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Selective Covalent Labeling of Tag-Fused GPCR Proteins on Live Cell Surface with a Synthetic Probe for Their Functional Analysis

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Abstract: Selective protein labeling with a small molecular probe is a versatile method for elucidating protein functions in living cells. In this paper, we report a covalent labeling method of tag-fused G-protein coupled receptor (GPCR) proteins expressing on cell surfaces utilizing small functional molecules. This method employs the selective and rapid reaction of a peptide tag and a molecular probe, which comprises the cysteine-containing short CA6D4x2 tag (CAAAAAADDDDGDDDD) and a tetranuclear Zn(II)-DpaTyr probe containing a reactive α-chloroacetyl moiety. The covalent labeling of tag-fused GPCRs such as bradykinin receptor (B2R) and acetylcholine receptor (m1AchR) selectively proceeded under physiological conditions during short incubation (10−30 min) with Zn(II)-DpaTyr probes bearing various functional groups. Labeling with fluorophore-appended Zn(II)-DpaTyr probes enabled visualization of the GPCRs on the surface of HEK293 cells by fluorescence. Labeling with the biotin-appended probe allowed introduction of a biotin unit into the GPCRs. This biotin label was utilized for fluorescence bioimaging studies and postlabeling blotting analysis of the labeled GPCRs by use of the specific biotin−streptavidin interaction. The utility of this labeling method was demonstrated in several function analyses of GPCRs, such as fluorescence visualization of the stimuli-responsive internalization of GPCRs and pH change in endosomes containing the internalized GPCRs.

Introduction

Membrane proteins on cell surfaces play significant roles in myriad biological processes. Most of these proteins are classified into several groups based on their functions such as membrane receptor protein, ion channel, or transporter. These proteins can transduct biological signals or transport biological substances from the outside to the interior of cells or vice versa in response to extracellular signals and maintain cell homeostasis.[1] The specific labeling of a protein with a functional molecule such as a luminescent marker, affinity handle, or activity modulator is one of the most powerful techniques for the functional analysis of these proteins. Unlike the use of fluorescence proteins or luciferases as genetically incorporated luminescent markers, labeling methods with small probes allow the post-translational introduction of a variety of functional molecules into a target protein at any appropriate time with controllable labeling efficiency. This approach significantly facilitates the functional analysis of proteins based on various analytical techniques.[2] Recently, a number of protein labeling methods using small molecular probes have been developed.[3] Most of these labeling methods rely on enzyme-catalyzed reactions, in which the enzyme is genetically incorporated into the target protein, thus, permitting specific reaction with the labeling probe. The most successful example to date is the SNAP-tag system developed by Johnsson et al., which employs an irreversible enzymatic reaction between O^6-alkylguanine-DNA alkyltransferase and O^6-benzylguanine derivatives.[4] Another protein labeling method utilizes an artificial molecular recognition system composed of a short peptide tag and a small molecular probe. The pioneering work of Tsien et al. led to the development of the first specific binding pair, consisting of a genetically encodable tetra-cysteine

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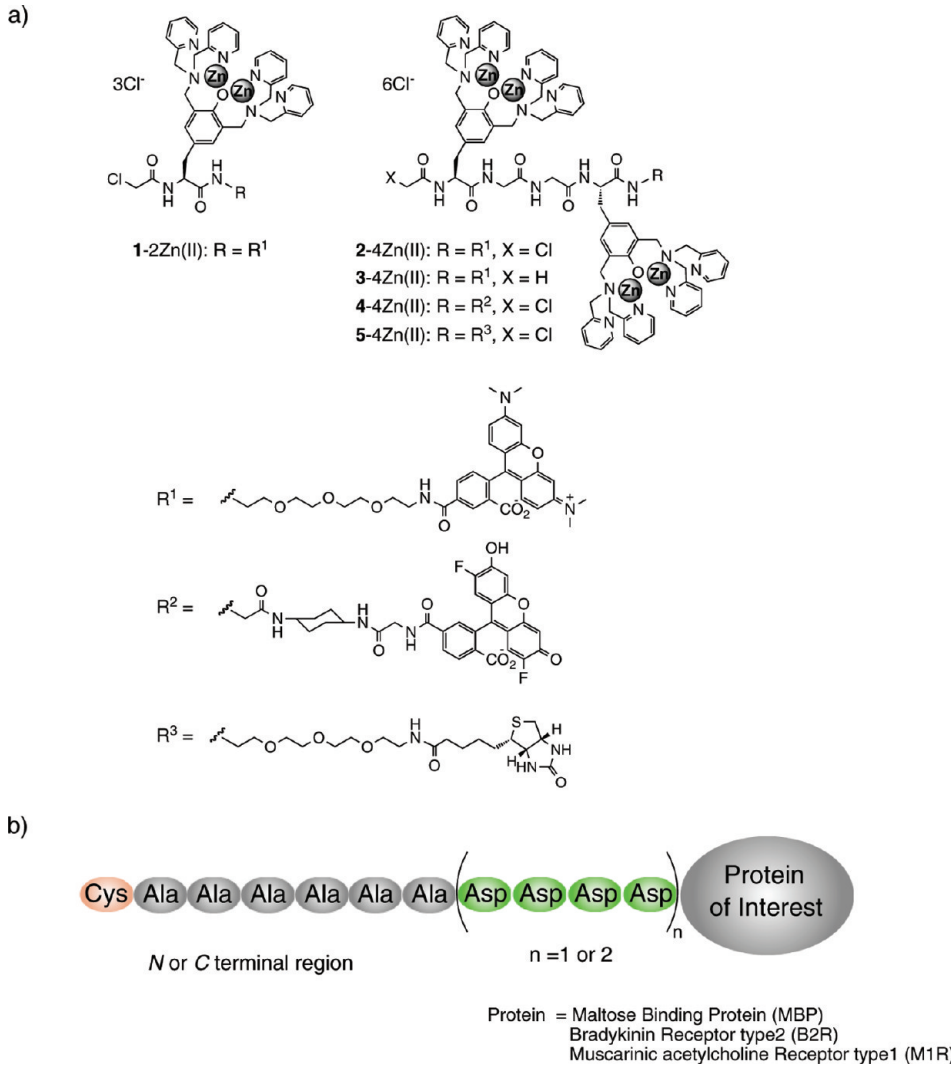

Figure 1. (a) Molecular design of the labeling probes. (b) Sequences of the CA6D4 and CA6D4x2 tag tethered to a protein of interest.

tag (-Cys-Cys-X-X-Cys-Cys-) and a bisarsenical probe (FlAsH).[5] More recently, several tag-probe pairs have been devised and applied to the functional analysis of membrane proteins, mainly for fluorescence bioimaging studies.[6,7] These labeling methods benefit from the small molecular size of both the tag and the probe: since these protein labels are small, they are less likely to perturb protein function and protein−protein interactions, in contrast to large fluorescent proteins and enzymes.

