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Fluorescence complementation via EF-hand interactions

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Abstract

Fluorescence complementation technology with fluorescent proteins is a powerful approach to investigate molecular recognition by monitoring fluorescence enhancement when non-fluorescent fragments of fluorescent proteins are fused with target proteins, resulting in a new fluorescent complex. Extension of the technology to calcium-dependent protein–protein interactions has, however, rarely been reported. Here, a linker containing trypsin cleavage sites was grafted onto enhanced green fluorescent protein (EGFP). Under physiological conditions, a modified fluorescent protein, EGFP-T1, was cleaved into two major fragments which continue to interact with each other, exhibiting strong optical and fluorescence signals. The larger fragment, comprised of amino acids 1–172, including the chromophore, retains only weak fluorescence. Strong green fluorescence was observed when plasmid DNA encoding complementary EGFP fragments fused to the EF-hand motifs of calbindin D9k (EF1 and EF2) were co-transfected into HeLa cells, suggesting that chromophore maturation and fluorescence complementation from EGFP fragments can be accomplished intracellularly by reassembly of EF-hand motifs, which have a strong tendency for dimerization. Moreover, an intracellular calcium increase upon addition of a calcium ionophore, ionomycin in living cells, results in an increase of fluorescence signal. This novel application of calcium-dependent fluorescence complementation has the potential to monitor protein–protein interactions triggered by calcium signalling pathways in living cells.

Keywords

EGFP; Fluorescence complementation; EF-hand; Calcium-dependent; Protein–protein interactions; EGFP fluorescent fragment

1. Introduction

The discovery of green fluorescent protein (GFP) from jelly-fish *Aequorea victoria* has opened a new era in biology, medicine, pharmaceuticals, drug discovery and material sciences (Akemann et al., 2001; Shimomura, 2005). The properties of GFP that allow for cofactor-independent chromophore formation make it possible to use this protein to examine physiological and pathological changes during cell or organ development (Chalfie, 1995; Tsien, 1998; Wouters et al., 2001) and to study protein–protein interactions, spatial and temporal changes associated with translocation and transport, proteolysis and protein phosphorylation in intact cells (Periasamy and Day, 1999; Belmont, 2001; Zhang et al., 2002; Zimmer, 2002; Lippincott-Schwartz and Patterson, 2003).

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GFP is a stable, soluble, globular protein with 238 amino acid residues (27 kDa). The chromophore of GFP is formed by cyclization of the tripeptide Ser65-Tyr66-Gly67 (Perozzo et al., 1988; Cody et al., 1993), located inside a β -barrel motif composed of 11 anti-parallel strands and a single central α -helix. Short helices cap the ends of the barrel (Ormo et al., 1996; Yang et al., 1996). Extensive hydrogen bond interactions both within the protein frame and with available water molecules have significant effects on the state of the chromophore folding (Wood et al., 2005). GFP exhibits absorption peaks at 400 and 470 nm and a fluorescent emission peak at 510 nm with a quantum yield of 0.72 when excited at 470 nm (Prendergast and Mann, 1978). Enhanced green fluorescent protein (EGFP), which includes the mutations F64L/S65T, has greater fluorescence intensity and thermo-sensitivity. Due to the chromophore's buried location in the tight β -barrel, GFP is very stable and highly resistant to denaturation and to various proteases (Ward and Bokman, 1982).

Extensive research has also been conducted to study the chromophore properties of fluorescent proteins (FPs) using proteolytic cleavage and deletion (Heim et al., 1994; Dopf and Horiagon, 1996; Li et al., 1997; Reid and Flynn, 1997; Kim and Kaang, 1998; Enoki et al., 2004). The lessons learned about chromophore properties in GFP have been applied to the development of various new sensors to monitor protein-protein interaction during cellular processes and protein folding (Cabantous and Waldo, 2006). Fluorescence complementation between FP fragments was detected in *Escherichia coli* (*E. coli*) through the interaction of anti-parallel leucine zippers (Magliery et al., 2005). Circularly permuted FP fragments, non-fluorescent in isolation, retain fluorescence when constrained in proximity by calmodulin (CaM) or M13 (Nagai et al., 2001). In yet another application, a bimolecular fluorescence complementation assay was developed to monitor interactions between bZIP and Rel family transcription factors based on a non-covalent assemblage of YFP fragments. In this case, multicolor fluorescence was achieved by complementation between fragments from different fluorescent proteins through the dimerization of the bZIP domains of Fos and Jun. This assay was used to track the location of gene transcription in living cells (Hu and Kerppola, 2003).

Novel in this report is the development of a fragment complementation system designed to be calcium-dependent, namely, one relying on the interaction between the EF-hand calcium binding motifs 1 and 2 (called EF1 and EF2, respectively) of calbindin D9k. These EF-hand motifs have a strong tendency to associate as heterodimers in the presence of calcium (Linse and Chazin, 1995; Julenius et al., 2002). We have fused EF-hands onto various fragments of EGFP to yield calcium-sensitive, fluorescent-complementary probes, which, intracellularly, are shown to respond to an influx of calcium by ionomycin. Here, we focus on the characterization of the probes: the design and engineering of tryptic and chymotryptic cleavage sites into EGFP; the elucidation of the precise cleavage sites; the identification of minimal domain for the chromophore; and the ability to co-transfect HeLa cells with plasmid DNA encoding EGFP fragments containing EF hand motifs, resulting in reconstitution of a strong fluorescent signal *in situ*.

