Stability of thioester intermediates in ubiquitin-like modifications

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Abstract: Ubiquitin-like modifications are important mechanisms in cellular regulation, and are carried out through several steps with reaction intermediates being thioester conjugates of ubiquitin-like proteins with E1, E2, and sometimes E3. Despite their importance, a thorough characterization of the intrinsic stability of these thioester intermediates has been lacking. In this study, we investigated the intrinsic stability by using a model compound and the Ubc9~SUMO-1 thioester conjugate. The Ubc9~SUMO-1 thioester intermediate has a half life of approximately 3.6 h (hydrolysis rate $k = 5.33 \pm 2.8 \times 10^{-5} \text{ s}^{-1}$), and the stability decreased slightly under denaturing conditions ($k = 12.5 \pm 1.8 \times 10^{-5} \text{ s}^{-1}$), indicating a moderate effect of the three-dimensional structural context on the stability of these intermediates. Binding to active and inactive E3, (RanBP2) also has only a moderate effect on the hydrolysis rate ($13.8 \pm 0.8 \times 10^{-5} \text{ s}^{-1}$ for active E3 versus $7.38 \pm 0.7 \times 10^{-5} \text{ s}^{-1}$ for inactive E3). The intrinsically high stability of these intermediates suggests that unwanted thioester intermediates may be eliminated enzymatically, such as by thioesterases, to regulate their intracellular abundance and trafficking in the control of ubiquitin-like modifications.

Keywords: Ubc9; SUMO; thioester; ubiquitin-like modifications; stability; E3 ligase

Introduction

Ubiquitin and ubiquitin-like post-translational modifications of proteins are rapid, dynamically regulated mechanisms to control protein functions.^{1–4} These modifications can attach a single or a chain of ubiquitin or ubiquitin-like proteins (Ubls) onto target proteins to modulate the life-spans, intracellular trafficking, and protein–protein interactions of modified proteins in the regulation of nearly every aspect of cellular functions. Thioester conjugates are formed as

intermediates in ubiquitin-like modifications. In these modifications, Ubl is activated by the E1 enzyme, which carries out an ATP-dependent adenylation of the C-terminus of Ubl. Then a thioester bond is formed between the catalytic Cys residue of E1 and the C-terminal glycine of Ubl. Next, the thioester-linked Ubl is transferred from E1 to E2, forming a second thioester intermediate between the C-terminus of Ubl and the catalytic cysteine of E2. In ubiquitination reactions catalyzed by HECT-domain-containing E3 ligases, thioester-bonded ubiquitin on E2 is transferred to the catalytic Cys on the HECT domain, thereby forming a third thioester intermediate before finally being transferred to target proteins. In modifications catalyzed by other E3 ligases, Ubl that is thioester bonded to E2 is transferred directly to a substrate, forming an isopeptide bond between the C-terminal glycine on Ubl and the ϵ -amino group of a lysine in the substrate. The modifications can be removed by isopeptidases, so that they are highly dynamic and tightly controlled.

Abbreviations: GC-DEG, a thioester model compound using Gly and Cys residues; SUMO, small ubiquitin-like modifiers; Ubl, ubiquitin-like proteins.

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Previous studies suggest that levels of the thioester intermediates in ubiquitin-like modifications are tightly regulated. For example, one study showed that the E2-ubiquitin thioester conjugate levels can vary significantly in response to external stimuli.⁵ Despite their functional importance, the intrinsic stability of thioester intermediates in ubiquitin-like modifications and the factors controlling their levels have not been systematically investigated. More than 10 ubiquitin-like modifications have been identified.⁴ In addition, in mammalian cells, there are more than 30 E2 enzymes that are involved in catalyzing ubiquitination. Some of these E2s are involved in catalyzing both ubiquitination and ubiquitin-like modifications.^{6,7} Furthermore, hundreds of E3 ligases have been identified. Thus, many different Ubl thioester intermediates can be formed. It is unclear whether thioester intermediates have varying stabilities resulting from the different E1, E2, and E3 proteins and UbIs involved. Moreover, many of the E2 and E3 proteins are in multi-protein complexes, interacting with other cellular factors or form homo- or hetero-dimeric E2 or E3 enzymes.^{8–13} However, it is not clear whether these noncovalent protein–protein interactions could also affect the stability of the thioester intermediates.

