Site-Specific, Orthogonal Labeling of Proteins in Intact Cells with Two Small Biarsenical Fluorophores

Alexander Zürn,[§] Christoph Klenk,[§] Ulrike Zabel,[§,#] Susanne Reiner,[§] Martin J. Lohse,*[,§,#] and Carsten Hoffmann[§]

Institute of Pharmacology and Toxicology, Rudolf Virchow Center University of Würzburg, Versbacher Strasse 9, 97078 Würzburg, Germany. Received September 7, 2009; Revised Manuscript Received January 19, 2010

The fusion of fluorescent proteins to proteins of interest has greatly advanced fluorescence microscopy, but is often limited by their large size. Here, we report site-specific, orthogonal labeling of two cellular proteins in intact cells with two small fluorescent dyes: fluorescein arsenical hairpin binder, FlAsH, and its red analogue, ReAsH, which bind to tetracysteine motifs. Development of a sequential labeling method to two different motifs, CCPGCC and FLNCCPGCCMEP, allowed site-specific labeling with FlAsH and ReAsH, respectively. Using the cell surface receptor for parathyroid hormone and its cytosolic binding protein, β-arrestin2, we show their selective visualization in intact cells and analyze their interaction by colocalization and fluorescence resonance energy transfer (FRET). We propose that this method may be widely applied to label intracellular proteins and to study their interactions in intact cells with minimal disturbance of their function.

INTRODUCTION

Fluorescence microscopy of cellular and subcellular structures generally requires the use of fluorophores. These fluorophores can be attached, for example, to antibodies in order to visualize specific proteins and structures. However, in intact cells this approach is mostly limited to cell surface proteins, because the labeled antibodies cannot pass the cell membrane. This situation has been greatly advanced by the discovery of the green fluorescent protein (GFP) and its many colored variants, which can be fused to proteins of interest and permit their visualization (*1*). To date, numerous variants of GFP and other fluorescent proteins, characterized by different fluorescent properties, have been described, and permit various fluorescence techniques to study the localization and interaction of proteins in intact cells, including fluorescence resonance energy transfer (FRET) (*2*). However, protein function may be disturbed by fusion with the ~27 kDa GFP molecule and limit their use for in vivo imaging (*3–5*). Moreover, GFPs are often fused to proteins via a flexible linker, and this together with the size of GFPs makes it difficult to predict the exact position of the central fluorophore in relation to the protein of interest.

Alternatives to GFPs include fusions with an O^6-alkylguanine-DNA-alkyltransferase (AGT), which binds covalently O^6-benzylguanine that can be chemically coupled with various fluorescent dyes. While this technique offers the advantage of using highly fluorescent dyes, the AGT tags are also of considerable size (~20 kDa). Depending on the dye used and its membrane permeability, this method can be used to label cell surface as well as intracellular proteins (*6–9*). Other enzymes that can be fused to proteins and permit labeling with a variety of fluorescent dyes include dihydrofolate reductase (together with fluorescent trimethoprim derivatives (*10*)) and a modified haloalkane dehalogenase designed to covalently bind to synthetic ligands (*11*).

A technique to minimize the size of the fluorescent dyes involves the insertion of an oligo-histidine tag into the target protein. This motif can form a complex with a nitrilo triacetic acid (NTA)-Ni^{2+}, which can again be coupled to fluorophores. By combining different fluorescently labeled NTA-variants, FRET measurements have been done with purified proteins (*12*). Labeling of proteins at the extracellular face of intact cells has also been shown, but the fluorophores did not cross the cell membrane and thus did not permit labeling of intracellular proteins (*13*). With sizes of 2−5 kDa, these complexes are much smaller than the GFP-variants or the AGT-tag, but their low affinity results in a low stability of the complex (*14*).

To circumvent these problems, a much smaller, chemical fluorescent label was reported as an alternative. The 700 Da fluorescein derivative FlAsH (fluorescein arsenical hairpin binder) and the resorufin derivative ReAsH (red arsenical hairpin binder) have been shown to bind to a tetracysteine motif (CCPGCC) with high affinity and specificity (*15, 16*). Such tetracysteine motifs can be genetically introduced into proteins. Labeling of tetracysteine-motif containing proteins requires a labeling step that results in a complex formation between the label and the tetracysteine motif. This needs to be followed by a washing procedure to minimize unspecific background staining. This labeling technique has been used for cytosolic as well as membrane proteins (*17, 18*). FlAsH can be used as an alternative to YFP and forms a FRET pair with CFP. Using FlAsH instead of YFP, we were able to rescue the functionality of a G-protein coupled receptor (GPCR), which was inactive when labeled with YFP in its third intracellular loop (*3*).

However, for FRET (or colocalization) studies, this approach still required CFP (or a similar protein) as a second label, and it would seem attractive to also replace this second label by a small one. Therefore, further studies have been undertaken to simultaneously label and visualize two different proteins within a cell using two small fluorophores. Different approaches for simultaneous labeling with FlAsH and ReAsH have been described. Robia et al. (*19*) cloned two CCPGCC sequences into the sarcoplasmic reticulum protein phospholamban and stained cells expressing this construct by simultaneous incubation with ReAsH and FlAsH; this resulted in random labeling of phospholamban with either FlAsH or ReAsH. Interestingly,

* Corresponding author. Institute of Pharmacology and Toxicology, University of Würzburg, Versbacher Str. 9, 97078 Würzburg. Phone: ++49 931 20148400, Fax: ++49 931 20148411, E-mail lohse@toxi.uni-wuerzburg.de.

[§] Institute of Pharmacology and Toxicology.

[#] Rudolf Virchow Center.

10.1021/bc900394j
Published on Web 04/29/2010

phospholamban, a small protein of 5 kDa, still formed pentamers after labeling, indicating that the protein had remained functional (*19*). Roberti et al. (*20*) performed nondirected labeling with the two small fluorophores of α-synuclein aggregates in vitro and in cells. Finally, Squier et al. presented a tetracysteine motif, CCKACC, with different binding rates and affinities for FlAsH and ReAsH compared to the CCPGCC motif; this allowed sequential labeling, but this approach has so far been done only with purified proteins (*21*).

