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A Homogeneous and Noncompetitive Immunoassay Based on the Enhanced Fluorescence Resonance Energy Transfer by Leucine Zipper Interaction

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Fluorescence resonance energy transfer (FRET) between two GFP variants is a powerful technique to describe protein−protein interaction in a biological system. However, it has a limitation that the two variants tethered to the respective proteins have to be in sufficient proximity upon binding, which is often difficult to attain by simple N- or C-terminal fusions. Here we describe a novel method to significantly enhance FRET between GFP variant-tagged proteins with the use of leucine zippers. For the homogeneous sandwich immunoassay of a high molecular weight antigen human serum albumin (HSA), two separate single-chain Fvs recognizing distant epitopes of HSA were respectively fused with fluorescence donor ECFP or acceptor EYFP, and FRET between the two was analyzed by fluorescence spectrometry. Because these two proteins did not give any detectable FRET upon antigen addition, we tethered each protein with a leucine zipper motif (c-Jun or FosB) at the C-terminus to help the neighborhood of the GFP variants. Upon antigen addition, the new pairs showed significant antigen-dependent FRET. By exchanging the binding domains, the method will find a range of applications for the assay of other proteins and their interactions in vitro or in vivo.

Green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria*, emits intrinsic intense and stable greenish fluorescence without any additional cofactors.[1] Since the advent of gene cloning, it has been used as a fluorescent tag. Fluorolabeling with GFP has several merits as compared with chemical labeling with generally used fluorescent dyes. The chief advantage is the utilization of the gene fusion technique. It enables one to prepare a fluorolabeled protein as a chimeric protein. Since GFP tolerates N- or C-terminal fusion with many kinds of proteins, it is possible to prepare an efficient and site-specific fluorolabeled protein. Also, genetic labeling facilitates the preparation of fluorolabeled protein by employing the quick *Escherichia coli* cultivation, which eliminates troublesome chemical labeling steps. Furthermore, for in vivo assay, because GFP and their chimera can be easily expressed in many kinds of organisms, fluorolabeled protein could be transferred into cells as a fusion gene, which obviates microinjection of the recombinant proteins. Due to these merits, many kinds of applications both in vivo and in vitro have been reported.[2] Furthermore, the developments of the color variants of GFP having distinct spectral properties have made it possible to exploit fluorescence resonance energy transfer (FRET) between the variants.[3,4]

FRET is a nonradiative exchange of energy between two fluorophores.[5] When the two fluorophores with their emission and excitation spectra overlapping are within or near Förster distance, R_0, the donor fluorophore transfers its excited-state energy to another acceptor fluorophore. Because this phenomenon occurs under physiological conditions, FRET has been widely used to study biological systems. One of the most popular applications among them is monitoring of protein−protein interaction. In the case of FRET based on GFP color variants (GFP-based FRET), the interaction between the proteins of interest fused to a donor or acceptor GFP variant could be quickly detected by monitoring the fluorescent spectra. Until now, a number of important protein−protein interactions in vivo have been verified by employing GFP-based FRET.[6,7] Recently, we have also shown that it could be applied to a homogeneous immunoassay, where antigen-induced neighboring of antibody V_H/V_L fragments was successfully monitored as a measure of antigen concentration.[8]

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Nevertheless, albeit these successes, GFP-based FRET is not always guaranteed to work. For example, in some cases, chimeric proteins might no longer interact with each other due to steric hindrance accompanied by the GFP fusion. This problem could be overcome by empirical trial of all the combinations of N- and C-terminal fusions of GFP color variants. However, in other cases, sufficient energy transfer might not be induced because the proximity of two GFP variants within the complex is not ensured by N- or C-terminal fusion of them. In such cases, since the efficiency of energy transfer depends on the inverse sixth power of the distance between the two fluorophores, a means to help them achieve proximity would significantly enhance the FRET signal.

Here we describe a potentially general method that can resolve these problems. We attempted to develop a homogeneous immunoassay of a protein antigen, human serum albumin (HSA) by employing GFP-based FRET. For this purpose, two separate single-chain Fvs (ScFvs) recognizing distant epitopes of HSA were each fused with fluorescence donor ECFP or acceptor EYFP, respectively. However, it was expected that the distance between the two GFP variants within the antigen−antibody complex (Ab−Ag−Ab′ complex) would not be sufficiently short for the FRET between them because of the large dimension of HSA and hence the possible distance between the two ScFv epitopes. Therefore, a careful molecular design of antibody−GFP chimera was attempted, considering the following points. First, a long flexible linker was placed between each ScFv and GFP color variant, to give the molecule sufficient flexibility. Second, each of them was C-terminally tethered with an artificial dimerization motif of leucine zippers, to pull the neighboring GFP color variants in closer proximity upon Ab−Ag−Ab′ complex formation. Leucine zipper motif in which five leucine residues are spaced periodically at every seven residues is conserved among all members of the *jun* and *fos* gene families[9] and is known to mediate their dimerization. In this assay, the two GFP variants with increased flexibility due to the flexible linker were expected to be juxtaposed by leucine zipper interaction within the complex, enabling sufficient FRET.

