Stabilization of native and functional membrane proteins for drug discovery

Anass JAWHARI, CALIXAR

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I- CALIXAR Technology background

II- Case Studies

A- Protein Production of stabilized native membrane proteins

- **GPCR**: Adenosine receptor stabilization, other GPCR
- **Ion channels**: M2 from influenza, other channels
- **Co-transporter**: KCC2

- Stabilization of already solubilized proteins

B- Protein Identification at the membrane

- BAG3 partner at Macrophage plasma membrane
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NATIVE ISOLATION APPROACH

NON DENATURING PROCESS
Innovative Detergent / surfactant based approach
(IP = 6 patents)

- No refolding
- No mutagenesis/ truncation/ fusion
- Stability improvement
- High purity
- Maintain Functional & structural integrities

Proprietary compounds and combinaison of commercial compounds
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Proprietary compounds and combinaison of commercial compounds
“Modify the chemical environment around the protein without modifying the protein”: innovative detergent/surfactant approach

CALIXAR proprietary compounds have four features

1- Solubilize and stabilize in the same time
2- Stabilize already solubilized proteins
3- Act as crystallization additive
4- Split virus particles
NATIVE EXTRACTION APPROACH

- Hardy D et al., 2016, BiochemSocTrans
- Chaptal V et al., 2017, Scientific report

A: Detergent micelle
B: Proteoliposome
C: Membrane scaffold protein
D: Lipoparticle

Detergent
Phospholipid
Membrane protein-helix
Scaffold protein
Polymer
SYSTEMATIC SOLUBILIZATION APPROACH

Relative quantification (96 well plates)

Mandon E et al., 2017, Analytical Biochem
SYSTEMATIC SOLUBILIZATION APPROACH

1- Insertion of biotinylated lipids into biological membranes

2- Isolation of biotinylated biological membrane

3- Membrane proteins solubilization

4- Solubilized membrane proteins separation

Non-solubilized proteins and membranes

Membrane proteins
streptavidine magnetic beads
detergent
lipids
biotinylated lipids
SOLUBILIZATION: EASY AND DIFFICULT TARGETS

Can be applied to easy and difficult to extract proteins
ROBUST SOLUBILIZATION METHOD

robust and reproducible method
Native (non-mutated) **Adenosine receptor A2A**: Class A GPCR involved in regulating myocardial blood flow/ hypertension, the regulation of glutamate and dopamine release. Good therapeutic candidate for insomnia, pain, depression, drug addiction and Parkinson’s disease.

2 Expression systems tested

1- **Yeast (Pichia Pastoris)**
GS115 & KM71 strains. YPD, 0.5% methanol induction, 21h of culture

2- **Insect cells**
Hi5, Sf9. 48h of culture
Conditions to purify A2A from plasma membrane/ internal membranes of Sf9/ yeast were obtained
1- RADIOBINDING (ZM241385)

Purified native A2A was functional (binding), homogenous and non-aggregated.
Purified native A2A was stable at RT & 37°C for a week.
Calixar condition / Native A2A

Classical condition (DDM) / Native A2A

Using the method recently developed (Ashok Y et al., 2015)

Purified native A2A was thermostabilized without any single mutation
Homogenous sample in negative stain EM

Collaboration: IGBMC Strasbourg University
FRAGMENTS SCREENING, HITS IDENTIFICATION BY NMR

First 10 fragments

Normal 1D

STD + prot

STD - prot

signal = ligand; no signal = no ligand

Collaboration: Lyon University
First 10 fragments

Normal 1D

WaterLOGSY + A2A

WaterLOGSY – A2A

signal >0 = ligand; signal <0 = no ligand

Collaboration: Lyon University
OTHER STABILIZED NATIVE GPCR

Electron microscopy

Thermal Shift assay

Tm=61.8°C

Stabilized and non aggregated native GPCR
Saturation ligand binding experiment

Kd = 150.2 pM

Functional and stabilized native GPCR
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Native (non-mutated) M2 ION CHANNEL

The M2 protein is a proton-selective tetrameric ion channel protein, integral part in the viral envelope of the influenza A virus. It is essential for viral replication; its transmembrane domain is highly conserved for all the human, swine, equine, and avian strains of influenza A virus.