We recently developed a complementary recognition pair composed of an oligo-aspartate tag (D4 tag; DDDD) and multinuclear Zn(II) complexes (Zn(II)-DpaTyrs) as a new tag-probe system for the selective labeling of membrane proteins.[7] Building upon this preliminary system, we further developed a method for covalently labeling a tag-fused protein with a Zn(II)-DpaTyr probe such as **1**−2Zn(II) (Figure 1).[8] In this method, the desired binding complex is formed and a specific nucleophilic reaction takes place between the cysteine residue incorporated into the reactive D4 tag (CA6D4 tag; CAAAAAAD-DDD) and the α-chloroacetyl group of the probe. This nonenzymatic labeling method, based on artificial tag-probe pairs, has several potential advantages over the previous enzyme-catalyzed labeling methods: there is no need to incorporate a large enzyme/protein domain into target proteins, a wider range of labeling reagents are available, and labeling conditions are independent of the enzyme properties. Although we demonstrated the utility of this covalent labeling method in the *in vitro* modification of soluble proteins, its applicability to cellular proteins and cell functional analyses was not successful. In this manuscript, we report that by utilizing the multivalency effect, our covalent labeling method is effective and selective for covalently modifying G-protein coupled receptor (GPCR) proteins fused with a short tag (14-amino acid) at their outer

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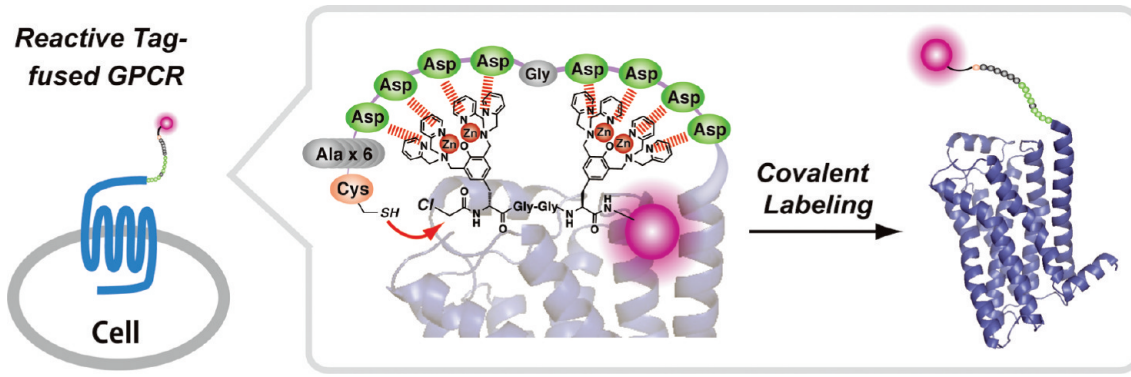

Figure 2. Selective covalent labeling of a tag-fused GPCR protein on the surface of living cells.

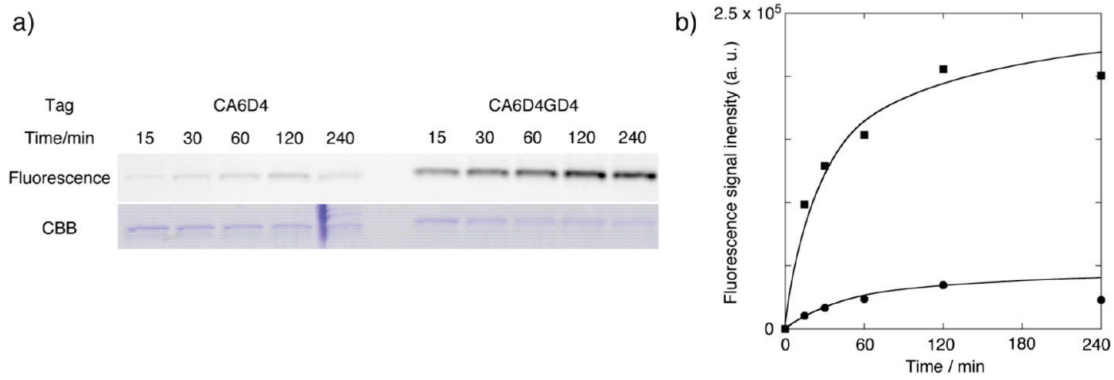

Figure 3. Comparison of the labeling efficiency of the two labeling reactions: MBP-CA6D4x2 with the dimeric probe **2**−4Zn(II) and MBP-CA6D4 with the monomeric probe **1**−2Zn(II). (a) In-gel fluorescence (upper) and CBB (lower) analyses of the labeling reactions. (b) Time-course plot of the fluorescence signal intensity of MBP protein labeled with **2**−4Zn(II) (■) and **1**−2Zn(II) (●). Conditions: 0.5 μM MBP protein, 2 μM probe in 50 mM HEPES buffer, 100 mM NaCl, and 100 μM DTT, pH 7.2, 4 °C.

membrane N-termini (Figure 2). The high specificity and stability of the covalent labeling, as well as the availability of various functional probes, allows for fluorescence imaging with various fluorophores as well as postlabeling analysis of the labeled GPCRs. We further demonstrate that this labeling method is applicable to several functional analyses of GPCRs, such as the fluorescence visualization of agonist-stimulated internalization of GPCRs and the subsequent pH change of the endosome interior containing the internalized GPCRs.

