Protein Imaging

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Fluorogenically Active Leucine Zipper Peptides as Tag–Probe Pairs for Protein Imaging in Living Cells**

*Hiroshi Tsutsumi, Wataru Nomura, Seiichiro Abe, Tomoaki Mino, Akemi Masuda, Nami Ohashi, Tomohiro Tanaka, Kenji Ohba, Naoki Yamamoto, Kazunari Akiyoshi, and Hirokazu Tamamura**

Artificial functional peptides are valuable tools in various fields of chemical biology. Small peptides, such as an oligohistidine tag (His tag), can be genetically incorporated into target proteins and used for purification of recombinant proteins, immobilization of proteins on microplates, and bioimaging of proteins on the surface of living cells with their complementary partner molecules, such as Ni^{II}–nitrilotriacetic acid complex (Ni^{II}-NTA).[1] Tsien and co-workers reported that pairs of tetracysteine motif peptides and biarsenical molecular probes, which specifically bind to tetracysteine peptides, are useful in the real-time fluorescence imaging of proteins in living cells.[2] Several pairs of other tag peptides/proteins and their specific ligands have also been reported.[3,4] In many cases, however, the bound/free (B/F) separation process of probes is necessary to avoid background emission from excess probe molecules. Fluorogenic tag–probe pairs can facilitate in distinguishing the labeled proteins from the free probes, without the B/F separation process. However, very few tag–probe pairs have been developed to date.[2a]

Engineered leucine zipper peptides, which have complementary selectivity and strong binding affinity, have been applied to tags for the affinity purification of expressed proteins, to anchors for immobilization of proteins on microplates, and to allosteric modulators of engineered enzyme activity.[5] Moreover, the hydrophobic cores of leucine zipper peptides can be engineered to form hydrophobic pockets in which small organic molecules can bind.[6] It is thought that selective binding of environmentally sensitive fluorescent dyes to these pockets inside the leucine zipper assembly might induce colorimetric changes and enhance their fluorescence intensity. The unique characteristics of leucine zipper peptides might enable production of fluorescent tag–probe pairs that are exchangeable. Herein, we describe the development of a fluorescent changeable tag–probe system based on artificial leucine zipper peptides, designated ZIP tag–probe pairs, and its application to the fluorescence imaging of ZIP tag–fused protein on the surface of living cells.

The design of ZIP tag–probe pairs is based on the crystal structure of an antiparallel coiled-coil trimer of a GCN4 mutant (Figure 1).[7] The probe peptide is an α-helical peptide with 4-nitrobenzo-2-oxa-1,3-diazole (NBD), an environmentally sensitive fluorescent dye, attached to the side chain of L-α-2,3-diaminopropionic acid, that is, Dap(NBD). Tag peptides are designed as antiparallel 2α-helical peptides linked through a Gly–Gly–Cys–Gly–Gly loop sequence. Two leucine residues, which are located at the positions complementary to the NBD in the probe peptide, are replaced by alanine or glycine so that hydrophobic pockets will be formed when the tag peptides bind to the probe peptide. Original tag peptides having two leucine residues at the complementary positions are designated as L2, and alanine- or glycine-substituted tag peptides are designated as A2 and G2, respectively.

In the UV/Vis analysis, the absorption spectra of the probe peptide changed on addition of A2 producing isosbestic points at 456, 403, and 333 nm, and thus the excitation wavelength was determined as 456 nm (Figure S1 in the Supporting Information). A fluorescence titration experiment revealed that the fluorescence spectra of the probe peptide changed remarkably with increasing A2 concentration. The emission maximum arising from the NBD dye showed a significant blue shift from 536 to 505 nm with a concurrent increase in the emission intensity (Figure 2a,b and Table 1). Such a spectral change clearly suggests that the NBD moiety of the probe peptide is located in the hydrophobic environment within the 3α-helical peptide bundle structure, which is supported by the previous report of Uchiyama et al.[8]

In the cases of L2 and G2, similar spectral changes were observed although the wavelength shifts and changes in fluorescence intensity were less than those in the case of A2 (Figure S2 in the Supporting Information and Table 1). As there is insufficient space to accommodate an NBD moiety in the complex of the L2–probe pair, the NBD moiety might bind only to the hydrophobic surface of two leucine residues of L2, thus causing the subtle fluorescence change. The

