Running title:

A real-time SUMO-binding assay

A real-time SUMO-binding assay for the analysis of the SUMO-SIM protein interaction network.

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Abstract

SUMO-interacting motifs (SIMs) play a central role in the fate of SUMO-modified proteins. Here we report a real-time SUMO-binding assay. It can be applied to the identification of SIMs and to screening for the identification of novel SUMO-binding proteins. Using this assay, we investigate the SIMs in SETDB1 and MCAF1, to gain insight into the assembly of SETDB1-MCAF1-mediated gene silencing.

Keywords: post-translational modification, SUMO, protein interaction, histone methyltransferase, chromatin

Article Outline

SUMO (small ubiquitin-related modifier) is a post-translational protein modifier. It is similar to ubiquitin in terms of its structure and its mechanism of conjugation to target proteins. SUMO is involved in many diverse cellular functions[1, 2]. Recent studies document diverse SUMO-interacting proteins that recognize conjugated SUMO moieties via SUMO-interacting motifs (SIMs), also known as SUMO-binding domains[1-5]. SIMs usually consist of a short stretch of hydrophobic amino acid residues followed by multiple acidic amino acid residues. By analogy to the relationship between ubiquitin and ubiquitin-interacting proteins, it has been speculated that SUMO-SIM protein interactions determine the fate of SUMOylated species.

Biochemical interactions between SUMO and SIM-containing proteins have, to date, been analyzed by the yeast two-hybrid assay and the glutathione S-transferase (GST)-pulldown/immunoblot assay combined with NMR spectrometry[3-5]. Neither procedure is complicated, but they are reasonably time consuming. For example, the yeast two-hybrid assay requires several days to grow yeast colonies and the GST-pull-down/immunoblot assay takes a minimum of 8 hours. In addition, such methods are not sufficiently sensitive to detect low-affinity interactions. For example, in the

two-hybrid assay, SIMs can easily dissociate from SUMO during yeast cell growth and in the GST-pulldown assay, dissociation can occur during the washing steps, potentially yielding false negative results.

We describe here, a fast, economical, simple and small-scale method to detect direct binding between SUMO and SIM-containing proteins. This method is based on the modification of a 'low-affinity protein interaction procedure' or the 'bead halo assay', described by Rexach and colleagues[6, 7]. It consists of only three steps prior to the assessment of binding (**Fig. 1A**): (i) preparation of beads attached with the protein of interest (~30 min), (ii) incubation with green fluorescent protein (GFP)-SUMO (~5 min), and (iii) observation of green fluorescence under the microscope (~10 min). It is simple, rapid, and detects low-affinity interactions in real-time under equilibrium binding conditions. Therefore, we refer to the assay as 'the real-time SUMO-binding assay'. This method is also cheap, as it requires only small quantities of glutathione-Sepharose beads, in addition to standard chemicals and buffers. Typically, only 30 particles of glutathione-Sepharose beads are used in a 4 µl reaction.

Fig. 1

We validated this method by examining GFP-SUMO-3 and GFP for their ability to bind immobilized GST-MBD1-containing chromatin-associated factor 1 (MCAF1)-SIM_{Wt} and GST-MCAF1-SIM_{Mut} [3-5]. Previous data have indicated that MCAF1 is a SIM-containing SUMO-3-interactive protein (Kd = ~1.3 µM)[5]. It was, therefore, expected that GST-MCAF1-SIM_{Wt} would give a positive result and that GST-MCAF1-SIM_{Mut} would give a negative result in the real-time SUMO-binding assay. A 50 ml culture of *Escherichia coli* (*E. coli*) carrying pET28-GFP-SUMO-3 was used to express recombinant SUMO-3 fused to GFP. The bacterial pellet was dissolved in 5 ml of phosphate-buffered saline (PBS, pH 7.0), followed by sonication at maximum output for 30 seconds on ice (TOMY UD201, Tokyo). The sonicated bacterial lysate was centrifuged at 9,100 × g for 5 minutes and the supernatant was stored at –20°C until use. It should be noted that purification of expressed GFP-SUMO-3 is not necessary for this assay, as the supernatant contains a large amount of full-length

recombinant GFP-SUMO-3 (approximately 0.01 mg GFP-SUMO-3/ml of bacterial lysate).

