Available online at www.sciencedirect.com
SCIENCE DIRECT®
Methods 36 (2005) 338–345
METHODS
www.elsevier.com/locate/ymeth

Preparation of enzymatically active recombinant class III protein deacetylases

Brian J.North [1], Bjoern Schwer [1], Nidhi Ahuja [1], Brett Marshall, Eric Verdin *

Gladstone Institute of Virology and Immunology, University of California, San Francisco, CA, USA

Accepted 30 March 2005
Available online 8 August 2005

Abstract

Class III histone deacetylases, or sirtuins, are homologous to the *Saccharomyces cerevisiae* transcriptional regulator SIR2. The class III enzymes are characterized by their dependence on nicotinamide adenine dinucleotide (NAD^+). This cofactor serves as an acetyl-group acceptor in the deacetylation reaction generating *O*-acetyl-ADP-ribose. Enzymatic activity of sirtuin can be measured in vitro using recombinant proteins purified from mammalian cells after overexpression or after purification from *Escherichia coli*. This review discusses protocols for the purification of enzymatically active human sirtuin 1, 2, and 3 and their activities on histone and nonhistone substrates.

Keywords: Histone deacetylase; Sirtuin; Sir2; Sirt; Hdac; Acetylation

1. Introduction

Reversible acetylation of histone and nonhistone proteins is emerging as a major mechanism for regulating protein function. This form of acetylation is distinguished from N-terminal acetylation of proteins by its reversibility and by its location on the ε-amino group of lysine residues within the peptide chain. While histones were the first proteins to be identified as acetylated, probably because of their abundance, acetylation is being identified in a growing number of nonhistone proteins. These proteins are involved in many cellular functions. They include nuclear, cytoplasmic, and even mitochondrial proteins, and one can confidently predict that acetylation is a pervasive modification involved in most biological functions. The level of acetylation of histone and nonhistone proteins is under the control of competing enzymatic activities of protein acetyltransferases and protein deacetylases.

Eighteen distinct human protein deacetylases have been identified. They are grouped into three classes based on their primary homology to three protein deacetylases in *Saccharomyces cerevisiae*. Class I histone deacetlyases (HDAC1, -2, -3, -8, and -11) are homologous to yRPD3, share a compact structure, and are predominantly nuclear proteins expressed in most tissues and cell lines (reviewed in [1]). Class II HDACs are homologous to yHDA1 and are subdivided in two subclasses, IIa (HDAC4, -5, -7, and -9 and its splice variant MITR) and IIb (HDAC6 and HDAC10), based on sequence homology and domain organization (for recent review, see [2]). While class I and II HDACs and their *S. cerevisiae* orthologs, yRPD3 and yHDA1, all share some degree of homology in their catalytic domain, class III HDACs are homologous to ySIR2 and show no homology to class I and II proteins. The class III enzymes are characterized by their dependence on nicotinamide adenine dinucleotide (NAD^+). This cofactor serves as an acetyl-group acceptor in the

* Corresponding author. Fax: +1 415 355 0855.
E-mail address: everdin@gladstone.ucsf.edu (E. Verdin).
[1] These authors contributed equally to the preparation of this review.

1046-2023/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2005.03.004

deacetylation reaction generating 2′-*O*-acetyl-ADP-ribose [3]. The properties of these enzymes have been described in detail in several recent, extensive reviews [2,4–6]. This review focuses on the experimental protocols that are used to prepare and purify the enzymatic activity associated with class III protein deacetylases.

2. Purification of enzymatically active class III HDACs

Class III HDACs have generally proven easier to manipulate than class I and II HDACs. While not all class III HDACs have histone deacetylase activity, proteins with high enzymatic activity can be purified by immunoprecipitation after transient or stable transfection in mammalian cells. Recombinant proteins can also be expressed in *Escherichia coli* in an enzymatically active form.

2.1. Mammalian cell culture systems

This method allows the purification of enzymatically active sirtuins directly from mammalian cells. While no qualitative or quantitative differences have been detected between sirtuins expressed in mammalian cells and *E. coli*, it can be safely assumed that proteins purified from mammalian cells more faithfully represent the sirtuins in their native environment. The presence of associated cofactors and proper post-translational modifications could contribute to differences in the activities of sirtuins from mammalian cells and *E. coli*.

2.1.1. Plasmid

Each of the class III human HDACs has been cloned into the pcDNA3.1 vector as a C-terminal fusion protein with the FLAG epitope.

