ABSTRACT  
Reversibly switchable fluorescent proteins (RSFPs) are GFP-like proteins that may be repeatedly switched by irradiation with light from a fluorescent to a nonfluorescent state, and vice versa. They can be utilized as genetically encodable probes and bear large potential for a wide array of applications, in particular for new protein tracking schemes and subdiffraction resolution microscopy. However, the currently described monomeric RSFPs emit only blue-green or green fluorescence; the spectral window for their use is thus rather limited. Using a semirational engineering approach based on the crystal structure of the monomeric nonswitchable red fluorescent protein mCherry, we generated rsCherry and rsCherryRev. These two novel red fluorescent RSFPs exhibit fluorescence emission maxima at ~610 nm. They display antagonistic switching modes, i.e., in rsCherry irradiation with yellow light induces the off-to-on transition and blue light the on-to-off transition, whereas in rsCherryRev the effects of the switching wavelengths are reversed. We demonstrate time-lapse live-cell subdiffraction microscopy by imaging rsCherryRev targeted to the endoplasmic reticulum utilizing the switching and localization of single molecules.

INTRODUCTION

Fluorescent proteins (FPs) glowing in all hues, have become indispensable research tools in cellular biology (1–3). Recently, novel FPs have been described which may be reversibly photoswitched between a fluorescent and a nonfluorescent state (4,5). Such reversibly switchable fluorescent proteins (RSFPs) have already been used for innovative protein tracking strategies (6), for fluorescence correlation spectroscopy at high molecular concentrations (7), and within different schemes to facilitate subdiffraction imaging in far-field optical microscopy (8–13).

In RSFPs, photoinduced reversible switching from the nonfluorescent to the fluorescent state, and vice versa, is achieved by irradiation with light of different wavelengths, where the light of one wavelength in addition to switching also excites fluorescence. A positive and a negative switching mode may be distinguished: In a positive-switching RSFP, the wavelength used to excite fluorescence switches the protein to the on-state. In case of a RSFP with a negative-switching mode, the wavelength used to excite fluorescence concurrently induces the on-to-off transition. In each case, the respective other transition is induced by light of a shorter wavelength. In contrast to photoactivatable fluorescent proteins (14), the photoswitching of RSFPs is reversible and the fluorophores may pass the switching cycle many times until they are photo-destroyed or transferred to a permanent non-switchable state.

Recent studies into the molecular switching mechanism of RSFPs demonstrated that a cis-trans isomerization of the chromophore is a key event in the switching process (15–19). In the RSFPs studied so far, the cis-conformation represents the fluorescent on-state, whereas the chromophore in the nonfluorescent off-state adopts the trans-conformation. The conformational changes are accompanied by different protonation equilibria of the chromophore (20–22). The different protonation equilibria determine the absorption spectra of the chromophore in both states. The on-state chromophores were found to be well ordered and relatively planar, whereas the off-state chromophores were more twisted and partially disordered, a condition likely to favor nonradiative decay of the excited chromophore.

The available monomeric RSFPs emit blue-green or green fluorescence (5,19,23). They all display negative-switching modes. Of these green fluorescent RSFPs, Dronpa and its variants are the most prominent (5,18,24), and have been used for several applications. The only known red fluorescent RSFP is asFP595 and its variants (4,25), but since asFP595 is an obligate tetramer, its practical use is limited. The current lack of monomeric RSFPs emitting yellow or red light prevents the utilization of RSFPs in dual-color experiments, representing a severe hurdle for their widespread use.

A promising area of application for RSFPs of various colors is far-field subdiffraction resolution microscopy. Every far-field fluorescence microscopy concept that has successfully outperformed diffraction is based on the switching between at least two molecular states, one of which is dark while the other gives a signal (13). Initial subdiffraction concepts following this pathway utilized reversible saturable optical linear (fluorescence) transitions (RESOLFT) with a beam of light featuring a local intensity minimum (26–29). The more recent superresolution concepts based on the readout of stochastically switched-on isolated single molecules have been termed PALM (10), STORM (30), fPALM...
MATERIALS AND METHODS

Mutagenesis

For site-directed random mutagenesis, the QuikChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) or a multiple-site mutagenesis approach using several degenerative primers were used (32). Error-prone random mutagenesis was essentially performed as described (33). For expression in Escherichia coli, the coding sequences were cloned into a pQE30 vector (Qiagen, Hilden, Germany).

