Analysis of Protein Stability by the Cycloheximide Chase Assay

Shih-Han Kao[1], Wen-Lung Wang[2], Chi-Yuan Chen[3], Yih-Leong Chang[4], Yi-Ying Wu[5], Yi-Ting Wang[6, 7, 8], Shu-Ping Wang[9], Alexey I Nesvizhskii[10, 11], Yu-Ju Chen[6, 7], Tse-Ming Hong[5] and Pan-Chyr Yang[2, 12, 13*]

[1]Research Center for Tumor Medical Science, China Medical University, Taichung, Taiwan; [2]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan; [3]Department of Nutrition and Health Sciences, Chang Gung University of Science and Technology, Taoyuan, Taiwan; [4]Department of Pathology and Graduate Institute of Pathology, National Taiwan University, Taipei, Taiwan; [5]Graduate Institute of Clinical Medicine, National Cheng Kung University, Taipei, Taiwan; [6]Chemical Biology and Molecular Biophysics Program, Taiwan International Graduate Program, Academia Sinica, Taipei, Taiwan; [7]Institute of Chemistry, Academia Sinica, Taipei, Taiwan; [8]Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan; [9]Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, USA; [10]Department of Pathology, University of Michigan, Ann Arbor, USA; [11]Department of Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI, USA; [12]Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan; [13]NTU Center of Genomic Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan

[*]For correspondence: pcyang@ntu.edu.tw

[Abstract] Comparison of protein stability in eukaryotic cells has been achieved by cycloheximide, which is an inhibitor of protein biosynthesis due to its prevention in translational elongation. It is broadly used in cell biology in terms of determining the half-life of a given protein and has gained much popularity in cancer research. Here we present a full cycloheximide chase assay in our laboratory using a lung adenocarcinoma cell line, CL1-5, as a model.

Materials and Reagents

A. Cell lines
   1. CL1-5/Flag-wtSlug stable cells
   2. CL1-5/Flag-4SA stable cells

B. Antibodies
   1. Flag M2 antibodies (Sigma-Aldrich, catalog number: F3165)
   2. β–actin antibodies (Sigma-Aldrich, catalog number: A5441)

C. Buffer and media
1. 100 mg/ml cycloheximide (CHX) stock solution (Sigma-Aldrich, catalog number: C7698)
2. RPMI-1640 medium (Life Technologies, Gibco®, catalog number: 11875-176)
3. Fetal bovine serum (FBS) (Life Technologies, Gibco®, catalog number: 10082147)
4. HEPES (Sigma-Aldrich, catalog number: H3784)
5. Streptomycin/Penicillin (5,000 U/ml) (Life Technologies, Gibco®, catalog number: 15070063)
6. Sodium pyruvate (Sigma-Aldrich, catalog number: P5280)
7. Sodium bicarbonate (Sigma-Aldrich, catalog number: S5761)
8. G418 (Sigma-Aldrich, catalog number: A1720)
9. Sodium chloride (Sigma-Aldrich, catalog number: S7653)
10. IGEPAL CA-630 (Sigma-Aldrich, catalog number:I8896)
11. Sodium orthovanadate (Sigma-Aldrich, catalog number: S6508)
12. Sodium fluoride (Sigma-Aldrich, catalog number: S7920)
13. Sodium pyrophosphate (Sigma-Aldrich, catalog number: 221368)
14. Protease inhibitor cocktail with EDTA (Roche, Catalog number: 04693116001)
15. BCA protein assay kit (Pierce, catalog number: 23225)
16. DTT (Sigma-Aldrich, catalog number: 43815)
17. SDS (Sigma-Aldrich, catalog number: L3771)
18. Glycerol (Sigma-Aldrich, catalog number: G5516)
19. Bromophenoblue (Sigma-Aldrich, catalog number: B5525)
20. Complete medium (see Recipes)
21. Protein lysis buffer (see Recipes)
22. 5x sample buffer (see Recipes)

Equipment

1. CO_2 incubator (Thermo Fisher Scientific, Forma series II)
2. 35-mm culture dishes (Corning, catalog number: 430165)
3. 1.5 ml tubes
4. Standard sonicator

Software

1. MetaMorph software (Molecular Devices)

Procedure

Determine the time course of cycloheximide chase beforehand. There can be great variation among proteins. For an unfamiliar protein, it is recommended to start with a 4-hour interval and chase till 24 h. Cycloheximide may cause cytotoxicity to certain cells if cells are exposed to it over 20-24 h. It has been known that Slug is a labile protein with a $T_{1/2}$ of about 40 min (Wang *et al.*, 2009). CL1-5 cells stably overexpressing Flag-tagged wtSlug and non-phosphorylatable Slug-4SA were prepared using lentiviral transduction (Kao *et al.*, 2014). Cells were grown in complete medium with 400 μg/ml G418. To compare the turnover of wtSlug and Slug-4SA, these stable cells were subjected to the cycloheximide chase assay.

