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Generation and Characterization of a Rat Monoclonal Antibody Specific for Multiple Red Fluorescent Proteins

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Abstract

Fluorescent proteins (FP) are widely used as *in vivo* reporter molecules and are available in multiple colors spanning almost the entire visible light spectrum. Genetically fused to any protein target, FPs offer a powerful tool to study protein localization and dynamics. After the isolation of the prototypical green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, a red fluorescent protein (DsRed) was discovered in the coral *Discosoma* sp. that provided a better spectral separation from cellular autofluorescence and allowed multicolor tracking of fusion proteins. However, the obligate tetramerization of DsRed caused serious problems for its use in live-cell imaging. Subsequent mutageneses of the red progenitor have resulted in several monomeric red FPs (mRFP1, mCherry, mOrange, mPlum, etc). These improved red FPs are characterized by higher brightness and photostability, complete chromophore maturation, and promise a wide variety of features for biological imaging and multicolor labeling. Here we report the generation and characterization of the first rat monoclonal antibody (MAb) against multiple red FPs, designated as multi-red 5F8. We demonstrate that multi-red 5F8 is a MAb with high affinity and specificity against the DsRed derivatives and corresponding fusion proteins, and that it is suitable for ELISA, immunoblotting, immunoprecipitation, and immunofluorescence assays. Applying our versatile antibody, one and the same red fluorescent protein tag can be used to perform not only microscopic studies, but also multiple biochemical assays.

Introduction

THE UNDERSTANDING OF COMPLEX biological systems is highly dependent on the ability to visualize participating factors in their cellular context. Fluorescent proteins (FP) are transcriptional and translational reporters commonly used in real-time imaging approaches where they help monitor the cellular distribution, interactions, and dynamics of targeted proteins *in vivo*. In the past few years the diversity of FPs has dramatically expanded from the prototypical green fluorescent protein (GFP)[(1)] isolated from the jellyfish *Aequorea victoria*, to its engineered mutants (enhanced eGFP) and additional spectral variants (cyan CFP, yellow YFP, etc.) thereof.[(2)]

The discovery of a red fluorescent protein (DsRed) in the coral *Discosoma* sp.[(3)] provided a new red-shifted reporter tool with better spectral separation from the cellular autofluorescence and allowed the simultaneous visualization of multiple fusion proteins in one cell. However, live-cell imaging and analysis of protein dynamics were limited due to the obligate tetrameric properties of the DsRed protein, leading to crosslinking, aggregation, and precipitation of the fusion protein. To circumvent the oligomerization of the fusion proteins, the tetrameric progenitor DsRed was re-engineered by targeted as well as random mutagenesis. The improved monomeric first-generation (mRFP1) and second-generation (mCherry, mPlum, mOrange, etc.) fluorescent proteins exhibit increased brightness, photostability, solubility, and complete chromophore maturation properties.[(2,4,5)] These re-engineered red FPs are therefore extremely useful in single- or multicolor fluorescence assays like fluorescence recovery after photobleaching (FRAP) or fluorescence resonance energy transfer (FRET).[(6,7)]

On the one hand, the development of new red fluorescent proteins has increased drastically and is still ongoing, and on the other hand, there is a lack of corresponding antibodies detecting these FPs in biochemical approaches. Currently there is a limited number of polyclonal and monoclonal an-

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tibodies raised against DsRed available. To extend the application range of FPs, especially to enable the detection of red fluorescent fusion proteins in biochemical approaches, we generated the first rat monoclonal antibody detecting multiple red FPs. We show here the suitability of this high-affinity and highly specific antibody for ELISA, immunoblotting, immunoprecipitation, and immunofluorescence assays. This first anti-multi-red FP antibody allows the simultaneous analysis of one and the same red fluorescent fusion protein via bioimaging as well as biochemical approaches.

Materials and Methods

Cell lines

Mouse C2C12 myoblasts and human HEK 293T embryonic kidney cells were cultured in DMEM containing 50 μg/mL gentamicin supplemented with 20% and 10% FCS, respectively.

