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Assays for investigating deSUMOylation enzymes (ms# CP-11-0260)

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Abstract

Post-translational modifications by the SUMO (Small Ubiquitin-like **MO**difier) family of proteins are recently discovered essential regulatory mechanisms. All SUMO proteins are synthesized as larger precursors that are matured by SUMO-specific proteases, known as SENPs, which remove several C-terminal amino acids of SUMO to expose the Gly-Gly motif. SENPs also remove SUMO modifications from target proteins, making this modification highly dynamic. At least six deSUMOylation enzymes, all of which are encoded by essential genes, have been identified in mammals. SENP1 has been shown to play an important role in the development of prostate cancer and in angiogenesis. This unit describes and discusses methods for characterizing the deSUMOylation enzymes. These assays enable the identification of inhibitors of these enzymes and investigate their mechanism of inhibition in order to develop research tools and future therapeutics.

Keywords

SUMO; SENP; Vinyl sulfone; FRET; Bioluminescence

INTRODUCTION

Post-translational modifications by the SUMO (Small Ubiquitin-like **MO**difier) family of proteins are reversible and highly dynamic modifications that regulate a wide range of cellular functions (Yeh, 2009). SUMO (or Sentrin)-specific proteases (SENPs) play key roles in SUMO modifications by catalyzing the maturation of SUMO proteins, as well as removing SUMO modifications from target proteins (Fig. 1). Identification of inhibitors of SENPs will greatly facilitate the elucidation of the roles of SENPs in cellular regulation and could provide lead molecules for the development of innovative therapeutics. This unit provides several protocols for investigating SENP activities in vitro and in cell lysates. Basic Protocol 1 describes an assay to evaluate SENP activities by Förster (or Fluorescence) resonance energy transfer (FRET). The method is sensitive and quantitative, and uses physiological substrates, but data analysis is not straightforward. The same FRET-based assay can be applied to a substrate containing a YFP-SUMO fusion protein and an ECFP-fusion target protein, such as RanGAP1. Such substrates can be made by a SUMOylation reaction in which the YFP-SUMO fusion and an ECFP-fused target protein are mixed with E1, E2, ATP and possibly an E3. Although such substrates require more steps to create than the YSE SUMO precursors, they provide information on the isopeptidase activity of a SENP, while the SUMO precursors reflect only the endopeptidase activity of SENP. The

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FRET assay can be adapted to study kinetics of SENP enzyme activity. Michealis–Menten constants can be derived from the initial rates across a YSE concentration range (20 nM–2 μM), as shown previously (Shen et al., 2006c). Alternate Protocol 1 uses the same substrates as Basic Protocol 1, but the reaction products are detected by SDS-PAGE, instead of FRET. Kinetic information can also be derived from this assay. The gel bands can be quantified using quantitative densitometry and can be converted to rates by establishing a standard curve with known substrate or product concentrations. This step will depend on the establishment of a proper substrate or product concentration range in which the staining method of choice provides a sufficient dynamic range for conversion from gel band intensity to sample concentration. The data can be fitted to a Michealis–Menten equation to derive the reaction constants (Reverter and Lima, 2006a). Basic Protocol 2 uses fluorogenic substrates that contain SUMO (SUMO-AMC). This assay is sensitive, convenient and quantitative. By varying the concentrations of SUMO-AMC, the release of AMC by SENPs, measured by the fluorescence intensity of AMC, can be directly used for steady state kinetic analysis (Kolli et al., 2010). Basic Protocol 3 is a bioluminescence-based assay with a pentapeptide substrate that contains the Gly-Gly motif. It is fast and quantitative. Although the substrate is not physiological and not specific for de-SUMOylation, the small substrate directly provides information on whether a candidate inhibitor targets the catalytic center. By varying the concentrations of the substrate and a candidate inhibitor, this protocol can also be adapted to conduct steady-state kinetics analysis. An advantage of this protocol over the FRET or fluorogenic assay is that it accommodates molecules (inhibitors) that are fluorescent and thus could interfere with the FRET or fluorogenic analysis. Another advantage of this method is the direct assessment of whether a candidate inhibitor interferes with the catalytic center or targets the surface required to bind the structured domain of SUMO. Basic Protocol 4 can be used to evaluate the specificity of the different SENPs and SUMO paralogues in cell lysates. The protocol involves using HA (hemagglutinin) - tagged SUMO1 or SUMO 2 modified with vinyl sulfone (1VS or 2VS). Immunoblotting is used to detect the preferential adducts of a SENP with 1VS or 2VS. With adduct formation between a specific SUMO paralogue, and a specific SENP, information on the preferential activity and specificity can be obtained.

