FG winds
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Immunoprecipitation (3)

Introduction

Systems such as UPS (ubiquitin-proteasome system) function in the degradation of intracellular proteins, and it is known that many proteins are involved in a complex manner. So far, proteomics analysis for elucidating the mechanism of UPS has been vigorously carried out, and many results have been obtained.

The proteasome is a huge enzyme complex that hydrolyzes ubiquitinated proteins, and the subunit of the proteasome, Rpt2, has undergone N-myristoylation modification in many eukaryotes, including budding yeast. Recent studies have shown that N-myristoylation of Rpt2 dynamically alters the localization of the proteasome in the nucleus and cytoplasm, which intricately affects UPS in intracellular localization.

In order to investigate the effect of N-myristoylation of Rpt2 on UPS, strains with mutations in the N-myristoylation site (Rpt2-G2A, Rpt2-G2Δ) and without mutations (Rpt2-WT) in Rpt2 of budding yeast proteasome were selected. A comprehensive ubiquitin proteomics analysis by mass spectrometry revealed that the Hsp70 family, Ssa1 and Sse1, involved in the transport of cytoplasmic misfolded proteins into the nucleus, showed a significant increase in ubiquitination in mutant strains. Therefore, the ubiquitination of Ssa1 in the wild strain and the two mutant strains was evaluated by IP-WB using Anti-Ssa1 antibody and Anti-ubiquitin antibody.

Result

0.25 ug of Anti-Ssa1 antibody was reacted with budding yeast lysate, and 25 ul of protein G beads from each company were added to recover the antigen-antibody complex, and Western blotting was performed after SDS-PAGE.

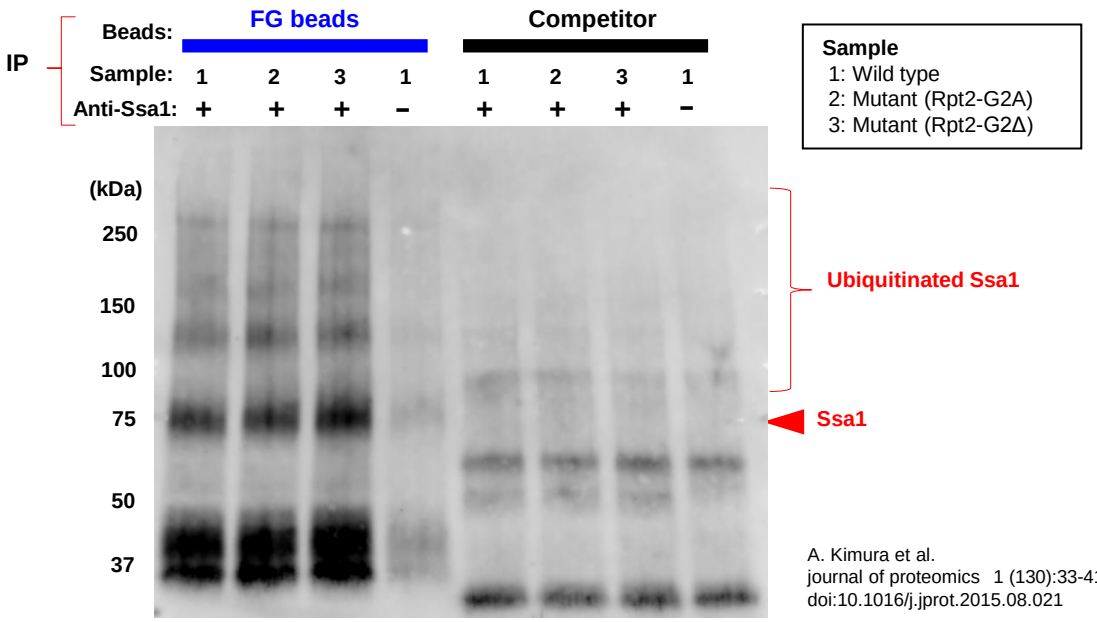
From the results of Western blotting, it was confirmed that ubiquitination of Ssa1 was increased in both mutant strains as compared with the wild strain, similar to the result of mass spectrometry. In addition, when the amount of Ssa1 contained in lysate was examined by mass spectrometry and Western blotting in three yeast strains, it was confirmed that there was no significant difference among the three yeast strains.

From this, it was confirmed that inhibition of N-myristoylation of Rpt2 has the effect of increasing ubiquitination of Ssa1.

In addition, the following points can be confirmed for FG beads and beads of other companies.

☆Ssa1 and ubiquitinated Ssa1 could not be recovered with the beads of other companies, but they could be recovered with a high recovery amount with the FG beads.

☆Other companies have the same configuration of the (+) and (-) fractions, but the FG beads have a high S/N ratio of (+) and (-).



Western blotting : Anti-ubiquitin

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Materials and method

■Materials

1. Protein G beads
2. *S.cerevisiae* lysate
3. Lysis buffer (20mM HEPES(pH8.0) , 9M Urea)
4. Protease inhibitor cocktail (from Nacalai Tesque)
5. PR-619 (Life Technologies)
6. Triton buffer (1% Triton-X100, 20mM Tris-HCl(pH7.4), 150mM NaCl, 2mM EDTA)
7. Anti-Ssa1 antibody (from Santa Cruz)
8. SDS sample buffer
9. Anti-mono- and polyubiquitinylated conjugates antibody (FK2H) HRP-conjugate (from Enzo Life Sciences)
10. PBS-T buffer (137mM NaCl, 2.7mM KCl, 8.1mM $Na_2HPO_4 \cdot 12H_2O$, 1.5mM KH_2PO_4, 0.1% Tween 20)
11. Blocking buffer (5% skim milk in PBS-T buffer)

■FG beads® information

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

■Method 1 (Lysate Preparation)

1. Yeast culture
Culture yeast for 3 days at 30ºC in synthetic complete medium lacking leucine (SC-Leu).

2. Harvest and homogenize
Centrifuge at 3000×g for 10min, wash twice in sterile water, and resuspende in lysis buffer with protease inhibitor cocktail and 50uM PR-619.
Vortex with glass beads and centrifuge to obtain lysate.

■Method 2 (Immunoprecipitation)

1. Lysate dilution
Add 9 volume of Triton buffer to lysate.

2. Reaction
Incubate lysate containing 125ug of total protein and 0.25ug of Anti-Ssa1 antibody at room temperature for 60min on microtube mixer.

3. Collect antigen-antibody complex
Add 25ul of Protein G beads, incubate at room temperature for 60min on microtube mixer.

4. Wash
Separate magnetically and remove supernatant.
Wash beads with Triton buffer 5 times.
Separate magnetically and remove supernatant.

5. Elution
Add SDS sample buffer and resuspend beads.
Incubate for 10min at 70ºC and remove the beads.

■Methods 3 (Western blotting)

1. Perform SDS-PAGE.
2. Transfer the protein from the gel to a PVDF membrane using Trans-Blot® Turbo™.
3. Block the membrane with the Blocking buffer for 120min at room temperature.
4. Dilute the Anti-ubiquitine antibody with the Blocking buffer to 1/1000.
5. Incubate the membrane for 120min at room temperature.
6. Wash the membrane with PBS-T buffer 3 times.
7. Detect with a chemiluminescence substrate.