2.1.2. Procedure

(1) To purify active enzyme, 293T cells are transiently transfected with the expression vector by the calcium phosphate DNA precipitation method. The DNA precipitate is left on the cells for 8–16 h, the medium is changed and the cells are placed back in culture for 24 h.
(2) Cells are washed twice in PBS by centrifugation and resuspended in fresh PBS. After the last centrifugation, the cell pellet is lysed in five packed cell pellet volumes of lysis buffer (50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) in presence of protease inhibitor cocktail (Complete; Roche Molecular Biochemicals, Indianapolis, IN) for 30 min at 4 °C with agitation. After lysis, cellular debris are cleared by centrifugation at 14,000*g* for 10 min at 4 °C and the supernatant is transferred to a precooled tube.
(3) The resulting lysates are immunoprecipitated with an anti-FLAG peptide antiserum. We favor an anti-FLAG antibody already conjugated to agarose beads (anti-FLAG M2 agarose affinity gel; Sigma). Protein concentration is measured in cell lysates with a detergent-compatible protein assay kit (DC protein assay kit, Biorad). Anti-FLAG M2 affinity gel should be added at 10 μl/ml of lysate and incubated for at least 2 h at 4 °C with constant agitation.
(4) After incubation, immune complexes are pelleted with the agarose beads by centrifugation at 6000*g* for 5 min at 4 °C. The supernatant should be carefully removed from beads so as to not disturb the pellet. The immune complexes are washed three times in lysis buffer [50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5% NP-40] for 15 min each at 4 °C with agitation. After each wash, the beads are pelleted and the supernatant is replaced with fresh lysis buffer. The immune complexes are then washed twice for 15 min each in the buffer used for the enzymatic assay (see below). The beads containing the immunoprecipitated material are now ready to use in the assay.

2.1.3. Notes

- Using this protocol, significant HDAC activity is detected with SIRT1, 2, 3, and 5, whereas SIRT2 is the sole sirtuin to show tubulin deacetylation activity (Fig. 1). These experiments suggest that the other SIRT proteins, SIRT4, 6, and 7, might target other substrates for deacetylation or that other cofactors, proteins, or small molecules are needed for their enzymatic activity. These enzymes may also function as mono-ADP ribosyltransferases, as recently demonstrated for another sirtuin [7].
- HEK 293T cells do not attach tightly to the plastic of the culture dish and tend to come off during the washes. We remove the cells from the plate by pipeting and transferring them into a conical tube.
- The lysis buffer does not give complete nuclear lysis in all cell types and should be supplemented with sonication if nuclear proteins are to be efficiently released into the lysate. Determination if nuclear lysis has occurred can be monitored by trypan blue staining to visualize both lysed cells and intact nuclei.

2.2. Expression in E. coli

Class III HDACs can be readily purified as enzymatically active protein after overexpression in *E. coli*. While this approach is generally more efficient at generating a large amount of enzymatic deacetylase activity, it has some limitations including the possible absence of regulatory cofactors and post-translation

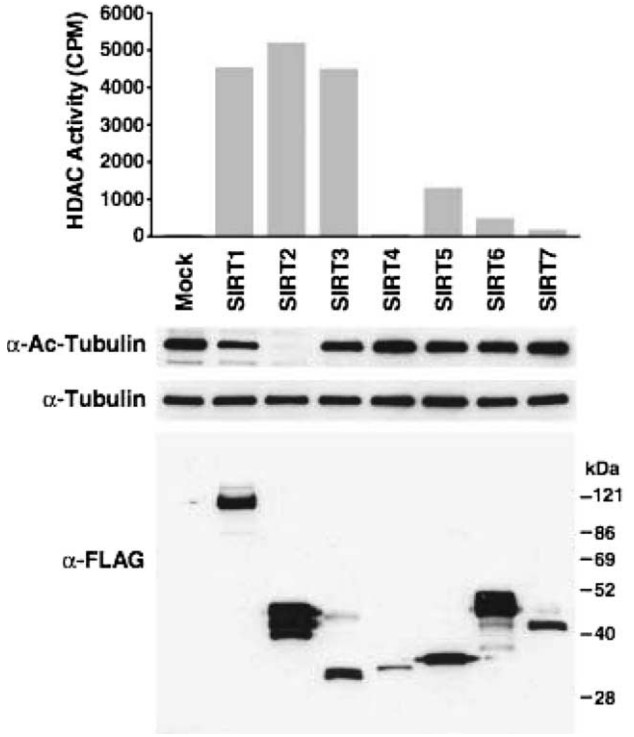

Fig. 1. Measurement of histone and tubulin deacetylase activity associated with human SIRT proteins. 293T cells were transfected with expression vectors for each of the human SIRT proteins. One half of the immunoprecipitated material was subjected to HDAC activity assay with a ^{3}H-acetylated histone H4 peptide. The other half of the immunoprecipitated protein corresponding to each SIRT-FLAG protein was incubated with total cellular lysate. The reaction products were separated by SDS–PAGE and visualized by Western blotting with antisera specific for acetylated tubulin, tubulin, and FLAG. Adapted with modifications from [18].

modifications. We have expressed SIRT1, 2, and 3 and obtained enzymatically active recombinant enzyme for each protein.