Despite these studies demonstrating the value of biarsenical dyes, site-specific labeling of two different proteins with FlAsH and ReAsH in intact cells has not yet been achieved. However, such specific labeling would be necessary to distinguish two different proteins by different labels in order to achieve the full power of fluorescence microscopy and FRET techniques. Therefore, we set out to develop such specific labeling of intracellular proteins. To do so, we utilized different tetracysteine motifs (*22*) and developed a selective and specific labeling protocol. We show that a cell surface and a cytosolic protein—the parathyroid hormone (PTH) receptor and its binding protein β-arrestin2—can be specifically labeled with FlAsH and ReAsH in the same cell and their known interaction (*23*) can then be visualized by colocalization and FRET studies.

EXPERIMENTAL PROCEDURES

Reagents. FlAsH and ReAsH are commercially available from Invitrogen as TC-FlAsH and TC-ReAsH, respectively. Human [$Nle^{8,18}$,Tyr^{34}]PTH(1−34), abbreviated as PTH(1−34), was obtained from Bachem. 1,2-Ethane dithiol (EDT) was from Sigma-Aldrich and British anti-Lewisite (BAL; 1,2-dimercaptopropanol) from Fluka. All other reagents were of the highest available grade from standard sources, generally from Sigma-Aldrich.

Molecular Biology. cDNA-sequences coding for a FLNCCPGCCMEP or a HRWCCPGCCKTF sequence (*22*) followed by those for CFP or YFP were fused to the very C-terminus of the A_{2A}-receptor cDNA. These constructs served for the analysis of the affinities for the twelve amino acid FlAsH binding motifs. The six amino acid motif CCPGCC was inserted in the third intracellular loop the A_{2A}-receptor after the position 215 (*3*), resulting in a sequence MESCCPGCCARS, and a CFP or YFP was fused to the receptor sequence after position 340.

Similarly, a FLNCCPGCCMEP motif (or a CCPGCC motif) was inserted into the C-terminus of the human PTH receptor following amino acid position 515. Likewise, a CCPGCC motif (or a FLNCCPGCCMEP motif) with a linker sequence was fused to the very C-terminus of β-arrestin2, resulting in an addition of KQMESCCPGCCARSTLQ (or KQMESFLNCCPGCCMEPARSTLQ), i.e., the same amino acid motifs as above. Site-directed mutagenesis was achieved using standard polymerase chain reaction techniques followed by DNA-sequencing for verification. For transient expression in mammalian cells, all cDNAs were cloned into the pcDNA3 vector (Invitrogen).

Cell Culture. HEK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 000 U/L penicillin and 100 mg/L streptomycin at 37 °C and 7% CO_2. For fluorescence measurements, cells were seeded on round polylysine-coated coverslips in 6-well plates 6 h prior to transfection. Cells were transfected using Effectene (Quiagen) according to the manufacturer's instructions. Assays were performed 48 h after transfection.

FlAsH and ReAsH Labeling. Transfected cells grown on polylysine-coated 24 mm coverslips to a confluence of about 70% were washed twice with 1 mL Phenol red-free Hank's balanced salt solution containing 1.8 g/L glucose (HBSS; Invitrogen) and incubated at 37 °C and 7% CO_2 for 1 h with 1 mL 2.5 μM ReAsH, 12.5 μM EDT in HBSS. Subsequently, cells were washed once with 1 mL HBSS. To reduce nonspecific labeling, cells were next incubated for 10 min at 37 °C and 7% CO_2 with 1 mL 250 μM BAL in HBSS and washed again with 1 mL HBSS. Subsequently, cells were incubated at 37 °C and 7% CO_2 for 1 h with 1 mL 250 nM FlAsH, 12.5 μM EDT in HBSS, washed again with HBSS, incubated for 10 min with 1 mL 250 μM EDT in HBSS, and finally washed twice with HBSS.

Determination of FlAsH and ReAsH Affinities. As a measure of the affinity of FlAsH and ReAsH to different binding motifs, we determined the concentration−response curves of the removal of the fluorophore by BAL. After labeling of transfected HEK cells, crude cell membranes were prepared by lysis with an Ultra-Turrax device and centrifugation at 120 000 × *g*. The membranes were resuspended in 500 μL of 50 mM Tris-buffer, pH 7.4, and FlAsH or ReAsH fluorescence were measured in a spectrofluorometer (Perkin-Elmer LS50B) in a 600 μL cuvette. The excitation wavelength for the CFP-FlAsH constructs was 436 nm and the emission spectrum was measured at 460−600 nm. For the YFP-ReAsH constructs, the excitation wavelength was 490 nm and the emission spectrum was measured at 500−700 nm. To determine the affinity of the fluorophores to the individual binding motifs, the concentration of BAL was increased stepwise. FRET between the two labels in a given construct was determined as the ratio between the emission of the donor (CFP or YFP; at 490 or 525 nm) and the emission of the acceptor (FlAsH or ReAsH; at 525 or 608 nm).

Confocal Microscopy. HEK cells were transfected with cDNA constructs coding for the PTH receptor or β-arrestin2, respectively, and labeled with FlAsH and ReAsH as described above. Coverslips were mounted using an Attofluor holder (Molecular Probes).

Images were taken on a Leica TCS SP5 confocal microscope with a 63 × 1.4 oil objective. For excitation of FlAsH, the 514 nm line of an argon laser was used, and fluorescence intensities were recorded from 520 to 560 nm. ReAsH was excited using a 594 nm laser line, and fluorescence intensities were recorded from 600 to 700 nm. Settings for image acquisition were kept constant at 1024 × 1024 pixel format and 400 Hz. Time series were recorded using the standard Leica software package. The pictures were taken sequentially at 3 min intervals. For the β-arrestin2 translocation, the time series were stopped after 9 min, and for receptor internalization, image acquisition was terminated after 18 min.

