Reduction-Triggered Fluorescence Probe for Peptide-Templated Reactions

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We developed a new nucleic acid-based fluorescence probe for protein detection. The method is based on the scission of an aptamer into two probes, which are then attached with a chemically reactive fluorogenic compound. The protein-dependent association of the two probes accelerates a reduction-triggered fluorogenic reaction and indicates the presence of the target protein, which is detected using a fluorescence readout. The fluorescence signal is generated *via* the deprotection of the azidomethyl group of fluorescein. The arginine-rich motif peptide of the human immunodeficiency virus-1 Rev protein was targeted by this type of probe. Emission was detected at 522 nm and was enhanced by about 19.4-fold in the presence of the target peptide. An oligonucleotide-based reduction-triggered fluorescence probe was successfully applied to the detection of the Rev peptide in solution.

Key words fluorogenic probe; peptide; aptamer; templated reaction

The detection and measurement of the levels of proteins are among the most fundamental and often performed experiments in biomedical research.[1—3)] Fluorescent proteins (*e.g.*, green fluorescent protein) exhibit important advantages that render them the most popular protein-labeling technique; these include high labeling specificity and simplicity of use. However, the use of fluorescent proteins requires genetic modification and thus cannot be applied to the detection of endogenous proteins. In addition, the large molecular weight of these proteins limits their application, as it can influence the structure and function of the target protein.[2,3)] On the other hand, antibody-based protein detection methodologies are commonly used in research and medical diagnostics.[1)] This method requires a careful washing step to remove unbound antibody and avoid background signal. Therefore, these methods are not well adapted to the rapid, high-throughput, and parallel detection of proteins.

A fluorogenic system of protein detection that requires no washing step is very useful, as it can be applied not only to simple protein detection but also to the imaging of cellular proteins. There are many reports on fluorogenic enzyme substrates based on small compounds; however, it is difficult to find specific enzyme substrates *via* the design of small compounds. Recently, a few oligonucleotide-based fluorogenic probes for sensing proteins have been reported.[4—10)] For example, the molecular beacon (MB), which was originally developed for nucleic acid detection, has been applied to protein detection. MB encodes a random sequence that targets single-strand-binding proteins[7,8)] or an aptamer sequence that targets the Tat protein.[9)] The binding of the target protein to MB induces the appearance of a fluorescence signal *via* the formation of an open structure. The alternative strategy is based on the scission of the DNA binding site of the protein into two DNA fragments modified by a fluorescent compound.[4—6)] When the target protein is present, the two fragments associate to emit a fluorescence resonance energy transfer (FRET) signal. In a similar strategy, two fluorophore-labeled aptamers associate with the target protein to emit a FRET signal.[10)] These strategies offer a temporary signal in the presence of the target protein. The consideration of a method that is able to offer the generation of a permanent signal seemed justified in some applications. In particular, it is potentially useful for signal amplification. Recently, Levy and Ellington reported a peptide-templated nucleic acid ligation, where the two halves of RNA aptamers bind to the target human immunodeficiency virus (HIV)-1 Rev peptide and then associate, which enhances the rate of chemical ligation of RNA half-molecules.[11)] However, no signal was emitted after the chemical ligation. In addition, this ligation strategy has the potential disadvantage on signal amplification by multiple chemical reaction due to the strong product inhibition.

Recently, we developed a reduction-triggered fluorescence (RETF) probe based on a new fluorogenic compound, *i.e.*, azidomethyl-protected fluorescein, for the detection of oligonucleotides in solution or even in living cells.[12—14)] Here, we applied the RETF system to a protein-templated chemical reaction for the detection of proteins. Unlike the previous system, the RETF probe generated a permanent fluorescent signal based on a chemical reaction and potentially minimized product inhibition because of the unligated chemical reaction. A motif consisting of two halves of an RNA aptamer was selected for the detection of the 17-mer arginine-rich motif (ARM) peptide originated from the HIV-1 Rev protein. The RETF probe was designed by introducing a fluorogenic compound into the RNA motif. The probe was successfully applied to the detection of the Rev protein in solution.

