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Fluorescent Indicators for Cyclic GMP Based on Cyclic GMP-Dependent Protein Kinase Iα and Green Fluorescent Proteins

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We describe herein fluorescent indicators for cyclic GMP (cGMP) in single living cells. cGMP-dependent protein kinase Iα (PKG Iα), a receptor for cGMP, was fused with blue- and red-shifted green fluorescent proteins (GFPs) to its N- and C-termini, respectively. Using PKG $I\alpha_{\Delta 1-47}$, in which the dimerization domain was deleted, fluorescence resonance energy transfer between the GFPs was found to increase upon cGMP-induced conformational change in PKG $I\alpha_{\Delta 1-47}$. We demonstrated that thus-developed fluorescent indicators reversibly responded to cGMP that was produced in nitric oxide-stimulated HEK293 cells. The present genetically encoded fluorescent indicators open a way not only for understanding the dynamics of cGMP signaling in single cells and organisms but also for discovering pharmaceuticals such as isoform-specific inhibitors for phosphodiesterases.

We have succeeded in filling the vacancy for cyclic GMP (cGMP) in an array of fluorescent indicators for major second messengers, such as Ca^{2+},[1,2] inositol 1,4,5-triphosphate,[3] diacylglycerol,[4] cAMP,[5,6] and cGMP. We describe herein genetically encoded fluorescent indicators for cGMP in single living cells.

Upon stimulating cells with various chemicals such as nitric oxide, peptide hormones, and toxins, cGMP is synthesized by catalytic conversion of GTP by soluble and particulate guanylyl cyclases.[7,8] cGMP acts as a signaling molecule via its receptor proteins, including cGMP-dependent protein kinases,[9−11] cyclic nucleotide-gated cation channels,[12] and cGMP-binding phosphodiesterases[13,14] and regulates various physiological processes such as relaxation of vascular smooth muscle cells, phototransduction in retinae, epithelial electrolyte transport, bone growth, and neuronal activity. Imaging the dynamics of cGMP in the single living cells is of interest not only to reveal the mechanism underlying the cellular and organ functions but also to discover or screen drugs such as phosphodiesterase inhibitors,[15] which control the intracellular [cGMP], from millions of candidate compounds. However, conventional radioimmunoassay of cell lysates prepared from millions of cells, exclusively used for analysis of total cGMP levels, does not meet those cell biological and pharmaceutical requirements.

Here we describe genetically encoded fluorescent indicators for cGMP. To construct the fluorescent indicators for cGMP, two functionally independent parts were chosen: one part is for selective molecular recognition for cGMP, and the other is for transducing the molecular recognition event to the generation or change in fluorescence signals. As a receptor for cGMP, cGMP-dependent protein kinase Iα (PKG Iα) was chosen on the basis of a previous small-angle X-ray scattering study in which cGMP-induced conformational change in PKG Iα was revealed.[16] As a transducer to detect the cGMP-dependent conformational change in PKG Iα, blue- and red-shifted mutants of green fluorescent proteins (GFPs), cyan fluorescent protein (ECFP), and yellow fluorescent protein (EYFP) were fused to N- and C-terminal ends of PKG Iα, respectively. ECFP and EYFP serve as the donor and acceptor fluorophores for fluorescence resonance energy transfer (FRET), of which intensity is a function of the proximity (R) and relative angular orientation (κ) between the fluorophores.[2] cGMP is expected to alter these R and/or κ values upon its binding to PKG Iα that is located between the two fluorophores. The change in these FRET parameters is expected to cause cGMP-dependent