Measurement of Intracellular Free Calcium Responses. HEK cells were grown on polylysine-coated 24 mm coverslips to a confluence of about 70%. The cells were transfected with PTH-receptor cDNA constructs using Effectene (Quiagen) according to the manufacturer's instructions. Assays were performed 48 h after transfection. To load the calcium indicator, cells were incubated for 35 min at 37 °C in loading buffer (10 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$) containing the calcium-sensitive dye, Fura-2 a.m. (1 μM), washed three times with loading buffer, and incubated at room temperature in the dark for 40 min. To determine Fura-2-fluorescence, an inverted fluorescence microscope was used (dichroic mirror, 455DCLP; filter set, 340 and 380 nm excitation filters and 520/20 emission filter), and the Ca^{2+} responses in single cells to stimulation with 10 nM PTH (1−34), added with a pipet, were measured by recording the Fura-2 fluorescence using *MetaMorph/MetaFluor* software (Visitron) and calculating the ratio of Fura-2 emission when excited at 340 nm and at 380 nm.

FRET Measurements with FlAsH and ReAsH. Dynamic FRET measurements using FlAsH as the donor and ReAsH as the acceptor were performed with a custom-built Zeiss Observ-

er.Z1 microscope equipped with a Visitron dual view, a DCLP 565 nm beam splitter, and 535/30 nm and 595/70 nm emission filters to monitor the FlAsH and ReAsH signals, respectively. An EXFO X-cite 120 lamp was used as the excitation source at a wavelength of 500 nm. Under these conditions, no direct excitation of ReAsH was measured. For bleedthrough of FlAsH into the ReAsH channel, a correction of 25% was determined in control experiments and applied. Data were measured using the *MetaFluor* software (Visitron), and *Origin6.1* (OriginLab) was used for analysis.

RESULTS

Affinity Measurements. In order to achieve selective binding of two small fluorophores to specific motifs, we aimed to identify motifs with different affinities toward FlAsH and ReAsH. Initially, we set out to assess the individual binding affinities of two recently described (*22*) 12-amino-acid high-affinity motifs (FLNCCPGCCMEP and HRWCCPGCCKTF). In order to study labeling and affinities in intact cells, we assessed these motifs in the context of the A_{2A} adenosine receptor, a prototypical G-protein coupled receptor (*3*). The two motifs were fused to C-terminus of the receptor, followed directly by CFP or YFP, respectively (Figure 1A). All constructs were expressed in HEK cells and showed specific membrane localization as measured by confocal microscopy (data not shown). In addition, to introduce a second label (for FRET studies) to a site with lower affinity, we expressed the similar A_{2A} adenosine receptor constructs carrying the core CCPGCC motif in their third intracellular loop (Figure 1A).

All constructs were labeled in HEK cells with either FlAsH or ReAsH, respectively, in order to allow intramolecular FRET between either CFP and FlAsH or YFP and ReAsH. Membranes were then prepared from the cells, and the emission spectra of each construct were measured. Figure 1B shows the emission spectra of a CFP- and FlAsH-labeled A_{2A} adenosine receptor (construct #5). The emission of both fluorophores, CFP (emission maximum at 480 nm) and FlAsH (emission maximum at 525 nm), were both clearly detectable in this spectrum when CFP was excited (436 nm). The strong emission of FlAsH under these conditions is indicative of FRET. Similarly, FRET was detected in membranes with an A_{2A} adenosine receptor labeled with YFP and ReAsH (construct #6; Supporting Information Figure 1).

FlAsH and ReAsH are fluorescent only when bound covalently to their tetracysteine motifs. British anti-Lewisite (BAL, dimercaptopropanol) can compete for binding and thus reduce their fluorescence signals (*22*). As a consequence, in the FRET-reporter constructs increasing BAL concentrations should decrease the emission of FlAsH or ReAsH, respectively, and because of ensuing lack of FRET increase the emission of the respective GFP-variant. This was indeed observed, with increasing concentrations of BAL causing the spectrum to shift progressively from two peaks of equal height (green curve) to one with a dominant CFP-emission and lacking FlAsH emission (cyan curve) (Figure 1B). The ratio between the emissions of CFP/FlAsH (or YFP/ReAsH) can be used to generate a kind of "competition curve" for BAL, and the IC_{50} value reflect the stability of FlAsH and ReAsH binding to the respective motif (i.e., a high IC_{50} value of BAL corresponds to a high stability).

These IC_{50} values were ~5.6 mM of BAL for FlAsH at the FLNCCPGCCMEP-motif, whereas the affinity for FlAsH at the HRWCCPGCCKTF motif was 2.3 mM of BAL. A clearly lower affinity for FlAsH was determined for the 6-amino-acid motif CCPGCC with 0.7 mM of BAL. For ReAsH, 3-fold lower affinities were determined for each motif, with IC_{50} values of 2.0 mM, 0.8 mM, and 0.2 mM of BAL, respectively (Supporting Information Table 1). Thus, the three tetracysteine motifs can be exploited for the development of a selective labeling protocol.

