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Antibody-based fluorescent and fluorescent ratiometric indicators for detection of phosphotyrosine

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Fluorescent indicators for protein phosphorylation are very important in not only fundamental biology but also biomedical applications. In this study, we developed novel fluorescent and fluorescent ratiometric indicators for detection of phosphotyrosine (pTyr) derivatives. A single-chain antibody variable fragment (scFv) against phosphotyrosine was fluorescent-labeled by incorporation of tetramethylrhodamine (TAMRA)-linked nonnatural amino acid at the N- or C-terminus. The TAMRA-labeled scFv showed fluorescence enhancement upon addition of pTyr-containing peptides based on antigen-dependent fluorescence quenching effect on TAMRA. The TAMRA-labeled scFv was further fused with enhanced green fluorescent protein (EGFP) to generate a double-labeled scFv for pTyr. In the absence of antigen, fluorescence resonance energy transfer (FRET) occurred from EGFP to TAMRA but TAMRA was quenched. The antigen-binding removed the quenching of TAMRA while FRET occurred without altering its efficiency. As a result of the FRET and antigen-dependent fluorescence quenching effect, the double-labeled scFv exhibited fluorescence ratio enhancement upon the antigen-binding. The fluorescent and fluorescent ratiometric indicators obtained in this study will become a novel tool for analysis of protein phosphorylation. Moreover, this strategy utilizes antibody derivatives, and therefore, can be easily applied to other antigen–antibody pairs to generate fluorescent ratiometric indicators for various target molecules.

[**Key words:** Single-chain antibody; Fluorescent ratiometric indicator; Protein phosphorylation; Cell-free translation; Nonnatural amino acid]

Protein phosphorylation is an important post-translational modification which affects protein structure and regulates various biological processes. In mammalian cells, serine, threonine, and tyrosine are reversibly phosphorylated in response to extracellular stimuli. For example, in signal transduction the autophosphorylation of Tyr in epidermal growth factor (EGF) receptor upon binding of EGF activates a kinase cascade that relays the signal to the transcriptional apparatus in the nucleus and triggers cell growth and differentiation. Abnormal protein phosphorylation may cause pathogenesis or even carcinogenesis. Therefore, methods for studies of protein phosphorylation are very important in not only fundamental biology but also biomedical applications.

To study protein phosphorylation, genetically-encoded fluorescent indicators for protein phosphorylation have been developed (1,2). Fluorescence resonance energy transfer (FRET)-based indicator consists of a substrate domain specific for the kinase of interest linked with a phosphorylation recognition domain through a flexible linker and sandwiched by cyan and yellow fluorescent proteins (CFP and YFP, respectively). Phosphorylation of the substrate domain promotes the binding of the substrate domain to the recognition domain and generates a large conformational change of the indicator which alters the distance and/or relative orientation of CFP and YFP to exhibit FRET change. The indicators are applicable to real-time monitoring of protein phosphorylation in live cells (3,4). Alternatively, phosphorylation-mediated assembly of a semisynthetic GFP-based indicator for protein kinases has been reported (5). The indicator consists of a kinase substrate peptide fused with 10th β-strand peptide of GFP and a truncated GFP without the 10th β-strand. Phosphorylation of the substrate peptide protects the GFP peptide from cleavage by carboxypeptidase and promotes the reconstitution of intact GFP.

Although the above indicators successfully monitored kinase activities, they cannot directly detect naturally occurring phosphorylated proteins. Most of strategies for detection of phosphorylated proteins are based on immunoassay using antibodies specific for phosphorylation sites. Anti-phosphorylation antibodies are widely utilized for various immunoassay including Western blotting and immunostaining (6–10). The use of antibodies allows us to detect various phosphorylated antigens; however, it requires tedious and time-consuming process including binding and washing steps. Moreover, it is difficult to detect antigens in a real-time manner.