In this study, using a synthetic model compound and the Ubc9~SUMO-1 thioester intermediate, we show that three-dimensional structures of proteins and protein–protein interactions have small effects on the stability of thioester intermediates in ubiquitin-like modifications, and that the thioester intermediates have intrinsically long life spans. As isopeptidases play critical roles in regulating the levels of Ubl-modified proteins, the high intrinsic stability of the thioester intermediates suggest that unwanted thioester intermediates may also be eliminated enzymatically.

Results

Effect of protein structures on the stability of the thioester bond

The structural properties of an E2-ubiquitin thioester conjugate, being central to ubiquitination, have been investigated previously.^{14–16} One study showed that the NMR linewidths of ubiquitin in a thioester conjugate with an E2 are similar to those of free ubiquitin,¹⁴ suggesting the structural and dynamic independence of ubiquitin in the conjugate with E2. However, ubiquitin in a thioester conjugate with another E2 appears to preferentially interact with specific surface of the E2.¹⁵ These studies indicated that the thioester conjugates between ubiquitin and different E2 enzymes may have different structural properties.

To investigate whether the three-dimensional structural contexts of Ubl and E2 affect the stability of thioester bonds, we measured the hydrolysis rate of an E2 (Ubc9)~SUMO-1 thioester in the absence and presence of 8M urea, which disrupted the three-dimen-

Figure 1. Hydrolysis of the E2~SUMO-1 thioester. (A) Gel image of the hydrolysis experiment. SUMO was detected by Western Blot with an anti-SUMO antibody, and visualized and quantified with Li-Cor Odyssey system. Both SUMO-1 and the Ubc9~SUMO-1 thioester are indicated. The amounts of SUMO-1 and Ubc9~SUMO-1 thioester were calibrated using a standard curve generated from the same Western Blot with known amounts of SUMO-1 protein on the same PVDF membrane transferred from a separate SDS-PAGE (lower panel). Asterisk indicates the samples treated with reducing SDS sample buffer and boiled for 5 min before loading on the gel. (B) Plot of relative amount of Ubc9~SUMO-1 thioester against time. Values were calculated by determining the relative intensities of the bands for the thioester.

sional structures of the proteins. Ubc9~SUMO thioester conjugate was formed as described in Methods. To initiate the hydrolysis of the thioester intermediate, the thioester conjugate was diluted 10 times into PBS, pH 7.4, or to 8M urea dissolved in PBS, pH 7.4. The hydrolysis experiment was carried out at room temperature and 10 μ L aliquots were withdrawn at various time points [Fig. 1(A)] and frozen in liquid nitrogen until all time points were taken. The samples were separated by SDS-PAGE under non-reducing conditions to preserve the thioester conjugate and detected with Western Blot. Ubc9 and SUMO can also form isopeptide bond,^{17,18} as shown by the aliquot taken at the end of the hydrolysis assay that was mixed with reducing SDS-loading buffer and heated to 95°C for 5 min to reduce the thioester bond. It appears that the isopeptide conjugate between Ubc9 and SUMO formed before the start of the hydrolysis experiment, because the samples in native conditions and denaturing conditions had similar amounts of isopeptide conjugates [Fig. 1(A)]. Thus these isopeptide conjugates, contributing to less than 20% of the band intensities, were subtracted from each time point [Fig. 1(B)]. Hydrolysis