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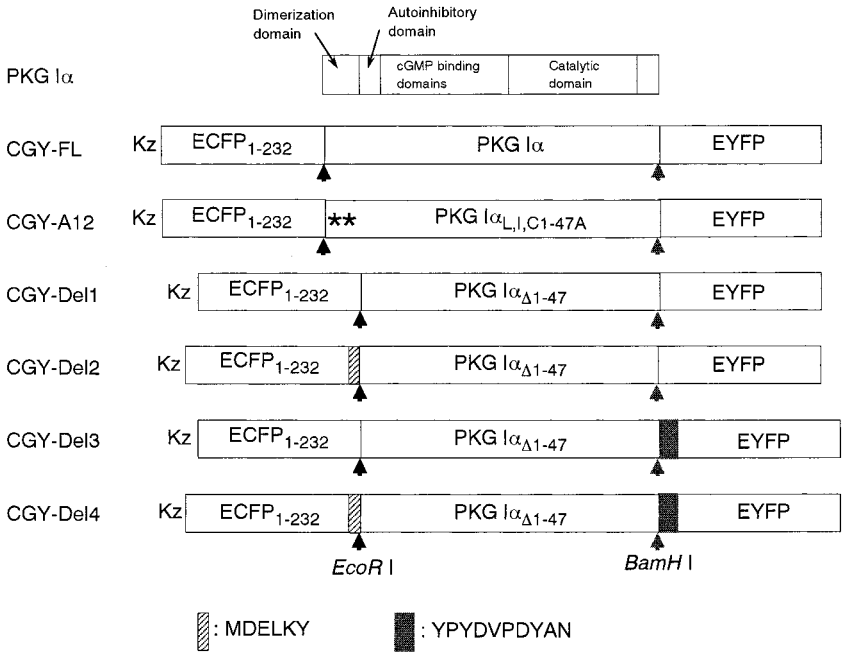

Figure 1. Schematic representations of domain structures of bovine cGMP-dependent protein kinase Iα (PKG Iα), CGY-FL, -A12, and -Del1-4. Two linker sequences are shown in the bottom. ECFP and EYFP are *Aequorea victoria* GFPs with mammalian codons and the following additional mutations: ECFP, F64L/S65T/Y66W/N146I/M153T/V163A/N212K, and EYFP, S65G/V68L/Q69K/S72A/T203Y. Kz is an abbreviation of a Kozak sequence, which allows optimal translation initiation in mammalian cells. Two asterisks in CGY-A12 indicate the mutation of all leucine, isoleucine, and cycteine residues within 1−47 residues in PKG Iα to alanine.

changes in the observed fluorescence spectra, which provides a selective and sensitive measure for intracellular concentration of cGMP, [cGMP]. The FRET between two different-color GFPs has been demonstrated to be powerful for monitoring Ca^{2+},[2,17] cyclic AMP,[6] and protease activity[18,19] in the cell. It also offers the potential for improving FRET-based fluorescent indicators replacing synthetic fluorophores such as fluorescein and rhodamine[20] with these color GFPs.

EXPERIMENTAL SECTION

Plasmid Construction. For constructing cDNAs of CGY-FL, -A12, and -Del1-4, fragment cDNAs of $ECFP_{1-232}$, $ECFP_{1-232}$ with a C-terminal linker sequence (MDELKY), PKG Iα, PKG $I\alpha_{L,I,C1-47A}$, PKG $I\alpha_{\Delta1-47}$, EYFP, and EYFP with an N-terminal linker sequence (YPYDVPDYAN) were generated by standard polymerase chain reaction to attach a Kozak sequence and restriction site shown in Figure 1. Each entire cDNA encoding CGY-FL, -A12, and -Del1-4 was subcloned at *Hin*dIII and *Xho*I sites of mammalian expression vector pcDNA3.1 (+) (Invitrogen Co., Carlsbad, CA).

Cell Culture and Transfection. Chinese hamster ovary (CHO-K1) and human embryonic kidney (HEK293) cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS) and in Dulbecco's modified eagle medium supplemented with 10% FCS, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids, respectively, at 37 °C in 5% CO_2. Cells were transfected with LipofectAMINE 2000 reagent (Life Technologies, Rockville, MD) in 24-well plates. Within 12−24 h after the transfection, the cells were spread onto glass bottom dishes for imaging.

Imaging of Cells. Before imaging, the culture medium was replaced with Hank's balanced salt solution. Within 3−5 days after the transfection, the cells were imaged at room temperature on a Carl Zeiss Axiovert 135 microscope with a cooled CCD camera MicroMAX (Roper Scientific Inc., Tucson, AZ), controlled by MetaFluor (Universal Imaging, West Chester, PA). The exposure time at 440 ± 10-nm excitation was 50 ms. The fluorescence images were obtained through 480 ± 15- and 535 ± 12.5-nm filters with a 40× oil-immersion objective (Carl Zeiss, Jena, Germany). To minimize an unavoidable increase in cGMP buffering as a result of overexpression of the cGMP indicators, cells expressing high concentration of the cGMP indicators were not used for imaging, as is the case for Ca^{2+} imaging reported previously.[17]

