FG winds
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Immunoprecipitation (4)

Introduction

Lipid molecules are important molecules that are deeply involved in the composition of cells, and their most important role is that they are 'the materials that make up the membrane' of cells and organelles. These membranes are formed by a lipid bilayer, and it is known that the outer and inner lipid molecules have completely different compositions and roles. By always properly adjusting this asymmetry, the transport, sensing, etc. of substances through the membrane can function normally.

The state change of lipid asymmetry is caused by the movement between the inside and outside layer of the lipid molecule called flip-flops, which involves a protein called flippases and floppases. Recent research has revealed a protein called Opt2, which has a structure that is significantly different from that of conventional flopases, and may be a new type of flopases.

Therefore, in order to examine the presence or absence of flop activity of Opt2, we purified and recovered Opt2. Opt2 is a membrane protein that is mainly localized in the Golgi membrane, but due to the vesicle formation of the membrane and the addition of HA tags to Opt2, it is possible to recover it by immunoprecipitation while it is embedded in the membrane.

Result

In order to recover Opt2 in its native state, lysate was first sonicated to divide the Golgi membrane into a number of vesicles to prepare vesicles containing Opt2. Next, using the HA tag attached to the N-terminal of Opt2, immunoprecipitation of vesicles containing Opt2 was performed using FG beads (protein G) and anti-HA tag antibody. Non-denaturing elution was performed by a competitive method using HA peptides. (Fig. 1) Then, a part of the eluted vesicles was subjected to SDS-PAGE and Western blotting to evaluate the purified sample. (Fig. 2)

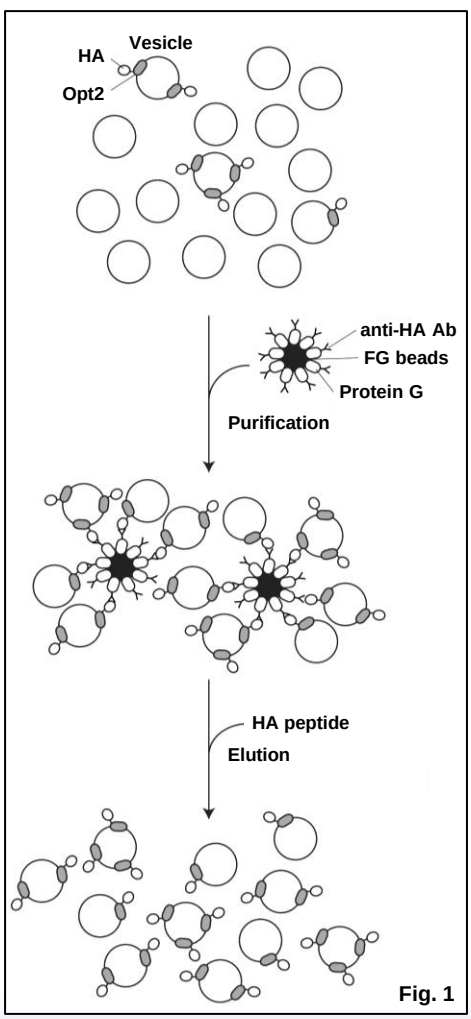

Fig. 1

From the results of Western blotting, it is possible to confirm that there is almost no Opt2 in the unbound fraction and many Opt2 in the elution fraction with respect to the Input fraction. In addition, in order to confirm the contamination (background) of non-specific vesicles in the eluted fraction, membrane proteins such as Pma1, Dpm1, Pho8, and Pep12 (localized to cell membrane, endoplasmic reticulum, vacuole, and endosome, respectively) was also evaluated, and it was confirmed that none of the proteins was present in the eluted fraction and most of them were present in the unbound fraction. Since Opt2 is also slightly localized in endosomes, Pep12 is slightly detected because Opt2 and Pep12 may coexist when the endosome membrane becomes vesicular.

In this experiment, the following were confirmed as the superiority of FG beads.

☆Opt2 was not recovered with the beads of other companies, but Opt2 could be recovered with a high recovery amount with the FG beads.

☆There was little non-specific adsorption, and vesicles containing Opt2 could be recovered with relatively high specificity.