According to previous studies, the order of the stability of leucine zipper interaction was found to be Jun/Fos > Jun/Jun > Fos/Fos.[10,11] Hence, by varying the combination of leucine zippers, we could control the interaction of two GFP variants within the Ab−Ag−Ab′ complex. In this study, we evaluated the effect of the leucine zipper interaction on antigen-dependent FRET by varying the combination of leucine zippers. Furthermore, using favorable pairs, we established a sandwich-type noncompetitive and homogeneous immunoassay for HSA.

EXPERIMENTAL SECTION

Apparatus. Fluorescence spectral measurement was performed using fluorescence spectrophotometer F-2000 (Hitachi, Tokyo, Japan) with a quartz crystal cuvette. Polymerase chain reactions were performed on a DNA thermal cycler PV-2000 (Takara, Kyoto, Japan). DNA sequencing was performed using DNA sequencer SQ-5500 (Hitachi, Tokyo, Japan).

Plasmid Construction. Two hybridoma cells producing mouse monoclonal antibodies specific for HSA, termed No. 11 and No. 13, were established previously. The cells were cultured in RPMI 1640 medium supplemented with 10% FCS. After 7 days, $\sim 10^7$ cells were collected by centrifugation. mRNA was isolated using a QuickPrep Micro mRNA purification kit (Amersham Biosciences, Tokyo, Japan), and a cDNA library was synthesized with a First-Strand cDNA synthesis kit (Novagen, Madison, WI). Using antibody-variable region-specific primer sets and the cDNA library as a template, single-chain Fv genes of both antibodies were amplified by PCR and cloned into pCANTAB-5E plasmid using the mouse ScFv module/recombinant phage antibody system (Amersham Biosciences). Competent *E. coli* TG1 cells are transformed with pCANTAB-5E containing antibody ScFv gene following standard protocol and spread on a SOB plate (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 0.95 g/L $MgCl_2$, 15 g/L agar) containing 100 μg/mL ampicillin and 2% glucose. After overnight culture, colonies were harvested and transferred to 5 mL of YT medium (8 g/L tryptone, 5 g/L yeast extract, 2.5 g/L NaCl, pH 7.0) containing 100 μg/mL ampicillin and 2% glucose. Bacteria were grown at 37 °C to an OD_{600} of 0.5. To rescue the phagemid, the cultures were left to stand at 30 °C for 1 h after addition of 6.0×10^{11} pfu M13KO7 helper phage (Stratagene, La Jolla, CA). Then 100 μL of the culture was transferred to a 5 mL of YT medium containing 100 μg/mL ampicillin and 50 μg/mL kanamycin. Following overnight culture at 30 °C, the culture was clarified by centrifugation and phage was precipitated using 2.5 M NaCl/20% PEG8000. Then it was resuspended in 8 mL of YT medium and stored at 4 °C until use. For the panning, we prepared immunotubes immobilized with antigen. HSA (Sigma, St. Louis, MO) was coated at a concentration of 100 μg/mL in PBS (1.48 g/L Na_2HPO_4, 0.43 g/L KH_2PO_4, 7.2 g/L NaCl, pH 7.2) in 5-mL immunotubes (Nunc) by incubation at 37 °C for 2 h. The tubes were blocked for 2 h at 37 °C with PBS containing 2% skim milk (MPBS), and rinsed 5 times with PBS. A 1.5-mL aliquot of YT medium containing $\sim 1.5 \times 10^{11}$ cfu phage was mixed with 1.5 mL of PBS containing 10% skim milk. Mixed solutions were incubated at room temperature for 10 min and added into immunotubes immobilized with HSA. Then they were left at 37°C for 1 h. Following 20 times washing with PBS containing 0.05% Tween-20, 2 mL of TG1 cells was added to the tubes and then incubated at 37 °C for 1 h. Serially diluted cultures were spread on SOB plates containing 100 μg/mL ampicillin and 2% glucose. Following overnight incubation at 37 °C, 20 clones were selected and phages for each clone were prepared in a similar manner as described above. The display efficiency of ScFvs on phage was determined by ELISA. The 200-μL aliquots of PBS containing HSA at concentrations of 0 or 100 μg/mL were added into microtiter plate wells. After incubation at 37 °C for 2 h, the microtiter plate wells were washed with PBS and blocked for 2 h at 37 °C with MPBS. Then the plate was rinsed 5 times with PBS. The 50-μL samples of phage solutions for each clone were mixed with 50 μL of MPBS. Mixed solutions were incubated at room temperature for 1 h and added into microtiter plate wells coated with or without HSA. After incubation at 37 °C for 2 h, wells were washed 5 times with PBS. Phage particles were detected with HRP-anti M13