Protein production and purification

Proteins were expressed in the E. coli strain BL21-CP-RIL and purified by Ni-NTA affinity chromatography (Ni-NTA Spin Kit, Qiagen) according to the manufacturer’s instructions. The purified proteins were concentrated by ultrafiltration and taken up in 10 mM Tris-HCl, 150 mM NaCl, pH 7.5.

Expression in mammalian cells

To target rsCherryRev to the lumen of the endoplasmic reticulum (ER), the coding sequence was amplified by PCR using the primers

\[
\begin{align*}
\text{CATCGAGATCTCGAGTCCGGACTTGTACAGCTCG} \\
\text{TCCATGCC}
\end{align*}
\]

The PCR fragments were digested with NheI and BglII and inserted into the vector pEgfp-Tub (Clontech, Mountain View, CA), replacing the EGFP sequence.

Spectroscopic analysis and optical switching

The absorption and fluorescence spectra of purified proteins were recorded with a Varian Cary 4000 UV/VIS spectrophotometer and a modified Varian Cary Eclipse fluorescence spectrometer (Varian, Palo Alto, CA), respectively. The absorption spectra were recorded at thermal equilibrium.

All switching curves were determined on colonies of live E. coli cells expressing the respective proteins. The photoswitching experiments were performed using a modified computer-controlled fluorescence microscope (Leica, Wetzlar, Germany) equipped with a 40× NA 0.6 air objective lens. The microscope was equipped with two 100 W Hg lamps delivering yellow light (550 ± 20 nm excitation filter, −2 W/cm²) and blue light (450 ± 20 nm excitation filter, −4 W/cm²). For photoswitching of both rsCherry and rsCherryRev, irradiation was performed in alternate turns with yellow light for 12 s and yellow + blue light for 0.5 s. The fluorescence was detected through the same objective lens and recorded by a photomultiplier tube (HR9306-0, Hamamatsu, Hamamatsu City, Japan) using a 600 nm long-pass detection filter (HQ 600 LP, AHF Analysentechnik, Tübingen, Germany).

The relaxation half-time \( t_{\text{relax}}^{1/2} \) from the ensemble on-state into the thermal equilibrium state was measured on colonies of E. coli cells expressing rsCherry or rsCherryRev, respectively. After switching into the on-state (to the maximal attainable fluorescence signal) with yellow light (550 ± 20 nm excitation filter, −6 W/cm²) for 2 s (rsCherry) or with blue light (450 ± 20 nm excitation filter, −4 W/cm²) for 2 s (rsCherryRev), the relaxation into the equilibrium state was followed at room temperature (22°C) in the dark by consecutive short measurements (0.02 s) with yellow light (−0.5 W/cm²) every 20 s or 2 s for rsCherry or rsCherryRev, respectively. For the determination of \( t_{\text{relax}}^{1/2} \) of rsCherryRev (Fig. 2 E), the E. coli colony utilized for the measurement was initially optically switched >10 times (see also Supplementary Material Fig. S3 in Data S1).

Dynamic light scattering analysis

Dynamic light scattering analysis was carried out using a DynaPro-MS/X (Wyatt Technology, Santa Barbara, CA) molecular-sizing instrument at 25°C. For the measurement, a 50 μL sample was passed through a filtering assembly containing a 0.2 μm filter into a 12 μL chamber quartz cuvette. The data were analyzed using the Dynamics 6.0 software. mCherry was taken as monomer as in Shaner et al. (3).

Size separation chromatography

The proteins (1 mg/mL⁻¹, in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) were analyzed on a Superdex 200 column with a Smart system (both Pharmacia, Uppsala, Sweden). Flow was set to 40 μL/min⁻¹ and the absorption at 280 nm was monitored continuously. Chromatography was performed at room temperature.
Brightness determination

We determined two different types of brightness values, based either on an ensemble of molecules or on single proteins. Ensemble brightness values were determined using the optical switching setup described above. We prepared solutions of purified proteins in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5 of equal concentrations (as determined by the absorption at 280 nm) and measured their fluorescence signal in the maximal attainable on-state (550 ± 20 nm excitation filter, −2 W/cm²) relative to mCherry.

The brightness of a single protein was determined on solutions of purified proteins using fluorescence correlation spectroscopy (see also Fig. S2 in Data S1) (34). Fluorescence correlation data were recorded on a confocal microscope setup equipped with a 561 nm laser for fluorescence excitation (Cobalt, Solna, Sweden) and analyzed as outlined previously (7). The single molecule brightness values were defined as the average fluorescence count rate per light-emitting single molecule diffusing through the confocal detection volume.