Results and Discussion

Design of Tag-Probe Pair for More Efficient Covalent Labeling. In our previous report, we conducted labeling reactions using a rather high concentration of the tag-fused protein (≥5 μM) *in vitro* because the binding affinity of the Zn(II)-DpaTyr probe **1**−2Zn(II) for the D4 tag (DDDD) is relatively weak (K_{app} = ∼10^5 M^{-1}).[8] We assumed that it would be necessary to enhance the binding affinity of the tag-probe pair in order to achieve effective labeling of a tag-fused GPCR that usually expresses on cell surfaces at rather low concentration. We previously reported that the binding affinity of the tag-probe pair was significantly enhanced by doubling the number of each binding site due to the multivalent effect.[7,9] For example, the binding affinity between the doubly repeated D4 tag (D4x2 tag; DDDDGDDDD) and the dimeric Zn(II)-DpaTyr probe **2**−4Zn(II) was increased up to 1.5 × 10^7 M^{-1}, which is over 100-fold stronger than the monomeric binding pair (K_{app} = ∼10^5 M^{-1}). Prior to the development of GPCR labeling on cell surfaces, labeling efficiency was preliminarily evaluated in *in vitro* reactions using soluble maltose binding protein (MBP) tethered to a CA6D4x2 tag (CAAAAAADDDDGDDDD, MBP-CA6D4x2) or a CA6D4 tag (CAAAAAADDDD, MBP-CA6D4) at its C-terminus, both of which have an optimized number of the alanine spacer (A6) between the reactive cysteine and the D4 sequence.[8] Figure 3 shows a comparison of the labeling efficiency of the two reactions, that is, MBP-CA6D4x2 with the dimeric probe **2**−4Zn(II) and MBP-CA6D4 with the monomeric probe **1**−2Zn(II), at a lower protein concentration (0.5 μM) than the previous labeling conditions which used ≥5 μM tag-fused protein. It is apparent by in-gel fluorescence analysis that the labeling reaction of MBP-CA6D4x2 with **2**−4Zn(II) rapidly proceeds and almost saturates within 60 min, even at low temperature (4 °C). This is in contrast to the monomer pair of MBP-CA6D4 and **1**−2Zn(II), in which the fluorescence intensity of the labeled MBP-CA6D4 is much lower, even after 240 min. These results indicate that the stronger binding of the dimer pair effectively facilitates the labeling reaction, whereas the interaction of the monomer pair is inefficient at lower concentrations due to the weak binding affinity (K_{app} = ∼10^5 M^{-1}). We, thus, decided to employ the dimer pair for covalent modification of GPCR proteins expressing on living cell surfaces.

Labeling of GPCRs on Living Cell Surfaces. Bradykinin receptor type2 (B2R) and porcine m1 type acetylcholine receptor (m1AchR) were used as target GPCRs for our covalent labeling method. B2R represents one of the most important GPCRs and

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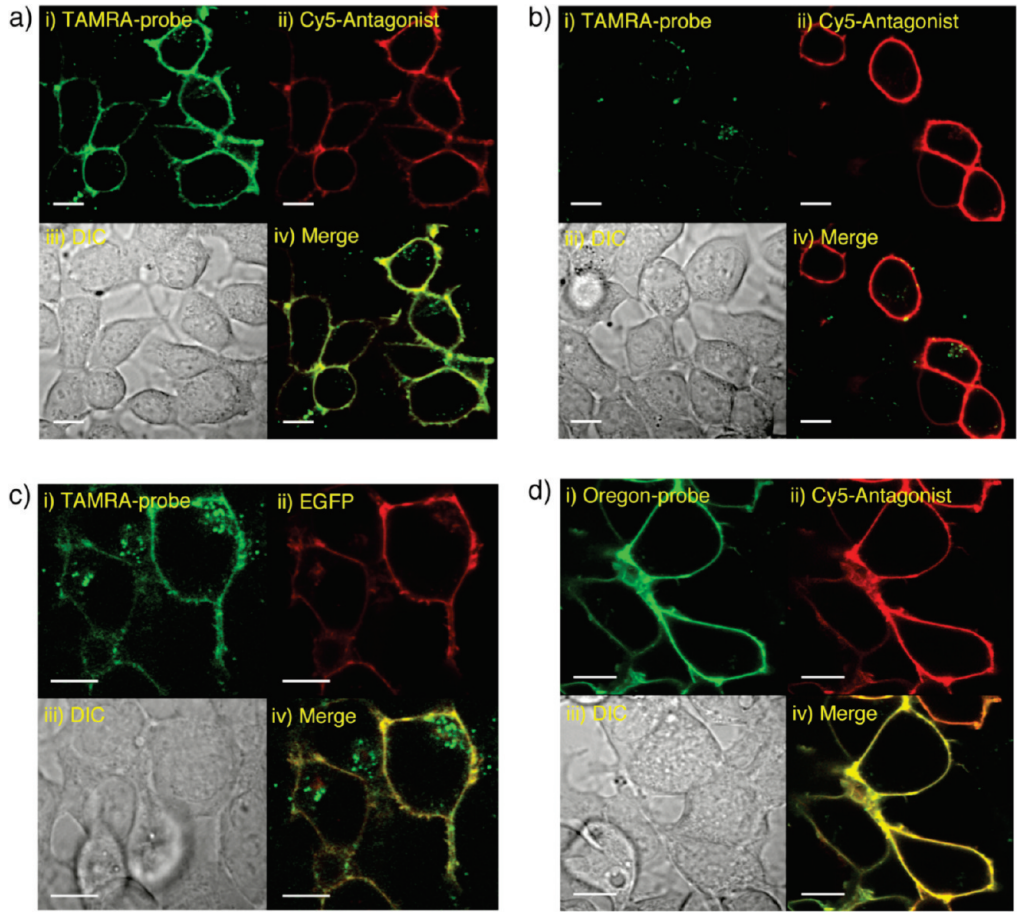

Figure 4. Covalent labeling of GPCR expression on the surface of HEK293 cells. Fluorescence imaging of CA6D4x2 tag-fused B2R (a), B2R lacking CA6D4x2 tag (b), and CA6D4x2 tag-fused m1AchR (c) by labeling with TAMRA-appended probe **2**−4Zn(II). (d) Fluorescence imaging of CA6D4x2 tag-fused B2R receptor by labeling with Oregon Green-appended probe **4**−4Zn(II). In each labeling experiment, the fluorescence images were obtained using two different channels corresponding to the probe (i) and a protein expression marker (ii) such as Cy5-appended antagonist peptide or EGFP. The transmission image is shown in (iii), and the overlay image of (i) and (ii) is shown in (iv). Scale bar is 10 μm.

