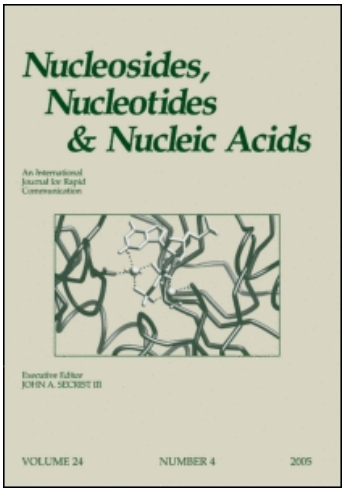

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Hasegawa, Tetsuya , Hagihara, Masaki , Fukuda, Masatora and Morii, Takashi(2007) 'Stepwise Functionalization of Ribonucleopeptides: Optimization of the Response of Fluorescent Ribonucleopeptide Sensors for ATP', Nucleosides, Nucleotides and Nucleic Acids, 26: 10, 1277 — 1281
To link to this Article: DOI: 10.1080/15257770701530491
URL: http://dx.doi.org/10.1080/15257770701530491

Nucleosides, Nucleotides, and Nucleic Acids, 26:1277–1281, 2007

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770701530491

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STEPWISE FUNCTIONALIZATION OF RIBONUCLEOPEPTIDES: OPTIMIZATION OF THE RESPONSE OF FLUORESCENT RIBONUCLEOPEPTIDE SENSORS FOR ATP

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□ *A stable complex of a peptide and RNA, ribonucleopeptide (RNP), provides a new framework to construct a macromolecular receptor for small molecules. The RNP receptor functionalized by a fluorophore-labeled Rev peptide exerts an optical signal associated with the ligand binding events. Replacing the Rev peptide of the ATP-binding RNP with a fluorophore-modified Rev peptide affords a fluorescent ATP sensor.*

Keywords RNA; peptide; ribonucleopeptide; fluorescent bisensor; in vitro selection

INTRODUCTION

The availability of receptors with appropriate affinity and specificity to the target has been expanded by in vitro selection[1] of RNA or DNA aptamers for targets ranging from small molecules to proteins or even cell membranes.[2] These macromolecular receptors composed of nucleic acids are chemically modified with reporter groups to convert into fluorescent sensors.[3–10] However, incorporating fluorophore-labeled nucleotides into the aptamers and chemical modifications of the aptamers with a fluorophore with suitable optical characteristics do not always guarantee successful construction of fluorescent biosensors responding at desirable wavelengths.

We have reported a strategy that enables isolation of fluorescent ribonucleopeptide (RNP) sensors with a variety of binding and signal-transducing characteristics, that is, high signal-to-noise ratios, detection wavelengths and concentration ranges for the ligand detection.[11] The modular structure of RNP is ideal for construction of a fluorescent sensor without introducing a

This work was supported in part by the Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan to T. M. (No. 17310125 and No. 17026019).

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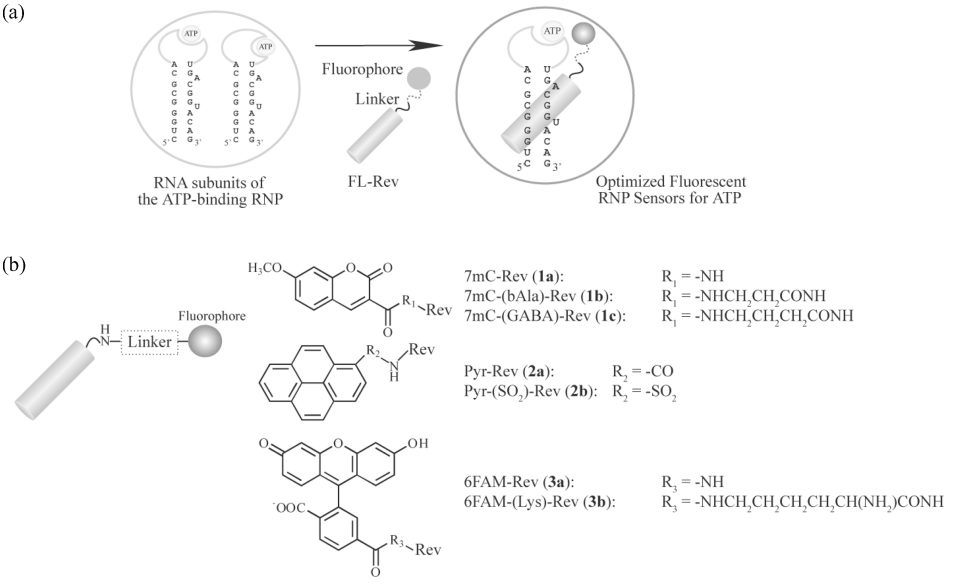