The assays described in this unit provide complementary information for the characterization of SENP activity. The biochemical assays described here can be used to conduct quantitative enzyme kinetic studies to examine inhibitory mechanisms, such as competitive, non-competitive, uncompetitive, and mixed inhibition mechanisms. Comparison of the inhibitory effects of a candidate inhibitor between a substrate containing the structured region of SUMO (i.e. YSE or SUMO-AMC) and a minimal substrate (DUB-Glo) allows the determination of whether the compound directly interferes with the catalytic center of a SENP, providing further mechanistic insights. These assays are also useful to establish whether a candidate inhibitor has specificity towards a specific SENP or a specific SUMO, or whether it differentially inhibits isopeptidase versus endopeptidase activities of a SENP. The biochemical assays described in this unit can also be adapted for high throughput screening to identify inhibitors of SENPs.

BASIC PROTOCOL 1 DETERMINATION OF SENP ACTIVITY BY FRET

In this assay, *Escherichia coli*-expressed substrates that contain premature (full-length) SUMO1 or SUMO2 (S), flanked by yellow fluorescent protein (Y) at one end and enhanced cyan fluorescent protein (E) at the other end will be used. This substrate will be referred to as YSE. The YSE fluorophores will participate in the FRET portion of the assay. When ECFP is excited by 405 nm light, it will emit light of ~ 480 nm, which is within the absorption range of YFP. If SUMO is not processed by SENP, the two fluorophores will be within 10 nm of each other (i.e., a distance where transfer is efficient, since Forster equation

defines that the transfer is a $1/r^6$ relationship, where r is the distance) allowing nonradiative energy transfer from excited ECFP, whose emission maximum is ~480 nm, to excite YFP and generating fluorescence emission in the range of 530 nm. If SUMO is cleaved by SENP1, the two fluorophores will not be close to each other, YFP will not be excited with 405 nm light, and therefore, the only light that will be emitted is from ECFP emission (Fig. 2A). We observe YFP/ECFP fluorescent ratios decrease with addition of SENP, symbolizing a disruption in the energy transfer of ECFP to YFP brought on by increasing distance between the two fluorophores as a result of SENP cleavage activity. (Fig. 2B).

Materials

Make dilutions using 50 mM Tris-HCL, pH 7.5, 150mM NaCl, and 5mM β -mercaptoethanol:

- a. YSE: 10 μ g/ μ L from a 7 mg/ml stock in 50 mM Tris-HCL pH 7.5 buffer
- b. SENP1: 100 ng/ μ L from 4 mg/ml stock in SENP buffer

(See supplementary protocol for YSE and SENP expression and purification)

6.25% DMSO (only needed as vehicle control for studies involving inhibitors, which are typically dissolved in 99.9% pure DMSO)

Milli-Q-purified water

384-well microtiter plates: flat, clear bottom, and black sides (Corning)

Plate reader with FRET capability: excitation wavelength 400–410, emission detection at 480 and 530 nm such as PHERAstar Plus (BMG LABTECH, Germany)

Set up for FRET reactions

1. A SUMO1 (or SUMO2) variation of YSE is placed in the 384 -well microtiter plate in Triplicate as seen in Fig. 2B with the cleaved assay in green and the uncleaved assay in black:

Cleaved:

- 6 μ L 50mM Tris /0.02%NaN₃
- 1 μ L YSE SUMO1 or 2 (1 μ g total)
- 2 μ L 6.25% DMSO (only needed for studies of inhibitors)
- 1 μ L SENP1 (10ng total)

Non-Cleaved Control

- 7 μ L 50mM Tris/0.02%NaN₃
- 1 μ L SUMO1 or 2 (1 μ g total)
- 2 μ L 6.25% DMSO (only needed for studies of inhibitors)

2. Continuous reading at 480 and 530 nm for up to 5 h or until reaction reaches completion, allowing the fluorescence intensity at each wavelength to be monitored with respect to time.
3. Complete cleavage of the substrate results in an E_{480}/E_{530} ratio below 0.9 (Fig. 2C) and uncleaved reactions display ratios of E_{480}/E_{530} greater than 2.0 (Fig. 2C)
4. An alternative to figure 2C would be to represent the data in terms of the percentage of substrates being cleaved. This process requires several steps of data

analysis and has been described in detail in a previous article (Tatham and Hay, 2009).

