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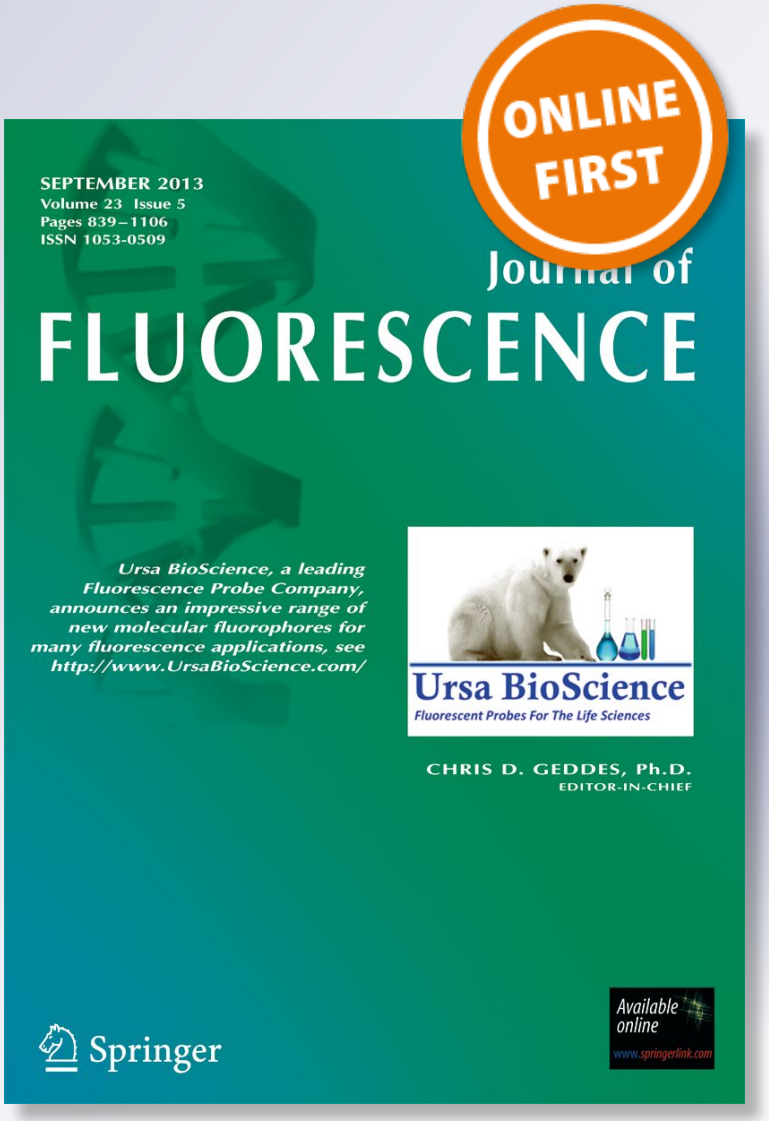
Journal of Fluorescence

ISSN 1053-0509

J Fluoresc
DOI 10.1007/s10895-017-2159-2



Springer

J Fluoresc
DOI 10.1007/s10895-017-2159-2

ORIGINAL ARTICLE

Substitution with a Single Cysteine in the Green Fluorescent Protein-Based Calcium Indicator GCaMP3 Enhances Calcium Sensitivity

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Received: 26 June 2017 / Accepted: 31 July 2017

Abstract Genetically encoded calcium indicators (GECI) such as GCaMP3 are attracting significant attention as a good option for measuring intracellular calcium levels. Recently, a modified GCaMP3 called dCys-GCaMP3 was developed by replacing two threonine residues with cysteines. dCys-GCaMP3 proved to be a better calcium indicator, but it was not clear how and why the two cysteine residues were able to enhance the protein's calcium sensitivity. The aim of the present study was to investigate the possible roles of these cysteine residues in dCys-GCaMP3. dCys-GCaMP3 (Thr330Cys;Thr364Cys) exhibited enhanced fluorescence intensity compared to the canonical GCaMP3 in calcium imaging experiments. However, substitution of a single residue at position 330 with cysteine (Thr330Cys) also afforded comparable sensitivity to GCaMP3. In contrast, the other single residue substitution at position 364 with cysteine (Thr364Cys) failed to enhance calcium sensitivity, showing that cysteine at position 330 is essential to improve calcium sensitivity. Thr330Cys substitution in the GCaMP3 or "Cys^{330}-GCaMP3" showed significantly reduced background fluorescence, and the fluorescence intensity was proportional to the amount of DNA used to transfect the cells used in the study. The substitute had to be cysteine, because replacement with other amino acids such as alanine, valine, and aspartate did not improve GCaMP3's calcium sensitivity. Cys^{330}-GCaMP3 outperformed a synthetic calcium-specific indicator, Fluo-3, in various calcium imaging experiments. Thus, the present study asserts that substituting the threonine at position 330 in GCaMP3 with cysteine is essential to enhance calcium sensitivity, and suggests that Cys^{330}-GCaMP3 can be used as a potent fluorescent calcium indicator to measure intracellular calcium levels.

