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Analysis of Sumoylation

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Summary Reversible attachment of SUMO (*s*mall *u*biquitin related *mo*difier) regulates a large number of proteins and plays an important role in processes such as transcriptional regulation, nucleo-cytoplasmic transport, genome integrity, and cell cycle progression. The steady state level of most sumoylated proteins is very low, presumably caused by strictly regulated modification and/or rapid cycles of modification and de-modification. This often causes a detection problem of sumoylation in vivo. One approach to overcome this obstacle is described here and involves enrichment of sumoylated proteins under denaturing conditions. After sumoylation is verified, addressing its functional consequences is the logical next step. This will benefit significantly from the availability of large quantities of modified protein. A protocol for efficient in vitro sumoylation of target proteins is described here. It makes use of an E3 ligase fragment that functions without target discrimination.

Key Words SUMO; His-SUMO1; Ni2+ pull down; E3-ligase; RanBP2 IR1+M

1 Introduction

Sumoylation involves an enzymatic cascade to form an isopeptide bond between the C-terminal glycine residue of SUMO and the ε-amino group of the substrate lysine. SUMO is first activated by the heterodimeric E1 activating enzyme Aos1/Uba2 (SAE1/SAE2), which catalyses adenylation of the SUMO C-terminal glycine and subsequently transfers the adenylate to a conserved cysteine resulting in an E1-SUMO thioester linkage. SUMO is then transferred to the E2 conjugating enzyme Ubc9, again forming a thioester. In the final step the modifier is conjugated to its substrate, a reaction that often requires a third class of enzymes, the E3 ligases *(1–3)*.

Steady state levels of sumoylation are usually very low, in part because of the presence of cysteine proteases (members of the Ulp family) that specifically remove SUMO from its conjugates *(1,3,4)*. These enzymes are distributed throughout the cell and are highly active, which often causes a problem in detection of endogenous

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sumoylation. Different methods to verify in vivo sumoylation have been described. One obvious approach combines immunoprecipitation of endogenous target protein with detection of a 15–20 kDa slower migrating modified form with anti-SUMO antibodies and vice versa. However, even if excellent antibodies are available and SUMO protease inhibitors like iodoacetamide or N-ethyl maleimide are included, this often is not sufficient to detect the marginal amounts of modified protein. Overexpression of either SUMO and/or the target protein can increase the level of modification. The method described here is based on a technique originally described for ubiquitin substrate verification *(5,6)*. Among others, Ron Hay's laboratory adapted this approach for sumoylation *(7–9)* and we successfully applied it in our lab *(10)*. The experimental strategy involves the enrichment of SUMO1 conjugates from a HeLa cell line stably expressing polyhistidine-tagged SUMO1 (kindly provided by Ron Hay), *(8)* on Ni^{2+} beads under denaturating conditions. This effectively inhibits the SUMO protease activity immediately upon cell lysis. Detection is performed by immunoblot analysis against the endogenous target protein and does not require as excellent antibodies as immunoprecipitation. A further advantage of this method is the possibility to easily increase the amount of starting material.

Once in vivo sumoylation has been demonstrated, understanding its functional consequences is the next step. Investigation of SUMO function involves mapping of the sumoylation site, often addressed by mutagenesis of potential lysines within the SUMO consensus motif (ψKxE, ψ is a bulky aliphatic and x any residue). This motif is recognized by the SUMO E2 in unstructured regions but not in α-helices *(11)*. However, the number of SUMO substrates modified on nonconsensus lysines is growing. A better way to identify modified lysines is by mass spectrometry. This requires relatively high amounts of modified protein but in vitro sumoylation of most substrates works only poorly in the absence of SUMO E3 ligases. Here, a tool is proposed that significantly enhances in vitro sumoylation as identified by analysing the SUMO E3 ligase RanBP2 *(10)*. A small fragment of this ligase designated IR1+M significantly enhances the SUMO transfer from the E2 to the target protein retaining its specificity for SUMO site recognition but not for substrate discrimination *(10)*. The obtained high yields of modified protein can be analysed by mass spectrometry but can also serve for further biochemical approaches to gain deeper insights into the SUMO function of a specific substrate.