[*] S. Abe, T. Mino, A. Masuda, Prof. K. Akiyoshi, Prof. H. Tamamura
Institute of Biomaterials and Bioengineering
Tokyo Medical and Dental University
Chiyoda-ku, Tokyo 101-0062 (Japan)
and
School of Biomedical Science, Tokyo Medical and Dental University
Chiyoda-ku, Tokyo 101-0062 (Japan)
Fax: (+81) 3-5280-8036
E-mail: tamamura.mr@tmd.ac.jp
Dr. H. Tsutsumi, Dr. W. Nomura, N. Ohashi, T. Tanaka
Institute of Biomaterials and Bioengineering
Tokyo Medical and Dental University
Chiyoda-ku, Tokyo 101-0062 (Japan)
Dr. K. Ohba, Prof. N. Yamamoto
AIDS Research Center, National Institute of Infectious Diseases
Shinjuku-ku, Tokyo 162-8640 (Japan)

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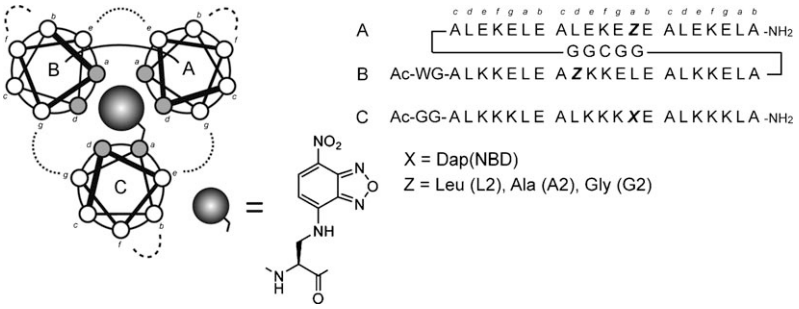

Figure 1. Structure and amino acid sequences of ZIP tag–probe pairs.

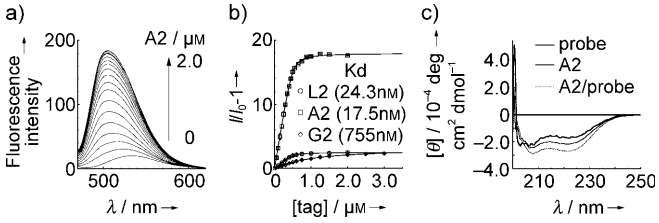

Figure 2. a) Fluorescence spectral change of the probe peptide upon addition of A2 at 25 °C in 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (pH 7.2, 100 mM NaCl): [probe] = 0.5 μM. b) Fluorescence titration curves of the probe peptide with L2, A2, and G2 at 516, 505, and 526 nm, respectively. I and I_0 represent the fluorescence intensity at various concentrations of tag peptides and the initial fluorescence intensity, respectively. c) Circular dichroism spectra of the A2 tag (solid line), the probe peptide (bold line), and their complex (dashed line) at 25 °C in 50 mM Tris–HCl buffer (pH 7.2, 100 mM NaCl).

Table 1: Emission maxima [nm] and $\Delta I_{max}/I_0$ values (in parentheses) of the probe peptide and tag–probe complexes, dissociation constants (K_d) [nM] between the tag and probe peptides, and the α-helix contents [%] of the probe, the tag peptides, and their complexes (in parentheses).

	Probe	L2	A2	G2
λ_{max} ($\Delta I_{max}/I_0$)	536 (–)	516 (2.7)	505 (17.9)	526 (2.5)
K_d[a]	–	24.3	17.5	755
α-helix content[b]	53	81 (78)	58 (71)	18 (41)

[a] Measurement conditions: 50 mM HEPES buffer solution (pH 7.2, 100 mM NaCl) at 25 °C, [probe] = 0.5 μM. [b] Measurement conditions: 50 mM Tris–HCl buffer solution (pH 7.2, 100 mM NaCl) at 25 °C; [tag], [probe], [tag–probe] = 1.0 μM. The α-helical contents were determined according to a standard method.[10]

wavelength shift and change in fluorescence intensity of the G2–probe pair were also small, which implies that the NBD moiety of the G2–probe peptide complex is located in a more hydrophilic area than those of the A2–probe peptide complex.

