Sumoylation and desumoylation assays for a chromatin-remodelling complex *in vivo* and *in vitro*

Jung Hwa Kim
Department of Biological Sciences, Research Center for Functional Cellulomics, Seoul National University

Keun Il Kim
Department of Biological Sciences, Research center for Women's disease, Sookmyung Women's University

Sung Hee Baek
Department of Biological Sciences, Research Center for Functional Cellulomics, Seoul National University

Method Article

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Abstract

Introduction

Small ubiquitin-like modifier \(SUMO) is a small molecule, but has a variety of regulatory functions in cells \[1-3]. SUMO modification is involved in transcriptional regulation, subcellular localization, and protein-protein interactions. SUMO conjugation requires sequential E1-dependent activation, E2-dependent conjugation, and E3-dependent ligation steps. Similar to protein phosphorylation, ubiquitination, acetylation, and methylation, SUMO conjugation and deconjugation are a dynamic processes. SUMOylating enzymes add SUMO to protein substrates, whereas deSUMOylating enzymes remove SUMO from SUMO-conjugated proteins. Many transcription factors and coregulators are SUMOylated and SUMOylation of these factors are mainly involved in transcriptiona repression mechanism \[4, 5]. SUMOylation and deSUMOylation assays are available to investigate the dynamics of SUMOylation/deSUMOylation process, and provide exciting ways to study transcriptional regulatory mechanisms. This protocol details both _in vivo_ and _in vitro_ SUMOylation/deSUMOylationg assays that provide combined molecular approaches to study cellular functions of chromatin remodelling complexes.

Reagents

SUMO1, reptin, SAE1/2 \(E1), Ubc9 \(E2), SENP1, SENP1C603A expression plasmids, PolyFect transfection reagent \(Qiagen), complete protease inhibitor cocktail \(Roche), Ni-NTA agarose beads \(Qiagen), TNT T7 quick-coupled reticulocyte lysate kit \(Promega), and protein A/G-sepharose beads \(Sigma).

Procedure

In vivo SUMOylation assay 1. HeLa cells grown in 35 mm dish were transfected with 0.3 mg of reptin, 0.6 mg of HisMax-SUMO1, and 0.6 mg of Ubc9 plasmids using PolyFect transfection reagents. 2. After 36 hrs, the cells were lysed in 500 ml of lysis buffer \(150 mM NaCl, 25 mM Tris-HCl at pH 7.8, 0.1 % Nonidet P40, and 1 mM EDTA) supplemented with complete protease inhibitor cocktail and 0.2 % SDS. 3. The crude extracts were sonicated briefly for 3 sec \(setting 3, Branson Sonifier 450), and were clarified by centrifugation at 13,000 rpm for 30 min. 4. The supernatants were incubated in 15 ml of Ni-NTA agarose \(50 % slurry) beads for 2 hrs, and the slurry was washed with 1 ml of lysis buffer four times. 5. Proteins were separated by SDS-PAGE and the existence of SUMOylated reptin and non-SUMOylated reptin was confirmed by immunoblotting with anti-reptin IgG or anti-SUMO antibody. **_In vitro_ SUMOylation assay** 1. SUMO1, GST-SAE1/2, and His-Ubc9 proteins were purified for the in vitro SUMOylation assay. 2. The assay was performed in a 10 ml of reaction volumes containing 2 mg of purified SUMO protein, 0.3 mg of purified SAE1/2 protein, 0.5 mg of purified Ubc9 protein, 0.2 ml of 100 mM ATP, 1X protease inhibitor cocktail, and an ATP regenerating system \(50 mM Tris-HCl at pH 7.6, 5 mM MgCl2, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml creatin kinase, and 0.6 U/ml inorganic pyrophosphatase) with 1 ml

of 35S-methionine-labelled in vitro-translated reptin protein. 3. Reaction products were incubated at 37 °C for 3 hrs, and analysed by autoradiography. **_In vivo_ deSUMOylation assay** 1. HeLa cells grown in 24-well plate were transfected with 0.05 mg of reptin, 0.15 mg of SUMO1, 0.15 mg of Ubc9, and 0.3 mg of SENP1 or SENP1C603A plasmids using PolyFect transfection reagents. 2. After 36 hrs, 100 ml of sampling buffer \(25 mM Tris-HCl, pH. 6.8, 2 % b-mercaptoethanol, 0.8 % \(w/v) SDS, 0.1 % bromophenol blue, and 10 % \(v/v) glycerol) was added to each well for the purpose of cell lysis. 3. Each 20 ml of cell lysate was loaded for SDS-PAGE followed by immunoblotting. **_In vitro_ deSUMOylation assay** 1. Each 10 mg of myc-SENP1 or SENP1 C603A was transfected to 293 cells grown in 100 mm plate using calcium phosphate transfection reagents. 2. After 48 hrs, cells were lysed with 500 ml of cell lysis buffer \(150 mM NaCl, 1 mM EDTA, 25 mM Tris-HCl, pH 7.8, 10 % Glycerol, 0.1 % Nonidet P-40, and protease inhibitor cocktal). 3. The lysates were incubated with anti-myc antibody for 2 hr, and 50 ml of 50 % protein A-Sepharose bead slurry was mixed with the lysates for an additional 1 hr. 4. Immunoprecipitated materials were washed three times with cell lysis buffer to eliminate non-specific binding, followed by incubation with 5 ml of in vitro SUMOylated reptin at 37 °C for 4 hrs. 5. Reaction products were incubated at 37 °C for 3 hrs, and analysed by autoradiography. **Isolation of SUMOylated and non-SUMOylated reptin chromatin remodelling proteins** 1. To isolate SUMOylated reptin protein, HeLa cells were transfected with Flag-reptin, Flag-Ubc9, and HisMax-SUMO1 plasmids. 2. After 48 hrs, cells were harvested and lysed with ice-cold phosphate-buffered saline \(PBS) containing 0.2 % Nonidet P-40 and 1 mM DTT. 3. The lysates were sonicated for 3 secs \(setting 3, Branson sofier 450), and the supernatant was taken after centrifugation. The supernatant was incubated with Ni-NTA agarose resin, and the resins were washed with PBS containing 0.2 % Nonidet P-40. 4. The bound materials were eluted with 300 mM imidazole. The eluted SUMOylated reptin were diluted with PBS containing 0.2 % Nonidet P-40 and 1 mM DTT and then incubated with anti-Flag M2 antibody-immobilized agarose beads. 5. The resulting immunoprecipitate was washed with PBS containing 0.2 % Nonidet P-40, followed by three washes with buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2 % Nonidet P-40, and 10 % glycerol. 6. The bound materials were eluted with 3X Flag peptide \(200 mg/ml) and used as SUMOylated Flag-tagged reptin proteins. 7. To prepare unmodified non-SUMOylated reptin protein, HeLa cells were transfected only with Flag-reptin plasmid, and the lysate was prepared as described above. The cell lysates were sonicated for 3 secs \(setting 3, Branson sonifier 450). The resulting supernatant was incubated with anti-Flag M2 antibody-immobilized agarose beads and Flag-tagged non-SUMOylated reptin was eluted as described above.

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