Video Article

In Vivo Detection and Analysis of Rb Protein SUMOylation in Human Cells

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Abstract

The post-translational modifications of proteins are critical for the proper regulation of intracellular signal transduction. Among these modifications, small ubiquitin-related modifier (SUMO) is a ubiquitin-like protein that is covalently attached to the lysine residues of a variety of target proteins to regulate cellular processes, such as gene transcription, DNA repair, protein interaction and degradation, subcellular transport, and signal transduction. The most common approach to detecting protein SUMOylation is based on the expression and purification of recombinant tagged proteins in bacteria, allowing for an *in vitro* biochemical reaction which is simple and suitable for addressing mechanistic questions. However, due to the complexity of the process of SUMOylation *in vivo*, it is more challenging to detect and analyze protein SUMOylation in cells, especially when under endogenous conditions. A recent study by the authors of this paper revealed that endogenous retinoblastoma (Rb) protein, a tumor suppressor that is vital to the negative regulation of the cell cycle progression, is specifically SUMOylated at the early G1 phase. This paper describes a protocol for the detection and analysis of Rb SUMOylation under both endogenous and exogenous conditions in human cells. This protocol is appropriate for the phenotypical and functional investigation of the SUMO-modification of Rb, as well as many other SUMO-targeted proteins, in human cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/56096/

Introduction

The accurate control of cell cycle progression in eukaryotic cells is based on a tight regulatory network, which ensures that particular events take place in an ordered manner[1,2]. One of the key players in this network is the retinoblastoma (Rb) protein, the first cloned tumor suppressor[1,3]. The Rb protein is thought to be a negative regulator of cell cycle progression, especially for the G0/G1 to S phase transition, and tumor growth[4,5]. Failure of Rb function either directly leads to the most common intraocular malignancy in children, retinoblastoma, or contributes to the development of many other types of cancer[5]. Moreover, Rb is involved in many cellular pathways including cell differentiation, chromatin remodeling, and mitochondria-mediated apoptosis[3,6,7].

Post-translational modifications play a pivotal role in the regulation of RB function[8,9]. Phosphorylation is one such modification, and it usually leads to Rb inactivation. In quiescent G0 cells, Rb is active with a low phosphorylation level. As cells progress through G0/G1 phase, Rb is sequentially hyper-phosphorylated by a series of cyclin-dependent protein kinases (CDKs) and cyclins, such as cyclin E/CDK2 and cyclin D/CDK4/6, which inactivate Rb and eliminate its ability to repress cell-cycle related gene expression[4,10]. Rb could also be modified by small ubiquitin-related modifier (SUMO)[11,12,13].

SUMO is a ubiquitin-like protein that is covalently attached to a variety of target proteins. It is crucial for diverse cellular processes, including cell cycle regulation, transcription, protein cellular localization and degradation, transport, and DNA repair[14,15,16,17,18]. The SUMO conjugation pathway consists of the dimeric SUMO E1 activating enzyme SAE1/UBA2, the single E2 conjugating enzyme Ubc9, multiple E3 ligases, and SUMO-specific proteases. Generally, nascent SUMO proteins must be proteolytically processed to generate the mature form. The mature SUMO is activated by the E1 heterodimer and then transferred to the E2 enzyme Ubc9. Finally, the C-terminal glycine of SUMO is covalently conjugated to the target lysine of a substrate, and this process is usually facilitated by E3 ligases. The SUMO protein can be removed from the modified substrate by specific proteases. A previous study by the authors of this paper revealed that SUMOylation of Rb increases its binding to CDK2, leading to hyper-phosphorylation at the early G1 phase, a process which is necessary for cell cycle progression[13]. We also demonstrated that the loss of Rb SUMOylation causes a decreased cell proliferation. Moreover, it was recently demonstrated that the SUMOylation of Rb protects the Rb protein from proteasomal turnover, thus increasing the level of Rb protein in cells[19]. Therefore, SUMOylation plays an important role in Rb function in various cellular processes. To further study the functional consequence and physiological relevance of Rb SUMOylation, it is important to develop an effective method to analyze the SUMO status of Rb in human cells or patient tissues.

