



Analysis of *in vitro* SUMOylation using bioluminescence resonance energy transfer (BRET)

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ARTICLE INFO

Article history:

Received 2 March 2009

Available online 14 March 2009

Keywords:

SUMO
Energy transfer
BRET
FRET
RanGAP
Luciferase

ABSTRACT

We demonstrated *in vitro* small ubiquitin-like modifier (SUMO)-mediated modification (SUMOylation) of RanGTPase activating protein-1 (RanGAP1) by using bioluminescence resonance energy transfer (BRET) for studying protein interactions. *Renilla* luciferase (Rluc) was fused to SUMO, and RanGAP1, the binding partner of SUMO, was fused to enhanced yellow fluorescence protein (EYFP). Upon binding of SUMO and RanGAP1, BRET was observed between EYFP (donor) and Rluc (acceptor) in the presence of E1 (Aos1/Uba2) and E2 (Ubc9) enzymes, whereas mutation (K524A) of RanGAP1 at its SUMO binding site prevented significant energy transfer. Comparing BRET and fluorescence resonance energy transfer (FRET) efficiencies using this *in vitro* model system, we observed that BRET efficiency was 3-fold higher than FRET efficiency, due to the lower background signal intensity of EYFP in the BRET system. Consequently, BRET system is expected to be useful for *in vitro* analysis of SUMOylation as well as studying other protein interactions.

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Introduction

Post-translational modification of proteins by small ubiquitin-like modifier (SUMO) is a key regulatory mechanism functioning during diversity cellular processes such as nuclear transport, gene expression, cell division, and endocytosis [1–3]. A number of SUMO protein binding targets have thus far been identified [4,5]. Despite increasing evidence correlating SUMOylation of targeted proteins with specific physiological effects, analysis of SUMOylation still relies mostly on gel electrophoresis, which is laborious, time-consuming, and is not amenable to high-throughput screening for target proteins or inhibitors.

With the recent progress of fluorescence-based techniques, much attention has been paid to resonance energy transfer (RET) techniques to characterize *in vitro* and *in vivo* protein–protein interactions [6,7], and these techniques allows simple and sensitive signal detection amenable to high-throughput screening. In particular, fluorescence resonance energy transfer (FRET) experiments have effectively detected the association of ubiquitin [8] or SUMO [9,10] with their target proteins. However, the full potential of FRET methods are often limited due to photobleaching, auto-

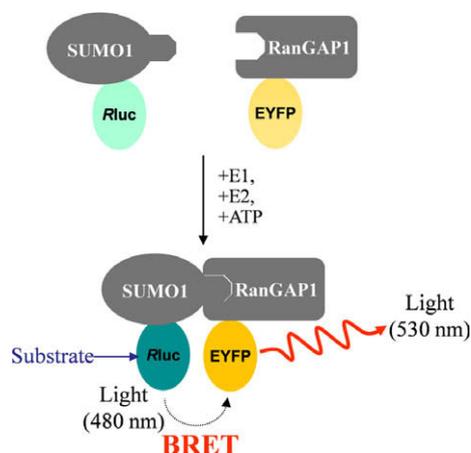
fluorescence and high residual excitation of the acceptor fluorophore [11], complications intrinsic to FRET systems. Bioluminescence resonance energy transfer (BRET) methods have been developed to overcome some limitations of FRET [12,13]. Compared to FRET, which often uses two fluorescent proteins, BRET methods do not require external excitation and therefore have relatively low background signal intensities, allowing for more sensitive detection of energy transfer during experiments.

While a few groups have used BRET to study protein–protein interactions [14–16], BRET methods have not been applied to study SUMOylation, a protein–protein interaction requiring multiple enzyme participants. Furthermore, the general utility of BRET methods has not been fully tested for studying multiple protein interactions that may exist within larger protein complexes. Therefore application of BRET to study SUMOylation provides a valuable model system for comparing BRET and FRET efficiencies during protein modification studies, as well as a guide for choosing between RET-based techniques for studying other biological systems, *in vitro* or *in vivo*.

Here we use BRET to measure an *in vitro* SUMOylation event. A known SUMO target, RanGTPase activating protein-1 (RanGAP1) is used. Components of the BRET system include *Renilla* luciferase (Rluc) as the energy donor and enhanced yellow fluorescence protein (EYFP) as the energy acceptor. Rluc was fused to SUMO and EYFP was fused to RanGAP1. This model system is depicted in Scheme 1. BRET efficiencies were determined in the presence of E1 (Aos1/Uba2) and E2 (Ubc9), enzymes required for SUMOylation of RanGAP1.