Expression constructs and purification of red fluorescent proteins (antigen preparation)

The full-length red FPs mRFP1, mCherry and mOrange, cloned into pRSETB bacterial expression vectors, adding an N-terminal histidine (His_6) tag were kindly provided by R.Y. Tsien (Dept. of Chemistry and Biochemistry, UCSD, La Jolla, CA).[4,8] The cDNA of mPlum was cut out of the pBAD bacterial expression vector (also provided by R.Y. Tsien[5]) and cloned into pRSETB similar to the other FPs. His-tagged red FPs were expressed in *E. coli* BL21(DE3) cells (Novagen, Darmstadt, Germany) and purified with the TALON Superflow Metal Affinity Resin system (Clontech, Saint Germain, France) under native conditions. The cell pellets of each 200 mL bacterial culture were resuspended in 20 mL TALON extraction buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole [pH 8.0]) and homogenized for 10 min. The lysate was cleared by centrifugation (12,000 *g*, 30 min, 4°C), and the supernatant was mixed with 2 mL of TALON Superflow Metal Affinity resin, pre-equilibrated in TALON extraction buffer. After incubation at 4°C for 2 h, the beads were washed with 50 mL TALON extraction buffer, and the red proteins were eluted by adding 150 mM imidazole to the TALON extraction buffer. Elution fractions were dialyzed against 1 × PBS and the purity of the eluted fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining. The protein concentrations were determined by absorption at 280 nm.

The mammalian expression constructs eGFP-PCNA and mRFP1-PCNA were described earlier.[9,10] The mCherry-PCNA, mPlum-PCNA, and mOrange-PCNA expression constructs were derived from the mRFP1-PCNA construct. The mRFP1 was removed by restriction enzyme digestion and was exchanged with the corresponding red FP variant. Inserts were verified by DNA sequencing.

Immunization, generation of hybridomas, and ELISA screening

The His-tagged red FPs were pooled and approximately 100 μg were injected both intraperitoneally (i.p.) and subcutaneously (s.c.) into Lou/C rats using CpG2006 (TIB MOLBIOL, Berlin, Germany) as adjuvant. After 8 weeks, a boost was given i.p. and s.c. 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed using polyethylene glycol 1500 (PEG 1500, Roche, Mannheim, Germany). After fusion, the cells were plated in 96-well plates using RPMI 1640 with 20% fetal calf serum, penicillin/streptomycin, glutamine, pyruvate, and non-essential amino acids (PAA, Cölbe, Germany) supplemented by aminopterin (Sigma, St. Louis, MO). Hybridoma supernatants were tested in a solid-phase immunoassay. Microtiter plates were coated overnight with His-tagged multi-red proteins at a concentration of 3–5 μg/mL in 0.1 M sodium carbonate buffer (pH 9.6). After blocking with non-fat milk (Frema, Neuform, Zarrentin, Germany), hybridoma supernatants were added. Bound rat MAbs were detected with a cocktail of biotinylated mouse MAbs against the rat IgG heavy chains, thus avoiding IgM MAbs (α-IgG1, α-IgG2a, α-IgG2b [ATCC, Manassas, VA], α-IgG2c [Ascenion, Munich, Germany]). The biotinylated MAbs were visualized with peroxidase-labeled avidin (Alexis, San Diego, CA) and o-phenylenediamine as chromogen in the peroxidase reaction. Multi-red 5F8 (rat IgG2a) was stably subcloned and further characterized.

Immunoblot analysis

For immunoblot analysis, serial dilutions (1.25–20 ng) of purified FPs (mRFP1, mCherry, mOrange, mPlum, and eGFP) were homogenized in SDS-containing loading buffer, separated on a 12% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). In addition, $\sim 1 \times 10^7$ HEK 293T cells, either mock treated or transiently transfected with expression vectors coding for fluorescent fusion proteins mRFP1-PCNA, mCherry-PCNA, mOrange-PCNA, mPlum-PCNA, and eGFP-PCNA, were harvested in ice cold PBS, washed twice, and incubated in 60 μL buffer (PBS, DNaseI [1.2 mg/mL], 2 mM PMSF, 5 mM MgCl) for 15 min on ice. Finally, the cells were homogenized in SDS-containing loading buffer. $\sim 5 \times 10^4$ cells/lane were separated on a 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Bio-Rad Laboratories). After protein staining with Ponceau S solution (Sigma), the membrane was blocked for 10 min with 3% milk in PBS containing 0.075% Tween-20. 0.2 μg of the multi-red antibody 5F8 was added and incubation continued for 1 h at room temperature. In addition a mouse monoclonal anti-GFP antibody (Roche) and a rat monoclonal anti-PCNA antibody (clone 16D10[11]) were used as primary antibodies. After washing with PBS containing 0.1% Tween-20, the blots were incubated with a secondary anti-rat IgG-HRP or anti-mouse Ig-HRP antibody (Jackson ImmunoResearch Europe). Immunoreactive bands were visualized with ECL Western Blot Detection reagents (GE Healthcare, Freiburg, Germany).