SUMOylation is a reversible, highly dynamic process. Thus, it is usually difficult to detect the SUMO-modified proteins under completely endogenous conditions. This paper presents a method to detect endogenous Rb SUMOylation. Furthermore, it shows how to detect exogenous Rb SUMOylation of both wild-type Rb and its SUMO-deficient mutation[11]. In particular, Jacobs *et al.* described a method to increase the SUMO modification of a given substrate specifically by Ubc9 fusion-directed SUMOylation (UFDS)[20]. Based on this method, this protocol describes how to analyze the forced SUMOylation of Rb and its functional consequences. Given that hundreds of SUMO substrates have been described previously and more putative SUMO substrates have been identified from many proteomic-based assays, this protocol can be applied to analyze the SUMO-modification of these proteins in human cells.

Protocol

1. Detection of Endogenous Rb SUMOylation at the Early G1 Phase

1. **Cell culture and cell cycle synchronization.**
    1. Maintain HEK293 cells in growth medium containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% Pen-Strep and 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 in an incubator.
    2. Synchronize the HEK293 cells at the G0 phase.
        1. Count the HEK293 cells using a hemocytometer and seed ~1.5 x 10^7 cells in a 15 cm dish with 25ml growth medium for 24 h before treatment. After reaching a confluence of 70% - 80%, aspirate the medium and wash the cells twice with 5 mL prewarmed phosphate-buffered saline (PBS).
        2. Add 25 mL DMEM containing 1% Penicillin-Streptomycin and incubate the cells at 37 °C for 72 h. Wash the cells off the plate using 3 mL ice-cold PBS. Transfer the cells into a new 5 mL tube.
        3. Subject a small portion of cells to cell cycle analysis by flow cytometry (step 1.2); collecting the remaining majority of the cells by centrifugation at 200 x g for 5 min at room temperature, and then store them in an ultra-lower temperature freezer until analysis of Rb SUMOylation.
    3. To obtain G1-phase cells, at the end of the G0 synchronization process, remove the DMEM and add back fresh growth medium, thus allowing the HEK293 cells to re-enter the cell cycle. Then, collect the cells at different G1 phases (early G1: 30 min; G1: 2h) for cell cycle analysis and further SUMO assay.
    4. Synchronize the HEK293 cells at the S phase using the double-thymidine block method.
        1. Grow HEK293 cells to a confluency of 50% and wash cells with prewarmed PBS. Then add growth medium supplemented with 2.5 mM thymidine for 18 h (first block).
        2. Remove the thymidine-containing medium, and then wash the cells twice with prewarmed PBS. Add fresh growth medium for 14 h to release the cells.
        3. Discard the medium with a pipette and add growth medium supplemented with 2.5 mM thymidine for another 18 h (second block) before conducting an analysis of the cell cycle and Rb SUMOylation.
    5. For the G2/M synchronization, plate ~1.5 × 10^7 HEK293 cells to a 15 cm dish with 25 mL growth medium for 24 h. Then add nocodazole to the medium until a final concentration of 400 ng/mL is obtained. Finally, incubate the cells for 16 h before conducting the analysis of the cell cycle and Rb SUMOylation.
2. **Cell cycle analysis by flow cytometry.**
    1. Re-suspend the synchronized HEK293 in PBS, and then fix the cell suspension using ice-cold 70% ethanol for 2 h at 4 °C. Note that to minimize cell clustering, add the cell suspension dropwise to the ice-cold 100% ethanol to obtain a final concentration of 70% ethanol while gently vortexing.
    2. Centrifuge the cells for 5 min at 500 x g, then carefully discard the supernatant and wash the cells twice with PBS. Repeat the centrifuge step.
    3. Add 500 µL PBS containing 50 µg/mL nucleic acid stain propidium iodide, 0.1% Triton X-100 and 1 µg/mL RNase A to the cells and mix well. Incubate the cells for 15 min at 37 °C.
    4. Store the samples at 4 °C until analysis by flow cytometry.
3. **Immunoprecipitation of endogenous Rb protein.**
    1. Prepare the HEK293 cell lysates.
        1. Lyse the synchronized HEK293 cells by gently re-suspending them in 1 mL of ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer (**Table 1**) containing freshly added 20 mM N-Ethylmaleimide, an isopeptidase inhibitor that could block SUMO proteases and stabilize SUMO conjugates.
        2. Further homogenize the cells on ice by Dounce homogenization, sonication or simply passing through 10 times through a 21 G needle attached on a 2 mL syringe. Then, incubate the cells on ice for 5 min.
        NOTE: The anionic detergent sodium dodecyl sulfate (SDS) in the RIPA buffer is crucial for the later determination of the Rb-SUMO, as it could eliminate the unspecific SUMO signal that is derived from the non-covalent interaction between the Rb protein and other non-Rb SUMO-species.
    2. Centrifuge the cell lysates at 18,000 x g for 30 min at 4 °C. Transfer the supernatants to new 1.5 mL micro-centrifuge tubes.
    NOTE: The protocol can be paused here. The proteins can be stored at -80 °C for at least 6 months.
    3. To prepare the input control of each sample for the later Western blot, save a small portion of the above-described supernatant to a new tube and store at -80 °C.
    4. To the above supernatants, add 1 µg of non-specific mouse immunoglobulin G (IgG) of the same species and isotype as the monoclonal Rb antibody, and 20 µL of 50% protein A/G-sepharose slurry. Then, incubate for 1 h at 4 °C with gentle rotation.