2.2.1. GST-SIRT1

2.2.1.1. Plasmid. A DNA fragment encompassing the full SIRT1 open-reading frame was amplified by PCR from a plasmid with primers containing *Bam*HI (forward primer) and *Sal*I (reverse primer) restriction sites. The digested DNA fragment was inserted into pGEX4T-1 cleaved with *Bam*HI and *Sal*I by ligation and confirmed by sequencing. This construct generates an N-terminal GST fusion protein with the SIRT1 protein.

2.2.1.2. Procedure

(1) Bacteria cultured in 250 ml LB–Amp medium to an absorbance of 0.6 are induced for 90 min with 0.5 mM IPTG. Cells are pelleted by centrifugation (7700*g* for 15 min) and supernatant was discarded. The bacterial pellet can be stored at −80 °C or processed immediately.
(2) Cell lysis: the pellet from a 250 ml culture is resuspended in 12.5 ml of ice-cold PBS, supplemented with 1 Complete Mini Protease Inhibitor Cocktail tablet (EDTA free, Roche), 1 mM EGTA, 1 mM PMSF, and 2 mM benzamidine. This is followed by addition of 1 μg/ml of lysozyme and 10 mM DTT.
(3) Cells are lysed by sonication on ice (5 × 15 s bursts at 60% power) and PMSF (1 mM) and benzamide (2 mM) are added again.
(4) This lysate is centrifuged at 35,000*g* for 10 min to remove insoluble material.
(5) We use 125 μL glutathione–Sepharose 4B (Amersham Biosciences) per 12.5 ml of sonicate for affinity purification. This is done by pipeting 167 μL of 75% bead slurry to a tube, washing with 1× PBS to remove the 20% ethanol storage solution, spinning, and decanting. Beads (125 μL) are resuspended in 12.5 ml of sonicate and incubated with gentle agitation at 4 °C for 1 h.
(6) This mixture is centrifuged at 500*g* for 5 min, the supernatant is decanted, and the matrix is resuspended in 1.25 ml PBS, 1 mM EGTA. This mixture is transferred to a column and washed with 7 ml PBS, 1 mM DTT to remove unbound proteins.
(7) The fusion protein is eluted by adding 125 μL aliquots (seven times) of elution buffer (10 mM glutathione in 50 mM Tris–HCl, pH 8.0, 1 mM DTT).
(8) Individual fractions are analyzed by SDS–PAGE, and fractions containing the highest amounts of recombinant protein are pooled, aliquoted, snap frozen, and stored at −80 °C.

2.2.2. 6× His-SIRT2

2.2.2.1. Plasmid. Full-length human SIRT2 cDNA was cloned into pHEX, a modified version of pGEX-2T (Pharmacia) in which the GST-encoding sequence was replaced with a hexahistidine-encoding sequence (6× His). The resulting protein is an N-tagged 6× His-SIRT2 fusion protein (see [8] for details). This vector is transformed in DH5α F'IQ bacteria (Gibco) for expression.

2.2.2.2. Procedure

(1) One liter of transformed bacterial culture is grown in LB–Amp medium to an absorbance of 0.6 (A_{600}), induced with 0.1 mM IPTG at 37 °C for 2 h, and pelleted.
(2) The pellet is resuspended in buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM imidazole-2 ml/g wet weight) and incubated on ice for 30 min in the presence of 1 mg/ml lysozyme. This mixture is sonicated on ice (four 10–15-s

bursts at 40–60% power) and centrifuged at 4 °C at 14,000*g* for 30 min.

(3) Supernatant (cleared lysate) is bound to Ni–NTA resin (1 ml of 50% Ni–NTA slurry for 4 ml of cleared lysate, batch method, Qiagen) on a rotary mixer at 4 °C for 60 min.
(4) The batch mixture is passed through a commercial column (Polyprep, BioRad) and the flow-through is saved. The resin bed is washed twice with 4 ml buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 20 mM imidazole).
(5) Bound proteins are eluted four times with 0.5 ml of elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 250 mM imidazole). SDS–PAGE analysis revealed that the second eluting fraction contains a majority of recombinant protein along with contaminating proteins.
(6) Ion-exchange chromatography is used to further purify the recombinant protein. The peak eluted fraction is shifted to ion-exchange buffer (50 mM Tris–HCl, pH 8.0, 0.2 mM DTT, 10% glycerol, and 50 mM NaCl) with a HiPrep26/10 desalting column (Pharmacia, 2.0 ml/min flow rate, 1.0-ml fractions).
(7) The peak fractions (as determined by UV monitoring or SDS–PAGE) are pooled (five to seven 1-ml fractions) and loaded on a Sepharose Q column and washed with buffer (50 mM NaH_2PO_4, pH 8.0, 30 mM NaCl, and 10 mM imidazole).
(8) Recombinant SIRT2 is eluted with a linear gradient of 50–1000 mM NaCl in buffer (50 mM Tris–HCl, pH 8.0, 0.2 mM DTT, and 10% glycerol). Under these conditions, SIRT2 elutes at ~265 mM NaCl. Peak fractions are pooled and concentrated using centrifuge concentrating spin columns (Centricon, MWCO 30 kDa). Recombinant protein is aliquoted and stored at −20 °C in storage buffer (50 mM Tris–HCl, pH 8.0, 265 mM NaCl, 0.2 mM DTT, and 10% glycerol).

