Improved Version of the Red Fluorescent Protein (drFP583/DsRed/RFP)

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ABSTRACT

GFP has established itself as a highly useful tool throughout many areas of modern biology. Recently, the novel fluorescent protein drFP583, also termed DsRed or RFP, was cloned from a coral of the Discosoma genus. The protein is only weakly homologous to GFP and has a red emission spectrum, which makes drFP583 an attractive candidate for in vivo double labeling together with GFP variants. However, wild-type drFP583 has several drawbacks, including inefficient folding of the protein, extremely slow maturation of the chromophore, and tetramerization even in dilute solutions. Here we report on important improvements to this reporter that lead to higher levels of fluorescent drFP583 species in the cell. We further characterized our best mutant for applications in yeast and mammalian cell biology.

INTRODUCTION

The red fluorescent protein (RFP) drFP583, also called DsRed, was first cloned in 1999 from a corallimorpharian *Discosoma* sp. (15). The protein shows only a weak amino acid sequence homology to the GFP of *Aequorea victoria* (approximately 23% identity). The tertiary structures of both proteins are strikingly similar, showing an 11-stranded β -barrel with a coaxial helix running through it (23,24,26). The core fluorophore of drFP583 is similar to GFP but becomes extended in a second oxidation step (10). Therefore, the formation of the red fluorophore proceeds via a green fluorescent intermediate. This final, enlarged chromophore results in the excitation and emission spectra of drFP583, with maxima at 558 and 583 nm respectively (15). This makes drFP583 an attractive tool as a protein tag or reporter gene together with available improved mutants of GFP (4,5,11) in multicolor in vivo labeling, fluorescence resonance energy transfer (FRET) experiments (14), and as a tag in several different species such as yeast (17,18) and *Drosophila* (20).

DsRed cannot yet meet all expectations because of some major caveats. There is a severe problem with the accumulation of drFP583-aggregates within the cells; furthermore, drFP583 displays very slow maturation of the chromophore (approximately two days) and a prominent tetramerization of the protein (1). Similar problems had to be solved for GFP (19). Faster chromophore maturation had been achieved

by site-directed mutagenesis of the chromophore residue Ser65 (11). Some other improvements to GFP were gained by molecular evolution using a DNA shuffling technique (5).

Here we report on how to obtain a mutant RFP, RedStar, which shows the vastly accelerated appearance of fluorescent protein species when expressed in vivo. Furthermore, we tested the application of this mutant by fusing it to several marker proteins in yeast and to a Golgi marker protein in mammalian cells.

MATERIALS AND METHODS

DNA and *E. coli* Techniques

The nucleic acid sequence of RFP with optimized codon usage for the yeast *Saccharomyces cerevisiae*, termed yRFP, was generated from the protein sequence of drFP583 (15). We applied the programs BACKTRANS-LATE and CODONFREQUENCY from the GCG Wisconsin Package (version 10.0) (lane BACKTR/CODON-FRQ), followed by hand-editing, to remove potentially destabilizing base pairs (lane yRFP). The yRFP DNA used for this study was then generated by full gene synthesis based on the published protein sequence using overlapping primers. Of the 226 codons in drFP583, 131 codons were modified to improve codon usage (58%).

The *E. coli* screening was performed using the pBAD/His A arabinose-inducible expression system (Invitrogen, Carlsbad, CA, USA). Constructed

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libraries (5) were transformed directly into Top10® *E. coli* cells (Invitrogen) and spread onto LB plates with 100 µg/mL ampicillin and 0.2% arabinose.

Yeast plasmids used were p415-Gal1-yRFP and p415-Gal1-RedStar. yRFP and RedStar were cloned as *Bam*HI/*Xho*I fragments in the *Bam*HI/*Xho*I sites of p415-Gal1 (16). Mammalian constructs yRFP and RedStar were fused to the C-terminus of the first 202 amino acids of rat GRASP65 as follows: the DsRed coding region was excised from p415-Gal1-yRFP or p415-Gal1-RedStar with *Bam*HI and *Xho*I, rat GRASP65 N-terminus with *Eco*RI and *Bam*HI (2), and the two fragments were triple ligated with pcDNA3.1+ (Invitrogen) cut with *Eco*RI and *Xho*I. The constructs were verified by restriction digestion and sequencing.