The FRET assay can be adapted to study kinetics of SENP enzyme activity. Michealis–Menten constants ([http://en.wikipedia.org/wiki/Michaelis–Menten_kinetics](http://en.wikipedia.org/wiki/Michaelis%E2%80%93Menten_kinetics)) can be derived from the initial rates across a YSE concentration range (20 nM–2 μM), as shown previously (Shen et al., 2006c).

The same FRET-based assay can be applied to a substrate containing a YFP-SUMO fusion protein and an ECFP-fusion target protein, such as RanGAP1. Such substrates can be made by a SUMOylation reaction in which the YFP-SUMO fusion and an ECFP-fused target protein are mixed with E1, E2, ATP and possibly an E3 (see the SUMOylation unit 10.6 for details). Although such substrates require more steps to create than the YSE SUMO precursors, they provide information on the isopeptidase activity of a SENP, while the SUMO precursors reflect only the endopeptidase activity of SENP.

ALTERNATE PROTOCOL 1 DETERMINATION OF SENP ACTIVITY USING A GEL-BASED ASSAY

Products obtained using Basic Protocol 1 can also be detected by SDS-PAGE. This assay is straightforward, and product formation is detected directly. In addition, this procedure is also feasible for studies of fluorescent inhibitors that interfere with FRET-based detection.

Materials

YSE
10X reaction buffer
Milli-Q-purified water
Human SENP catalytic domain (320 nM stock)
SENP buffer
3X sample buffer
Heat block or bath at 37°C
0.5 mL microcentrifuge tubes
Ice bucket with ice
4–12% Bis-Tris gel (Life technologies)
NuPAGE MOPS running SDS buffer (Invitrogen)
SDS-PAGE apparatus (Invitrogen)

Set up of the activity assay

1. Equilibrate heating block or water bath to 37 °C.
2. Prepare SENP at a concentration of 32nM with the SENP buffer.
3. Prepare 20 μl reactions in 0.5 mL sterile micro centrifuge tubes on ice.
 - 2 μl of 10X reaction buffer
 - 2 μl of 320 nM SENP1 or SENP2
 - 16 μl 50 μg/ml YSE

Milli-Q-purified H₂O up to 20 μ l

4. Mix samples by tapping the tube and transfer to heated block or bath for 15 min.
5. After 15 min, transfer tubes onto ice and stop reaction with 7 μ l sample buffer.
6. Resolve reaction with a 4–12% gradient Bis-Tris gel with equilibration at 120 V for 10 min and resolving at 168 V for 55 min.
7. The gel can be stained with a Coomassie-based procedure (Instant blue, Expedeon (San Diego, CA) (Fig. 3).

Kinetic information can also be derived from this assay. Substrate and/or inhibitors may be titrated to establish a concentration range in the presence of a constant SENP concentration. The gel bands can be quantified using quantitative densitometry and can be converted to rates by establishing a standard curve with known substrate or product concentrations. This step will depend on the establishment of a proper substrate or product concentration range in which the staining method of choice provides a sufficient dynamic range for conversion from gel band intensity to sample concentration. The data can be fitted to a Michealis–Menten equation to derive the reaction constants (Reverter and Lima, 2006a).

BASIC PROTOCOL 2 *IN VITRO* ASSAY OF SENP ACTIVITIES USING SUMO-7-AMINO-4-METHYLCOUMARIN (SUMO-AMC) AS A SUBSTRATE

SUMO-AMC is a fluorogenic substrate that is useful for studying the enzymatic activities of SENPs. This fluorogenic assay is ideal for kinetic studies as the fluorescence that occurs from the release of the fluorophore AMC by SENP is directly related to product formation. Therefore, data analysis is more straightforward than the ratiometric FRET assay described in Basic Protocol 1. The fluorogenic assay does not require a FRET reader. However, the substrate is not as physiologically relevant as those described in Basic Protocol 1.