2.2.2.3. Notes

- This protocol yields a recombinant SIRT2 protein with high enzymatic activity and >90% purity as determined by SDS–PAGE (not shown).
- Using a linear gradient of imidazole (20–500 mM) for elution of SIRT2 bound to the Ni–NTA resin has yielded a significantly purer recombinant preparation (John Denu, University of Wisconsin, Madison, Wisconsin, personal communication).
- Examples of enzymatic reactions performed with SIRT1, 2, and 3 proteins expressed in *E. coli* are shown in Fig. 2.

2.2.3. GST-tagged SIRT2

2.2.3.1. Plasmid. SIRT2 has also been expressed using a GST fusion protein (N-terminal) using the same vector

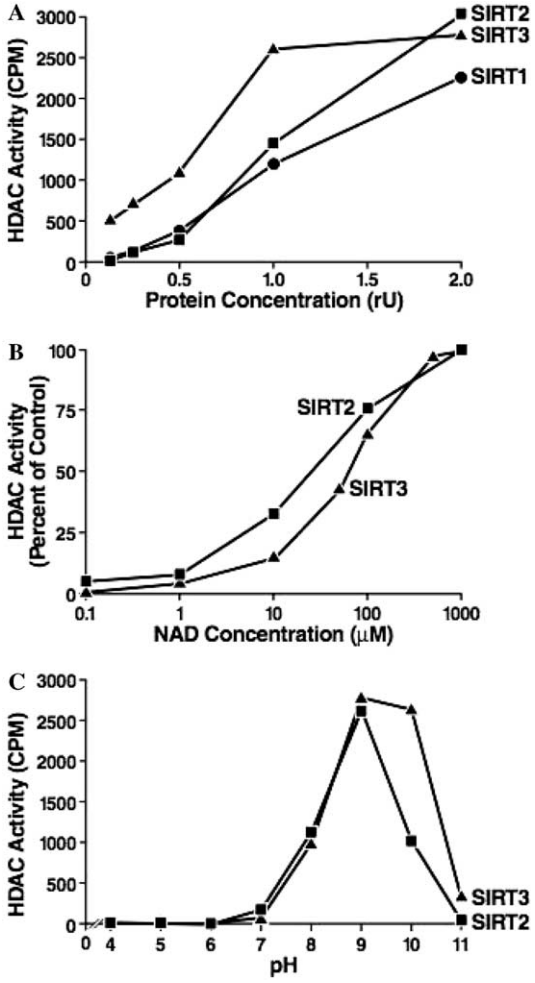

Fig. 2. Measurement of HDAC activity associated with SIRT1, 2, and 3 expressed in *E. coli*. (A) The enzymatic activity of recombinant GST-SIRT1, 6× His-SIRT2 and 6× His-SIRT3 on a ^{3}H-acetylated histone H4 peptide was measured in the presence 1 mM NAD^+ at different concentrations of recombinant protein (rU = relative units). (B) The enzymatic activity of recombinant 6× His-SIRT2 and 6× His-SIRT3 on a ^{3}H-acetylated histone H4 peptide was measured in the presence of increasing concentrations of NAD^+ (0, 1, 10, 100, 1000 μM). (C) The activity of 6× His-SIRT2 and 6× His-SIRT3 on a ^{3}H-acetylated histone H4 peptide was measured in a range of pH [4–11] in the presence of 1 mM NAD^+.

(pGEX-4T3, Amersham) as described above for SIRT1. The construct is transformed in *E. coli* BL21 (DE3) cells.

2.2.3.2. Procedure

- A single colony of transformed cells is inoculated into 10 ml LB medium and allowed to incubate overnight at 37 °C with vigorous shaking. The following morning, the starter culture is used to inoculate 1 L LB medium and the culture is allowed to grow at 37 °C with vigorous shaking until the absorbance at 600 nm reaches 0.6. The culture is then induced with 1 mM IPTG and allowed to grow for 4 h at 30 °C with shaking.

