Green Fluorescent Protein-like Proteins in Reef Anthozoa Animals

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ABSTRACT. **Green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* has become an important tool in molecular and cellular biology as a transcriptional reporter, fusion tag, and biosensor. Most significantly, it encodes a chromophore intrinsically within its protein sequence, obviating the need for external substrates or cofactors and enabling the genetic encoding of strong fluorescence. Mutagenesis studies have generated GFP variants with new colors, improved fluorescence and other biochemical properties. In parallel, GFPs and GFP-like molecules have been cloned from other organisms, including the bioluminescent sea pansy *Renilla reniformis* and other non-bioluminescent Anthozoa animals. In the jellyfish and sea pansy, the GFPs are coupled to their chemoluminescence. Instead of emitting the blue light generated by aequorin and luciferase, the GFPs absorb their energy of primary emission and emit green light, which travels farther in the sea. In contrast, GFP-like proteins in reef Anthozoa are thought to play a role in photoprotection of their symbiotic zooxanthellae in shallow water; they transform absorbed UV radiation contained in sunlight into longer fluorescence wavelengths (Salih, A., Larkum, A., Cox, G., Kuhl, M., and Hoegh-Guldberg, O. 2000. Nature, 408: 850–853). In this review, I will describe both the biological and practical aspects of Anthozoan GFP-like proteins, many of which will be greatly improved in utility and commercially available before long. The ubiquity of these molecular tools makes it important to appreciate the interplay between sunlight and GFP-like proteins of Anthozoan animals, and to consider the optimal use of these unique proteins in biological studies.**

Key words: GFP/ bioluminescence/ Anthozoa/ RFP/ photoconversion

Emergence of a family of GFP-like proteins

Although spectral variants with blue, cyan, and yellow-ish-green emissions were generated from *Aequorea* GFP, none exhibited emission maxima longer than 529 nm (Tsien, 1998). The discovery of GFP-like proteins from Anthozoa significantly expanded the range of colors available for biotechnological applications. Matz *et al.* (1999) cloned six anthozoan naturally fluorescent proteins (FPs) all with 26–30% identity to *Aequorea* GFP; and one of the new FPs, drFP583, or DsRed, as it is known commercially, was cloned from a red *Discosoma* species and showed red-shifted excitation and emission spectra. Even more recently, the family of "GFP-like proteins" has been expanded

further (Labas *et al.*, 2002; Matz *et al.*, 2002). To date, about 30 significantly different members have been deposited into the sequence database, as summarized in Table I. Despite a modest degree of sequence identity, all GFP-like proteins are likely to share the same general β-can fold, and possess intrinsic chromophores. Although most GFP-like proteins are FPs, some members display only intense absorption without fluorescence emission and are called chromoproteins (CPs). Both FPs and CPs should be considered major determinants of the coloration of reef anthozoan animals (Lukyanov *et al.*, 2000).

Red and far-red FPs

Proteins that fluoresce at red or far-red wavelengths (RFPs) are of specific interest because eukaryotic cells and tissues have a markedly reduced autofluorescence at longer wavelengths. Also, RFPs can be used in combination with other fluorescent proteins of shorter wavelengths for multicolor labeling or fluorescence resonance energy transfer (FRET) experiments (Mizuno *et al.*, 2001). Currently,

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  Abbreviations: GFP, green fluorescent protein; UV, ultra violet; CP, chromo protein; FRET, fluorescence resonance energy transfer; FRAP, fluorescence recovery after photobleaching.

Table I.