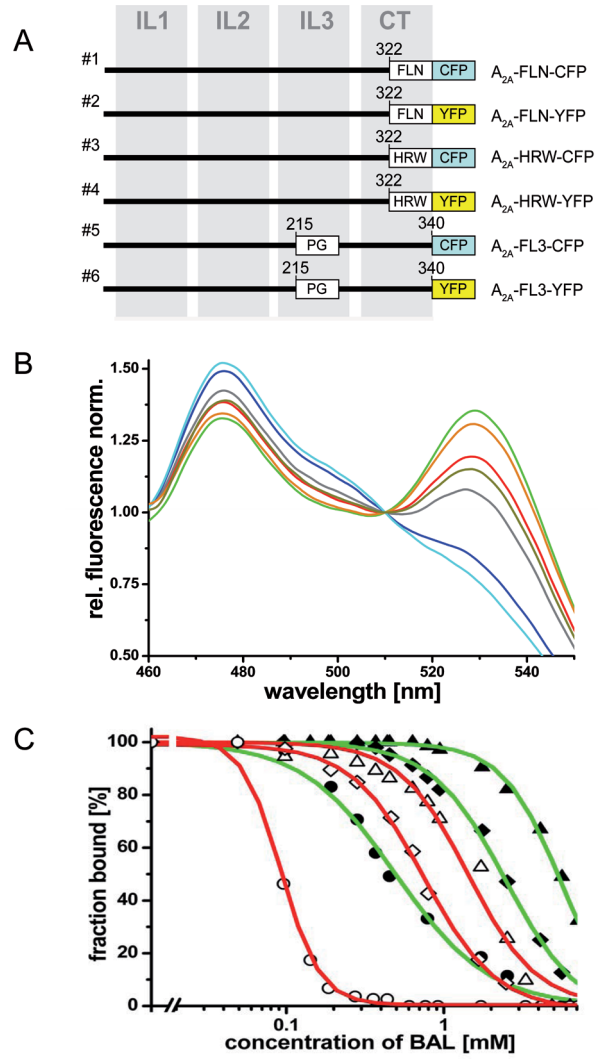

Figure 1. Characterization of FlAsH and ReAsH binding motifs in A2A-adenosine receptors. (A) Schematic representation of the different constructs. CFP or YFP were attached C-terminally after the indicated amino acid position. The 12-amino-acid tetracysteine motifs are denoted "FLN"for FLNCCPGCCMEP and "HRW" for HRWCCPGCCKTF. The 6-amino-acid tetracysteine motif CCPGCC is denoted "PG". Additional numbers denote the sites (amino acid number) of motif insertion. IL and CT denote intracellular loops and C-terminus, respectively. (B) Fluorescence emission spectrum of FlAsH-labeled A2A-FL3-CFP (construct #5) and disruption of FRET by increasing concentrations of BAL (0−4 mM), measured with 436 nm excitation. (C) Reduction of ReAsH and FlAsH binding to the different tetracysteine motifs by BAL. Shown is the fraction of the indicated motifs occupied by the respective label (FlAsH green curves, ReAsH red curves) as a function of the BAL concentration used to displace binding. Data are derived from experiments as shown in (B). Symbols denote: open circle, A2A-Fl3-YFP (construct #6), filled circle, A2A-Fl3-CFP (construct #5); open diamond, A2AHRW-YFP (construct #4); filled diamond, A2A-HRW-CFP (construct #3); open triangle, A2A-FLN-YFP (construct #2); filled triangle, A2A-FLN-CFP (construct #1). Data are representative measurements of three independent experiments. IC_{50} values are given in Supporting Information Table 1.

Selective Labeling in Intact Cells. In order to develop a double labeling protocol, we used two proteins, localized in two different cellular compartments in resting cells, that display a well-described, agonist dependent interaction: the cell surface parathyroid hormone receptor (PTH receptor, a G-protein coupled receptor) and its cytosolic binding protein β-arrestin2 (*23*). The motif with the highest affinity, FLNCCPGCCMEP, was inserted into the C-terminus of the PTH receptor, and the motif with the lowest affinity, CCPGCC, was fused to the