FIGURE 1 (a) A scheme illustrates a strategy to optimize fluorescent RNP sensors. Combination of the RNA subunit of the RNP receptor for ATP and a fluorophore-labeled Rev or a fluorophore- labeled Rev peptide with a linker generates combinatorial fluorescent RNP libraries, from which RNP sensors with desired optical and/or binding properties are screened. (b) Structures of fluorescent Rev peptides used in this study.

fluorophore-labeled nucleotide to the RNA aptamer (Figure 1). Adenosine 5′-triphosphate (ATP)-binding RNP receptors were obtained by in vitro selection of a library of stable RNP complexes[12,13] consist of HIV Rev-peptide and its target RNA sequence RRE.[14] The RNA subunit of RNP was utilized to construct a ligand-binding cavity by in vitro selection. The RNA subunit of the ATP-binding RNP and a Rev peptide modified with a fluorophore group formed a stable fluorescent RNP complex that showed an increase in the fluorescence intensity upon binding to ATP. Combination of a pool of RNA subunits obtained from the in vitro selection of ATP-binding RNPs and a fluorophore-modified peptide subunit afforded fluorescent RNP library, from which RNP sensors with expedient optical and binding properties were screened in a convenient manner.

RESULTS AND DISCUSSION

Fluorophores are often sensitive to a subtle microenvironmental change. By changing a tethering position of the fluorophore, or by introducing a linker with appropriate length between the amino-terminal of Rev and the fluorophore, fluorescent response of the resulting RNP sensor could be modulated. To test this, Rev derivative peptide with an additional β-alanine linker or γ-aminobutyric acid linker at the amino-terminal were modified

with 7-methoxycoumarin-3-carboxylic acid to afford 7mC-(GABA)-Rev (**1b**) and 7mC-(GABA)-Rev (**1c**). A derivative of Pyr-Rev was synthesized by modifying the Rev peptide with pyrene sulfonyl chloride (Pyr-(SO_2)-Rev, **2b**). A derivative of 6FAM-Rev was synthesized by an addition of a Lys residue at the *N*-terminal of Rev followed by modification with 6-FAM at the ε-amino group of the Lys residue (6FAM-(Lys)-Rev, **3b**).

Each fluorophore-labeled Rev (**1a**, **1b**, **1c**, **2a**, **2b**, **3a**, and **3b**) and RNA complex was placed individually on a multi-well plate, and was evaluated by the change of fluorescence intensities in the absence or presence of ATP (0.1 mM) by using a microplate reader. Relative ratios of fluorescence intensity (I/I_0) in the absence (I_0) and the presence (I) of ATP for fluorescent RNPs with (**1a-c**), (**2a-b**), and (**3a-b**) monitored at 390, 390, and 535 nm, respectively, were summarized in Figure 2. Fluorescent RNP libraries derived from the fluorescent Rev peptide of **2b** and **2c** revealed a pattern of I/I_0 ratios different from that of the 7mC-Rev derived RNP library (Figure 2a). Fluorescent RNP of **1b** with A6, A8, A24, A28, and A35 showed improved I/I_0 ratios. The observed I/I_0 ratios of fluorescent RNP of **2b** were much higher than that of the original fluorescent RNP derived from **2a**, as typically shown for the RNP with A02, A25, A26, A28, A34, and A35 (Figure 2b). Comparison of the observed I/I_0 ratios of fluorescent RNP derived from **3a** and **3b** revealed that **3b** afforded fluorescent RNPs with much higher I/I_0 ratios than **3a**, as typically shown for A6, A9, A17, and A24 (Figure 2c).

Even using the same fluorophore, the I/I_0 ratio of fluorescent sensor was affected by a subtle change in the attaching chemistry of the fluorophore on the peptide. Thus, derivatizing the fluorophore-attaching position of the Rev peptide also increased a diversity of the fluorescent RNP pool. The degree to which the fluorescence intensity changed upon binding to ATP varied with each fluorescent RNP. These results confirmed the difficulty in predicting the efficiency of optical response of the fluorescence sensor, and demonstrate the advantage of the above strategy to obtain usable fluorescence sensors.