Table I. Hydrolysis Rate Constants for GC-DEG and Ubc9~SUMO Thioester

	Phosphate buffer (s ⁻¹)	8M urea (s ⁻¹)
Gly-Cys-DEG ^a	2.2 ± 0.2 (×10 ⁻⁵)	2.2 ± 0.2 (×10 ⁻⁵)
Ubc9~SUMO-1 thioester	5.33 ± 2.8 (×10 ⁻⁵)	12.5 ± 1.8 (×10 ⁻⁵)
IR1M•Ubc9~SUMO-1	13.8 ± 0.8 (×10 ⁻⁵)	N/A
IR1MASBM•Ubc9~SUMO-1	7.38 ± 0.7 (×10 ⁻⁵)	N/A

^a The structure is shown as compound 8 in Fig. 2.

rate was reproducibly higher under the denaturing condition than under native condition [Table I, Fig. 1(B)]. The differences in hydrolysis rates indicated that the thioester bond is more stable in the native structural environment. However, to rule out any effect of the high concentration of urea on thioester hydrolysis, a model compound was designed and synthesized, as described below.

Hydrolysis of a model thioester compound

To calibrate the effect of urea on thioester hydrolysis and understand the influence of three-dimensional structures of proteins on the stability of the thioester intermediates, we synthesized a model compound [**8** in Fig. 2] using Gly and Cys residues, referred to as GC-DEG, which mimicked the thioester intermediates in ubiquitin-like modifications. The synthesis route is illustrated in Figure 2 with details given in the Methods section. The product, **8**, was confirmed by mass spectrometry and NMR spectroscopy.

To measure the hydrolysis rate, a concentrated GC-DEG sample was prepared by dissolving lyophilized GC-DEG in 35 µL d₆-DMSO at 920 mM as determined by 1D-NMR spectrum.¹⁹ The hydrolysis rate of the model compound, at a concentration of 4.6 mM, was measured at 25°C in PBS in D₂O with 0.5% residual DMSO, pH 7.5. To assess the effect of denaturing conditions on the hydrolysis rate of the thioester bond, we performed NMR analysis of hydrolysis of 4.6 mM GC-DEG in a buffer containing PBS and 8M Urea in H₂O, pH 7.5. We expected the structural properties of the model dipeptide to be similar under native conditions and in 8M urea. The hydrolysis rate was obtained by continuously monitoring the decrease in peak intensities of the CH₂ groups of the Cys in GC-DEG over time, as hydrolysis of the thioester bond significantly affected their chemical shifts. Nine time points were acquired over 342 min, with the first data point acquired 14 min after addition of GC-DEG to the aqueous buffer [Fig. 3(A)]. For the sample in 8M urea, 12 points were acquired over 377 min, with the first data point acquired 21 min after addition of GC-DEG to the aqueous buffer. Figure 3(B) shows a representative fit of the Cys-CH₂ peak intensity versus time. The hydrolysis rates under both native and denaturing conditions were obtained by linear fitting of the data shown in Figure 3(B) and presented in Table I. These data suggest that 8M urea has no influence on the intrinsic hydroly-

sis rate of the thioester bond. In addition, the model compound hydrolyzed slower than the protein conjugate in 8M urea, consistent with previous findings that covalent structures affect thioester hydrolysis.^{20,21}

Effect of non-covalent protein-protein interactions on the stability of the Ubc9~SUMO-1 thioester

As shown previously, the IR1-M domain of RanBP2 functions as an E3 and interacts with both SUMO-1 and Ubc9.^{22–25} To test the effect of the E3, we examined the hydrolysis rate of the Ubc9~SUMO-1 thioester in the presence of the IR1-M domain of RanBP2. Another version of the E3, which had a deletion of the SUMO-binding motif (SBM, also known as SUMO-interacting motif, SIM) from IR1-M, referred to as IR1-M-ΔSBM, was also tested.