Keywords Calcium dye · Genetically encoded calcium indicators · GCaMP3 · Calcium imaging · Fluo-3

Introduction

Calcium plays a pivotal role in living systems as a second messenger, relaying signals from extracellular environment into intracellular region through actions of various receptors, ion channels and/or enzymes. Thus, intracellular calcium levels are frequently measured to monitor target molecules that may be involved in various pathophysiological conditions. To this end, calcium indicators are used widely in a broad spectrum of research areas. Synthetic dyes, which are small molecule-based calcium indicators such as Fluo-3, are known to emit fluorescence when calcium is bound to the dye [1]. However, these synthetic dyes do have limitations; additional incubation time is necessary for the dye to be loaded into the cells, and once the synthetic dye enters the cells, cells often try to expel the dye, making long-term and repeated measurement very challenging [2].

To circumvent these limitations of synthetic dyes, a different class of calcium indicators called genetically encoded calcium indicators (GECI) has been developed. GCaMP is a very good example of this class of indicators [2, 3]. Basically, GCaMP is a fusion product of three different proteins; circularly permutated enhanced GFP moiety

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Published online: 08 August 2017

which gives fluorescence signal, calmodulin domain as a sensor for calcium, and M13 which serves as a transducer [4]. The advantage of GCaMP is that it does not emit fluorescence in the absence of calcium because of the defect in the barrel structure introduced by circular permutation. Since the time GCaMP has been developed, there were various attempts to maximize the calcium sensitivity and minimize the disadvantages of GCaMP [5–8]. As a result, numerous GCaMP variants have been developed. GCaMP3 is one of the popular variants, which has stronger fluorescence than the other GCaMP variants [9, 10].

GCaMP3 too has its drawbacks such as high background fluorescence from dead or damaged cells [11]. To overcome this problem, a novel variant of GCaMP3 called dCys-GCaMP3 had been developed. dCys-GCaMP3 is engineered by substituting two residues in GCaMP3 with cysteine (Thr330Cys;Thr364Cys) [11]. Although the authors speculated that the enhanced sensitivity of dCys-GCaMP3 was probably due to putative cysteine–cysteine disulfide bond, they did not offer definitive evidence to support their hypothesis. Moreover, it was not clear why two cysteine substitutions are required, since the canonical GCaMP3 already contains many cysteine residues. Therefore, the aim of the present study was to investigate the importance of the cysteine substitutions in the enhancement of the calcium sensitivity of GCaMP3.

We also investigated the possible roles of the threonine residues at positions 330 and 364 in GCaMP3, in addition to investigating the effect of substituting these residues with cysteine. Finally, the utility of the new GCaMP3 variant in calcium imaging in combinations with receptors and/or ion channels was examined by comparing its calcium sensitivity to that of a synthetic dye, Fluo-3.

Materials and Methods

Genes

GCaMP3 (Plasmid #22692), which has been subcloned into the backbone of pEGFP-N1 expression vector, was purchased from Addgene (Cambridge, MA, USA). Coding sequences of *TRPA1* (NM_007332.2), *TRPV1* (NM_080704.3), *MrgprA3* (NM_153067.2), and *MrgprC11* (NM_207540) were subcloned in pcDNA3.1. All the constructed vectors were sequenced, to confirm that the sequences of the inserts showed 100% identity with GenBank sequences.

Site-Directed Mutagenesis of GCaMP3

Various point mutations were introduced using site-directed mutagenesis kit, following manufacturer's protocol (MutaDirect™ Site Directed Mutagenesis Kit, iNtRON, Seongnam, Korea). Primer sequences used in the present study are summarized in Table 1.