MATERIALS AND METHODS

Synthesis of oligopeptides and fluorophore coupling reactions, nucleic acids preparations, preparation of ribonucleopeptide receptors were performed as described previously.[11]

Fluorescence Measurements on the Microplate

96-Well fluorescence measurements were performed on a Wallac ARVOsx 1420 multilabel counter. A binding solution (100 μl) containing 1 μM of fluorescent RNP in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM

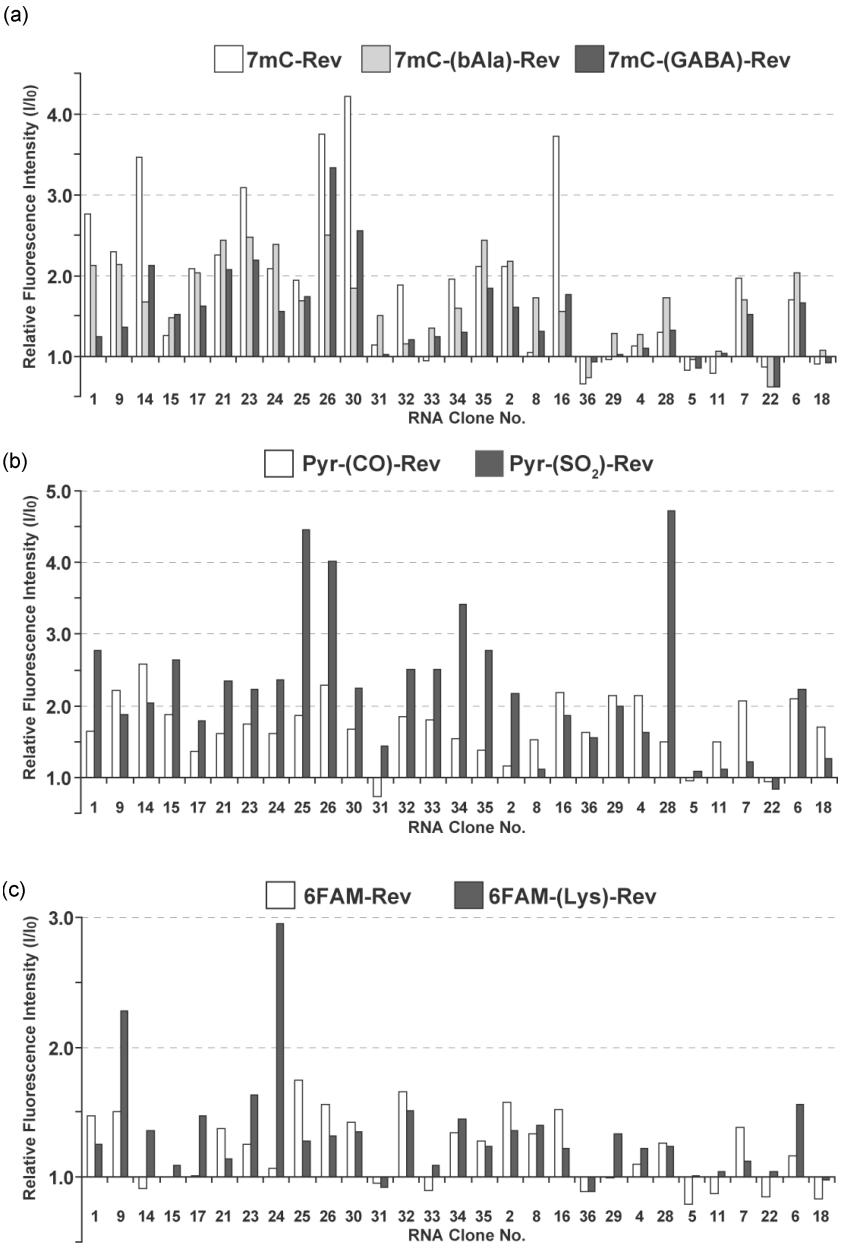

FIGURE 2 Relative fluorescence intensity changes (I/I_0) of RNPs upon ATP binding are shown in the bar graphs for the fluorescent RNPs derived from (a) 7mC-Rev (**1a**), 7mC-(bAla)-Rev (**1b**) and 7mC-(GABA)-Rev (**1c**); (b) Pyr-Rev (**2a**) and Pyr-(SO_2)-Rev (**2b**); (c) 6FAM-Rev (**3a**) and 6FAM-(Lys)-Rev (**3b**).

$MgCl_2$ with indicated concentration of ligand was gently swirled for a few minutes and allowed to sit for 30 minutes at 20°C. Emission spectra were measured with an appropriate filter set for each fluorophore. Images of the fluorescence intensity of wells were obtained by using the Wallac 1420 software version 2.00. Color-coded images of fluorescent intensity of each well are obtained by using the Wallac 1420 software version 2.00.

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