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(Pharmacia Biotech) and developed with *o*-phenylenediamine. The plasmids containing ScFv gene were isolated from positive clones detected by ELISA. *Eco*RV and *Hin*dIII sites were introduced to the N- and C-termini, respectively, of isolated ScFv DNA fragments by standard PCR using forward (5′-CCCCCGATATCCAGGTSA-ARCTGCAGCAGTCAGG-3′) and reverse (5′-CCCCCAAGCTT-CGCCCGTTTGAKYTCCAGCTTGG) primers (cleavage sites for *Eco*RV and *Hin*dIII are underlined). The amplified fragments were digested with *Eco*RV and *Hin*dIII. The DNA fragment encoding the flexible linker $(GGGGS)_4$ (FL4) was prepared from pET32/EBFP−FL4-EGFP[12] by digesting with *Hin*dIII and *Not*I. The DNA fragment encoding EYFP was prepared from the plasmid pET32/VL-EYFP[13] by digesting with *Not*I and *Xho*I. The fragment encoding ECFP was prepared from pECFP-C1 (Clontech, Polo Alto, CA) by PCR as previously described.[8] DNA fragment encoding mouse c-Jun leucine zipper motif (LzJun) was prepared from plasmids pJac-1 [14] (distributed by RIKEN DNA Bank, Japan) by standard PCR using primers 5′−CCCCCCTCGAGGGTGGC-CGGATCGCTCGGCTAGAGG and 5′- CCCCCGGATCCGTCGAC-GAATTCAGTGGTTCATGACTTTCTGCTTAAGCTGTG (cleavage sites for *Xho*I and *Sal*I are underlined). An amplified DNA fragment of LzJun was digested with *Xho*I and *Sal*I. The fragment encoding mouse FosB leucine zipper motif (LzFos) was prepared from the plasmid pBSKS−FosB[15] (distributed by RIKEN DNA Bank, Japan) by standard PCR using primers 5′-CCCCCCTC-GAGGGTGGCCTGACAGATCGACTTCAGGCGG and 5′- CCCCCG-GATCCGTCGACGAATTCAGTGGGCCACCAGGACAAACTC (cleavage sites for *Xho*I and *Sal*I are underlined). An amplified DNA fragment of LzFos was digested with *Xho*I and *Sal*I. Prepared DNA fragments, the *Eco*RV−*Hin*dIII fragments encoding scFv11 or scFv13, the *Hin*dIII−*Not*I fragment for flexible linker, and the *Not*I−*Xho*I fragments for ECFP or EYFP were cloned into the multiple cloning site of pET32b(+). In addition, the *Xho*I and *Sal*I fragments for c-Jun or FosB leucine zipper motif were cloned into the *Xho*I site of pET32b(+). Consequently, six expression plasmids, pET32/ScFv11-FL4-ECFP-LzJun (p11CJ), pET32/ScFv11-FL4-ECFP-LzFos (p11CF), pET32/ScFv11-FL4-ECFP (p11C), pET32/ScFv13-FL4-EYFP-LzJun (p13YJ), pET32/ScFv13-FL4-EYFP-LzFos (p13YF), and pET32/ScFv13-FL4-EYFP (p13Y), were prepared (Figure 2).

Expression and Purification of Recombinant Protein. For the expression of chimeric proteins, pET TRX Fusion System 32 (Novagen), a fusion expression system with *E. coli* thioredoxin (*trxA*), was employed to enhance the solubility of the expressed proteins in *E. coli* cytoplasm. For recombinant protein purification from *E. coli* lysate, we used *E. coli* OrigamiB (λDE3, pLysS) (Novagen), a strain lacking the thioredoxin reductase gene (*trxB*) and glutathione reductase gene (*gor*) as a host. The bacteria were transformed with each of six expression plasmids and selected on LB agar plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar) containing 50 μg/mL ampicillin, 12.5 μg/mL tetracycline, 15 μg/mL kanamycin, and 34 μg/mL chloramphenicol. For all the cultivations thereafter, LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) containing 50 μg/mL ampicillin, 12.5 μg/mL tetracycline, 15 μg/mL kanamycin, and 34 μg/mL chloramphenicol was used. A 1.5-L aliquot of the medium was inoculated with a 5-mL overnight culture at 30 °C of each strain and cultured at 30 °C for 7 h. At OD_{600} of 0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM to induce the expression of the fusion proteins and the cells were further cultured for 12 h at 16 °C. Harvested cells were resuspended in the sonication buffer (50 mM Tris-HCl, 50 mM NaCl, 2 mM EDTA, pH 8.0) and disrupted by freeze/thaw and sonication. The six supernatants were obtained by centrifugation at 20 kg for 20 min at 4 °C. Purifications of expressed proteins were performed by HSA affinity chromatography to collect active proteins, which has sufficient affinity to HSA. The supernatants were applied to a 2-mL column of HSA−Sepharose made from CNBr-activated Sepharose 4B (Amersham Biosciences) and HSA (Sigma) according to the manufacturer's protocol. The column was washed with 20 bed volumes of the sonication buffer and then the bound materials were eluted with 5 volumes of 0.1 M Na_2HPO_4−NaOH, pH 12.0. Each fraction of 1 mL was immediately neutralized with 100 μL of 1 M Tris-HCl, pH 6.8. The fractions with sufficient fluorescence activity were dialyzed against 25 mM Tris-HCl, 0.1 M NaCl, pH 8.0, and stored at −80 °C until use. In the case of recombinant protein purification from the inclusion body, we used *E. coli* Origami (λDE3; Novagen) as a host. Recombinant proteins were expressed and purified in a similar manner except that LB medium (or LB agar plate) without chloramphenicol and a final concentration of 0.1 mM IPTG were used. The pellets of inclusion bodies were obtained by centrifugation at 20 kg for 20 min at 4 °C and resuspended in the sonication buffer containing 3 M urea. Then they were dialyzed against the sonication buffer. After dialysis, HSA affinity chromatography was performed as described above. Purified proteins were stored at −80 °C until use. Each protein concentration was determined by microBCA assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) as standard.