RESULTS AND DISCUSSION

Design and Expression of cGMP Indicators, CGYs. Mammalian PKG Iα is composed of two identical monomers. Each monomer in the dimeric kinase contains four types of functional domains, as shown in Figure 1. The N-terminal dimerization domain is composed of a leucine/isoleucine zipper motif.[21,22] In the absence of cGMP, PKG Iα displays a kinase inactive "closed" conformation, in which its catalytic center is occupied by an autoinhibitory domain. Upon binding of two cGMPs to the cGMP binding domains, a marked conformational change is induced by which the autoinhibitory domain is removed from the catalytic

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center and PKG Iα is converted into a kinase active "open" form in its conformation.[16,23] However, because the overall structure of PKG Iα has not been solved by X-ray diffraction or NMR, *R* and *κ* values in the tandem fusion protein of ECFP, PKG Iα, and EYFP, termed CGY, are unpredictable, in both the absence and the presence of cGMP. We have designed three constructs, CGY-FL, -A12 and -Del1, as fluorescent indicators for cGMP, as shown in Figure 1. CGY-FL contains a full-length PKG Iα. In contrast, the leucine/isoleucine zipper motifs of the dimerization domains in CGY-A12 and in CGY-Del1 are mutated (L, I, C1−47A) and deleted (Δ1−47), respectively, to avoid *inter*molecular FRET, which might possibly occur by dimerization of the indicators and complicate the fluorescence signal changes based on *intra*molecular FRET that is modulated by cGMP.

CHO-K1 cells were transfected by a standard lipofection method with cDNAs encoding, respectively, CGY-FL, -A12, and -Del1 inserted in mammalian expression vectors. Figure 2a shows fluorescence images of the transfected cells taken by using emission filters for ECFP (480 ± 15 nm) and EYFP (535 ± 12.5 nm), showing unexpected accumulation of CGY-A12 in the nucleus. CGY-FL and CGY-Del1 were excluded from the nucleus; however, in about 30% of CHO-K1 cells expressing CGY-Del1, fluorescence from CGY-Del1 was observed also in the nucleus (data not shown). The leucine/isoleucine zipper motif in PKG Iα may have a role to direct its localization in the extranuclear compartment.

Intramolecular FRET Is Sensitive to cGMP-Induced Conformational Changes in CGYs. To evaluate the responses of the indicators for cGMP, CHO-K1 cells expressing the indicators were stimulated with 8-Br-cGMP, which is widely used as a membrane-permeable and phosphodiesterase-resistant analogue of cGMP. Figure 2b shows pseudocolor images of ratios of 480 ± 15 nm to 535 ± 12.5 nm emissions before (time, 0 s) and at different time points after the addition of 1 mM 8-Br-cGMP. Figure 2c shows time courses of the emission ratio changes for the indicators in the cytosol. No detectable change in the emission ratio was observed for CGY-FL (Figure 2b,c). In addition, fluorescecne intensities for ECFP and EYFP were not affected by stimulating with 8-Br-cGMP (data not shown). In contrast, a dose of 8-Br-cGMP produced a significant decrease in the emission ratio for the CGY-Del1-expressing cell (Figure 2b,c), in which reciprocal changes in ECFP and EYFP fluorescence intensities in the cytosol were observed, as shown in Figure 2d. These results indicate that FRET between ECFP and EYFP increases upon binding of 8-Br-cGMP to CGY-Del1. In the case of CGY-A12, 8-Br-cGMP caused a change in the emission ratio, as shown in Figure 2b,c; however, the maximal change in this ratio was about one-third of that for CGY-Del1.

Intramolecular FRET between GFPs in their double fusion protein is sensitive to both *R* and *κ* values, due to their more rigidly fixed chromophores,[2,17,24] than to using conventional organic fluorophores,[5,20] which suggests that by inserting small linker sequences between the GFP mutants and PKG $I\alpha_{\Delta 1-47}$, the change in the emission ratio for CGY-Del1 may be improved. Several constructs containing linker sequences between PKG $I\alpha_{\Delta 1-47}$ and GFPs were tested, three of which are schematically shown in Figure 1 as CGY-Del2, -Del3, and -Del4. The initial emission ratios and the emission ratio changes of CGY-Del2, -Del3, and -Del4 for 8-Br-cGMP are shown and compared to those of CGY-FL, -A12, and -Del1 in Figure 2e. In CHO-K1 cells, CGY-Del2, -Del3, and -Del4 responded to 8-Br-cGMP; however, the linker sequences, MDELKY to the N-terminus of PKG $I\alpha_{\Delta 1-47}$ and/or YPYDVP-DYAN to the C-terminus of PKG $I\alpha_{\Delta 1-47}$, gave smaller effects for 8-Br-cGMP than were expected on both the initial emission ratios and the changes in the emission ratio. In view of the high initial emission ratios for CGY-FL, -A12, and -Del1-4, ECFP and EYFP seem not to be closely located in the absence of cGMP, due to the large size of PKG Iα, which decreases the sensitivity of FRET efficiency for the initial difference in the *R* value caused by the small linker insertion. Substantial deletion of PKG Iα may improve the emission ratio change of the indicator, although the responses of CGY-Del1-4 are already enough for the cGMP imaging.

