RESEARCH COMMUNICATION

Far-red fluorescent tag for protein labelling

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Practical applications of green fluorescent protein ('GFP')-like fluorescent proteins (FPs) from species of the class Anthozoa (sea anemones, corals and sea pens) are strongly restricted owing to their oligomeric nature. Here we suggest a strategy to overcome this problem by the use of two covalently linked identical red FPs as non-oligomerizing fusion tags. We have applied this approach to the dimeric far-red fluorescent protein HcRed1 and have demonstrated superiority of the tandem tag in the *in vivo* labelling of fine cytoskeletal structures and tiny nucleoli. In addition, a possibility of effective fluorescence resonance energy transfer ('FRET') between enhanced yellow FP mutant ('EYFP') and tandem HcRed1 was demonstrated in a protease assay.

Key words: DsRed, green fluorescent protein (GFP), HcRed, tandem tag.

INTRODUCTION

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its enhanced mutants are indispensable tools for the *in vivo* visualization of gene expression, protein localization, dynamics and interaction. Unfortunately, a main limitation of GFP-based labelling is the restricted diversity of fluorescent colours. Consequently, the discovery of GFP-like fluorescent proteins (FPs) of different colours from species of the class Anthozoa (sea anemones, corals and sea pens) [1–4] has excited considerable interest worldwide. The red FP drFP583 (commercial name DsRed) has been intensively investigated and widely used over the past 2 years. Previous studies highlighted the tendency of this protein to form strong tetramers [5–8], which is a major drawback of DsRed. Several other Anthozoa-derived FPs also appear oligomeric [4,8,9]. FP oligomerization strongly restricts their application as fusion tags for target proteins, since it frequently results in improper folding and functioning of the tagged partners, as well as intensive intracellular aggregation [7,10].

The first progress towards non-oligomerizing FPs was made on the far-red protein HcRed, which was transformed into a dimeric form [4] and is commercially available as HcRed1 (ClonTech). Very recently, when the present experimental work was already completed, a paper describing monomeric DsRed mutant mRFP1 was published [11]. This protein was generated by multiple steps of molecular evolution that included both site-directed and random mutagenesis (in total 33 substitutions compared with DsRed).

In the present paper we describe a far-red fluorescent tag that may be generally used for protein labelling. The tag is based on two head-to-tail linked identical HcRed1 copies. Since purified HcRed1 forms dimers in solution, we hypothesize that covalently linked copies of this protein should form intramolecular dimers, which may be used as a non-oligomerizing tag *in vivo*.

EXPERIMENTAL

Plasmid construction

pEGFP-actin (ClonTech) was used as the parent vector for the construction of all fusion plasmids. HcRed1, HcRed, M355NA and DsRed2 coding regions were cloned into this vector between *Age*I and *Bgl*II restriction sites, in lieu of the enhanced GFP (EGFP)-coding region. To create tandems, the second copy of the corresponding FP was cloned into these plasmids (between the first FP and β-actin genes) using the *Bgl*II and *Eco*RV restriction sites. All plasmids contained the four amino acid linker Arg-Thr-Arg-Ala between FP and actin. In the case of tandems, FP genes were separated by the four amino acid linker Arg-Ser-Pro-Gly. The fibrillarin coding region was amplified with the primers 5′-GGTGCTCGAGCCATGAAGCCAGGATTCAG and 5′-GGTGGGATCCTCAGTTCTTCACCTTGGGGG (restriction sites are underlined) using Marathon-Ready Human Liver cDNA (ClonTech) as a template, and cloned between *Xho*I and *Bam*HI restriction sites, in lieu of the actin-coding region.

For prokaryotic expression of FP, full-length coding regions were cloned into the pQE30 vector (Qiagen). Proteins fused to an N-terminal His_6 tag were expressed in *Escherichia coli* XL1 Blue strain (Invitrogen) and purified using the TALON metal-affinity resin (ClonTech). Gel-filtration analyses were performed as described in [4].

Cell culture

L929 and HEK293 cells were obtained from the American Type Culture Collection (A.T.C.C.) and cultured in standard

Abbreviations used: EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; FP, fluorescent protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; LP, long-pass filter; ROI, region of interest; t-HcRed1, tandem HcRed1.