Immunoprecipitation

For immunoprecipitation, $\sim 1 \times 10^7$ HEK 293T cells, either mock treated or transiently transfected with expression vectors coding for fluorescent fusion proteins mRFP1-PCNA, mCherry-PCNA, mOrange-PCNA, mPlum-PCNA, and eGFP-PCNA, were harvested in ice cold PBS, washed twice, and subsequently homogenized in 200 μL lysis buffer (20 mM Tris/HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 0.5% NP40). After a centrifugation step (10 min, 14,000

g, 4°C), the supernatant was adjusted with dilution buffer (20 mM Tris/HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF) to 500 μL. 50 μL were mixed with SDS-containing sample buffer (referred to as input [I]). For immunoprecipitation, 4 μg of the multi-red antibody 5F8 were added and incubated 1 h at 4°C. For pull down of immunocomplexes, 30 μL of dilution buffer equilibrated protein G agarose beads (GE Healthcare) were added and incubation continued for 1 h. After centrifugation (2 min, 5000 *g*, 4°C) 50 μL of the supernatant was collected (referred to as nonbound/or flowthrough [F]) while the remaining supernatant was removed. The beads were washed twice with 1 mL dilution buffer containing 300 mM NaCl. After the last washing step, the beads were resuspended in 100 μL SDS-containing sample buffer and boiled for 10 min at 95°C. For immunoblot analysis, 1% of the input and the flowthrough as well as 10% of the bound (B) fraction were separated on a 10% SDS–PAGE, electrophoretically transferred to a nitrocellulose membrane, and subjected to immunoblot analysis as described above.

Immunofluorescence

For immunostainings, C2C12 cells were grown on coverslips and transfected transiently with mRFP-PCNA, mCherry-PCNA, mOrange-PCNA, and mPlum-PCNA using