Figure 2f illustrates the responses of CGY-Del1 for 8-Br-cGMP and 8-Br-cAMP in CHO-K1 cells, which were in concentration-dependent manners for both cyclic nucleotide analogues. Approximate concentrations of the cyclic nucleotide analogues that give half-maximum responses were 10^{-6} M and 10^{-4} M for 8-Br-cGMP and 8-Br-cAMP, respectively. The cyclic nucleotide selectivity of the CGY-Del1 response thus obtained is similar to that of native and wild-type PKG Iα activation that was reported previously.[25] By using a baculovirus-mediated large-scale expression system, in vitro studies to evaluate the exact binding constants of CGYs for cGMP and cAMP may be possible.[25] From these results, we conclude that FRET from ECFP to EYFP in CGY-Dels can be a selective measure for the cellular concentration of cGMP, and the physiological concentration of cAMP does not interfere with this cGMP detection.

Imaging of Nitric Oxide-Stimulated Production of cGMP. We next examined whether the indicators thus developed respond to intracellular cGMP generated upon stimulation of the living cells with nitric oxide (NO). HEK293 cells[26] were transfected with cDNA of CGY-Del1. To activate the soluble guanylyl cyclase in the cell, NOC-7,[27] a NO-releasing compound, was added to give its final extracellular concentration of 500 μM. NOC-7 releases NO spontaneously in a rate-controlled manner. Figure 3a shows time courses of the NOC-7-induced changes in the emission ratio for CGY-Del1 in HEK293 cells. A significant and fast decrease in the emission ratio was followed by a slower increase in the ratio. The latter increase in the emission ratio disappeared when the cells were pretreated with 100 μM zaprinast,[28] a selective inhibitor for cGMP-specific phosphodiesterase, although the initial fast decrease in the emission ratio was not affected. Pretreatment of the cells with 10 μM ODQ,[29] a selective inhibitor for NO-sensitive guanylyl cyclase, completely suppressed the change in the emission ratio elicited earlier by stimulating with the NO donor.