2. Materials and methods

2.1. Construction of EGFP variants and fragments

A DNA sequence encoding a cleavable linker (EEEIREAFRVFDKDGNGYISAAELRHVMTNL) was inserted into pET28a plasmid encoding EGFP at Glu172 using the polymerase chain reaction (PCR) technique. The resulting variant, expressed in *E. coli*, was denoted EGFP-T1. The DNA sequences encoding the large (amino acid residues 1–172) and small (amino acid residues 173–238) EGFP fragments (not including the engineered EF-hand motif III of CaM) were fused with the whole EF1 (KSPEELKGIFEKYAAKEGDPNQLSKEELKLLQLTEFPSLLKGP) and EF2 (STLDELFEELDKNGDGEVSFEFQVLVKKISQ) motifs of calbindin D9k, or with partial

EF1 (KSPEELKG) and partial EF2 motifs (STLDELFE), respectively, and cloned into pcDNA3.1 (+) vector for mammalian cells. The resulting variants containing whole EF-hand motifs or partial EF-hand motifs were designated N-EGFP-EF1, C-EGFP-EF2, N-EGFP-EF1p and C-EGFP-EF2p. A linker (GGSGSGSS) was interposed to connect the N-EGFP fragment and EF1, or the C-EGFP fragment and EF2 in order to improve the flexibility of EF-hand motifs. The newly synthesized plasmid DNAs were ligated with T4 DNA ligase, transformed into DH5 α competent cells grown in Luria-Bertani (LB) media containing kanamycin or ampicillin, and then purified with a QIAprep Spin Miniprep Kit (Qiagen, USA). The constructed plasmid DNA was verified through automated DNA sequencing.

2.2. Expression and purification of EGFP variants

EGFP variants with inserted cleavable linkers were expressed in *E. coli* BL21 (DE3). A single colony was inoculated into 20 ml of LB media with 30 μ g kanamycin/ml at 37 °C with agitation at 200 rpm overnight and then transferred to 1 L of LB media with 30 μ g kanamycin/ml. The cell cultures were induced with 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when O.D._{600 nm} reached 0.6 and allowed to grow at 30 °C for another 16–20 h. To purify the proteins, cell pellets were resuspended in 10 ml of lysis buffer (20 mM Tris, 10 mM NaCl, 0.1% Triton X-100, pH 8.8) and sonicated to disrupt cell membranes. The solution was centrifuged at 20,000 $\times g$ for 20 min, and the supernatant was filtered and injected into a histidine-chelating column preloaded with 0.1 M nickel sulfate solution for fast protein liquid chromatography (FPLC). After washing with buffer A (50 mM phosphate, 250 mM NaCl, pH 7.4), the bound protein was eluted with a gradient of imidazole from 0 to 0.5 M in phosphate buffer. The purity of the fractions was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein collected from FPLC was dialyzed with 10 mM Tris buffer with 1 mM DTT at pH 7.4 to remove imidazole. The concentration of purified protein was determined by UV absorbance at 280 nm applying an extinction coefficient of 21,890 M⁻¹ cm⁻¹.

2.3. SDS-PAGE analysis for EGFP-T1 cleavage

The protease sensitivity and cleavage status of EGFP-T1 were monitored by 15% SDS-PAGE. Usually trypsin, chymotrypsin or thrombin was added to 15 μ M EGFP-T1 to a final protein to enzyme ratio (w/w) of 100:1, and the digestion was conducted in each of the respective optimal buffers at room temperature. Different ratios of protein and trypsin (w/w; 2000:1, 1000:1, 200:1 and 100:1) were used to investigate the effects of enzyme concentration. Aliquots were taken at different times for SDS-PAGE. EGFP-T1, not subjected to protease digestion but incubated at room temperature, was evaluated in parallel as a control. The optical density of bands with molecular mass of 20 kDa in SDS-PAGE was determined using Scion Image software to determine the cleavage kinetics of EGFP-T1.

2.4. Separation of major fragments

After protease digestion of EGFP-T1, the mixture of products retained strong green fluorescence. In order to investigate cleavage sites and the source of fluorescence, a method combining FPLC and high-pressure liquid chromatography (HPLC) was utilized, which was able to separate protein fragments under denaturing conditions. 6 M urea was added to the digestion products of EGFP-T1 and heated in a 90 °C water bath for 5 min to denature protein fragments. The denatured protein fragments were injected into an equilibrated “Hitrap Sephadex 75” size-exclusion column and eluted with 10 mM Tris buffer containing 6 M urea at pH 7.4. The major fragments were collected and further purified by reversed-phase HPLC on a Whatman C₄ column eluted with a mobile phase gradient of 0–80% of acetonitrile in 0.1% TFA over 30 min. The fractions with purified protein fragments were lyophilized in a vacuum centrifuge for analyses of protein sequence, mass spectroscopy and spectral properties.