1. Seed about 6 x 10^5 cells in 35-mm dishes and have them incubated in a CO_2 incubator overnight.
2. After 12-h incubation, remove the medium and add complete medium with 300 μg/ml* cycloheximide (dissolved in DMSO) into each dish.
   *The concentration of cycloheximide depends on the cell line used. For CL1-5 cells, 300 μg/ml cyclcoheximide does not cause apoptosis within the time course of the chase assay. It is recommended to start a cell line test with different concentrations of cycloheximide (ranging from 50-300 μg/ml) for a least 8-h chase. For transfected cells, to avoid the sub-efficient cycloheximide concentrations, the expression of tagged proteins should be determined in the absence or presence of different concentrations of cycloheximide by Western blot at 24 h after transfection.
3. Prepare the protein lysis buffer with freshly-added protease inhibitor. About 200 μl of lysis buffer is needed for one 35-mm dish.
4. Lyse t = 0 h cells with the protein lysis buffer and store the lysates in a -80 °C freezer.
5. Collect lysates at other time points (t = 1, 2, 3, 6 h) according to the experimental design.
6. After all the lysates have been collected, sonicate the protein lysates with a sonicator for 10 times (1 sec/time) on ice.
7. Centrifuge lysates at 12,000 rpm at 4 °C for 30 min.
8. Collect and transfer the supernatants to new tubes with corresponding labels.
9. Determine protein concentrations by the BCA protein assay kit.
10. Take 50 μg of proteins, add 5x sample buffer, and incubate samples at 100 °C for 10 min.
11. Analyze the results by the SDS-PAGE assay and Western blotting (anti-Flag M2 antibodies with 1:5,000 dilution; β-actin antibodies with 1:10,000 dilution).
12. Quantify the Western bands of Flag and β-actin in triplicates using MetaMorph Software or other equivalent softwares.

Quantification

Quantification was achieved by the MetaMorph software in this case. An analysis guide should be referred to for a first-time user. Basically, regions of measurement are selected and threshold is defined. Draw region of interest on top of the western blot bands and quantitate its total grey signal (this can be done in Image J and any other image analysis software). The values of total thresholded areas are measured; a ratio of Flag-Slug to its β-actin is then calculated. The final Slug protein turnover rate at each time point is the percentage of Slug/β-actin at t = 0 of each experimental group. A plot can be created afterwards.

Representative data

Western blots and quantification images have been published in Oncogene (Kao *et al.*, 2014). For details, please refer to Figure 2f at Kao *et al.* (2014).

Recipes

1. Complete medium
   RPMI-1640
   10% FBS
   15 mM HEPES (pH 7.01)
   100 U/ml streptomycin/penicillin
   1 mM sodium pyruvate
   44 mM sodium bicarbonate
2. Protein lysis buffer
   20 mM Tris (pH 7.5)
   100 mM NaCl
   1% IGEPAL CA-630
   100 mM Na_3VO_4
   50 mM NaF
   30 mM sodium pyrophosphate
3. 5x sample buffer
   250 mM Tris-HCl (pH 6.8)
   500 mM DTT
   10% SDS
   50% glycerol
   0.1% bromophenoblue

Acknowledgments

This work was supported by grants from the National Science Council, Taiwan (NSC99-2628-B-006-031-MY3 NSC101-2325-B-006-018, NSC100-2321-B-002-071 and NSC101-2321-B002-068), National Taiwan University, Taiwan (10R71601-2), and National Institute of Health, USA (R01-GM-094231, to AIN). SP Wang is supported by a Human Frontier Science Program long-term fellowship. This protocol was adapted from previous work published in *Oncogene* (Kao *et al.*, 2014).

References

1. Kao, S. H., Wang, W. L., Chen, C. Y., Chang, Y. L., Wu, Y. Y., Wang, Y. T., Wang, S. P., Nesvizhskii, A. I., Chen, Y. J., Hong, T. M. and Yang, P. C. (2014). GSK3beta controls epithelial-mesenchymal transition and tumor metastasis by CHIP-mediated degradation of Slug. *Oncogene* 33(24): 3172-3182.
2. Wang, S. P., Wang, W. L., Chang, Y. L., Wu, C. T., Chao, Y. C., Kao, S. H., Yuan, A., Lin, C. W., Yang, S. C., Chan, W. K., Li, K. C., Hong, T. M. and Yang, P. C. (2009). p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. *Nat Cell Biol* 11(6): 694-704.