5. Centrifuge the samples at 3,000 x g for 3 min at 4 °C. Carefully collect the supernatants without disturbing the beads, and transfer them to new 1.5 mL micro-centrifuge tubes. To each of the samples, add 5 µL Rb primary antibody and 40 µL of 50% protein A/G-sepharose slurry. Then, incubate overnight at 4 °C with gentle rotation.
6. Collect the beads by centrifugation at 3,000 x g for 3 min at 4 °C, and carefully remove the supernatant by pipetting. Note that in order to avoid bead loss, do not aspirate the beads dry.
7. Wash the beads four times with 1 mL of the RIPA buffer. Each time, mix the tubes well by rotation them at 4 °C for 15 min. Collect the beads by low speed centrifugation at 3,000 x g for 3 min at 4 °C and then discard the supernatants.
8. After removing supernatants from the final wash, re-suspend the beads in 30 µL 1x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (**Table 1**) and mix well.
    1. Incubate the tubes at 100 °C in a heat block for 10 min. Centrifuge the samples at 12,000 x g for 1 min to pellet the beads. Carefully collect the supernatants without disturbing the beads, and transfer them to 1.5 mL micro-centrifuge tubes.
9. Analyze the samples by 4% - 20% gradient SDS-PAGE gel and Western blot or store them at -20 °C for later use.

2. Analysis of 6XHis-tagged Exogenous Rb SUMOylation in Human Cells

1. **Cell culture and transfection.**
    1. Seed ~6 × 10^6 HEK293 cells in a 10 cm dish and incubate for 24 h in growth medium under normal cell culture conditions to acquire 75%-85% confluent cells. Before transfection, remove the growth medium and add 6 mL of prewarmed reduced serum medium (see **Table of Materials**) and then place the dishes back into the incubator.
    2. For the Rb constitutive SUMOylation assay, add 15 µg each of the 6XHis-tagged Rb-Ubc9-WT, Rb-Ubc9-C93S, Ubc9-WT and Ubc9-C93S plasmids in 500 µL reduced serum medium in 1.5 mL micro-centrifuge tubes, respectively.
        1. To analyze the SUMO-conjugation of wild type Rb and its SUMOylation-defective mutant, mix 10 µg of either 6XHis-tagged Rb-WT or Rb-K720R plasmid together with GFP-SUMO1 (10 µg) in 500 µL reduced serum medium in 1.5 mL micro-centrifuge tubes[13].
    3. Meanwhile, in a separate tube, mix 50 µL transfection reagent (see **Table of Materials**) in 500 µL reduced serum medium, and incubate at room temperature for 5 min.
    4. Fully combine the two separate tubes described above, and incubate at room temperature for 15 min. Add the transfection reagent/plasmid complexes to the cells and continue to culture for 8 h at 5% CO_2 and 37 °C.
    5. Replace reduced serum medium with growth medium, and incubate the cells under normal culture conditions for 48 h after transfection.
2. **Nickel affinity pull down of the 6XHis-tagged Rb protein.**
    1. Lyse the transfected HEK293 cells and extract the total proteins as described Section 1.3.1.
    2. Centrifuge the cell lysates at 18,000 x g for 30 min at 4 °C. Carefully collect the supernatants and transfer them to clean tubes. NOTE: The protein can be frozen at this point for more than 6 months at -80 °C.
    3. To prepare the input control of each sample for the later Western blot, save a small portion of the supernatants described above to a new tube and store at -80 °C.
    4. Wash 25 µL of 50% nickel nitrilotriacetic acid (Ni-NTA) agarose beads with RIPA buffer twice in a 1.5 mL micro-centrifuge tube for each sample. Collect the beads by centrifugation at 3,000 x g for 3 min at 4 °C.
    5. Add 1 M imidazole to each sample to obtain a final concentration of 10 mM. Then, add each sample to the tube containing the prepared Ni-NTA agarose beads.
    6. Incubate the beads and the lysates for 2 h at 4 °C with gentle rotation. Spin the beads at 3,000 x g for 3 min at 4 °C, and carefully remove the supernatants by pipetting. To avoid bead loss, do not aspirate the beads dry.
    7. Wash the beads with 1 mL of wash buffer containing 20 mM imidazole (**Table 1**). Each time, mix the tubes well by rotation at 4 °C for 15 min. Collect the beads by spinning at 3,000 x g for 3 min at 4 °C, and discard the supernatants.
    8. After the final wash, add 30 µL of elution buffer containing 250 mM imidazole (**Table 1**) to each sample and flick to mix. Then, incubate for 20 min at 4 °C to elute the proteins.
    9. Mix each sample with 6× SDS-PAGE loading buffer (**Table 1**). Then, incubate the tubes at 100 °C in a heat block for 10 min.
    10. Centrifuge at 12,000 x g for 1 min to pellet the beads. Carefully collect the supernatants without disturbing the beads, and transfer the samples to 1.5 ml micro-centrifuge tubes. The samples can be analyzed by Western blot or stored at -20 °C for later use.