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Scheme 1. Schematic representation of SUMOylation of RanGAP1 based on BRET system. The generation of BRET signal is dependent on conjugation of the SUMO1 protein with the target (RanGAP1) via the energy-consuming E1/E2 enzyme reactions. Once the recombinant SUMO1 is sensitized via a substrate-induction, the strong emission of acceptor EYFP-tagged RanGAP1 is generated by the energy transfer from the donor to the acceptor.

Materials and methods

Plasmid construction. The cDNA sequence encoding the *Renilla* luciferase was amplified from the plasmid pHRI-CMV (Promega) by using polymerase chain reaction (PCR). The forward primer (5'-CGC GGA TCC ATG GCT TCC AAG GTG TAC GAC CCC-3') included a *Bam*HI cloning site while the reverse primer (5'-CGG GGT ACC CTG CTC GTT CTT CAG CAC GCG CTC-3') included a *kpn*I site. The cDNA sequence encoding the SUMO-1(G) protein was amplified from a pGEX4T-1-SUMO-1 plasmid (kindly provided by the Molecular Genetics Lab in KAIST), employing two primers with *kpn*I and *HIND*III sites (forward 5'-CGG GGT ACC ATG TCT GAC CAG GAG GCA AAA C-3' and reverse 5'-GTA CCC AAG CTT CTA AAC TGT TGA ATG ACC CC-3'). To construct a fusion gene of Rluc-SUMO-1 inside of a pRSET-A expression vector (Invitrogen), the gene for Rluc was first cloned into the pRSET-A vector between the *Bam*HI and *kpn*I restriction enzyme sites. Next the gene for SUMO-1 was cloned into the same pRSET-A vector, already containing Rluc, between the *kpn*I and *HIND*III restriction enzyme sites, creating a pRSET-A vector containing a Rluc-SUMO-1 fusion protein with an upstream hexahistidine tag.

Human RanGAP1 and Ubc9 cDNAs were obtained by reverse transcription PCR using total RNA extracts from HeLa cells as templates. The gene for RanGAP1 domain protein (RanGAP1D or RanGAP1^{418–587}) was amplified by PCR and cloned into a pGEX4T-1 vector (Amersham Biosciences) between the *Bam*HI and *Sall* restriction enzyme sites. The gene for Ubc9 was also cloned into a pGEX4T-1 vector between the *Sall* and *Not*I restriction enzyme sites. cDNA encoding RanGAP1D with a single mutation (K524A) was generated by PCR-directed mutagenesis and subcloned into a pGEX4T-1 vector between the *Bam*HI and *Sall* restriction enzyme sites. The plasmid pGEX4T-3, containing the E1 gene encoding heterodimeric SUMO-activating enzyme (SAE1/SAE2), was a kind gift from Dr. Hay (University of St. Andrews, UK).

To construct the fusion gene for enhanced cyan fluorescence protein (ECFP)-SUMO1 in an *Escherichia coli* expression vector, cDNAs containing the ECFP coding sequence were amplified from the pMAL-C2X-ECFP plasmid, provided by the KAIST Bioengineering Laboratory, by using PCR with two primers (forward 5'-GAT GGA TCC ATG GTG AGC AAG GGC-3' and reverse 5'-CTT GTA CAG CTC GTC-3'). The SUMO1 fragment was also generated from pGEX4T-1-SUMO1 by using PCR with two primers (forward 5'-GAC GAG CTG TAC AAG GGA GGT ATG TCT GAC CAG GAG-3' and

reverse 5'-GTT AGT CGA CTC ATC AAC CCC CCG TTT GTT C-3'). This was followed by amplifying ECFP-SUMO1 from the above template (ECFP and SUMO1) by using overlapping PCR with two primers (forward 5'-GAT GGA TCC ATG GTG AGC AAG GGC-3' and reverse 5'-GTT AGT CGA CTC ATC AAC CCC CCG TTT GTT C-3'). The PCR product was cloned into pQE30 expression vector (Qiagen) between the *Bam*HI and *Sall* restriction enzyme sites.