Yeast Techniques

The media and basic yeast techniques were performed as previously described (17). Primers used were RFP-C (5'-TACCTCTATACTTTAACGTCAAGGAGAAAAACTATATCT-AGAACTAGTGGATCCGTATG-3'; the *Bam*HI site is underlined, and the START codon is bolded) and RFP-D (5'-GGATGTGGGGGAGGGCGTG-AATGTAAGCGTGACATAACTAAT-TACATGACTCGAGTTAC-3'; the *Xho*I site is underlined, and the STOP codon is bolded). Plasmids similar to p415-Gal1-yRFP, but containing the *E. coli* optimized variants, were constructed and used as templates for PCR during the first round of DNA shuffling. For library construction, PCR products obtained with primers RFP-C and RFP-D were used for transformation together with *Bam*HI- and *Xho*I-digested plasmid p415-Gal1 (16) in yeast strain ESM356-1 (*MATa ura3-53 leu2Δ1 his3Δ200 trp1Δ63 GAL+*). The transformants were selected on plates with synthetic medium containing glucose and lacking leucine. Nearly all transformants (97%) contained plasmid with DsRed inserted between the *Bam*HI and *Xho*I sites of p415-Gal1 because of homologous recombination between the overhangs provided by the primers RFP-C and RFP-D, which are homologous to the ends of the gapped plasmid p415-Gal1.

Fluorescence measurement in

yeast cells. Cells expressing enhanced GFP (4), yRFP, and RedStar were grown overnight on synthetic medium containing 2% galactose and 2% raffinose to saturation. Fluorescence was measured in 4×10^8 cells on an agar plate, using the Fluoroskan Ascent FL fluorescence plate reader (Thermo Lab-systems, Helsinki, Finland) and an adapter for the agar plates and indicated wavelength combinations (see Figure 2B). Chromosomal tagging of the *HHF2*, *MYO1*, *MYO5*, and *SPC42* genes was performed using the PCR-based gene-tagging strategy (22) and yRFP-kanMX4, respectively. RedStar-kanMX4 modules were constructed as previously described for the enhanced GFP-kanMX4 module (12).

Mammalian Cell Culture and Techniques

Hela cells were plated at 30% confluency on glass coverslips or 2-cm dishes in 2 mL DMEM with 10% FCS 24 h before transfection and then grown at 37°C in 5% CO₂. GRASP65-DsRed expression plasmids were transfected with Effectene™ (Qiagen, Valencia, CA, USA), 0.4 µg DNA were diluted into 100 µL buffer EC (from the kit), 3.2 µL enhancer reagent were added, and the tube was vortex-mixed for 1 s and then left for 5 min at room temperature. Effectene (10 µL) was then added, and the tube was vortex-mixed for 10 s. After 10 min at room temperature, the DNA-Effectene complexes were directly added to the cells, swirling gently to mix. After the times indicated, the cells were fixed in either -20°C methanol for 4 min or lysed in SDS-PAGE sample buffer for analysis by Western blot analysis. Fixed coverslips were mounted in Mowiol® 4-88 (Calbiochem-Novabiochem, San Diego, CA, USA) containing 1 µg/mL DAPI. Western blots were probed with affinity-purified rabbit anti-DsRed and mouse anti-α-tubulin (Sigma, St. Louis, MO, USA). Fluorescence images were collected on an Axioplan-2 (Carl Zeiss, Thornwood, NY, USA) equipped with a 63× Plan-Apochromat objective with a numerical aperture of 1.4, a halogen light source (HB103; Carl Zeiss), and a charge-coupled device camera (Roper Scientific, Trenton,

NJ, USA). For DsRed, a Cy3 filter set (Chroma Technology, Brattleboro, VT, USA) was used.

Various Methods

Protein extraction from yeast for Western blot analysis was performed as previously described (12). The antibodies used were affinity-purified rabbit anti Spc42p (7) and affinity-purified rabbit anti yRFP.

