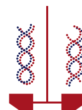

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Development of a fluorescent protein-antibody Förster resonance energy transfer probe for the detection and imaging of osteocalcin

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Fluorescence-based biosensor probes, especially those based on Förster resonance energy transfer (FRET) between fluorescent protein (FP) variants, are widely used to monitor various biological phenomena, often detecting ligand-induced association of the receptor domains. While antibodies are fertile sources of specific receptors for various biomolecules, their potential has not been fully exploited. In this study, we used a fluorescent probe comprising FP-fused antibody variable region fragments to detect a bone metabolism biomarker, osteocalcin (BGP), by using fluorescence spectrometry/microscopy. Because the association between the two proteins increases in the presence of antigen BGP or its C-terminal peptide, the increased antigen in a sample can be monitored as a FRET efficiency increase, based on the open sandwich fluoroimmunoassay principle. The results clearly indicated that the FP-antibody FRET probe could be used as a diagnostic reagent to measure levels of BGP in the clinically relevant concentration range and to image BGP produced from live osteoblast cells.

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[**Key words:** Förster resonance energy transfer; Fluorescent protein; Noncompetitive; Homogenous immunoassay; Bioimaging]

Biomarkers are measurable substances in the body that represent the specific state of biological processes, pathogenic processes, or pharmacologic responses (1). Thus, the detection of biomarkers is important for diagnosis, clinical endpoint measurement and disease progression monitoring. Conventional immunoassays such as sandwich enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay are well-established methods to detect biomarkers, with high sensitivity and specificity (2). However, these heterogeneous immunoassays usually require multiple rounds of incubation and washing steps, making it difficult to apply them to point-of care devices. In addition, low molecular weight antigens are barely detected by sandwich ELISA. Thus, a rapid detection method for diverse biomarker species including small molecules is in great demand for applications such as high-throughput screening and early-stage diagnosis.

Previously, an immunoassay based on the interchain interaction of the antibody variable region (V_H–V_L), open sandwich ELISA (OS-ELISA), has proven to be a powerful method to quantify low molecular weight antigens (3). On the other hand, the efficiency of Förster resonance energy transfer (FRET) is affected by the distance and orientation of fluorescence donor and fluorescence acceptor. It provides a powerful approach to instantly monitor the spatial relationship and/or conformation change of interesting molecules in a homogeneous solution. In order to minimize the limitations of OS-ELISA, open sandwich fluoroimmunoassay (OS-FIA) with fluorescein-labeled (V_H) and rhodamine-labeled (V_L) antibody variable region was established (4). However, the performance of OS-FIA was greatly affected by the variations in fluorescence labeling efficiency. To overcome this problem, fluorescent protein (FP) pair-based OS-FIA was designed to enable label-free OS-FIA (5,6). With OS-FIA, significant change in FRET efficiency depending on the antigen (hen egg lysozyme or phosphotyrosine) concentration was observed. However, the sensitivity attained for hen egg lysozyme was much lower than that of OS-ELISA.

Osteocalcin, also known as bone Gla protein (BGP), is a 49-residue polypeptide (MW = 5.8 kDa), is a known biomarker of bone formation. Its expression varies during osteoblast differentiation, reaching the maximum level in mature osteoblasts. BGP is also produced by osteosarcoma cells after vitamin D3 (7) and vitamin K (8) stimulation. Therefore, it serves as a significant biomarker of osteoblast differentiation (9) and bone formation (10). Variations in blood BGP levels represent different clinical conditions, which can be used for diagnosis of bone metabolism diseases (11). For example, while the normal blood BGP concentration in healthy humans is 0.6 nM (0.13–1.4 nM), it is 3.9 nM (0.96–11.7 nM) and 8.2 nM (0.35–61.4 nM) in primary hyperparathyroidism and renal osteodystrophy, respectively (12). In this study, we demonstrated detection of BGP by an FP-fused antibody FRET probe that can be easily prepared by an *Escherichia coli* expression system. This probe can be used as a diagnostic reagent to measure biomarkers in a homogeneous reaction and to monitor BGP levels produced by live osteoblast cells.

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MATERIALS AND METHODS

Materials The affinity-matured anti-BGP antibody V_H clone R4A10 was prepared as described previously (13). The plasmids pCyPet-His and pYPet-His were kindly provided by Dr. PS Daugherty. The nucleotide sequences of DNA primers used in this study are summarized in Table 1. KOD-plus-neo DNA polymerase and In-Fusion advantage PCR cloning kits were purchased from Takara Bio (Otsu, Shiga, Japan). Ligation High ver. 2 was from Toyobo (Osaka, Japan). PureYield plasmid miniprep kit was purchased from Promega (Madison, WI). TALON metal affinity resin was obtained from Takara Bio. C-terminal peptide from BGP (BGP-C7, NH_2-RRFYGPV-COOH, MW = 894) was purchased from Lifetein (Hillsborough, NJ, USA). Human bone osteosarcoma epithelial U2OS cell was a kind gift by Dr. Masaki Inagaki, Aichi Cancer Center Research Institute. The water used in the study was purified with Milli-Q (Millipore Japan, Tokyo, Japan).

