Published in final edited form as:

Methods. 2014 January 15; 65(2): 201–206. doi:10.1016/j.ymeth.2013.09.012.

Analysis of PTP1B Sumoylation

Sayanti Saha and Jonathan Chernoff*

Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA

Keywords

signal transduction; protein tyrosine phosphatases; phosphorylation; posttranslational modification; sumoylation

Introduction

Protein tyrosine phosphorylation is a well-known post-translational modification that modulates key biological processes such as cell proliferation, cell cycle, cell migration, immune response and apoptosis. Its dynamic and reversible nature is regulated by the opposing forces of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) [1]. Dysregulation of PTPs are associated with several human diseases including diabetes, cancer and inflammation [2].

PTP1B has attracted particular attention, as it was one of the first PTPs to be identified and extensively characterized, and because it plays a vital role in insulin signaling and in transformation. PTP1B is a 435-acid (aa) protein, consisting of an N-terminal catalytic phosphatase domain (residues 1–300) followed by a regulatory region of about 80–100 residues and a membrane localization domain (residues 400–435) that localizes the enzyme to the cytoplasmic face of the endoplasmic reticulum (ER) (Figure 1) [3]. PTP1B is subjected to spatio-temporal regulation by its localization to ER and several post-translational modifications such as oxidation, phosphorylation, sumoylation, and proteolysis. Regulation of PTP1B by these post-translational modifications has been well described elsewhere [4]. Here, we review various methodologies used to study PTP1B sumoylation.

Sumoylation is a post-translational modification characterized by covalent attachment of small ubiquitin-like modifier (SUMO) peptide to a target protein. The SUMO peptide consists of approximately 100 aa, resulting in a molecular weight of ~11 kDa. The SUMO protein sequence is highly conserved across eukaryotic organisms, is ubiquitously expressed in all tissues and has been implicated in wide array of cellular processes, such as transcription, nuclear transport, DNA repair, and cell cycle [5]. Moreover, sumoylation can alter localization, activity, stability of the target protein and its interaction with other proteins. SUMO modification is a reversible process that involves the covalent attachment of SUMO to the lysine residue in the consensus sequence Ψ KXE/D, where Ψ is a hydrophobic residue in a target protein [6]. However, it should be noted that alternative

© 2013 Published by Elsevier Inc.

*To whom correspondence should be addressed: Jonathan Chernoff, Cancer Biology Program, Fox Chase Cancer Center, 333 Cottman Ave, Philadelphia, PA 19111, USA, Tel.: (215) 728 5319; Fax: (215) 728 3616; Jonathan.Chernoff@fccc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

SUMO attachment sequences have also been described [7]. The SUMO peptide is initially translated as a precursor, with a short sequence extending past a conserved GG motif at the C-terminus and proteolytic cleavage of this sequence by SUMO-specific iso-peptidases (sentrin-specific proteases; SENPs) converts SUMO to its mature form. Thereafter, mature SUMO undergoes a three step process: (1) SUMO is activated by the SUMO activating enzyme E1 in an ATP-dependent reaction, (2) active SUMO is transferred to the catalytic Cys residue of the SUMO conjugating E2 enzyme Ubc9, and (3) SUMO is conjugated to a Lys residue in the substrate by its C-terminal Gly residue with the help of an E3 ligase (PIAS) (Figure 2). Sumoylation is a reversible process counterbalanced by desumoylation, a process mediated by specific cysteine (Cys) proteases such as Ubiquitin-like protein-specific protease (Ulp) or sentrin-specific proteases (SENPs). These desumoylating enzymes deconjugate SUMO from the substrate and replenish a pool of free SUMO that can be used for additional rounds of sumoylation [5].

1. PTP1B Sumoylation

We identified PIAS1, SUMO E3 ligase as PTP1B interacting protein by a yeast two-hybrid assay [8]. This interaction was confirmed by co-immunoprecipitation studies, using wild-type PTP1B mouse embryonic fibroblasts (MEFs) or *Ptp1b*-null MEFs. We then showed that PTP1B is in fact sumoylated in cells, mainly at the inner nuclear envelope, and that this process is regulated by insulin as well as by the cell cycle [8, 9]. Importantly, sumoylation of PTP1B decreases the catalytic activity of this phosphatase, demonstrating the potential importance of this posttranslational modification.