Experimental

Synthesis of Unmodified Oligonucleotides Oligonucleotides were synthesized on a 0.2 μmol scale on a DNA/RNA synthesizer (H-8-SE; Gene World) using standard phosphoroamidite coupling chemistry. Deprotection and cleavage from the controlled pore grass (CPG) support was carried out by incubation in concentrated ammonia : EtOH (=3 : 1) for 8 h at 55 °C and then triethylamine trihydrofluoride at room temperature for 12 h. The reacted oligonucleotides were collected by buthanol precipitation and quantitated by UV absorbance using the nearest-neighbor approximation to calculate molar absorptivities.

3′-Azidomethyl Protected Fluorescein (BAF)-Conjugated Oligonucleotide (BAF Probe 1, 2 and 3) The bromoacetyl group of BAF was reacted with the phosphorothioate group on the oligodeoxynucleotides (ODNs). For 3′-phosphorothioate sequences, the 3′-phosphate CPG was sulfurized by the sulfurizing reagent (Glen Research) after the first nucleotide was added. Seventy-five nanomoles of the 3′-phosphorothioate oligonu-

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cleotide in 50 μl of 400 mM triethylammonium bicarbonate buffer were shaken for 5 h at room temperature with 750 nmol of BAF in 200 μl of dimethylformamide. The reacted products were collected by ethanol precipitation. Next, the products were purified by reverse-phase HPLC (0—60% acetonitrile/50 mM triethylammonium acetate gradient).

BAF probe 1: MALDI-TOF MS *m/z*: 3764.7 (Calcd for $C_{122}H_{138}N_{42}O_{77}P_{10}S$ 3764.5).

BAF probe 2: MALDI-TOF MS *m/z*: 4456.3 (Calcd for $C_{142}H_{162}N_{52}O_{91}P_{12}S$ 4454.6).

BAF probe 3: MALDI-TOF MS *m/z*: 4462.0 (Calcd for $C_{142}H_{162}N_{52}O_{91}P_{12}S$ 4454.6).

5′-Triphenylphosphine (TPP)-Linked Oligonucleotide (TPP Probe) Carboxy-triphenylphosphine (TPP) NHS ester was reacted with 5′-amino-modified oligonucleotide. 5′-Aminomodifier C6 (Glen Research) was used to prepare 5′-aminomodified oligonucleotide. Fifty nanomoles of the 5′-amino-modified oligonucleotide in 135 μl of 185 mM sodium tetraborate (pH 8.5) was shaken for 12 h at room temperature with 2 μmol of TPP NHS ester in 115 μl of dimethylformamide. The reacted products were collected by ethanol precipitation. Next, the collected products were purified by reverse-phase HPLC (0—60% acetonitrile/50 mM triethylammonium acetate gradient).

MALDI-TOF MS *m/z*: 7852.8 (Calcd for $C_{245}H_{300}N_{92}O_{160}P_{24}$: 7833.2). A peak corresponding to the oxidized product (+O) was also seen and presumed to arise from oxidation during purification.

5′-Dithiothreitol (DTT)-Linked Oligonucleotide (DTT Probe) Dithiol phosphoroamidite (DTPA; Glen Research) was used to prepare 5′-DTT-modified oligonucleotide. Deprotection and cleavage from the CPG support was carried out by the standard method. Following deprotection, the oligonucleotide was purified by reverse-phase HPLC (0—60% acetonitrile/50 mM triethylammonium acetate gradient) and quantitated by UV absorbance. To cleave the disulfide bond, 10 nmol of purified oligonucleotide was treated with 100 μl of 100 mM tris(2-carboxyethyl)phosphine (TCEP) and 100 mM DTT in 1×TB buffer (pH 6.0). The reacted products were collected by ethanol precipitation, and then preserved in 100 μM DTT solution.

MALDI-TOF MS *m/z*: 7583.0 (Calcd for $C_{224}H_{280}N_{91}O_{160}P_{23}S_2$: 7580.0).