Materials

SUMO-AMC (Boston Biochem, 50 μ g unit)
Assay buffer
Milli-Q-purified water
SENP1 or SENP2
SENP buffer
96- or 384-well microtiter plates
Fluorometer (380 nm excitation and 460 nm emission wavelengths)

Set up of the reactions in well plates

1. Varying concentrations of SUMO1 or SUMO2 variations of SUMO-AMC (50 nM – 50 μ M) in assay buffer are placed in the 96- (or 384-) well microtiter plate.
2. SENP (15 pM – 50 nM as needed) is added to reaction mixture.
3. The reaction is kept at 37 °C and the increase in fluorescence is monitored at 460 nm by fluorometry with an excitation wavelength of 380 nm. Details of this assay are provided by the venders of SUMO-AMC.

By varying the concentrations of SUMO-AMC, the release of AMC by SENPs, measured by the fluorescence intensity of AMC, can be directly used for steady state kinetic analysis

(Kolli et al., 2010). The reaction rates can be directly converted from fluorescence intensity in steady-state kinetic analysis.

BASIC PROTOCOL 3 QUANTITATIVE DETERMINATION OF SENP ACTIVITIES BY A BIOLUMINESCENT-BASED ASSAY

In this assay, the substrate is based on a short peptide (Z-RLRGG-amino-luciferin; carboxylbenzyl-Arg-Leu-Arg-Gly-Gly-luciferin). Luciferins, derived from fire flies, are a class of small-molecule substrates that when oxidized by the enzyme luciferase produce oxyluciferin and energy in the form of light. The GG motif on the substrate is recognized by SENPs (Fig. 4A and B). C-terminal cleavage at GG by a SENP will yield free luciferin that can be detected quantitatively by coupling to a luciferase reaction and reading the output with a luminometer. The output, in relative light units (RLU), is directly proportional to the cleavage product (Fig. 4a). An inhibitor of a particular SENP will cause a decreased in the RLU output of this SENP catalyzed cleavage of the peptide-luciferin substrate, and the more potent inhibitor will cause greater reduction of the RLU output.

Materials

DUB-Glo Protease Assay 50mL kit (containing the substrate at 4 mM concentration and allows for 1000 assays at 50 μ l/assay in 96-well plates; Promega)

Opaque 96-well microtiter plate (Costar, Corning Incorporated, Corning NY)

1.5 ml pop-top microcentrifuge tubes (Denville)

Well plate Luminometer (Spectra max M5, Molecular devices, Sunnyvale CA)

Human SENP1 or SENP2 catalytic domain expressed and purified from *E.coli* (50 nM final concentration from a 3.2 μ M stock)

SENP buffer

Softmax Pro software 5.4 (Molecular devices, Sunnyvale CA)

GraphPad Prism 5.04 (GraphPad Software Inc.)

Set up of the reactions in 96-well microtiter plate

1. Following protocols provided with the kit, prepare the luciferase substrate mixture.
2. Set up the luminometer parameters.
 - a. Turn on the Spectra max M5.
 - b. On the Softmax Pro software, click on the “setting” interface. Select luminescence (RLU) with a typical integration of 500 ms, and select “96-well standard opaque” for “assay plate type”.
3. On ice, dilute SENP with SENP buffer to a 100 nM concentration.

Note the limits of detection of each protease suggested by the manufacturer. If inhibitors are being evaluated, add these in varying concentrations in different 1.5 ml pop-top microcentrifuge tubes (typically in the nM- μ M range) to a fixed SENP concentration.
4. Add 50 μ l of the mixture prepared in Step 1 to the microtiter plate wells.
5. Add 50 μ l of the SENP (with and without inhibitor) prepared in Step 3 and mix with a 200 μ l micropipette (see Fig. 4C for well plate set up).

6. Incubate at room temperature or 37 °C for up to 30 min for maximal luciferase output as recommended by the kit and take a reading with the luminometer. However, make sure the production of RLU output is still linearly proportional to time in order to estimate initial rates for enzyme kinetic analysis.
 - a. After plate selection, you can scroll down to the “Wells to read” section and highlight the section of the plate to be analyzed.
 - b. By this time the Spectra max M5 will finish calibration and opens its well plate drawer. Place well plate in this drawer and press the drawer button to close it.
 - c. Exit the parameter software by hitting the “ok” button and click the “read” link on the top left corner of the user interface to collect readings.
7. The values are in relative light units (RLU) that can be plotted against the concentration of inhibitors to get a dose response curve.