2 Materials

2.1 Cell culture

1. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum, puromycin (2μg/mL), penicillin/streptomycin.
2. Tissue culture dishes 150 × 25 mm (Falcon)

2.2 Lysis and Ni^{2+}-pull down

Unless stated otherwise all buffers are prepared fresh and supplemented with 1 m*M* β-mercaptoethanol and 10 m*M* iodoacetamide.

1. Lysis Buffer: 6*M* guanidine hydrochloride, 100 m*M*, NaH_2PO_4 10 m*M* Tris-HCl, pH 8.0.
2. Wash buffer 1: 8*M* urea, 100m*M* NaH_2PO_4, 10 m*M* Tris-HCl, 10 m*M* imidazole, pH 8.0.
3. Wash buffer 2: 8*M* urea, 100 m*M* NaH_2PO_4, 10 m*M* Tris-HCl, 10 m*M* imidazole, pH 6.3.
4. Elution buffer: 8*M* urea, 100 m*M* NaH_2PO_4, 10 m*M* Tris-HCl, 10 m*M* imidazole, pH 4.5.
5. Ni^{2+}- ProBond Resin (Invitrogen).
6. Laemmli buffer: 25 m*M* Tris, 192 m*M* glycine, and 0.1% SDS, bromophenol blue, and 100 m*M* DTT in aqueous solution.
7. Micro Bio-Spin Chromatography Columns (Biorad - 732–6204).
8. Standard SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2.3 Purification of RanBP2 IR1+M

1. Competent bacterial strain BL 21 DE3.
2. RanBP2 IR1+M cloned in pGEX-2T (10).
3. Glutathione Sepharose TM 4B (Amersham Biosciences).
4. Thrombin Cleavage Capture Kit, Novagen.
5. Buffer 1: 50 m*M* Tris-HCl, pH 8, 300 m*M* NaCl supplemented with 1 m*M* PMSF, 1μg/mL each of aprotinin, leupeptin, pepstatin and 1 m*M* DTT.
6. Transport buffer (TB): 20 m*M* HEPES, 110 m*M* KOAc, 2 m*M* $Mg(OAc)_2$, 1 m*M* EGTA pH 7.3 supplemented with 1 μg/mL each of aprotinin, leupeptin, pepstatin and 1 m*M* DTT.
7. LB with ampicillin (100 μg/mL).
8. Lysozyme (SIGMA).
9. Micro Bio-Spin Chromatography Columns (Biorad - 732–6204).
10. Standard SDS-PAGE (15%).

2.4 In vitro SUMOylation with RanBP2 IR1+M

1. Recombinant proteins: SUMO, E1 and E2 *(12)*, more detailed in *(16)*, RanBP2 IR1+M, and target protein.
2. Shift buffer: Transport buffer (TB): 20 m*M* HEPES, 110 m*M* KOAc, 2 m*M* $Mg(OAc)_2$, 1 m*M* EGTA pH 7.3 supplemented with 1 μg/mL each of aprotinin,

leupeptin, pepstatin, 1 m*M* DTT, 0.05% (v/v) Tween, and 0.2 mg/mL ovalbumin grade VI (Sigma). Aliquots can be stored at −20°C.
3. ATP: 100 m*M* ATP, 100 m*M* $Mg(OAc)_2$, 20m*M* HEPES, titrate pH 7.4 with 10*N* NaOH.
4. Heat block.
5. Standard SDS-PAGE.

3 Methods

3.1 Cell culture and lysis

1. Grow three 150 × 25 mm dishes of His-SUMO1 expressing HeLa cells to 80% confluency (*see* **Note 1** and **2**). As negative control use the same amount of wt HeLa cells.
2. Wash the cells twice with ice cold 1 × PBS.
3. Lyse the cells in 6 mL lysis buffer (2 mL/plate) scrape and transfer them into an appropriate tube.
4. Incubate the lysate for 30 min at room temperature and sonicate the sample reduce viscosity.
5. Centrifuge the lysate for 1 h at 100,000*g* to remove the cellular debris that can cause unspecific binding.
6. Use the cleared supernatant for the pull down (*see* **Note 3**).

3.2 Ni^{2+} -Pull Down

After denaturing cell lysis, 2 subsequent rounds of pull down are performed to reduce unspecific binding.