2. PTP1B Sumoylation Assays

There are three main methods to produce, detect, and analyze sumoylated proteins. These are: a) co-expression of a suspected target of sumoylation (*e.g.*, PTP1B) with epitope-tagged SUMO in mammalian cells, followed by immunoprecipitation and immunoblot (Figure 3B); b) expression of the target protein and sumoylation enzymes in *E. coli*; and c) *in vitro* sumoylation using recombinant proteins. The first method is simple to set up and is most useful in rapidly determining if a target protein can be sumoylated, whereas the second and third methods are most useful in producing larger amounts of sumoylated protein that may be needed for functional or structural studies. All three systems can be used to determine the site(s) of sumoylation.

2.1 Mammalian cell culture systems

In our system, we find it most useful to use immortalized, *Ptp1b*-null MEFs [10], as these lack endogenous PTP1B and thus signals are easy to discern, especially if site-directed mutants are to be studied.

2.1.1 Plasmids—For these assays, full-length human PTP1B cDNA has been cloned into the pCMV6-HA vector [11], while T7-tagged SUMO-1 or SUMO-1-QT (a form of SUMO-1 that cannot be conjugated to proteins [12] has been cloned into the pmRFP vector [8, 12]. Both these vectors employ the CMV promoter to drive expression and can be used to achieve high levels of expression in many mammalian cell types, including MEFs.

2.1.2 Procedure

1. *Ptp1b*-null MEFs are maintained at 37°C in 10-cm dishes in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin and 200 mg/ml hygromycin B. Transfection is

carried out using Lipofectamine-2000 (Gibco BRL) according to the manufacturer's recommendations.

2. 48 h post-transfection, cells are washed x2 with ice-cold PBS, then lysed with in ice-cold lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl₂, 1% Nonidet P-40, 0.75% sodium deoxycholate, 1 mM dithiothreitol) supplemented with 1:100 diluted protease inhibitor cocktail and phosphatase inhibitors I and II (Sigma, St Louis, MO) and 20 mM N-ethylmaleimide (NEM) (Sigma). After lysis, cellular debris is cleared by centrifugation at 14,000g for 10 min at 4°C and the supernatant is transferred to a pre-cooled Eppendorf tube.
3. Protein concentrations are determined by Bradford assay, and 450 µg whole cell lysate are incubated with 10 µl anti-HA polyclonal antibodies or 3 µl anti-T7 monoclonal antibodies for 2 h, then collected on Protein A/G beads. The immune complexes are washed three times in lysis buffer for 10 min each at 4°C with agitation. Immunocomplexes are eluted in SDS sample buffer, resolved by SDS-PAGE, transferred onto a PVDF membrane and visualized using secondary antibodies conjugated to alkaline phosphatases.

2.1.3 Notes

- The inclusion of NEM is critical to prevent loss of sumoylation due to cysteine-based SENP isopeptidases that are abundant in cellular lysates.
- The order of the immunoprecipitation can be reversed: immunoprecipitate T7-SUMO-1, then immunoblot for HA-PTP1B.
- The appearance of a ladder of immunoreactive bands above the immunoprecipitated protein suggests that either that multiple sites are sumoylated or that poly-sumo chains are formed. Each sumoylation increases the apparent molecular mass by about 15,000 Da.
- The assay can also be carried out in other cell types such as NIH-3T3, HEK-293, COS-1, or HeLa cells.
- As an alternative approach, especially if large amounts of sumoylated proteins are desired, Jakobs *et al.* have described a clever system to augment production of sumoylated protein in eukaryotic cells [13]. The method, borrowed from the ubiquitin field, is simple: fuse your cDNA to Ubc9 and express the hybrid protein in cells. In doing so, the protein of interest is brought into close proximity to the E2 SUMO conjugating enzyme (*i.e.*, Ubc9) and results in E3-independent sumoylation. Expression of such fusion proteins in mammalian cells can induce >40% sumoylation on the target protein. Importantly, since the general level of cellular sumoylation is not altered, other endogenous proteins are unaffected. Equally important, Jakobs *et al.* showed that the known sumoylation sites in four test proteins was faithfully reproduced (though, of course, at much higher levels) when the proteins were fused to Ubc9.