The dissociation constants of the probe peptide toward L2, A2, and G2 were estimated from the fluorescence titration curves by analysis with a nonlinear least-squares curve-fitting method[9] (Table 1). L2 and A2 showed high affinity, comparable to that of a normal antigen–antibody interaction, for the probe peptide. In general, the hydrophobicity of leucine zipper peptides is essential for their self-assembly and L2, for example, is more hydrophobic than A2 because it has two leucine residues. However, the binding affinity of the A2–probe pair is slightly superior to that of the L2–probe peptide pair, indicative not only of the hydrophobic interaction but also of the steric complementarity between A2 and the probe peptide, which is critical for the strong binding affinity. The binding affinity of G2 for the probe peptide is much lower than that of L2 or A2. Since a glycine residue generally destabilizes an α-helical structure, the structure of the G2–probe pair might be less stable than those of the L2– and A2–probe peptide pairs.

Circular dichroism (CD) spectra revealed that L2 and A2 tags, the probe peptide, and their complexes form α-helical structures (Figure 2c and Figure S3 in the Supporting Information). The probe peptide showed a CD spectral pattern typical of α-helical structures with negative maxima at 208 and 222 nm. L2, A2, and their complexes with the probe peptide also showed CD spectral patterns typical of α-helical structures. The α-helical content of A2 is lower than that of L2, which indicates that A2 forms a less stable α-helical structure than L2. However, the α-helix content of the A2–probe complex is higher than those of A2 or the probe peptides alone, which suggests that the A2–probe pair forms a stable 3α-helical bundle structure. Furthermore, the enhanced α-helical structure of the A2–probe complex is nearly equal to that in the L2–probe complex, which indicates that A2 can form a stable 3α-helical leucine zipper structure with the probe peptide. The CD spectrum of G2, however, shows a random-coil pattern and the α-helix content of the G2–probe complex is only 41 %. These results imply that the mutation of the leucine or alanine residues to glycine causes the destabilization of the structure of G2 and of the G2–probe complex, and it is thought that this is the reason why the G2–probe pair has a lower binding affinity than the A2–probe pair.

The fluorescence titration experiment and the CD spectra suggest that formation of a stable α-helical structure with a hydrophobic pocket is necessary for high binding affinity and fluorogenic activity. A2 forms a stable α-helical structure with a pocket that can accommodate NBD and it is thought, therefore, that the combination of the A2 tag and the probe leads to expression of the remarkable fluorescence activity. In addition, the A2–probe pair showed the same fluorescence spectral change in the cell lysate solution (Figure S7 in the Supporting Information), thus indicating that A2 is the most appropriate partner of the probe peptide as a fluorogenic tag–probe pair for protein imaging in vivo.

Next, we investigated whether our ZIP tag–probe system is available for the fluorescence imaging of proteins in living cells. CXCR4 was chosen as a model protein. CXCR4 is one of the 7-transmembrane G-protein coupled receptors, a member of a chemokine receptor family.[11] The A2 tag is genetically fused at the N terminus of CXCR4, which is an extracellular region, through the $(Gly\text{-}Ser)_6$ linker sequence. The A2-tag-fused CXCR4 is transiently expressed on the surface of Chinese hamster ovary K1 (CHO-K1) cells, and double labeling experiments of the A2-tag-fused CXCR4 using a fluorescent CXCR4 antagonist with tetramethylrhod-

amine (TAMRA)[12] and the probe peptide with the NBD dye were performed. The A2-tag-fused CXCR4 was specifically stained with red fluorescence by TAMRA-appended CXCR4 antagonist (Figure S8a in the Supporting Information). Then, the sequential labeling of the A2-tag-fused CXCR4 was performed using the probe peptide. Before removal of the probe peptide, a bright green fluorescence was observed on the surface of cells in the presence of excess probe peptide, but the fluorescence resulting from this peptide was not observed in CHO-K1 cells without expression of the A2-tag-fused CXCR4 (Figure 3b). Fluorescence arising from the TAMRA-appended CXCR4 antagonist was also observed (Figure 3a), which suggests that the binding events between the A2 tag and the probe peptide, and between CXCR4 and the TAMRA-appended CXCR4 antagonist, are independent of each other. The fluorescence image derived from the probe peptide was merged well with the fluorescence image of the TAMRA-appended CXCR4 antagonist (Figure 3c,d). After removal of the probe peptide by the exchange of culture medium, similar fluorescence images were also observed (Figure S9 in the Supporting Information). These results suggest that CXCR4 can be successfully visualized using our ZIP tag–probe system without removal of excess probe molecules. This ZIP tag–probe system is consequently a useful fluorescence-imaging tool for proteins in living cells.