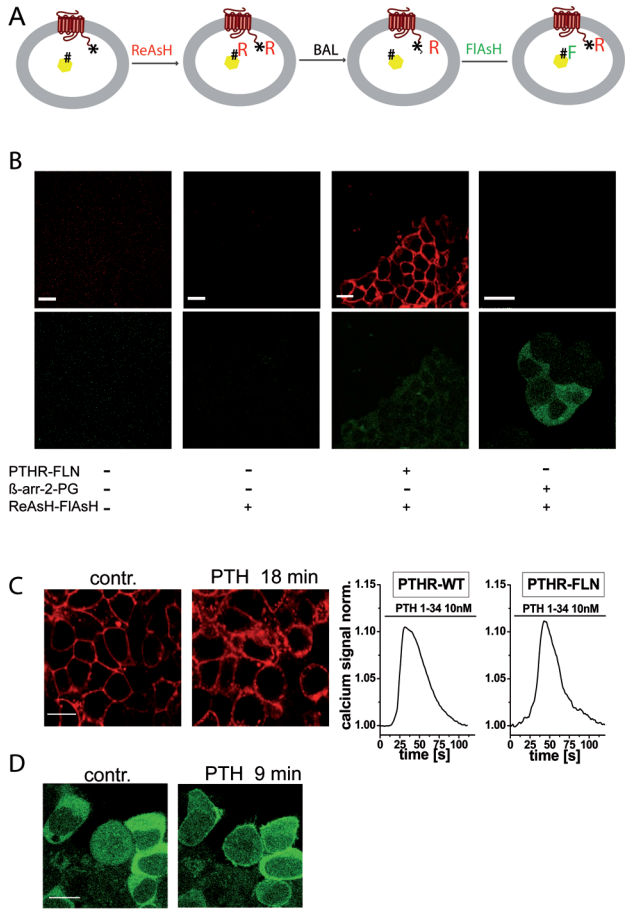

Figure 2. Specific labeling of the PTH receptor and β-arrestin2 in intact cells with ReAsH and FlAsH. (A) Schematic representation of the labeling protocol. Initially, the FLNCCPGCCMEP motif in the PTH receptor (*) as well as the CCPGCC motif in β-arrestin2 (#) were both covalently labeled with ReAsH (R). In a washing step with BAL, ReAsH was selectively removed from the CCPGCC motif in β-arrestin2. Finally, the latter motif was again labeled with FlAsH (F). (B) Specificity and completeness of labeling. The upper row of panels shows the ReAsH emission (594 nm laser line excitation, 600−700 nm emission); the lower row shows the FlAsH fluorescence (514 nm laser line excitation, 520−560 nm emission). The panels (from left to right) show nontransfected HEK cells before (left) and after (second from left) the labeling protocol (as depicted in (A)), HEK cells expressing only the PTH receptor construct (second from right) or the β-arrestin2 construct (both labeled as depicted in (A)). (C) Functional integrity of the ReAsH labeled PTH receptor. The left panels show receptor internalization in HEK cells in response to PTH (1 μM, 18 min; right) compared to unstimulated controls (left). The right panels show Ca^{2+}-responses to PTH (measured with Fura-2) in HEK cells transiently expressing wild-type or FLNCCPGCCMEP labeled receptors. (D) Functional integrity of FlAsH-labeled β-arrestin2. HEK cells transiently expressing unlabeled PTH receptors and FlAsH-labeled β-arrestin2 were stimulated with PTH (1 μM, 9 min; right) to induce translocation of β arrestin2 to the plasma membrane. White scale bars represent 10 μm.

C-terminus of β-arrestin2. HEK cells expressing both constructs were sequentially labeled with FlAsH and ReAsH with various protocols, resulting in the following optimal procedure (Figure 2A; see Experimental Procedures for further details). First, cells were incubated with 2.5 μM ReAsH for 1 h and then washed with 250 μM BAL for 10 min. This BAL concentration left >95% of FLNCCPGCCMEP motif in the PTH receptor bound with ReAsH, while almost all ReAsH was removed from the CCPGCC motif in β-arrestin2 (compare open circles and open triangles in Figure 1C). Subsequently, cells were incubated for another hour with 250 nM FlAsH, which allowed full labeling of the CCPGCC motif, while the FLNCCPGCCMEP motif remained "protected" by the already bound ReAsH. Finally, cells were subjected to a washing procedure using 250 μM 1,2-ethane dithiol (EDT) to reduce nonspecific labeling.

The specificity of this labeling protocol was ascertained in various control experiments (Figure 2B). First, we showed that background labeling, i.e., when no tetracysteine containing proteins were transfected, was negligible (Figure 2B, second from left), and in fact, fluorescence in both channels was not much different from totally unlabeled cells (Figure 2B, left). Furthermore, we confirmed that the two labels were bound to the correct sites, i.e., that indeed ReAsH already bound to the FLNCCPGCCMEP motif would protect this motif from subsequent labeling with FlAsH. To do so, we transfected HEK cells with the PTH receptor-FLNCCPGCCMEP construct alone and subjected these cells to the labeling protocol. Figure 2B (second from right) shows that indeed this resulted in clear cell surface staining with ReAsH, but essentially no labeling with FlAsH. This illustrates that despite its higher affinity FlAsH does not displace ReAsH from the FLNCCPGCCMEP motif and confirms the site specificity of the labeling procedure. Conversely, when HEK cells were transfected with the β-arrestin-2-CCPGCC construct alone and then labeled, this resulted in clear labeling of the cytosolic β-arrestin-2 with FlAsH but essentially no labeling with ReAsH (Figure 2B, right). Protein synthesis ongoing during the labeling process did not appear to cause a significant resynthesis of the first labeling motifs, as evidenced by very low background staining (Figure 2B, second from left), low FlAsH binding to receptors nearing the ReAsH motif (Figure 2B, second from right) and a lack of improvement when the labeling was done in the presence of the protein synthesis inhibitor cycloheximide (not shown). Taken together, these data indicate both negligible background staining and highly specific, orthogonal labeling of the two tetracysteine motifs with FlAsH or ReAsH, respectively.

The functional integrity of the labeled proteins after this labeling procedure was verified along several lines. First, the ReAsH-labeled PTH receptors were localized at the cell surface and internalized in response to stimulation with PTH (Figure 2C), indicating an intact activation switch and internalization process of the receptor construct. Second, the ReAsH-labeled receptors generated intracellular Ca^{2+} signals that were not different from the signals mediated by wild-type receptors (Figure 2C), indicating intact coupling to G-proteins and downstream signaling. Finally, FlAsH-labeled β-arrestin2 translocated from the cytosol to the cell surface in response to stimulation of the PTH receptor (Figure 2D), indicating an intact interaction between the two proteins and normal β-arrestin2 behavior (*23*).

Co-Localization and FRET Measurements. A number of experiments were then carried out to assess whether this site-specific labeling protocol allowed the study of protein colocalization and protein−protein interactions, using again the PTH receptor/β-arrestin2 interaction as a model. HEK cells, expressing PTH receptor-LNCCPGCCMEP and β-arrestin2-CCPGCC, were labeled as above. Analysis by confocal microscopy with the appropriate wavelengths showed the ReAsH-labeled receptors at the cell surface and the FlAsH-labeled β-arrestin2 in the cytosol. Stimulation of the cells with PTH caused translocation of β-arrestin to the cell surface, and this was visualized by an overlay of the ReAsH and FlAsH emissions (Figure 3A).

Scanning of such cells expressing the two labeled proteins allowed a quantification of the ReAsH label at the cell surface and of the cytosolic (control) vs cell surface (PTH-stimulated) localization of β-arrestin2 (Figure 3B). While the PTH receptors were primarily localized to the cell surface under both conditions, β-arrestin2 was evenly distributed throughout the cell under control conditions, but showed a preferential localization

