p62/SQSTM1-based biochemical methods for the analysis of autophagy progression in mammalian cells

Peidu Jiang, Noboru Mizushima

METHOD

1. Remove and discard the spent cell culture media from the culture dish or plate. Gently add Dulbecco's phosphate-buffered saline (DPBS) to avoid disturbing the cell layer, and rock the dish back and forth several times.
2. Remove and discard the wash solution from the dish. Add 0.05% trypsin-EDTA to the dish. Gently rock the dish to allow complete coverage of the cell layer with the trypsin solution. Place the dish in a humidified incubator at 37°C, 5% CO_2 for approximately 2–5 min. Note that the actual incubation time varies with the cell line used.
3. Add the equivalent of 2 volumes (twice the volume used for trypsin-EDTA) of pre-warmed complete DMEM to the dish. Mix the medium by pipetting it over the cell layer surface several times.
4. Transfer the cells to a 15-ml centrifuge tube and centrifuge at 200× *g* for 5 min. Resuspend the cell pellet in pre-warmed complete DMEM.
5. Dilute the cell suspension to the appropriate seeding density to obtain an 80–90% confluent dish the next day, pipet the appropriate volume into new cell culture dishes, and return the cells to the incubator. Allow the cells to adhere to the dish during overnight culture.
6. Gently wash the cells 3 times with DPBS and culture them in either complete DMEM or starvation medium in the presence or absence of 100 nM bafilomycin A_1 for 2 h in a humidified incubator at 37°C, 5% CO_2.
7. Place the cell culture dishes on ice and wash the cells with ice-cold PBS. Aspirate the PBS, then add ice-cold cell lysis buffer approximately 1 ml per 10^7 cells (cell lysis buffer containing 1% Triton X-100 is generally used in our laboratory).
8. Scrape adherent cells from the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microcentrifuge tube. Vortex the tube and leave cells on ice for 30 min.
9. Spin at 16,000× *g* for 20 min in a 4°C pre-cooled centrifuge. Transfer the supernatant to a fresh microcentrifuge tube kept on

ice, and discard the pellet.

10. Take a small volume (*e.g.*, 25 µl) of lysate to perform a protein assay. Prepare BCA working reagent by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B (50:1, Reagent A:B). Pipette 25 µL of each diluted BSA standard or unknown sample replicate into a microplate well. Add 200 µL of the BCA working reagent to each well and mix plate thoroughly on a plate shaker. Place the plate at 37°C for 30 min. Measure the absorbance at or near 562 nm on a multimode plate reader. Determine the protein concentration for each cell lysate using a BSA standard curve.
11. Add 6× sample buffer to the remaining volume of cell lysate. Boil each cell lysate immediately in sample buffer at 100°C for 5 min. Centrifuge the boiled lysates in microcentrifuge tubes at 16,000× *g* in a microcentrifuge for 5 min. Samples can be stored frozen at −20°C for several months.
12. Prepare SDS-polyacrylamide stacking and separation gels according to a standard protocol.
13. Load equal amounts of protein (10–20 µg per lane) into each well of the SDS-PAGE gel, along with molecular weight markers. Run the gels according to the manufacturer's suggestion.
14. Electrotransfer to PVDF or nitrocellulose membrane according to the equipment and membrane being used.
15. After transfer, briefly rinse the membrane in DDW, immerse it in a sufficient amount of Ponceau S staining solution and stain for 2–5 min on a shaker at room temperature to assure that equal amounts of proteins were loaded and efficiently transferred to the membrane. Destain the membrane in DDW until the background becomes clean.
16. Transfer the membrane into Western blot blocking buffer containing 5% skim milk and incubate it at room temperature for 1 h.
17. Remove the blocking solution, and add primary antibody diluted in fresh blocking solution. Note that each primary antibody requires different dilutions. We use anti-p62 and anti-β-actin antibodies at 1:2000 dilutions.
18. Incubate the membranes with primary antibodies at room temperature for 1 h, or at 4°C overnight.
19. After incubation with the primary antibodies, remove the antibody solution, and wash the membranes 3 times in membrane wash buffer on a rocking platform, for 5 min per wash. Incubate the membrane in the HRP-conjugated secondary antibody solution for 30–60 min at room temperature on a rocking platform.