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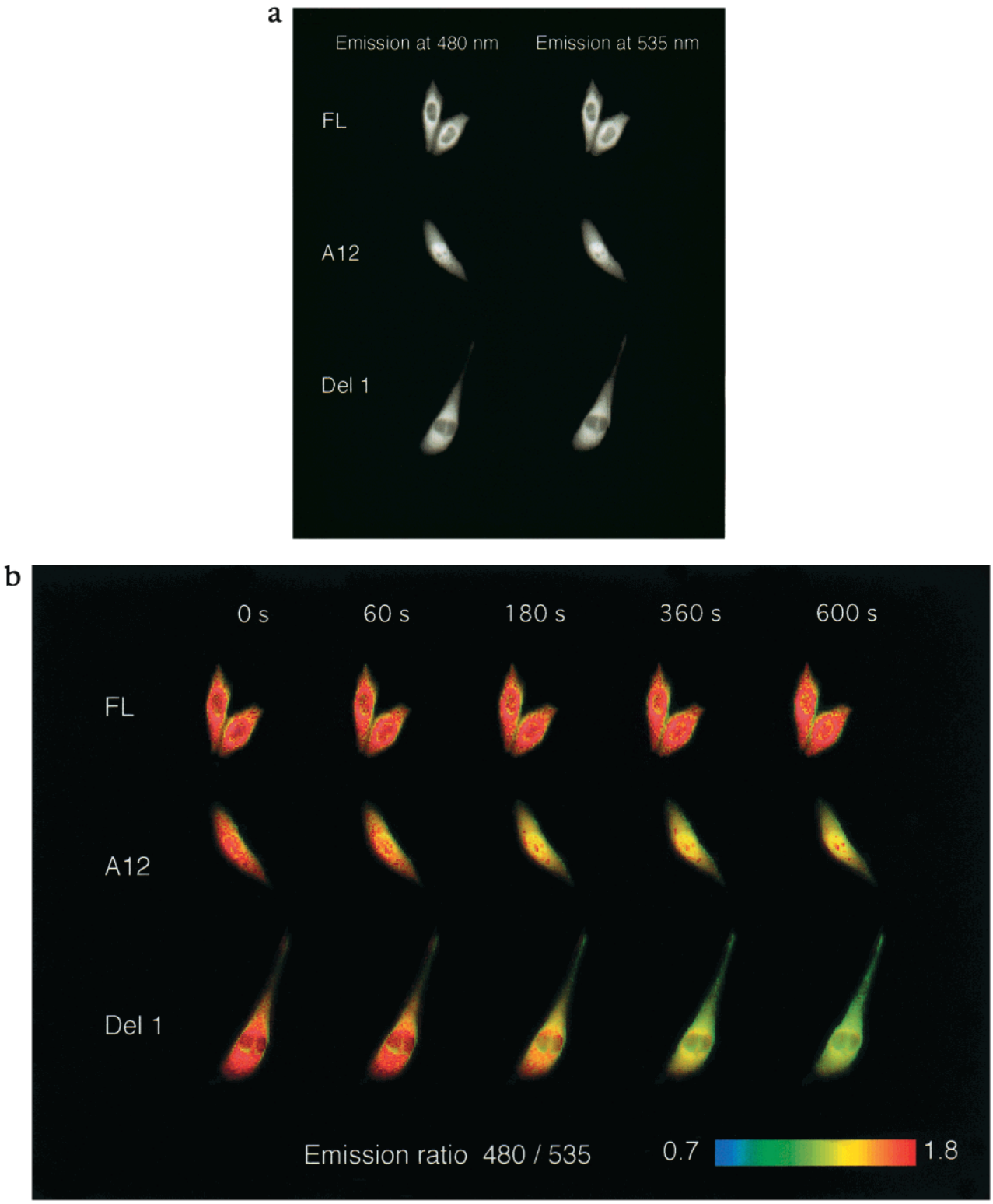
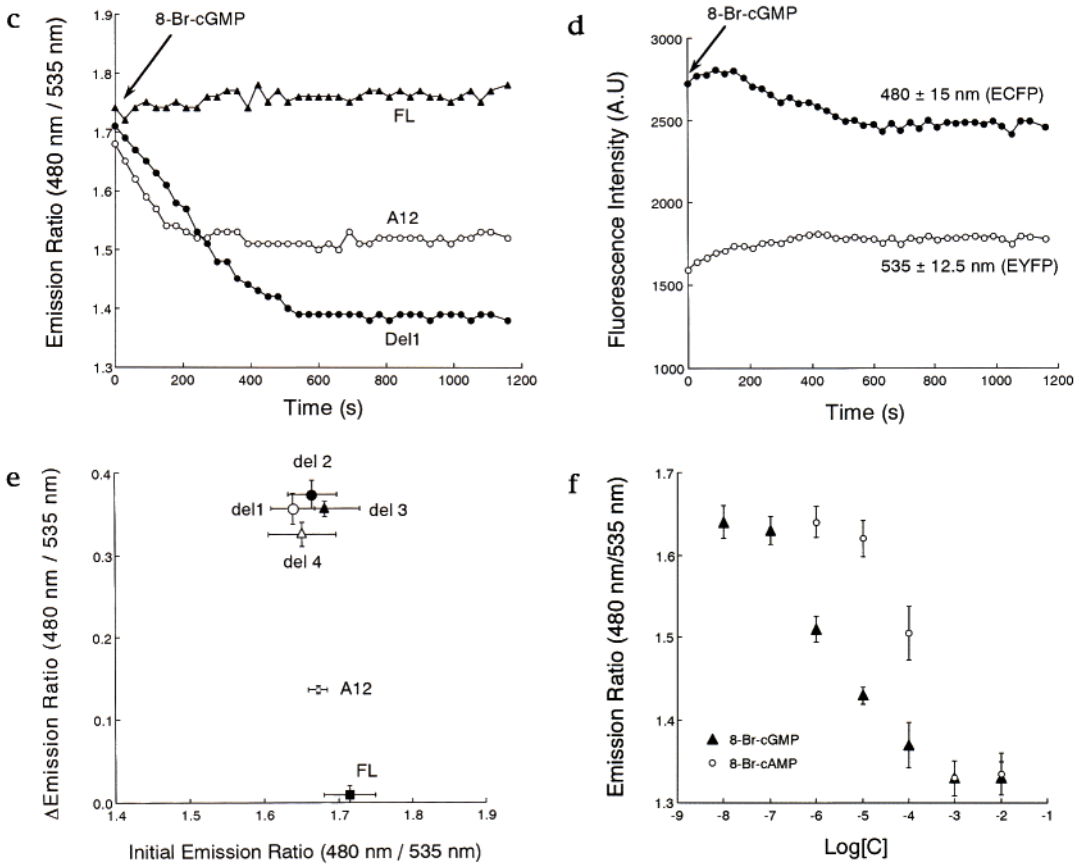