Wide-field imaging

For imaging of *E. coli* cells, rsCherry and rsCherryRev were expressed in BL21-CP-RIL. *E. coli* cells expressing either of these proteins were mixed and imaged with a Leica DM6000 epifluorescence microscope (Leica, Wetzlar, Germany). The microscope was equipped with a 100 × NA 1.40 oil immersion objective lens and a DPCI550 FX camera (Leica). The 100 W Hg lamp delivered yellow light (546 ± 6 nm excitation filter, −4 W/cm²) or blue light (470 ± 20 nm excitation filter, −5 W/cm²). Fluorescence was recorded using a 600 ± 20 nm bandpass detection filter. The *E. coli* cells were imaged with the following irradiation scheme:

1. Switching for 1 s with yellow light (rsCherry: On; rsCherryRev: Off).
2. Imaging with yellow light excitation (60 ms).
3. Switching for 1 s with blue light (rsCherry: Off; rsCherryRev: On).
4. Imaging with yellow light excitation (60 ms).

For the imaging of rsCherryRev expressed in PtK2 cells, the same microscope as described above equipped with a BGR-Filter Cube (Leica), delivering yellow light (570 ± 10 nm excitation filter, −15 W/cm²) or blue light (495 ± 7.5 nm excitation filter, −6 W/cm²) was employed. A 640 ± 20 nm bandpass filter was used for detection. The following irradiation scheme was employed:

1. Switching for 1.1 s with blue light (rsCherryRev: On).
2. Imaging with yellow light excitation (160 ms).
3. Switching for 1.1 s with yellow light (rsCherryRev: Off).
4. Imaging with yellow light excitation (160 ms).

PALMIRA microscopy

The arrangement of the wide-field imaging setup utilized for subdiffraction PALMIRA microscopy was essentially as described earlier (12). In brief, the beam of a continuous-wave, diode-pumped solid-state laser running at 561 nm (Cobolt Jive 50; Cobolt, Stockholm, Sweden) was intensity-controlled by an acousto-optical tunable filter (AA-AOTF-6C, Pegasus Optik, Wallenhorst, Germany) and expanded by a telescope. The beam polarization was converted from linear to circular by a quarter wave-plate and subsequently coupled into a commercial wide-field microscope (DM IRB, Leica Microsystems, Mannheim, Germany). We achieved uniform epillumination of an area of ~10-μm diameter by underilluminating the back aperture of the objective lens (HCX PL APO 100×/1.4 oil, Leica Microsystems). The fluorescence light was collected by the same lens, separated from the excitation light using a dichroic filter (zUV/561rpc, AHF Analysentechnik, Tübingen, Germany) and imaged onto an electron multiplying charge-coupled device camera (IXON-Plus DU-860, Andor Technology, Belfast, Northern Ireland).

The detected wavelength range was limited to the emission spectrum of rsCherryRev by a bandpass filter (620 ± 30 nm, Chroma Technology, Rockingham, VT). Before data acquisition, the majority of the proteins were switched to a dark state by illuminating the sample for ~1 min with 20 kW/cm² of the 561-nm laser light.

For each PALMIRA image, 20,000 frames were continuously recorded at a rate of 500 frames/s using 561-nm laser-light illumination at 20 kW/cm². The background in each frame was estimated by applying a low-pass filter to the data. The planar positions of the individual rsCherryRev proteins in the background-corrected frames were determined by an implementation of Hogbom’s classical CLEAN algorithm (35) in conjunction with a mask-fitting algorithm of the Airy spot as detailed previously (12).

The final PALMIRA images were obtained by depicting each fluorescent protein exhibiting >20 detected photons in a new frame as a Gaussian with a standard deviation corresponding to the respective localization accuracy (10). The epifluorescent (diffraction-limited) counterpart images to the superresolved PALMIRA images were determined by summing up all the signals of the background-corrected frames and interpolated to a pixel size of 40 × 40 nm. For display, we accounted for the comparatively high dynamic range in the images by selecting a color table which emphasizes dim features.