has been extensively studied with respect to its fundamental cell biology, and as a therapeutic target for treating diseases.[10] For the labeling experiment, a human B2R tethered to a CA6D4x2 tag (-CAAAAAADDDDGDDDD-) at the exoplasmic N-terminus was transiently expressed in HEK293 cells. We employed TAMRA-appended probe **2**−4Zn(II) for the labeling reaction; this probe is cell impermeable and, thus, suitable for labeling cell surface proteins. Short incubation (10 min) of the cells with **2**−4Zn(II) (1 μM) enabled fluorescence imaging of the tag-fused B2R on the cell surfaces. However, this fluorescence intensity was significantly diminished upon washing with HBS buffer containing a high concentration of pyrophosphate (PPi, 1 mM), a strong competitive binder for Zn(II)-DpaTyrs. This result indicates that the **2**−4Zn(II) probe binds reversibly through metal−ligand interactions, and does not form a stable covalent bond with the cysteine residue of the tag. After a series of trials, we obtained durable fluorescence images when labeling was conducted after short treatment (10 min) with the reducing agent, tris-carboxyethylphosphine (TCEP) (Figure 4). The effectiveness of this reductive pretreatment strongly suggests that the cysteine residue of the tag, which would be deactivated under extracellular oxidative conditions, is reactivated as a nucleophile. Fluorescence remained after extensive washing with a high concentration of inorganic pyrophosphate (1 mM), indicating that B2R was covalently labeled. A 10-min labeling period was thus sufficient to obtain a fluorescence signal of B2R by confocal laser-scanning microscopy. The labeling specificity for B2R was confirmed by co-staining with a B2R antagonist peptide appended with a Cy5 fluorophore. Fluorescence labeling detectable by the Cy5 and TAMRA channels predominantly overlapped, as shown in Figure 4a. A control labeling experiment with B2R lacking the CA6D4x2 tag produced negligible fluorescence labeling in the TAMRA channel (Figure 4b), which was quantitatively evaluated to be less than 3% relative to the intensity observed in Figure 4a. Another control labeling experiment using the tag-fused B2R and a nonreactive probe lacking an α-chloroacetyl group **3**−4Zn(II) also afforded no detectable fluorescence labeling in the TAMRA channel (Figure S1). The selective fluorescence detection of the tag-fused B2R by **2**−4Zn(II), together with the two control experiments, indicated that, on living cell surfaces, the covalent labeling reaction occurs site-specifically between the tag fused to B2R and the α-chloroacetyl group of the probe. The cell viability assay using trypan blue revealed that the cells maintain a high viability rate (>98%) within 24 h after the labeling with **2**−4Z(II) (Figure S2), indicating that the labeling treatment with TCEP and the probe did not exhibit significant cell toxicity and cell death induction.

This labeling method is applicable to another GPCR, m1AchR,[11] which has a CA6D4x2 tag and an enhanced green

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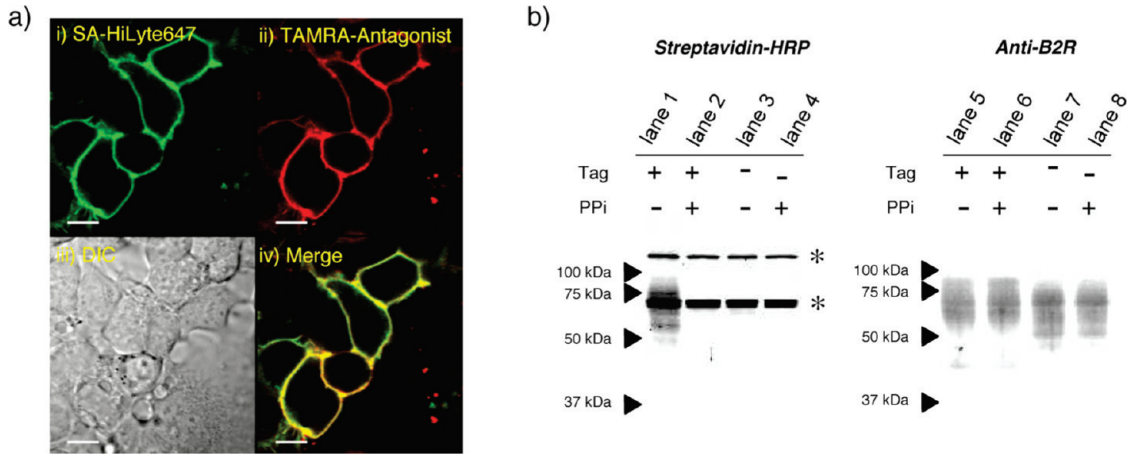

Figure 5. (a) Fluorescence imaging of CA6D4x2 tag-fused B2R by labeling with biotin-appended probe **5**−4Zn(II) and subsequent visualization with SA-HiLyte647. The fluorescence images were obtained using two different channels corresponding to HiLyte647 (i) and TAMRA appended to the B2R antagonist (ii). The transmission image is shown in (iii), and the overlay image of (i) and (ii) is shown in (iv). Scale bar is 10 μm. (b) Blotting analyses of biotin-labeled B2R by chemiluminescence detection using streptavidin−HRP conjugate (lanes 1−4) and Western blotting using anti-B2R antibody (lanes 5−8). The two bands marked with asterisk derive from endogenous biotinated proteins. The labeling reaction was conducted in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of PPi (1 mM).

fluorescent protein (EGFP) domain at the exoplasmic N-terminus. Fluorescence labeling of m1AchR with **2**−4Zn(II) was carried out by the same procedure as used for B2R labeling. As shown in Figure 4c, clear fluorescence labeling in the TAMRA channel was detected on the surface of HEK293 cells expressing m1AchR, which co-localized well with EGFP fluorescence. In the control experiment, HEK293 cells expressing m1AchR which lacked the CA6D4x2 tag showed negligible fluorescence labeling of **2**−4Zn(II) (Figure S3), demonstrating again the tag-specific labeling of GPCRs.