3. Western Blot

1. Load the immunoprecipitation or pull down samples obtained from the previous steps onto 4-20% gradient SDS-PAGE gels. Conduct electrophoresis at 120 V for 90 min to separate the proteins.
2. Transfer the proteins from the gel to a polyvinylidene difluoride (PVDF) membrane by electroblotting at 300 mA for 90 min at 4 °C using the tank transfer method. Block the PVDF membrane in block buffer containing 5% nonfat milk (**Table 1**) for 1 h at room temperature.
3. Incubate the membrane with primary antibody in antibody dilution buffer containing 3% bovine serum albumin (BSA) (**Table 1**) overnight at 4 °C. For primary antibodies, use a working concentration of 0.5 µg/mL (anti-SUMO1 antibody), or a dilution of 1:2000 (anti-Rb and anti-Tubulin antibodies), or 1:5,000 (anti-GFP and anti-His antibody).
4. Wash the membrane 3x for 10 min each time using 1x tris-buffered saline with Tween (TBST) buffer (**Table 1**). Incubate the membrane with species specific Horseradish Peroxidase (HRP)-conjugated secondary antibodies diluted 1:5,000 in block buffer containing 5% nonfat milk (**Table 1**). Wash the membrane 3x for 10 min each time using 1x TBST buffer.
5. Incubate the membrane with enhanced chemiluminescence (ECL) working solution. Cover the membrane with plastic wrap and expose it to X-ray film depending on the strength of signal.

Representative Results

To detect endogenous Rb SUMOylation during cell cycle progression, this study first synchronized HEK293 cells at five different stages of the cell cycle (G0, early G1, G1, S, and G2/M) as described in the protocol section of this paper. The quality of synchronization was confirmed by using the nucleic acid stain with propidium iodide followed by flow cytometry analysis (**Figure 1**). Next, the cells were collected and lysed by denaturing RIPA buffer.The SUMO proteases inhibitor, N-Ethylmaleimide, was added to a final concentration of 20 mM to preserve the native SUMO signal during the experiments. After immunoprecipitation of the endogenous Rb species under denaturing conditions to block the non-covalent unspecific interaction, and the following western blot using an anti-SUMO1 antibody, the presence of the SUMO signal was specifically detected at the early G1 phase (**Figure 2**). Although the global SUMOylation was enhanced at the S/G2/M phase, at the time point of Rb SUMOylation, it had not changed (**Figure 2**, input panel). This finding suggests that the SUMOylation of Rb is not simply the consequence of altered global SUMO conjugation activity. Thus, these results show that the protein immunoprecipitation and Western blot analysis described in this paper allow for the detection of SUMOylation of the endogenous Rb protein.

