Supporting Information

Development of green fluorescent protein-based cAMP indicators with expanded dynamic range and various working ranges

Sohei Hiasa[1], Takeru Fujimori[1], Saki Aiki[2], Hiroshi Ueda[3], Takashi Tsuboi[2]*, Tetsuya Kitaguchi[3]*

[1]School of Life Science and Technology, Department of Life Science and Technology, Tokyo Institute of Technology 4259 Nagatsuta-cho, Midori-ku, Yokohama-shi, Kanagawa, 226-8501, Japan
[2]Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
[3]Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama-shi, Kanagawa, 226-8503, Japan,

* E-mail: kitaguct-gfp@umin.ac.jp, takatsuboi@bio.c.u-tokyo.ac.jp

Experimental Procedures

Chemicals

cAMP and cGMP were purchased from Sigma-Aldrich (St. Louis, MO, USA), Forskolin from Tokyo Chemical Industry (Tokyo, Japan), 3-isobutyl-1-methylxanthine, 8-Br-cAMP and adenosine from Merck Millipore (Darmstadt, Germany), ATP, ADP and AMP from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Plasmid construction

The DNA fragment of GFP, restructured from circularly permuted (cp) GFP in G-GECO[1], a green fluorescent genetically-encoded Ca^{2+} indicator, was modified by PCR to contain KpnI and BglII restriction enzyme sites between N144 and S146 and cloned into the pRSET-A vector (Thermo Fisher Scientific, Massachusetts, USA). The cDNA for the mouse cAMP-dependent protein kinase (PKA) type I-beta regulatory subunit[2] (NP_001240819.1) was obtained from adult mouse brain total RNA by RT-PCR. The region of the cDNA encoding the cAMP binding domain (amino acids 92-248) derived from site A, was ligated at the KpnI/BglII site of the GFP DNA. To improve the fluorescence intensity (FI), the N- and C-terminus linker lengths were optimized by inserting various lengths (N-terminus 0 to 9 amino acids; C-terminus 0 to 10 amino acids) of leucine zipper sequences[3] between the GFP and the cAMP binding domain sequences. The plasmids encoding mutant indicators with different linker lengths at the N- and C-terminus of the cAMP binding domain were transformed into *Escherichia coli* JM109 (DE3) (Promega, Madison, USA), and these lysates were subjected to examine the FI change. The plasmid DNA of the mutant showing the maximum response was selected as a template for subsequent random mutagenesis. To further improve the FI change by introducing random mutations into the amino acid sequences around the linkers, PCR was performed using two degenerate primers containing NNK and MNN mixtures of bases. Plasmids carrying site-directed random mutations around linkers were transformed into JM109 (DE3), and around 50 colonies (empirically ~15 kinds of amino acid coverage) were picked. The plasmid of mutant with the maximum response was selected as the next template for the molecular evolution cycle of random mutagenesis, and this cycle continued until the response was more than 3-fold. Then, to adjust the half-maximal effective concentration (EC_{50}) of Green Falcan by changing the affinity to cAMP, PCR was performed using two primers containing NNK and MNN designed to the region around R211 of PKA, which is important for cAMP binding[4]. The mutants with various EC_{50} (1, 3, 10 μM) were selected by the dose-response curve using a four-parameter logistic curve fitting. For live-cell imaging in mammalian cells, Green Falcan DNA sequences were subcloned into the pcDNA3.1 (-) vector (Thermo Fisher Scientific, Massachusetts, USA). To improve the solubility of the expressed proteins, the super acidic

region of mouse amyloid β precursor protein (amino acids 190-286) was fused to the N-terminus of the indicators. For subcellular localization, the nuclear localization signal (nls； PKKKRKV) fused to the N-terminus of the indicators was derived from SV40.