By varying concentrations of the substrate and a candidate inhibitor (see Fig. 4D for well plate set up), kinetic parameters of V_{max} , K_i , and K_m can be extracted. The accumulated RLU values versus the log of the concentration can be applied to the GraphPad Prism module for enzyme kinetics.

BASIC PROTOCOL 4 SENP LABELING WITH HAEMAGGLUTININ (HA) TAGGED SUMO VINYL SULFONE (VS)

This protocol can be used to determine the specificity of a candidate inhibitor for different endogenous and recombinant SENPs. HA-SUMO1-VS (1VS) or HA-SUMO2-VS (2VS) are irreversible active site binders of SENPs. Interaction between SENPs and HA-SUMO-VS leads to the formation of a SUMO-VS-SENP adduct. Immunoblotting with an anti-HA antibody allows for the observation of the specificity from the formation of SENP-SUMO-VS adducts (Kolli et al., 2010).

Materials

HeLa cells (ATCC, maintained with Complete DMEM)
 DMEM (Dulbecco's modified Eagle's medium) (Invitrogen, Carlsbad CA)
 Penicillin, streptomycin (Invitrogen, Carlsbad CA)
 Fetal bovine serum (FBS) (Invitrogen, Carlsbad CA)
 Phosphate Buffered Saline (PBS)
 Lysis buffer (at 4 °C)
 3X Sample buffer
 Blocking buffer (Odyssey, Licor)
 PBST
 Anti-SENP1 rabbit monoclonal primary antibodies (Epitomics, Burlingame CA)
 Anti-SENP2 rabbit monoclonal primary antibodies (Epitomics, Burlingame CA)
 Anti-SENP3 rabbit monoclonal primary antibodies (Epitomics, Burlingame CA)
 Anti-SENP5 mouse monoclonal primary antibodies (Epitomics, Burlingame CA)
 Anti-HA mouse monoclonal primary antibodies (Sigma, Missouri)

Anti-mouse secondary antibodies (IgG (H+L) HRP-conjugated) (Promega)
Anti-rabbit secondary antibodies (IgG (H+L) HRP-conjugated) (Promega)
HA-SUMO1-VS (Boston Biochem, Cambridge MA)
HA-SUMO2-VS (Boston Biochem, Cambridge MA)
100 mm Tissue culture plates (Corning, Corning NY)
1.5 ml pop-top microcentrifuge tubes (Denville)
Cell scraper (BD Biosciences)
Ice bucket with ice
Tabletop microcentrifuge at 4 °C (Eppendorf, model No. 5417R)
Sonicator XL with a micro tip probe (Misonix, Farmingdale, NY)
Boiling water bath
10-well 4–12% gradient Bis-Tris gel (Life technologies)
NuPAGE MOPS running SDS buffer (Invitrogen)
SDS-PAGE apparatus (Invitrogen)
Trans blot turbo transfer system apparatus (Bio-rad)
0.2 µM PVDF trans blot turbo membrane (Bio-rad)
Super signal west Pico chemiluminescent substrate (Thermo scientific)
Blue X-ray Film (Bioland)

Set up for SENP labeling

1. Incubate 50 µl of cell lysate, which contains SENPs, or recombinant SENPs (100 nM) with 1–5 µM of 1VS or 2VS for 15 min at 25 °C.
2. Stop reaction with 17 µl of sample buffer and boil for 5 min.
3. Resolve reaction by loading 30 µl in a 10-well 4–12% gradient Bis-Tris gel with equilibration at 120 V for 10 min and resolving at 168 V for 55 min all in the SDS-PAGE apparatus.
4. Detect SUMO-SENP covalent adducts using Western blots.

With the HA antibody as a probe, SUMO-SENP adducts can be observed because their molecular weights are increased. Similarly, an anti-SENP antibody can also detect the adduct of a specific SENP with a particular SUMO1-VS or SUMO2-VS.

SUPPLEMENTARY PROTOCOL

YSE and SENP protein expression and purification

Materials

Luria Broth (Merck): prepared according to the manufacturer's manual and stored in 10–1000 ml volumes at 4 °C after autoclaving.

Kanamycin: dissolved in distilled water to 50 mg/ml, 0.22 µm filter-sterilized and stored in 100–1000 µl aliquots at –20 °C (single use).

Isopropyl- β -D-thiogalactopyranoside (IPTG): prepare 1M stock solution, 0.22 μ m filter-sterilize, and store in -20°C . Thaw just before use, and add to Luria broth to 0.5–1 mM.