Cell Culture and Gene Transfection

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and Zell Shield (Minerva biolabs, Berlin, Germany). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. The cells were subcultured every 3 or 4 days with complete media. Cells were transfected with appropriate genes using FuGENE® HD Transfection Reagent (Promega, Madison, WI, USA) or ViaFect™ Transfection Reagent (Promega, Madison, WI, USA) according to manufacturer's protocol. Calcium imaging experiments were performed 24 h after transfection.

Calcium Imaging

Intracellular calcium was measured using a calcium imaging technique with an inverted microscope (ECLIPSE Ti-U; Nikon, Tokyo, Japan) by a previously described method. HEK293T cells were cultured on a lysine-coated eight-well chamber (Lab-Tek; Naperville, IL, USA). The cells were transfected with genes of interest. A day after transfection, the culture medium was replaced with normal bath solution NBS [140 mM NaCl, 5.0 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, and 5.5 mM HEPES (pH 7.4)]. For some experiments, the cells were incubated with 5 μM Fluo-3 AM (Invitrogen, Carlsbad, CA, USA) in the

Table 1 Primer sequences for site-directed mutagenesis of GCaMP3

Name	Forward	Reverse
Thr330Cys	CGGGGATGGGACAATATGCACCAAGGAGCTGGGGACG	CGTCCCCAGCTCCTTGGTGCATATTGTCCCATCCCCG
Thr364Cys	GCCGACGGTGACGGCTGCATCGACTTCCCTGAGTTC	GAACTCAGGGAAGTCGATGCAGCCGTCACCGTCGGC
Thr330Ala	CGGGGATGGGACAATAGCAACCAAGGAGCTGGGGACG	CGTCCCCAGCTCCTTGGTTGCTATTGTCCC ATCCCCG
Thr330Val	CGGGGATGGGACAATAGTCACCAAGGAGCTGGGGACG	CGTCCCCAGCTCCTTGGTGACTATTGTCCCATCCCCG
Thr330Asp	CGGGGATGGGACAATAGACACCAAGGAGCTGGGGACG	CGTCCCCAGCTCCTTGGTGTCTATTGTCCCATCCCCG

presence of 0.1% Pluronic F-127 (Invitrogen, Carlsbad, CA, USA). After incubation, the medium was washed out and appropriate compounds were added to induce calcium influx. The excitation wavelength was 488 nm and emission wavelength was 515 nm for measuring the calcium-specific fluorescence. Microscopic images were acquired at 1.5-s intervals, using Nikon NIS Elements software. Changes in intracellular calcium levels are expressed as F/F_0 ratio, where F_0 is the initial fluorescence intensity and F is the fluorescence intensity after the calcium influx. The ratio was calculated using ImageJ program (National Institutes of Health (NIH), Bethesda, MD, USA) with custom-made scripts.

Statistical Analysis

All data were presented as mean ± SEM. In calcium imaging data, total cell numbers counted from at least three separate experiments were represented as *n*. For comparison among more than three groups, one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post-test was used.

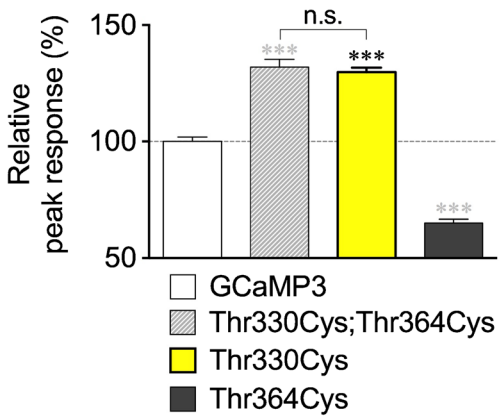

Fig. 1 Comparison of relative fluorescence intensities in response to 100 μM AITC treatment of HEK293T cells transfected with different GCaMP3 variants that also transiently express TRPA1 ion channels. Relative to the peak fluorescence intensity of canonical GCaMP3, which was set to100% (empty bar, 100.0 ± 1.882%, $n = 2230$), the peak response of Thr330Cys;Thr364Cys was 131.8 ± 3.381%, (diagonal stripe bar, $n = 821$). Single substitution variant, Thr330Cys, also showed peak response (yellow bar, 129.7 ± 1.930%, $n = 2371$) similar to that of Thr330Cys;Thr364Cys, whereas Thr364Cys substitution exhibited decreased response (black bar, 65.02 ± 1.596%, $n = 1904$). *** $p < 0.001$ after ANOVA with Dunnett's post-test compared to GCaMP3 as a control. n.s: not significant