This protein labeling method allowed for the introduction of various functional molecules into the tag-fused GPCR. When we used Zn(II)-DpaTyr probe **4**−4Zn(II), which contains Oregon Green 488 as the fluorophore, labeling of tag-fused B2R with **4**−4Zn(II) proceeded smoothly to provide clear fluorescence images in the Oregon Green channel (Figure 4d). Proteins labeled with distinct fluorescence colors are essential for multicolor imaging in cells. The biotin-appended probe **5**−4Zn(II) is also available for GPCR labeling; this was confirmed by treatment of biotin-labeled B2R with streptavidin modified with the near-infrared HiLyte647 dye, SA-HiLyte647. A bright and clear fluorescence image was obtained due to strong florescence from the multiple HiLyte647 dye molecules appended to streptavidin (Figure 5a). The biotin unit is also useful for the postlabeling analysis of GPCRs. For example, HEK293 cells displaying labeled B2R were lysed and subjected to biotin blotting. Figure 5b shows a distinctive smear band in the range from 75 to 50 kDa (lane 1), which was coincident with the Western blotting pattern obtained using anti-B2R antibody (lane 5).[12] Control labeling experiments using PPi as an effective inhibitor of the tag-probe binding (lanes 2 and 4) or B2R lacking a CA6D4x2 tag (lanes 3 and 4) gave negligible bands upon biotin blotting analysis. These results indicate that the labeling reaction with **5**−4Zn(II) specifically occurs with tag-fused B2R. It is noteworthy that such postlabeling analysis is practically impossible with reversible labeling based on noncovalent tag-probe interactions, clearly demonstrating the advantage of the present method in protein analyses.

Evaluation of Labeling Efficiency of GPCR. Careful analysis of fluorescence images of living cells allowed quantitative evaluation of the labeling rate of GPCRs. After treatment of HEK293 cells transiently expressing B2R with **2**−4Zn(II), the pixel-by-pixel scatterplot between two fluorescence channels corresponding to TAMRA of the probe and Cy5 of the B2R antagonist peptide gave a distribution graph (Figure 6a), which was analyzed by linear curve-fitting to give the slope value. Since the slope, which is the averaged fluorescence intensity of the probe based on the antagonist fluorescence, increased as the labeling reaction proceeded, we can simply define the slope as the relative labeling ratio (r). The r values obtained at different labeling times are shown in Figure 6b. For the labeling reaction between the CA6D4x2 tag-fused B2R with **2**−4Zn(II), the r value increased significantly in a time-dependent manner and reached a plateau after 30 min. The change in the r value agrees well with a theoretical saturation curve based on pseudo-first-order reaction. After 60 min, reductive treatment with TCEP followed by a second labeling with a different probe, **3**−4Zn(II), did not alter fluorescence labeling of B2R, suggesting that the labeling reaction with **2**−4Zn(II) was essentially quantitative.[13] In contrast, for the first labeling reaction with the nonreactive probe, **3**−4Zn(II), which lacks an α-chloroacetyl group, a negligible change was induced in the r value during the reaction,

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(13) To confirm the labeling reaction efficiency for the overexpressed tag-fused B2R in live cells, we quantitatively evaluated its expression number using fluorescence microscopy analysis (Supporting Information, Table S1, and Figure S4). On the basis of the local concentration of B2R on the cell surface, the number of B2R was estimated to be $6.3 \pm 1.3 \times 10^4$/cell, which is comparable to a reported value (1.43×10^4/cell) evaluated in a cell line of HEK293 stably expressing the native B2R (Lamb, M. E. et al., *Biochem. J.* **2001**, *355*, 741.). In the fluorescence microscopy analysis for the quantification, the strong fluorescence of the Cy5-appended B2R antagonist was selectively detected on the cell surface, indicating that B2R is not homogeneously distributed in solution. Indeed, we evaluated the local concentration of B2R on the cell surface as 25.0 ± 5.2 μM, whereas its formal concentration in solution is calculated to be a significantly lower value (57 ± 12 nM), suggesting that B2R is concentrated by ca. 500-fold on the cell surface. These results may imply that the substantially high concentration of B2R on the cell surface largely contributes to the efficient labeling of B2R.

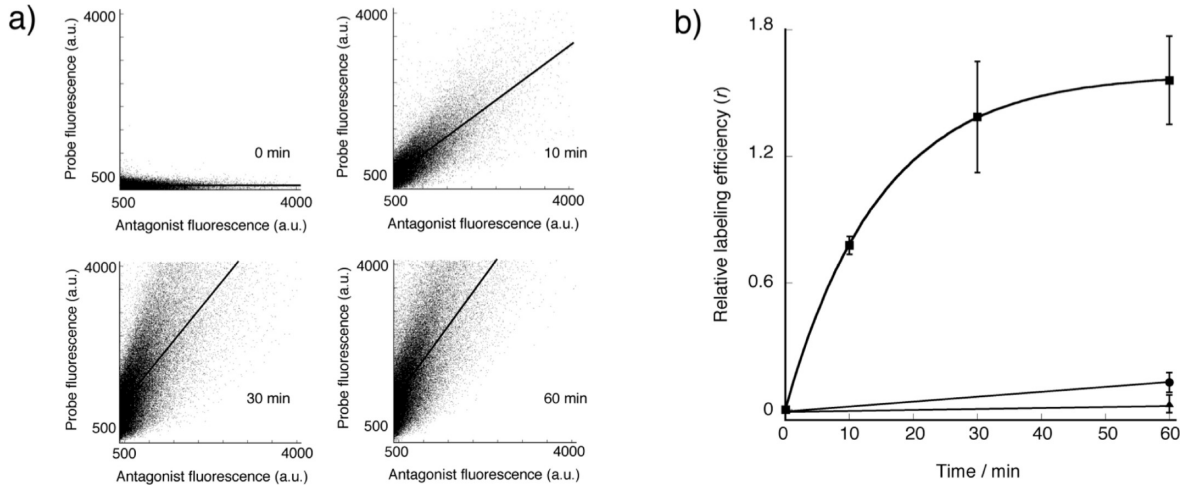

Figure 6. (a) Pixel-by-pixel scatterplot analysis of fluorescence images of cells using two fluorescence channels corresponding to TAMRA appended to **2**−4Zn(II) and Cy5 appended to the B2R antagonist peptide. Linear curve-fitting analysis of the scatterplot gives the slope value defined as the relative labeling efficiency (r) at each labeling time (0, 10, 30, and 60 min). (b) Time-course of the relative labeling efficiency (r) in the labeling reaction of tag-fused B2R with **2**−4Zn(II) (■), **1**−2Zn(II) (●), and **3**−4Zn(II) (▲).