1. During centrifugation of the lysate prepare the Ni^{2+}-bio spin column with each 200 µL (*see* **Note 4**) of Ni^{2+}-beads for the SUMO1 expressing cell line and the control cells, respectively.
2. Wash the beads 3 times with each 1 mL Lysis buffer.
3. Transfer the cell extracts carefully onto the column (flow through can be collected to control depletion of His-SUMO1 conjugates).
4. Wash the column 4 times with each 1 mL of Wash buffer 1.
5. Wash the column 4 times with each 1 mL of Wash buffer 2.
6. Elute His-SUMO1 with 1 mL Elution buffer into a new tube.
7. Adjust the eluate to pH 8 with NaOH and control pH with pH paper.
8. Add 50 µL washed Ni^{2+}-beads to the eluate and incubate the sample for 1 h at room temperature on a rotating wheel.
9. Apply the lysate/beads mixture onto new bio spin column.
10. Wash the beads 4 times with each 1 mL Wash buffer 1.

11. Elute His-SUMO1 conjugates with 100 µL hot 1 × Laemmli buffer into a new tube.
12. Separate the sample by standard SDS- polyacrylamide gel electrophoreses.
13. Detect the protein of interest by standard Western blotting (RanGAP1 sumoylation is demonstrated as example in Figure 9.1A, (right panel). An anti-SUMO1 (*see* **Note 5**) blot can be performed as control (Fig. 9.1A left panel).

3.3 Purification of RanBP2 IR1+M

1. Transform pGEX-RanBP2 IR1+M into the bacterial *Escherichia coli* strain BL21(DE3).
2. Use a single colony to inoculate 5-mL overnight culture in LB/Amp.
3. Dilute 2 mL of the overnight culture 1:50 in LB/Amp and grow for 2–3 h at 37°C to OD600 < 0.6.

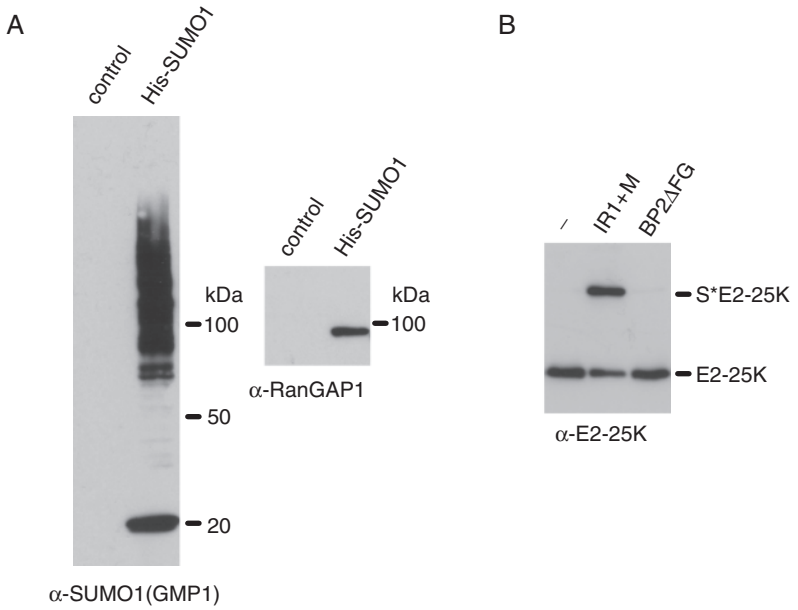

Fig. 9.1 (**A**) Ni^{2+} pull down from a His-SUMO1 expressing HeLa cell line as described here: Enrichment from His-SUMO1 conjugates in 2 rounds of Ni^{2+} pull down, elution with 1× Laemmli buffer and separation on 5–20% SDS – PAGE. Detection was performed with α-SUMO1 (GMP1) antibodies (left panel) and α-RanGAP1 antibodies (right panel), respectively. (**B**) In vitro sumoylation assay with E2-25K as SUMO substrate: A 20 µL reaction of E2-25K (450 n*M*, Boston Biochem), SUMO1 (5 µ*M*), E1 (70 n*M*), E2 (25 n*M*) and 0.5 m*M* ATP with or without RanBP2 IR1+M (300 n*M*) or RanBP2ΔFG (8 n*M*) was incubated at 30°C for 30 min. Samples were stopped with 2× Laemmli buffer and separated on a 12.5% SDS-PAGE. Detection was performed by immunoblotting using α-E2-25K antibodies