2.2 Sumoylation in *E. coli*

PTP1B sumoylation can also be studied by a recently developed bacterial expression system as shown in Figure 3A [14]. This system involves co-transformation of *E. coli* with a His-tagged or GST-tagged expression vector designed to express the protein of interest (here, PTP1B) and a second vector encoding sumoylation E1 and E2 enzymes, plus SUMO-1.

2.2.1 Plasmids—pET28-PTP1B 403 (encoding aa 1-403)[8] and pT-E1E2S1 (a plasmid expressing Aos1, Uba2, Ubc9 and SUMO-1)[14] are used to co-transform chemically competent BL21 (DE3) cells.

2.2.2 Buffers

Lysis Buffer: bacterial lysis buffer BPER (Pierce), supplemented with 1 mM EGTA, 1 mM PMSF, 10 µg/ml aprotinin, and 20 mM NEM, added just before use.

Elution buffer: 10 mM imidazole in 50 mM Tris-HCl, pH 8.0, 1 mM DTT.

2.2.3 Protein induction and purification

1. After co-transformation, the bacteria are plated on an LB plate containing chloramphenicol (50 µg/ml) and kanamycin (25 µg/ml) to select for both plasmids. A single colony is selected and cultured in LB media containing chloramphenicol and kanamycin at 37°C with shaking for 12 h (OD₆₀₀ ~1.0), followed by induction with 0.2 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation for another 12 h at 25°C, the bacteria are pelleted by centrifugation.
2. Cell lysis: the pellet from a 250 ml culture is resuspended in 12.5 ml of ice-cold Lysis buffer for 10 min at 4°C.
3. The lysate is centrifuged at 35,000g for 10 min to remove insoluble material.
4. We use 250 µL Ni-NTA (Qiagen) resin per 12.5 ml of lysate for affinity purification. This is done by pipeting 500 µL of 50% bead slurry to a tube, washing x2 with PBS to remove the 20% ethanol storage solution, spinning, and decanting. Beads (250 µL) are resuspended in 12.5 ml of sonicate and incubated with gentle agitation.
5. This mixture is centrifuged at 500g 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.
6. The fusion protein is eluted by adding 125 µL aliquots (seven times) of Elution Buffer.
7. Individual fractions are analyzed by SDS-PAGE and/or immunoblot, and fractions containing the highest amounts of sumoylated recombinant protein are pooled, aliquoted, snap frozen, and stored at -80°C.

2.2.4 Notes

- A similar bacterial sumoylation system has been described by Mencía and de Lorenzo [15], but this system requires co-transformation of bacteria with three separate plasmids, whereas the system described here requires only two. In either system, different origins of replication, and different antibiotic resistance genes are used to enable co-transformation.
- Other T7-compatible bacterial strains (*e.g.*, Tuner (Novagen), C41 (Lucigen), JM109(DE3) (Promega), *etc*) can also be used to produce sumoylated proteins.
- pGEX vectors, or other bacterial expression vectors, can also be used, provided that they employ a pBR322 or pUC based origin of replication, as opposed to p15A, which is used in the pTE1E2S1 plasmid.
- The expression of an E3 ligase such as PIAS1 is not required for sumoylation in *E. coli*.