2.5. Trypsin cleavage site identification in EGFP-T1

To investigate trypsin cleavage sites in EGFP-T1, the molecular mass of digested products was measured by Matrix-assisted Laser Desorption/Ionisation-Mass Spectrometry (MALDI-MS) (Applied Biosystems), and the N-terminal sequence was analyzed by Edman degradation (Applied Biosystems). The cleavage sites were deduced from the molecular mass of digested fragments and their N-terminus.

2.6. Spectral analysis of fragments from EGFP-T1 trypsin digestion

Optical properties of P20 fragment were monitored with a UV-1601 spectrophotometer (Shimadzu Scientific Instruments Inc., Japan) and a fluorescence spectrophotometer (Photon Technology International Inc., Canada). The UV-vis spectra of the fragments were scanned from 600 to 200 nm in 10 mM Tris, 1 mM DTT, pH 7.4. The UV-vis spectra at different pH were monitored by adding 0.5 M NaOH into 1 ml of P20 solution in 10 mM Tris, 1 mM DTT at pH 7.32 to achieve final NaOH concentrations of 1.49, 2.98, 4.46, 5.93, 7.39 and 8.84 μ M. The final pH was 10.82. Fluorescence spectra of P20 were measured in the emission region of 500–600 nm at an excitation wavelength of 490 nm.

2.7. Cell culture and transfection

HeLa cells were grown on glass coverslips ($0.5\text{--}1.0 \times 10^6$ cells/dish) in 35 mm culture dishes at 37 °C with 5% CO₂ in a humidified incubation chamber. The culture medium was Dulbecco's Modified Eagles Medium (DMEM) (Sigma, St. Louis) with 44 mM NaHCO₃, pH 7.2, and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U penicillin/ml and 0.1 mg streptomycin/ml (Pen/Strep). After cells were seeded and grown overnight, N-EGFP-EF1 and C-EGFP-EF2 were co-transfected into HeLa cells with lipofectamine-2000 (Invitrogen Life Technologies) in serum-free Opti-MEM (Gibco Invitrogen) according to the manufacturer's instructions. For transfection, 2 μ g of N-EGFP-EF1 or 2 μ g of N-EGFP-EF2 DNA were added with 2 μ l of lipofectamine-2000. Following incubation at 37 °C for 6 h, the medium containing DNA and lipofectamine complex was removed and replaced by DMEM enriched with FBS and Pen/Strep. The cells were then grown 24–48 h in a humidified chamber with 5% CO₂ at 30 °C before fluorescence microscope imaging. Individual transfection with N-EGFP-EF1 or co-transfection with N-EGFP-EF1p and C-EGFP-EF2p was used as controls.

2.8. Fluorescence imaging

HeLa cells with single- or co-transfected EGFP fragments containing EF-hand motifs were imaged following expression for 24 or 48 h. Cell imaging was performed using a 40 $\times$ oil objective on a Zeiss Axiovert 200 inverted microscope connected to a CCD camera (AxioCam HRc). Excitation from the light source (FluoArc, Zeiss) was passed through a FITC filter set (excitation wavelength: 480 ± 20 nm; emission wavelength: 510 ± 20 nm). Axiovision software was used to acquire serial images over a specific time course to gauge the progress of calcium-dependent protein–protein interactions. During image acquisition, ionomycin and calcium with final concentrations of 1 μ M and 5 mM, respectively, were added to plates containing of N-EGFP-EF1 and C-EGFP-EF2 co-transfected cells to adjust intracellular Ca²⁺ flux and concentration.

3. Results and discussion

3.1. Design, expression and purification of modified EGFP variants

To create EGFP variants that can be cleaved to yield a smaller fragment containing the chromophore, we inserted a linker based on the helix-loop-helix motif containing potential cleavage sites for trypsin and chymotrypsin at position Glu172 in EGFP. Such helix-loop-helix motif allows a good solvent accessibility to facilitate the efficient cleavage by proteases. Fig.

1 shows a model structure of EGFP with the cleavable linker in an exposed loop at position 172. After PCR amplification of the modified gene, the corresponding protein, termed EGFP-T1, was expressed in *E. coli* BL21 (DE3) in LB-media and purified as noted in Section 2. The modified protein exhibited strong fluorescence, indicating that the presence of the linker did not suppress the fluorescence of EGFP.

3.2. EGFP-T1 digestion

Fig. 2A shows that intact EGFP-T1 (34 kDa) was first cleaved by trypsin into three fragments, with molecular masses of 23, 20 and 8 kDa, visible already at 1 h. The 23 kDa fragment was further digested to 20 kDa after 24 h. With similar kinetics, chymotrypsin first cleaved EGFP-T1 into three fragments of 21, 20 and 10 kDa; the 21 kDa band was then converted to 20 kDa band at ≥ 6 h. Exhaustive cleavage (24 h) of EGFP-T1 by either chymotrypsin or trypsin resulted in two stable fragments, a larger one, approximately 20 kDa, designated “P20”, and a smaller one, approximately 8 kDa, designated “P8” (trypsin) or 10 kDa, designated “P10” (chymotrypsin).