Complete protease inhibitor tablets (Roche): dissolve the desired number of tablets in the lysis buffer just before use.

Imidazole: dissolve in distilled water to 1 M and stored at -20°C .

Phosphate-buffered saline (PBS).

Lysis buffer: PBS with 0.3 M NaCl, 5 mM imidazole, 5 mM β -mercaptoethanol and complete protease inhibitors.

Wash buffer: PBS with 0.3 M NaCl, 5 mM imidazole, 1 mM PMSF, 1 mM benzamidine, 5 mM β -mercaptoethanol.

Elution buffer: PBS with 0.3 M NaCl, 250 mM imidazole, 1 mM PMSF, 1 mM benzamidine, 5 mM β -mercaptoethanol.

Dialysis buffer: 50 mM Tris-HCl, pH7.5, 10 mM NaCl, 2 mM β -mercaptoethanol.

Escherichia coli BL21DE3 transformed with the following plasmid DNAs:

YSE fusion proteins and SENP

- pHIS-TEV-30a-YFP-SUMO-1(1–101)-ECFP

- pHIS-TEV-30a-YFP-SUMO-2(1–103)-ECFP

Production of SENP and YSE Fusion Proteins—YSE fusion proteins were expressed from the pHIS-TEV-30a plasmid. Fluorescent proteins were expressed in *Escherichia coli* BL21 (DE3). Purification was by nickel affinity chromatography followed by anion-exchange chromatography. Protein concentrations were determined from the known extinction coefficients of YFP (Venus) and ECFP and the measured optical density of protein samples at 515 nm and 435 nm, respectively.

The catalytic domain of human SENP1 and SENP2 were generated by IPTG expression in BL21 (DE3) *Escherichia coli* (OD₆₀₀ = 0.8; 0.1 mM IPTG) for 4 hr at 30°C . Purification was by nickel affinity chromatography followed by dialysis (50mM Tris-HCl (pH 8.0), 200 mM NaCl and 2mM β -mercaptoethanol). Concentration was determined using the Bradford assay.

REAGENTS AND SOLUTIONS

Complete DMEM

Prepare in DMEM and sterilize through a 0.2- μ m filter. Store at 4°C for 1 year.

DMEM culture media (Invitrogen Carlsbad, CA)

10% (vol/vol) Fetal bovine serum (Invitrogen (Carlsbad, CA)

1% (wt/vol) Non-essential amino acids (Irvine scientific, Santa Ana, CA)

1% (wt/vol) Glutamine (Invitrogen)

1% (wt/vol) Penicillin-streptomycin (Invitrogen)

10X reaction buffer

Stable at room temperature, may become cloudy if there is bacterial contamination. Store for 6 months

200 mM Hepes

500 mM NaCl

30 mM MgCl

Bring to pH 7.5

SENP buffer

Prepare 500 mL. Store at 4°C for up to 1 year.

50 mM Tris-HCl

150 mM NaCl

Fresh DTT should be added to a final concentration of 10 mM to preserve SENP activity during the assay.

Lysis buffer

Store in 500 ml aliquots at 4°C for 1 year.

Fresh DTT added to 1 mM final concentration

50 mM Tris/HCl, pH 7.4

5 mM MgCl₂

250 mM sucrose

2 mM ATP

Phosphate-buffered saline (PBS)

Should be stored at 4 °C to minimize bacterial growth for a month. A cloudy solution indicates bacterial growth, and should be discarded.

13.7 mM NaCl

0.27 mM KCl

0.43 mM Na₂HPO₄

0.14 mM KH₂PO₄

Bring to pH 7.4

Phosphate-buffered saline – Tween-20 (PBST)

This should be stored at 4 °C to avoid bacterial growth.