- NEM is not strictly required in the lysis buffer, as *E. coli* lack SENP-like protease activities.
- Expected yields of sumoylated protein depend on the expression levels that can be achieved for the gene under study, but, in favorable circumstances, can exceed 5 mg/L. It is possible to achieve near quantitative yields of sumoylated protein.
- If one wishes to study other forms of SUMO, SUMO-2 or SUMO-3 can be used in place of SUMO-1 by modifying the pTE1E2S1 vector.
- In the case of PTP1B, the hydrophobic C-terminal 32 amino acids are omitted to facilitate expression of soluble protein in bacteria.
- The sites of sumoylation can be determined by either using site directed mutagenesis to replace consensus site lysine residues with arginine residues, or by using mass spectrometry. If the latter approach is used, it may be useful to modify the pTE1E2S1 vector such that SUMO-1 T95R is used in place of wild-type SUMO-1. The mutant form is simpler to map following trypsin digestion, as it will leave a di-glycine tag on the sumoylated protein, whereas wild-type SUMO-1 has no lysine or arginine residues in its C-terminus [16].

2.3 Sumoylation in vitro using purified enzymes

As it is possible to produce large amounts of purified components of the sumoylation machinery, one can incubate a protein such as recombinant PTP1B in presence of recombinant E1, E2, and SUMO-1, along with a suitable energy regeneration system, to produce sumoylated proteins *in vitro* [17].

The E1 enzyme is composed of two subunits, Aos1 and Uba2, which can be purified separately, then added together, or co-expressed in *E. coli* and co-purified. The latter procedure generally yields higher levels of active enzyme, ~1 mg/L of culture. The rest of the components – the E2 enzyme Ubc9, SUMO-1, and PTP1B – are produced separately using conventional methods, as outlined below.

2.3.1 Plasmids—pET11d-Uba2, pET28a-Aos1, pET23a-Ubc9 [17], pRH-SUMO-1 [15] and pGEX2T-PTP1B 1-403 [18].

2.3.2 Protein induction and purification: E1

2.3.2.1 Buffers

Lysis buffer: 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 10 mM Imidazole.

Wash buffer: 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM β -mercaptoethanol, and 1 μ g/ml each of aprotinin, leupeptin, and pepstatin.

Elution buffer: 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 300 mM imidazole, 1 mM β -mercaptoethanol, and 1 μ g/ml each of aprotinin, leupeptin, and pepstatin.

S200 buffer: 50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, and 1 μ g/ml each of aprotinin, leupeptin, and pepstatin.

Q buffer 1: 50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, and 1 μ g/ml each of aprotinin, leupeptin, and pepstatin.

Q buffer 2: 50 mM Tris, pH 7.5, 1 M NaCl, 1 mM DTT, and 1 μ g/ml each of aprotinin, leupeptin, and pepstatin.

Transport buffer (TB): 110 mM KOAc, 20 mM HEPES, pH 7.3, 2 mM Mg(OAc)₂, 1 mM EGTA, 1 mM DTT, and 1 μ g/ml each of leupeptin, pepstatin, and aprotinin.

2.3.2.2 Procedure

1. pET28a-Aos1 and pET11d-Uba2 are co-transformed into BL21(DE3) and used to inoculate 500 ml of LB with 50 µg/ml ampicillin and 30 µg/ml kanamycin. Cells are grown for 18 h at 37°C, then collected by centrifugation, resuspended in 2 liter fresh medium, and induced for protein expression with 1 mM IPTG.
2. Grow cells for 6 h at 25°C, then harvest by centrifugation at 4000g in a suitable rotor. Resuspend cell pellet in 50 ml BPER (Pierce) containing 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 µg/ml each of aprotinin, and 50 mg lysozyme (Sigma), on ice for 1 h. Bacterial debris is removed by centrifugation for 1 hr at 4°C at 100,000g. His-Aos1 and associated Uba2 are purified by adding 6 ml Ni-NTA resin (Qiagen) equilibrated in lysis buffer, including protein inhibitors and β-mercaptoethanol for 1 h at 4°C. The resin is transferred into a column and washed extensively with cold wash buffer until no more protein elutes from the column, as determined by A280 readings. Proteins are then eluted with 3 volumes elution buffer collected as 2 ml fractions. Protein containing fractions are combined and concentrated to 2–5 ml using a centrifugal device (e.g., 30K-Millipore concentrator). The concentrated protein is then passed through a 20 µm filter (Millipore) to remove particulates and then purified by gel filtration using a FPLC S200 preparative gel filtration column equilibrated in S200 buffer. 5 mL fractions are collected and analyzed by SDS–PAGE. Fractions that contain both His-Aos1 (migrates at ~40 kDa) and Uba2 (migrates at ~90 kDa despite a predicted size of ~72 kDa) are combined and applied for further purification on a 1ml MonoQ anion-exchange column (Pharmacia FPLC, 1 ml). Elution from the MonoQ column is accomplished using a linear gradient from 50 to 500 mM NaCl (generated from Q buffers 1 and 2). Fractions (0.5 ml) are collected and analyzed by SDS–PAGE. Fractions containing approximately equimolar levels of His-Aos1 and Uba2 are combined and dialyzed against TB. The enzyme is aliquoted (at 5 µl aliquots) and stored at 80°C. Dilutions are routinely done in TB buffer containing 0.05% Tween 20 and 0.2 mg/ml ovalbumin.