In conclusion, we have developed a new functional peptide pair with fluorogenic activity based on leucine zipper peptides. The alanine-substituted tag peptide A2 binds strongly to a probe peptide, and this binding is accompanied by a dramatic fluorescent colorimetric change from weak yellow to bright green. In addition, we have demonstrated that the fluorescence imaging of A2-tag-fused CXCR4, which is a membrane-bound protein, is successfully achieved by the probe peptide. Recently, Yano et al. reported that two α-helical leucine zipper tag–probe pairs are useful fluorescence imaging tools for membrane-bound proteins.[13]

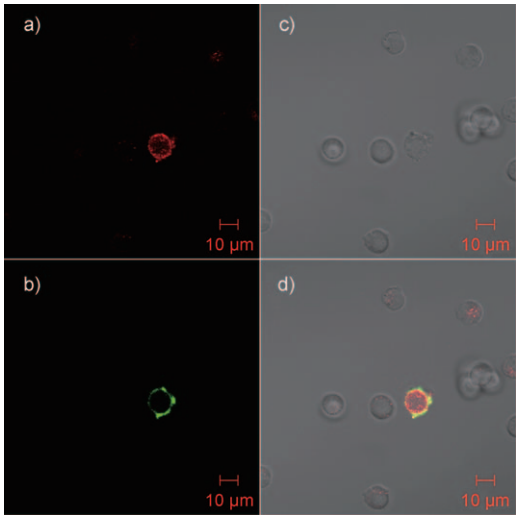

Figure 3. Sequential labeling of A2-tag-fused CXCR4 by the probe peptide after labeling by TAMRA-appended CXCR4 antagonist. a) Fluorescence image derived from TAMRA (excitation: 543 nm, emission filter: >560 nm). b) Fluorescence image derived from NBD (excitation: 458 nm, emission filter: 505–530 nm). c) Differential interference contrast. d) Merged image of (a)–(c).

Our ZIP tag–probe pairs have, in addition, fluorogenic activity which might facilitate the real-time imaging of proteins without the necessity to remove excess probe molecules. Thus, ZIP tag–probe pairs would become valuable imaging tools for target proteins in living cells.

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[1] a) K. Terpe, *Appl. Microbiol. Biotechnol.* **2003**, *60*, 523–533; b) M. Hedhammar, T. Gräslund, S. Hober, *Chem. Eng. Technol.* **2005**, *28*, 1315–1325; c) E. G. Guignet, R. Hovius, H. Vogel, *Nat. Biotechnol.* **2004**, *22*, 440–444; d) C. R. Goldsmith, J. Jaworski, M. Sheng, S. J. Lippard, *J. Am. Chem. Soc.* **2006**, *128*, 418–419; e) C. T. Hauser, R. Y. Tsien, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 3693–3697.

[2] a) B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **1998**, *281*, 269–272; b) G. Gaietta, T. J. Deerinck, S. R. Adams, J. Bouwer, O. Tour, D. W. Laird, G. E. Sosinsky, R. Y. Tsien, M. H. Ellisman, *Science* **2002**, *296*, 503–507.

[3] a) K. M. Marks, M. Rosinov, G. P. Nolan, *Chem. Biol.* **2004**, *11*, 347–356; b) A. Ojida, K. Honda, D. Shinmi, S. Kiyonaka, Y. Mori, I. Hamachi, *J. Am. Chem. Soc.* **2006**, *128*, 10452–10459; c) I. Chen, Y.-A. Choi, A. Y. Ting, *J. Am. Chem. Soc.* **2007**, *129*, 6619–6625; d) H. M. O'Hare, K. Johnsson, A. Gautier, *Curr. Opin. Struct. Biol.* **2007**, *17*, 488–494.