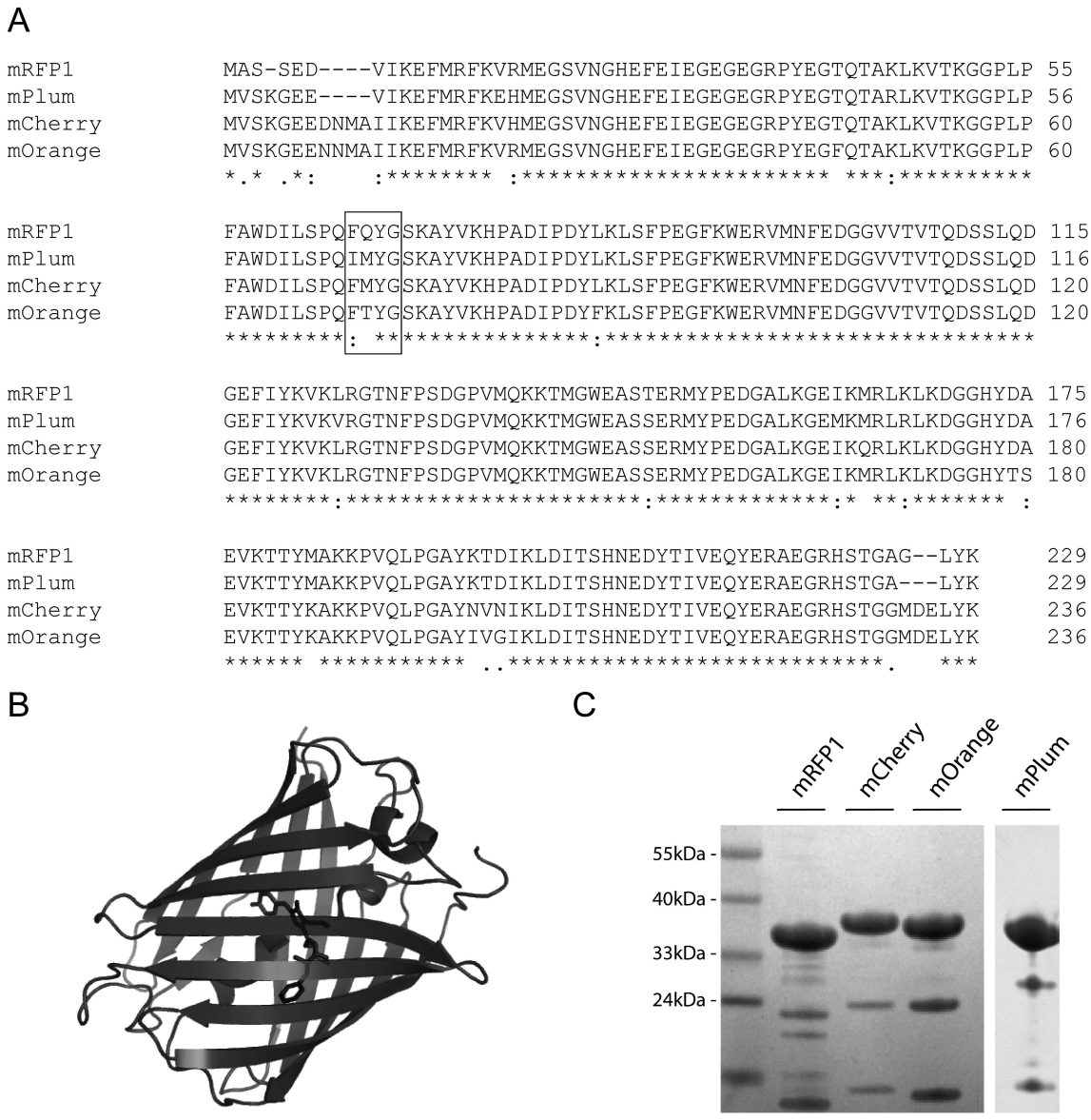

FIG. 1. Multiple red fluorescent proteins (FPs). (**A**) Sequence alignment of the red FPs mRFP1, mPlum, mCherry, and mOrange. Identical residues are indicated by asterisks; highly conserved residues are marked with two vertical dots and lesser-conserved residues with a single dot below the sequence. The chromophore core is boxed. (**B**) Crystal structure of the mCherry monomer (PDB 2H5Q).[12] The beta-barrel shaped structure (gray) encircles the central alpha-helix containing the embedded chromophore core (dark gray, sticks). The image was generated using PyMOL.[13] (**C**) Purified FPs mRFP1, mCherry, mOrange, and mPlum were subjected to a 15% SDS-PAGE and stained with Coomassie. The molecular weight markers are indicated on the left.

polyethylenimine (PEI Sigma, St. Louis, MO). Cells were fixed with 3.7% formaldehyde in PBS. As primary antibody, multi-red 5F8 (diluted 1:20 [6–10 μg/mL]) in PBS containing 2% BSA) was used. The primary antibody was detected with a secondary anti-rat IgG antibody (diluted 1:400 in PBS containing 2% BSA) conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR). Cells were counterstained with DAPI and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Images of the cells were obtained using a widefield epifluorescence microscope (Axiophot 2, Zeiss, Oberkochen, Germany) using a 63x/1.4 NA Plan-Apochromat oil immersion objective.

Results and Discussion

Generation of a rat MAb against multiple red fluorescent proteins

In vivo imaging has benefited greatly from the possibility of visualizing and tracking proteins by creating genetically encoded fusions with a red or green fluorescent protein tag (fusion tagging).

mOrange, mRFP1, mCherry, and mPlum are the most frequently used red fluorescent reporters, exhibiting an excitation and emission maxima ranging from 548 to 590 nm and 562-649 nm, respectively.[4] The widespread chromophore spectra of the red FPs result from simple rearrangements of the chromophore cavity or autonomous multi-step post-translational modifications that include one or more covalent modifications to the polypeptide backbone.[12] The amino acid sequences as well as the three-dimensional structures of the four red DsRed descendants are highly conserved (Fig. 1A and B). As described previously,[4] the GFP-type termini of the DsRed variants result in a higher tolerance to N- and C-terminal fusions. The red chromophore is directly encoded in the amino acid sequence (Fig. 1A, box). Figure 1B shows the crystal structure of mCherry (PDB 2H5Q) with the typical β-barrel shape (gray), which stably encircles the central alpha-helix containing the embedded chromophore core (F70, M71, Y72 to G73, black).