2.3.3 Protein induction and purification: E2—The E2 enzyme Aos9 is not epitope tagged, and thus is purified by conventional column chromatography. Yields can approach 10 mg/L bacterial culture.

2.3.3.1 Buffers

Buffer 1 (B1): 50 mM Na-phosphate, pH 6.5, 50 mM NaCl, 1 mM dithiothreitol (DTT), and 1 µg/ml each of aprotinin, leupeptin, and pepstatin.

Buffer 2 (B2): 50 mM Na-phosphate, pH 6.5, 300 mM NaCl, 1 mM DTT, and 1 µg/ml each of aprotinin, leupeptin, and pepstatin.

Transport buffer (TB): 110 mM KOAc, 20 mM HEPES, pH 7.3, 2 mM Mg(OAc)₂, 1 mM EGTA, 1 mM DTT, and 1 µg/ml each of leupeptin, pepstatin, and aprotinin.

2.3.3.2 Procedure

1. pET23a-Ubc9 is transformed into *E. coli* strain BL21(DE3). A single colony is used to inoculate a 20 ml overnight culture. This preculture is then used to inoculate 2 liters of LB/Amp medium. At an OD₆₀₀ of 0.6 (after approximately 2 h at 37°C, 250 rpm), 1 mM IPTG is added and the cells are grown for a further 4 h at 37°C before harvesting.

2. The cells are resuspended in 60 ml B1 and subjected to one freeze–thaw cycle (-80°C). Once thawed, 0.1 mM PMSF is added, and the bacterial debris is removed by centrifugation at 1 h 100,000g at 4°C .
3. The 100,000g supernatant is applied to a 10-ml SP Sepharose column equilibrated in B1 and flow through is discarded. After washing the column with 30 ml B1, Ubc9 is eluted with B2. 15 fractions of 2 ml are collected and analyzed by SDS–PAGE for the presence of Ubc9, as assessed by Coomassie Blue staining.
4. Ubc9-containing fractions are combined and concentrated to ~2 ml using a centrifugal concentrator (5K; Centricon, then purified by gel filtration using a preparative S200 FPLC column (Pharmacia). The column is equilibrated in TB, 2 ml of the protein solution is injected per run, and 5-ml fractions are collected. Ubc9 fractionates with an apparent molecular mass of 20 kDa. Fractions containing Ubc9 are checked for purity by SDS–PAGE, pooled, aliquoted, and stored at -80°C .

2.3.4 Protein induction and purification: His-SUMO-1—His-SUMO-1 is prepared essentially as described in section 2.3.2, using pRH-SUMO-1 in place of pET28a-Aos1 and pET11d-Uba2, and selecting BL21 (DE3) bacteria in 50 $\mu\text{g/ml}$ ampicillin. Expected yields are ~5 mg/L bacterial culture.

2.3.5 Protein induction and purification: Gst-PTP1B 403—The plasmid pGEX2T-PTP1B 1-403 encodes all but the hydrophobic C-terminal tail of PTP1B. Expected yields are ~5 mg/L bacterial culture.

2.3.5.1 Buffers: Lysis buffer: PBS, 1% Triton X-100. Immediately prior to use, add 2 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, and 25 $\mu\text{g/ml}$ PMSF.