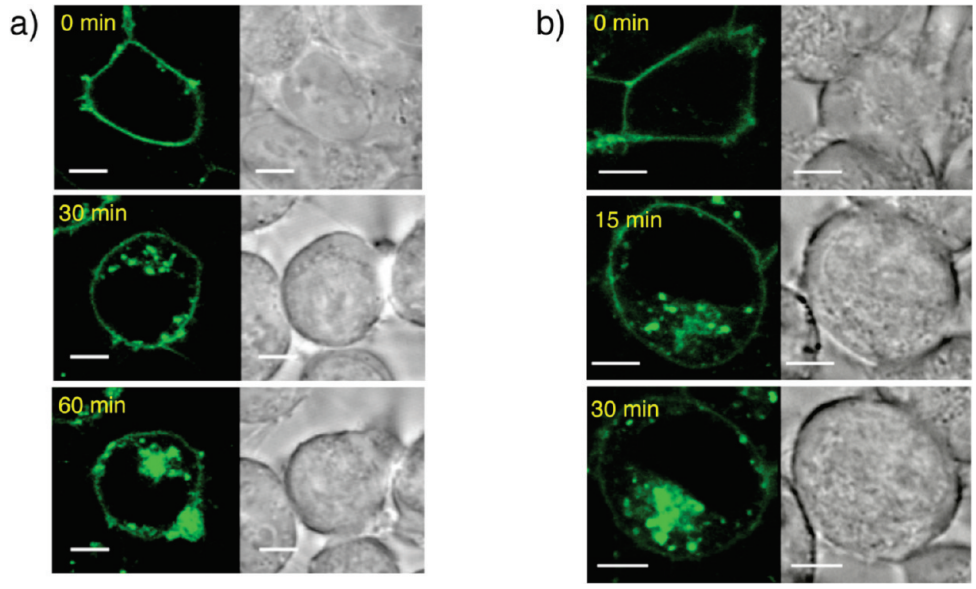

Figure 7. Time-lapse fluorescence imaging of the internalization of CA6D4x2 tag-fused B2R (a) and m1AchR (b) labeled with **2**−4Zn(II) by agonist stimulation with bradykinin (200 nM) and calbachol (100 μM), respectively. Scale bar is 5 μm.

indicating no labeling of B2R. A slight increase in the r value was observed during the labeling reaction with the monomeric probe **1**−2Zn(II) after 60 min ($r = 0.13$), but this value is approximately 12-fold less than that obtained with **2**−4Zn(II) after the same reaction time ($r = 1.56$). This result indicates that the labeling efficiency of the monomer-type probe **1**−2Zn(II), is much lower than that of the dimer probe **2**−4Zn(II), consistent with the *in vitro* labeling experiment using the tag-fused MBP a s shown in Figure 3. We, thus, concluded that the strong binding between the CA6Dx2 tag and a tetranuclear Zn(II)-DpaTyr probe such as **2**−4Zn(II), supported by the multivalency effect, is essential for the efficient covalent labeling of B2R expressing on cell surfaces.

Fluorescence Visualization of GPCR Internalization. It is well-known that many GPCRs expressing on the cell surface migrate into the cytosol as components of endosome particles, induced by strong stimulation with the corresponding agonists. This phenomenon, called internalization, is an important regulatory mechanism of GPCR-mediated cell signaling.[14] We examined fluorescence visualization of GPCR internalization to demonstrate the utility of this labeling method for analyzing dynamic cell functions involving GPCR. Figure 7a shows time-lapse imaging of agonist-induced internalization of CA6D4x2 tag-fused B2R labeled with TAMRA-appended probe **2**−4Zn(II) in HEK293 cells. Cell stimulation with bradykinin (200 nM), a native agonist peptide of B2R,[15] clearly induced the intracellular movement of fluorescent endosome particles containing labeled B2R after 30 min; by 60 min, these particles were localized in the cytosol. Such dynamic fluorescence change was not observed during the same time frame (60 min) in the absence of

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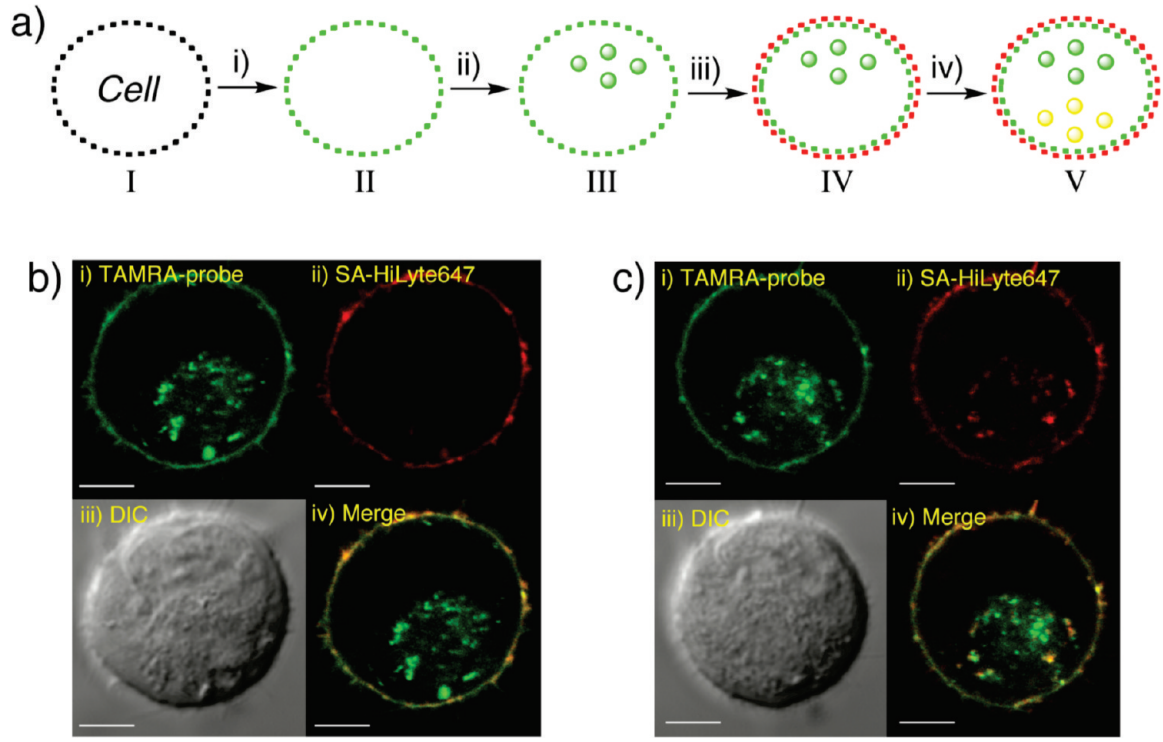