RESULTS

Generation of the red fluorescent reversibly photoswitchable protein rsCherry

To generate photoswitchable proteins emitting red fluorescence, we decided to use the fluorescent protein mCherry as a starting point. mCherry is a monomeric red fluorescent protein (Em.: 586 nm/ Exc.: 611 nm) with very good photostability that has been successfully employed in a number of live cell studies (3).

Like the chromophores of most fluorescent proteins, the chromophore of mCherry adopts a cis-conformation (PDB: 2H5Q) (36). Likewise, the on-state chromophores of all RSFPs analyzed so far are in the cis-position, whereas their respective off-state chromophores adopt a trans-position (16,17,19). Since the photoinduced cis-trans isomerization of the chromophore is a structural key event in the switching of RSFPs, we reasoned that to render mCherry switchable, it would be required to make the trans-position accessible to the chromophore. Modeling of a trans chromophore into the mCherry structure without adjusting the rest of the structure immediately revealed that Ile161 (all amino-acid numbering according to the DsRed sequence) occupies the space required by the chromophore (Fig. 1), thereby explaining the fact that the chromophore of mCherry is exclusively found in the fluorescent cis-conformation. We reasoned that exchange of Ile161 by smaller amino-acid residues might make the trans position accessible to the chromophore and thus may facilitate photoinduced reversible switching.

We performed saturated site-directed mutagenesis at position 161. This resulted in mCherry-Ile161Ser. In *E. coli* colonies expressing this variant, fluorescence could be switched-off to ~70% of the initial brightness by irradiation with light of 450 nm. Irradiation with 550 nm brought the fluorescence back to the starting value. To improve the re-

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rsCherry and rsCherryRev conformations. The inset table lists all amino-acid residue exchanges in mCherry (Table S1 and Fig. S1 in Data S1). In the fluorescent chromatography verified that rsCherry is a monomer in solution (Table S1 and Fig. S2 in Data S1), which highlights rsCherry’s potential for single-molecule-based fluorescence experiments. An ensemble of mCherry molecules expressed in E. coli adopt a thermal equilibrium at which they exhibit 32% of the maximum fluorescence level. Once pushed to its maximum fluorescence brightness, the ensemble relaxes back into thermal equilibrium with a relaxation time of $t_{\text{on-relax}} \sim 40$ s (Fig. 2 D). Under the employed light intensities, the protein shows relatively fast switching kinetics with a $t_{1/2}^{\text{on}} \sim 3$ s and a $t_{1/2}^{\text{off}} \sim 0.05$ s. Altogether, rsCherry is a genuine monomeric red fluorescent RSFP with a positive-switching mode.

Reversing the wavelengths required for switching of rsCherry

During the optimization of rsCherry, we found that exchange of a glutamine residue at position 163 against a methionine reversed the effects of the wavelengths that induce the photoswitching. In rsCherry-Gln$^{163}$Met, light of 450 nm builds up the on-state while irradiation with 550 nm, which is also used to probe fluorescence, largely depopulates the fluorescent states. Therefore, rsCherry-Gln$^{163}$Met exhibits a negative-switching mode.

Further rounds of optimization resulted in mCherry-Glu$^{144}$Val, Ser$^{146}$Cys, Ile$^{161}$Ser, Gln$^{163}$Met, Val$^{177}$Phe that maintained a negative switching mode but is improved considerably in the dynamic range of the fluorescence signal between the on- and the off-state (Fig. 2 B). We denote this variant rsCherryRev (for reversibly switching Cherry with reversed switching characteristics).

Like mCherry, rsCherryRev is a monomeric protein (Table S1 and Fig. S1 in Data S1). The absorption and fluorescence emission spectra of rsCherryRev and rsCherry in their respective equilibrium states are almost superimposable (Fig. 2 C, Table 1). Like rsCherry, in the ensemble of purified proteins, the switched-on rsCherryRev is rather dim, with an on-state ensemble fluorescence signal of $\sim 10\%$ compared to mCherry. Nonetheless, the brightness value of a single rsCherry molecule in the on-state is comparable to that of a single mCherry molecule (Table S1 and Fig. S2 in Data S1), which highlights rsCherry’s potential for single-molecule-based fluorescence experiments. An ensemble of mCherry molecules expressed in E. coli adopt a thermal equilibrium at which they exhibit 32% of the maximum fluorescence level. Once pushed to its maximum fluorescence brightness, the ensemble relaxes back into thermal equilibrium with a relaxation time of $t_{\text{on-relax}} \sim 40$ s (Fig. 2 D). Under the employed light intensities, the protein shows relatively fast switching kinetics with a $t_{1/2}^{\text{on}} \sim 3$ s and a $t_{1/2}^{\text{off}} \sim 0.05$ s. Altogether, rsCherry is a genuine monomeric red fluorescent RSFP with a positive-switching mode.