4. Add 1 m*M* IPTG to induce protein expression and incubate for another 3 h at 37°C.
5. Harvest the cells by centrifugation at 3,000g.
6. Resuspend the cell pellet in 1.5 mL Buffer 1 supplemented with 1 m*M* PMSF, 1 µg/mL each of aprotinin, leupeptin, pepstatin and 1 m*M* DTT.
7. Subject sample to one cycle of freeze-thawing in liquid nitrogen (or −80°C).
8. Transfer the solution to an appropriate ultracentrifuge tube, add 1 mg/mL lysozyme and incubate the sample for 1 h on ice.
9. Centrifuge the sample at 100,000g for 1 h.
10. Meanwhile, add 200 µL glutathione beads into a Micro Bio-Spin Chromatography Column.
11. Wash the beads 3 times with 1 mL Buffer 1.
12. Apply the supernatant (*9*) carefully to the column.
13. Wash the column 3 times with each 1 mL Buffer 1.
14. Wash the column 3 times with each 1 mL TB.
15. Add 200 µL TB buffer and 1 µL biotinylated Thrombin and incubate overnight at 4°C.
16. Centrifuge the column and collect the flow through.
17. Incubate the flow through with 10 µL prewashed streptavidin beads to remove the biotinylated Thrombin.
18. Spin the sample to pellet the beads, aliquot the supernatant and shock-freeze the aliquots in liquid N2 and store them at −80°C.
19. Determine the protein concentration and analyse the sample on a 15% SDS-PAGE.

3.4 In vitro SUMOylation with RanBP2 IR1+M

1. Mix target protein (450 n*M* to 1 µ*M*) (*see* **Note 6**) with SUMO1 (5 µ*M*) (*see* **Note 7**), E1 (70 n*M*), E2 (25 n*M*), RanBP2 IR1+M (300 n*M*) and 0.5 m*M* ATP (*see* **Note 8**). As control include a sample without RanBP2 IR1+M (shows E3 independent reaction) and/or one without ATP (shows no shift).
2. Incubate the reactions for 30 min at 30°C.
3. Stop the reaction either by adding 2x Laemmli buffer (for SDS-PAGE) or by ATP depletion with 1 U Apyrase (SIGMA) or by blocking the E1 activity with 10 m*M* EDTA (for biochemical analysis) (*see* **Note 9**).
4. Separate the sample on an appropriate SDS-PAGE.
5. Detect the target protein and its sumoylated form either by Western blot (an example is demonstrated in Fig. 9.1B) or by Coomassie stain.

4 Notes

1. The amount of His-SUMO1 expressing HeLa cells to start with depends on the detection level of your antibodies and on the expected steady state level of sumoylation for the target protein and can therefore easily be scaled up- or down.

2. Usage of $Ni2^{+}$-pull down has also been described for verification of substrate sumoylation in yeast (e.g., (13, 14)).
3. To determine the amount of modified versus unmodified protein, precipitate (TCA or Chlorophorm/Methanol-) an aliquot of the lysate.
4. High excess of $Ni2^{+}$-beads over His-tagged protein increases the background (The QIAexpressionist handbook from Qiagen is highly recommended as trouble-shooting guide for $Ni2^{+}$-pull downs).
5. Antibody to detect mammalian SUMO1: mouse monoclonal anti-GMP1 (anti-SUMO1) from Zymed
6. Usage of deletion fragments in the in vitro sumoylation assay is problematic because it can lead to wrong conclusions regarding the SUMO modification site (Lysines hidden in the full length protein can be exposed or small fragments become unstructured).
7. Recently, a SUMO mutant (SUMOT95R) that allows easier identification of sumoylated peptides by mass spectrometry analysis has been described (15). One may consider to use this mutant in the in vitro sumoylation assay for SUMO site identification.
8. Ovalbumin in the shift buffer is not required for assays using high protein concentration (but it is strongly recommended for dilutions to low concentrations, like for enzymes)
9. For studying the function of a sumoylated protein the modified protein has to be separated from the unmodified after the in vitro reaction. For small proteins this can be performed e.g. by separation on a Sephadex 75 FPLC column (11). Larger proteins can, e.g., be separated in a 2-step procedure by using a tagged SUMO that allows the separation of the modified from the unmodified form by pull down. In a second step the free SUMO is removed from the conjugate by gel-filtration.

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