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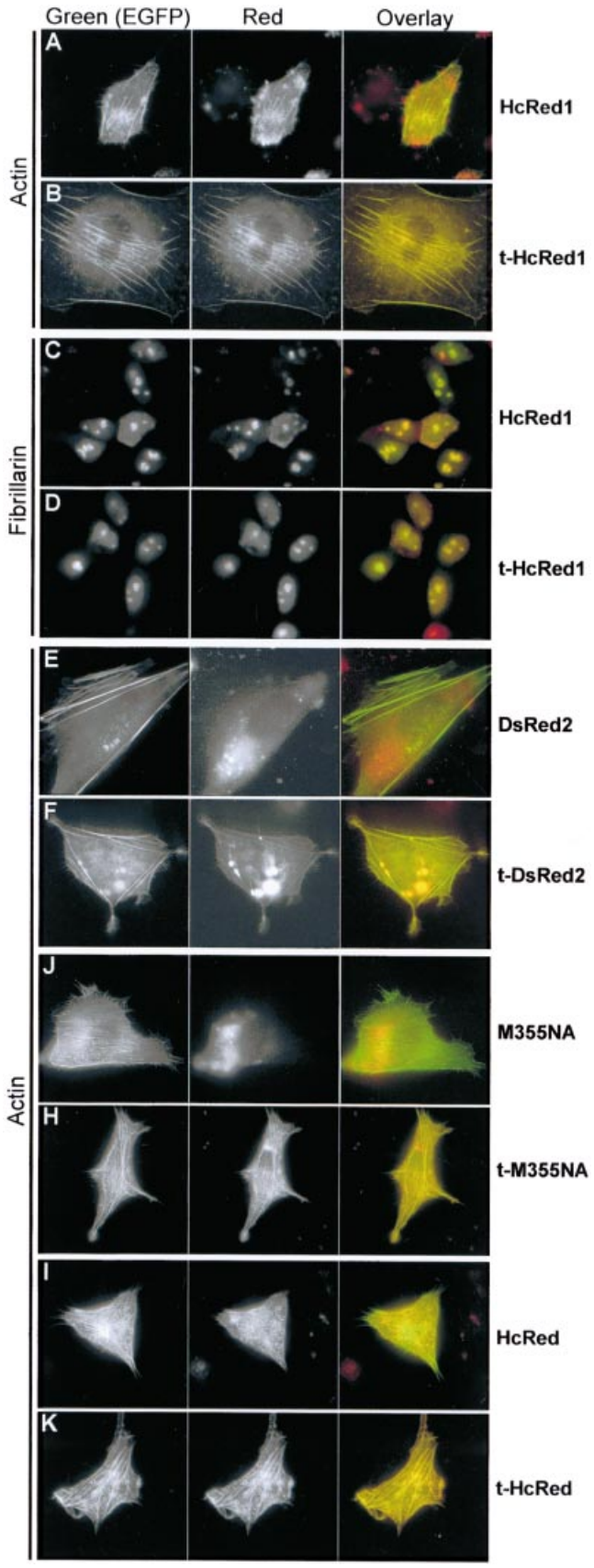

Dulbecco's modified minimal essential medium (Invitrogen) supplemented with 10 % (v/v) fetal bovine serum (Sigma). Cells grown to 50–70 % confluence on 18 mm × 18 mm coverslips in 35-mm-diameter dishes (Falcon) were transfected with the above plasmids, using LIPOFECTAMINE PLUS™ (Invitrogen) or FuGENE 6 (Roche) reagents for L929 or HEK293 cells respectively. After transfection (48 h), cells were washed with Dulbecco's PBS and fixed with 4 % (w/v) paraformaldehyde in PBS for 30 min. For fluorescence microscopy, coverslips were mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.).

Fluorescence microscopy and image analyses

Images of fixed cells were acquired with an ORCA-ER digital camera (Hamamatsu, Bridgewater, NJ, U.S.A.) attached to an Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with Plan Apo 100 × /1.40-numerical-aperture oil-immersion objective, using standard GFP/FITC (excitation at 460–505 nm; emission at 510–560 nm), G-2A [excitation at 510–560 nm; emission at long-pass filter (LP) 590 nm] and TxRed (excitation at 540–580 nm; emission at 600–660 nm) filter sets for EGFP, M355NA and HcRed or HcRed1 respectively. Exposure times of 0.24–4.08 s and the high-resolution 1 × 1 binning mode of the camera were employed. Digital images were deconvoluted, pseudocoloured, superimposed and assembled in Adobe Photoshop.

