Mitochondrial fractionation isolation using SORVALL LLYNX6000

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Abstract

In Tokushima National Hospital we primarily conduct clinical studies on nerves, myopathy and other fundamental research. In the clinical research department that I belong to, the study of the molecular mechanism of neurodegenerative diseases is conducted as fundamental research. I have worked as study assistance every day in the clinical research department from March of last year. I engage in cell fractionation. It is difficult to isolate mitochondria conventionally. I purchased an ultracentrifuge instrument for the laboratory. The cell fractionation that I learned crushes cells from a cultured cell and is a method to fractionate by size, shape, and difference in density, and to isolate each intracellular component. However, the isolation that cytoplasm fraction got mixed with mitochondrial fraction, and was good was difficult.
We purchased a new SORVALL LYNX6000 instrument. With this, mitochondrial fractionation was more remarkable than with the traditional approach, and purity rose. We want to continue our efforts in therapeutic drug development in the future while learning new techniques.

Key words Parkin, Klokin 1, mitochondria, biogenesis, Super Speed Centrifuge

Introduction

We conducted a functional analysis of the cause gene parkin of familial Parkinson's disease. We obtained a very important finding about the intracellular localization of the parkin protein. The mitochondrial fractionation isolation requires a centrifugal force of 10,000xg or more. Our laboratory did not have the facilities, and a centrifugal force of 2,330xg was used. And we did not get along well. We purchased a SORVALL LYNX6000 supercentrifuge instrument and performed mitochondrial fractionation isolation.

Materials and Methods

Cell culture

COS-1 (a cell line derived from kidney cells) was cultured at 37°C (5% CO2) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-incubated fetal bovine serum (FBS).

Homogenate

The following 3 steps were carried out.

1. Wash 1-3x10^8 cells in phosphate buffered saline twice.
2. Suspend the cells in 1ml homogenate buffer containing 0.25M Sucrose, 25mM KCl, 5mM MgCl2, 1 mM PMSF, 20mM Gricine-KOH (pH 7.8) using 20 strokes of a Degital homogenizer (IUCHI Japan).
3. Centrifuge the homogenate at 244xg for 10min to pellet the nuclei and supernatant the mitochondria and cytosol.

Preparation of nuclei, mitochondria and cytosol

Preparation of nuclei, mitochondria and cytosol were carried out using OptiPrep™ Applications according to the manufacture's protocol [1]. The following steps were the brief procedures.

For isolation of nuclei

1. Produce a crude nuclear pellet by centrifugation at 860xg for 10min repert.
2. Mix 300ul volumes of the sample (resuspended nuclear pellet) and buffer; 50%(w/v) iodixanol: mix 5 vol. of 60% inodixanol with 1 vol.of homogenate buffer.
3. Underlayar the sample with 300ul of the 30% iodixanol and 35% iodixanol. After we centrifuged at 13.800xg 20min, we collected the band of nuclei at the 30%-35% iodixanol interface.

For isolation of mitochondria and cytosol

1. Centrifuge the homogenate at 244xg 10min to pellet the nuclei. Aspirate and retain the supernatant.
2. Samples were re-homogenized with the homogenate buffer and we repeat the centrifugation twice.
3. Resuspend this pellet in gradient solutions and centrifugation [2] using SORVALL LYNX6000 (50.000x g 3～4h).
4. Harvest the mitochondria which band just above the upper interface.
5. Supernatant was centrifuged at 3.800xg for 5 min and the supernatant was centrifuged at 13.800xg for 10 min. The cytosolic fraction was obtained from the final supernatant.

Immunoblotting

We used two antibodies. Tom20 (monoclonal antibody (F-10): (Santa Cruz Biotechnology, Inc, Dallas, USA, polyclonal antibody (FL-145): Santa Cruz Biotechnology, Inc, Dallas, USA). The sample was separated by 4-20% SDS-PAGE, and processed for immunoblotting followed by visualization using the ECL method [3] and both stained with Anti-Tom20 diluted to 1:1500.

Results and Discussion

As shown in Figure1, Tom20, which was a mitochondrial marker, was detected only in the mitochondrial fraction. We want to continue our efforts in therapeutic drug development in the future while learning new techniques.

References

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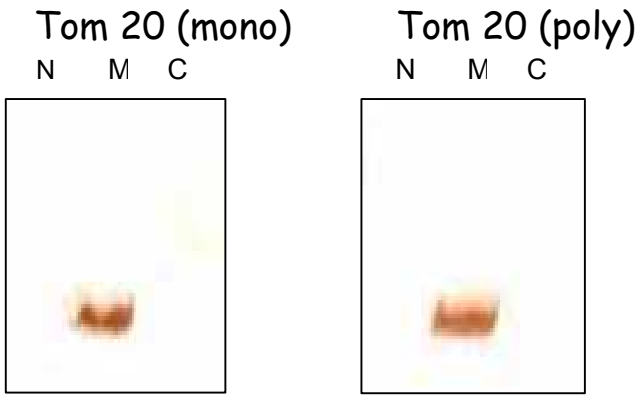

Figure 1. Cellular fractionation and immunoblotting using anti-Tom 20 antibody

N, nucleus; M, mitochondria, Cy, cytosol