Fluorescence Resonance Energy Transfer Assay. The FRET response of each chimeric protein pair after the addition of antigen was evaluated as follows. The mixture of two chimeric proteins, Trx-ScFv11-FL4-ECFP-LzJun (11CJ)/Trx-ScFv13-FL4-EYFP-LzFos (13YF), Trx-ScFv11-FL4-ECFP-LzJun (11CJ)/Trx-ScFv13-FL4-EYFP-LzJun (13YJ), Trx-ScFv11-FL4-ECFP-LzFos (11CF)/Trx-ScFv13-FL4-EYFP-LzFos (13YF), and Trx-ScFv11-FL4-ECFP (11C)/Trx-ScFv13-FL4-EYFP (13Y), was prepared at a concentration of 20 nM in the assay buffer (50 mM Tris-HCl, 50 mM NaCl, 0.1% gelatin, pH 8.0). HSA was serially diluted to varying concentrations in the assay buffer, and 20 μL of each dilution was added to 180 μL of each chimeric protein mix. After 60-min incubation at room temperature, the emission spectra were measured with 433-nm excitation at room temperature.

Dose−Response Curves. Two chimeric protein pairs, 11CF/13YJ and 11CJ/13YJ, were used for producing dose−response curves. The mixture of two chimeric proteins were prepared at a concentration of 20 nM in the assay buffer. HSA was serially diluted to varying concentrations in assay buffer, and then 20-μL aliquots of antigen solutions were added to 180 μL of each chimeric protein mix. After 60-min incubation at room temperature, the emission spectra were measured with 433-nm excitation

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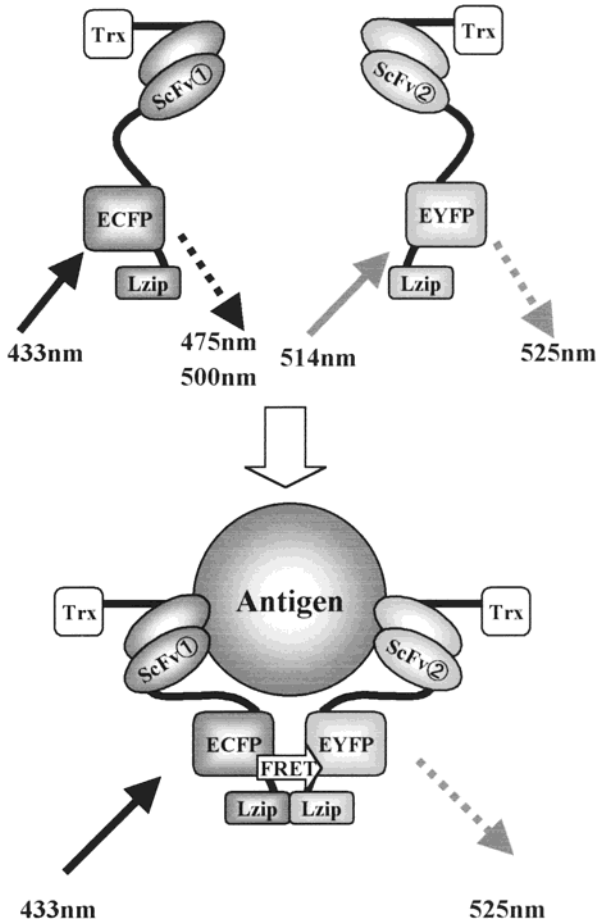

Figure 1. Principle of the assay. Without antigen, the two chimeric proteins remain monomeric; thus, FRET between them is negligible. The addition of antigen−antibody reactions and subsequent leucine zipper dimerization, accompanied by the FRET from ECFP to EYFP.

at room temperature. The fluorescence emission ratio I(525 nm)/I(475 nm) was calculated to evaluate FRET. The assays were carried out in triplicate. The detection limit was determined as a HSA concentration of which the mean I(525 nm)/I(475 nm) − 2SD was higher than the mean blank I(525 nm)/I(475 nm) + 2SD.

RESULTS AND DISCUSSION

Principle of the Assay and Chimeric Protein Design. The principle of this assay is schematically shown in Figure 1. This assay employs two chimeric proteins, one is composed of a ScFv that binds to an epitope of the antigen, a flexible linker (FL4), and a GFP color variant ECFP with a C-terminally tethered leucine zipper motif, and the other is composed of another ScFv that binds to another epitope of the antigen, a flexible linker (FL4), and another GFP color variant EYFP tethered with the same or another leucine zipper motif. In the absence of the antigen, the major parts of these two chimeric proteins in a concentration below the dissociation constant K_d of leucine zipper interaction are expected to remain dissociated; hence, the FRET from ECFP to EYFP would not be observed. On the other hand, in the presence of antigen, these two chimeric proteins bind to the two separate epitopes, which then help the leucine zipper motifs to dimerize with each other due to their increased local concentration. Upon this leucine zipper dimerization, ECFP (donor) and EYFP (acceptor) are expected to be located within closer proximity; hence, the FRET would be induced. Since the extent of FRET gives a good measure of the fraction of the Ab−Ag−Ab′ complex, we would be able to homogeneously determine the antigen concentration.