Results

A Single Amino Acid Residue Substitution at Position 330 in GCaMP3 with Cysteine is Essential and Sufficient to Improve its Calcium-Specific Fluorescence Intensity

We first examined whether dCys-GCaMP3 (Thr330Cys;Thr364Cys) truly exhibited enhanced calcium sensitivity. To this end, HEK293T cells were co-transfected with dCys-GCaMP3 (0.5 μg) and TRPA1 (0.5 μg), followed by treatment with allyl isothiocyanate (AITC), a specific agonist of TRPA1, to induce calcium influx. As shown in Fig. 1, dCys-GCaMP3 indeed showed higher relative peak intensities (131.8 ± 3.381%, $n = 821$), in response to 100 μM AITC treatment, compared to the canonical GCaMP3 (Control, 100.0 ± 1.882%, $n = 2230$).

We further investigated which of the substitutions is critical for the enhancement of calcium sensitivity. HEK293T cells expressing TRPA1 were transfected with single substitution variants, in which threonine either at position 330 (Thr330Cys) or 364 (Thr364Cys) were replaced with cysteine. The results showed that a single substitution, Thr330Cys, yielded peak responses (129.7 ± 1.930%, $n = 2371$) comparable to that of the variant with double substitution. In contrast, single substitution at position 364 (Thr364Cys) failed to enhance calcium sensitivity beyond that of control (65.02 ± 1.596%, $n = 1904$). These results indicate that Thr330Cys substitution is essential to enhance the calcium sensitivity of GCaMP3. It further indicates that Thr364Cys substitution may not be necessary for improving the calcium sensitivity of GCaMP3.

Cys^{330}-GCaMP3 Exhibits Improved Calcium Indicator Characteristics Compared to GCaMP3

We then further investigated the overall characteristics of Thr330Cys substitution in the GCaMP3 or "Cys^{330}-GCaMP3" as a calcium indicator. When HEK293T cells expressing TRPA1 were treated with 100 μM AITC, the fluorescence time-course of Cys^{330}-GCaMP3 was similar to that of canonical GCaMP3 (Fig. 2a). More specifically, Cys^{330}-GCaMP3 showed an instantaneous rise in fluorescence intensity upon AITC treatment, and gradually declined after reaching the peak. Although the time-course profiles of Cys^{330}-GCaMP3 and canonical GCaMP3 are similar, the peak intensity of Cys^{330}-GCaMP3 was approximately two times stronger than that of canonical GCaMP3. Thus, these data illustrate that Cys^{330}-GCaMP3 has gained in calcium sensitivity without any change in the time-course.

Advantages of Cys^{330}-GCaMP3 were even more distinct when fluorescent microscopic images were directly compared to those of canonical GCaMP3, as shown in Fig. 2b. Elevated fluorescence intensity after AITC ("+AITC") treatment of the cells expressing Cys^{330}-GCaMP3 was