Fig. 2B shows semi-quantitative measurements of cleavage rates of EGFP-T1 by trypsin, as monitored by integrating the optical density of the P20 band with time. Relative to the final optical density of the P20 band, P20 fragment formation with trypsin reached 20% at 1 h, and 50% at 6 h; with chymotrypsin, 40% at 1 h and 80% at 6 h. In order to examine the effects of trypsin concentration on the kinetics of hydrolysis of EGFP-T1, trypsin was added at different weight ratios (2000:1, 1000:1, 200:1, and 100:1; protein/trypsin). The final cleavage patterns were consistent although rates were changed (Fig. 3A). At the slowest rate, an intermediate product (31 kDa) was observed at ≤ 12 h, which was precursor to the 8 and 23 kDa bands (Fig. 3B). The cleavage order with trypsin is summarized as: intact protein 34 kDa $\rightarrow$ 31 kDa, then 31 kDa $\rightarrow$ 23 + 8 kDa, and finally $\rightarrow$ 20 + 8 kDa. The implied fragments of ~ 3 kDa are not visible on the gels.

3.3. Generation and identification of trypsin cleavage sites in EGFP-T1

To allow positive identification of the cleavage sites by sequence analysis and mass spectrometry and to investigate their optical properties, we purified the two major fragments from EGFP-T1 digestion by FPLC and HPLC. The molecular masses of “P20” and “P8” were 20,842.06 and 7968.58 Da, as determined by MALDI-MS. Thus, although there are 33 potential trypsin cleavage sites and 27 chymotrypsin sites in this protein, under non-denaturing conditions, cleavage by trypsin is limited and specific at the desired, engineered sites.

The N-terminal sequence of P20 is MVSKGEE, as determined by Edman degradation. Apparently the His-tag was removed by trypsin, consistent with the observation that P20 does not bind to Hitrap nickel chelator. In fact, the intermediate product (31 kDa) observed during trypsin digestion corresponds to the removal of His-tag at the last Arg in the tag (cf. Fig. 1) (Battistutta et al., 2000). Amino acid sequence analysis of P8 reveals its N-terminal as HVMTNL, located in the inserted exiting helix of the cleavage motif. The calculated molecular mass for the fragment from HVMTNL in F-helix to the C-terminal is 7946.90 Da, 21.68 Da less than the observed molecular mass of 7968.58 Da, presumably due to a Na^+ adduct. The calculated mass from MVSK (N-terminus of P20 less His-tag) to the Arg at position -3 in the E-helix is 20,844.68 Da, which matches, within error, the mass observed in MALDI-MS at 20,842.06 Da. A slightly heavier fragment, from MVSK to Arg at position 2 of the F-helix (calculated mass 22,581.58 Da) apparently corresponds to the transient 23 kDa band seen in SDS-PAGE.

All of these results are consistent with the three cleavage steps deduced from SDS-PAGE experiments (Figs. 2 and 3): a fragment of 31 kDa appearing as the first step, a fragment of 23

kDa as the second step and a fragment of 20 kDa as the third step; hence the designations of cleavage order are given in Fig. 1 and the fragment boundaries are tabulated in Table 1. Similar digestion trends and results were obtained from chymotrypsin digestion.

Although several groups have attempted to identify short fragments for complementing fluorescent chromophore in fluorescent proteins through deletion, truncation and protease digestion and amino acid residues 7–229 of GFP have been shown to be essential for the fluorescence of the protein, these studies did not report the optical properties of the products (Cody et al., 1993; Dopf and Horiagon, 1996; Li et al., 1997; Kim and Kaang, 1998; Chiang et al., 2001; Flores-Ramirez et al., 2007). Our task has been different and we have inserted cleavable sequences fused to a helix-loop-helix motif at loop location 172. This task permitted us not only to investigate minimal fluorescent domain to be comprised of residues 1–172 of EGFP (i.e. not counting the His-tag N-terminal) with competent chromophore, but also to investigate the sites and kinetics of proteolysis and to characterize the optical properties of the resultant fragments.

3.4. Two major fragments cleaved from EGFP-T1 remain associated under physiological conditions

The proteolytically cleaved EGFP-T1 fragments have been identified to contain chromophore structure in the larger fragment (P20 fragment), and both cleaved EGFP-T1 fragments exhibited a strong tendency to interact with each other under physiological conditions, as judged by several observations. First, to examine the strength of interaction, digested sample mixtures, both without thermal denaturation and with denaturation by heating at 95 °C, were loaded on the same SDS-PAGE. The sample digested under physiological conditions and not subjected to additional thermal denaturation has only a single band fluorescing green and with a molecular weight corresponding to uncleaved EGFP-T1, while the digested sample subjected to thermal denaturation exhibits two bands with molecular masses of 20 and 8 kDa. The ambient temperature and SDS concentration within the gel are insufficient to disassociate the digested fragments without strong thermal denaturation. Moreover, when tryptic digestion mixtures of EGFP-T1 are separated either on Sephadex 75 size-exclusion column or on ion-exchange columns (Hitrap Q) eluted with salt gradients under physiological conditions, the two major fragments with molecular masses of 20 and 8 kDa (observed in SDS-PAGE) co-elute, we know these to be non-covalent complexes. Based on this association of EGFP fragments under physiological conditions, we presumed this would be a non-covalent reassembly of the non-fluorescent fragments into fluorescent-competent protein complexes. Furthermore, we also presumed this reassembly would be contingent on calcium concentration, and, hence, manipulable.