We used an experimental design that minimized complications caused by loss of the Ubc9~SUMO-1 thioester by modification of the E3 ligase [Fig. 4(A)]. E3 ligases are frequently self-modified in ubiquitination and ubiquitin-like modifications, although the functions of E3 self-modifications remain largely unclear. In this experiment, the Ubc9~SUMO-1 thioester was bound to either the IR1-M or the IR1-M-ΔSBM domains, which were immobilized on microtiter plates [Fig. 4(A)]. Hydrolysis of the thioester released SUMO-1 from the immobilized protein complex, and the released SUMO-1, which was conjugated to Alexa680 at residue Cys52, could be detected in the supernatant. A dot blot of two-fold serial dilutions of Alexa680-SUMO-1 showed that the dye offers high sensitivity and accuracy for quantification [Fig. 4(B)]. Furthermore, labeling SUMO-1 with Alexa680 at residue Cys52 did not affect the enzymatic reactions of sumoylation (data not shown). This experimental design ensured that release of SUMO-1 would only be due to hydrolysis of the Ubc9~SUMO-1 thioester conjugate, but not due to SUMO-1 modification of the E3 ligase. SUMO-1 by itself has very low affinity for IR1-M, and it was not detectable in a pull-down experiment (data not shown); therefore, it is unlikely that most SUMO-1 bound to the immobilized protein complex was in its free, unconjugated form. In fact, addition of DTT resulted in the release of a large amount of SUMO-1 from the immobilized protein complex into the supernatant [Fig. 4(C)], suggesting that most of the SUMO-1 bound to the immobilized protein

Figure 2. Synthesis of the model compound. Details are given in Methods.

complex was in the Ubc9~SUMO-1 thioester form. In the absence of DTT, SUMO-1 was gradually released into the supernatant from hydrolysis of the Ubc9~SUMO-1 thioester [Fig. 4(C)], and although the thioester hydrolysis was measurable for both the complex with IR1-M and the complex with IR1-M-ΔSBM, it was faster in the presence of IR1-M [Fig. 4(C,E), Table I]. SDS-PAGE analysis of the supernatants in non-reducing conditions indicated that the released SUMO-1 was in its free form, but not in thioester form (data not shown). Similar background SUMO concentrations were detected at the beginning of the hydrolysis measurement, which may reflect the small amount of residual free SUMO-1 that was not completely eliminated by the washing step. Taken together, these data

suggest that the active E3 ligase can enhance the reactivity of the Ubc9~SUMO-1 thioester bond.

Discussion

In this study, we have carried out a systematic characterization of the thioester intermediates in ubiquitin-like modifications. Hydrolysis of several thioester model compounds were studied previously including ethyl *p*-nitrothiolbenzoate, *s*-hippurylthioglycolic acid, and *s*-ethyl monothiolsuccinate.^{20,21} Under physiological conditions, the hydrolysis rates of these compounds range from 10^{-5} to 10^{-7} s⁻¹, and can be catalyzed by nucleophile-containing compounds, such as imidazole or OH⁻. These previous studies also showed that thioester hydrolysis can be influenced by covalent

Figure 3. Hydrolysis of the model compound. (A) NMR spectra recorded under native conditions showing the resonances of CH₂ group of the Cys residue. The sharp peak at 3.6 ppm is from an unidentified impurity. The quartet peaks around 3.65 and 3.32 ppm are from the CH₂ group before hydrolysis. The upper spectrum was obtained 14 min after addition of the sample to aqueous solution, and the lower spectrum was acquired after 342 min. The boxed region was integrated to extract the hydrolysis rate. (B) Plot of the relative amount of the original compound against time, as determined by analysis of the intensities of the Cys-CH₂ peak. The hydrolysis rate was deduced from linear fitting.

structural contexts. Thus, we synthesized compound **8** (Fig. 2) to best mimic the thioester in ubiquitin-like modifications and to rule out any effect of 8M urea in catalyzing thioester hydrolysis. The slower hydrolysis rate of the Ubc9~SUMO-1 thioester under native condition than under denaturing condition indicates that the protein structure itself has a modest stabilizing effect, which would prevent wasteful hydrolysis.