EYFP-RanGAP1D was also constructed from pMAL-C2X-EYFP and pGEX4T-1-RanGAP1 plasmids by using overlapping PCR. The primers were subsequently used to generate the DNA fragments for EYFP (forward 5'-GAT GGA TCC ATG GTG AGC AAG GGC-3' and reverse 5'-CTT GTA CAG CTC GTC-3'), RanGAP1D (forward 5'-GAC GAG CTG TAC AAG GGA GGT AAC ACT GGG GAG CCA-3' and reverse 5'-GTT AGT CGA CCT ACT AGA CCT TGT ACA G-3'), and EYFP-RanGAP1D (forward 5'-GAT GGA TCC ATG GTG AGC AAG GGC-3' and reverse 5'-GTT AGT CGA CCT ACT AGA CCT TGT ACA G-3'). EYFP-RanGAP1D^{K524A} was amplified from pGEX4T-1-RanGAP1D^{K524A} and subcloned into the pQE30 expression vector, similarly to the procedure followed for EYFP-RanGAP1D.

Protein expression and purification in *E. coli*. Unless stated otherwise, *E. coli* BL21(DE3) cells transformed with either pRSET-A, pGEX4T-1/3 or pQE30 vector were grown at 37 °C in 500 mL Luria-Bertani broth containing 100 µg mL⁻¹ ampicillin with reciprocal shaking (180 rpm/min) until optical density reached 0.5. For induction of fusion protein expression, 0.5 ~1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Duchefa Biochemie) was added to cultures, which were further incubated at 20 °C for 16 h (or 30 °C for 3 h) after IPTG administration, allowing time for fusion proteins to be expressed. A GST-fused heterodimeric SAE1/SAE2 protein was over-expressed in *E. coli* Rosetta cells by induction with 0.1 mM IPTG at 30 °C for 3 h.

Cells used for protein expressions were harvested by centrifugation and suspended in 25 mL of lysis buffer (20 mM Tris-Cl, 0.5 mM EDTA, 1× protease inhibitor cocktail (Roche), pH 8.0), followed by sonication (a pulse length of 1 min × 4 times). After centrifugation (12,000g) for 30 min at 4 °C, supernatants were collected and further purified by using either a Glutathione SepharoseTM 4B column (Amersham Biosciences) or Ni-NTA Superflow column (Qiagen), according to the manufacturers' instructions. GST-fused proteins GST-SUMO1, GST-SAE1/SAE2 and GST-E1 were eluted from a Glutathione column equilibrated in 50 mM Tris-HCl (pH 7.5) containing 30 mM glutathione and 120 mM NaCl. The GST-cleaved forms of Ubc9 and SUMO1 were acquired by on-column digestion with thrombin and removal of thrombin according to the protocols provided by manufacturers. For the purification of hexahistidine tagged proteins (Rluc-SUMO1, ECFP-SUMO1, EYFP-RanGAP1D and EYFP-RanGAP1D^{K524A}), 5 mL of the supernatant was mixed with 1 mL of 50% Ni-NTA Superflow (Qiagen) equilibrated in 20 mM Tris-Cl buffer. The mixture was incubated for 1 h at 4 °C with reciprocal shaking to promote efficient binding. For purification, the lysate-Ni-NTA mixture was loaded into a column (1.5 × 5 cm) with the bottom outlet capped, and the flow-through sample was collected just after removing the bottom outlet. The column was rinsed twice with the washing buffer (20 mM Tris-Cl, pH 8.0, 300 mM NaCl, 20 mM imidazole) and the protein was eluted from the column using elution buffer (20 mM Tris-Cl, pH 8.0, 300 mM NaCl, 250 mM imidazole). The eluted fractions were analyzed by SDS gel electrophoresis and the purified fractions of all proteins were concentrated and exchanged into 20 mM HEPES buffer by using a Microcon (YM-10, YM-30, or YM-100, Millipore), selected according to the molecular weight of each protein. All purified proteins were divided into separate aliquots and then stored at 70 °C. Protein concentrations were determined by using the Bradford method according to the manufacturer's instructions.