As a model antigen, we chose HSA. HSA comprises 585 amino acids with a molecular mass of 66.5 kDa. HSA is a helical protein with turns and extended loops and resembles a heart shape, with approximate dimensions of 80 × 80 × 30 Å.[16] Taking the large dimension of this antigen into consideration, normally we would not expect two GFP variants to approach close enough to induce FRET within the Ab−Ag−Ab′ complex when they were just fused to two separate monoclonal antibodies, because the Förster distance R_0 between the two GFP variants ECFP and EYFP was reported to be ∼50 Å.[17] A possible way to allow the donor and acceptor GFP variants to approach to a closer proximity within the Ab−Ag−Ab′ complex is the insertion of flexible linker. Therefore, we introduced a linker with the amino acid sequence $(GGGGS)_4$ between ScFv and GFP variant. Since the distance between successive α-carbon atoms is 3.8 Å and this linker is flexible, possibly the two GFP variants might approach each other (or dimerize) within the Ab−Ag−Ab′ complex. However, since the affinity between two GFP is weak (K_a ∼10^4/M),[18] we considered that the introduction of the flexible linker per se is not enough to induce successful FRET. Therefore, to locate and keep the donor and acceptor GFP variants within closer proximity, we introduced a leucine zipper motif to the C-terminus of the GFP variant moiety with GlyGly as a linker. Upon leucine zipper dimerization within the Ab−Ag−Ab′ complex, the GFP variants would be juxtaposed, leading to the observation of sufficient FRET between them.

As a pair of antibodies for this assay, we prepared and used two anti-HSA mouse monoclonal antibodies termed No. 11 and No. 13. Although their epitopes have not been determined yet, we thought this antibody combination to be suitable because we could sensitively measure HSA concentration in the sandwich ELISA using immobilized No. 11 IgG and No. 13 Fab′−peroxidase conjugate (data not shown).

For the bacterial expression of the antibodies' chimera, we constructed ScFv genes for No. 11 and No. 13 antibodies and displayed them on M13 phages using a phage antibody cloning system. The resultant phage display libraries for No. 11 and No. 13 antibodies were panned on the immobilized HSA. After one round of panning, individual colonies were isolated and analyzed by ELISA for their specific binding to HSA. As a result, we could obtain several positive clones from the phage display libraries for both No. 11 and No. 13 antibodies. According to the nucleotide sequencing, all these clones had in-frame the framework regions and the complementarity determining region sequences. We also confirmed that the N-terminal amino acid sequences (20aa) estimated from the V_H and V_L gene sequences perfectly corresponded to the N-terminal amino acid sequences determined by amino acid sequence analysis of the heavy- and the light-chain

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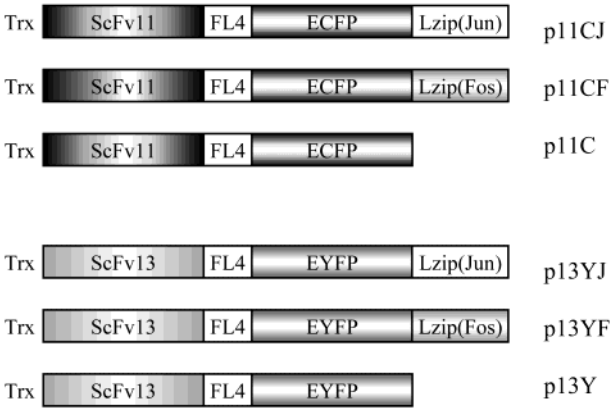

Figure 2. Structures of the chimeric proteins. pET32/ScFv11-ECFP-LzJun (p11CJ), pET32/ScFv11-ECFP-LzFos (p11CF), pET32/ScFv11-ECFP (p11C), pET32/ScFv13-EYFP-LzJun (p13YJ), pET32/ScFv13-EYFP-LzFos (p13YF), and pET32/ScFv13-EYFP (p13Y) were constructed to express the fusion proteins with thioredoxin (Trx). Transcription is driven by T7 promoter.

fragments derived from both No. 11 and No. 13 antibodies (data not shown).

To construct and express the chimeric proteins in *E. coli* according to the molecular design described above, DNA fragments coding for ScFv (ScFv11 or ScFv13), a flexible linker (FL4), GFP variants (ECFP or EYFP), and leucine zippers (LzJun or LzFos) were fused after the thioredoxin tag of pET32 vector in this order. Consequently, we constructed six vectors to express Trx-ScFv11-FL4-ECFP-LzJun (11CJ), Trx-ScFv11-FL4-ECFP-LzFos (11CF), Trx-ScFv11-FL4-ECFP (11C), Trx-ScFv13-FL4-EYFP-LzJun (13YJ), Trx-ScFv13-FL4-EYFP-LzFos (13YF), and Trx-ScFv13-FL4-EYFP (13Y) (Figure 2).