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by fluorescence correlation spectroscopy. At thermal equilibrium the fluorescence signal of rsCherryRev is <10% of the maximal attainable signal, hence the majority of the molecules adopt the off-state (Fig. 2 E). rsCherryRev displays faster switching kinetics than rsCherry, which is also reflected in its less stable on-state: It relaxes with a relaxation time \( t_{\text{on-relax}}^{1/2} \approx 13 \) s from the on-state into the thermal equilibrium (Fig. 2 E). Interestingly, the relaxation kinetics change when the proteins are repeatedly switched, indicating a complex photophysical behavior involving additional states (Fig. S3 in Data S1). Switched-off rsCherryRev molecules exhibit only 5% of the maximal fluorescence signal, so that rsCherryRev displays a favorable dynamic range of fluorescence switching.

The fluorescence of single E. coli colonies on agar plates expressing rsCherryRev may be photoswitched hundreds of times (Fig. 2 B and Fig. 3). We found that for the first \( \sim 100 \) switching cycles the recorded maximum fluorescence level increased, while the residual fluorescence in the off-states remained at an almost constant value. For a further \( \sim 100 \) switching cycles, the increase in fluorescence apparently counteracted the photobleaching, before the fluorescence signal decreased (Fig. 3 A). The increase of the fluorescence signal during consecutive switching cycles appears to be an irreversible process, since 4 h of darkness during a switching series did not alter the maximal fluorescence (Fig. 3 B). This points to a light-induced structural rearrangement of rsCherryRev upon photoswitching. We observed an increase in the fluorescence with successive switching cycles only in rsCherryRev variants containing a cysteine residue at position 146, showing that the mutation Ser\(^{146}\)Cys is crucial to this phenomenon. The molecular mechanism is, however, unclear.

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Absorpt. max. (nm)</th>
<th>Emission max. (nm)</th>
<th>Ensemble brightness*</th>
<th>Single-molecule brightness†</th>
<th>Switch-on half-time ( r_{1/2}^{\text{on}} ) (s)‡</th>
<th>Switch-off half-time ( r_{1/2}^{\text{off}} ) (s)§</th>
<th>On-state relaxation half-time ( t_{\text{relax}}^{1/2} ) (s)</th>
<th>Fluor. at equil.</th>
<th>Residual fluor. in the off-state</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry</td>
<td>586</td>
<td>611</td>
<td>1</td>
<td>1</td>
<td>n.a.§</td>
<td>n.a.§</td>
<td>n.a.§</td>
<td>100%</td>
<td>n.a.§</td>
</tr>
<tr>
<td>rsCherry</td>
<td>572</td>
<td>610</td>
<td>-0.1</td>
<td>-1</td>
<td>3.0</td>
<td>0.05</td>
<td>40</td>
<td>32%</td>
<td>15%</td>
</tr>
<tr>
<td>rsCherryRev</td>
<td>572</td>
<td>608</td>
<td>-0.1</td>
<td>-1</td>
<td>0.05</td>
<td>0.7</td>
<td>13</td>
<td>8%</td>
<td>5%</td>
</tr>
</tbody>
</table>

*Purified proteins were concentrated to the same 280-nm absorption and fluorescence was measured relative to mCherry.
†Determined by fluorescence correlation spectroscopy; the same protein solutions as in the first footnote were used, diluted for the measurements.
‡Measured with yellow light 350 ± 20 nm (4 W/cm\(^2\)) and blue light 450 ± 20 nm (4 W/cm\(^2\)).
§Not applicable; mCherry is a nonswitchable protein.
Hence, rsCherryRev is a bona fide switchable red fluorescent protein with a substantial depth of fluorescence modulation, exhibiting negative-switching for up to several hundred switching cycles in ensemble measurements.