2.3.5.2 Procedure

1. Inoculate one colony of each bacterial strain expressing each construct (GST alone, GST fusion proteins) into individual 5-ml aliquots of LB broth containing appropriate antibiotic selection. Grow overnight at 37°C with shaking.
2. Inoculate 1 liter of LB containing the antibiotic selection with the 5 ml overnight culture from Step 1. Grow the cultures at 37°C with shaking to an OD_{600} of 0.5–1.0 (3–6 h).
3. Induce expression of the protein by adding IPTG to a final concentration of 0.1 mM.
4. Incubate the cultures for an additional 3 hours at 37°C with shaking.
5. Centrifuge the bacterial culture at 3500g for 20 minutes at 4°C and discard the supernatant. Resuspend the pellet in 20 ml of PBS lysis buffer.
6. Sonicate the bacterial suspension on ice, in three, short 10-second bursts, alternating with 10 seconds of resting on ice.
7. Centrifuge the lysate at 12,000g for 15 minutes at 4°C and transfer the supernatant to a fresh tube.
8. Add 5 ml of a 50:50 slurry of Glutathione-Sepharose beads in PBS lysis buffer and incubate for 30 min at 4°C , rotating the tube end over end to ensure mixing.
9. Centrifuge the samples at 750g for 1 minute at 4°C to pellet the beads. Remove the supernatant and wash the beads in 5 ml of ice-cold PBS with protease inhibitors.

10. Centrifuge the samples at 750g for 1 minute at 4°C to pellet the beads. Remove the supernatant.
11. Add 5 ml of ice-cold PBS with protease inhibitors. Resuspend the beads by gentle mixing and pour the slurry into a disposable chromatography column.
12. Wash column with 5 ml PBS with protease inhibitors.
13. Elute the fusion protein by adding 5 ml of cold (0°C-4°C) 50 mM Tris-Cl (pH 8.0) containing 20 mM reduced glutathione.
14. Collect ~0.5-ml fractions of the eluate in microcentrifuge tubes.
15. Dialyze eluted fractions against 20 mM Tris-HCl, pH 8.0, 20% glycerol, 1 mM EDTA, 2 mM DTT.
16. Measure protein content of eluted fractions, and test those with high protein content for purity using SDS/PAGE and Coomassie staining. The apparent molecular mass should be ~70 kD (26 kD GST moiety plus ~44 kD for PTP1B 403).
17. Store protein at 4°C for short periods or in small aliquots at -80°C.

2.3.6 Sumoylation Reaction

2.3.6.1 Buffers

10X ATP regeneration mix: 20 mM ATP, 100 mM creatine phosphate disodium salt, 35 U/ml creatine kinase and 6 U/ml inorganic pyrophosphatase.

10X SUMO buffer: 500 mM Tris-Cl (pH 7.5) and 50 mM MgCl₂

2.3.6.2 Procedure

1. Assemble 20 µl sumoylation reactions on ice, adding the following components in the following order: 10x SUMO buffer, 500 ng Uba2/Aos1, 2 µg Ubc9, 2 µg His-SUMO-1, 1 µg His-PTP1B 1-403, and 2 µL of 10X ATP regeneration mix.
2. Incubate at 30°C for 1–3 h.
3. Mix 20 µL 2X SDS/PAGE sample buffer with 20 µL reaction and boil for 5 min.
4. Analyze by immunoblot with anti-His and/or anti SUMO-1 antibodies.

2.3.7 Notes

- If desired, larger amounts of sumoylated proteins can be produced by scaling up the reaction 10x or 100x.
- As the efficiency of sumoylation may be low, sumoylated protein can be purified using sequential glutathione-agarose and Ni-NTA agarose chromatography, in either order.

3. Rapid PTP1B Activity Assay

As a quick method to assess the effects of sumoylation on PTP1B activity, it is easiest to use the chromogenic substrate *p*-nitrophenyl phosphate [19], which produces a yellow color upon the release of P_i with concomitant formation *p*-nitrophenol. It should be noted that such assays are difficult to perform using mammalian cell lysates, as the NEM that must be added to preserve sumoylation also interferes with PTP activity by modifying the catalytic cysteine of these enzymes. While in principle it might be possible to reactivate PTPs following their purification in NEM-containing buffers, in practice it is more straightforward to adapt the *E. coli* or the *in vitro* sumoylation system for this purpose.