Expression and Purification of Chimeric Proteins. Each fusion protein was expressed in *trxB*$^-$ *gor*$^-$ *E.coli* Origami B (λDE3, pLysS) harboring each expression vector. At first, we attempted to purify the recombinant proteins from the cell lysate. However, after HSA affinity chromatography, the samples for ScFv11 derivatives, 11CJ, 11CF, and 11C, contained a large fraction of truncated proteins without fluorescent activity, and the yields of the ScFv13 derivatives, 13YJ, 13YF, and 13Y, were very poor. Therefore, the purification of the recombinant proteins from the inclusion body was attempted. In this case, although the amounts of the ScFv13 derivatives were modestly increased, the contamination of the truncated form of ScFv11 derivatives was dramatically decreased. We estimated the purity of the proteins by SDS−PAGE. As shown in Figure 3, each chimeric protein was mainly full-length product (11C and 13Y: 74 kDa. 11CJ, 11CF, 13YJ and 13YF: 78 kDa). Although a small amount of truncated form was detectable (43 kDa), we judged that all these were applicable to the assay because they had sufficient fluorescent activity of ECFP or EYFP (date not shown). Final yields of 100−700 μg/L of culture for the ScFv11 derivatives and 20−100 μg/L of culture for the ScFv13 derivatives were obtained.

Change in Fluorescence Spectra due to Addition of Antigen. To confirm antigen-dependent FRET between two chimeric proteins, fluorescence spectra after antigen additions were measured. At first, we examined the combination of 11CJ/13YF. The affinity of Jun and Fos leucine zipper heterodimerization determined using synthetic peptides was reported to be 1.0×10^{-7} M.[11] Considering the affinity constant, chimeric protein concentration of 2.0×10^{-8} M was used to prevent antigen-independent leucine zipper dimerization. As a result, they showed large changes in the fluorescence emission spectra after additions of HSA (Figure 4A). With increasing concentration of HSA (to a final concentration of 150 nM), a small decrease in emission at 475 nm (ECFP emission maximum) and a large increase at 525 nm (EYFP emission maximum) were observed. Also, similar changes of emission spectra were observed with the combination of 11CF/13YJ (data not shown). When FRET between ECFP and EYFP occurs, ECFP (donor) excited-state energy is nonradiationally transferred to EYFP (acceptor). Therefore, these observed changes of emission spectra are considered the result from the energy transfer between ECFP and EYFP in a HSA-dependent manner.

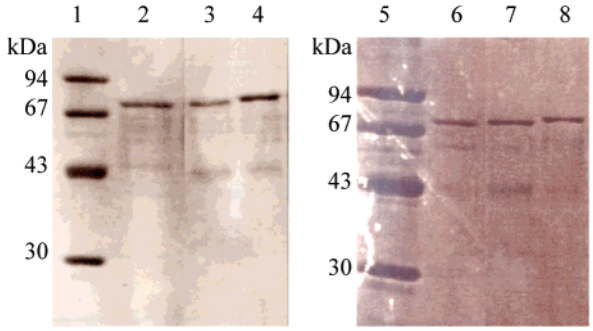

Figure 3. SDS−PAGE showing the purification of chimeric proteins after HSA affinity chromatography. Lanes 1 and 5, molecular weight markers; lane 2, 11C; lane 3, 11CJ; lane 4, 11CF; lane 6, 13Y; lane 7, 13YJ; lane 8, 13YF. Proteins were stained with Coomassie Brilliant Blue.

Upon the addition of 15.0 μM HSA solution, which contains excess antigens over antibodies, the change in fluorescence emission spectrum is less than that with the addition of 1.5 μM HSA solution. We reasoned that each chimeric protein (Ab or Ab′) bound to separate HSA; thus, the ratio of the Ab−Ag−Ab′ complex mediated by leucine zipper dimerization would be decreased. Since this phenomenon is frequently observed in a one-step sandwich immunoassay, the result also suggests that these changes in the fluorescence emission spectra are consequences of antigen−antibody interaction. Because leucine zipper dimerization depends on the concentration of the two, we consider that a higher concentration of chimeric proteins would cause antigen-independent leucine zipper dimerization. In fact, a higher concentration of these chimeric proteins induced considerable FRET without antigen (data not shown). Therefore, it is necessary to lower the concentration of these chimeric proteins than the dissociation constant of Jun−Fos heterodimerization.

Similar changes in the fluorescence emission spectra were obtained with the combination of 11CJ/13YJ (Figure 4B). In this case, the extent of FRET was less than that with the combination of 11CJ/13YF. Jun−Jun homodimer was reported to be less stable than Jun−Fos heterodimer.[10] Therefore, these results suggest that antigen-dependent FRET signals depend on the strength of leucine zipper dimerization.

In contrast to the results shown above, the combination of 11CF/13YF showed no change in the fluorescence emission spectra after addition of HSA (Figure 4C). This was identical to the control experiment without leucine zipper (Figure 4D). With