Alternate highlighting of rsCherry and rsCherryRev in E. coli

Next, we imaged a mixed culture of E. coli cells, expressing either rsCherry or rsCherryRev (Fig. 4). To discriminate between cells expressing the different RSFPs, a sequence of irradiation events bringing them in and out of the on-state was employed. To this end, the cells were first irradiated with light of 546 nm to switch rsCherry on and simultaneously rsCherryRev off. Second, the cells were imaged with 546 nm irradiation and the fluorescence was detected between 580 and 620 nm. In the next step, the sample was irradiated with 470 nm light to transfer rsCherry into the off-state and rsCherryRev into the on-state. In the last step, the fluorescence signal was recorded as before. Since switching of both proteins is reversible, the imaging sequence could be repeated several times (Fig. 4). Because an ensemble of rsCherry molecules exhibits a residual fluorescence signal after switching off that corresponds to ~15% of its maximum signal (Table 1), the E. coli cell expressing rsCherry is clearly visible even when the proteins are switched off. Hence, rsCherryRev, due to its more favorable dynamic range between the on- and the off-state, appears to be more suitable for most imaging applications that rely on photoswitching with good contrast.

Application of rsCherryRev in mammalian cells as a cellular marker

Therefore, we next tested rsCherryRev for its performance when expressed in mammalian cells. To this end we targeted rsCherryRev to the endoplasmic reticulum (ER) by fusing it to an N-terminal ER targeting signal and a C-terminal retention sequence. Cells expressing this construct displayed bright fluorescence and were not affected in their viability (Fig. 5 A). The fluorescence was reversibly switchable by irradiating the live cells with blue (495 ± 7.5 nm) and yellow (570 ± 10 nm) light. We also fused rsCherryRev to α-Tubulin resulting in a fluorescently-labeled microtubule network underscoring the fact that rsCherryRev is functionally monomeric (Fig. 5 A) and can be readily utilized for imaging applications.

Live-cell PALMIRA

To demonstrate the utility of rsCherryRev for subdiffraction resolution imaging in living mammalian cells, we imaged the rsCherryRev-labeled ER using the PALMIRA mode (12). The intricate ER is a dynamic polygonal array of interconnected tubules that spreads throughout the cell (37,38). The diameter of individual tubules is frequently well below the diffraction barrier and hence many of the features of the reticulum are difficult or even impossible to resolve using conventional far-field microscopy.

To visualize the ER within a living cell we captured 60,000 frames lasting 2 ms each without pause. We divided them into three groups of 20,000 frames to visualize the movement of the ER over the course of the image acquisition period. The resulting effective acquisition time of 40 s per image allowed us to follow movements of the network at the nanoscale (Fig. 5 B). Movements taking place within the recording time are averaged out in the resulting image. The chosen frame rate was long enough to capture (in >90% of all events) all detectable photons emitted from a molecule in its temporary fluorescent on-state in one frame. Utilizing all events exhibiting at least 20 photons, the achieved resolution was <75 nm in the optical plane as demonstrated on small structures of the

FIGURE 3 rsCherryRev exhibits an increase in the maximal fluorescence signal upon repeated photoswitching. (A and B) Fluorescence signals of 300 switching cycles recorded on E. coli colonies expressing rsCherryRev are shown. Photoswitching was accomplished by alternating irradiation with yellow light (550 ± 20 nm, 12 s) and yellow together with blue light (450 ± 20 nm, 0.5 s). In panel B, the switching sequence was interrupted for 4 h after cycle 150.

FIGURE 4 Imaging of E. coli cells expressing rsCherry or rsCherryRev. The cells were imaged according to the following sequence: 1), Irradiation with yellow light (546 ± 6 nm, 1 s) to switch rsCherry (rsCh) in the on-state and rsCherryRev (rsChRev) in the off-state. 2), Imaging with yellow light (60 ms). 3), Irradiation with blue light (470 ± 20 nm, 1 s) to switch rsCherry in the off-state and rsCherryRev in the on-state. 4), Imaging with yellow light. Shown are two cycles. Scale bar: 3 μm.
ER (Fig. 5 C). At this resolution, structures of the ER can be delineated at the nanoscale which are not resolvable by conventional microscopy (Fig. 5 D). These results clearly show that rsCherryRev, visualized in the PALMIRA mode, is appropriate to reveal the organization of nanosized organelles within living mammalian cells.

DISCUSSION

In this study, we generated two novel monomeric red fluorescent photoswitchable proteins based on the nonswitchable fluorescent protein mCherry. The new RSFPs rsCherry and rsCherryRev exhibit positive and negative switching modes, respectively. rsCherryRev is suitable for protein labeling in mammalian cells. Targeted to the endoplasmic reticulum, rsCherryRev could be utilized for time-resolved subdiffraction resolution microscopy in living mammalian cells.