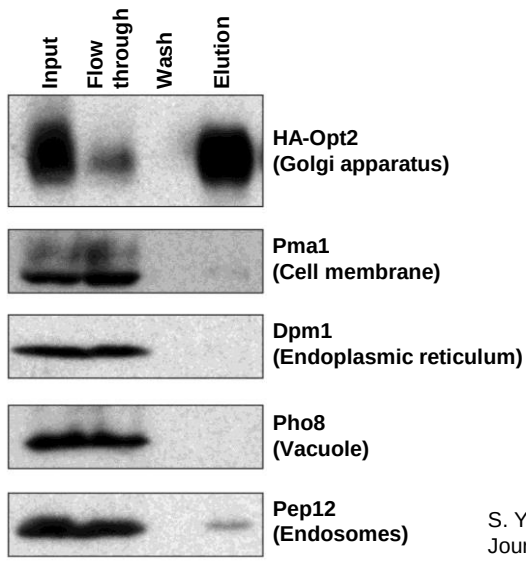

Fig. 2

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Materials and method

■Materials

1. Protein G beads
2. *S.cerevisiae* cells expressing HA-Opt2
3. PBS(-)
4. Lysis buffer (PBS(-), 1 mM PMSF, 1 mM DTT, 1x protease inhibitor cocktail)
5. Wash buffer (10 mM HEPES-NaOH(pH7.9), 50 mM KCl, 0.2 mM EDTA, 10%(v/v) glycerol)
6. Anti-HA-tag antibody (TANA2; from MBL)
7. Anti-Pma1 antibody (yN-20; from Santa Cruz)
8. Anti-Dpm1 antibody (5C5A7; from Thermo Fisher)
9. Anti-Pho8 antibody (gift from Prof. Y. Ohsumi (Tokyo Institute of Technology))
10. Anti-Pep12 antibody (2C3G4; from Thermo Fisher)
11. HA peptide buffer (1 mg/mL HA peptide (from MBL) in PBS(-))
12. TBS-T buffer (TBS(-), 0.1% Tween 20)
13. Blocking buffer (5% skim milk in TBS-T buffer)
14. Anti-mouse antibody HRP conjugate (from GE Healthcare)
15. Anti-rabbit antibody HRP conjugate (from GE Healthcare)

■FG beads® information

Product name	Protein G beads
Product number	TAS8848N1173
Storage temperature	2-8˚C
Storage buffer	10 mM HEPES(pH7.9), 50 mM KCl, 1 mM EDTA, 10% glycerol
Size of beads	190 nm ± 20 nm
IgG binding capacity	>100 ug mouse IgG /mg of beads

■Method 1 (Lysate Preparation)

1. Yeast culture
Culture yeast cells to log phase in YPD medium (1% yeast extract, 2% pepton, 2% glucose).

2. Harvest and homogenize
Collect cells by centrifugation and wash with PBS(-).
Resuspend cells with Lysis buffer, and lysis them by vigorously mixing with glass beads for 10 min at 4˚C.
Transfer the lysate to new tube without taking beads.

3. Vesiculate membrane
Sonicate the lysate (10 sec x 3) to vesiculate the membrane.
Centrifuge at 13,000 *g* for 15 min.
Ultracentrifuge the supernatant at 100,000 *g* for 30 min.
Remove the supernatant, resuspend the pellet with Lysis buffer, and sonicate (10 sec x 2) (“Vesiculated membrane”).

■Method 2 (Immunoprecipitation)

1. Beads preparation
Transfer 1 mg (50 ul) of beads to a tube.
Wash beads with 200 ul ice-cold PBS(-) twice.

2. Bind Antibody
Add antibody (50 ug in 200 ul PBS(-)) to beads.
Mix for 30 min at room temperature.
Wash beads bound to antibody with Wash buffer 3 times, and store at 4˚C in 200 ul Wash buffer.

3. Immunoprecipitation
Add 100 ul beads bound to the antibody to “Vesiculated membrane”, and incubate overnight at 4˚C with rotation.

4. Wash
Separate beads magnetically from the suspension, and collect a part of supernatant for immunoblot analysis (Unbound fraction).
Wash beads 3 times with PBS(-).
Optional*: Resuspend beads with HA peptide buffer and stand for 5 min at 4˚C. Separate magnetically and collect supernatant.
*, In some cases, vesicules containing HA-tagged protein may be released from the beads during this short period of incubation. If so, omit this process.

5. Elution
Resuspend beads with HA peptide buffer and incubate for 8 h at 4˚C with rotation.
Separate the beads magnetically and collect supernatant (Elution fraction).

■Methods 3 (Western blotting)

1. Perform SDS-PAGE.
2. Transfer proteins from the gel to a PVDF membrane.
3. Block the membrane with Blocking buffer for 60 min at room temperature.
4. Dilute the each primary antibody with the Blocking buffer. (Anti-HA; 1/2,000, Anti-Pma1; 1/1,000, Anti-Dpm1; 1/500, Anti-Pho8; 1/1,000, Anti-Pep12; 1/1,000)
5. Incubate the membrane for 60 min at room temperature.
6. Wash the membrane with TBS-T buffer 3 times.
7. Diluted the Secondary antibody with TBS-T buffer to 1/7,500, and incubate the membrane for 60 min at room temperature.
8. Wash the membrane with TBS-T buffer 3 times.
9. Detect with a chemiluminescence substrate.