Fluorescence Measurement Reactions on the peptide template were performed in 1.2 ml of Tris–HCl buffer (50 mM, pH 8.0) containing 50 or 0 mM KCl, 0.13% Triton X-100 with HIV-1 ARM (100 or 50 nM) or peptide 1 (100 nM), BAF probe (100 or 50 nM), and TPP probe (50 or 100 nM) or DTT probe (100 nM) at 37 °C. The increase of fluorescence intensity produced by reduction of azidomethyl group on BAF probe was continuously monitored at time intervals. Reactions were observed by fluorescence spectrometry (FP-6500; JASCO). Fluorescence spectra were measured under the following conditions: excitation, 490 nm; emission, 522 nm.

Results

The Rev protein of HIV-1 interacts tightly and specifically with RNA molecules that form a short stem-internal loop-stem structure, *i.e.*, the Rev-binding element (RBE).[15—20] The 17-amino acid arginine-rich motif (ARM) of Rev forms an α-helical conformation and retains binding to RBE.[11,15] On the other hand, an anti-Rev RNA aptamer, which was developed using an *in vitro* selection technique, binds more tightly to Rev protein than wild-type RBE (Fig. 1). The aptamer that retained a stem-internal loop-stem structure similar to that of RBE was responsible for the specific and high-affinity interaction with the ARM peptide (Kd, *ca.* 1 nM). Based on this aptamer, we designed RETF probe sequence using the two halves of the RNA aptamer (Fig. 1B).[11] One probe carried bisazidomethyl-protected fluorescein (BAF), and another probe carried a reducing reagent, *e.g.*, triphenylphosphine (TPP) or dithiothreitol (DTT). These two probes formed a complex with the target ARM, which triggered a fluorogenic reaction. In the first step of the reaction using the BAF and TPP probe pair, an unstable intermediate containing an aza-ylide bond was formed and quickly hydrolyzed. Consequently, the azide group of BAF was reduced to an amino group, and the TPP group was transformed into the corresponding phosphorus compound. The resulting amino hemiacetal group was quickly hydrolyzed and yielded an unmasked phenol group; the product emitted a fluorescence signal (Fig. 2). The use of the BAF and DTT probe pair resulted in a reduction reaction that yielded a fluorescent product without an observable intermediate. Two BAF probes of different sizes were designed, one containing 10 bases (BAF probe 1) and the another containing 12 bases (BAF probe 2), to avoid undesired background signal caused by the formation of an RNA complex between the probes in the absence of ARM (Fig. 3). BAF probe 3 with scramble sequence was designed not to bind with ARM peptide for the control experiment. BAF probes 1, 2 and 3 were synthesized by modification of the 3′ end of the RNA aptamer, where the bromoacetyl group of BAF was reacted with the phosphorothioate group present at the 3′ end of the 10 or 12 bases-long RNA molecule (Fig. 4A). The TPP probe was generated *via* the conjugation of 2′-carboxytriphenylphosphine with the 5′ amino linker of a 23 bases-long RNA probe, *via* amide-bond formation. The DTT probe was generated *via* the addition of DTT at the 5′ end of a 23 bases-long RNA molecule, using commercially available phosphoramidite reagents. In addition, we synthesized the peptide 1 as negative control which is not targeted by the aptamer (Fig. 1A). The sequence

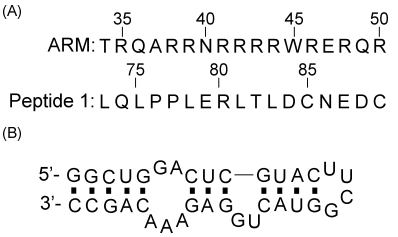

Fig. 1. (A) Sequence of the 17-mer HIV-1 Rev ARM (Residues 34—50 of the HIV-1 Rev Protein) and Peptide 1 (Residues 73—89 of the HIV-1 Rev Protein) and (B) Sequence and Secondary Structure of the Anti-Rev Aptamer