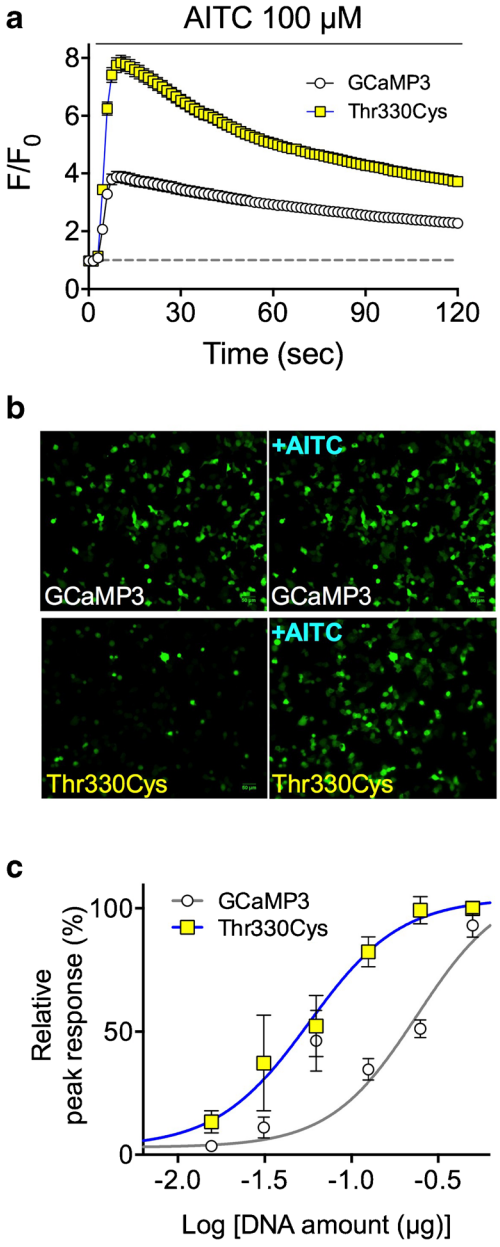

Fig. 2 Cys^{330}-GCaMP3 showed enhanced calcium sensitivity than canonical GCaMP3. **a** A time-course graph of fluorescence intensity of either Cys^{330}-GCaMP3 (Thr330Cys, yellow rectangle) or canonical GCaMP3 (empty circle) in HEK293T cells transfected with 0.125 μg of the plasmid containing the appropriate insert. Intracellular calcium levels increased after treatment with 100 μM AITC, a specific agonist of TRPA1 ion channel. F/F_0 indicates relative peak intensity, where F indicates fluorescence intensity at certain time point, and F_0 denotes initial fluorescence intensity. Dotted line drawn for $F/F_0 = 1$, indicates the absence of any changes in fluorescence intensities. **b** Representative calcium imaging data comparing before (left) and after (right) 100 μM AITC ("+AITC") treatment of HEK293T cells expressing TRPA1, transfected with either GCaMP3 (top) or Cys^{330}-GCaMP3 (Thr330Cys, bottom). Notice the high background fluorescence in GCaMP3 (top left) even before AITC treatment and the strong increase of fluorescence after AITC treatment in cells with Cys^{330}-GCaMP3. **c** DNA amount vs. response curves of canonical GCaMP3 (empty circle) and Cys^{330}-GCaMP3 (Thr330Cys, yellow rectangle). Notice clear left-shift of the Cys^{330}-GCaMP3 curve, which normally implies increased potency

evident when compared to that of cells expressing canonical GCaMP3 (control). Moreover, it was also observed that the initial fluorescence intensity (before AITC treatment) in many cells with Cys^{330}-GCaMP3 was remarkably lower, implying that there is significantly reduced background fluorescence. This is important since the background noise is often a reflection of dead or damaged cells, which may further attenuate calcium sensitivity. Conversely, canonical GCaMP3 (control) not only exhibited relatively higher initial background fluorescence, but also showed minimal calcium sensitivity after AITC treatment.

When ranges of different DNA amounts of Cys^{330}-GCaMP3 were used in transfection, the plot of [DNA amount] vs. [AITC response] was of typical sigmoidal shape, which is characteristic of a classical dose–response curve (Fig. 2c). Moreover, the curve of Cys^{330}-GCaMP3 was undoubtedly left-shifted compared to that of canonical GCaMP3 (Fig. 2c), which implies that calcium sensitivity of Cys^{330}-GCaMP3 is more potent than that of canonical GCaMP3. Taken together, these results demonstrate that Cys^{330}-GCaMP3 exhibits improved characteristics as a calcium indicator compared to GCaMP3.

Replacing Threonine with Cysteine at Position 330 is Required to Enhance Calcium-Specific Fluorescent Intensity of GCaMP3

We further examined whether the amino acid replacing the threonine at position 330 has to be specifically cysteine, to improve the calcium sensitivity of GCaMP3. To this end, GCaMP3 variants with different amino acids at position 330 such as alanine, aspartate, and valine were constructed.

As shown in Fig. 3, none of these substitutions improved calcium sensitivity, when HEK293T cells expressing TRPA1 were treated with 100 μM of AITC. Specifically, GCaMP3 variants with alanine (Thr330Ala, $91.64 \pm 1.410\%$, $n = 1363$) and valine (Thr330Val, $93.27 \pm 1.169\%$, $n = 2011$) exhibited even lower calcium sensitivity than canonical GCaMP3. Although aspartate substitution at position 330 (Thr330Asp, $106.8 \pm 1.462\%$, $n = 1597$) resulted in slightly higher calcium sensitivity than that of canonical GCaMP3, it was not comparable to that of Cys^{330}-GCaMP3 ($129.7 \pm 2.970\%$, $n = 4129$). Although not all amino acids were tested as substituents of threonine at position 330, it appears that substitution of threonine at position 330 with cysteine is indeed required to enhance the calcium sensitivity of GCaMP3.