This study has also shown that noncovalent protein–protein interactions has a small effect on the stability of the E2~SUMO thioester. The binding site of the IR1-M domain of RanBP2 is approximately 30 Å away from the thioester bond. It was shown previously that the IR1-M-ΔSBM construct, which lacks the SUMO-binding motif, cannot stimulate SUMOylation, but the IR1-M domain can function as a E3 ligase and

dramatically enhance SUMOylation.²⁵ Here, we found that both IR1-M and IR1-M-ΔSMB can slightly stimulate the hydrolysis of the E2~SUMO-1 thioester conjugate, although IR1-M induced a slightly faster hydrolysis rate. Thus, it is unlikely that the E3 ligase activity of IR1-M is related to its effects on destabilizing the thioester. E2 and E3 ligases often function in noncovalent complexes with other proteins.^{8–13} These noncovalent protein–protein interactions can potentially affect the stability of the thioester intermediates. A recent study has shown that a novel E3 ligase may also have thioesterase activity.²⁶ Result from this study suggests that thioesterase activity is not a general property of all E3 ligases.

The slow hydrolysis rate of the model compound and the Ubc9~SUMO-1 thioester relative to the life spans

Figure 4. RanBP2 binding increased the hydrolysis of the Ubc9~SUMO thioester. (A) Schematic of the experimental design. When hydrolysis occurs, the fluorescent SUMO-1 is released from the plate wall into the supernatant. (B) Dot blot of two-fold serial dilutions of Alexa680-SUMO-1 (1000–7.8 pg). (C) Dot blot of hydrolyzed (released) Alexa680-SUMO-1 from Ubc9~SUMO-1 thioester immobilized on microtiter plates precoated with IR1-M-ΔSBM domain (top) or IR1-M domain (bottom). Supernatants (5 μ L) taken at the indicated incubation times or supernatants (5 μ L) taken from 10 min incubations in the presence of 20 mM DTT were blotted onto PVDF membrane and analyzed with an Odyssey infrared imaging system. (D) Linear regression fit of integrated intensities against Alexa680-SUMO-1 amounts for dot blot in (B). (E) Plot of relative amount (%) of released Alexa680-SUMO-1 against time, as determined by analysis of the integrated intensities of the dot blot shown in (C). The percentages of released Alexa680-SUMO-1 from microtiter plates precoated with IR1-M-ΔSBM or IR-1M were calculated relative to the amount of Alexa680-SUMO-1 released after incubation with DTT (set as 100%).

of short-lived cellular proteins suggests that ubiquitin-like modifications may initiate from thioester intermediates that are already present in cells instead of always starting from adenylation. The various thioester conjugates of different UbIs and E1, E2, or E3s are likely to have similar stabilities because of the modest structural effect that was observed in this study. Consistent with their stability, a previous study showed that E2~ubiquitin thioester conjugates have significantly increased amounts upon heat shock, an external stimulus that often triggers post-translational modifications, such as ubiquitination.⁵ In addition, intracellular trafficking of E2~ubiquitin thioester has also been reported.²⁷

The robust stability of these intermediates suggests that unwanted thioester intermediates may be eliminated enzymatically, such as by thioesterases. Thioesterases have been reported to hydrolyse unwanted thioester intermediates for other systems, such as nonribosomal peptide synthesis,²⁸ but they are largely unknown for ubiquitin-like modifications. Like isopeptidases that remove UbI from modified proteins in the regulation of UbI modifications levels, such thioesterases may be important for controlling the levels of intermediates in the regulation of ubiquitin-like modifications.

In summary, we have shown that the thioester intermediates in ubiquitin and ubiquitin-like modifications have long life spans, and their stability is only slightly influenced by the three-dimensional structural context and by interactions with other proteins. The intracellular trafficking and enzymatic hydrolysis of the thioester intermediates may be involved in the regulation these post-translational modifications.

