Enhanced Sensitivity of FRET-Based Protease Sensors by Redesign of the GFP Dimerization Interface

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Real-time imaging of molecular events in living cells is important for understanding the basis of physiological processes and diseases. [1–3] Genetically encoded sensors that use fluorescence resonance energy transfer (FRET) [4] between two fluorescent proteins are attractive in this respect because they do not require cell-invasive procedures, can be targeted to different locations in the cell, and are easily adapted through mutagenesis and directed evolution approaches. [5–7] Following the pioneering work of Roger Tsien and others, on genetically encoded protease and calcium sensors, FRET-based imaging probes have been developed for many other small molecules and cell signaling events. [8–13] In these probes, conformational changes in a sensor domain are translated into a change in energy-transfer efficiency between donor and acceptor fluorescent domains, which is detected by a change in the ratio of donor and acceptor emission. This ratiometric response is independent of the sensor concentration, which is an important advantage of FRET-based sensors. In practice, however, most FRET-based sensors display only a relatively small difference in emission ratio upon activation. Improvement of these ratiometric changes has been recognized as an important prerequisite for use of these sensor systems in high-throughput applications based on fluorescence plate readers and fluorescence assisted cell sorting (FACS). [14,15]

Recently a pair of CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) variants, CyPet and YPet, respectively, have been reported that were optimized for FRET through a process of directed evolution. [16] When incorporated in a protease sensor, a 20-fold change in emission ratio was observed upon cleavage of a flexible peptide that linked CyPet and YPet, compared to only a fourfold change for the same construct with enhanced CFP (ECFP) and enhanced YFP (EYFP) domains. However, the mechanism behind their remarkable FRET properties has remained unclear. A total of eighteen mutations were introduced in the course of their development, many of which were at the exterior of the protein, at a large distance from the fluorophore. Moreover, no large differences in quantum yield or extinction coefficient were reported; this suggests that the photophysical properties of the fluorescent proteins were not significantly altered. We therefore hypothesized that the increase in FRET observed for CyPet and YPet could be due to an enhanced tendency to interact when connected by a peptide linker. The parent green fluorescent protein (GFP) has a known tendency to dimerize, [17] and analysis of the mutations in YPet have identified two residues, S208F and V224L, that are present at the dimer interface, as shown by the X-ray structure of the GFP dimer. Here, we show that introduction of just these two mutations in both fluorescent domains of ECFP–linker–EYFP constructs results in a fourfold increase in the EYFP-to-ECFP emission ratio, which yields a 16-fold change in emission ratio upon protease cleavage of the peptide linker (Figure 1). Additional biophysical evidence is provided, which shows that the mutations indeed result in formation of an intramolecular complex.

The effects of various mutations were tested by using CLY9, a fusion protein in which ECFP was connected to EYFP by a long, flexible peptide linker containing nine GGSGGS repeats. All proteins reported here were expressed in E.coli and purified by using Ni-affinity and size-exclusion chromatography (see the Supporting Information). We recently showed that the amount of energy transfer in “wild-type” CLY9 can be quantitatively understood when the linker is assumed to behave as a random-coil. [18] Both the amount of energy transfer and EYFP anisotropy were found to be consistent with the absence of any intramolecular domain interactions. Figure 2 shows the emission spectra obtained after excitation of ECFP at 420 nm for wild-type CLY9 and variants of CLY9 containing the S208F

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Figure 1. Improvement of FRET-based protease sensors based on the promotion of intramolecular association of enhanced cyan and yellow fluorescent protein (ECFP and EYFP, respectively). A) In the classical sensor design the linker assumes a random-coil distribution, which results in intermediate energy transfer. B) Introduction of S208F and V224L mutations in both domains promotes intramolecular-complex formation, which results in an increase in energy transfer prior to cleavage. The interaction between the mutants of ECFP and EYFP is weak enough to result in dissociation of ECFP and EYFP after proteolytic cleavage.
and/or V224L mutations in both fluorescent protein domains. Introduction of the S208F mutation resulted in a substantial increase of the EYFP/ECFP emission ratio ($R_{527/475}$) from 2.8 to 6.6. Mutation V224L alone showed almost no increase in energy transfer ($R_{527/475}$ 3.3), but a large synergistic effect was observed for the combination of V224L and S208F mutations, which resulted in a fourfold increase in emission ratio for the S208F/V224L mutant ($R_{527/475}$ 10.9) compared to CLY9. To provide evidence that this increase in energy transfer was due to intramolecular-complex formation between the fluorescent domains, a lysine was introduced at position 206. This A206K mutant is known to prevent dimerization of GFP and GFP variants, and would therefore be expected to counter the effect of the S208F/V224L mutations. [6,17] Figure 2 shows that this was indeed the case: the emission ratio of the A206K/S208F/V224L mutant was only slightly higher than that of the wild-type CLY9 protein. If complex formation is the result of high effective protein concentration dissociation of the complex is expected upon cleavage of the peptide linker. This hypothesis was tested by monitoring the emission ratio over time after addition of proteinase K. All measurements were performed in Tris-HCl (50 mM), NaCl (100 mM), glycerol (10%, v/v), Tween-20 (0.05%, v/v), at pH 8.0. Figure 2A) shows normalized emission spectra in A) were normalized for protein concentration.