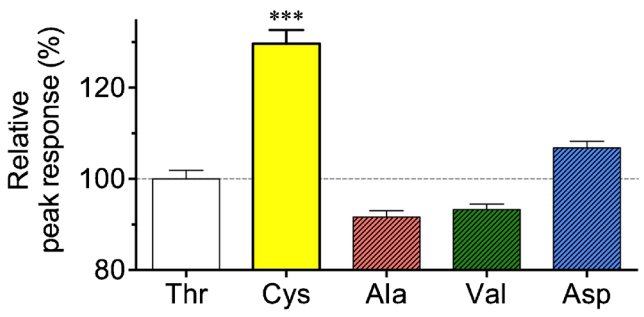

Fig. 3 Comparison of relative peak intensities of variants with different substitutions at position 330 in GCaMP3. Calcium influx was stimulated by 100 μM AITC treatment of HEK293T cells expressing TRPA1. Relative to the peak intensity of control (Thr, empty bar), which was set as 100%, only Cys^{330}-GCaMP3 (Cys, yellow bar, 129.7 ± 2.970%, $n = 4129$) showed remarkably increased peak intensity, whereas Thr330Ala (Ala, red bar, 91.64 ± 1.410%, $n = 1363$), Thr330Val (Val, green bar, 93.27 ± 1.169%, $n = 2011$), Thr330Asp (Asp, blue bar, 106.8 ± 1.462%, $n = 1597$) variants did not show any difference compared to control (Thr). *** $p < 0.001$ in ANOVA with Dunnett's post-test compared to Thr as a control

Calcium-Specific Fluorescence Intensity of Cys^{330}-GCaMP3 is Higher Than That of Fluo-3

Finally, the calcium sensitivity of Cys^{330}-GCaMP3 was compared to that of Fluo-3, a widely-used synthetic calcium-specific fluorescent dye. As shown in Fig. 4a, Cys^{330}-GCaMP3 showed significantly brighter and stronger fluorescence intensity than Fluo-3, upon induction of calcium influx in HEK293T cells expressing TRPA1. This superior efficacy of Cys^{330}-GCaMP3 was also observed in HEK293T cells expressing a different ion channel, TRPV1, treated with 100 μM capsaicin, a specific agonist of TRPV1 (Fig. 4b). To further verify the efficacy of Cys^{330}-GCaMP3 under different conditions, a G-protein-coupled receptor of $G\alpha_{q/11}$ category, MrgprC11, was expressed in HEK293T cells [12]. Again, Cys^{330}-GCaMP3 outperformed Fluo-3, following treatment of the cells with BAM8-22, an endogenous peptide known to be a specific agonist of MrgprC11 (Fig. 4c). Lastly, Cys^{330}-GCaMP3 responded even better than Fluo-3 upon chloroquine (1 mM) treatment (Fig. 4d), which requires the action of both receptor (MrgprA3) and ion channel (TRPA1) to induce calcium influx.

In the light of all the above results, it can be concluded that Cys^{330}-GCaMP3 outperforms Fluo-3 under various calcium imaging conditions. Therefore, we believe that Cys^{330}-GCaMP3 is a superior alternative that can replace classical calcium-specific fluorescent dyes in various calcium imaging experiments.

Discussion

The present study discovered that a single amino acid replacement Thr330Cys, but not Thr364Cys, was