- The cells are harvested by centrifugation. The cell pellet is resuspended in 18 ml PBS, 1% Triton X-100, and the cells are sonicated on ice (five 10 s bursts at 40% power).
- Cell debris is removed by centrifugation and 300 μl glutathione– Sepharose 4B resin (equilibrated with PBS containing Triton X-100) is added to the cleared lysate. The resin is allowed to incubate with the lysate at 4 °C for 30 min. Beads are washed twice with 10 ml PBS–1% Triton X-100 followed by two washes with PBS. The beads may be resuspended in PBS with 10% glycerol and stored at −20 °C at this step or eluted as described below.
- SIRT2 is eluted from the column with 5–10 column volumes of the elution buffer (10 mM reduced glutathione, pH 8.0). If removal of the GST tag is necessary, fusion proteins may be cleaved with thrombin either while bound to glutathione–Sepharose or in solution after elution. To remove a GST tag after elution of protein from the column, 10 U of thrombin is added to 1 mg of eluted protein, and incubation is allowed at room temperature for 2–16 h. To cleave GST-SIRT2 bound to the column, 80 U of thrombin is incubated with each ml of glutathione–Sepharose bed volume, and incubation is allowed at room temperature for 2–16 h. After incubation, three bed volumes of PBS are added to the column to elute SIRT2. Thrombin will co-elute with SIRT2 and maybe removed by affinity chromatography using a benzamidine–Sepharose column.

2.2.3.3. Notes

- High-level expression of SIRT2 has been achieved with this procedure. The GST-SIRT2 fusion protein purified by this procedure is >90% pure as determined by SDS–PAGE (not shown).
- We have observed that the enzymatic activity of GST-SIRT2 is lower compared to the 6× His-tagged SIRT2 (data not shown). The larger size of the GST-tag may interfere with the enzymatic activity of SIRT2.

2.2.4. 6× His-SIRT3

We previously reported that SIRT3 is proteolytically processed after its import into the mitochondrial matrix [9]. N-terminal sequencing of SIRT3 protein immunoprecipitated from human cells established that the proteolytic processing of SIRT3 occurs between amino acids Ser101 and Ile102 (B. Schwer and E. Verdin, unpublished observations). Based on these results and the finding that in vitro-synthesized SIRT3 can be activated by proteolytic processing [9], we constructed an expression vector that expresses a truncated SIRT3 protein corresponding to the mature SIRT3 protein detected in mammalian cells.

2.2.4.1. Plasmid. The region comprising the coding sequence for SIRT3 amino acids 101–399 was cloned into the bacterial expression vector pTrcHis (Invitrogen) to yield an N-terminal 6× His-tagged SIRT3, pTrcHis-$SIRT3_{101–399}$. In the following section the recombinant 6× His-tagged SIRT3 protein expressed from pTrcHis-$SIRT3_{101–399}$ will be referred to as rSIRT3m, indicating that it corresponds to the enzymatically active mature SIRT3 protein.

2.2.4.2. Procedure

- To express rSIRT3m, the plasmid pTrcHis-$SIRT3_{101–399}$ is transformed into DH5α cells. The rSIRT3m protein is purified under native conditions by Ni–NTA affinity chromatography (Qiagen). One liter of transformed bacterial culture is grown in LB–Amp to an absorbance of 0.6 (A_{600}), induced with 1 mM IPTG at 37 °C for 2 h, and pelleted.
- The pellet is resuspended in buffer (50 mM NaH_2PO_4, pH 8.0, 300 NaCl, 10 mM imidazole-2 ml/g wet weight) and incubated on ice for 30 min in the presence of 1 mg/ml lysozyme. This mixture is sonicated on ice (four 10–15-s bursts at 40–60% power) and centrifuged at 4 °C at 14,000*g* for 30 min.
- Supernatant (cleared lysate) is bound to Ni–NTA resin (1 ml of 50% Ni–NTA slurry for 4 ml of cleared lysate, batch method, Qiagen) on a rotary mixer at 4 °C for 60 min.
- The batch mixture is passed through a commercial column (Polyprep, BioRad) and the flow-through is saved. The resin bed is washed twice with 4 ml buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 20 mM imidazole).
- Bound proteins are eluted four times with 0.5 ml of elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 250 mM imidazole). SDS–PAGE analysis revealed that the second eluting fraction contains a majority of recombinant protein along with contaminating proteins.
- Ion-exchange chromatography is used to further purify the recombinant protein. The rSIRT3m protein is concentrated and its buffer changed to a new buffer (50 mM Tris–HCl, pH 8.0, 0.2 mM DTT, 10% glycerol, and 50 mM NaCl) using a spin concentration device (Vivaspin 6, MWCO 5000, Vivascience) according to the manufacturer's instructions.
- After buffer exchange, protein is loaded on a Sepharose Q column and washed with five column volumes of ion-exchange buffer (50 mM Tris–HCl, pH 8.0, 0.2 mM DTT, 10% glycerol, and 50 mM NaCl).
- Recombinant SIRT3m is eluted with a linear gradient of 50–1000 mM NaCl in buffer (50 mM Tris–HCl, pH 8.0, 0.2 mM DTT, and 10% glycerol).