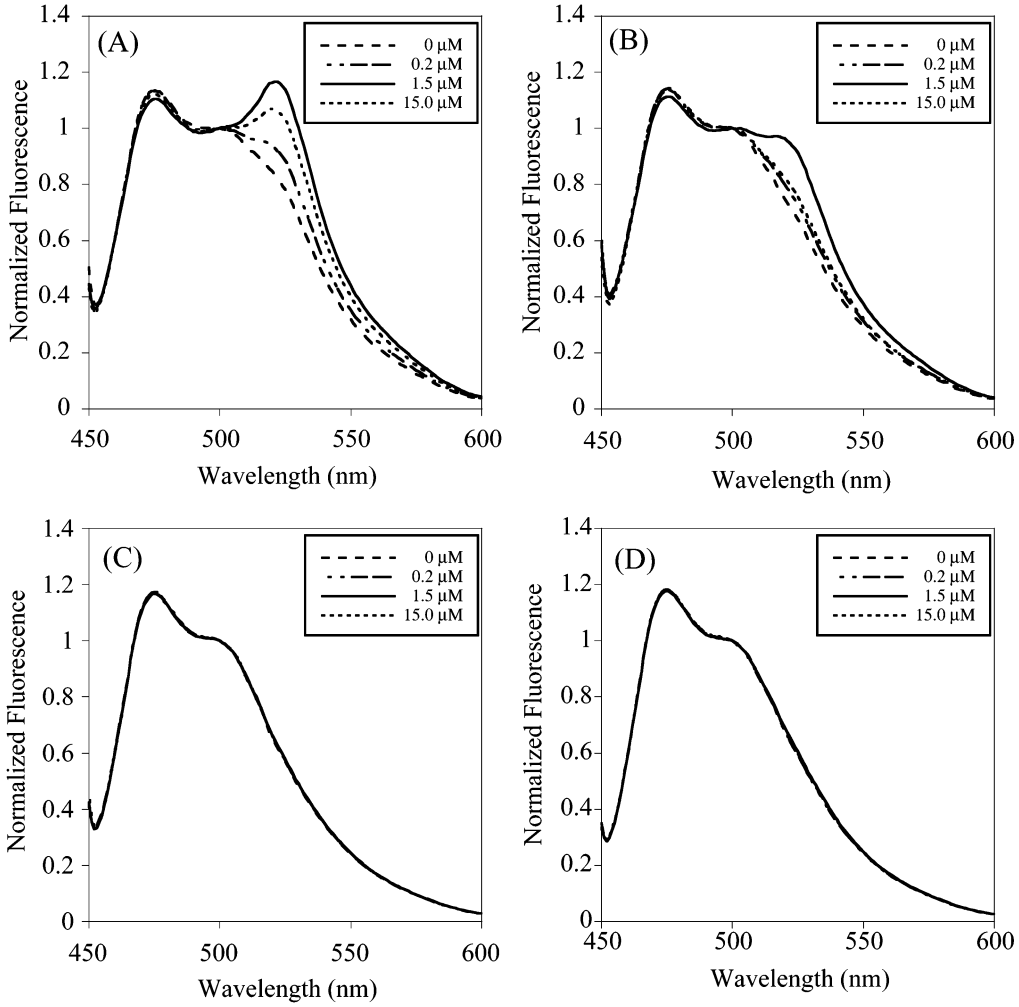

Figure 4. Change in fluorescence spectra by FRET due to the addition of HSA. Normalized fluorescence spectra 1 h after addition of 0, 0.2, 1.5, and 15.0 μM HSA solutions to the combination of (A) 11CJ/13YF, (B) 11CJ/13YJ, (C) 11CF/13YF, and (D) 11C/13Y are shown. These data of fluorescence intensity are normalized at 500 nm.

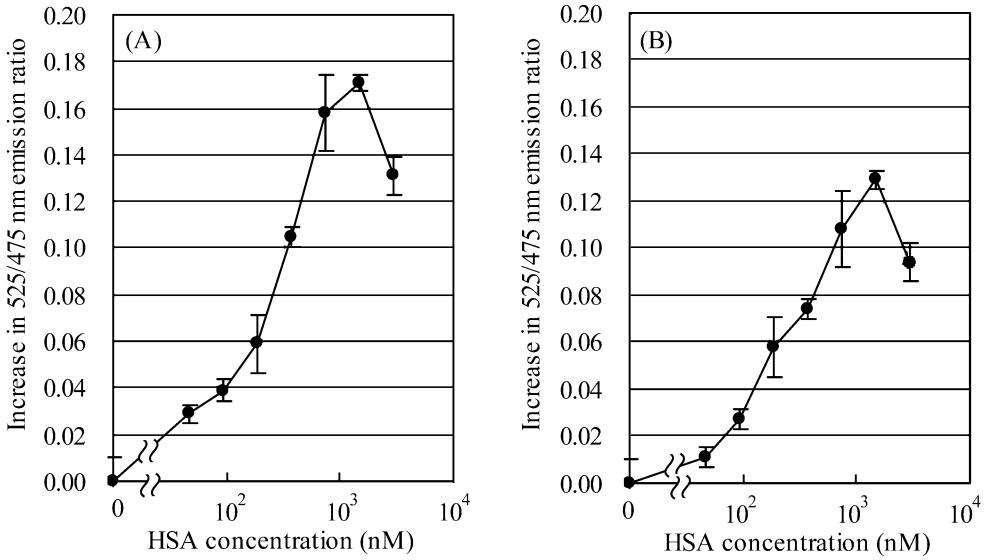

Figure 5. Dose−response curves for HSA using the combination of 11CF/13YJ (A) and the combination of 11CJ/13YJ (B). Data are average ± one standard deviation (n = 3).

regard to the Fos leucine zipper, it is reported that it forms a relatively unstable homodimer with a calculated dissociation constant of 6 μM.[10] Therefore, some degree of strength of leucine zipper dimerization might be necessary to dimerize within the Ab−Ag−Ab′ complex. Thus, two chimeric proteins that possessed strong dimerization activity showed antigen-dependent FRET.