Figure 2. Response of CGY-FL, -A12 and -Del1-4 for 8-Br-cGMP in CHO−K1 cells: (a) Fluorescence images of the cells expressing CGY-FL, -A12, and -Del1, taken using emission filters for ECFP (480 ± 15 nm) and EYFP (535 ± 12.5 nm), when excited at 440 ± 10 nm; (b) Pseudocolor images of ratios of 480 ± 15 nm to 535 ± 12.5 nm emissions before (time 0 s) and at different time intervals after the addition of 1 mM 8-Br-cGMP, obtained from the CHO-K1 cells expressing CGY-FL, -A12, and -Del1; (c) Changes in the ratios of emissions from CGY-FL (▲), -A12 (○), and -Del1 (●) in the cytosol, each when stimulated with 8-Br-cGMP (Figure 2b); (d) Kinetics of cytosolic fluorescence at 480 ± 15 nm (ECFP, ●) and 535 ± 12.5 nm (EYFP, ○) from the CGY-Del1-expressing CHO-K1 cell, each when stimulated with 8-Br-cGMP (Figure 2b); (e) Comparison of the initial emission ratios and the changes in the emission ratios for CGY-FL, -A12, and -Del1-4, each when stimulated with 8-Br-cGMP; (f) Response of CGY-Del1 for various concentrations of 8-Br-cGMP (▲) and 8-Br-cAMP (○) in CHO-K1 cells. CGY-Del1-expressing cells were incubated with 8-Br-cGMP or 8-Br-cAMP for 3−6 h, and the emission ratio in the cytosol was measured on the fluorescence microscope. The results are the means and standard deviations of emission ratios from five different cells (mean ± SD).



Similar results were obtained by using CGY-Del2−4 (data not shown). These results indicate that CGY-Dels sufficiently work as the fluorescent indicators for [cGMP] in the single living cells.

Can the fluorescent indicators CGY-Dels then reversibly detect the fluctuation of intracellular [cGMP]? As shown in Figure 3b,c, an administration of 500 μM NOC-7 to the CGY-Del1-expressing HEK293 cell caused a rapid decrease in the emission ratio, and the subsequent recovery of the ratio to the initial level was observed. The later increase in the emission ratio is due to the degradation of cGMP by cGMP-specific phosphodiesterase, as was pharmacologically examined using zaprinast in Figure 3a. Upon a subsequent dose of 1 mM 8-Br-cGMP, a phosphodiesterase-resistant analogue of cGMP, the recovered emission ratio was decreased again (Figure 3b,c). By using zaprinast instead of 8-Br-cGMP, a similar re-decrease in the recovered emission ratio was observed (Figure 3c). These results demonstrate the reversible response of CGY-Del1 to fluctuating concentration of cGMP in the single living cells.

Figure 3d shows the response of CGY-Del1 to differing concentrations of NO, 0.01−100 μM NOC-7, in HEK293 cells. At higher concentrations of NO, 10 and 100 μM NOC-7, cytosolic concentration of cGMP similarly increased transiently and decreased nearly to the initial level; however, at lower concentrations of NO, 1 and 0.1 μM NOC-7, the responses of CGY-Del1 were quite different from those at higher concentrations of NO. At 1 μM NOC-7, the rate of cGMP accumulation was faster than that at a higher concentration of NOC-7, and the recovery rate of the emission ratio was slower, which resulted in more prolonged duration of cGMP accumulation. At 0.1 μM NOC-7, the change in cytosolic concentration of cGMP was found to be not monotonic but, rather, was oscillating. Intracellular regulation of the activities of guanylyl cyclase and cGMP-phosphodiesterase should be responsible for the complex changes in [cGMP] observed in Figure 3d. For example, rapid desensitization of soluble guanylyl cyclase at higher concentration of NO[30] and allosteric or PKG-mediated phosphorylation-dependent regulation of cGMP-phosphodiesterase[15] were recently discussed. The present indicators for cGMP are, thus, expected to be used for discovering and understanding the cellular dynamics of cGMP, such as the cGMP oscillation, and for discerning cGMP signaling pathways by combining its use with general molecular biological techniques.