Under these conditions, SIRT3m elutes at ~370 mM NaCl.

- Peak fractions are pooled and concentrated using Vivaspin concentrators (Vivascience, MWCO 5000). Recombinant protein is aliquoted and stored at −20 °C in storage buffer (50 mM Tris–HCl, pH 8.0, 265 mM NaCl, 0.2 mM DTT, and 10% glycerol).

2.2.4.3. Notes

- This protocol yields an enzymatically active deacetylase with similar NAD^{2+} dependency and pH sensitivity as recombinant SIRT2 (Fig. 2). As expected for a class III protein deacetylase, rSIRT3m is inhibited by nicotinamide (data not shown).

2.3. In vitro translated protein

As discussed above, SIRT3, the closest relative of SIRT2, localizes to mitochondria [9,10]. After import into the mitochondrial matrix, SIRT3 is proteolytically processed and becomes activated as an NAD^{2+}-dependent protein deacetylase [9]. The activation process of SIRT3 occurring within the mitochondrial matrix can be reconstituted in vitro by incubation of in vitro synthesized full-length SIRT3 with recombinant yeast mitochondrial processing peptidase (MPP). The following section describes this protocol in detail.

2.3.1. Procedure

- For in vitro synthesis of SIRT3 protein, the TNT Coupled Reticulocyte Lysate System (Promega) is used. All steps are carried out according to the manufacturer's instructions: 25 µl of TNT rabbit reticulocyte lysate is mixed with 2 µl of TNT reaction buffer, 0.5 µl amino acid mixture minus leucine, 0.5 µl amino acid mixture minus methionine, 1 µl RNasin ribonuclease inhibitor, 1 µg plasmid DNA (pcDNA3.1+ expression vector containing the SIRT3 cDNA downstream of T7 promoter), and 1 µl TNT T7 RNA polymerase. The reaction is brought to a total reaction volume of 50 µl with nuclease-free water. The reaction is incubated at 30 °C for 60–90 min.
- The in vitro-translated full-length SIRT3 protein is incubated with recombinant yeast MPP in cleavage buffer (1 mM dithiothreitol, 1 mM $MnCl_2$, and 10 mM Hepes–KOH, pH 7.4) for 45 min at 27 °C. A detailed protocol for the preparation and purification of bacterially expressed recombinant yeast MPP has been reported [11]. If necessary, the proteolytically processed and enzymatically active SIRT3 is further purified by standard chromatographic techniques or by immunoprecipitation.

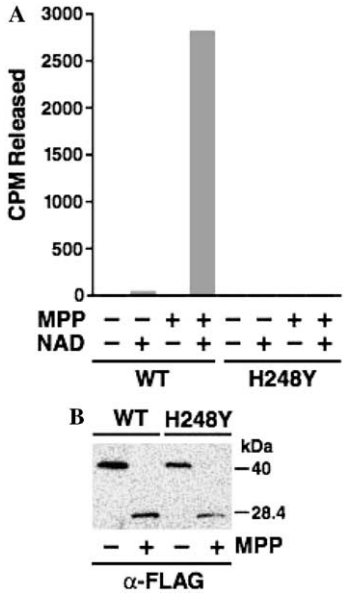

Fig. 3. Proteolytic processing of SIRT3 by MPP leads to its enzymatic activation. (A) SIRT3-Flag wild-type (WT) or the catalytically inactive mutant SIRT3-H248Y-Flag (H248Y) was synthesized in vitro and subsequently processed by recombinant yeast MPP. Flag-tagged proteins were immunoprecipitated with anti-Flag M2-agarose beads and analyzed for deacetylase activity with an acetylated H4 histone peptide in the presence or absence of NAD^+ (1 mM). (B) Western blot analysis of immmunoprecipitates used in the deacetylase assay shown in (A) indicates that both proteins are processed in the presence of MPP. Adapted with modifications from [9].

2.3.2. Notes

- Unprogrammed reticulocyte lysates contain a high level of TSA-sensitive histone deacetylase activity. Further purification of the translated SIRT3 to measure its enzymatic activity is recommended. Alternatively, it should be feasible to conduct the enzymatic assay in the presence of TSA (1 µM), a specific inhibitor of class I and II HDACs.
- In vitro translated SIRT3 shows NAD^+-dependent deacetylase activity after processing by MPP (Fig. 3). A mutant SIRT3 carrying a substitution H248Y can be used to prove that the enzymatic activity detected is originating from SIRT3 and not from a contaminant from the reticulocyte lysate or the MPP preparation. This mutant is processed by MPP as efficiently as wild-type SIRT3, but remains catalytically inactive after MPP processing (Fig. 3).