Figure 8. Pulse-chase visualization of internalized B2R involving endosomes. (a) Schematic representation of the labeling procedure (i−iv) and the merged fluorescence image in each step (I−V); (i) labeling of CA6D4x2 tag-fused B2R with **2**−4Zn(II) and **5**−4Zn(II), (ii) first internalization induced by bradykinin, (iii) fluorescence visualization of the biotin-labeled B2R with SA-HiLyte647, and (iv) second internalization induced by bradykinin. (b and c) Fluorescence images of labeled B2R in steps IV (b) and V (c). The images were obtained using two different channels corresponding to TAMRA appended to **2**−4Zn(II) (i) and SA-HiLyte647 (ii). The transmission image is shown in (iii), and the overlay image of (i) and (ii) is shown in (iv). Scale bar is 5 μm.

stimulation (Figure S5), indicating that the phenomena shown in Figure 7a is agonist-dependent internalization. Agonist-induced internalization of m1AchR was also clearly visualized (Figure 7b). In the time-lapse imaging, many fluorescent endosome particles containing m1AchR labeled with **2**−4Zn(II) were gradually internalized into the cytosol of HEK293 cells upon stimulation with carbachol (100 μM).[16] These two examples demonstrate that our protein labeling method is useful for fluorescence monitoring of GPCR internalization. Significantly, fundamental functions such as agonist binding ability are not noticeably compromised, and the agonist-induced conformational changes involving stimulus-responsive oligomerization, essential to GPCR internalization, are also unaffected. To further confirm whether the labeling with the probe exerts any influence on the B2R function, we measured the receptor activated Ca^{2+} signaling of the HEK293 cells. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which was monitored by the ratio of the fura-2 emission excited at 340 and 380 nm (ex340/ex380), increased upon the addition of bradykinin (100 nM). The ($[Ca^{2+}]_i$) responses observed in the B2R labeled with **2**−4Zn(II) were nearly identical to those in the nonlabeled B2R (Figure S6), indicating that the labeling with the probe does not interfere with the original activity of B2R in the intracellular signaling. Interestingly, only a subtle $[Ca^{2+}]_i$ response was observed in the case of the B2R fused with EGFP at the exoplasmic N-terminus, indicative of functional impairment of the B2R against the agonist stimulation. These results suggest an advantage of the present labeling method over fluorescent proteins fusion method in the point of less disturbance of protein function, which might be ascribed to the smaller size of the tag and probe pair than EGFP.

We next performed pulse-chase visualization of the internalized B2R by labeling using two different probes (Figure 8). First, the CA6D4x2 tag-fused B2R expressing on HEK293 cells was simultaneously labeled with the TAMRA-appended probe **2**−4Zn(II) and the nonfluorescent biotin-appended probe **5**−4Zn(II) (step II in Figure 8a). The cells were then treated with bradykinin to induce agonist-dependent internalization (step III in Figure 8a) followed by SA-HiLyte647 to fluorescently visualize B2R labeled with **5**−4Zn(II). B2R on the cell surfaces was detectable by both the TAMRA (green) and HiLyte647 (red) fluorescence channels, whereas the endosome particles containing the internalized B2R were selectively visualized by TAMRA fluorescence inside the cells (step IV in Figure 8a,b). A second bradykinin stimulation was then conducted to induce the second internalization (step V in Figure 8a), followed by detection of the newly internalized endosomes by fluorescence of HiLyte647 (red) inside the cells (Figure 8c-ii). In the merged image of the two fluorescence channels (Figure 8c-iv), the first internalized endosomes were visualized as green, whereas the second internalized endosomes were selectively visualized as yellow particles due to co-labeling of B2R with both green and red fluorophores. Therefore, pulse-chase visualization using two different probes allows for fluorescent discrimination of B2R-containing endosomes internalized at different times.

Fluorescence Detection of Interior Environment of Endosome. It is known that the interior environment of endosomes is different from the environment in the cytosol. For example, endosomal pH gradually decreases from neutral to pH 5−6 between the early and late stages of endocytosis.[17] Fluorescence analysis of the interior environment of endosomes

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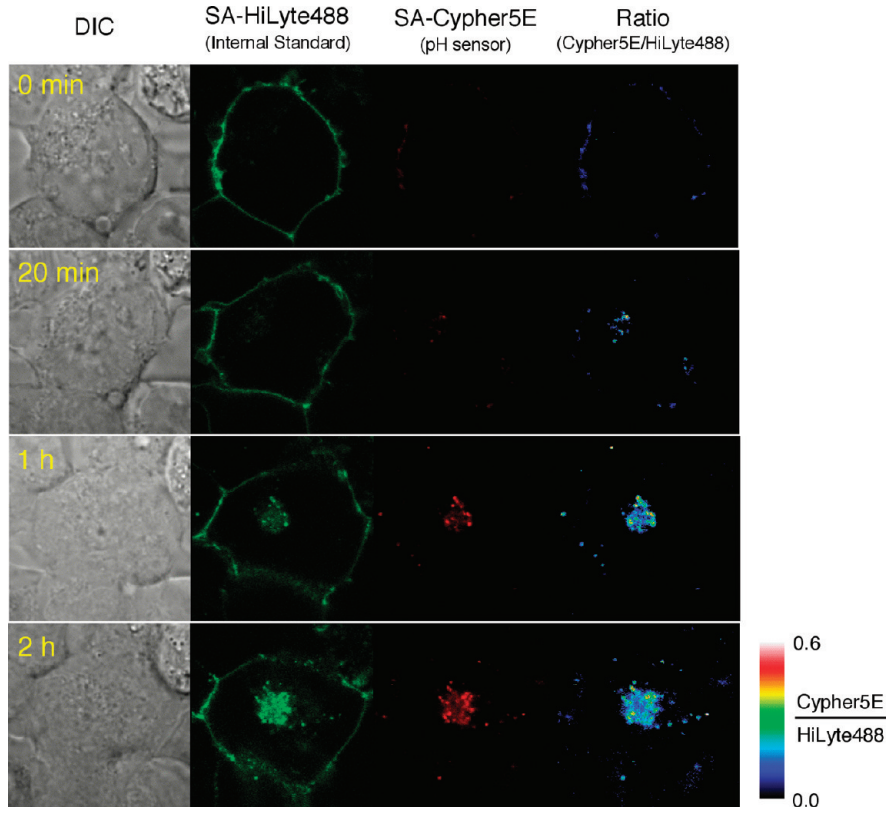