20. Rinse the membranes 6 times for 5 min each with membrane wash buffer. Mix the required amounts of ECL solutions in a 15-ml centrifuge tube. Add enough ECL solutions to cover the surface of the membranes. Incubate at room temperature for 1–2 min.
21. Remove excess reagent and cover the membrane in transparent plastic wrap. Capture the chemiluminescent signals using an LAS 4000 mini (or conventional X-ray films).

MATERIALS

Mouse embryonic fibroblasts (MEFs)
Dulbecco's modified eagle's medium (DMEM), high glucose (Sigma-Aldrich, cat. no. D6546)
Fetal bovine serum (FBS), sterile filtered (Equitech-Bio, lot. no. SFBM30-2566)
L-glutamine, 200 mM solution (Life technologies, cat. no. 25030-081)
Penicillin-streptomycin, liquid (Life technologies, cat. no. 15070-063)
Dulbecco's phosphate-buffered saline (DPBS, 10×) (Life technologies, cat. no. 14200-075)
D-MEM (high glucose) with sodium pyruvate, without amino acids (Wako, cat. no. 048-33575)
0.05% Trypsin-EDTA (1×), phenol red (Life technologies, cat. no. 25300-062)
Albumin standard, 2.0 mg/ml (Thermo Scientific, cat. no. 23209)
BCA protein assay reagent A (Thermo Scientific, cat. no. 23228)
BCA protein assay reagent B (Thermo Scientific, cat. no. 23224)
Tris(hydroxymethyl)aminomethane, nuclease and protease tested (Nacalai tesque, cat. no. 35434-34)
Sodium lauryl sulfate granular (SDS, Nacalai tesque, cat. no. 02873-75)
Glycerol (Wako, cat. no. 075-00616)
Bromophenol blue (Wako, cat. no. 021-02911)
(+/−)-Dithiothreitol (DTT, Wako, cat. no. 048-29224)
Precision plus protein dual color standards (Bio-Rad, cat. no. 161-0374)
Ponceau S (MP Biomedicals, cat. no. 190644)
Trichloroacetic acid (TCA, Wako, cat. no. 204-02405)
5-Sulfosalicylic acid dihydrate (Wako, cat. no. 190-04572)
Skim milk powder (Wako, cat. no. 198-10605)
Anti-p62 (SQSTM1) pAb (MBL, cat. no. PM045)
Monoclonal anti-β-actin antibody produced in mouse (Sigma-Aldrich, cat. no. A5316)

Peroxidase-conjugated affiniPure goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, cat. no. 111-035-144)
Peroxidase-conjugated affiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, cat. no. 315-035-003)
SuperSignal west pico stable peroxide solution (Thermo Scientific, cat. no. 1856135)
SuperSignal west pico luminol/enhancer solution (Thermo Scientific, cat. no. 1856136)
Immobilon Western HRP substrate peroxide solution (Millipore, cat. no. WBKLS0500)
Immobilon Western HRP substrate luminol reagent (Millipore, cat. no. WBKLS0500)
Dimethyl sulfoxide (DMSO, Wako, cat. no. 045-24511)
Bafilomycin A_1 (Wako, cat. no. 023-11641)

EQUIPMENT
Milli-Q Integral 5 (Millipore)
CO_2 incubator, model: MCO-17AIC (SANYO Electric Biomedical)
Tissue culture dish (Corning)
Tissue culture plate (Corning)
15 ml centrifuge tube (Corning)
5 ml serological pipet (Corning)
1.5 ml microcentrifuge tube (BioUniverse)
High speed refrigerated micro centrifuge, model: MX-100 (TOMY Seiko)
EnSpire multimode plate reader, Model: 2300-00J (PerkinElmer)
Real power, model: BP-4 (Bio Craft)
PowerPac HC (Bio-Rad)
Trans-Blot SD semi-dry transfer cell (Bio-Rad)
ImageQuant LAS 4000 mini (GE Healthcare)

REAGENT PREPARATION
Regular cell culture medium
Supplement the DMEM cell culture medium with FBS to a final concentration of 10% (vol/vol) and antibiotic solution to a working concentration of 50 U/ml penicillin and 50 µg/ml streptomycin. Fresh L-glutamine is also added to a working concentration of 2 mM. Store the supplemented cell culture medium at 4°C. Preheat the culture medium to 37°C in a water bath before use in cell culture experiments.