To detect the forced SUMOylation of the Rb protein, this study generated a constitutive SUMOylated Rb construct by fusing Ubc9, the sole SUMO E2 ligase, to its C-terminal allowing for efficient and selective SUMOylation of Rb (**Figure 3A**). The loss-of-function mutation of Ubc9, C93S, is also fused to the C-terminal of Rb to serve as a control (**Figure 3A**). To further strengthen the specificity of the SUMO-Rb signal, non-fused Ubc9 alone was constructed as well. All four of the His-tagged plasmids were transfected into the HEK293 cells for 48 h before they were lysed in RIPA buffer supplemented with 20 mM N-Ethylmaleimide. All the proteins were then subjected to a pull down assay, as described in the protocol section of this paper. The eluted Rb proteins were analyzed by Western blot. The Ubc9 fusion-directed SUMOylation of Rb was detected using an anti-SUMO1 antibody (**Figure 3B**, SUMO1 panel), whereas the Ubc9 defective mutation, C93S, failed to produce this signal. Note that the highly efficient SUMO-modification caused by Ubc9-fusion leads to a higher molecular weight band that could even be directly detected by using the Rb antibody, and it corresponds to the SUMO signal (**Figure 3B**, Rb panel). Moreover, Ubc9 alone did not cause any SUMO conjugation, further confirming the Rb-specific SUMOylation (**Figure 3B**).

Because SUMO1 is conjugated to lysine 720 of the Rb protein[11], to further analyze the Rb SUMOylation, this study generated a SUMO-deficient mutation by replacing this lysine residue with an arginine (K720R). To facilitate the detection of SUMO-conjugation of the two transiently expressed Rb proteins, a GFP-SUMO1 construct was added to enhance the intracellular SUMO signal. His-tagged wild type or mutant Rb was co-transfected into HEK293 cells together with GFP-SUMO1, followed by pull down assay and analysis of Rb-SUMO1 conjugation capability. As observed, the K720R mutant totally abolished the SUMO-modification of Rb, further strengthening the proposed method's ability to detect the Rb SUMOylation (**Figure 4**).

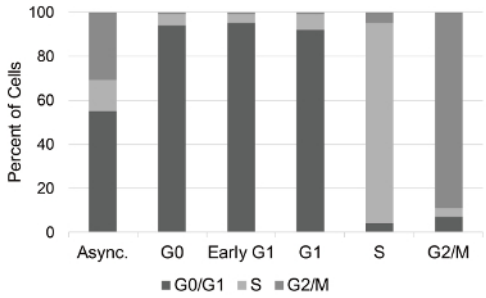

Figure 1: Validation of the cell cycle synchronization assay by flow cytometry. HEK293 cells were cultured and synchronized at the G0, early G1, G1, S, and G2/M phases of the cell cycle as described in this protocol. Cell cycle distributions were determined by fluorescence-activated cell sorting (FACS). The represented data shows an example of the quantitative analysis of the FACS assay (n = 1). Please click here to view a larger version of this figure.

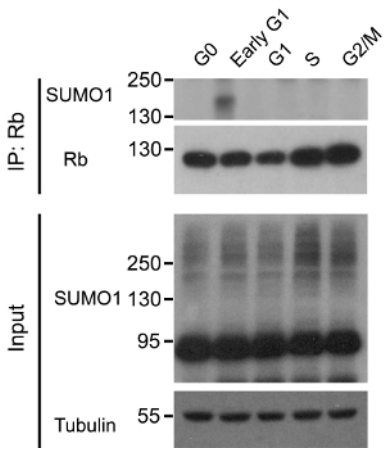

Figure 2: Rb is SUMOylated at early G1 phase. HEK293 cells were synchronized at different phases of the cell cycle as described in this protocol. The cells were collected and lysed in RIPA buffer supplemented with 20 mM N-Ethylmaleimide. The input controls were loaded on a 4% - 20% SDS-PAGE gradient gel, transferred, and blotted with anti-SUMO1 and anti-Tubulin, as indicated. The remaining cell lysates were then subjected to immunoprecipitation using anti-Rb antibody at a dilution of 1:200. The resulting eluents were separated by 4% - 20% SDS-PAGE gradient gel and immunoblotted using anti-SUMO1 antibody and anti-Rb antibody. This experiment was repeated twice with the same result. This figure has been modified with permission[13]. Please click here to view a larger version of this figure.