3. Enzymatic substrates and reaction

A large variety of substrates and assay systems have been used to detect the deacetylase activity of class I, II, and III enzymes. A recent method uses purified histone acetyltransferase to acetylate isolated nucleosomes

in vitro with high specific activity [12]. This method offers the significant advantage of a physiologically relevant substrate (chromatin) and should prove useful for examining the activity of HDACs on their natural substrates. A variety of novel nonradioactive assays, based on fluorescent oligopeptides [13,14] or on an acetylated lysine derivative [15,16], have been developed recently. Since an extensive description of these substrates is beyond the scope of this review, we describe a sensitive assay using a peptide corresponding to the N-terminal extremity of histone H4 acetylated in vitro.

3.1. Histone substrate procedure

- Immunoprecipitated material and recombinant SIRT2 are resuspended in 100 μl of SIRT2 deacetylase buffer (50 mM Tris–HCl, pH 9.0, 4 mM $MgCl_2$, and 0.2 mM DTT) and 10,000 cpm of [^{3}H]histone H4 peptide substrate. This substrate is prepared by in vitro acetylation of a histone H4 N-terminal peptide (amino acids 1–25) with radiolabeled acetylCoA and recombinant PCAF.
- Enzymatic reactions are started by adding NAD^+ are incubated for 2 h at room temperature with agitation and are stopped by adding 25 μl of 100 mM HCl and 160 mM acetic acid. Released acetate is extracted in 500 μl ethyl acetate by vortexing for 15 s. After centrifugation at 14,000*g* for 5 min, 400 μl of the ethyl acetate fraction is mixed with 5 ml of scintillation fluid and counted.

3.2. Notes

- The product of the enzymatic reaction catalyzed by class III HDAC is not acetate but acetylADP ribose [17]. It is therefore not entirely clear why the acetate release assay described above functions with these enzymes. Free acetate may be released from acetylADP ribose under these acidic experimental conditions.

3.3. Nonhistone protein enzymatic assays for class III HDACs

Sirtuins target nonhistone proteins for deacetylation [4]. Furthermore, the localization of SIRT3 in the mitochondrial matrix also implies that this enzyme targets a nonhistone substrate in that organelle. Interestingly, SIRT4, 5, 6, and 7 have little detectable activity on a histone peptide substrate (see Fig. 1), an observation that could indicate that they either target nonhistone proteins for deacetylation or function primarily as ADP-ribosyltransferases, as recently documented for a trypanosome sirtuin [7]. Given the documented ability of sirtuins to deacetylate nonhistone proteins, we describe the harvesting of known sirtuin targets in an acetylated state for use in in vitro deacetylation reactions below.

Acetylated proteins can be detected either by pulse labeling of living cells with radiolabeled acetate or by Western blotting with antibodies specific for the acetylated state. The latter procedure can be performed either with a nonspecific antiacetylated lysine antiserum or with an antiserum specific for the acetylated form of a given protein. Such antibodies have been developed for a number of acetylated proteins and represent the most convenient reagents to study acetylation. However, antiacetylated lysine antisera represent an excellent alternative, in particular if the protein of interest is abundant or if it can be immunoprecipitated using another specific antiserum. The relative abundance of the protein in conjunction with the detection method available will dictate whether prior immunoprecipitation of the target is necessary. For instance, tubulin is a highly abundant acetylated protein that is targeted for deacetylation by SIRT2. An antiserum specific for anti-acetylated tubulin is available and allows for the direct detection of acetylated tubulin without any immunoprecipitation step. However, less abundant proteins may require immunoprecipitation of either the endogenous protein or of epitope-tagged proteins to enhance the detection of the acetylated form. Tubulin is discussed below as an example of a highly abundant protein for which a specific acetylated tubulin antibody is available. The sirtuin to be used in the assay can be either purified recombinant or immunoprecipitated from transfected cell culture systems as described above.