3.5. Optical properties of fluorescence-competent EGFP-T1 fragments

Fig. 4A shows the absorption spectra of purified P20 fragment compared with those of uncleaved EGFP-T1 and EGFP-wt. A maximum absorbance for EGFP-wt at 488 nm was observed in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.4), while the intact EGFP-T1 exhibited two absorbance peaks at 398 and 488 nm. Purified P20 has an absorbance maximum at 383 nm under identical Tris buffer conditions at pH 7.4, similar to the maximum absorption of EGFP-wt at pH 3.36. The maximum absorbance peak gradually shifts to 354 nm at a final pH of 10.8 with the addition of NaOH (Fig. 4B), suggesting that under physiological conditions the chromophore of P20 exists largely in the neutral state (Brejc et al., 1997; Tsien, 1998).

The P20 fragment has a maximum fluorescence excitation at 490 nm with a shoulder at 470 nm and a fluorescence emission at 505 nm (Fig. 5A and B). Both excitation and emission maxima are somewhat blue-shifted compared with the uncleaved EGFP-wt and EGFP-T1, both of which show an emission maximum at 510 nm from a single excitation at 490 nm. Consistent

with previous reports of short, protease-derived peptide fragments containing a chromophore (chromopeptides) (Shimomura, 1979), the optical properties of P20 fragment were blue-shifted from 397 to 383 nm and exhibited only weak absorbance at 490 nm compared with EGFP-wt or EGFP-T1. The absorption spectrum of P20 is essentially identical to that of EGFP-wt under acid-denaturing conditions (pH 3.36). In addition, the absorbance at 383 nm decreases with a simultaneous increase at 454 nm as pH increases (Fig. 4B). Such pH-dependence indicates that the P20 chromophore exists mainly in a neutral state. The P20 absorption spectrum under neutral conditions is reminiscent of the spectrum reported by Niwa et al. (1996) of a fragment of GFP (residues 63–69) containing the chromophore.

The fluorescence properties of the P20 fragment are a main excitation peak at 490 nm with a shoulder at 470 nm and maximum emission at 505 nm. This excitation maximum is slightly blue-shifted compared to EGFP-wt, and its associated emission intensity is approximately 87-fold lower than that of intact EGFP-T1. These results indicate a low quantum yield, near 0.04, for P20. In contrast, the short GFP peptides examined by Niwa et al. (1996) showed no fluorescence under physiological conditions. These lysyl endopeptidase fragments have no detectable fluorescence in organic solvent or aqueous mixtures, and, in particular, the heptapeptide fragment containing the cyclic tripeptide fluoresces only with low intensity even in ethanol glass at a temperature of 77 K. The absorbance and fluorescence properties of the isolated P20 fragment are very similar to that of bacterially expressed and refolded EGFP fragment (residues 1–158), which has an excitation peak at 490 nm and emission peak at 505 nm (Demidov et al., 2006).

3.6. Fluorescence complementation of EGFP fragments with EF-hand motifs from calbindin D9k in HeLa cells

It is important to note that the isolated EGFP-T1 fragment, P20, encompassing EGFP residues 1–172, has fluorescence 87-fold weaker than both tryptic fragments P20 and P8 in non-covalent association. This observation leads us to hypothesize that the EGFP-T1 fragments might serve as competent sensors for the calcium-dependent association of EF-hand motif 1 (EF1) and motif 2 (EF2) from calbindin D9k. These motifs are known to exhibit a strong tendency to dimerize (Linse and Chazin, 1995). To test this hypothesis, we fused EF1 and EF2 to the carboxyl end of the N-terminal fragment of EGFP and to the amino end of the C-terminal fragment of EGFP, respectively (Fig. 6). These constructs are denoted as N-EGFP-EF1 and C-EGFP-EF2. In an effort to improve the flexibility of EF-hands in both fragments, a linker (GGSGSGSS) was interposed between the EGFP and calbindin moieties. To explore the minimal requisites for the expected association, “N-EGFP-EF1p” and “C-EGFP-EF2p” were also constructed, similar to the above, but with incomplete flanking helices.

In order to track fluorescence complementation of EGFP fragments *in vivo*, genes for N-EGFP-EF1 and C-EGFP-EF2 were co-transfected into HeLa cells (cf. Section 2) and protein expression was allowed to continue for 24–48 h. Fluorescence images were acquired using an epifluorescence microscope with excitation at 488 nm for 50 ms exposure time. HeLa cells co-transfected with both EGFP fragments revealed strong fluorescence (Fig. 7A). In contrast, no significant fluorescence evolved, even at 1000 ms exposure time, when cells were transfected with N-EGFP-EF1 alone (Fig. 7B), or co-transfected with truncated N-EGFP-EF1p plus C-EGFP-EF2p (Fig. 7C).