In vitro SUMOylation. *In vitro* SUMOylation was performed using expressed SUMO1, RanGAP1, and other components (E1 and E2

enzymes) as described elsewhere [17,18]. The reaction was little modified by using the fusion proteins Rluc-SUMO1 and EYFP-RanGAP1D instead of SUMO-1 and RanGAP1. As a negative control, EYFP-RanGAP1D^{K524A} and E1/E2-deficient conditions were used and the reaction products analyzed by SDS–PAGE. The reactions were incubated at 37 °C for 3 h in a standard test tube, and performed in a total volume of 100 µL with the following reagent concentrations: 2 µM Rluc-SUMO1, 2 µM EYFP-RanGAP1D (or EYFP-RanGAP1D^{K524A}), 0.3 µM SAE1/SAE2, 0.3 µM Ubc9, 5 mM MgCl₂, 50 mM NaCl, 5 mM ATP, and 0.1 mM dithiothreitol (DTT) in 50 mM HEPES buffer (pH 7.4).

Measurement of bioluminescence and fluorescence. Spectral measurement of bioluminescence of each reaction mixture was performed by using a multi-well plate reader (SpectraMax M5, Molecular Devices, U.S.A.). After 1 µL of coelenterazine-*h* (Promega), the substrate of the Rluc enzyme, was added to each reaction mixture, the resulting solution was promptly transferred into a 96-well plate and measured automatically without excitation within at most 5 min after addition of coelenterazine-*h*. A final concentration of coelenterazine-*h* was 5 µM (ca. 2 µg mL⁻¹) per well. To image the *in vitro* SUMOylation reaction mixtures, we used a luminescent image analyzer (LAS-3000, Fujifilm, Japan), selecting the optimal filter set for FRET or BRET imaging. For imaging using the BRET system, coelenterazine-*h* (final concentration of 5 µM) was added to each reaction mixture just prior to fluorescence measurement. Bioluminescence images were obtained by integrating the fluorescence signal intensities collected during six 10-s measurement intervals (60-s total measurement time). The signal intensities for BRET or FRET fluorescence experiments were analyzed using an imaging software (Multi Gauge ver 3.0, Fujifilm), and the mean values and standard deviations were calculated from duplicate experiments.

Calculation of BRET or FRET efficiency. Excitation-free bioluminescence was obtained immediately after substrate addition, whereas FRET fluorescence was obtained after direct excitation of reaction mixtures at 460 nm. Donor only systems (without energy transfer acceptors) were used as controls in the BRET and FRET experiments. BRET efficiency was calculated from the ratio of the BRET emission to total emission while FRET efficiency was calculated from the ratio of FRET emission to total emission of donor and acceptor as followings:

$$\text{BRET efficiency} = \frac{Em_{515}}{Em_{\text{Total}}} - \frac{Em_{515}(\text{Cont})}{Em_{\text{Total}}(\text{Cont})} \quad (1)$$

where Em_{515} and $Em_{515}(\text{Cont})$ are the emission at 515 nm from sample and donor-only control group, respectively; Em_{Total} and $Em_{\text{Total}}(\text{Cont})$ are total emission at all wavelengths from sample and donor-only control, respectively.

$$\text{FRET efficiency} = \frac{Em_{460/515}}{Em_{460/515} + Em_{460/480}} - \frac{Em_{460/515}(\text{Cont})}{Em_{460/515}(\text{Cont}) + Em_{460/480}(\text{Cont})} \quad (2)$$

where $Em_{460/515}$ and $Em_{460/515}(\text{Cont})$ are the emission at 515 nm for 460 nm excitation from sample and donor-only control group, respectively; $Em_{460/480}$ and $Em_{460/480}(\text{Cont})$ are the emission at 480 nm for 460 nm excitation from sample and donor-only control groups, respectively.

Results and discussion

Overview of SUMOylation

As Scheme 1 illustrates, SUMOylation of a target protein, RanGAP1, was monitored by using a BRET system. As tagging proteins,

Renilla luciferase (Rluc) was fused to the N-terminal of SUMO1 while enhanced yellow fluorescent protein (EYFP) was fused to the N-terminal of RanGAP1. In this BRET system, Rluc-tagged SUMO1 (donor) typically generates its own bioluminescence at 480 nm in the presence of the Rluc substrate, coelenterazine-*h*. As SUMOylation occurs with the assistance of enzymes, the donor and acceptor proteins in the energy transfer process are brought into close proximity. Consequently, a resonance energy transfer from the donor, Rluc-SUMO1, to the acceptor, EYFP-RanGAP1, takes place, resulting in strong EYFP emission at 530 nm. SUMO, similarly to ubiquitin, is covalently attached to target proteins through a reaction cascade that involves an E1 activating enzyme, the heterodimer Aos1/Uba2, and an E2 conjugating enzyme, Ubc9 [3].