Construction of the FRET probe The V_H fragment of anti-BGP Fab expression vector pUQ1H(KTM219) was replaced with that of R4A10 using restriction enzymes *Age*I and *Xho*I, to yield pET-FabQ(R4A10). The resulting plasmid was digested with *Nde*I and *Eag*I, to yield DNA encoding Fd cDNA, which was inserted into pET30b vector, resulting in pET_Fd(BGP). The remaining fragment encoding the light chain was self-ligated, resulting in pET_Lch(BGP). The cDNAs for CyPet on pCyPet-His was amplified using the primers CyPetYPetNdeBack and CyPetG3S1AgeFor, and inserted into *Nde*I- and *Age*I-digested pET_Fd(BGP) using an In-Fusion PCR cloning kit, resulting in pCyPet-G3S1-Fd(BGP). Similarly, the cDNAs for YPet on pYPet-His was amplified using the primers CyPetYPetNdeBack and YPetG3S1SpeFor, and inserted into *Nde*I- and *Spe*I-digested pET_Lch(BGP) plasmid using an In-Fusion PCR cloning kit, resulting in pYPet-G3S1-Lch(BGP). To construct CyPet-V_H fusion protein, pCyPet-G3S1-Fd(BGP) was digested with *Age*I and ligated with V_H (BGP) fragment amplified using primers VH(KTM)AgeBack and JH1XhoINotI, to yield pCyPet-G3S1-V_H(BGP). To construct YPet-V_L fusion protein, pYPet-G3S1-Lch(BGP) was digested with *Spe*I and *Bam*HI, and ligated to the V_L(BGP) fragment amplified by using the primers YPetInnerBack and VLbgpBamFor, to yield pYPet-G3S1-V_L(BGP).

FRET probe expression and purification *E. coli* SHuffle T7 Express lysY cells harboring either pCyPet-G3S1-V_H(BGP) or pYPet-G3S1-V_L(BGP) were grown in 200 mL Luria broth supplemented with either kanamycin (50 μg/mL) or ampicillin (100 μg/mL), respectively, at 30°C until the A_{600} reached 0.5–0.6. Cultured cells were treated with 0.4 mM isopropyl-thio-β-galactopyranoside for 16 h at 16°C to induce protein expression, before harvesting by centrifugation. The cells with CyPet-V_H proteins were suspended in 15 mL of extraction buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0) and subjected to sonication. After centrifugation (10,000 ×*g* for 20 min), the clear lysate was rotated with 0.1 mL TALON metal affinity resin (Takara Clontech, Otsu, Shiga, Japan) for 2 h. The resin was poured into a gravity column, and washed thrice with 5 mL extraction buffer containing 5 mM imidazole. The CyPet-V_H protein was eluted with 0.5 mL of elution buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole, pH 7.0).

For the cells containing YPet-V_L protein, the cleared lysate was incubated with 0.1 mL anti-Flag antibody agarose slurry (Wako, Osaka, Japan) for 2 h, and washed with 10 mL phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The protein was eluted with 0.2 mL PBS containing 150 μg/mL Flag (DYKDDDDK) peptide.

The purified proteins were analyzed on 10 % SDS-PAGE and stained with a Quick-CBB staining kit (Wako). Fluorescence image of unboiled protein samples were obtained using a transilluminator with excitation wavelength around 500 nm (Gelmieru, Wako). Precision Plus Protein Dual Color Standards (Bio-Rad, Heracures, CA, USA) were used as protein standards.

Fluorescence immunoassay The probes, CyPet-V_H and YPet-V_L (final concentration, 50 nM each) were mixed with indicated concentration of BGP-C7 peptide in PBS, and incubated at 25°C for 20 min. The fluorescence spectra were recorded from 440 nm to 640 nm using an excitation wavelength of 430 nm at 25°C, with the slit widths set to 5 nm each, using a fluorescence spectrometer (model FP-8500, Jasco, Tokyo, Japan). The fluorescence intensity ratio F_{525}/F_{475} was calculated as an index of FRET efficiency.

The dose–response curves in OS-FIA were fitted to a four parameter equation, $y = a + (b - a)/(1 + (x/c)^d)$ using Kaleida Graph ver. 4.5.2 (Synergy Software, Reading, PA, USA). The EC_{50} values were estimated based on the *c* in the formula. The limit of detection (LOD) was obtained as the estimated antigen concentration that shows the mean blank value + 3 standard deviations (SD).

TABLE 1. Nucleotide sequences of primers used in this study.