3.3.1. Procedure

- Cells are lysed in buffer (50 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5% NP-40, and 150 mM NaCl) and the final protein concentration is adjusted to 10 mg/ml.
- The sirtuin immunoprecipitated material or recombinant protein is mixed with 50 μg of cell lysate (containing the acetylated tubulin substrate) and 1 mM NAD^+ in a final volume of 100 μl (50 mM Tris–HCl, pH 9.0, 4 mM $MgCl_2$, and 0.2 mM DTT). NAD^+ is added last to initiate the reaction. Reactions are carried out at room temperature for 2 h on a vortex shaker and stopped by addition of 20 μl 6× SDS–PAGE buffer. If an immunoprecipitated sirtuin is used, the beads are pelleted by centrifugation at 14,000*g* for 10 min, 10–20 μl of the supernatant is separated on two SDS–PAGE gels that are transferred to nitrocellulose membranes. One membrane is probed with the anti-acetylated tubulin antibody (Clone 6-11B-1, Sigma) to detect acetylated tubulin, and the other membrane is probed with anti-tubulin antibody to control for equal loading of tubulin across reactions. (Fig. 1).

3.3.2. Notes

- If the level of tubulin acetylation is low (this depends on cell type), pretreatment of cells for 2 h with 400 nM trichostatin A (TSA) will cause significant tubulin hyperacetylation due to inhibition of HDAC6, which also deacetylates tubulin. However, TSA will not affect SIRT enzymatic activity, since sirtuins are insensitive to TSA.
- This protocol can be adapted to the study of other acetylated proteins. For less abundant targets, or when an anti-acetyl-lysine antibody is used to detect the acetylated protein, it is recommended that the target protein be immunoprecipitated before the enzymatic assay.
- There are a number of commercially available anti-acetylated lysine antibodies. We recommend testing a new potential acetylated protein with several antibodies since each antibody recognizes different acetylated proteins with different efficiencies. If the level of acetylation is low, treatment of cells with nicotinamide (20 mM) or TSA (400 nM), or both, for 16 h before harvest and immunoprecipitation is likely to increase the amount of acetylated protein in the lysate significantly.

Acknowledgments

We thank Maki Hirao and Julian Simon (Fred Hutchinson Cancer Center) for providing us with the GST-SIRT1 construct. We thank Sarah Sande for help with the preparation of the manuscript, John Carroll and Jack Hull for graphics, and Stephen Ordway and Gary Howard for editorial assistance. We apologize to colleagues whose work could not be cited due to space constraints. Our research is supported by the NIH, The J. David Gladstone Institutes, and The Sandler Foundation. Eric Verdin is a Senior Scholar of the Ellison Medical Foundation.

References

[1] W. Fischle, V. Kiermer, F. Dequiedt, E. Verdin, Biochem. Cell Biol. 79 (2001) 337–348.
[2] E. Verdin, F. Dequiedt, H.G. Kasler, Trends Genet. 19 (2003) 286–293.
[3] M.T. Borra, F.J. O'Neill, M.D. Jackson, B. Marshall, E. Verdin, K.R. Foltz, J.M. Denu, J. Biol. Chem. 277 (2002) 12632–12641.
[4] B.J. North, E. Verdin, Genome Biol. 5 (2004) 224.
[5] G. Blander, L. Guaremte, Annu. Rev. Biochem. 73 (2004) 417–435.
[6] P. Marks, R.A. Rifkind, V.M. Richon, R. Breslow, T. Miller, W.K. Kelly, Nat. Rev. Cancer 1 (2001) 194–202.
[7] J.A. Garcia-Salcedo, P. Gijon, D.P. Nolan, P. Tebabi, E. Pays, EMBO J. 22 (2003) 5851–5862.
[8] R.A. Frye, Biochem. Biophys. Res. Commun. 260 (1999) 273–279.
[9] B. Schwer, B.J. North, R.A. Frye, M. Ott, E. Verdin, J. Cell Biol. 158 (2002) 647–657.
[10] P. Onyango, I. Celic, J.M. McCaffery, J.D. Boeke, A.P. Feinberg, Proc. Natl. Acad. Sci. USA 99 (2002) 13653–13658.
[11] P. Luciano, S. Geoffroy, A. Brandt, J.F. Hernandez, V. Geli, J. Mol. Biol. 272 (1997) 213–225.
[12] P.A. Wade, P.L. Jones, D. Vermaak, A.P. Wolffe, Methods Enzymol. 304 (1999) 715–725.
[13] K. Hoffmann, R.M. Soll, A.G. Beck-Sickinger, M. Jung, Bioconjug. Chem. 12 (2001) 51–55.
[14] D. Wegener, F. Wirsching, D. Riester, A. Schwienhorst, Chem. Biol. 10 (2003) 61–68.
[15] B. Heltweg, M. Jung, Arch. Pharm. (Weinheim) 335 (2002) 296–300.
[16] B. Heltweg, M. Jung, Anal. Biochem. 302 (2002) 175–183.
[17] K.G. Tanner, J. Landry, R. Sternglanz, J.M. Denu, Proc. Natl. Acad. Sci. USA 97 (2000) 14178–14182.
[18] B.J. North, B.L. Marshall, M.T. Borra, J.M. Denu, E. Verdin, Mol. Cell 11 (2003) 437–444.