Fluorescence-resonance-energy-transfer (FRET) assay

A pQE30-based plasmid encoding a triple fusion HcRed1–HcRed1–enhanced yellow fluorescent protein (EYFP) was constructed. The EYFP-coding region was amplified from the pEYFP-N1 vector (ClonTech). An amino acid linker, RTRAP-AGIEGR, between the second HcRed1 and EYFP was introduced by PCR (recognition site for Factor Xa is underlined). The purified protein was digested with Factor Xa (Promega) in buffer containing 100 mM NaCl, 2 mM $CaCl_2$ and 20 mM Tris/HCl, pH 8.0. Fluorescence spectra (excitation at 490 nm) before and after digestion were measured using a Carry Eclipse Fluorescence Spectrophotometer (Varian, Sunnyvale, CA, U.S.A.). Excitation (460–490 nm) and emission (LP 510 nm LP 610 nm) filters were used with an Olympus SZX12 stereomicroscope to visualize protein samples (inset to Figure 2 below).

RESULTS AND DISCUSSION

Initially we tested the tandem HcRed1 construct (t-HcRed1) in a prokaryotic expression system. Several amino acid linkers of

Figure 1 Fluorescence microscopy of cells expressing FP-tagged actin or fibrillarin

Cells (L929 or HEK239 in the case of actin and fibrillarin labelling, respectively) were simultaneously co-transfected with EGFP-labelled and red FP-labelled targets. The left column corresponds to the green fluorescent signal from EGFP (desired fluorescent image). The middle column corresponds to the red signal from FPs. The right column represents an overlay of the left and middle images pseudocoloured in green and red respectively. Target proteins are designated on the left. Red FP tags are indicated on the right. (**A**) HcRed1–actin; (**B**) t-HcRed1–actin; (**C**) HcRed1–fibrillarin; (**D**) t-HcRed1–fibrillarin; (**E**) DsRed2–actin; (**F**) t-DsRed2–actin; (**J**) M355NA–actin; (**H**) t-M355NA–actin; (**I**) HcRed–actin; and (**K**) t-HcRed–actin are shown.

Table 1 Comparison of various β-actin fusion constructs expressed in L929 fibroblasts

At least ten quantified and normalized by intensity in 0–255 scale cellular images for each construct and five regions of interest (ROI) per cell were analysed. Very low, low, medium, high and very high grades used for the comparison mean respectively: (A) up to 5, 10, 15, 20 or more small aggregates per cell (large aggregates were considered as respective by area amount of small ones); (B) 0–51, 52–102, 103–153, 154–204 and 205–255 of average pixel intensity in ROIs located at actin bundle (judged from EGFP pattern) as compared with ROIs in empty dark field (no cell image); (C) The same as (B), but measured in ROIs located in cytoplasm without any visible structures; (D) More than 5, 4 or 5, 3 or 4, 2 or 3 and 1 or 2 EGFP versus FP averaged pixel intensity ratio in arbitrary generated intracellular ROIs.

FP fused to β-actin ...	EGFP	HcRed1	t-HcRed1	DsRed2	t-DsRed2	M355NA	t-M355NA	HcRed	t-HcRed
(A) Formation of aggregates	Very low	Medium	Very low	High	Medium	High	Medium	High	Low
(B) Labelling of actin structures	High	Medium	High	Very low	Medium	Very low	High	Low	High
(C) Cytoplasmic background	Low	Medium	Low	High	Low	High	Low	High	Low
(D) Total overlay with EGFP	–	Medium	Very high	Very low	Medium	Very low	High	Low	High