[4] a) A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, K. Johnsson, *Nat. Biotechnol.* **2003**, *21*, 86–89; b) A. Gautier, A. Juillerat, C. Heinis, I. R. Corrêa, Jr., M. Kindermann, F. Beaufils, K. Johnsson, *Chem. Biol.* **2008**, *15*, 128–136; c) J. Yin, F. Liu, X. Li, C. T. Walsh, *J. Am. Chem. Soc.* **2004**, *126*, 7754–7755; d) G. V. Los, A. Darzins, N. Karassina, C. Zimprich, R. Learish, M. G. McDougall, L. P. Encell, R. Friedman-Ohana, M. Wood, G. Vidurgiris et al., *Promega Cell Notes* **2005**, *11*, 2–6.

[5] a) B. Tripet, L. Yu, D. L. Bautista, W. Y. Wong, R. T. Irvin, R. S. Hodges, *Protein Eng.* **1996**, *9*, 1029–1042; b) K. Zhang, M. R. Diehl, D. A. Tirrell, *J. Am. Chem. Soc.* **2005**, *127*, 10136–10137; c) S. Yuzawa, T. Mizuno, T. Tanaka, *Chem. Eur. J.* **2006**, *12*, 7345–7352.

[6] a) I. Obataya, S. Sakamoto, A. Ueno, H. Mihara, *Biopolymers* **2001**, *59*, 65–71; b) M. K. Yadav, J. E. Redman, L. J. Leman, J. M. Alvarez-Gutiérrez, Y. Zhang, C. D. Stout, M. R. Ghadiri, *Biochemistry* **2005**, *44*, 9723–9732.

[7] B. Lovejoy, S. Choe, D. Cascio, D. K. McRorie, W. F. DeGrado, D. Eisenberg, *Science* **1993**, *259*, 1288–1293.

[8] S. Uchiyama, T. Santa, K. Imai, *J. Chem. Soc. Perkin Trans. 2* **1999**, 2525–2532.

[9] T. Kuwabara, A. Nakamura, A. Ueno, F. Toda, *J. Phys. Chem.* **1994**, *98*, 6297–6303.

[10] a) Y.-H. Chen, J. T. Yang, K. H. Chau, *Biochemistry* **1974**, *13*, 3350–3359; b) P. J. Gans, P. C. Lyu, M. C. Manning, R. W. Woody, N. R. Kallenbach, *Biopolymers* **1991**, *31*, 1605–1614; c) D. Y. Jackson, D. S. King, J. Chmielewski, S. Singh, P. G. Schultz, *J. Am. Chem. Soc.* **1991**, *113*, 9391–9392.

[11] a) S. G. Ward, J. Westwick, *Biochem. J.* **1998**, *333*, 457–470; b) H. Tamamura, H. Tsutsumi, W. Nomura, T. Tanaka, N. Fujii, *Expert Opin. Drug Discovery* **2008**, *3*, 1155–1166.

[12] W. Nomura, Y. Tanabe, H. Tsutsumi, T. Tanaka, K. Ohba, N. Yamamoto, H. Tamamura, *Bioconjugate Chem.* **2008**, *19*, 1917–1920.

[13] Y. Yano, A. Yano, S. Oishi, Y. Sugimoto, G. Tsujimoto, N. Fujii, K. Matsuzaki, *ACS Chem. Biol.* **2008**, *3*, 341–345.

Communications

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H. Tsutsumi, W. Nomura, S. Abe, T. Mino, A. Masuda, N. Ohashi, T. Tanaka, K. Ohba, N. Yamamoto, K. Akiyoshi, H. Tamamura* ——— ■■■■–■■■■

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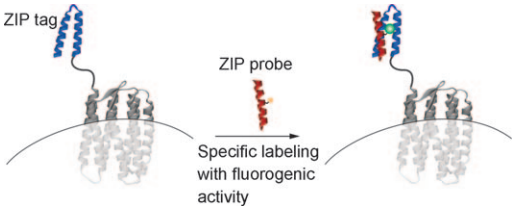

All zipped up: Pairs of leucine zipper peptides with a solvatochromic fluorescent dye show fluorogenic activity through formation of a 3α-helical bundle structure. Such peptides enable the in situ fluorescence imaging of proteins in the presence of excess fluorescent probe (see picture). Imaging of membrane-bound protein is demonstrated using these zipper peptides as functional tag–probe pairs.