CONCLUSIONS

Fluorescence imaging is one of the most powerful techniques available for observation of the intracellular dynamics involving second messengers, exemplified by fluorescent indicators for Ca^{2+},[1,2] cAMP,[5,6] and inositol 1,4,5-triphosphate[3]. However, fluorescent indicators for cGMP, one of the pivotal second messengers, have been left undeveloped. The analysis of cGMP has, therefore, exclusively depended upon conventional radioimmunoassay of cell lysates prepared from millions of cells, which makes it difficult to probe the spatial and temporal dynamics of cGMP in the living cells. To overcome this limitation, we developed fluorescent indicators for cGMP. Tandem fusion proteins of ECFP, PKG I$\alpha_{\Delta 1-47}$, and EYFP were demonstrated to work as fluorescent indicators for cGMP, in which FRET was reversibly increased and decreased upon binding and dissociation of cGMP, respectively (Figure 4).

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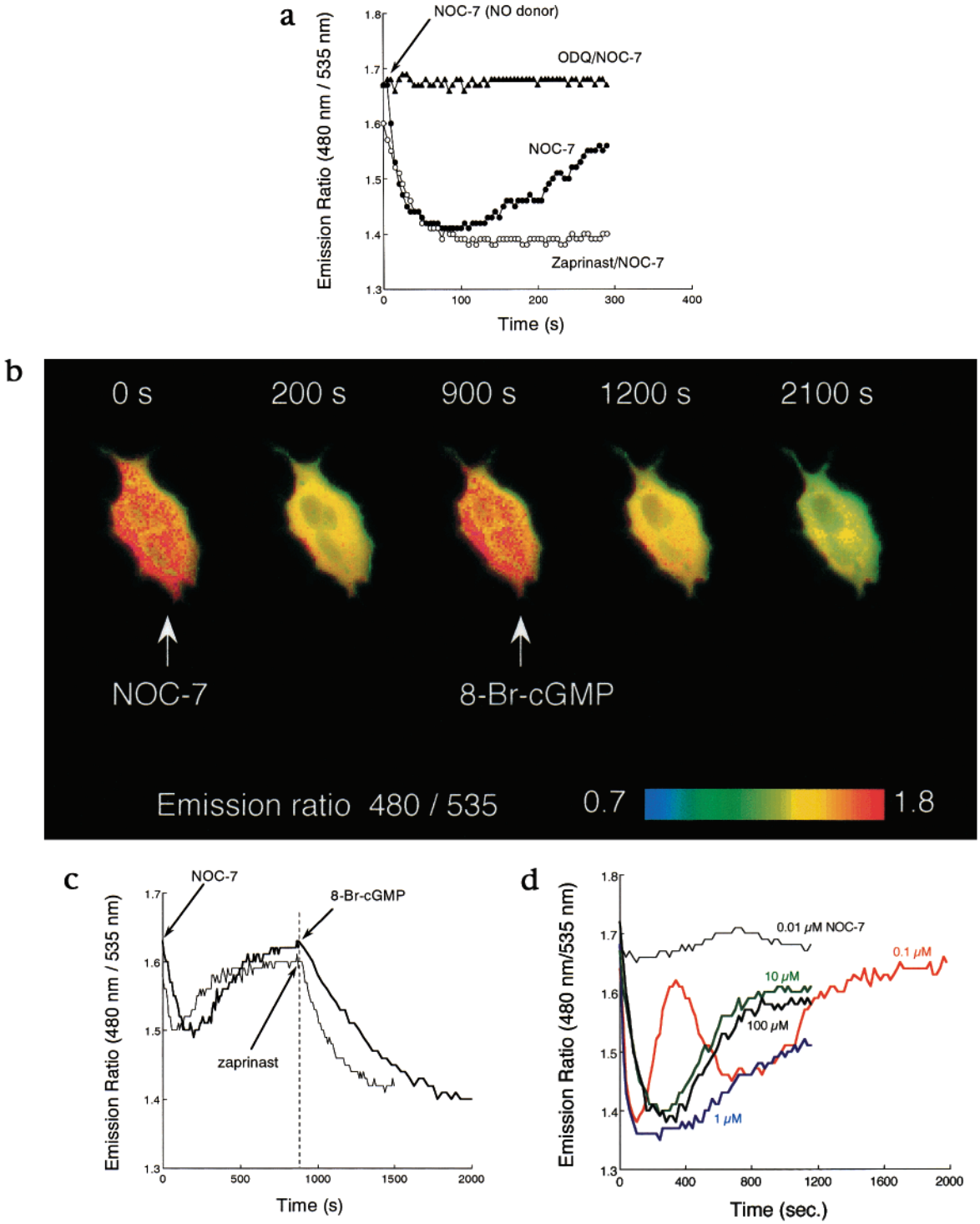