RESULTS

Screening in *E. coli*

DrFP583, when expressed in *E. coli*, forms aggregates that contain large fractions of nonfluorescent protein (unpublished observation). To improve the properties of the protein, we constructed a yeast codon optimized drFP583 gene (see Materials and Methods). This gene, named yRFP, was used to screen for brighter variants of the protein in *E. coli* using recursive cycles of DNA shuffling (5), followed by the selection of the brightest mutants. In total, we performed six cycles of screening, using the best mutants from the previous round as input for the new round. For the second, third, fifth, and sixth rounds, we used the 40 best clones from the previous round. For the fourth round, we used the three best clones from the third round. For each cycle, we screened about 20 000 colonies. Selection was performed in two ways. First, we picked the 200 brightest colonies by investigating the colonies on the plates using a Fuji LAS-1000 fluorescence imager (Fuji, Tokyo, Japan). The excitation wavelength was 488 nm, and fluorescence emission above 540 nm was acquired. Data processing (finding of the brightest colonies) was performed using the LAS-1000 software. These colonies were tested again and investigated visually. About 20% of the 200 colonies showed green or orange colors, instead of the bright red color obtained by the expression of drFP583. The brightest red fluorescent clones were again selected, following growth in liquid medium in 24-well plates for 24 h and investigation at three different wavelength combinations (488/527,

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488/590, and 544/590 nm, respectively), using the Fluoroskan Ascent FL fluorescence plate reader. After six rounds, the brightest mutant was 70- to 80-fold brighter than the original yRFP. Analysis of the best clones by SDS-PAGE and Coomassie® staining showed that a significant fraction of the total improve-

ment was due to a roughly 10-fold higher expression level of the mutant proteins versus yRFP (data not shown). This improvement was not due to improved codon usage but depended on the introduced amino acid exchanges (data not shown). The best mutant found in this screen (termed "MUT59":

R18K; V97I; S113T; F125L; M183K; P187Q; T203I) was then expressed in yeast and found to be six times brighter than the wild-type protein, while the expression level was exactly the same as for yRFP. This confirms that part of the improvements achieved in *E. coli* were host independent.

Figure 1. Sequence alterations and quantification of the brightness of RedStar in relation to DsRed/yRFP. (A) Alignment of the DNA and amino acid (aa) sequences of the various genes and proteins generated during the course of this study. Lane drFP583/yRFP: amino acid sequence of the indicated proteins. Note that yRFP has an additional glycine residue at position 2 (see Materials and Methods and Utility Model DE200 01 395.5). Lanes RedStar res. RedStar/aa: DNA and amino acid sequences of RedStar, as determined by sequencing the RedStar mutant obtained in this study. Only the residues differing from yRFP are shown. (B) The comparison of the fluorescence present in yeast cells following expression of enhanced GFP (4), yRFP, and RedStar for 16 h (Materials and Methods). Fluorescence measurements were performed at the indicated wavelength combinations. Inlay: yRFP and RedStar were detected in these cells by Western blot analysis. (C) The expression and appearance of fluorescent species of yRFP and RedStar in yeast. After induction of protein expression for 3 h, the appearance of fluorescence in the yeast cells was followed during the indicated period under conditions in which further protein synthesis was blocked. The average measurements of three individual clones are shown. Fluorescence measurements were performed with an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

Table 1. Comparison of Enhanced GFP, yRFP, and RedStar in Fusion with Some Marker Proteins in Yeast

Protein	Type of Protein	Localization	Enhanced GFP ^a	DsRed	RedStar
Hhf2p	Histone H4	Chromosomal DNA (22)	good ^b	toxic ^c	toxic ^c
Myo1p	Myosin	Ring at bud neck (13)	good	n.d.	n.d.
Myo5p	Myosin	Cortical actin patches (9)	good	n.d.	OK, faint
Spc42p	Structural protein	Yeast centrosome (6)	good	n.d.	OK

n.d., no specific signal was detected.
^aThe yeast enhanced GFP variant (S65G and S72G) (4).
^bHistone H4 is expressed from two different genes. If only one gene carries an enhanced GFP fusion (this case *HHF2*), then the cells show only mild growth defects. If *HHF2-GFP* is the sole source for Histone H4, *HHF2-GFP* is nonfunctional.
^cThe toxic effects of *HHF2-DsRed* (and RedStar) in the presence of a wild-type copy suggest that it is dominant lethal.