Phosphate-buffered saline

0.15% (v/v) Tween 20 (Sigma)

3X Sample Buffer

2.4 ml 1 M Tris-Cl, pH 6.8

3 ml 20% SDS 3 ml

Glycerol (100%)
1.6 ml β -mercaptoethanol
0.006 g Bromophenol blue
Store in 1 ml aliquots at -20°C

Assay Buffer

50 mM Tris-HCl, pH 7.8
100 $\mu\text{g/ml}$ ovalbumin
10mM DTT

COMMENTARY

Background Information

SUMOylation is initiated and ended by the activities of SENPs (Fig. 1). SUMO proteins are synthesized as large precursors that are cleaved by SUMO-specific proteases, known as SENPs. After cleavage, SUMO is conjugated to other cellular proteins by forming an isopeptide bond with a Lysine side chain on target proteins through the catalysis of three enzymes, known as E1 (activating enzyme, a heterodimer of SAE1 and SAE2), E2 (conjugation enzyme, Ubc9) and one of ~ten E3 ligases. SUMO modifications are highly dynamic, and can also be removed by SENP proteins. There are at least three members of the SUMO family (SUMO-1, -2, and -3) that can conjugate to other cellular proteins through this chain of enzyme catalysis. The initial step in the SUMOylation pathway is the cleavage of a SUMO precursor by the SENP endopeptidase activity, and the last step is the removal of SUMO modifications by the isopeptidase activity of the SENP proteins. Mammals have six SENP proteins, SENP1, 2, 3, 5, 6 and 7, all of which are essential. Some SENPs have endopeptidase activities (SUMO maturation), but all SENPs possess isopeptidase (reversal of SUMOylation) activities. SENPs share a conserved C-terminal catalytic domain that is a cysteine protease of approximately 250 amino acids, while the N-terminal regions of SENPs are not well conserved and are responsible for substrate specificity and localization (Bailey and O'Hare, 2004). SENP1 and SENP2 have nuclear localization signals and nuclear export signals along with other potential regulatory motifs (Bailey and O'Hare, 2004). Both SENP3 and SENP5 localize to the nucleolus, but SENP5 translocates to mitochondria during mitosis (Gong and Yeh, 2006; Nishida et al., 2000). SENP6 and SENP7 both localize to the nucleoplasm (Cheng et al., 2006; Mukhopadhyay et al., 2006; Shen et al., 2009). In terms of isopeptidase activities, SENP1 and 2 catalyze cleavage of both SUMO1 and SUMO2/3 modifications (Shen et al., 2006b; Xu and Au, 2005). However, based on results obtained using the catalytic domains of both SENPs (Reverter and Lima, 2006b; Shen et al., 2006a), SENP1 appears to have greater specificity towards SUMO1-modified substrates, while SENP2 appears to have greater specificity towards SUMO2/SUMO3-modified substrates. The other four SENPs appear to be isopeptidases specific to SUMO2 and SUMO3 (Gong and Yeh, 2006), while SENP6 and SENP7 specifically process poly-SUMO2/SUMO3 chains (Mukhopadhyay et al., 2006; Shen et al., 2009).

Among the SENPs, the functions of SENP1 appear to be the most relevant to cancer therapy. SENP1 regulates the stability of hypoxia-inducible factor 1 α (HIF1 α) during hypoxia (Cheng et al., 2007; Xu et al., 2010). Hypoxia induces SUMOylation of HIF1 α , which promotes its binding to a ubiquitin ligase, leading to its ubiquitination and degradation. SENP1 plays a key role in angiogenesis of aggressive tumors, and SENP1 deficiency enhances SUMOylation of HIF1 α , resulting in reduced hypoxia-induced transcription of HIF1 α -dependent genes such as vascular endothelial growth factor and glucose transporter

1. SENP1 is also highly expressed in human prostate cancer specimens and regulates androgen receptor (AR) activities (Bawa-Khalfe et al., 2010; Cheng et al., 2006; Kaikkonen et al., 2009). Androgen induces rapid and dynamic conjugation of SUMO-1 to AR. SENP1 over-expression induces transformation of normal prostate gland tissue and facilitates the onset of high-grade prostatic intraepithelial neoplasia. Thus, SENP1 is a potential target for developing new cancer therapies by inhibiting angiogenesis and expression of tumor promoting genes, and the availability of SENP1-specific inhibitors will greatly facilitate the discovery of SENP1's role in other cellular functions and disease processes. Although the other SENPs are also essential, their roles in cellular regulation are less well understood.

Critical parameters and troubleshooting

In all assays, the critical portions are dependent on the concentration, purity and activity of the SENPs. Unless otherwise directed, all proteins used in the assay should be stored in microliter aliquots at -80°C . Before cold storage, the aliquots can be purged with Argon gas to displace oxygen because oxidation causes the loss of SENP activity. Also, DTT at a final concentration of 10 mM must be added with each SENP aliquot thaw, followed by incubation at 25°C for 15 min. It is important to confirm the enzymatic activity of purified recombinant SENP proteins, such as using a DUB-Glo kit.