Figure 3. Reversible response of CGY-Del1 for fluctuating concentration of cGMP in the living cells: (a) Changes in the emission ratio for CGY-Del1 in the cytosol of HEK293 cells when stimulated with 500 μM NOC-7 (▲, the cell is pretreated with 10 μM ODQ; ○, the cell is pretreated with 100 μM zaprinast.). (b) Pseudocolor images of 480/535-nm emission ratios before (time 0 s) and at various time points after the addition of 500 μM NOC-7. At 900 s after addition of NOC-7, 8-Br-cGMP was added to give its final extracellular concentration of 1 mM. (c) Changes in the emission ratios for CGY-Del1 in the cytosol of HEK293 cells when stimulated with 500 μM NOC-7. At 900 s after addition of NOC-7, 1 mM 8-Br-cGMP (bold line) or 100 μM zaprinast (thin line) was added. (d) Response of CGY-Del1 to various concentrations of NO, 0.01−100 μM NOC-7.

The cGMP indicators presented in this paper are genetically encoded, allowing further improvement of the indicators by standard mutagenesis techniques. The intracellular location of the indicator should easily be controlled by fusing with signal sequences or with proteins of interest such as guanylyl cyclases,[7,8] cyclic nucleotide-gated cation channels,[12] or anchoring proteins of PKG Iα.[31] On the basis of previous mutagenesis studies regarding the binding domains for cyclic nucleotides,[25,32] cGMP/

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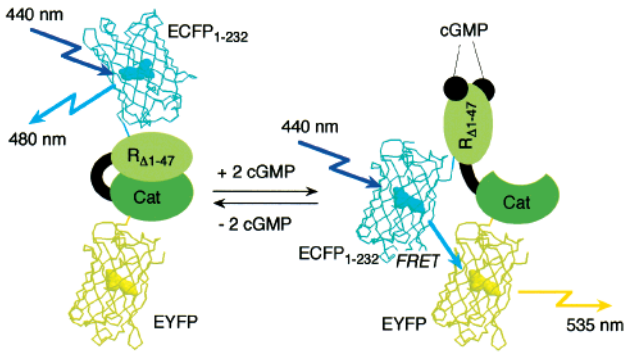

Figure 4. Schematic diagram showing how to detect cGMP on the basis of FRET between ECFP and EYFP. The cylindrical β-can structure and the central chromophore of GFPs are drawn according to the crystal structure of *Aequorea victoria* GFP. The precise overall structure of PKG I$\alpha_{\Delta 1-47}$ and the relative orientations of the GFPs are not known; however, small-angle X-ray scattering data have revealed a marked conformational change in PKG Iα upon cGMP binding.[16]

cAMP selectivity of the indicator may be changed, which suggests a possibility for the development of cAMP indicators consisting of single polypeptides, not the complex of GFPs-fused catalytic and regulatory domains of cyclic AMP-dependent protein kinase, as was previously reported.[6] Kinase-deficient mutants of the CGY-Dels may be more physiological, although no toxic effects were observed on proliferation rates of cells expressing the CGY-Dels. The present indicators for cGMP are expected to contribute to cell-based high-throughput screening[33,34] of potent and isoform-specific inhibitors for phosphodiesterases. In addition, transgenic animals expressing the indicator for cGMP may enable nondestructive and continuous monitoring of [cGMP] levels in tissues and organs of interest before and after the administration of pharmaceuticals.

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