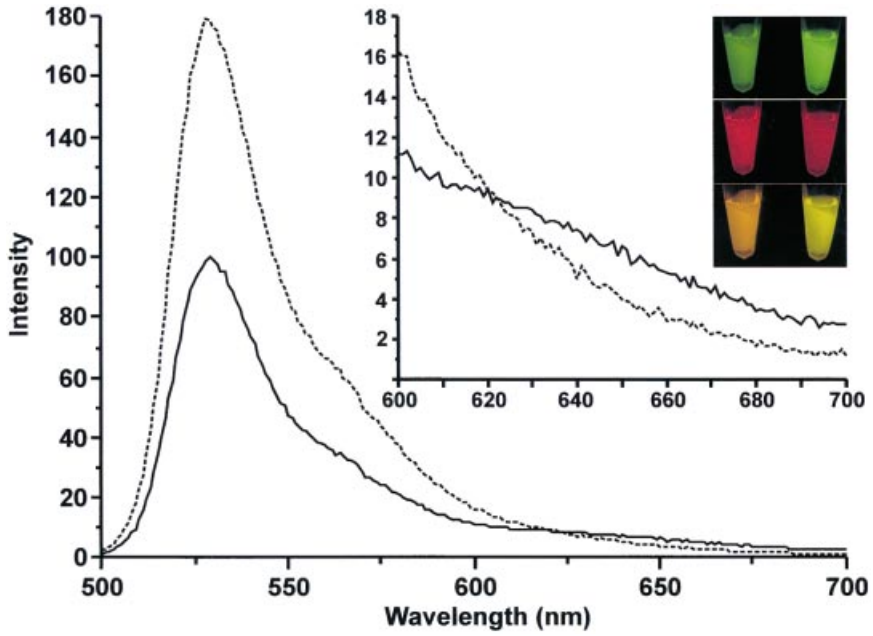

Figure 2 Illustration of FRET between EYFP and t-HcRed1

Emission spectra (excitation at 490 nm) of purified triple fusion HcRed1–HcRed1–EYFP before (continuous line) and after (broken line line) Factor Xa digestion. Dissociation of EYFP and HcRed resulted in a significant increase in yellow fluorescence and decrease in red fluorescence due to elimination of FRET. The inset zooms in at the 600–700 nm region. The small inset in the right upper corner shows tubes with non-digested (left) and digested (right) protein samples visualized by fluorescence stereomicroscopy. The upper row depicts excitation by blue light and the detection of green emission. The middle row represents excitation by blue light and the detection of red emission. The bottom row represents superimposition of the upper and middle images. Increase in the green signal and the simultaneous decrease in the red signal after digestion caused by FRET elimination are clearly distinguishable.

different lengths and compositions between monomers were examined. The best results in terms of rate and completeness of protein maturation and fluorescence brightness were obtained with a four amino acid linker, Arg-Ser-Pro-Gly, which was subsequently used in all further constructs. Purified protein t-HcRed1 is characterized by a far-red fluorescence spectrum (excitation at 590 nm, emission at 637 nm), a very high absorption coefficient (160000 $M^{-1} \cdot cm^{-1}$) and low quantum yield (0.04). Thus the brightness of t-HcRed1 is comparable with that of mRFP1, which possesses a lower absorption coefficient (44000 $M^{-1} \cdot cm^{-1}$), but higher quantum yield (0.25) [11]. *E. coli* colonies expressing t-HcRed1 possessed brighter fluorescence and more rich purple coloration in comparison with colonies with singleton HcRed1. It appears that protein dimerization occurs more effectively between closely linked rather than free monomers. Gel-filtration chromatography revealed the same mobility for HcRed1 and t-HcRed1. Both proteins appeared to be 'dimers', as evident from the similar single peaks observed between the tetrameric DsRed and monomeric EGFP peaks (results not shown). Thus linked HcRed1 forms intramolecular, but not intermolecular, dimers, and may therefore be utilized as a monomeric tag for fusion partners.

To examine the properties of t-HcRed1 in eukaryotic cells, we fused it to cytoplasmic β-actin and the nucleolar protein fibrillarin. A series of plasmids expressing one or two copies in tandem of HcRed1 linked with actin or fibrillarin was constructed. Corresponding EGFP-tagged functional fusion proteins [12,13] were used as positive controls to visualize desired fluorescent patterns. Simultaneous transient co-transfection of cells with two plasmids expressing EGFP- and HcRed1-tagged fused constructs allowed the comparison of green and red fluorescent images within the same cells, and thus the estimation of any

imperfections in the red tags used (Figure 1). In fusion with actin, the singleton HcRed1 tag produced rather high levels of cytoplasmic aggregation within L929 fibroblasts, although some filaments and stress fibres were partially visible (Figure 1A; Table 1). In contrast, the pattern of actin structures with the t-HcRed1 tag was very similar to that with EGFP-actin (Figure 1B). t-HcRed1 and EGFP labelling of fibres, cellular cortices and processes were practically indistinguishable.