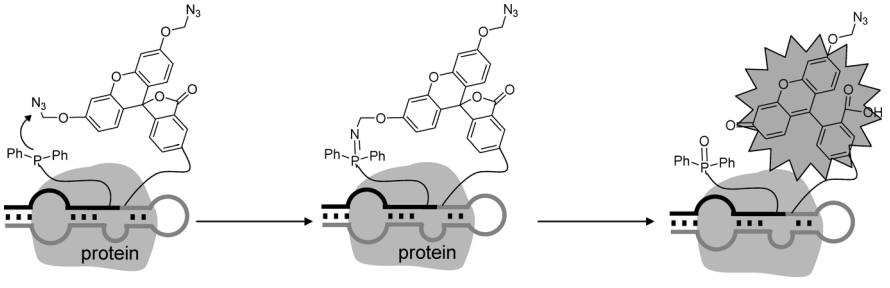

Fig. 2. Illustration of the Azidomethyl Reduction Reaction, Which Generated a Fluorescent Signal in the Presence of the Target Protein

of peptide 1 exists in C terminal region of the Rev protein at amino acid position 73 to 89 that includes the sequence of nuclear export signal.[21)]

First, we tested whether the BAF probe was able to generate a fluorescence signal *via* the reduction of its azidomethyl group. No significant fluorescence with excitation at 490 nm was observed for BAF probe 1 (Fig. 5). The addition of a high concentration of DTT to the solution yielded a strong fluorescence emission at around 522 nm. The emission was enhanced *ca.* 78-fold. Thus, we confirmed that reducing reagents can transform the BAF probe into a fluorescent form by intermolecular reaction and that the RNA structure does not affect the fluorescence.

Second, we tested whether the RETF probe is able to detect the target ARM peptide and produce a fluorescence signal (Fig. 6). A Rev-aptamer binding assay was carried out using Tris–HCl buffer (pH 8.0) containing 50 mM KCl.[16,17,19)] First, we followed the reported condition for our experiment (Fig. 6). The incubation of BAF probe 2 (50 nM) with the TPP probe resulted in a fluorescence signal at 522 nm, which increased in the presence of the target ARM peptide *via* the reduction of the azidomethyl group of BAF probe 2 over a period of 30 min. However, BAF probe 1 did not yield the desired fluorescence signal in the presence of the target ARM peptide. We therefore used BAF probe 2 in all subsequent experiments. We noted that a slight increase in fluorescence was observed, even in the absence of the ARM peptide (Fig. 7A). To solve this problem, we tested additive reagents, which included polyethylene glycol (PEG), bovine serum albumin (BSA) and Triton X-100 to reduce the undesired molecular interactions that caused the false signal. Triton X-100 slightly improved the background fluorescence signal. Next, we tested a reaction buffer without KCl. This experimental condition successfully reduced the background signal (Fig. 7A). Thus, the reaction buffer without KCl was used in subsequent experiments. The use of BAF probe 2 (100 nM) with the TPP probe led to an efficient increase in fluorescence intensity in the presence of the target ARM peptide, over a

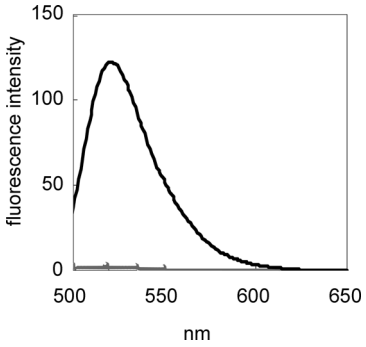

Fig. 5. Fluorescent Emission Spectra with Excitation at 490 nm

The reaction was carried out using the following conditions: 50 nM BAF probe 1 with 100 mM (black line) or 0 mM DTT (gray line) in buffer (50 mM Tris–HCl (pH 8.0)).