The generation of these two novel RSFPs was based on the assumption that a fluorescent protein can be made switchable, provided the chromophore is allowed to undergo a cis–trans isomerization. In mCherry, the chromophore adopts a cis-conformation and it apparently cannot access the trans-conformation because the required space is occupied by a bulky isoleucine residue. We found that upon exchange of this residue by a smaller serine residue (Ile161Ser), the protein gained switching capabilities. This strongly indicates that in rsCherry, which is an improved mCherry-Ile161Ser variant, the on-state chromophore adopts a cis-conformation, and the off-state chromophore a trans-conformation.

rsCherry exhibits positive-switching, so that irradiation with yellow light, which also elicits fluorescence, transfers the protein from the off- to the on-state. Blue light induces the reverse switching direction. Introduction of Gln163Met reversed the effects of the switching wavelengths, rendering Gln163Met the key mutation in rsCherryRev. The mCherry structure suggests that in rsCherryRev the amino-acid residue at position 163 is located between the chromophoric p-hydroxyphenyl ring in the cis- and the hypothetical trans-position. Hence, this residue is in a prime position to influence the chromophore and its immediate environment. Two
explanations as to how the residue exchange Gln$^{163}$Met may result in a reversion of the wavelengths may be envisioned. First, it may influence the chromophore cavities in a way that irradiation with 450 nm now induces the transition from the trans- to the cis-conformation and that 550 nm light induces the reverse effect. Alternatively, Gln$^{163}$Met may render the cis-position nonfluorescent and the trans-position fluorescent. In the latter case, the direction of the light-induced conformation changes would remain the same as in rsCherry. We found that reintroduction of an isoleucine residue at position 161 into a switchable rsCherryRev variant (mCherry-Glu$^{144}$Val, Ile$^{161}$Ser, Gln$^{163}$Met, Val$^{177}$Phe) resulted in a nonswitchable, but fluorescent protein (Fig. S4 in Data S1). An isoleucine at position 161 most likely prevents the chromophore from adopting a trans conformation. This observation supports the view that in rsCherryRev the on-state chromophore adopts a cis-conformation as well. The absorption spectra of rsCherry and rsCherryRev at pH 7.0 displayed a major peak at 572 nm, corresponding to the deprotonated (anionic) state of the chromophore. At pH 5.0, both proteins show an additional absorption peak at 464 nm (Fig. S5 in Data S1), corresponding to the neutral state (1,39). Altogether, these findings strongly support the view that the switching mechanism of rsCherry and rsCherryRev follows similar lines as described for other RSFPs (16,17,21). The irradiation with blue light apparently induces the conversion from a little populated neutral cis-(rsCherry) or trans-(rsCherryRev) state, respectively, into the corresponding other state. Irradiation with yellow light induces the isomerization from the predominantly deprotonated trans-(rsCherry) or cis-(rsCherryRev) state in the other direction.

Both rsCherry and rsCherryRev display rather low ensemble but excellent single-molecule brightness values compared to mCherry. We found that chromophore maturation appears to be only slightly diminished in rsCherry and rsCherryRev, as compared to mCherry (to ~40% and ~70%, respectively; see Fig. S6 in Data S1). Therefore, at least under the wide-field illumination that is used to determine the ensemble brightnesses, an incomplete activation, potentially due to competing transitions into other nonfluorescent states, is likely to explain a substantial part of the reduced ensemble brightness values of rsCherry and rsCherryRev. Possibly, the activation is more efficient using high light intensities, which would explain the unexpected bright images (Fig. 5 A) when using more intense irradiation.

In this study, we demonstrated the utilization of rsCherryRev for time-lapse live-cell subdiffraction microscopy based on the switching and localization of single-molecule emitters. rsCherryRev, like its relative rsCherry, has been generated by mutagenesis on the basis of a previously well-characterized nonswitchable fluorescent protein. It can easily be envisioned that further RSFPs with diverse photophysical properties may be generated along the mutagenesis strategies outlined here. Hence, the addition of the now available red RSFPs may initiate a collection of reversibly switchable fluorescent proteins glowing in all hues that will foster new developments in live-cell multicolor subdiffraction resolution microscopy, based on RESOLFT (13,29) or single-molecule superresolution concepts and related schemes (10,12,30,31), to address a multitude of problems in cell biology.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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