The differences between singleton and tandem HcRed1 tags used in conjunction with fibrillarin in HEK293 cells were not so obvious. HcRed1–fibrillarin displayed the correct pattern in the majority of cells, although about 20 % of cells still exhibited high levels of cytoplasmic red fluorescence that did not correspond to EGFP–fibrillarin distribution (Figure 1C). On the other hand, practically all dual-colour labelled cells demonstrated very similar green and red fluorescent signals in the case of t-HcRed1 tag (Figure 1D). The doubled size of the tandem tag had no effect on the high-level expression in cells, proper folding of filamentous actin, and tagging to fine actin structures or the transport of fusion proteins to the nuclei. On the basis of these results, we conclude that the t-HcRed1 construct is useful for protein tagging to the same extent as EGFP.

We further applied this approach to three other tetrameric red FPs. One is DsRed2, the commercially available improved (non-aggregating and fast folding) version of DsRed [9,14]. The second, HcRed, is the most far-red fluorescent mutant of the non-fluorescent chromoprotein hcCP [4]. The third, M355NA, is non-aggregating and the brightest red mutant of the chromoprotein as FP595 [9]. These FPs in both singleton and tandem forms were fused to β-actin and expressed in L929 cells, together with the EGFP–actin control. As expected, all three singleton FPs displayed manifestly incorrect patterns of localization with extremely high cytoplasmic aggregation (Figures 1E, 1J and 1I; Table 1). In contrast, FP-tandem proteins adequately labelled filamentous actin structures. In the latter case, the major patterns of actin structures were clearly distinguishable, although the degree of labelling and contrast between actin filaments and the background cytoplasmic signal was still lower than that in EGFP-actin images (Figures 1F, 1H and 1K). Some non-specific aggregate formation was additionally observed with all three FP-tandems.

Recombinant t-DsRed2, t-M355NA and t-HcRed tandem proteins expressed in *E. coli* were indistinguishable from their parental singletons in gel-filtration experiments, where they migrated as tetramers (results not shown). This indicates that each tandem protein molecule forms an intramolecular 'dimer' consisting of two covalently linked FP-barrels, and two of these dimers combine into the 'tetrameric' structure. Consequently, these tandem proteins behave as dimeric tags. This may be the main reason for the superiority of DsRed2, M355NA and HcRed tandems over singletons in protein labelling.

Taking into account the importance of FRET applications [15–20], we used a simple model system to demonstrate FRET between EYFP and t-HcRed1 proteins. A pQE30-based plasmid encoding triple fusion t-HcRed1–EYFP and containing the Factor Xa protease cleavage site within the linker between the second HcRed1 and EYFP was constructed. We expected to detect spectral changes upon Factor Xa digestion, owing to FRET elimination, analogous to the well-documented examples for pairs of GFP mutants of different colours [15,17]. Indeed, incubation of purified fusion construct with Factor Xa led to a gradual increase in the yellow emission peak at 528 nm and simultaneous decrease in red emission at 650 nm with an isosbestic point at 625 nm. Protease digestion resulted in an 80 % increase in yellow fluorescence and 30 % decrease in red fluorescence (Figure 2). The ratio of donor to acceptor fluorescence changed by 2.5-fold, which was comparable with FRET levels between other FP pairs [7,15,16,18,19]. Control experiments demonstrated no spectral changes after digestion with Factor Xa of EYFP and t-HcRed1 individually or when mixed (results not shown).

Our results indicate that the novel far-red t-HcRed1 may be successfully used in a similar manner to GFP and its mutants in numerous biotechnological and cell biological applications. Owing to vivid spectral differences between the GFP variants used for double-labelling, the additional introduction of far-red t-HcRed1 together with recently introduced monomeric red mRFP1 [11] should potentially permit tetracolour applications.

Our data additionally demonstrate the usage of t-HcRed1 as the FRET acceptor for other FP donors, such as EYFP. This supports its application in the development of more deep-tissue-penetrable far-red FRET-based assays to study *in vivo* protease activities and protein–protein interactions, and create intracellular sensors. Moreover, emission and excitation spectral overlays of GFP variants as well as red mRFP1 and far-red HcRed1 tandems should allow the simultaneous use of two independent FRET pairs of FPs in a single living cell.

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