 3 (M3) was solubilized from GeneBLAzer expression cell line using the GPCR Extraction & Stabilization Reagent and three formulations where the stabilization reagent was omitted or substituted with stabilizers that are commercially available . Radioligand binding was then performed on fresh extracts. Extracts made using the GPCR Extraction & Stabilization Reagent (Stabilizer 1) show a >2 fold increase in activity after 10 day cold storage when compared to the formulations substituted with other stabilizers.*

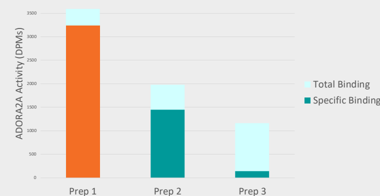

Figure 4: Superior functionality compared to SMALP*. Adenosine receptor 2A (ADORA2A) was solubilized from mouse brain tissue using the whole cell lysate method and GPCR Extraction & Stabilization Reagent (Prep 1), and the SMALP method with either Lipodisq 3.1 (Prep 2) or Amphipol (Prep 3) for polymer stabilization. Radioligand binding was then performed on fresh extracts. Extracts made using the GPCR Extraction & Stabilization Reagent have >2 fold specific activity from endogenously expressed ADORA2A when compared to the SMALP preps.*

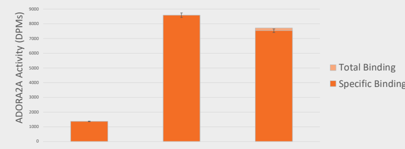

Figure 5: Stabilization of receptor activity throughout purification*. Adenosine receptor type 2A was solubilized from mouse brain tissue (Endogenous) and Expi293 cells expressing ADORA2A-GFP-6xHis (Expressed). ADORA2A-GFP-6xHis was then purified using Pierce Ni-NTA Magnetic Agarose (Purified). Radioligand binding was performed on the two extracts and the purified samples at varying protein loads. Comparison of the three extracts shows the advantage of using an expression system, and the ability to specifically bind adenosine in both whole cell lysate and purified samples.*

CONCLUSIONS

Here we report:

- Using optimized ratios of detergent and stabilizer for GPCR extraction not only efficiently solubilizes GPCRs better than other methods (i.e. membrane prep, SMALP), but also stabilizes the receptor.
- Receptor stability is preserved immediately after extraction as well as extended during cold storage, when using a non-denaturing detergent in conjunction with a stabilizer.
- Extraction using the GPCR Extraction and Stabilization Reagent allows for the assessment of receptor activity in both a whole cell lysate and a purified sample.
- With evolving techniques and technologies, such as native mass spectrometry and cryo-EM, extraction of a stable receptor will be essential. With the improved extraction efficiency, solubility and preserved activity obtained with this method, researchers will have a greater breadth and flexibility in their GPCR research and targeted therapeutic studies

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