Anticipated Results

The anticipated results of the FRET experiment (Protocol 1) are shown in Figure 2C. The YFP/ECFP fluorescence ratio before and after SENP addition can be used to extract the reaction rate. Figure 3 shows a representative result of Alternative Protocol 1, which can be obtained without a FRET detection instrument and is not interfered by fluorescent compounds. Typically the YSE construct is about 75kDa and cleavage with SENPs, yields 2 main products. Quantitative information of YSE cleaved can be derived by quantifying the gel bands by densitometry or image processing software like ImageJ. Protocol 2 can be used for real time monitoring of the reaction, and this assay is easily adapted for HTS. A sample result of a luminescence experiment (Protocol 3) is shown in Figure 4. In this experiment, the change in the RLU is dependent on the addition of SENPs to cleave the aminoluciferin substrate. The RLU values can be used in a HTS to screen SENP inhibitors as well as to determine the kinetics of the activity. An example of labeling SENP with SUMO-VS (Protocol 4) is described by Kolli *et al.* (Kolli, 2010). With SUMO-1-VS and SUMO-2-VS, this protocol allows for the determination of substrate specificity of SENPs. One can blot for a SENP upon reaction with SUMO1 or 2-VS, and observation of a molecular weight increase in the SENP band, due to formation of adducts, indicates reactivity of the SENP towards the particular SUMO conjugates. For example, SENP6 preferentially forms adduct with SUMO2-VS suggesting its preferential enzymatic activity for SUMO2/3 conjugates.

Time Considerations

The assays described in Basic Protocols 1–3 are easy to set up, and results can be obtained within half a day. The Coomassie gel assay, which involves SDS-PAGE, gel staining and destaining, can be completed within 24 h. Protocol 4 requires 3–4 days, including growing cells for a day, followed by cell extraction, and SDS-PAGE. Immunoblotting with antibodies and visualization requires another day.

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Figure 1.

Illustration of the role of SENPs in SUMOylation. SUMO is synthesized as a precursor and C-terminally processed by the endopeptidase activity of SENP1 and SENP2. The conjugation to proteins involves three types of enzymes, generally known as E1, E2, E3 (described in unit 10.6). The cleavage of SUMO from its target substrate, termed “deSUMOylation,” is catalyzed by the isopeptidase activity of all SENPs. An isopeptidase cleaves an isopeptide bond, consisting of an amide linkage between the carboxyl terminus of one protein (SUMO) and a lysyl ε-NH₂ group of another (target). Abbreviations: E1 = activating enzyme, E2 = conjugation enzyme (Ubc9), and E3 = ligases.

Figure 2.

FRET-based assay. (A) Schematic illustration of a FRET-based assay to detect SENP activity on YSE cleavage. Y= Yellow fluorescent protein (YFP), S = SUMO1 or 2, E = Enhanced cyan fluorescent protein (ECFP). (B) Schematic of well plate loading with SENP (green) and without SENP (black). (C) Fluorescent ratios of SUMO1 or SUMO2 YSE with or without cleavage by SENP1. YSE (1 μ g each) was mixed with (10 ng) or without SENP1. Fluorescent Emission Ratio (480 nm/530 nm) were compared to the control. SENP1 activity results in a change in the FRET signal that represents a change in the distance between the two fluorophores. Measurements are ratio averages of two trials each done in duplicates.

Figure 3.

Assessment of SENP1 and 2 endopeptidase activity using YFP- SUMO1-ECFP (YSE) as a substrate. SENP1 and SENP2 (32 nM) and YSE substrate (~50 μ g/ml) were incubated at 37 $^{\circ}$ C for 15 min. The reaction products were analyzed by SDS-PAGE and Coomassie-based staining.

Figure 4. Quantitative determination of the activity of SENP1 and SENP 2 using the bioluminescent assay. (A and B) The DUB-Glo substrate at 40 μ M was incubated with or without SENP1 (A) and SENP2 (B) at 50 nM in a 96-well format. The mixture was incubated at 37 $^{\circ}$ C for 30 min, followed by analysis of the relative light unit (RLU) output. (C) Schematic of a 96-well plate for the analysis of inhibitors of SENP. (D) Schematic of a 96-well plate for the analysis of enzyme kinetics.