Antibody or small molecules targeting the M2 proton channel are promising therapeutic candidates for treating influenza virus infections as universal vaccine.

- Mandon E et al., 2016, Protein Expression & Purification
- Acharya R et al. PNAS 2010;107:15075-15080
Purified Native M2 was homogenous and tetrameric

Mandon E et al., 2016

Mandon E et al., 2016
NATIVE ISOLATION OF M2 ION CHANNEL (Influenza)

Purified Native M2 was inserted in planar lipid bilayer and could show a pH dependent ion channel activity.

Mandon E et al., 2016
NATIVE ISOLATION OF M2 ION CHANNEL (Influenza)

Specific channel inhibition

Mandon E et al., 2016
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**KCC2** is a **K-Cl co-transporter** plays multiple roles in the physiology of central neurons.

KCC2 regulates **intraneuronal chloride homeostasis**, KCC2 strongly influences the efficacy and polarity of the chloride-permeable γ-aminobutyric acid (GABA) type A and glycine receptor (GlyR) mediated synaptic transmission.

It is critically involved in many neurological diseases including **brain trauma, epilepsies, neuropathic pain, autism and schizophrenia**.

**KCC2 has 12 TM and exist at oligomer** most probably dimer.
KCC2 was isolated as monomer and dimer

➢ Agez M et al., to be submitted
KCC2 monomers and dimers were separated by SEC

Agez M et al., to be submitted
MOLECULAR ARCHITECTURE OF KCC2 MONOMER: EM

KCC2 monomers are observed in EM

Agez M et al., to be submitted
KCC2 dimers are observed in EM
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After solubilization and purification in OG

Strong thermostabilization effect was obtained for the Bacteriorhodopsin

Normalized Absorbance (560 nm)

25°C

37°C

CALXGLYCOSIDE
LMNG
A835
FA3
OG

CALXGLYCOSIDE amphotilphilic calixarene glycoside
Patented, 2015 (WO2015158575)
STABILIZATION OF NATIVE GPCRs

1- Solubilization

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1 2 3 4 5 6 7 8 9

CHS
cholesteryl hemisuccinate

CALXChol
novel steroidic calixarene derivatives
Patent submitted

2- Radio-ligand binding

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Functional /binding stabilization effect on native GPCR
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**BAG3** (Bcl-2 associated athanogene 3) is released by pancreatic ductal adenocarcinoma (PDAC) cells and **activates macrophages through a specific unknown receptor**.

BAG3-activated macrophages secrete factors that stimulate PDAC cell proliferation.

**CALIXAR native solubilization of BAG3/receptor complexes**

**Pull-down of BAG3** to enrich in native BAG3/receptor complexes

**Identification of BAG3 receptor by mass spectrometry**

In collaboration with: **Biouniversa s.r.l.** (Fisciano, Italy)
IFITM2 (Interferon-induced transmembrane protein 2) was identified as the specific receptor of BAG3 in macrophages.

Results were confirmed by:

- Complementary Co-IP approach of IFITM2

- RNAi approach

IFITM2 silencing abrogated BAG3-induced signaling in macrophages (IL-6 release, AKT and p38 phosphorylation)

We could solubilize and/or stabilize native MPs while maintaining their structural and functional integrities.

This was applied to human GPCRs, ion channels, transporters, bacterial and viral targets (not shown) from different biological membranes.

The applications are drug screening, structural biology and antibody discovery (not shown).

Same approach is used for partner identification (protein/protein) and deorphanization (protein/ligand).
CALIXAR (Lyon, France)

Native and functional membrane proteins isolation and characterization

Joint-Venture
CALIXAR
University of Avignon
CHEM2STAB

Synthesis and physical-chemical characterization of amphiphilic molecules