The SIM-containing protein of interest was expressed in *E. coli* as a recombinant GST-fusion protein and was then incubated with glutathione-Sepharose 4B beads (GE Healthcare, Buchinghamshire, UK). We used 10 µl of packed beads for bacterial lysate prepared from 10 ml of bacterial culture. This results in GST-fusion proteins immobilized at high concentrations on the surface of the Sepharose beads. (Approximately 80–90 µg of GST-fusion proteins were bound to 10 µl of packed beads). The beads were resuspended as a 50% slurry in PBS and a 2 µl portion of the slurry, in which approximately 30 particles of beads were included, was mixed, on a 76 × 26 mm Micro Slide Glass, (thickness 1.2 mm, Matsunami, Osaka), with 2.0 µl of bacterial lysate containing recombinant GFP-SUMO-3. As soon as the incubation started, the beads were viewed in real-time under equilibrium conditions using an Eclips E66 fluorescence microscope (Nicon, Tokyo). When GFP-SUMO-3 binds to an immobilized GST-fusion protein of interest, the interaction is visible through GFP filters as a halo of fluorescence around the beads. As shown in **Fig. 1B** and **C**, GFP-SUMO-3 showed strong green fluorescence around the Sepharose beads when incubated with GST-MCAF1-SIM_{Wt} beads. By contrast, incubation of GST-MCAF1-SIM_{Mut} beads with GFP-SUMO-3 did not show any green fluorescence localized to beads. As a further control, we demonstrated that GFP protein alone, with no SUMO-moiety, showed no specific binding signals (data not shown). These results indicate that this method is applicable to detect the interaction of SUMO with the SIM in MCAF1. It should be noted that we also demonstrated that this method can determine protein binding to SUMO-1 as well. For instance, GFP-SUMO-1 localized efficiently to the beads containing Ran binding protein 2 (RanBP2)/Nucleoporin 358 (Nup358), a well-characterized SIM-containing polypeptide that binds to SUMO-1 (data not shown).

We next applied this method to characterize the interaction between SUMO-3 and a histone methyltransferase, SET domain bifurcated 1 (SETDB1). We chose this protein, because we had previously isolated partial cDNA fragments of SETDB1 in a yeast

two-hybrid screen using a GAL4-SUMO3-SUMO3 fusion protein as bait (our unpublished data). Furthermore, another group has recently reported the interaction between SUMO, and SETDB1, suggesting that SETDB1 is a SUMO-binding protein in chromatin-based gene expression[8]. In addition, SETDB1 forms a heterodimer with MCAF1 and a complex of MCAF1 with SETDB1 modulates the histone methylase activity of SETDB1, converting it from an H3-K9 dimethylase to a trimethylase[9]. Therefore, characterization of the binding between SUMO and SETDB1 is likely to contribute to the understanding of the regulation of methyltransferase activity by MCAF1 and to the function of the methyltransferase complex in the context of gene silencing and formation of heterochromatin.

Fig. 2

To determine the region responsible for binding to SUMO2/3, we generated a series of deletion mutants of recombinant SETDB1 fused to GST and performed the real-time SUMO-binding assay (**Fig. 2A** and **B**). To this end, twelve amino acid residues ($SETDB1_{120-131}$), which show similarity to the canonical SIM, a short stretch of hydrophobic amino acid residues followed by multiple acidic amino acid residues[1-5], were found to directly interact with GST-SUMO-3 (**Fig. 2A**). Moreover, we found that the point-mutants in which isoleucine (I) at 122, 123, or 125 was substituted for alanine (A) had remarkably reduced affinity to SUMO-3, suggesting that these amino acid residues were important for SUMO-binding (**Fig. 2B**). It should be noted that GST-d1-1-5-1, but not a SIM-deleted mutant (GST-d1-1-5-delta SIM), bound to SUMO-3 in the *in vitro* pulldown assay (**Fig. 2D**), confirming the *in vitro* interaction observed in the real-time SUMO binding assay described above. Although the structural basis for the interaction between SUMO-3 and the SIM in SETDB1 requires further elucidation, our results indicate a direct molecular linkage of the SUMO pathway with, not only MCAF1, but also SETDB1.

In conclusion, we describe a method which improves upon current *in vitro* SUMO binding assays and we show application of this method, referred to as the real-time SUMO binding assay, for the characterization of MCAF1 and SETDB1, thereby giving an insight into SUMOylation-induced gene silencing. Since this method is simple, rapid and cheap, it

may be particularly applicable for screening novel SUMO-binding proteins, as well as for biochemical analysis of the SUMO-SIM protein interaction network. It is also highly applicable to large-scale screening approaches for the identification of drug(s) that can inhibit or enhance SUMO-SIM interaction.

Acknowledgments

We thank Ms. Yuka Fujimitsu for helping the in vitro binding experiments. This work was supported by grants to H.S from the Naito Foundation and from The Japanese Government Ministry of Education, Culture, Sport, Science and Technology.

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Figure Legends.

Figure 1. Detection of SUMO-SIM interaction by the real-time SUMO binding assay.

(A) Schematic representation of the procedure for the real-time SUMO binding assay.