These results would seem to indicate that N-EGFP-EF1 by itself cannot fold to form the mature chromophore necessary for emitting strong fluorescence, even though it encompasses the entire chromophore sequence. Moreover, co-transfection of N-EGFP-EF1p and C-EGFP-EF2p, with incomplete EF-hand motifs, also fails to support fluorescence complementation, presumably due to the inability of the incomplete EF-hand motifs to dimerize. Maturation of a chromophore

capable of full fluorescence complementation was achieved only by fusion of the two EGFP fragments with complete EF-hand motifs (EF1 and EF2) from calbindin D9k.

Since this dimerization is known to be calcium-dependent, we next sought to test the effects of calcium on the fluorescence complementation in living cells. To do so, we needed to modulate calcium concentration and to monitor the time courses of the development of fluorescence in living cells. To modulate intracellular $[Ca^{2+}]$, we used 1 μ M ionomycin, a calcium ionophore permitting influx of extracellular calcium. The cell images were acquired through an epifluorescent microscope operated in time-course mode to follow consecutive additions of ionomycin and 5 mM Ca^{2+} . Fig. 8 traces the fluorescence signal through a 20% increase following the addition of ionomycin, and a further 10% increase upon addition of 5 mM calcium. (Initial calcium concentration in the culture medium was 1 mM.) We interpret these phenomena to mean that the extent of fluorescence complementation of EGFP fragments fused to EF-hand motifs from calbindin D9k is enhanced by increasing intracellular Ca^{2+} concentration, presumably exerted through the calcium-mediated dimerization of EF-hand motifs.

Fluorescence complementation between fragments of fluorescent proteins is based on the non-covalent reassembly of two fragments with weak or no fluorescence to result in the restoration of fluorescence, which was developed with the goal of detecting specific interactive biochemical processes or various types of molecular recognition in living cells. The attractive attributes of the approach are to visualize protein–protein interactions or DNA hybridization, to monitor conformation change upon maltose binding or metal-dependent GFP reconstitution, to screen protein expression in different cellular environments and to enhance protein folding and solubility in living cells (Taniuchi et al., 1986; Ozawa et al., 2000; Hu and Kerppola, 2003; Hynes et al., 2004; Wilson et al., 2004; Magliery et al., 2005; Cabantous and Waldo, 2006; Demidov et al., 2006; Jeong et al., 2006; Mizuno et al., 2007). Novel to our studies is exploitation of the calcium-dependent heterodimerization of the EF-hand motifs from calbindin D9k to reunite EGFP fragments. EF-hand proteins contain a helix-loop-helix calcium binding motif and represent one of the major types of calcium binding proteins. They have been found in all organisms and rank as the 5th most common binding motif in eukaryotic genomes. More than 3000 calcium binding proteins are estimated to exist in eukaryotes, and another 500 in prokaryotes (Zhou et al., 2006). Upon stimulation by first messenger agonists, such as ATP, intercellular calcium concentration can rise rapidly. Trigger proteins with paired EF-hand motifs, such as CaM and troponin C (TnC), sense this rise and regulate more than 100 biological processes downstream. Other proteins with EF-hand motifs, such as calbindin D9k and parvalbumin, function as buffers to maintain calcium ion concentration. EF-hand motifs in proteins are normally coupled to form a compact domain. For example, a relatively small unit of paired EF-hand motifs exists in calbindin D9k, where it contributes greatly to the cooperativity of calcium binding and functions as an on/off switch over a narrow range of free calcium concentrations.

Isolated EF-hand motifs have a strong tendency to dimerize. EF-hand motif III from skeletal TnC has been shown to dimerize in the presence of calcium (Delbaere et al., 1989; Shaw et al., 1990). Other EF-hands, such as that from rabbit skeletal TnC, from the C-terminus of E6-binding protein (E6bp4), the third motif from parvalbumin of silver hake and the N-terminal EF-hand from human S100B protein have also been shown to form dimers (Kay et al., 1991; Revett et al., 1997; Chen et al., 1998). Particularly high affinities for dimerization are found among the EF-hand motifs of calbindin D9k. Linse's research group has measured affinity between motif 1 (residues 1–43) and motif 2 (residues 44–75) at $3.6 \times 10^{11} M^{-1}$ in the presence of calcium (Berggard et al., 2001). They find the heterodimerization constant to exceed the homodimerization constant by more than six orders of magnitude (Julenius et al., 2002). Some 11 hydrophobic core residues are responsible for the dimerization and the resultant stability of

the intact domain. Moreover, the dimerization of EF-hand motifs in calmodulin results in a dramatic change of calcium binding affinity shown as dissociation constant from sub-millimolar level to micromolar level due to the cooperation between different EF-hand motifs (Jaren et al., 2002; Ye et al., 2005; VanScyoc et al., 2006). Thus the approach described in this paper has a large potential for monitoring calcium-dependent interactions of proteins.