3.1 pNPPase assay

3.2 Buffer

2x phosphatase buffer: 50 mM Hepes (pH 7.4), 1 mM EDTA, 10 mM DTT, 200 µg/ml BSA.

3.2.1 Procedure

1. Assemble 20 µl pNPPase reactions on ice, adding the following components in the following order: 10 µl 2x phosphatase buffer, 4 µl 250 mM pNPP, and ~ 500 ng GST-PTP1B 403.
2. Incubate at 30°C for 5–30 min.
3. Stop reaction by adding 500 µl 1N NaOH.
4. Measure absorbance at 405 nm. If absorbance is >1, one can reduce the amount of added PTP or reduce the time of the assay.
5. Enzyme specific activity (µmoles/min µg) = $50 \text{ (vol)} \times \text{OD}_{405\text{nm}} \times 1/\text{time (min)} \times \text{enzyme (µg)} \times 1/18,000$ (molar extinction coefficient).

3.2.2 Notes

- P_i , released as a result of PTP activity can also be measured using a malachite green assay [20].
- pNPP is a convenient, but relatively non-specific substrate. Phospho-peptides or ^{32}P -labelled protein substrates may also be used.

Concluding Remarks

Given the number of available approaches, it is relatively straightforward to determine if a protein of interest such as PTP1B can be sumoylated. Similarly, it is not difficult to determine the site(s) of sumoylation, by replacing consensus site lysines with arginine residues and/or by using mass spectrometry approaches. Assessing the effects of sumoylation can be more challenging, especially for PTPs, as the stoichiometry for sumoylation is typically low and the conditions required for isolating sumoylated proteins from mammalian cell extracts preclude measurements of PTP catalytic activity. It should also be noted that most sumoylation is spatially confined within or near the nucleus [21], so localized concentrations of modified PTP1B could have effects on particular, restricted populations of substrates [9]. However, the effects of sumoylation on PTP activity are readily achievable using the bacterial or *in vitro* approaches, which facilitate production of large amounts of sumoylated protein. Such approaches could also be used to produce proteins for structural studies, thus giving mechanistic insights into the regulation of PTP1B or other modified PTPs by sumoylation.

References

1. Tonks NK, Neel BG. Combinatorial control of the specificity of tyrosine phosphatases. *Curr Opin Cell Biol.* 2001; 13(2):182–95. [PubMed: 11248552]
2. Hunter T. Signaling--2000 and beyond. *Cell.* 2000; 100(1):113–27. [PubMed: 10647936]
3. Frangioni JV, et al. The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell.* 1992; 68(3):545–60. [PubMed: 1739967]
4. Yip SC, Saha S, Chernoff J. PTP1B: a double agent in metabolism and oncogenesis. *Trends in biochemical sciences.* 2010; 35(8):442–9. [PubMed: 20381358]
5. Johnson ES. Protein modification by SUMO. *Annu Rev Biochem.* 2004; 73:355–82. [PubMed: 15189146]