Analysis of *in vitro* SUMOylation using SDS–PAGE

To detect conjugation of SUMO to its target, we examined the relative migration of SUMOylation reaction products on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). To construct BRET couples, SUMO and its target protein, RanGAP1D, the carboxyl-terminal domain (418–587) of full-length RanGAP1, were tagged with the BRET donor, Rluc, and RET acceptor, EYFP, respectively. As a control, we also eliminated the SUMOylation interaction by mutating Lys 524 in the RanGAP1D to Ala via site-direct mutagenesis (RanGAP1D^{K524A}). Lys 524 is a required part of the consensus motif $\psi\text{K}\chi\text{E}$ recognized by the E2 enzyme during SUMOylation. Additional controls included performing the SUMOylation reaction without the target protein or without E1/E2 enzymes.

Fig. 1 shows that the SUMOylation reaction worked as expected. Rluc-SUMO1 was well conjugated with EYFP-RanGAP1D in the presence of E1/E2 enzymes and additives (ATP, salt, and detergent), producing a new band for the SUMOylation product in lane 4 of representative gel. The molecular weights of Rluc-SUMO1 and EYFP-RanGAP1D are 53 kDa and 47 kDa, respectively, as confirmed by gel electrophoresis. We presume that the new band corresponding to a molecular weight of approximately 100 kDa (lane 4) is consistent with the expected molecular weight of the covalently bound SUMOylation product between Rluc-SUMO1 and EYFP-RanGAP1D.

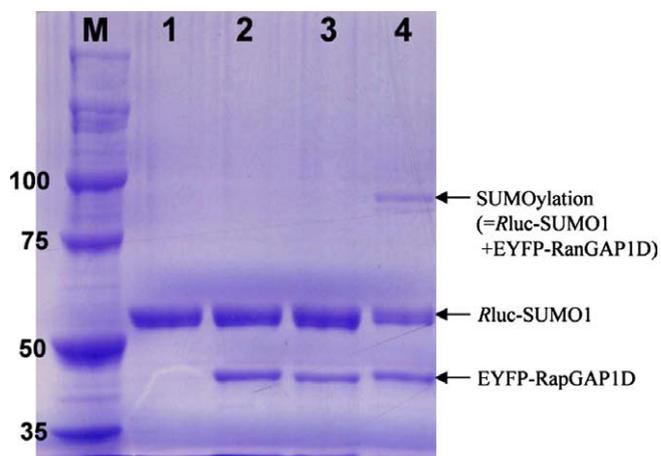


Fig. 1. SDS–PAGE analysis of SUMOylation using BRET couples. Rluc-tagged SUMO1 was incubated with recombinant EYFP-RanGAP1D (lane 4) or its mutant (EYFP-RanGAP1D^{K524A}, lane 3). As other negative controls, SUMOylation was performed in the absence of EYFP-RanGAP1D (lane 1) and E1/E2 enzymes (lane 2). The arrows indicate the position of expressed proteins and SUMOylated conjugates, and molecular weight standard (M) was displayed in far left lane. The reaction mixture was incubated for 2 h at 37 °C in the presence of additives containing 5 mM MgCl₂, 50 mM NaCl, 5 mM ATP, and 0.1 mM DTT in 50 mM HEPES.

Mutant EYFP-RanGAP1D^{K524A} showed no 100 kDa band with Rluc-SUMO1 (lane 3), confirming that Lysine 524 of RanGAP1D is necessary for SUMOylation, as mentioned earlier [19,20], and verifying that SUMOylation in our *in vitro* system occurs where expected on the RanGAP1D target protein in the absence of a L524A mutation. Other control lanes without EYFP-RanGAP1D (lane 1) or E1/E2 enzymes (lane 2) also did not display the SUMOylation product band. These results clearly demonstrate that SUMOylation of tagged RanGAP1D can be successfully accomplished *in vitro*.