It is this binding tenacity which lies at the root of the strong fluorescent signal from HeLa cells co-transfected with genes for N-EGFP-EF1 and C-EGFP-EF2 (cf. Fig. 7). Clearly, a well-matured chromophore exists in these cells. With the incomplete EF-hand motifs, N-EGFP-EF1p and C-EGFP-EF2p, weak fluorescent signal is observed. Further, in line with the known characteristics of calbindin EF-hand dimerization *in vitro*, we also note an enhancement of fluorescence following calcium influx upon addition of ionomycin. On the other hand, experiments like that of Fig. 7 suggest that the rate and extent of fluorophore maturation reflect ambient calcium concentration; therefore, it seems unlikely that a subsequent decrease in calcium would result in a decrease in the probe's fluorescent signal. As constituted with calbindin modules, the system explored here is a sort of "latching" sensor, as opposed to a real-time sensor. Such latching sensors have been proven to be useful in probing cellular actions. Further, applications to monitor the subcellular Ca^{2+} signalling process can be achieved by adding signal peptide (Zou et al., 2007). Because different cellular compartments have different calcium concentration ranges, we have conducted the fluorescence complementation of EGFP fragments in cytosol via EF-hand interactions through normal cytosol calcium concentration and calcium influx with ionomycin induction and extracellular calcium with 5 mM. Therefore, the fluorescence complementation can be accomplished from μM to mM level. In the future, in order to further improve the sensitivity of fluorescence complementation based on calcium signal change, we will conduct fluorescence complementation in various subcellular compartments at their different calcium concentration ranges, which will not only examine the sensitivity of calcium-dependent fluorescence complementation molecular probes in living cells, but also can track calcium signal pathways in different subcellular compartments through multiple fluorescence complementation at the same time.

Fluorescence complementation *versus* FRET-based calcium sensors, the latter using trigger proteins such as CaM and TnC, represents a productive strategy to monitor intracellular calcium. The idea is to employ a pair of fluorescent proteins in which energy transfer depends on conformational change triggered by calcium-dependent protein complexation (Miyawaki et al., 1997). Recent efforts in our laboratory (Zou et al., 2007) have produced a single-molecule, non-FRET calcium sensor, targeted to the ER and with a calcium binding constant commensurate with the millimolar concentration of Ca^{2+} , typical of the ER. This was achieved by grafting a calcium binding motif into a sensitive spot in GFP, where calcium binding elicits a conformational change, which, in turn, modulates fluorescence from the modified GFP. CaM and TnC are major participants in calcium signalling systems. Artificial complexation of these molecules to achieve FRET may conceivably perturb the signalling systems they are intended to monitor. We suggest that systems, such as those described in this paper, based on calcium-dependent *in situ* assembly or conformational change of "by-stander" proteins will significantly enlarge the repertoire of tools aimed at understanding the crucial events of intracellular calcium flux and signalling.

4. Conclusions

A new approach of fluorescence complementation technology with fluorescent proteins was developed by grafting a trypsin cleavage site onto EGFP, named as EGFP-T1. Under physiological conditions, both fragments of EGFP-T1 remain associated and give a strong fluorescence signal, although the isolated larger fragment, residues 1–172, contains the chromophore and retains weak fluorescence. Strong green fluorescence was observed when

plasmid DNA encoding EGFP fragments containing EF hand motifs from calbindin D9k were co-transfected into HeLa cells, indicating that chromophore maturation and fluorescence complementation can be achieved through the assembly of two (well-characterized and stable) EGFP fragments as a result of calcium-dependent dimerization from EF-hand fragments. Moreover, a significant increase in fluorescence was observed following treatment with ionomycin, which facilitates calcium influx into living cells. We believe this strategy opens new potentials to study protein–protein interactions related to calcium signalling pathways in living cells.

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Fig. 1.

The structural model of EGFP-T1 with a protease-cleavable linker. The structural model of EGFP-T1 containing a protease-cleavable helix-loop-helix motif from calmodulin, generated via Modeller and PyMol software, depicts the cleavable linker, in red, grafted at position 172 in the loop region of EGFP. His-tag is shown in cyan. The ordinal designations (1st, 2nd, and 3rd) are based on trypsin cleavage kinetics experiments, described in the text. Following exhaustive trypsin digestion, two major fragments (P20 and P8) result, shown in green and yellow, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2.

The cleavage patterns and kinetics of EGFP-T1 by various proteases. (A) The cleavage patterns of EGFP-T1 by various proteases were identified via SDS-PAGE. Trypsin, chymotrypsin or thrombin cleavage of EGFP-T1 following digestion for 0, 1, 6, and 24 h was analyzed by SDS-PAGE. Lane M is protein molecular weight markers. Lane 1 is the control, EGFP-T1 not subjected to protease cleavage. Lanes 2, 3 and 4 are the samples of EGFP-T1 subjected to digestion at room temperature by trypsin, chymotrypsin, and thrombin, respectively. The ratio of protein-to-enzyme was 100:1 (w/w). (B) The rate of cleavage of EGFP-T1 is semi-quantitatively measured based on the formation of P20 fragment, using optical density integrated over its band, following EGFP-T1 digestion with trypsin (▨) or chymotrypsin (▩).

Fig. 3.