BAF probe1: 5'-CUGGACUCG**dT**-BAF-3'

BAF probe2: 5'-GGCUGGACUC-G**dT**-BAF-3'

BAF probe3: 5'-CAGGUGGCCU**dT**-BAF-3'

TPP probe: 5'-TPP-ACUUCGGUACUGGAGAAACAGCC-3'

DTT probe: 5'-DTT-ACUUCGGUACUGGAGAAACAGCC-3'

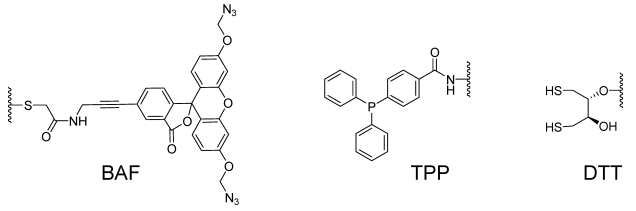

Fig. 3. Sequence of the Probes

BAF probes 1, 2 and 3 were generated by conjugation with BAF at the 3′ end. The TPP probe was generated by the addition of TPP at the 5′ end. The DTT probe was generated by the addition of DTT at the 5′ end. The stem regions in secondary structure of the aptamer were underlined.

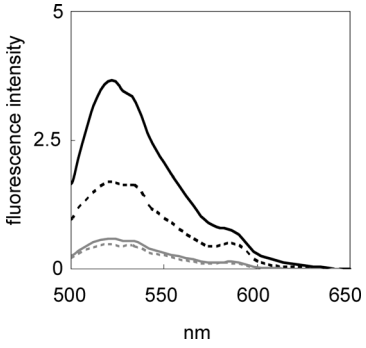

Fig. 6. Fluorescent Emission Spectra with Excitation at 490 nm

The reaction was carried out using the following conditions: 50 nM TPP probe with 50 nM BAF probe 1 (gray) or BAF probe 2 (black) and 50 nM (solid line) or 0 nM (dashed line) ARM in buffer (50 mM Tris–HCl (pH 8.0), 50 mM KCl).

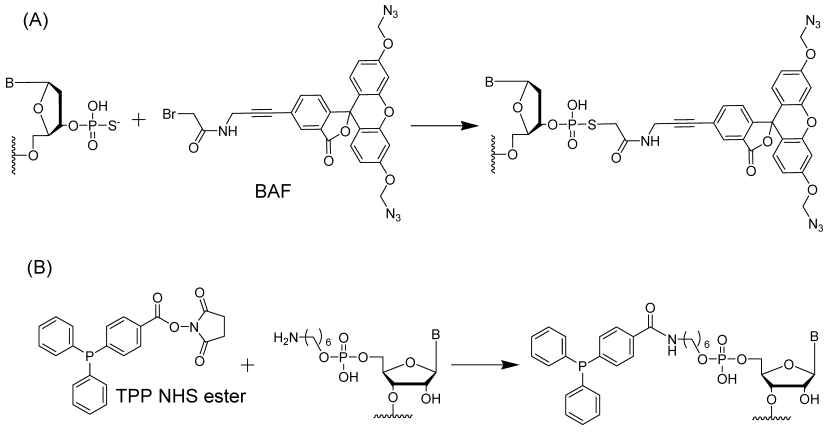

Fig. 4. (A) Reaction of 3′-Phosphorothioate with BAF and (B) Reaction of 5′-Amino Group with the TPP NHS Ester

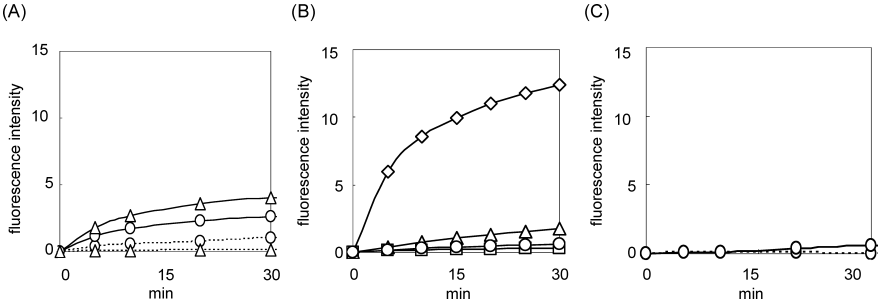

Fig. 7. Time-Course Analysis of the Fluorescence Intensity of the Reaction of BAF Probe with the TPP or DTT Probes