Dose−Response Curves. Based on these results, dose−response curves for the detection of HSA using the combination 11CF/13YJ or 11CJ/13YJ were drawn. At first, to evaluate the response time of assay, time-dependent change in FRET was investigated with 11CF/13YJ combination. As a result, it took ∼1 h to obtain significant FRET signal in this assay (data not shown). Therefore, fluorescence measurements were performed 1 h after antigen injection. Dose−response curves are shown in Figure 5. In both cases, the measuring ranges are 2 orders of HSA concentration. The detection limits of 50 nM for 11CF/13YJ and 100 nM for 11CJ/13YJ were obtained. The coefficient of variation at each HSA concentration is below 2%.

The blank value of 11CF/13YJ ($I_{525}/I_{475} = 0.83$) is higher that that of 11CJ/13YJ ($I_{525}/I_{475} = 0.67$). We reasoned that the antigen-independent FRET is induced by Jun/Fos leucine zipper interaction in the assay conditions. Compared with the blank value of 11CJ/13YJ, the blank values of 11CF/13YJ or 11CJ/13YF tend to change depending on chimeric protein concentration because Jun/Fos leucine zipper interaction is more stable than Jun/Jun leucine zipper interaction.

In the sandwich ELISA using immobilized No. 11 IgG and No. 13 Fab′−peroxidase conjugate, we could measure HSA concentrations in the range of 15 pM−15 nM. Considering the higher performance of the ELISA, there seems to be a room to improve the assay. Although the ELISA would be more sensitive because the detection signals could be amplified by enzyme, we suspected the low binding activity of chimeric proteins to HSA affected these performances. Generally, compared with whole IgG, single-chain Fv has lower binding activity because it has no constant domains that contribute to the stability. In fact, BIAcore analysis of 11C and 13Y showed that the binding activities of both single-chain Fvs to HSA, as association constants, were ∼100 fold lower than those of whole IgG (data not shown). With regard to the lower affinity of both chimeric proteins, we reasoned that not only "single chaining" of Fv but also N- and C-terminal fusion of ScFv affects their binding activity. Therefore, we suspect that further improvement in sensitivity and assay speed might be possible with the use of an antibody fragment with higher binding activity, such as Fab or Fab′.

Although the sensitivity and the dynamic range of the assay are worse than those of the ELISA for the present, this method is superior to the ELISA with regard to the simple operation. The ELISA requires some washing steps to separate unbound antibody−enzyme conjugate. Furthermore, it requires at least two incubation steps for antigen−antibody reaction and color development. These processes are very tedious and time-consuming. On the other hand, the operation of this assay is very simple because it requires only one incubation step. In this assay, we merely need fluorescence measurement after injection of sample into the chimeric protein mixture.

Besides the simple operation, this method also enables simple preparation of assay reagents. Generally, chemical labeling of antibody requires a lot of time and trouble to purify labeled antibody. On the other hand, the gene fusion technique and employing the quick *E. coli* cultivation with lower culture cost compared to hybridoma cell cultivation described in this study enable simple preparation of labeled antibody with 1:1 stoichiometry and site-specific labeling.

Although we have only expected that the donor and the acceptor fluorophores would be close within antigen−antibody complex in conventional FRET immunoassay, the use of flexible linker and leucine zippers will allow the method to assay many other high molecular weight antigens even in case the distance between two epitopes is far and FRET does not occur without using leucine zippers. Once the genes for two separate antibodies recognizing different epitopes are obtained, we will easily assay many high molecular weight antigens with this method. In addition, we can see great possibility in this method. For example, we envisage the application of the method to monitoring the intracellular antigen concentration in vivo, because both two chimeric proteins can be expressed in living cells.

CONCLUSION

We prepared six carefully designed chimeric proteins, 11CJ, 11CF, 11C, 13YJ, 13YF, and 13Y. With these molecular sensors, we demonstrated that the use of an appropriate combination of leucine zippers enabled FRET-based homogeneous and noncompetitive immunoassay for HSA. The strategy presented here is applicable not only to the immunoassay but also to the analysis of various protein−protein interactions. The results of the present study indicate that the method enables homogeneous detection of a ternary protein complex. By substituting ScFvs with other protein domains that bind other common proteins, we may monitor the formation of protein complex consisting of more than two members.

Abbreviations: Fv, antibody variable region; Trx, thioredoxin; ScFv, single-chain Fv; FL4, flexible linker with the amino acid sequence $(GGGGS)_4$; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; LzJun, c-Jun leucine zipper motif; LzFos, FosB leucine zipper motif; 11CJ, Trx-ScFv11-FL4-ECFP-LzJun; 11CF, Trx-ScFv11-FL4-ECFP-LzFos; 11C, Trx-ScFv11-FL4-ECFP; 13YJ, Trx-ScFv13-FL4-EYFP-LzJun; 13YF, Trx-ScFv13-FL4-EYFP-LzFos; 13Y, Trx-ScFv13-FL4-EYFP; Ab−Ag−Ab′ complex, antigen−antibody complex; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; HSA, human serum albumin; BSA, bovine serum albumin; IPTG, isopropyl β-D-thiogalactopyranoside; MPBS, PBS containing 2% skim milk; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS−PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; V_H, antibody heavy-chain variable region; V_L, antibody light-chain variable region;

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