(B) The entire amino acid sequences of MCAF1-SIM_{Wt} and MCAF1-SIM_{Mut} fused to GST are shown. The position of the mutation (I to A) is underlined. Indicated on the *right* are aliquots of immobilized GST-MCAF1-SIM_{Wt} and GST-MCAF1-SIM_{Mut}, as analyzed by SDS-PAGE and stained with Coomassie blue. Note that equal amounts of GST-MCAF1-SIM_{Wt} and GST-MCAF1-SIM_{Mut}, to be used for the assay in B, were immobilized on the beads.

(C) Bacterial lysate containing 0.01 mg/ml of either GFP-SUMO-3 (*1st* and *2nd* columns) or GFP (*3rd* and *4th* columns) was mixed with the bead-immobilized GST-MCAF1-SIM_{Wt} (*1st* and *3rd* columns) or the bead-immobilized GST-MCAF1-SIM_{Mut} (*2nd*and *4th* columns). An aliquot of the recombinant GFP-SUMO-3 or GFP resolved by SDS-PAGE and stained with Coomassie blue is shown. GFP signal localized to the beads indicates a positive SUMO-SIM interaction (*upper panel*). *Lower-panels* show the phase-contrast images of the Sepharose beads.

Figure 2. Detection of a SIM in SETDB1 by the real-time SUMO binding assay.

(A) Schematic representation of human SETDB1 and a summary of the real-time SUMO binding assay. SETDB1 deletion constructs are represented as *thin black lines*. The *Table* on the right summarizes the interactions of each deletion mutant with GFP-SUMO-3 in the real-time SUMO binding assay. + indicates significant binding, - indicates negligible binding. *Numbers* correspond to amino acid residues in SETDB1. The amino acid sequence of $SETDB1_{120\text{-}131}$ (d1-1-5-1) is represented as SIM. The amino acid residues which are important for SUMO-3 binding are indicated by *dots*.

(B) The real-time SUMO binding assay. Bacterial lysate containing 0.01 mg/ml of

GFP-SUMO-3 was mixed with bead-immobilized GST-SETDB1 deletion mutant proteins. Aliquots of the recombinant GST fusion proteins resolved by SDS-PAGE and stained with Coomassie blue are shown. The mixtures were imaged under a fluorescent microscope.

(C) GST-d1-1-5-1-I122A, -I123A and -I125A mutants abolish SUMO-3-binding in the real-time SUMO binding assay. Bacterial lysate containing 0.01 mg/ml of GFP-SUMO-3 was mixed with bead-immobilized GST- d1-1-5-1 mutant proteins as indicated. An aliquot of each recombinant GST fusion protein, resolved by SDS-PAGE and stained with Coomassie blue, is shown.

(D) GST-d1-1-5-1, but not GST--d1-1-5-deltaSIM, binds His_6-SUMO-3 in the *in vitro* pulldown assay. GST-d1-1-5-1 or GST--d1-1-5-deltaSIM was incubated with beads containing 5 μg of His_6-SUMO-3. Following incubation, pull-down assays were carried out[4)], and proteins associated with the beads were analyzed by immunoblot analysis using anti-GST antibody. Coomassie Brilliant Blue (CBB) stained gel is shown at the bottom to show amount of the input proteins.