The complementation of live cell imaging data with biochemical studies of one and the same red-labeled fusion protein is limited due to sparely available and applicable antibodies against red FPs. Here, we present the first rat monoclonal antibody against a set of red FPs useful in microscopic as well as in biochemical approaches.

To generate a multi-red antibody, the cDNAs of the full-length red FPs mRFP1, mCherry, mOrange, and mPlum with an N-terminal His_6 tag were expressed separately in *Escherichia coli* and purified under native conditions for antigen production. To test the purity of the eluted fractions, the purified proteins were subjected to SDS-PAGE followed by Coomassie Blue staining (Fig. 1C). The recombinant proteins were highly soluble and the yield of purified protein was 1~5 mg/mL.

In order to generate monoclonal antibodies against a multiple set of red FPs and due to their high sequence similari-

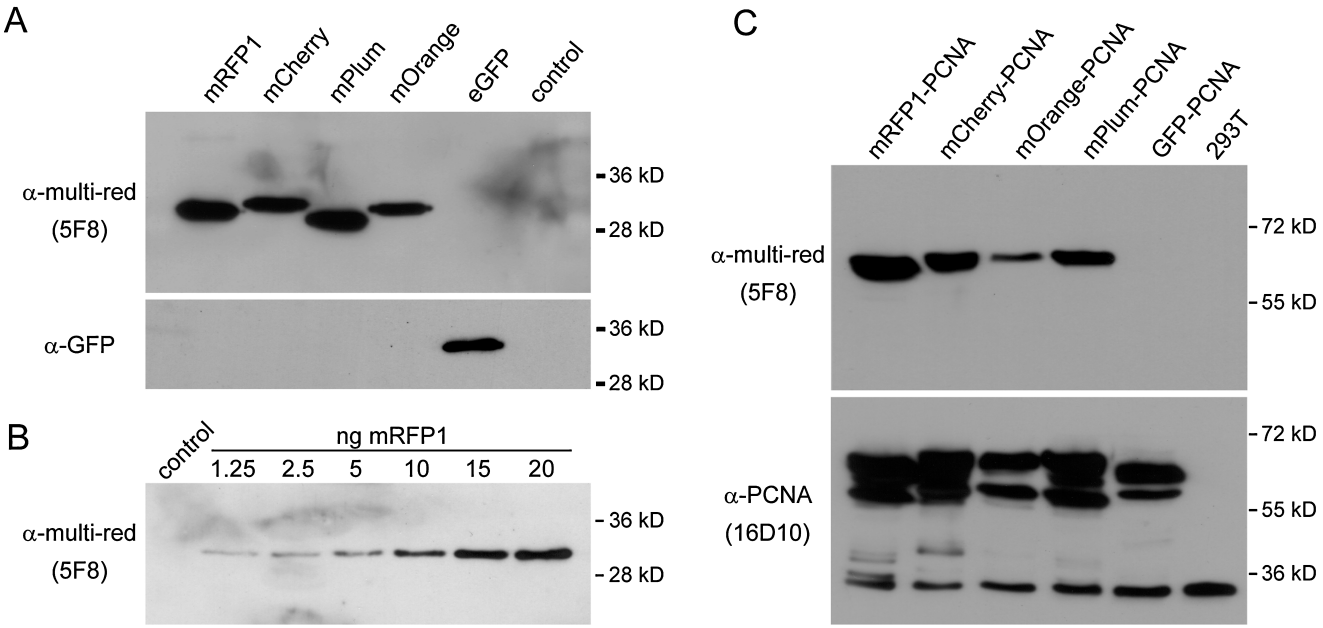

FIG. 2. Immunoblot analyses to test the specificity and affinity of the rat monoclonal multi-red antibody 5F8. (**A**) Multi-red immunoblot. 20 ng of purified red FP, eGFP, and *E. coli* protein extract (control) were loaded and subjected to SDS-PAGE. Immunostaining was performed with either the anti-multi-red 5F8 (**A,** upper panel) or with an anti-GFP antibody (**A,** lower panel). The molecular weight markers are indicated on the right. (**B**) A serial dilution of purified mRFP1 protein and *E. coli* protein extract (control) were loaded to test the affinity of the multi-red antibody. The mRFP1 protein migrates at ~32 kDa. The detection limit of the multi-red MAb ranges between 1.25 and 2.5 ng of purified mRFP1. (**C**) Immunoblot of red fluorescent fusion proteins mRFP-PCNA, mCherry-PCNA, mOrange-PCNA, and mPlum-PCNA. Soluble protein extracts of HEK 293T cells expressing the red-PCNA fusions, GFP-PCNA or mock treated (293T control) were loaded on SDS-PAGE. Immunostaining was performed with the multi-red 5F8 antibody (**C,** upper panel). Detection of PCNA was used to control for expression and loading (**C,** lower panel). The molecular weight markers are indicated on the right.

ties, all purified red FPs were pooled in equal amounts and were used to immunize Lou/C rats. A panel of hybridomas was generated by fusing spleen cells from immunized animals with the myeloma cell line P3X63-Ag8.653. The antibodies produced by the hybridomas were initially screened in a solid-phase immunoassay (indirect ELISA). Plates coated with His_6-tagged pooled red FPs were incubated with the hybridoma supernatants containing monoclonal antibodies. Specific antigen binding was detected with a mixture of biotinylated mouse MAbs against the rat IgG heavy chains. In a next step, 17 positive hybridoma supernatants were screened by an immunoblot assay using whole cell extracts from human HEK 293T transiently transfected with multiple red fusion proteins (data not shown). One antibody designated as multi-red 5F8 was selected, established, and expanded. The isotype of the specific antibody was found to be rat IgG2a.