Protein expression and purification

Green Falcans DNA in the pRSET-A vector were transformed into *Escherichia coli* JM109 (DE3) cells, cultured in 400 mL LB medium with 50 mg/μL ampicillin at 20℃ for 4 days and harvested by centrifugation. The harvested cells were suspended in phosphate-buffered saline (PBS; pH 7.4) with 0.5% (v/v) Triton-X and 40 μg/mL lysozyme, and lysed by French press (Constant Systems Ltd, Daventry, UK). After centrifugation of the lysate, the supernatant containing the Green Falcans protein was purified by TALON® Metal Affinity Resin (TaKaRaBio, Shiga, Japan). After washing three times with 10 mM imidazole, the proteins were eluted from the resin with 300 mM imidazole. To remove the imidazole, the eluate was applied to PD-Miditrap G-25 (GE Healthcare, Chicago, USA) in 150 mM KCl, 50 mM HEPES-KOH (pH 7.4), 0.5% (v/v) Triton-X. The purified protein was stored at -80℃.

***In vitro* spectroscopy**

The excitation and emission spectra of Green Falcans were measured using a spectrophotometer (F2700, Hitachi, Tokyo, Japan). To examine the dose-response relationship, various concentrations of cAMP and cGMP (from 0.001 to 300 μM) were applied to 0.3 μM purified Green Falcan protein. Relative FI was calculated by dividing by the FI of the peak in the absence of cAMP. To obtain the EC_{50} value and dynamic range of the Green Falcans for cAMP and cGMP, a four-parameter logistic curve fitting was performed using QtiPlot (IONDEV SRL, Bucharest, Romania). For cAMP, the minimum FI in fitting was normalized to 1. For cGMP, the minimum FI in fitting was normalized to 1, and the maximum FI was normalized to the maximum FI for cAMP in the fitting. To examine specificity, cAMP analogues were applied at 10 times the concentration of each EC_{50} value to 0.3 μM purified Green Falcan proteins. The absorption spectra of 50 μM Green Falcan proteins were measured using a spectrophotometer (V-730BIO, JASCO Corporation, Tokyo, Japan) with or without 100 μM cAMP.

Cell culture and transfection

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (High Glucose) with L-glutamine, sodium pyruvate, penicillin and streptomycin (Wako, Osaka, Japan), 10% (v/v) heat-inactivated fetal bovine serum (GE Healthcare, Chicago, USA). These cells were subcultured in 35 mm glass-bottomed dishes at 37℃ with 5% CO_2 for live-cell imaging. Plasmids of each Green Falcans were transfected to

these cells with Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instruction. Then, HeLa cells were cultured at 37℃ overnight. After changing the medium, HeLa cells were cultured at 30℃ to 32℃ for at least 30 h to allow maturation of indicator chromophores.

Live-cell imaging

Live-cell imaging was performed using a fluorescence inverted microscope (IX70, Olympus, Tokyo, Japan) equipped with an oil immersion ×40 objective lens (UApo/N 340, ×40, NA=1.35, Olympus), a cooled CCD camera (Cool SNAP HQ^2, Photometrics), a mercury lamp, an excitation filter of 460-480 nm, an emission filter of 495-540 nm and a dichroic mirror of 485 nm (U-MGFPHQ, Olympus). For dual-color imaging, the DA/FI/TR-3X3M-C Sedat filter set (OPTO-LINE, Inc., Saitama, Japan) was changed by using an HF110 high-speed filter wheel (Prior Scientific, Cambridge, UK). Fluorescence images were acquired every 5 s using Metafluor software (Molecular devices, Sanjose, USA). FI was measured by manually surrounding each cell with a region of interest (ROI) using ImageJ software (https://imagej.net/software/fiji/downloads)[5]. After subtracting the FI of a cell-free area, relative FI was calculated by dividing by the mean of the FI captured over 1 or 2 min before administering reagents. For statistical analysis, a student t-test was performed using R (https://www.r-project.org/)[6].