The emission of fluorescence at 522 nm was monitored by excitation at 490 nm. The reaction was carried out using the following conditions: (A) 50 nM BAF probe 2, 50 nM TPP probe with 50 mM (circles) or 0 mM (triangles) KCl and 50 nM (solid line) or 0 nM (dashed line) ARM in buffer (50 mM Tris–HCl (pH 8.0)). (B) 100 nM TPP probe with 100 nM BAF probe 2, 100 nM ARM (diamonds); 100 nM BAF probe 2, 0 nM ARM (circles); 100 nM BAF probe 3, 100 nM ARM (squares); 100 nM BAF perobe 2, 100 nM peptide1 (triangles) in buffer (50 mM Tris–HCl (pH 8.0), 0.13% Triton X-100). (C) 100 nM BAF probe 2, 100 nM DTT probe with 100 nM (solid line) or 0 nM (dashed line) ARM in buffer (50 mM Tris–HCl (pH 8.0)).

30 min period. In contrast, little fluorescence was observed in the absence of the target peptide (Fig. 7B). The signal/background ratio with or without target peptide reached 19.4-fold. The best sensitivity was achieved in this condition. On the other hand, the case of scramble BAF probe 3 indicated no significant increase in fluorescence. In addition, peptide 1 that is not aptamer target showed little fluorescence. Thus, the reaction of RETF probe could be accelerated by template effect of specific RNA/peptide complex. The DTT probe was also tested: the incubation of BAF probe 2 (100 nM) with the DTT probe in buffer resulted in a very low reaction speed and in a very slight increase in fluorescence in the presence of the target peptide over a period of 30 min, although background fluorescence was not observed in the absence of the target (Fig. 7C). Therefore, we concluded that the BAF/TPP probe pair offers the best sensitivity.

Discussion

We have demonstrated that the RETF probes can detect the ARM peptide by yielding a simple fluorescence readout. To date, a few fluorogenic probes based on oligonucleotides have been developed for protein detection, which include MB and fluorescence-labeled split oligonucleotides. The fluorogenic mechanism of these methods is based on FRET.[4—10] The fluorescence signal appears temporarily *via* a conformational change of the probe. In contrast, the fluorogenic mechanism of our RETF probe was based on a chemical reaction that was activated by reduction. The fluorescence signal is permanent because of the generation of a fluorescent product. This method has the advantage that the reaction occurs on the target in multiple times and that the fluorescence signal is amplified. The fluorogenic reaction proceeds only under the existence of specific RNA/peptide complex (Fig. 7B). The signal/background ratio was enhanced about 19.4-fold (for the TPP probe) in the presence of the target ARM peptide. However, this enhancement is similar to that observed for MB (*ca.* 14-fold)[9] and for two-aptamer-based MB (*ca.* 22-fold).[10] The current RETF RNA probe did not offer signal amplification, as the phosphine group of the TPP probe tended to be oxidized to phosphine oxide (triphenylphosphine oxide) in solution. In our previous study, the RETF DNA probe led to the successful amplification of the signal during oligonucleotide detection. In comparison, we found that the TPP RNA probe was more oxidizable than the TPP DNA probe. To circumvent this problem and obtain signal amplification using the RETF probe, we need to use a more stable reducing reagent (*e.g.*, triphenylphosphine-3,3′ and 3″-trisulfonic acid trisodium salt[22]) or a DNA probe.

In conclusion, we succeeded in applying the RETF system to the detection of peptides. The design of the RETF probes is not limited to the ARM peptide. Aptamers generated *via in vitro* selection allow us to design RETF probes for a variety of target proteins.

Acknowledgement H.A. and Y.I. were financially supported by MEXT (Ministry of Education, Culture, Sports, Science and Technology of Japan) and NEDO (New Energy and Industrial Technology Development Organization of Japan), and by MEXT, respectively. This work was also supported by a Grant-in-Aid for Scientific Research on Priority Areas "Lifesurveyor" from MEXT. We are grateful to the support of BSI's Research Resources Center for mass spectrum analysis.

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