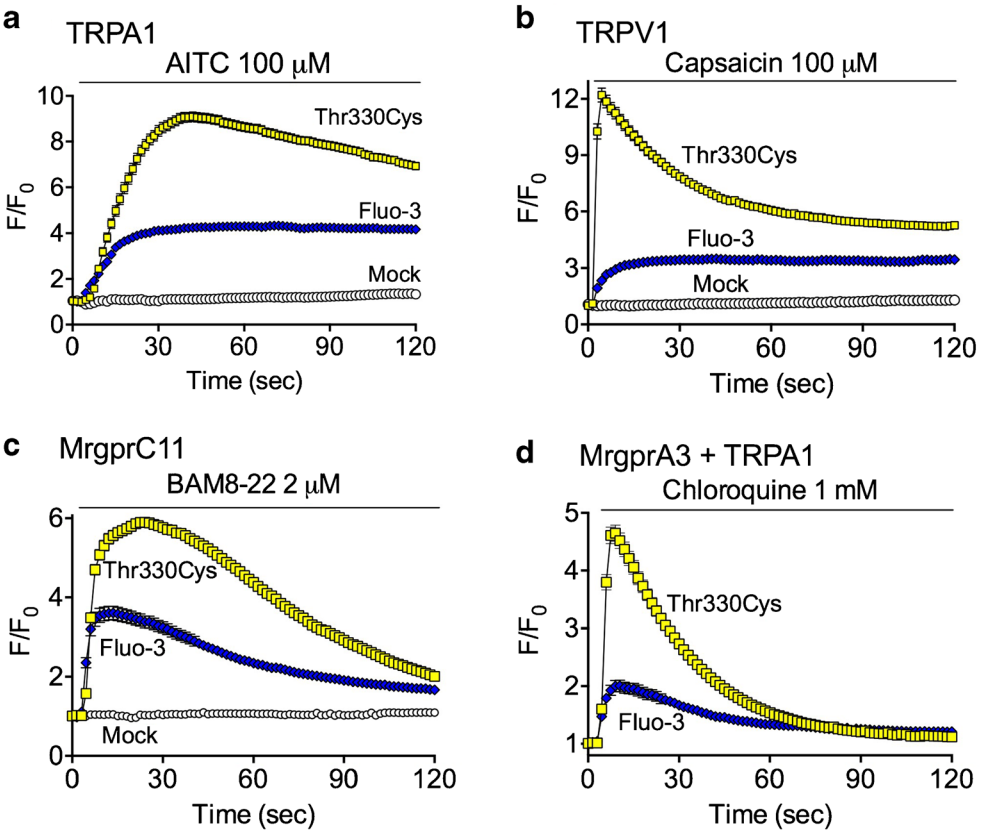

Fig. 4 Comparison of time-course graphs of Cys^{330}-GCaMP3 (Thr330Cys, yellow rectangle) and Fluo-3 (blue diamond). HEK293T cells were transiently transfected with different genes such as *TRPA1* (**a**), *TRPV1* (**b**), *MrgprC11* (c), and co-transfection with *MrgprA3* and *TRPA1* (**d**). Each group was treated with its specific agonist such as AITC (**a**), capsaicin (**b**), BAM8-22 (**c**), and chloroquine (**d**). Mock indicates HEK293T cells transfected with empty pcDNA3.1 vector (empty circle)

sufficient and essential to improve the calcium sensitivity of GCaMP3. This finding is rather unexpected in the light of a previous study which reported that two cysteine substitutions (Thr330Cys;Thr364Cys) were required to enhance the calcium sensitivity of GCaMP3.

It should first be recalled that cysteine is a very unique amino acid. Cysteine is the only amino acid that has a sulfhydryl group (−SH) in its structure. Moreover, cysteine is known to play a significant role in protein structure by forming a covalent disulfide bond (–S–S–) with another cysteine, which affects the folding and stability of proteins. The previous study, referred to earlier, concluded that the improved calcium sensitivity was attributable to the putative disulfide bond formed between the two cysteine substituents. However, in the present study, it was found that the substitution of even a single threonine with cysteine at position 330, yielding Cys^{330}-GCaMP3, resulted in the enhancement of the calcium sensitivity of the single substitution variant, comparable to that of dCys -GCaMP3, which has two threonine residues at positions 330 and 364 substituted with cysteine. Considering that canonical GCaMP3 does not have any cysteine residues in its calmodulin domain, Cys^{330}-GCaMP3 is not likely to form an internal disulfide bond. There was a similar attempt to modify calmodulin domain by introducing double substitutions with cysteine [13]. Double substitution greatly improved the overall activity of calmodulin. However, similar improvement was found even with a single residue substitution with cysteine, which is consistent with our results.

Therefore, it is likely that the enhancement observed in the case of Cys^{330}-GCaMP3 is attributable to yet unknown mechanisms other than the putative disulfide bond. A plausible theory is that the enhancement is associated with the EF hand of calmodulin domain in Cys^{330}-GCaMP3. Calmodulin has 4 different EF hand motifs, each of which contains a calcium-binding loop or EF loop [14]. EF loop sequences are composed of 12 conserved amino acids, to which a calcium ion binds in a pentagonal bipyramidal manner [6]. Among the 12 amino acids in the EF loop, 6 residues at positions 1 (+X), 3 (+Y), 5 (+Z), 7 (−Y), 9 (−X) and 12 (−Z) are strongly involved in calcium binding [15–17]. These residues are relatively conserved throughout the species. For instance, position +X is mostly aspartate, position +Y is either an aspartate or asparagine, and position −Z is glutamate. Thus, it is likely that these residues are critical for calmodulin function. In fact, when glutamate in position −Z was replaced by different amino acids, calcium binding affinity declined [18].