6. Bernier-Villamor V, et al. Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. *Cell*. 2002; 108(3):345–56. [PubMed: 11853669]
7. Knipscheer P, et al. Ubc9 sumoylation regulates SUMO target discrimination. *Mol Cell*. 2008; 31(3):371–82. [PubMed: 18691969]
8. Dadke S, et al. Regulation of protein tyrosine phosphatase 1B by sumoylation. *Nat Cell Biol*. 2007; 9(1):80–5. [PubMed: 17159996]
9. Yip SC, Cotteret S, Chernoff J. Sumoylated protein tyrosine phosphatase 1B localizes to the inner nuclear membrane and regulates the tyrosine phosphorylation of emerin. *Journal of cell science*. 2012; 125(Pt 2):310–6. [PubMed: 22266903]
10. Haj FG, et al. Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatase-1B. *J Biol Chem*. 2003; 278(2):739–44. [PubMed: 12424235]
11. Liu F, Chernoff J. Suppression of oncogene-mediated transformation of rat 3Y1 fibroblasts by protein tyrosine phosphatase 1B requires a functional SH3-ligand. *Mol Cell Biol*. 1998; 18:250–259. [PubMed: 9418872]
12. Zhao Y, et al. Broad spectrum identification of cellular small ubiquitin-related modifier (SUMO) substrate proteins. *J Biol Chem*. 2004; 279:20999–1002. [PubMed: 15016812]
13. Jakobs A, et al. Ubc9 fusion-directed SUMOylation (UFDS): a method to analyze function of protein SUMOylation. *Nat Methods*. 2007; 4:245–50. [PubMed: 17277783]
14. Uchimura Y, et al. Overproduction of eukaryotic SUMO-1- and SUMO-2-conjugated proteins in *Escherichia coli*. *Anal Biochem*. 2004; 331(1):204–6. [PubMed: 15246018]
15. Mencía M, de Lorenzo V. Functional transplantation of the sumoylation machinery into *Escherichia coli*. *Protein Expr Purif*. 2004; 37:409–18. [PubMed: 15358364]
16. Knuesel M, et al. A Method of Mapping Protein Sumoylation Sites by Mass Spectrometry Using a Modified Small Ubiquitin-like Modifier 1 (SUMO-1) and a Computational Program. *Mol Cell Proteomics*. 2005; 4:1626–1636. [PubMed: 16020427]
17. Bossis G, et al. A fluorescence resonance energy transfer-based assay to study SUMO modification in solution. *Methods Enzymol*. 2005; 398:20–32. [PubMed: 16275316]
18. Liu F, Chernoff J. Protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor. *Biochem J*. 1997; 327(Pt 1):139–45. [PubMed: 9355745]
19. Chernoff J, et al. Characterization of a phosphotyrosyl protein phosphatase activity associated with a phosphoserine protein phosphatase of Mr = 95,000 from bovine heart. *The Journal of biological chemistry*. 1983; 258(12):7852–7. [PubMed: 6305959]
20. Lorenz U. Protein Tyrosine Phosphatase Assays. *Curr Protoc Immunol*. 2011; (Unit 11.7)
21. Watts FZ. SUMO modification of proteins other than transcription factors. *Semin Cell Dev Biol*. 2004; 15:211–20. [PubMed: 15209381]

Figure 1. Structure of PTP1B

Schematic representation of PTP1B. PTP1B consists of an N-terminal catalytic domain (green), central proline-rich domain (brown), and a C-terminal ER targeting domain (grey). PTP1B is regulated by sumoylation at the lysine residues in proline rich domain. The lysine residues known to be sumoylated (K73, K335, K347 and K389) have been indicated.

Figure 2. Sumoylation cycle

SUMO-1 is initially transcribed as an inactive precursor, which is cleaved by SENP proteases to expose the C-terminus di-glycine motif. This mature form of SUMO-1 is then activated by the E1 enzyme in the presence of ATP. The activated SUMO-1 is then transferred to the E2 conjugating enzyme. E2 and E3 mediate the Sumoylation of the target protein. Desumoylation is mediated by SENP proteases.

Figure 3. PTP1B sumoylation assays

A) pT-E1E2S1/2 vector expresses the SUMO machinery including SUMO-E1, SUMO-E2 and SUMO-1/SUMO-2 under control of the T7 promoter. When the pET28a-based vector was cotransformed with pT-E1E2S1/S2, PTP1B expressed from the pGEX vector was modified by either SUMO-1 or SUMO-2 in *E. coli*. PTP1B was both mono-sumoylated and poly-sumoylated. B) *Ptp1b*-null MEFs were either transfected with HA-PTP1B or T7-SUMO-1, alone or together. Cells were lysed and immunoprecipitated using anti-HA or anti-T7 antibodies. Western blot was performed with anti-T7 or anti-HA antibodies to detect sumoylated forms of PTP1B. PTP1B consisted of both mono-sumoylated and poly-sumoylated forms.