An antibody-based fluorescent indicator for homogeneous competitive immunoassay has been reported to detect phosphorylated proteins (11). This indicator consisted of two components; a phosphorylated peptide conjugated with CFP and leucine zipper, and an Fab fragment against phosphorylated protein (extracellular signal-regulated kinase) fused with YFP and leucine zipper. FRET efficiency was significantly enhanced by the antigen–antibody binding and the interaction of the leucine zippers in the absence of phosphorylated antigen. On the other hand, FRET signal decreased

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in the presence of phosphorylated antigen due to the competitive binding of phosphorylated antigen to the antibody which induced the dissociation of the two components. Although this strategy is potentially available for detection of various phosphorylated antigens, it requires careful design of the synthetic phosphorylated peptide so that the binding affinity to the synthetic peptide must be lower than that to the target antigen.

To develop a simple and widely-applicable fluorescent biosensor for protein phosphorylation, we utilized Quenchbody technology (12). Quenchbody has been developed by N- or C-terminus specific fluorescence labeling of single-chain antibody variable fragment (scFv) and Fab fragments. In the absence of antigen, the fluorophore is in close proximity to Trp residues located at the interface between the variable domains (V_H and V_L) of scFv, thus, fluorescence is quenched by Trp. In the presence of antigen, this quenching is removed because the tight binding of V_H and V_L prevents the interaction of fluorophore and Trp residues. Therefore, fluorescence is significantly enhanced in an antigen concentration dependent manner. Quenchbody has been proved to have a wide range of applications in detecting not only small molecules but also peptides and proteins with high sensitivity including phosphoserine residue (13).

Here, we developed Quenchbody against phosphotyrosine (pTyr) residue by site-specific fluorescence labeling with tetramethylrhodamine (TAMRA) using a nonnatural amino acid mutagenesis (Fig. 1A) (14–16). Fluorescence response of the fluorescent indicators was investigated for several pTyr-containing peptides.

In addition, we developed here a fluorescent ratio indicator based on FRET and Quenchbody technology. Although Quenchbody is a powerful method for detection of various antigens based on fluorescence intensity change, fluorescence intensity of the single-fluorescent-labeled Quenchbody is also dependent on the concentration of this indicator in measuring samples. Therefore, it is a difficulty in the application of this technique to detect antigens under heterogeneous conditions such as in living cells where the local concentration of the indicator cannot be controlled. To solve this disadvantage, we fused enhanced green fluorescent protein (EGFP) to TAMRA-labeled anti-phosphotyrosine scFv (Fig. 1B). An expected mechanism of the fluorescent ratio indicator is as follows: The double-labeled scFv exhibits FRET from EGFP to TAMRA but TAMRA is quenched in the absence of antigen. As a consequence, the double-labeled scFv mainly emits green fluorescence. In the presence of antigen, however, the quenching effect is eliminated and the double-labeled scFv emits both green and red fluorescence.

While most FRET indicators require FRET change induced by large conformational changes of protein scaffold, the present strategy can detect fluorescence ratio change even if FRET does not change. We have already demonstrated that site-specifically double-labeled maltose-binding protein exhibits fluorescence ratio change based on the same mechanism (17). The present study will enable us to detect antigens in a ratiometric manner and expand the utility of Quenchbody technology.

MATERIALS AND METHODS

Materials Primers for PCR were custom synthesized by Invitrogen (Life Technologies Japan). QIAquick PCR purification kit and QIAquick gel extraction kit were purchased from Qiagen (Venlo, Netherlands). In-Fusion HD Cloning kit was from Clontech (CA, USA). KOD-Plus DNA polymerase and Thermo T7 RNA polymerase were purchased from Toyobo (Osaka, Japan). T4 RNA ligase was obtained from Takara Bio (Otsu, Japan). *Nde*I, *Hin*dIII restriction enzymes, Quick Ligase, Prestained protein marker (7–175 kDa) and T7 RNA polymerase were from New England BioLabs (MA, USA). *Escherichia coli* S30 extract for linear templates, pGEMEX-1 vector, and MagneHis Ni-particles were from Promega (WI, USA). Zeba desalting spin columns (7K MWCO) were from ThermoFisher Scientific (MA, USA). EGF receptor substrate 2 (DADE-pY-LIPQQG), insulin receptor (1142–1153) (TRDI-pY-ETD-pY-pY-RK) were obtained from GenScript (NJ, USA), pp60src SH2 domain-binding peptide (Ac-pYEEIE) was from Bachem (Bubendorf, Switzerland).