Methods

Synthesis of the model compounds

The following compounds were used for the synthesis of the model compound: *N*- α -Fmoc-*S*-*t*-butylthio-*L*-cysteine, (*N*- α -9-fluorenylmethoxycarbonyl-*S*-tertbutylthio-*L*-cysteine) and *N*- α -*t*-Boc-glycine (*N*- α -tert-butylthio-*L*-cysteine) (Novabiochem; Pittsburgh, PA); DEG-Wang resin (100–200 mesh, substitution: 0.7 mmol/g) (Tianjin Nankai Hecheng Science & Technology, Tianjin, China); acetonitrile (MeCN), dichloromethane (DCM), and *N,N'*-dimethylformamide (DMF) (Fisher Scientific); ethanedithiol, *N*-methylimidazole, piperazine, and pyridine (Sigma-Aldrich); acetic anhydride (Ac₂O) (Mallinckrodt Baker; Phillipsburg, NJ);

1-hydroxybenzotriazole (HOBt) (Chem-Impex International; Wood Dale, IL); dicyclohexylcarbodiimide (DCC) (SynPep; Dublin, CA); ethanol (EtOH) (Pharmco Products.; Shelbyville, KY); and trifluoroacetic acid (TFA) (Halocarbon Products; River Edge, NJ).

The model compound was synthesized as follows (Fig. 2). A solution of *N*- α -Fmoc-S-t-buthylthio-*L*-cysteine (**1**) (5.6 mmol in 2 mL DMF) was treated with DCC (5.6 mL of 1M solution in DCM), and HOBt (5.6 mmol in 2 mL DMF). The dicyclohexylurea precipitate was removed by filtration. The resulting filtrate was added to the polystyrene DEG-Wang resin (1 g) and left at room temperature for 24 h. The resin was then filtered, washed sequentially with DMF, MeCN, and DCM and dried *in vacuo*, producing resin **2**. Piperazine (6%) in EtOH/DMF (1:6, v/v) was added (3 mL) and the resulting mixture was stirred for 30 min. The resin was filtered and then washed with DMF and EtOH in turn. The supernatant and the washes were combined. Absorption at 300 nm was measured and loading of compound **1** was estimated to be 0.525 mmol/g of the resin. The *N*-terminal amino group and the remaining, unreacted hydroxyl groups on the resin were acetylated by treatment with a solution of Ac₂O and *N*-methylimidazole in pyridine. The mixture was shaken at room temperature for 2 h and washed. To cleave the S-tBu protecting group and generate a free sulfhydryl group, resin **4** was treated with ethanedithiol (5 mL) in DMF (5 mL) for 12 h and then washed, yielding resin **5**. Resin **5** was reacted with an active ester generated by the reaction of *N*- α -t-Boc-glycine (**6**) (1.05 g, 6.0 mmol) with DCC (6 mL of 1M solution in DCM) and HOBt (0.81 g, 6 mmol in 2 mL DMF), and left at room temperature overnight. The resulting resin **7** was treated with 50% TFA in DCM (5 mL) and kept at room temperature for 1 h. The resin was then washed and filtered. Filtrates and washes were collected and concentrated *in vacuo*. The desired product (**8**; GC-DEG) was purified by HPLC using a Zorbax CombipHT C8 column (21.2 $\times$ 150 mm) and an MeCN/water gradient (0.5–20%) with 0.1% TFA at a flow rate of 1.75 mL/min. The final product was confirmed by mass spectrometry and NMR spectroscopy.

NMR studies

NMR spectra were acquired using a Bruker Avance 500 MHz spectrometer equipped with a triple resonance probe and triple-axis pulsed field gradient. Pre-saturation was used for water suppression for the 1D spectra of samples dissolved in D₂O. For the sample dissolved in PBS in 8M urea, 1D spectra were acquired using a W5 watergate sequence²⁹ with addition of pre-saturation to suppress the large NH₂ signal from urea. Integration of the NMR resonances was performed using Bruker Topspin 2.1 software.