References

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Supplementary figure

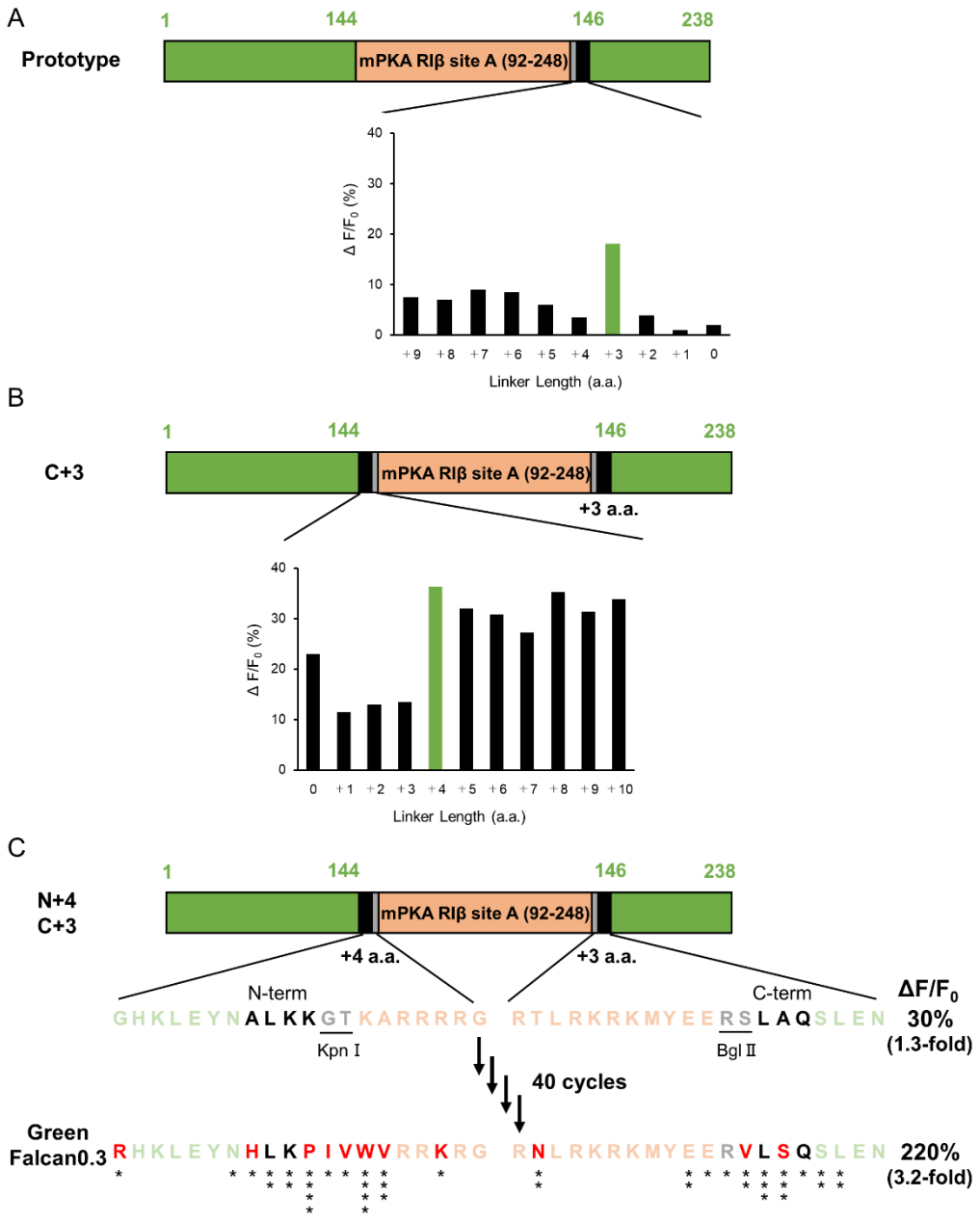

Figure S1. Screening by the crude Green Falcan protein (A, B) Diagram of the indicators under development, and the screening results of various constructs with modified linker lengths at (A) the C-terminus and (B) the N-terminus. Responses to 100 μM cAMP are shown as $\Delta F/F_0$. (C) The amino acid sequences around the linker and introduced point mutations. Asterisks indicate where random mutations were performed, and red letters indicate mutated amino acids.