Immunoblotting and immunoprecipitation with the multi-red 5F8 antibody

For a first functional analysis, we tested the ability of the multi-red antibody 5F8 to detect a set of red FPs in immunoblotting. 20 ng of the purified red FPs mRFP1, mCherry, mPlum, and mOrange were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane (Fig. 2A). To investigate the specificity of the multi-red antibody for only red FPs, we included in our Western blot analysis the green fluorescent protein eGFP as well as *E. coli* cell extract as negative control. The 5F8 antibody recognized all DsRed derivatives but not the unrelated eGFP, and confirmed that the generated antibody is a real multi-red FP-specific antibody. To determine the affinity of the multi-red antibody 5F8 in immunoblotting, a serial dilution of purified mRFP1 ranging from 1.25 to 20 ng was separated in SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with 0.2 μg multi-red 5F8 antibody (Fig. 2B). The monoclonal antibody was able to detect 1.25 ng of mRFP1 protein. As expected, this signal was not present in the *E. coli* extract, which was used as a negative control.

Figure 2A and 2B demonstrates that the multi-red antibody 5F8 recognized multiple purified red FPs in Western blot. Nevertheless, the main application area of the multi-red antibody 5F8 is the detection of red-labeled fusion proteins in cell extracts. Soluble protein extracts from equal numbers of human HEK 293T cells, either transiently transfected with the red- or green-labeled PCNA fusion proteins or mock treated, were separated by SDS-PAGE and transferred onto an nitrocellulose membrane. As shown in Figure 2C, multi-red 5F8 was able to detect red-labeled fusion proteins with a molecular weight of ~70 kDa. Moreover, the absence of any cross-reacting bands underlined the highly specific antigen binding of this antibody. As loading control and to verify the high specificity of the multi-red 5F8 antibody in immunoblot, we took advantage of our recently published anti-PCNA rat MAb 16D10[11] and detected the PCNA in the fusion proteins as well as the endogenous PCNA (lower panel). Besides the endogenous PCNA at ~31 kDa, two additional bands were detectable. The upper band at ~70 kDa co-localized with the anti-multi-red MAb signal, whereas the lower band at ~55 kDa represents an additional degradation product of the corresponding fusion protein. The weaker signal of mOrange-PCNA could be explained by differences in expression levels apparent in the anti-PCNA control blot. These data illustrated the high specificity and affinity of the multi-red 5F8 antibody for immunoblot applications.