Construction scFv genes DNA sequence of heavy and light chains of anti-pTyr antibody 4G10 was obtained from the National Center for Biotechnology Information USA (NCBI) Nucleotide database (18). Accession numbers for heavy

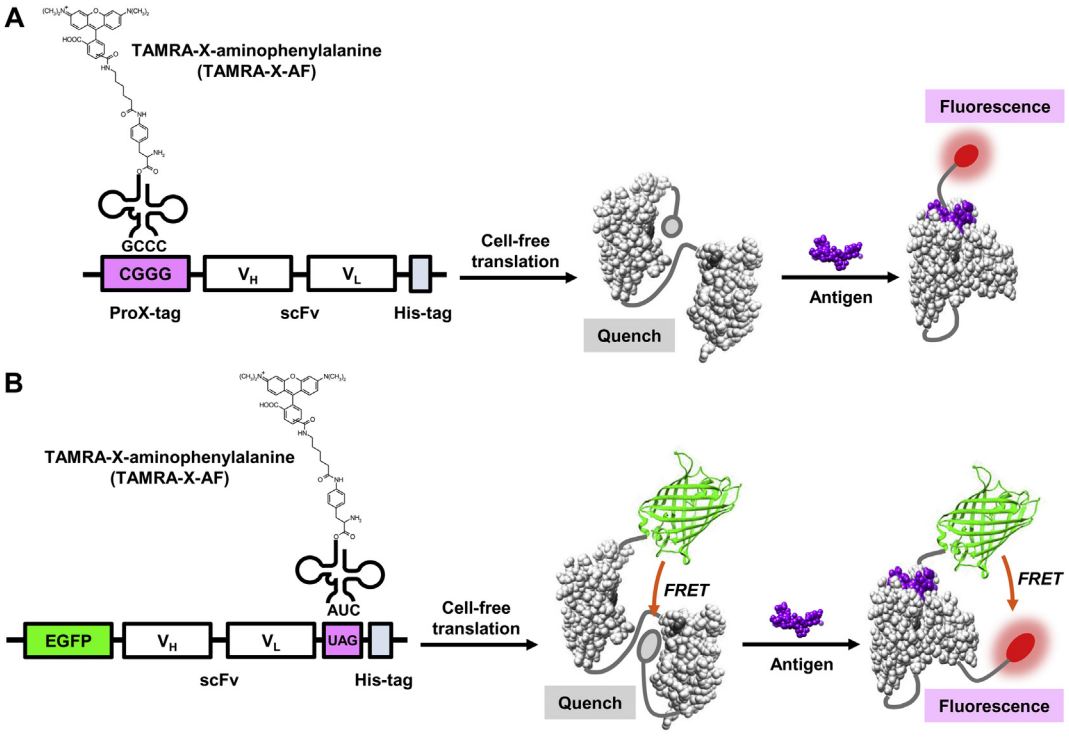

FIG. 1. (A) Schematic illustration of synthesis of TAMRA-labeled scFv and detection of pTyr-containing antigen based on antigen-dependent removal of fluorescence quenching effect on TAMRA. (B) Schematic illustration of synthesis of double labeled scFv in which EGFP is fused to the N-terminus of scFv and detection of pTyr-containing antigen based on FRET from EGFP to TAMRA and antigen-dependent removal of fluorescence quenching effect on TAMRA.