Screening in *S. cerevisiae*

The two best mutants (MUT59 and a different but not sequenced mutant) from the *E. coli* screen were used to find further improved mutants using additional rounds of DNA shuffling (5), this time followed by screening in *S. cerevisiae*. We used plasmid p415-Gal1 (16) that contains the galactose-inducible Gal1 promoter. Libraries were constructed directly in yeast by making use of the efficient homologous recombination system present in the organism (see Materials and Methods). For each round of DNA shuffling, libraries with roughly 4×10^6 independent plasmids were obtained. The initial screening was done with a FACScalibur™ (BD Biosciences, San Jose, CA, USA) and an attached cell sorter after 3–6 h of induction of protein expression. About 3% of the brightest cells (488/595 nm) were gated to the sorter, collected, and plated on medium that induced the promoter. After colony formation (growth for 2.5 days at 30°C), the colonies were assayed using the LAS-1000, as described earlier for the *E. coli* screen. For post-selection, round patches obtained by dropping cells from cultures grown on galactose-containing medium onto agar plates were further investigated using the Fluoroskan Ascent FL and an adaptor for agar plates. Again we found about 20% of the clones with green-shifted fluorescence properties.

In total, we performed five rounds of selection. The brightest mutant from the last screen was called RedStar. Compared with MUT59, RedStar also carried the G2S substitution and a reversion of the S113T mutation. In addition, there were several silent base-pair exchanges. Figure 1A shows an alignment of the DNA and amino acid sequences of drFP583, yRFP, and RedStar. Figure 1B shows the fluorescence measurements of enhanced GFP, yRFP, and RedStar. As can be seen, the 544/590 nm fluorescence for RedStar is about 20-fold brighter than that of yRFP. The expression levels (Western blots in Figure 1B) of RedStar were roughly 2- to 3-fold higher than for yRFP when quantified using the LAS-1000 (data not shown). We addressed the kinetic aspects of fluorescent protein formation of yRFP versus RedStar in an experiment where we expressed the proteins for 3 h by the addition of 2% galactose and then stopped protein synthesis by addition of 2% glucose for 5 min, followed by washing the cells with cycloheximide. We then followed fluorescence formation in patches of 4×10^8 cells on agar plates containing cycloheximide (10 μ M) over two days. Figure 1C shows that the initial fluorescence detected in the RedStar mutant was roughly 5-fold higher than for yRFP. Within the first 6 h after shutting off protein synthesis, RedStar fluorescence increased in this experiment to

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about 20 times the fluorescence of yRFP. The ratio of RedStar:yRFP fluorescence remained constant; although, for both proteins, the fluorescence continued to increase.

Analysis of the RedStar Mutant in Yeast

We tested the RedStar mutant by fusing it to several well-known marker proteins in *S. cerevisiae*. These constructs were compared with identical fusions containing yRFP or enhanced GFP (4). The outcome of this investigation is summarized in Table 1. Figure 2A shows pictures of cells expressing yRFP and RedStar fused to Spc42p. In cells that have been grown to saturation over night, the characteristic Spc42p-yRFP dot-like, spindle pole body staining was only detected in a few cells. The visible spindle pole bodies represented old spindle pole bodies that had been created several generations ago, while the younger spindle pole bodies had not yet developed fluorescence (17). In contrast, RedStar staining was detectable in all cells, although the intensity of the signal was different from cell to cell (Figure 2A). Occasionally, cells with two spindle pole bodies (two dots per cell; cells undergoing mitotic cell division), one of which appearing fainter than the other, could be seen. Figure 2B shows that protein levels of the Spc42p-yRFP and Spc42p-RedStar fusions were identical.

Analysis of the RedStar Mutant in Mammalian Cells

To test the RedStar mutant in mammalian cells, we fused it to the first 202 amino acids of the peripherally associated Golgi apparatus protein GRASP65 (2). We then compared the expression profiles and fluorescent properties of the identical constructs made with either the yRFP or RedStar mutant. Figure 2C shows images of HeLa cells transfected for 18 h with the yRFP and mutant RedStar proteins fused to GRASP65. Images were obtained under identical conditions to allow the comparison of the relative levels of fluorescence in the fixed cells. The RedStar mutant gave between 10 and 20 times brighter fluorescence than yRFP

under these conditions (Figure 2C) and also in unfixed live cells (data not shown). Western blot analysis of the transfected cells revealed that similar

levels of the two fusion proteins were expressed (Figure 2D), which indicated that the difference in fluorescence was not due to different expression levels.