To further test the specificity of multi-red 5F8 to recognize the native, folded red fusion proteins, we performed im-

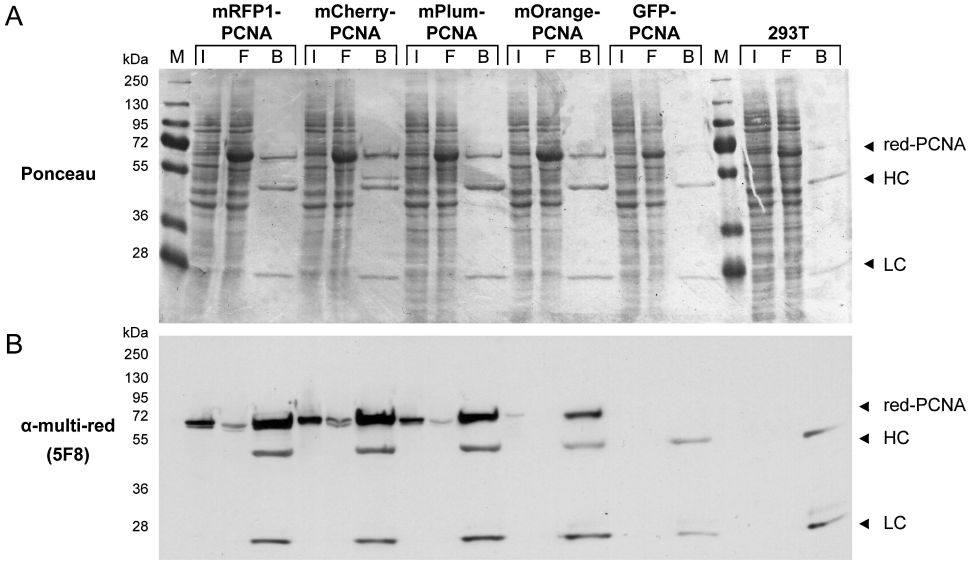

FIG. 3. Immunoprecipitation of red fluorescent fusion proteins. Human HEK 293T cells were transiently transfected with either red PCNA fusions, GFP-PCNA fusion or mock treated (293T control). Soluble cell extracts were subjected to immunoprecipitation analysis using the multi-red antibody 5F8. One percent of input (I) and flowthrough (F) fractions and 10% of the bound (B) fractions were subjected to SDS-PAGE followed by immunoblot analysis. (**A**) Successful immunoprecipitation and blotting were confirmed via Ponceau staining. The molecular weight markers are indicated on the left. (**B**) Precipitated red PCNA fusions were subsequently detected by immunostaining with multi-red 5F8. Fusion proteins as well as denatured heavy (HC) and light chains (LC) of the IgGs are marked by arrowheads.

munoprecipitation assays. To this end, we used the same set of different fluorescently labeled PCNA fusions from whole cell lysates of transiently transfected human HEK 293T cells. After incubation of the soluble cell extract with the antibody followed by extensive washing, the bound protein was eluted and subjected to SDS-PAGE electrophoresis and immunoblotting (Fig. 3). The Ponceau S staining of the nitrocellulose membrane (Fig. 3A) demonstrated the efficient and clean immunoprecipitation with the multi-red 5F8 antibody, shown by distinct bands in the bound fractions. The protein band of ~70 kDa subsequently reacted with the multi-red 5F8 antibody (Fig. 3B). The two lower bands (~25 kDa, ~50 kDa) correspond to the heavy and light chains of the denatured IgGs. No unspecifically precipitated proteins could be observed in the bound fraction of the negative controls GFP-PCNA and mock-treated 293T cells. A quantitative analysis of the non-bound (F) fraction revealed a nearly complete depletion of red PCNA fusion from human cell extracts after immunoprecipitation with multi-red 5F8. This result showed that the multi-red 5F8 antibody recognized its epitope under native conditions, when it is part of larger nuclear complexes.

We verified the immunoprecipitation data with the anti-PCNA antibody 16D10[11] and detected the PCNA in the red-labeled fusion proteins after immunoprecipitation and immunoblot (data not shown). The high pulldown efficiency of multi-red 5F8 provides a significant improvement for the analysis of all kinds of multiple red fluorescent fusion proteins.