Primer	Nucleotide sequence (5′–3′)
CypetYpetNdeBack	AAGAAGGAGATACATATGGTGTCTAAAGGTGAAGAATTATTC
CypetG3S1AgeFor	TCCAGCTGTACCTCACCGGTTGAACCGCCACCTTTGTAC AATTCATCCATACC
YpetG3S1SpeFor	GTGAGCTCAATGTCACTAGTTGAACCGCCACCTTTGTAC AATTCATCCATACC
VH(KTM)AgeBack	GGAATTCACCGGTCAAGTAAAGCTGCAGCAGTC
JH1XhoINotI	GGAATTCGCGGCCGCGCTCGAGACGGTGACCGTGGT
YpetinnerBack	GACTGCTGCTGGTATTACCGA
VLbgpBamFor	GGCGGATCCACCACGTTTGATTTCAAGCTTGG

Fluorescence imaging of agarose beads with an immobilized probe YPet-V_L protein (1.8 μg) was incubated with 20 μL anti-Flag antibody beads at 4°C for 1 h. Following incubation, the beads were washed three times by PBS to remove unbound YPet-V_L protein. Next, CyPet-V_H protein (3 μg) and 10 μM of BGP-C7 peptide in 100 μL PBS were incubated with the beads at 4°C. Part of reaction mixture was kept as no-wash sample and the remaining mixture was washed by PBS. The sample diluted with PBS was transferred to a 35-mm glass bottom dish (Matsunami Glass, Tokyo, Japan) for imaging. The differential interference contrast (DIC), CFP (440DF20 excitation and 480DF30 emission filters), YFP (490DF20 excitation and 535DF25 emission filters), FRET (440DF20 excitation and 535DF25 emission filters with a 455DRLP dichroic mirror), and RFP (BP545-580 excitation and BA610IF emission filters) images were captured by fluorescence microscopy IX71 (Olympus, Tokyo, Japan). The images were obtained using the software HCImage (Hamamatsu Photonics, Shizuoka, Japan) equipped with ImaGEM EM-CCD camera with exposure of 0.2 s and sensitivity gain of 4.

Live imaging of osteocalcin-producing osteoblasts using FP-antibody FRET probe U2OS cells (2×10^4 cells/well) were seeded in a triple-well glass-based dish (AGC Technoglass Co. Ltd., Tokyo, Japan) in DMEM medium (Wako) supplemented with 10% fetal bovine serum (FBS, Japan Bioserum, Fukuyama, Japan) and 1% penicillin/streptomycin at 37°C with 5% CO_2. The cells were washed three times with PBS and incubated in serum-free DMEM for 24 h, and subsequently incubated for 36 h in phenol red-free DMEM containing 0.1% FBS and 100 nM vitamin D_3. Next, equal concentration (5 nM each) of FRET probes (CyPet-V_H and YPet-V_L) were added to the cultured cells and incubated at room temperature for 30 min. The differential interference contrast (DIC), CFP, YFP and FRET images were observed by fluorescence microscopy. The exposure time and sensitivity gain were set to 1 s and 4 s, respectively. For control, similar procedures were followed without adding vitamin D_3.

Intracellular localization of FP-antibody FRET probe U2OS cells were prepared as above, either treated or not treated with vitamin D3, and CellLight Early Endosomes-RFP, BacMam 2.0 (Molecular Probes, Thermo-Fisher Scientific) was added according to the manufacturer's instructions, 16 h before adding the FRET probes as above. DIC, YFP and RFP images were observed by fluorescence microscopy, and the localization of each probe was investigated.

RESULTS AND DISCUSSION

Construction of FP-fused antibody FRET probe The affinity-matured anti-BGP antibody V_H clone R4A10 used in this study was selected from a randomized V_H library of anti-BGP antibody KTM219 (14), which showed antigen-dependent interaction with V_L fragment with high sensitivity in OS-ELISA (13). In other words, the V_H fragment of R4A10, as well as that of KTM219, does not associate with the V_L fragment in the absence of antigen BGP, but does associate in the presence of BGP or its C-terminal fragment BGP-C7. Hence, FP-fused antibody FRET probe for BGP was constructed by tethering a donor FP to the V_H fragment, and an acceptor FP to the V_L fragment, respectively (Fig. 1A). The two FPs were tethered to the N-terminus of each V fragment via a short linker, respectively. The shorter inter-terminal distance between the N-termini (~3.5 nm) rather than that between the C-termini (~4.5 nm) of antibody V regions was expected to be favorable to attain higher FRET efficiency upon complexation with BGP antigen. Also, an optimized cyan–yellow FP pair for FRET, CyPet and YPet (15), was used as a donor and an acceptor, respectively. It is worth noting that the dissociation constant of two FPs derived of *Aquorea victoria* is around 110 μM, which is too large to affect dimerization behavior of the fusion proteins at submicromolar range (16).

Expression and purification of FRET probe CyPet-V_H and YPet-V_L proteins were expressed individually in *E. coli* SHuffle T7 Express lysY, which is engineered to express proteins with disulfide bonds in the oxidative cytoplasm. The two cell extracts were purified to near homogeneity with metal affinity resin or anti-Flag antibody beads. The purified FRET probes were analyzed on 10% SDS-PAGE and stained with Coomassie Brilliant Blue (Fig. 1B). When samples were loaded without boiling, the fluorescence

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