Amino acid- and serum-free starvation medium
Use D-MEM (high glucose) with sodium pyruvate, without amino acids (Wako, cat. no. 048-33575) directly. Store the starvation

medium at 4°C. Preheat the starvation medium to 37°C in a water bath before use in cell culture experiments.

PBS for cell culture

Dilute 10× DPBS in a 1:10 ratio with deionized distilled water (DDW) and autoclave (121°C, 30 min). Store this solution at room temperature.

Bafilomycin A_1 stock solution (1000×)

Prepare a 100-µM bafilomycin A_1 solution in DMSO. Store it in 50-µl aliquots at −20°C and protect it from freeze-thaw cycles.

Sample buffer (6×)

Combine 3.75 ml 1M Tris-HCl (pH 6.8), 6 ml glycerol, 1.2 g SDS, 0.93 g DTT, and 6 mg bromophenol blue. Add DDW to a total volume of 10 ml. Store at −20˚C in 0.5-ml aliquots.

Ponceau S staining solution

Mix 0.2% (wt/vol) Ponceau S, 3% (wt/vol) trichloroacetic acid, and 3% (wt/vol) 5-sulfosalicylic acid dihydrate in DDW. Store this solution at room temperature.

TROUBLESHOOTING TIPS

1. The incubation time of cells in starvation medium should not be more than 2 h because the expression level of p62 is restored to basal levels during prolonged starvation in some types of cell lines such as MEFs and HepG2 cells, which may lead to underestimation of autophagic activity (Sahani *et al*, 2014).

2. The typical result of p62 degradation assay was shown in Figure 1. Lane 1 shows the expression level of p62 in MEF cells under normal growth conditions. The amount of p62 decreased in lane 3 (amino acid and serum starvation conditions) compared to that in lane 1, suggesting that p62 degradation occurs in response to starvation. Moreover, p62 expression was almost restored to a basal level in lane 4 (starvation plus bafilomycin A_1 treatment). The difference in p62 levels between lane 3 and lane 4 indicates the amount of p62 that is degraded inside the autolysosome under starvation conditions (*i.e.*, starvation-induced autophagic activity).

3. One important pitfall of this assay is that the expression level of p62 can change at the transcriptional level due to many factors. For example, as described above, p62 is upregulated at the

transcriptional level during prolonged starvation (more than 2 h), at least in MEFs and HepG2 cells (Sahani *et al*, 2014). Thus, it is important to confirm that the p62 mRNA level has not changed if the p62 protein level is used as an indicator of autophagy flux.

REFERENCES

M.H. Sahani, E. Itakura, N. Mizushima, Autophagy 10 (2014) 431-441.

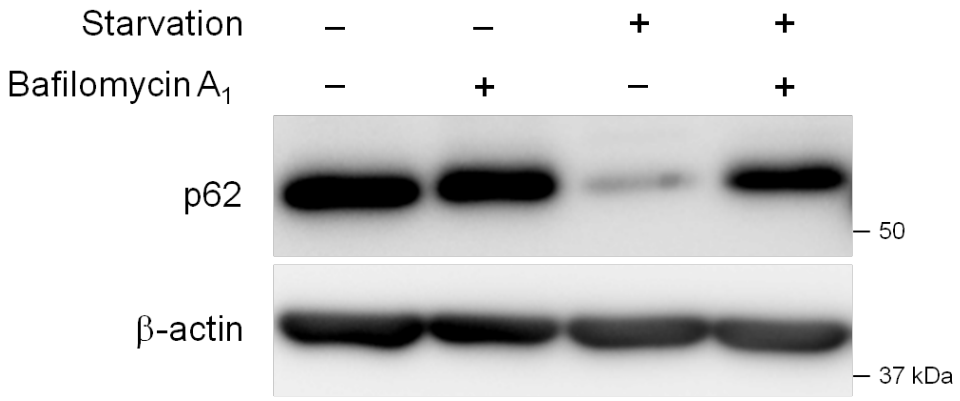

Figure 1. Steady-state levels of p62 expression. MEF cells were cultured in regular DMEM culture medium (lane 1), DMEM containing 100 nM bafilomycin A_1 (lane 2), DMEM without amino acids and serum (starvation medium, lane 3), starvation medium containing 100 nM bafilomycin A_1 (lane 4) for 2 h. Cell lysates were subjected to immunoblot analysis with p62 and β-actin antibodies.