Protein ID (original ID)	GenBank accession #	Taxonomy Genus species (Class, Sub-class, Order)	Excitation maxima, nm	Emission maxima, nm	Color
amajGFP (amFP486)	AF168421	*Anemonia majano* (Anthozoa, Zoantharia, Actiniaria)	458	486	
dstrGFP (dsFP483)	AF168420	*Discosoma striata* (Anthozoa, Zoantharia, Corallimorpharia)	456	484	
clavGFP (cFP484)	AF168424	*Clavularia sp.*(Anthozoa, Alcyonaria, Alcyonacea)	443	483	
GFP	M62653	*Aequorea victria* (hydrozoa, …, hydroida)	395, 471	508	
cgigGFP	AY037776	*Condylactis gigantea* (Anthozoa, Zoantharia, Actiniaria)	399, 482	496	
hcriGFP	AF420592	*Heteractis crispa* (Anthozoa, Zoantharia, Actiniaria)	405, 481	500	
ptilGFP	AY015995	*Ptilosarcus sp.*(Anthozoa, Alcyonaria, Pennatulacea)	500	508	Green
rmueGFP	AY015996	*Renilla muellenri* (Anthozoa, Alcyonaria, Pennatulacea)	498	510	
zoanGFP (zFP506)	AF168422	*Zoanthus sp.*(Anthozoa, Alcyonaria, Zoanthidea)	496	506	
asulGFP (asFP499)	AF322221	*Anemonia sulcata* (Anthozoa, Zoantharia, Actiniaria)	403, 480	499	
dis3GFP	AF420593	*Discosoma sp. 3* (Anthozoa, Zoantharia, Corallimorpharia)	503	512	
dendGFP	AF420591	*Dendronephthya sp.* (Anthozoa, Alcyonaria, Alcyonacea)	494	508	
mcavGFP	AY037769	*Montastraea cavemosa* (Anthozoa, Zoantharia, Scleractinia)	506	516	
rfloGFP	AY037772	*Ricordea florida* (Anthozoa, Zoantharia, Corallimorpharia)	508	518	
scubGFP1	AY037767	*Scolymia cubensis* (Anthozoa, Zoantharia, Acleractinia)	497	506	
scubGFP2	AY037771	*Scolymia cubensis* (Anthozoa, Zoantharia, Acleractinia)	497	506	
zoanYFP (zFP538)	AF168423	*Zoanthus sp.* (Anthozoa, Zoantharia, Zoanthidea)	494, 528	538	Yellow
DsRed (drFP583)	AF168419	*Discosoma sp. 1* (Anthozoa, Zoantharia, Corallimorpharia)	558	583	
dis2REP (dsFP593)	AF272711	*Discosoma sp. 2* (Anthozoa, Zoantharia, Corallimorpharia)	573	593	
zoan2RFP	AY059642	*Zoanthus sp. 2* (Anthozoa, Zoantharia, Zoanthidea))	552	576	Orange-Red
cpFP611	AY130757	*Entacmaea quadricolor*	559	611	
mcavRFP	AY037770	*Montastraea cavernosa* (Anthozoa, Zoantharia, Scleractinia)	508, 572	520, 580	
rfloRFP	AY037773	*Ricordea florida* (Anthozoa, Zoantharia, Corallimorpharia)	506, 566	517, 574	
Kaede	AB085641	*Trachyphyllia geoffroyi*	508, 572	518, 582	
asulCP (asCP)	AF246709	*Anemonia sulcata* (Anthozoa, Zoantharia, Actiniaria)	568	none	
hcriCP (hcCP)	AF363776	*Heteractis crispa* (Anthozoa, Zoantharia, Actiniaria)	578	none	Purple-Blue
cgigCP (cgCP)	AF363775	*Condylactis gigantea* (Anthozoa, Zoantharia, Actiniaria)	571	none	
cpasCP (cpCP)	AF383155	*Condylactis passiflora* (Anthozoa, Zoantharia, Actiniaria)	571	none	
gtenCP (gtCP)	AF383156	*Goniopora tenuidens* (Anthozoa, Zoantharia, Scleractinia)	580	none	

GFP-like proteins. Protein names are indicated according to the nomenclature suggested by Labas *et al*. (2002). Copyright 2002 National Academy of Science, U.S.A.

the RFPs that are commercially available from Clontech were derived from two native GFP-like proteins. First, DsRed (drFP583) is a native FP with an excitation and emission maxima at 558 and 583 nm, respectively (Matz *et al*., 1999). Second, a far-red FP has been generated by mutagenesis from a native CP, cgCP, which absorbs at 571 nm (Gurskaya *et al*., 2001). The resultant mutant FP, commercially available as HcRed1 (Clontech), has excitation and emission maxima at 588 and 618 nm, respectively. Despite this significant red-shift, HcRed1 shows a rather low molar extinction coefficient ($20,000$ $M^{-1}cm^{-1}$) and quantum yield (0.015). So far, the reddest FP of all native GFP-like proteins is eqFP611, cloned from the sea anemone *Entacmaea quadricolor* (Wiedenmann *et al*., 2002). This protein also exhibits a large Stokes shift; it absorbs at 559 nm and emits at 611 nm.

Advantages of DsRed

Extensive investigation of the fluorescent and biochemical properties of DsRed has revealed several striking features (Baird *et al*., 2000; Mizuno *et al*., 2001). DsRed has impressive brightness and stability against pH change, denaturants, and photobleaching. Brightness can be expressed as the product of the molar extinction coefficient and quantum yield. Precise determination of the molar extinction coefficient and quantum yield of wild-type DsRed has been somewhat difficult, with large discrepancies reported by various investigators (Matz *et al*., 1999; Baird *et al*., 2000; Patterson *et al*., 2001). The values depend upon many factors, such as protein purity or maturation. The most updated values measured using fully mature DsRed are $72,500$ M^{-1} cm^{-1} for the molar extinction coefficient and

0.68 for the fluorescence quantum yield (Patterson *et al.*, 2001). Together with its resistance to photobleaching and pH changes over a wide range, from 4.5 to 12, the behavior of mature DsRed is comparable to that of rhodamine dyes.