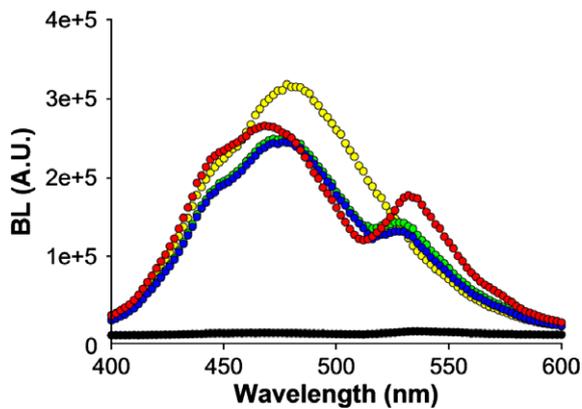


Fig. 2. Emission spectra of *in vitro* SUMOylation based on BRET. Purified Rluc-SUMO1 was used as an energy donor in BRET. The SUMOylation of the acceptor was compared between recombinant EYFP-RanGAP1D (red) and its mutant type (EYFP-RanGAP1D^{K524A}, blue) in the presence of E/E2 enzymes and additives. Other control set were also displayed with donor only (yellow), without E1/E2 enzymes (green), and with no tagged SUMO (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Analysis of *in vitro* SUMOylation using BRET and FRET

After SUMOylation of Rluc-SUMO1 to EYFP-RanGAP1, spectral changes were observed (Fig. 2) showing significantly greater emission intensity at 530 nm (EYFP emission) coupled with a lower signal at 460 nm (Rluc emission), in other words, SUMOylation brought the donor and acceptor close enough together in space to allow BRET. In contrast, the use of the mutant SUMOylation target EYFP-RanGAP1D^{K524A} (Fig. 2, blue) or the absence of E1/E2 enzymes (Fig. 2, green) showed relatively small changes in emission intensity at 530 nm. As other negative controls, Rluc-SUMO1 alone did not generate the emission shoulder at 530 nm (Fig. 2, yellow), and the SUMOylation product between free SUMO1, without fusion to Rluc, and EYFP-RanGAP1D showed no emission in BRET (Fig. 2, black). These results confirm that SUMOylation via the active binding domain of RanGAP1 enables the BRET from Rluc to EYFP.

We also attempted SUMOylation on a multi-well plate to check the feasibility of high-throughput screening for SUMOylation events. For comparison, FRET couples, ECFP-SUMO1 and EYFP-RanGAP1D, were introduced. The BRET and FRET efficiencies were calculated according to Eqs (1) and (2) (Fig. 3). The greatest energy transfer efficiency was observed for the SUMOylation product of the Rluc-SUMO1 with EYFP-RanGAP1D (Fig. 3A). During BRET experiments, bioluminescence of the donor, Rluc, was observed equally in all of the wells (Fig. 3A, a1 and a4), whereas fluorescence intensity the BRET acceptor, EYFP, was strongly observed only in well of a4. In particular, the change in energy transfer between the recombinant EYFP-RanGAP1D and its mutant type EYFP-RanGAP1D^{K524A} was clearly discerned (Fig. 3A, a3 and a4), again indicating that the consensus binding site including K524 of RanGAP is necessary for SUMOylation, which is required to observe BRET. In the case of FRET data (Fig. 3B), the fluorescent intensities from