Plasmids

Expression constructs for SUMO E1 (SAE1/SAE2), Ubc9, and wild-type and C52A mutant SUMO-1_{1–97} were

made as described previously.^{23,30–32} cDNAs encoding the IR1-M domain (2629–2710) and the IR1-M- Δ SBM (2637–2710) domain of RanBP2 followed by His₆-tags at the C-termini were amplified by PCR, and inserted into the NdeI and BamHI sites of pET11a+ (Novagen). All constructs were confirmed by DNA sequencing.

Expression and purification of recombinant proteins

Purification of human SUMO-1 (C52A), SUMO E1, and Ubc9 used previously described protocols.^{23,30,31} RanBP2 IR1-M and IR1-M- Δ SBM were expressed in the *E. coli* BL21(DE3) strain, purified using Ni-NTA columns (Qiagen) and dialyzed against PBS containing 1 mM DTT.

Ubc9~SUMO-1 thioester hydrolysis assay

Formation of Ubc9~SUMO thioester complex was achieved by incubating 10 μ M SUMO-1-C52A with 15 μ M Ubc9 in the presence of 1 μ M SAE1/SAE2, 2 mM ATP, and 5 mM MgCl₂ for 30 min at 37°C. The reaction was stopped by adding 50 mM EDTA and 0.5 U/mL apyrase to remove Mg²⁺ and ATP. To initiate the hydrolysis, the above mixture was diluted 10 times into PBS, pH 7.4, or 8M urea dissolved in PBS, pH 7.4. The hydrolysis assay was conducted at room temperature, and 10 μ L aliquots were withdrawn at 2, 5, 10, 20, and 30 min and frozen in liquid nitrogen until all time points were taken. All samples were mixed with nonreducing SDS-loading buffer. To calculate the portion of Ubc9-SUMO conjugate that were not thioester complex, another aliquot was taken at the end of the hydrolysis assay and mixed with reducing SDS-loading buffer containing DTT and heated to 95°C for 5 min. The samples were separated by SDS-PAGE, transferred to PVDF membrane, and bands containing SUMO-1 protein were detected with Western Blot using monoclonal antibody anti-SUMO-1 (Abgent), and visualized and quantified with Li-Cor Odyssey system. The amounts of SUMO-1 and Ubc9~SUMO-1 thioester were calibrated using a standard curve generated with the same Western Blot of known amounts of SUMO-1 protein on the same PVDF membrane transferred from a separate SDS-PAGE.

Fluorescent Alexa680-SUMO-1 was prepared by modifying purified SUMO-1 C52A with succinimidyl ester Alexa-Fluor680 reactive dye followed by gel filtration according to the manufacturer's protocol (Molecular Probes). Alexa680-SUMO-1 had similar conjugation activities as unmodified SUMO-1 (data not shown). Microtiter plates were coated with IR1-M or the inactive IR1-M- Δ SBM and then blocked with bovine serum albumin (BSA), as described earlier. Then, 2 μ g E1 (0.18 μ M), 6 μ g Ubc9 (3.3 μ M), 20 μ g Alexa680-SUMO-1 (15 μ M), 1 mM ATP, and 5 mM Mg²⁺ in 100 μ L blocking buffer (2% BSA and 0.1% Tween20 in PBS) was added to each well and incubated at room temperature for 1 h. This allowed the

Ubc9~SUMO-1 thioester to form and bind to the RanBP2 domains immobilized on the microtiter plates, and sumoylation of the two RanBP2 domains was allowed to reach saturation. After each well was washed five times with 150 μ L blocking buffer, 100 μ L PBS with or without 20 mM DTT was added and the release of hydrolyzed Alexa680-SUMO-1 into the supernatant over time was monitored by removing supernatant from the wells at the indicated times. At each time point, supernatant (5 μ L) was blotted directly onto a PVDF membrane, along with two-fold serial dilutions of standard Alexa680-SUMO-1 (1000–7.8 pg). The fluorescent Alexa680-SUMO-1 on the membrane was detected and quantified using the Odyssey infrared imaging system (Li-Cor BioTech.).

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