The effects of trypsin concentrations on digestion pattern and kinetics of EGFP-T1. (A) The effects of trypsin concentration on the digestion pattern of EGFP-T1 were analyzed by SDS-PAGE. EGFP-T1 samples digested with various concentrations of trypsin for 0, 1, 6, 12, 24, and 48 h at room temperature were visualized in SDS-PAGE. M is protein molecular weight markers. NP is the sample of EGFP-T1 not subjected to trypsin digestion, which serves as a control. Lanes 1, 2, 3 and 4 are the digests at EGFP-T1/trypsin ratios of 2000:1, 1000:1, 200:1 and 100:1, respectively. (B) Rates of cleavage of EGFP-T1 are semi-quantitatively measured based on the formation of the P20 fragment measured as optical density of its band, and normalized to that at 48 h. EGFP-T1/trypsin ratios of 2000:1 (▨), 1000:1 (▩), 200:1 (▧) and 100:1 (■), respectively.

Fig. 4.

The characteristics of UV-vis spectra of the P20 fragment. (A) The characteristics of continuous UV-vis spectra of the P20 fragment (◆) were compared with those of EGFP-wt (●), EGFP-T1 (■) and acid-denatured EGFP-wt (◇) at pH 3.36. The maximum absorption of the P20 fragment is at 383 nm, similar to that of acid-denatured EGFP-wt. Native EGFP-wt and EGFP-T1 exhibited less pronounced peaks at 383 nm. (B) The characteristics of UV-vis spectra of the P20 fragment at various pHs were also measured after serial addition of small volumes of NaOH. The maximum visible absorption of the P20 fragment at pH 7.32 (●) gradually shifted from 383 to 454 nm, the sole peak of the P20 fragment at the final pH 10.82 (△).

Fig. 5.

Fluorescence excitation and emission spectra of the P20 fragment. Excitation spectra (A) and emission spectra (B) of 1 μ M EGFP-wt (●), 1 μ M EGFP-T1 (■) and 10 μ M P20 (◆) were obtained in Tris buffer (10 mM Tris, pH 7.4), revealing that the P20 fragment has a maximum excitation peak at 490 nm, similar to EGFP-wt, but with a more pronounced shoulder at 470 nm. EGFP-T1 has excitation peaks at 490 and 398 nm. The maximum wavelength of fluorescence emission of EGFP-wt and EGFP-T1 occurred at 508 nm while the maximum emission of the P20 fragment was slightly blue-shifted to 505 nm.

Fig. 6.

The design of fluorescence complementation induced with calcium-dependent protein-protein interactions. The design is diagrammed, based on EGFP fragments fused to EF-hand motifs of calbindin D9k. C-terminus of N-EGFP fragment is connected to EF-hand motif 1 (EF1) following Glu172 and EF-hand motif 2 (EF2) is connected to N-terminus of C-EGFP fragment before Asp173 with an extended GGSGSGGS linker. N-EGFP-EF1p and N-EGFP-EF2p, with truncated flanking helices, were also constructed similarly to explore the minimal requisites for the expected association. Helix-loop-helix structures (EF1 and EF2 from calbindin D9k) are highlighted in blue, red and blue, respectively. The partial EF-hand motifs are highlighted in purple. GGSGSGGS linker is shown in black. The connection sites of Glu172 in the C-terminus of N-EGFP fragment and Asp173 in the N-terminus of C-EGFP fragments are highlighted in green and cyan color, respectively. Fluorescence complementation is postulated to result from the reconstitution of both EGFP fragments guided by the calcium-dependent dimerization of EF1 and EF2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 7.

Fluorescence complementation confirmation of EGFP fragments in living cells through co-transfection. The strong fluorescence complementation in HeLa cells through (A) the co-transfection of N-EGFP-EF1 and C-EGFP-EF2 under excitation of 488 nm proceeded via the dimerization of EF-hand motifs and was monitored by fluorescence microscopy with 50 ms exposure time. The weak fluorescence in HeLa cells with (B) individual transfection of N-EGFP-EF1, and (C) co-transfection of N-EGFP-EF1p and C-EGFP-EF2p at excitation of 488 nm with 1000 ms exposure time.

Fig. 8.

The effects of calcium on fluorescence complementation of EGFP fragments in living cells. The change in fluorescence signal was monitored in the HeLa cells upon co-transfection with the genes for N-EGFP-EF1 and C-EGFP-EF2 before and after promotion of calcium influx by ionomycin, and with extracellular addition of calcium. The fluorescence signal is increased by about 20% with addition of 1 μ M ionomycin and another 10% with the addition of 5 mM calcium.

Table 1

Molecular mass of fragments produced by trypsin digestion of EGFP-T1.

Sequence start point	Sequence end point	Observed mass (Da)	Calculated mass (Da)
Intact protein			34,451.80
MVSKGE	C-terminal	31k (SDS-PAGE)	30,520.46
MVSKGE	SAAELR	23k (SDS-PAGE)	22,581.58
HVMTNL	C-terminal	7,968.58 (MALDI)	7,946.90
MVSKGE	EEEIREAFR	20,842.06 (MALDI)	20,844.68