Attempts to eliminate drawbacks of GFP-like proteins

Some GFP-like proteins, including DsRed, have exhibited the following significant drawbacks that have limited their utility: 1) slow and incomplete maturation of the chromophore, 2) formation of obligate tetramers, and 3) a tendency to aggregate.

The red chromophore of DsRed requires an additional autocatalytic modification of its GFP-like chromophore (Gross *et al.*, 2000). Slow maturation precludes the use of DsRed as a reporter of gene expression or protein synthesis on short time scales. Also, incomplete maturation gives rise to residual green fluorescence, preventing the combined use of GFPs in dual-color labeling experiments. Only modest improvements in maturation have been provided with the commercially available DsRed2, but more recently, engineered variants of DsRed known as T1 (Bevis and Glick, 2002) and E57 (Terskikh *et al.*, 2002), have overcome the problem of the slow and incomplete maturation. Conversely, slow maturation can be utilized to analyze the history of gene expression. A new mutant of DsRed, E5, changes its color from green to red over time (Terskikh *et al.*, 2000). This feature makes it possible to use the ratio of the green to red emission peaks as an estimate of the time elapsed since the expression of the reporter gene was turned on. Thus, E5 functions as a fluorescent timer to give temporal and spatial information regarding target promoter activity.

Oligomerization does not limit the use of DsRed to report gene expression or to mark cells, but does preclude its use in fusion protein applications. Very recently, Campbell *et al.* (2002) reported successful engineering of monomeric RFP (mRFP1) from DsRed. Their approach employed site-directed mutagenesis to break the tetrameric structure followed by random mutagenesis to rescue red fluorescence. There are some differences in the behavior of mRFP1 and DsRed. mRFP1 matures over 10 times faster, so that it shows similar brightness in living cells despite its lower molar extinction coefficient, fluorescence quantum yield, and photostability. Also, the excitation and emission peaks of mRFP1 are 584 and 607 nm, respectively, which should confer better spectral separation from signals of other FPs. This work offers great promise regarding the conversion of other oligomeric fluorescent proteins into monomers. All the Anthozoan GFP-like proteins characterized thus far have been found to form obligate tetramers. The far red variant, HcRed1, has also been engineered to form dimers (Gurskaya *et al.*, 2001), while its parent cgCP is probably an obligate tetramer. The reddest native FP, epFP611, was shown to be monomeric, but only at low concentrations and in the presence of detergent (Wiedenmann *et al.*, 2002).

Aggregation may impede all possible applications due to considerable cellular toxicity. Although the molecular mechanisms of the aggregation of GFP-like proteins remain unclear, two models have been proposed. First, the aggregation may result from the oligomerization of GFP-like FPs. If host proteins are also oligomeric, fusion to FPs may result in cross-linking into massive aggregates. In this case, the problem of aggregation would be solved using the monomeric versions of FPs. Second, electrostatic or hydrophobic interactions between FPs may cause aggregation. Yanushevich *et al.* (2001) have successfully generated non-aggregating mutants by removing some basic residues located near the N-termini of several FPs, including DsRed, zFP506, zFP538, amFP486, and asFP595. This finding suggests that electrostatic interaction is responsible for the aggregation of these FPs. It also may be possible to make non-aggregating mutants by removing hydrophobic side chains on the surface of the tetrameric complex. It should be noted that *Renilla* GFP does not aggregate as a result of its dimerization; its hydrophobic patch is hidden in the dimerization interface, so that the surface of the dimer becomes hydrophilic.

Thermotolerance of Anthozoan GFP-like proteins

DsRed matures more efficiently at 37°C than at room temperature, in contrast to *Aequorea* GFP, which folds preferentially at lower temperatures (Mizuno *et al.*, 2001). Such a difference can be explained by the temperature of the water in which the organisms live; *Aequorea victoria* is found in the Pacific Northwest, while *Discosoma* is native to the Indo-Pacific Ocean. Also, the tight tetramerization of wild-type Anthozoan proteins probably evolved to maximize thermotolerance in the presence of intense tropical sunlight.