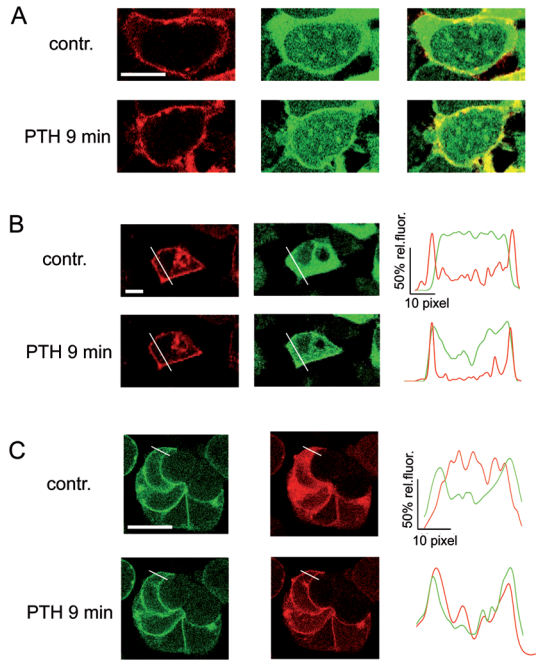

Figure 3. Interaction of ReAsH-labeled PTH receptors with FlAsH-labeled β-arrestin2 in HEK cells. (A) PTH-induced colocalization of receptors and β-arrestin2 at the plasma membrane. HEK cells expressing both ReAsH-labeled PTH receptors (FLNCCPGCCMEP motif) and FlAsH-labeled β-arrestin2 (CCPGCC motif) are shown prior to stimulation (top) or 9 min after stimulation with PTH (1 μM, bottom). Shown are the images of ReAsH emission (left), FlAsH emission (center), and the corresponding overlay images. The yellow color in the overlay images indicates co-localization of FlAsH and ReAsH labeled proteins. (B) PTH-induced translocation of β-arrestin2 to the plasma membrane represented by relative distribution of fluorescence intensity. FlAsH or ReAsH emission coming from labeled β-arrestin2 or labeled PTH receptor was quantified along the indicated white line before (top right) and after (bottom right) stimulation of the cells with PTH as in (A). The relative fluorescence intensity traces indicate a uniform distribution of FlAsH under control conditions and clear plasma membrane preponderance after receptor stimulation. (C) Same experiment as in (B), but with β-arrestin2 equipped with the FLNCCPGCCMEP motif and labeled with ReAsH, and the PTH receptor with the lower-affinity CCPGCC motif labeled with FlAsH. PTH-induced translocation of β-arrestin2 to the plasma membrane represented by confocal images (left) and relative distribution of fluorescence intensity (right). The relative fluorescence intensity traces indicate a uniform distribution of ReAsH under control conditions, and clear cell surface preponderance after receptor stimulation.

at the cell surface after stimulation with PTH. These experiments document that ReAsH and FlAsH can be used for selective labeling of specific proteins and to visualize their intracelluar (co)localization.

To demonstrate the versatility of our technique, we exchanged the tetracysteine tags, i.e., β-arrestin2 was equipped with the FLNCCPGCCMEP sequence (ReAsH motif), and the PTH receptor with the lower affinity CCPGCC sequence (FlAsH motif). Labeling was done as before and resulted in clear cytosolic ReAsH-labeling of β-arrestin2 and cell surface labeling of the PTH receptor with FlAsH (Figure 3C). The ReAsH-labeled β-arrestin2 again translocated to the cell surface upon receptor stimulation as visualized by confocal imaging (Figure 3C, left) and fluorescence quantification (Figure 3C, right).

To show that FRET can be measured between FlAsH and ReAsH, we first studied intramolecular FRET and labeled again an A_{2A}-adenosine receptor (as in Figure 1), carrying a CCPGCC sequence (FlAsH motif) in the third intracellular loop, and a FLNCCPGCCMEP sequence at the C-terminus, which was labeled at both sites as before. Partial acceptor photobleaching (550 nm, 500 s) caused an increase of donor fluorescence by almost 10%, indicative of FRET (Supporting Information Figure 2). Ratiometric quantification of agonist-induced changes in FRET was then used to study the dynamics of the interaction between β-arrestin2 and PTH receptors and to show that direct protein−protein interactions were the basis for their colocalization in cells stimulated with PTH (Figure 4). HEK cells transfected and labeled as in Figure 3A were excited at the appropriate wavelength for FlAsH (500 nm) and observed under wide-field conditions equipped to simultaneously image emission of FlAsH (535/30 nm) and ReAsH (595/70 nm). Addition of the agonist PTH caused a decrease of the FlAsH fluorescence, while the ReAsH fluorescence increased in a reciprocal manner with the same time course (Figure 4, left). As a consequence, the FRET ratio increased (Figure 4, right) and the time course of this increase corresponded to the previously described kinetics of the PTH receptor/β-arrestin2 interaction (*23, 24*). This behavior is indicative of the progressive development of FRET and illustrates that site-selective labeling with ReAsH and FlAsH can be used for specific FRET measurements.

DISCUSSION

Labeling of intracellular proteins with GFP and other fluorescent proteins variants has become an extremely popular tool in fluorescence microscopy. However, the limitations mentioned above have prompted a search for alternatives, most

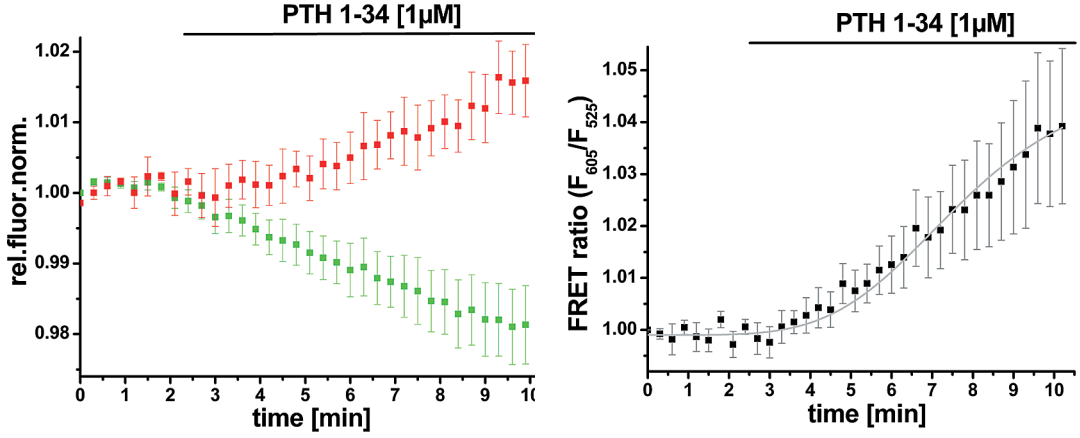

Figure 4. FRET between ReAsH-labeled PTH receptors (FLNCCPGCCMEP motif) and FlAsH-labeled β-arrestin2 (CCPGCC motif) in HEK cells. Exposure to PTH (1 μM) causes a decrease in FlAsH emission and a simultaneous increase in ReAsH emission (left), resulting in an increase in the emission ratio (595 nm/535 nm), which is indicative for FRET (right). Every time point shows the mean of five independent measurements and the SEM.