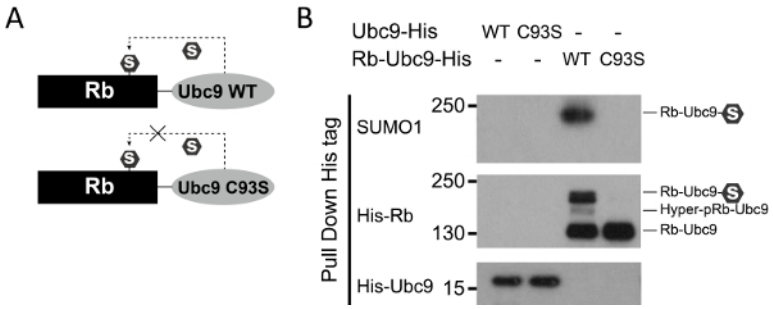

Figure 3. Detection of the SUMO-modification of Rb-Ubc9 fusion protein. (**A**) Diagram of the Ubc9 fusion-directed SUMOylation (UFDS) constructs of Rb. (**B**) Constitutive SUMOylation of Rb caused by UFDS. HEK293 cells transiently transfected with His-tagged UFDS constructs were lysed in RIPA buffer with 20 mM N-Ethylmaleimide. The total lysate of each sample was incubated with Ni-NTA agarose beads to pull down the His-tagged Rb-Ubc9 fusion proteins or Ubc9 (used as the negative control), respectively. The purified proteins were separated by 4% - 20% SDS-PAGE gradient gel and immunoblotted with anti-SUMO1 and anti-His antibodies. His-Rb: 6XHis tagged Rb-Ubc9 fusion proteins; His-Ubc9: 6XHis tagged Ubc9 only. Rb-Ubc9: unmodified Rb-Ubc9; Rb-Ubc9-S: SUMOylated Rb-Ubc9; Hyper-pRb-Ubc9: hyper-phosphorylated Rb-Ubc9. The results shown in the figure are representative of three independent trials. This figure has been modified with permission[13]. Please click here to view a larger version of this figure.

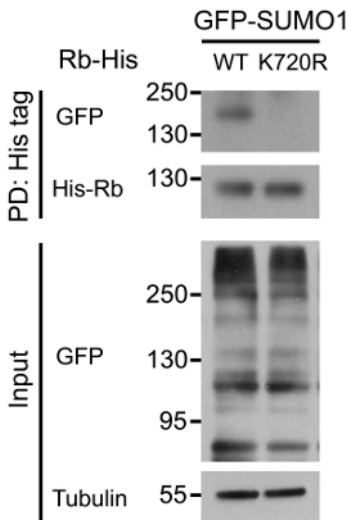

Figure 4. The SUMO-deficient K720R mutation reduces the SUMOylation of Rb. HEK293 cells were transiently co-transfected with the wild type or mutant Rb-His constructs together with GFP-SUMO1. The cells were collected and lysed in RIPA buffer supplemented with 20 mM N-Ethylmaleimide. A small portion of the lysates was directly analyzed by Western blot as the input control. The rest of the lysates were incubated with Ni-NTA agarose beads to pull down the His-tagged Rb proteins as described in this protocol, and they were immunoblotted with anti-SUMO1 and anti-His antibodies. Note that the lysine-to-arginine mutation at 720 of Rb totally abolishes the SUMO modification of this protein. The experiment was conducted once with a similar result to that previously reported[11]. This figure has been modified with permission[13]. Please click here to view a larger version of this figure.

Solution	Components	Comments
RIPA Lysis Buffer	50 mM Tris, pH = 8.0; 150 mM NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% SDS	Add protease inhibitor cocktail and N-ethylmaleimide immediately before use.
Wash Buffer	50 mM NaH_2PO_4 pH = 8.0; 300 mM NaCl; 20 mM imidazole	Used for Pull down assay only
Elution Buffer	50 mM NaH_2PO_4 pH = 8.0; 300 mM NaCl; 250 mM imidazole	Used for Pull down assay only
6x Loading Buffer	0.5 M Tris, pH = 6.8; 30% glycerol; 10% SDS; 5% β-mercaptoethanol; 0.1% bromphenol blue	Store at -20 °C
10x Running Buffer	0.25M Tris, pH 8.6; 1.9M Glycine; 1% SDS	To make 1x Running Buffer: mix 100 ml 10x Running Buffer with 900 mL ddH_2O
10x Transfer Buffer	0.25 M Tris, pH 8.3, 1.9 M Glycine	To make 1x Transfer Buffer: mix 100 mL 10x Transfer Buffer with 200 ml Methanol and 700 mL ddH_2O
10x TBS Buffer	In 1 L of ddH_2O: 24.08 g Tris pH 7.4; 80 g NaCl	To make 1x TBST Buffer: mix 100 mL 10x TBS Buffer with 900 mL ddH_2O and 1 mL of Tween-20
Blocking Buffer	1x TBST with 5% nonfat dry milk	Prepare the buffer immediately before use
Antibody Dilution Buffer	1x TBST with 3% BSA and 0.02% sodium azide	This Buffer could be stored at 4 °C for at least 6 month

Table 1: Solutions and buffers.