Immunofluorescence stainings of red-labeled PCNA fusions with the monoclonal antibody

To investigate whether the multi-red antibody 5F8 is also suitable to detect red-labeled PCNA fusions *in situ*, we performed immunofluorescence stainings of transiently transfected C2C12 cells. PCNA, as a key component of the nuclear replication machinery, accumulates at sites of DNA replication, the so-called replication foci.[9] Fluorescently labeled PCNA fusion proteins are widely used in microscopy to identify replication foci and to distinguish S-phase stages (cell cycle marker). The results of the immunofluorescence analyses with the multi-red antibody 5F8 are summarized in Figure 4. C2C12 cells, transiently transfected with either mRFP1-PCNA, mCherry-PCNA, mPlum-PCNA, or mOrange-PCNA expression constructs, were fixed with formaldehyde and stained with the multi-red antibody 5F8. Subcellular localization of the red-PCNA fusions are shown in the left panel, antibody specific signals are shown in the middle panel, and the distribution of AT-rich heterochromatic regions are highlighted by DAPI staining (right panel). Typical late S-phase cells, containing few horse shoe-like foci representing late replicating heterochromatin, are shown in Figure 4A. The antibody staining showed a complete co-localization with all the red PCNA fusion proteins. No cross-reaction was observed in untransfected C2C12 cells (Fig. 4B). Methanol fixation yielded similar results (data not shown). The immunofluorescence stainings demonstrated that multi-red 5F8 specifically recognized its epitope also in the complex cellular environment.

In summary, we have generated the first rat MAb with a high specificity and affinity for multiple red fluorescent proteins (mRFP1, mCherry, mPlum, mOrange) and associated fusions. We could demonstrate that multi-red 5F8 is highly suitable for ELISA, immunoblotting, immunoprecipitation, and immunofluorescence assays. With this versatile antibody, we provide a valuable tool for the simultaneous analysis of one and the same red fluorescent fusion protein via bioimaging and biochemical approaches.

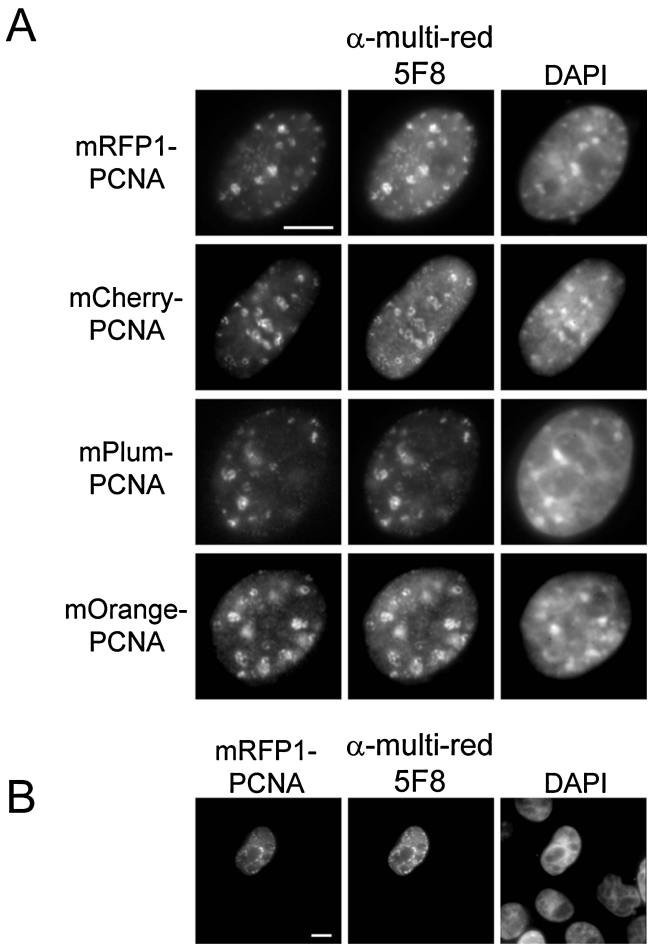

FIG. 4. Immunofluorescence staining of the cell cycle marker protein red PCNA with the multi-red 5F8 antibody (**A and B**). Shown are wide-field fluorescence images of mouse C2C12 myoblasts transiently transfected with mRFP1-PCNA, mCherry-PCNA, mPlum-PCNA, or mOrange-PCNA (**A**). Cells were fixed with formaldehyde. The subcellular cell cycle–dependent distribution of the red PCNA fusions (left panel) and the immunofluorescence staining with the multi-red 5F8 antibody (middle panel) are shown. The DNA was counterstained with DAPI (right panel). Non-transfected cells are used to control for antibody specificity (**B**). Scale bars, 5 μm.

Acknowledgments

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