Color variation of an Anthozoan GFP-like protein

Ando *et al.* (2002) have serendipitously discovered that a green-emitting FP they cloned from the stony coral *Trachyphyllia geoffroyi* may be useful as an optical cell marker. The discovery occurred when they accidentally left a test tube of the FP on a lab bench overnight, and found the next day that it had turned red. The FP was named Kaede, the Japanese word for "maple leaf". Kaede emits bright green fluorescence after synthesis, but changes to a bright and stable red fluorescence efficiently upon irradiation with UV or violet light. Selectively exposing some cells expressing the protein to UV light could allow cellular marking, with the added benefit that the excitation wavelength required for the protein will not induce photoconversion.
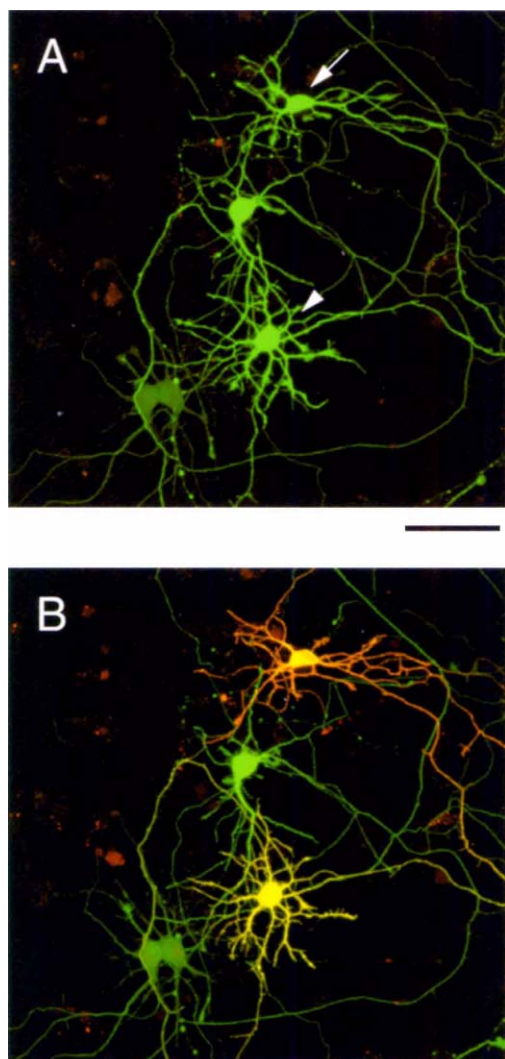
Fig. 1. Optical marking of individual neurons in a hippocampal primary culture. (A) One day after transfection with cDNA encoding Kaede, a novel fluorescent protein, the top and bottom neurons (indicated by an arrow and arrowhead) were illuminated in their cell bodies using a violet laser diode (405 nm) for 3 and 1 sec, respectively. (B) After 3 minutes, the red and yellow neurons, together with adjacent green neurons, were imaged simultaneously using confocal microscopy with 488/543 nm excitation and merged, which allowed the colors to be appreciated through a microscope. Scale bar, 50 μm.

The mobility of a fluorescence-labeled molecule has been assessed using a specific photobleaching technique called fluorescence recovery after photobleaching (FRAP) (Lippincott-Schwartz *et al.*, 2001). Acquisition of a series of images after photobleaching in a small region of the cell is useful for qualitative description of the diffusion of the fluorescence-labeled molecule. For studying cell lineage, organelle dynamics and protein trafficking, however, optical marking is preferred; individual cells, organelles and proteins can be optically labeled with unique colored markers. Three methods of optical marking have become commonplace, and are based upon photo-induced alteration of the excitation or emission spectra of certain fluorescent proteins such as wild-type *Aequorea* GFP and DsRed (Yokoe and Meyer, 1996; Elowitz *et al.*, 1997; Marchant *et al.*, 2001). Some limitations of these methods have been recognized, however, including dimness and instability of the photoconverted product and the unavailability of simple, efficient or specific illumination for marking. Conversely, Kaede exhibits bright emission, permanent photoconversion, and the ability to be photoconverted using a UV lamp or laser.

To test Kaede's ability to mark living cells, the protein was expressed in a dense culture of neurons and glial cells. Individual green cells are hard to identify because their processes are inter-entangled (Fig. 1A). Focusing a pulse of 405 nm light from a laser diode (Nichia company) on the body of a neuron for a short time produced red-emitting Kaede that then spread over the entire cell. It was possible to distinguish its axon and dendrites from those belonging to other cells, and, with a confocal microscope, contact sites between neurons were clearly visualized (Fig. 1B). While the fluorescent marker appears useful for optically marking cells, Kaede tends to aggregate much like wild-type DsRed, probably due to its obligate tetramerization. This makes the wild-type protein an unlikely candidate for protein studies. Attempts are being made to develop a monomer of the fluorescent protein in much the same way that the DsRed monomer was recently engineered (Campbell *et al.*, 2002).

The study by Ando *et al.* not only demonstrates the benefits of a newly-cloned fluorescent protein, but addresses the molecular mechanisms for the color variation occurring within an individual *Trachyphyllia geoffroyi*. When exposed to sunlight, the tentacles and disk turn a shade of red in proportion to the degree of Kaede photoconversion. Then, they revert to green as newly-synthesized Kaede is added. This mechanism may be responsible for the variety of color in stony corals.

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