Discussion

This paper describes a protocol to detect and analyze the endogenous SUMOylation of Rb in human cells. As this method is specifically focused on the endogenous Rb protein without any alternation of global SUMO-related signal, it is an important tool for investigating Rb-SUMO modification under completely natural physiologic circumstances.

To achieve this aim, it is important to keep in mind that: 1) although SUMO comprises four isoforms (SUMO1-4, each encoded by different genes)in comparison to ubiquitination, all the SUMO species are much less abundant; 2) for most SUMO target proteins, only a small portion of a given protein is SUMOylated at steady state, which is the outcome of the constant competition between the enzymes involved in SUMO conjugation and deconjugation[14,21]; and 3) SUMO targets can undergo rapid cycles of SUMOylation and deSUMOylation. For example, recent data obtained by the authors of this current paper demonstrate that Rb-SUMO1 exists only for a very short window of time in the early G1 phase, which is consistent with the conception that SUMOylation is a reversible, highly dynamic process[13]. All these facts make it challenging to directly detect the endogenous SUMOylation of a given protein. Thus, to successfully detect this, it is vital to identify the exact conditions under which

this modification occurs. For instance, in this proposed protocol, the timing of the cell cycle synchronization is crucial for the detection of Rb SUMOylation. An optimized experimental procedure is also important for the successful detection of the low-level SUMO-modified species of a given protein. For example, proper sonication or passing-through needles could prevent the formation of sticky and viscous components due to genomic DNA, thus proper sonication can facilitate total protein extraction. Moreover, a good monoclonal antibody with high affinity to the target protein could be helpful for improving the quantity and specificity of immunoprecipitation. In summary, the proposed method is important for further exploring the physiological conditions under which endogenous Rb is SUMOylated, which is the premise of the following overexpression-based functional assay.

To amplify the SUMO signal of a given protein, it is common to co-overexpress this protein as well as other SUMO-related proteins (usually Ubc9 and SUMO protein) in cells. Although a simple Western blot using an antibody against the substrate is the easiest way to find the higher molecular weight, SUMOylated form of this protein, we failed to detect the SUMO-Rb signal with this method. Thus, this paper only focused on a method to detect the SUMO-modification of the precipitated exogenous Rb protein. Moreover, this method described how to artificially enhance or eliminate the SUMO-conjugation of Rb. As the sole E2 ligase, Ubc9 has been found to directly transfer SUMO to specific targets[22]. Thus, by fusing to the C terminal of Rb, Ubc9 significantly promotes the SUMO-modification of Rb. Using this method, the authors of this paper successfully demonstrated that SUMOylation of Rb sufficiently promoted its own phosphorylation by increasing its binding to CDK2[13]. Moreover, to study the functional consequences of the loss of Rb SUMOylation, the authors generated a SUMO-deficient Rb construct, as previously reported[11]. By using the method proposed in this current paper, it was shown that SUMO-Rb is necessary for normal cell cycle progression and cell proliferation[13].

In addition to SUMO1, SUMO2 and SUMO3 also play important roles in protein SUMOylation[14,17]. SUMO2 and SUMO3 are often referred to as SUMO2/3 because they are closely related and share 97% identity. SUMO1 shares a 50% similarity with SUMO2/3. It is widely accepted that SUMO1 is responsible for normal cell physiological functions and maintenance, whereas SUMO-2/3 is predominantly involved in cell stress responses[15,17,23,24]. Given that Rb could also be SUMOylated by SUMO2/3 with unknown function[12], the method proposed in this study is still suitable for the detection and analysis of this modification of Rb. In addition to Rb, the methods described here can be widely applied to the detection and functional analysis of the SUMOylation of a variety of target proteins.

Disclosures

The authors have nothing to disclose.

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