Position 330 in GCaMP3 corresponds to −X position of the first EF loop or "EF1", whereas 364 corresponds to the −Y position of the second EF loop or "EF2". According to a recent study on calmodulin, backbone carbonyl group of the −Y position in EF loop provides oxygen atom to calcium ion [19]. This position in EF2 is strongly conserved throughout various species, indicating the importance of threonine at this position [19]. Although detailed mechanisms are still elusive, it is speculated that Thr364Cys substitution might have affected calcium binding at the −Y position, resulting in significantly decreased calcium sensitivity of this variant, compared to that of the canonical GCaMP3 (Fig. 1).

In contrast, position 330, which corresponds to the −X position in EF1, is as strongly conserved across species as the −Y position in EF2 [19]. Halling et al., pointed out that different amino acids appear at the −X position in EF loop in different species. However, the most common amino acid found at the −X position in EF1 in various species is either threonine or serine [19]. Interestingly, the amino acid at the −X position in EF1 does not directly bind to calcium ion, but to the water molecule that bridges the two [20]. Since threonine and serine are polar amino acids that often participate in hydrogen bonding, the water molecule should be kept in position via hydrogen bonding. If the −X position in EF1 is replaced by a charged or hydrophobic amino acid that does not participate in hydrogen bonding, calcium sensitivity would be decreased. Indeed, substitution of threonine at position 330 with hydrophobic (Thr330Ala, Thr330Val) or negatively-charged (Thr330Asp) amino acids did not result in any improvement in calcium sensitivity, as can be seen from Fig. 3.

Another possible explanation could be related to pK_a values. Cysteine, threonine, and aspartate have ionizable side chains. The pK_a values of their side chains are 8.18 (sulfhydryl of cysteine), 13 (hydroxyl of threonine), and 3.65 (carboxyl of aspartate), respectively [21]. Assuming that the normal cellular pH is approximately 7.3, most of the hydroxyl group of threonine (pK_a 13) will be protonated based on acid dissociation characteristics. On the other hand, sulfhydryl group of cysteine (pK_a 8.18) in 10% of the protein molecules will exist in deprotonated form (S^-). Therefore, it could be expected that the bond with the water molecule at −X position in EF1 will be stronger with cysteine than with threonine. On the other hand, most of aspartate (pK_a 3.65) will be negatively charged at intracellular pH. Reid and Hodges proposed the acid-pair hypothesis, according to which calcium affinity of the EF hands is affected by the presence of negatively charged residues in chelating positions [15]. This hypothesis also predicts that the presence of additional carboxyl groups would decrease the calcium affinity of the binding site because of electrostatic repulsion. Therefore, increasing the number of negatively charged residues such as aspartate in EF1 may only decrease the stability of the protein. The present study also found that Thr330Asp substitution did not contribute to an increase in the calcium sensitivity of GCaMP3, as shown

in Fig. 3. It is conceivable that substituting the threonine at position 330 with cysteine results in adequate negative charge that provides optimized interaction with the water molecule, which in turn might have improved the calcium sensitivity of Cys^{330}-GCaMP3. Further in-depth studies are essential to completely elucidate the underlying mechanism of the effect of Thr330Cys substitution in GCaMP3. Additional factors may contribute to the calcium binding affinity of EF hand, which include the hydrophobicity of the loop, flanking residues, the nature of the chelating residues within the loop, and electrostatic interactions [15].

Although GECI has been developed to improve the monitoring of the intracellular calcium levels, most researchers are still using conventional synthetic dyes such as Fluo-3. Therefore, it is important to compare the calcium sensitivity of the synthetic dye with that of GECI. The present study found that Cys^{330}-GCaMP3 outperformed Fluo-3 in non-automatic, manual calcium imaging studies. Considering the ease of use, improved calcium sensitivity and most importantly economic advantages, Cys^{330}-GCaMP3 can be used as an alternative fluorescent calcium indicator that has higher sensitivity than not only that of canonical GCaMP3 but also that of conventional synthetic dyes such as Fluo-3, for the measurement of intracellular calcium levels.

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