notably smaller labels, an issue that appears particularly important when labeling small and/or multifunctional proteins (*25, 26*). To achieve labeling of intracellular proteins with small labels in intact cells, the technique of tetracysteine motif labeling with FlAsH (or ReAsH) appears at present the most attractive approach, due to the small size of the labels (~0.7 kDa). An additional, and particularly useful property of these dyes is that they become fluorescent only when bound to these tetracysteine motifs (*2, 15*). Analogues with brighter fluorescence have also been synthesized (*27, 28*) and will add to the attractiveness of this technology. Applied to GPCRs, FlAsH-labels have not only been used to report agonist-induced conformational changes of receptors (*3, 29*), but also to pick up differences in agonist-induced changes in different positions of the third intracellular loop (*30*), illustrating the versatility of this label and the usefulness of its small size and the fixed binding to its recognition sequence.

In FRET studies using FlAsH, usually CFP (or a similar variant) is employed as the second label. Attempts to measure FRET between FlAsH and ReAsH have been reported, but they were based on nonselective, i.e., random labeling of tetracysteine motifs by the two labels (*20*). Such random labeling does not permit the identification of different proteins by specific labels; furthermore, it reduces the signal/noise ratio and complicates the molecular interpretation of FRET changes. With an isolated protein in vitro, Wilkins et al. (*31*) have recently achieved site-specific labeling with FlAsH using protection of sites with photocaged amino acids, but this approach has so far been used only with in vitro translated proteins and a single label.

To overcome these problems, we have developed here a sequential labeling procedure that results in site-specific, orthogonal labeling of two different tetracysteine motifs in intact cells. Applying this procedure to a cell surface GPCR and its cytosolic binding protein, β-arrestin2, we observed that the labeled proteins retained essential functions. Furthermore, we showed that the labeling was clearly site-specific, as evidenced by the cell surface vs cytosolic localization of the two labels and their distinct movements upon receptor stimulation. Finally, the specific labeling permitted the demonstration of the agonist-dependent interaction of the two proteins by co-localization and by FRET between the labels; the kinetics of this translocation and interaction agree with the well-known time course, which occurs over several minutes (*24, 32*).

The labeling procedure resulted in virtually no background labeling, but essentially complete labeling of the two sites, as evidenced by our inability to label the "ReAsH-site" with FlAsH after the ReAsH labeling step (see Figure 2B). The specificity and wide applicability of this method should also make the development of FlAsH analogues with improved fluorescent properties an attractive goal, to achieve wider variability of colors and combinations of different fluorophores. Our data suggest that with this method specific labeling of intracellular proteins may be generally achievable and may permit the study of intracellular proteins with minimal disturbance of their function.

ACKNOWLEDGMENT

We thank Moritz Bünemann, Kristina Lorenz, Joachim Schmitt, and Gregory Harms for helpful discussions and Nicole Ziegler for expert technical assistance. This work was funded by the Deutsche Forschungsgemeinschaft (SFB487 TPA1; to MJL and CH) and the European Research Council (Advanced Investigator Grant to MJL).

Supporting Information Available: Table 1 reflectsBAL concentrations that were needed to reduce binding of FlAsH or ReAsH to the indicated tetracystein motif by 50%. Figure 1 shows fluorescence excitation and emission spectra of YFP and ReAsH. Figure 2 shows photobleaching of a FlAsH and ReAsH labeled A_{2A}-PG3-FLN33 construct. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Tsien, R. Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem. 67*, 509–44.

(2) Shaner, N. C., Steinbach, P. A., and Tsien, R. Y. (2005) A guide to choosing fluorescent proteins. *Nat. Methods 2*, 905–909.

(3) Hoffmann, C., Gaietta, G., Bünemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilardaga, J. P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. (2005) A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat. Methods 2*, 171–176.

(4) Lisenbee, C. S., Karnik, S. K., and Trelease, R. N. (2003) Overexpression and mislocalization of a tail-anchored GFP redefines the identity of peroxisomal ER. *Traffic 4*, 491–501.

(5) Andresen, M., Schmitz-Salue, R., and Jakobs, S. (2004) Short tetracysteine tags to beta-tubulin demonstrate the significance of small labels for live cell imaging. *Mol. Biol. Cell 15*, 5616–22.

(6) Gautier, A., Juillerat, A., Heinis, C., Correa, I. R., Jr., Kindermann, M., Beaufils, F., and Johnsson, K. (2008) An engineered protein tag for multiprotein labeling in living cells. *Chem. Biol. 15*, 128–36.

(7) Gronemeyer, T., Chidley, C., Juillerat, A., Heinis, C., and Johnsson, K. (2006) Directed evolution of O6-alkylguanine-DNA alkyltransferase for applications in protein labeling. *Protein Eng. Des. Sel. 19*, 309–16.

(8) Keppler, A., Pick, H., Arrivoli, C., Vogel, H., and Johnsson, K. (2004) Labeling of fusion proteins with synthetic fluorophores in live cells. *Proc. Natl. Acad. Sci. U.S.A. 101*, 9955–9.