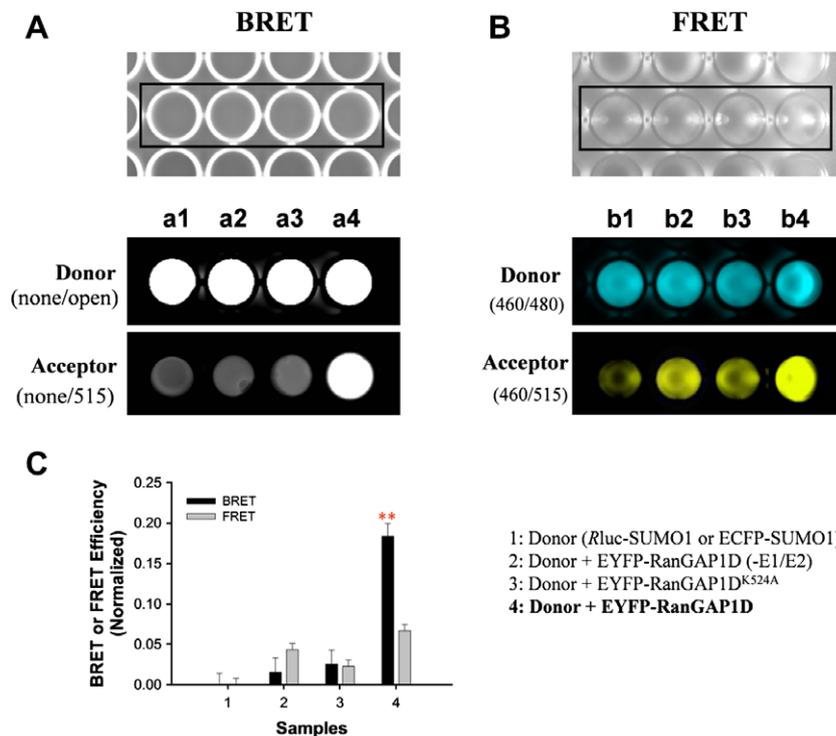


Fig. 3. Imaging of *in vitro* SUMOylation based on the BRET (A) and the FRET (B), and the corresponding energy transfer efficiencies (C). Multiwell plate images were obtained from a reaction solution in a 96-well plate. Purified Rluc-SUMO1 and ECFP-SUMO1 were used as energy donors in BRET and FRET, respectively. The SUMOylation of the acceptor was compared between recombinant EYFP-RanGAP1D (well 4) and its mutant type (EYFP-RanGAP1D^{K524A}, well 3) in the presence of E1/E2 enzymes and additives. Other control set were also displayed with donor only (well 1) and without E1/E2 enzymes (well 2). Bioluminescence was measured immediately after adding the substrate (coelenterazine-*h*). The error bars indicate the standard deviations in duplicate experiments.

the donor ECFP-SUMO1 and its conjugated acceptor EYFP-RanGAP1D (Fig. 3B, b4) did not seem to generate significant energy transfer, when compared to control wells (Fig. 3B, b1, b2, and b3). Specifically, the absence of E1/E2 enzymes during SUMOylation (Fig. 3B, b2) showed high EYFP intensity, similarly to well of b4, but this intensity presumably resulted from the high background signal that is generally caused by cross talk (abnormal response of the acceptor to the donor excitation) during FRET experiments. It is indeed well known that BRET systems offer the advantage of lower background noise compared to FRET systems [6,21]. Consistent with this advantage and data shown in Fig. 3C, the maximum BRET efficiency (18.4%, asterisk) we observed was approximately 3-fold higher than the maximum FRET efficiency (6.7%) observed, suggesting that BRET systems may be more effective than FRET systems for screening *in vitro* post-translational modifications such as SUMOylation.

Considering some disadvantages of FRET, namely, autofluorescence, photobleaching and cross-talk, BRET may be preferred for high-throughput *in vitro* or cellular assays. It was reported that BRET has a great potential for use during high-throughput immunoassays [22] or protease assays [23]. These papers demonstrated that the sensitivity of BRET is 40- to 50-fold higher than that of FRET in these assays. Based on our results and these published reports, we believe that BRET-based approaches can be used to study SUMOylation, other protein modifications, and intermolecular interactions with high sensitivity.

In conclusion, we developed an *in vitro* BRET-based detection system of SUMOylation that is confirmed by gel electrophoresis and that performs better than an analogous FRET system designed to detect SUMOylation under identical conditions. Resonance energy transfer took place when the donor molecule, Rluc-SUMO1, was conjugated to the acceptor molecule, EYFP-RanGAP1D, with the assistance of E1/E2 enzymes, whereas mutated EYFP-RanGAP1D^{K524A} resulted in negligible energy transfer. Without requiring any external photoexcitation, our BRET system showed 3-fold higher RET efficiency than an almost identical FRET system. We anticipate that the BRET-based analysis will be useful for studying post-translational modification of proteins with high sensitivity.

Acknowledgments

This work was supported by the Pioneer Research Program for the Converging Technology, the Nano Science & Technology Program (M10503000868-08M0300-86810), the Nano/Bio Science & Technology Program (2005-01291), the Brain Korea 21 Program, and WCU program (R31-2008-000-10071-0) of MEST, the Korea Health 21C R&D Project (0405-MN01-0604-0007) of MIHWAF, and the Next-Generation New-Technology Development Program for MKE.

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