Figure 2. Expression of yRFP and RedStar fusion proteins in yeast and mammalian cells. (A) Yeast strains expressing chromosomal *SPC42* fusions to either yRFP or RedStar were grown on synthetic medium containing raffinose as a carbon source. Red fluorescence pictures were acquired using a standard Cy3 filter set. (B) Identical amounts of proteins extracted from these cells were further analyzed by Western blot analysis using anti-Spc42p and anti-yRFP antibodies. Lanes and proteins are as indicated in the figure. Two independent clones were tested for each strain. (C) The localization of GRASP65-yRFP and GRASP65-RedStar fusions in HeLa cells after 18 h of transfection (see Materials and Methods). Photographs of representative cells are shown. (D) Levels of GRASP65-DsRed and GRASP65-yRFP protein 6, 12, and 18 h after transfection were analyzed by Western blot analysis using anti-yRFP antibodies.

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DISCUSSION

The primary goal of the study was to improve RFP for its use in eukaryotic cells, particularly in yeast. To be generally applicable in this organism, a fluorescent protein has to fulfill several criteria. First, the protein has to be very bright because of the small size of yeast cells. Second, because of the short cell-division cycle of the yeast *S. cerevisiae* (90–180 min, depending on the growth media and carbon source), the protein has to develop its fluorescence very rapidly so that new protein pools can be readily detected. While DsRed meets the first criterion in theory (1), the very long maturation time of the chromophore prevents it from being used as a general tool. Additional factors (tetramerization and aggregation/solubility) can further affect the usefulness of a fluorescent protein.

The DNA shuffling procedure used in our study to generate stepwise improvements of the properties of yRFP was successful in *E. coli*, and the screening in yeast improved the best *E. coli* version another three times. These improvements did not seem to be the result of an accelerated rate by which the chromophore was formed. Nevertheless, RedStar shows an initial acceleration for the formation of fluorescent protein species (Figure 1C). As a result, cells expressing RedStar are approximately 20-fold more fluorescent than cells expressing yRFP. Because many different factors inside a cell regulate the biosynthesis of functional and the degradation of nonfunctional proteins, one has to see the improvements of RedStar in context with the environment within a specific cell. Explanations such as “improved folding” alone are insufficient to describe the changes.

This has been demonstrated for the cycle3 mutant of GFP (5,8).

The only way to estimate the usefulness of a fluorescent protein for a specific biological application is to perform the experiment. To evaluate further the improvements of RedStar, we fused it to different well-known yeast marker proteins. We observed that RedStar is not yet good enough or as good as enhanced GFP to allow reliable detection of fusion proteins. However, for two of them, we were able to see fluorescence at the expected locations inside the cells, which was clearly not the case with yRFP (Table 1 and Figure 2A). The toxic effects and lack of detection of some of the RFP fusions could also result from the strong tendency of RFPs to oligomerize (1). This could be the reason why yRFP/RedStar, when fused to one of the two Histone H4 (*HHF2*) genes in yeast, leads to

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a dominant negative phenotype, while enhanced GFP does not (Table 1). The 10- to 20-fold improved brightness of RedStar versus yRFP was also confirmed in mammalian cells (Figure 2C).

In parallel to our study, other studies were performed that improved wild-type DsRed. One study demonstrated a 3-fold improvement (20) caused by a single mutation (S197Y), while the DsRed2 variant (BD Biosciences Clontech, Palo Alto, CA, USA) showed a 2-fold improved brightness, according to the manufacturer. Only recently, another group reported the identification of a DsRed mutation with accelerated chromophore maturation properties but reduced overall fluorescence compared to wild-type DsRed (3), and yet another group reported an aggregation-free variant of DsRed (25). Ongoing additional studies might address other drawbacks of the protein such as its strong tetramerization (21). Combining the mutations found in all these and future studies will probably be the best way to obtain finally an enhanced GFP-like (or even much better) RFP for yeast and other organisms.

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