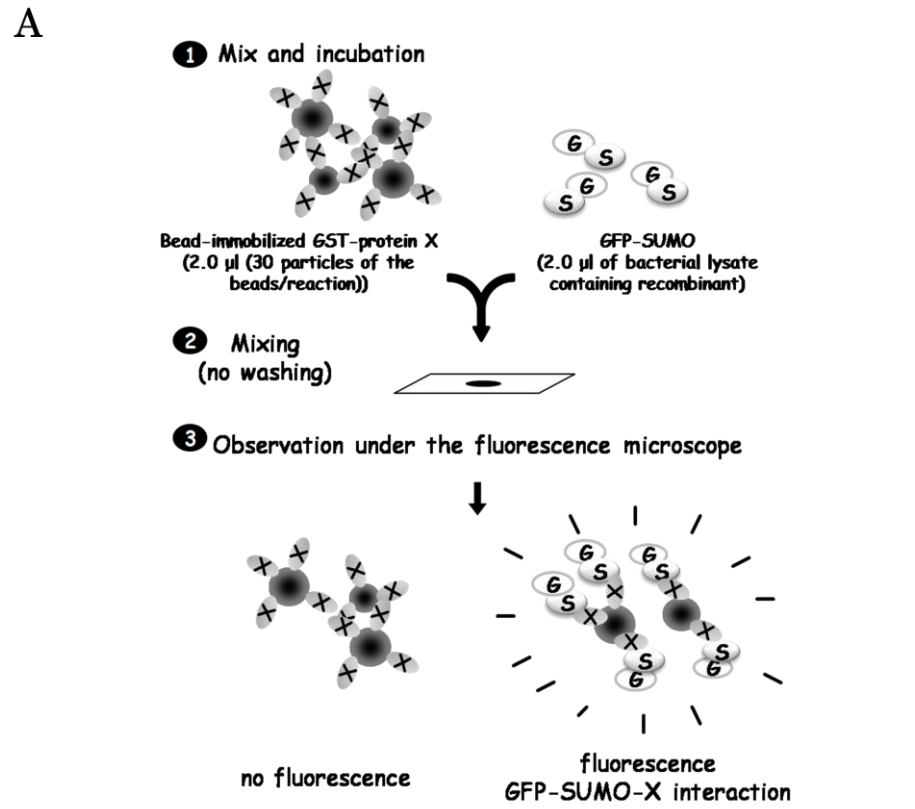

Figure 1. Tanaka & Saitoh

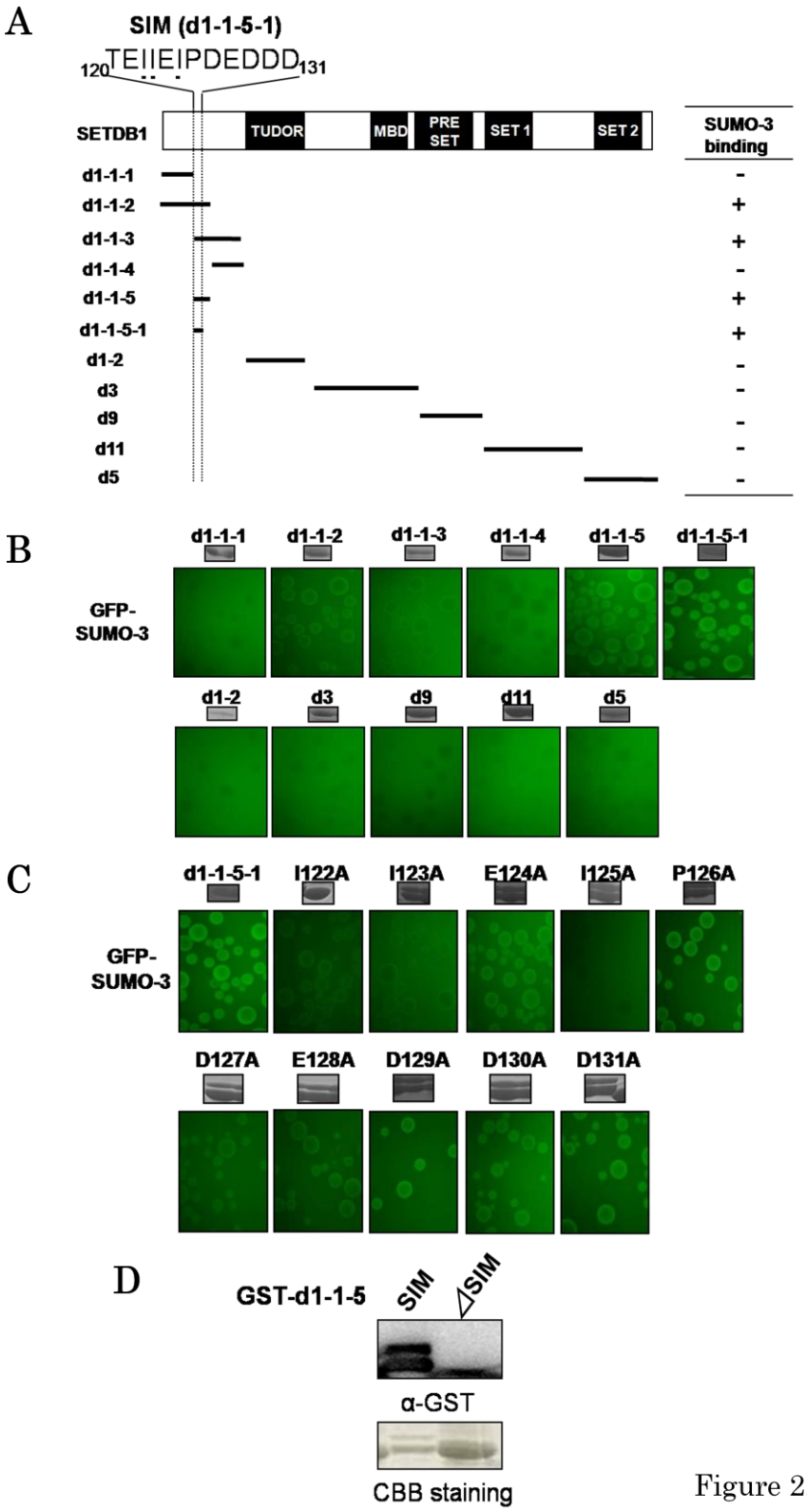

Figure 2. Tanaka & Saitoh

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