(9) Maurel, D., Comps-Agrar, L., Brock, C., Rives, M. L., Bourrier, E., Ayoub, M. A., Bazin, H., Tinel, N., Durroux, T., Prezeau, L., Trinquet, E., and Pin, J. P. (2008) Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. *Nat. Methods 5*, 561–7.

(10) Miller, L. W., Cai, Y., Sheetz, M. P., and Cornish, V. W. (2005) In vivo protein labeling with trimethoprim conjugates: a flexible chemical tag. *Nat. Methods 2*, 255–7.

(11) Los, G. V., and Wood, K. (2007) The HaloTag: a novel technology for cell imaging and protein analysis. *Methods Mol. Biol. 356*, 195–208.

(12) Kapanidis, A. N., Ebright, Y. W., and Ebright, R. H. (2001) Site-specific incorporation of fluorescent probes into protein: hexahistidine-tag-mediated fluorescent labeling with ($Ni^{(2+)}$:nitrilotriacetic Acid (n)-fluorochrome conjugates. *J. Am. Chem. Soc. 123*, 12123–5.

(13) Guignet, E. G., Hovius, R., and Vogel, H. (2004) Reversible site-selective labeling of membrane proteins in live cells. *Nat. Biotechnol. 22*, 440–4.

(14) Soh, N. (2008) Selective chemical labeling of proteins with small fluorescent molecules based on metal-chelation Methodology. *Sensors 8* (2), 1004–102.

(15) Griffin, B. A., Adams, S. R., and Tsien, R. Y. (1998) Specific covalent labeling of recombinant protein molecules inside live cells. *Science 281*, 269–72.

(16) Adams, S. R., Campbell, R. E., Gross, L. A., Martin, B. R., Walkup, G. K., Yao, Y., Llopis, J., and Tsien, R. Y. (2002) New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J. Am. Chem. Soc. 124*, 6063–76.

(17) Nikolaev, V. O., Hoffmann, C., Bunemann, M., Lohse, M. J., and Vilardaga, J. P. (2006) Molecular basis of partial agonism at the neurotransmitter α_{2A}-adrenergic receptor and G_i-protein heterotrimer. *J. Biol. Chem. 281*, 24506–11.

(18) Jost, C. A., Reither, G., Hoffmann, C., and Schultz, C. (2008) Contribution of fluorophores to protein kinase C FRET probe performance. *ChemBioChem 9*, 1379–84.

(19) Robia, S. L., Campbell, K. S., Kelly, E. M., Hou, Z., Winters, D. L., and Thomas, D. D. (2007) Forster transfer recovery reveals that phospholamban exchanges slowly from pentamers but rapidly from the SERCA regulatory complex. *Circ. Res. 101*, 1123–9.

(20) Roberti, M. J., Bertoncini, C. W., Klement, R., Jares-Erijman, E. A., and Jovin, T. M. (2007) Fluorescence imaging of amyloid formation in living cells by a functional, tetracysteine-tagged alpha-synuclein. *Nat. Methods 4*, 345–51.

(21) Chen, B., Cao, H., Yan, P., Mayer, M. U., and Squier, T. C. (2007) Identification of an orthogonal peptide binding motif for biarsenical multiuse affinity probes. *Bioconjugate Chem. 18*, 1259–65.

(22) Martin, B. R., Giepmans, B. N., Adams, S. R., and Tsien, R. Y. (2005) Mammalian cell-based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. *Nat. Biotechnol. 23*, 1308–1314.

(23) Vilardaga, J. P., Frank, M., Krasel, C., Dees, C., Nissenson, R. A., and Lohse, M. J. (2001) Differential conformational requirements for activation of G proteins and the regulatory proteins arrestin and G protein-coupled receptor kinase in the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein. *J. Biol. Chem. 276*, 33435–43.

(24) Vilardaga, J. P., Bünemann, M., Krasel, C., Castro, M., and Lohse, M. J. (2003) Measurement of the millisecond activation switch of G protein-coupled receptors in living cells. *Nat. Biotechnol. 21*, 807–12.

(25) Robia, S. L., Flohr, N. C., and Thomas, D. D. (2005) Phospholamban pentamer quaternary conformation determined by in-gel fluorescence anisotropy. *Biochemistry 44*, 4302–11.

(26) Marks, K. M., and Nolan, G. P. (2006) Chemical labeling strategies for cell biology. *Nat. Methods 3*, 591–6.

(27) Cao, H., Xiong, Y., Wang, T., Chen, B., Squier, T. C., and Mayer, M. U. (2007) A red cy3-based biarsenical fluorescent probe targeted to a complementary binding peptide. *J. Am. Chem. Soc. 129*, 8672–3.

(28) Spagnuolo, C. C., Vermeij, R. J., and Jares-Erijman, E. A. (2006) Improved photostable FRET-competent biarsenical-tetracysteine probes based on fluorinated fluoresceins. *J. Am. Chem. Soc. 128*, 12040–1.

(29) Vilardaga, J. P., Nikolaev, V. O., Lorenz, K., Ferrandon, S., Zhuang, Z., and Lohse, M. J. (2008) Conformational cross-talk between α_{2A}-adrenergic and μ-opioid receptors controls cell signaling. *Nat. Chem. Biol. 4*, 126–31.

(30) Zürn, A., Zabel, U., Vilardaga, J. P., Schindelin, H., Lohse, M. J., and Hoffmann, C. (2009) Fluorescence resonance energy transfer analysis of α_{2A} -adrenergic receptor activation reveals distinct agonist-specific conformational changes. *Mol. Pharmacol. 75*, 534–41.

(31) Wilkins, B. J., Yang, X., and Cropp, T. A. (2009) Photochemical control of FlAsH labeling of proteins. *Bioorg. Med. Chem. Lett. 19*, 4296–8.

(32) Krasel, C., Bunemann, M., Lorenz, K., and Lohse, M. J. (2005) β-Arrestin binding to the β_2-adrenergic receptor requires both receptor phosphorylation and receptor activation. *J. Biol. Chem. 280*, 9528–35.

BC900394J