Figure 2. A) Fluorescence emission spectra ($\lambda_{ex}=420$ nm) of CLY9 and its mutant variants before cleavage with proteinase K. B) Emission ratios ($R_{527/475}$) of wild-type and mutant CLY9 proteins monitored over time after addition of proteinase K (0.01 Unit/mL). All measurements were performed in Tris-HCl (50 mM), NaCl (100 mM), glycerol (10%, v/v), Tween-20 (0.05%, v/v), at pH 8.0, by using ~1 μM protein concentrations. Emission spectra shown in A) were normalized for protein concentration.

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Figure 3. A) Elution profiles of wild-type CLY9 and various mutants on a Sepharose 12 size-exclusion chromatography column, equilibrated with Tris-HCl (50 mM), NaCl (150 mM), EDTA (1 mM), glycerol (10%, v/v), Tween-20 (0.05%, v/v), pH 8.0, at 22 °C. Error bars show the standard deviation of two independent experiments.

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To gain independent evidence that the increase in FRET observed in the S208F and S208F/V224L mutants was due to intramolecular association of ECFP and EYFP, size-exclusion chromatography (SEC) was used to study the effect of these mutations on the oligomerization state and hydrodynamic volume of CLY9. Wild-type CLY9 elutes as a single peak with an apparent molecular weight of 83 kDa. This value is higher than expected based on its true molecular weight of 60 kDa; this indicates that CLY9 adopts an extended conformation that deviates from the globular shape of the reference proteins (Figure 3A). CLY9-S208F and CLY9-S208F/V224L also elute as single peaks but at apparent molecular weights of 63 and 67 kDa, respectively, which is much closer to the true molecular weight of these mutants. Thus, these mutations induce a conformational change to a more compact, globular structure; this provides independent evidence that intramolecular association of ECFP and EYFP is responsible for the observed increase in FRET. The elution time of the V224L mutant was similar to that of wild-type CLY9, which is consistent with the marginal increase in FRET observed for this mutant. The apparent molecular weight of 76 kDa observed for CLY9-A206K/S208F/V224L was also in agreement with the observed decrease in FRET.
V224L mutations are also present in YPet, our results suggest that have been developed in recent years. Since the S208F and ported here will also be applicable to the many color variants among derivatives of GFP we expect that the mutations redesigns. Given the high sequence and structural homology rational application of these mutations in future FRET-sensor association constant of this interaction as this will allow more FRET upon activation. We are currently trying to determine the protease sensors and other sensors that display a decrease in transitions can be an attractive strategy for improving FRET-based demonstration that promoting intramolecular domain interaction 208 by the much more hydrophobic phenylalanine result- tion interface by substitution of the hydrophilic serine at posi- FRET measurements and SEC.

In conclusion, we have shown that redesign of the dimerization interface by substitution of the hydrophilic serine at position 208 by the much more hydrophobic phenylalanine resulted in intramolecular complex formation of ECFP and EYFP, and a large increase in energy transfer. Although introduction of V224L by itself did not enhance the intramolecular interaction of ECFP and EYFP, it did induce a further increase in energy transfer in the presence of the S208F mutation, possibly due to a slight reorientation of the two domains. These results demonstrate that promoting intramolecular domain interactions can be an attractive strategy for improving FRET-based protease sensors and other sensors that display a decrease in FRET upon activation. We are currently trying to determine the association constant of this interaction as this will allow more rational application of these mutations in future FRET-sensor designs. Given the high sequence and structural homology among derivatives of GFP we expect that the mutations reported here will also be applicable to the many color variants that have been developed in recent years. Since the S208F and V224L mutations are also present in YPet, our results suggest that (part of) the improved FRET properties of CyPet and YPet could also be due to intramolecular-complex formation.

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