Figure 9. Time-lapse fluorescence detecting endosomal pH change using a pH-responsive fluorophore, Cypher5E, linked to tag-fused B2R. Each column shows a transmission image, the fluorescence channel for HiLyte488, the fluorescence channel for Cypher5E, and the ratio image of the two fluorescence channels (CypHer5E/HiLyte488) from left to right.

by incorporation of environmentally sensitive probes is possible using our GPCR labeling method. We attempted to ratiometrically detect the change in endosomal pH using a pH-responsive fluorophore (Figure 9). After introducing a biotin unit into tag-fused B2R by **5**−4Zn(II), simultaneous labeling with two different streptavidins (SAs) (appended to pH-responsive CypHer5E or to pH-insusceptible HiLyte488) was conducted to afford the dual-emission pH sensing system. Initially (0 h), clear fluorescence due to HiLyte488 was observed on the cell surfaces, whereas no fluorescence of CypHer5E was detected because the fluorophore existed in the nonfluorescent proton-free form (pK_a = 7.3) under the slightly basic buffered conditions (pH 8.0).[18] After internalization stimulated by bradykinin, fluorescence of CypHer5E inside the cells gradually intensified over a 2-h period, co-localizing with that of HiLyte488. This result indicates that SA-CypHer5E fluorescently senses the decrease in endosomal pH due to the formation of the fluorescent protonated form. The acidic pH shift inside the endosome particles was also detectable by ratiometric analysis of the two fluorescence images (CypHer5E/HiLyte488), which more precisely visualizes the distribution of acidic endosomes inside cells compared to the single channel image due to CypHer5E.

Conclusion

In conclusion, we demonstrated a useful labeling method for selective covalent modification of GPCR proteins expressing on cell surfaces. The advantages of this nonenzymatic labeling method include (i) a simple labeling procedure with a short reaction time (10−30 min); (ii) flexible availability of the functional molecular probes, which enables fluorescence imaging of GPCRs with various fluorophores and postlabeling biotin-blotting analysis; and (iii) small molecular size of the tag and probe, which allows agonist-induced internalization without significant perturbation of GPCR functions. We also demonstrated the applicability of this labeling method to fluorescence analysis of GPCR internalization, and analysis of environmental changes occurring in the interior of endosomes. To our knowledge, our labeling method is the first to allow functional modification of cell surface proteins by a nonenzymatic reaction between a short peptide tag and a small molecular probe. Moreover, this labeling method has potential for other cell functional analyses, such as detection of GPCR oligomerization by using a labeling probe with a photo cross-linking unit and analysis of dynamic structural changes in membrane-bound proteins using ESR probes. In addition to such fundamental researches, the present GPCR labeling and imaging in living cells may be expectedly useful for more practical applications such as convenient high-throughput screening of drug candidates targeting GPCR-involved cell functions. Research along these lines is currently underway.

Experimental Section

General Methods. Unless otherwise noted, all proteins/enzymes and reagents were obtained from commercial suppliers and used

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without further purification. Streptavidin-HiLyte647 and HiLyte488 were obtained from AnaSpec, Inc. Bradykinin and carbachol were obtained from Wako Pure Chemical Industries.

Covalent Labeling of the CA6D4-Tagged MBP and CA6D4x2-Tagged MBP. A solution of MBP (500 nM in 50 mM HEPES, 100 mM NaCl, 0.1 mM DTT, pH 7.2) was mixed with **1**−2Zn(II) or **2**−4Zn(II) (2 μM in final concentration). The mixture was incubated at 4 °C for 2 h, in which 40 μL of the mixture was sampled at the appropriate time and treated with Laemmli buffer to quench the labeling reaction. The sampling solutions were heated at 95 °C for 3 min, and subjected to SDS-PAGE. The in-gel fluorescence analysis was performed with LAS-4000 lumino image analyzer (FUJIFILM) by EPI mode (520 nm excitation, 575DF20 filter).

Cell Culture and Recombinant Protein Expression in HEK293 Cells. HEK293 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. A subculture was performed every 3−4 days from subconfluent (<80%) cultures using a trypsin−EDTA solution. Transfection of cDNA plasmids was carried out in a 35 mm glass-bottomed dish (Iwaki) using Lipofectamine LTX (Invitrogen) according to a general protocol. The cells were subjected to labeling experiments after 44−52 h of the transfection.

General Procedure for GPCR Labeling and Fluorescence Imaging. The HEK293 cells expressing the tag fused B2R ($\sim 1 \times 10^6$) were incubated with 1 mL of HEPES-buffered saline (HBS, containing 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 11.5 mM glucose, 20 mM HEPES, adjusted to pH 7.4 with NaOH) containing 0.5 mM TCEP for 10 min at room temperature. After removing TCEP solution, the cells were treated with 1 mL of HBS (pH 7.4) containing the labeling probe (0.5 μM) for 10 min at room temperature. The probe solution was removed and the cells were washed with 1.0 mL of Phosphate Buffered Saline (PBS) containing 1 mM PPi to remove the unreacted probe. After being filled with PBS containing Cy5-appended B2R antagonist peptide (0.5 μM), the cells were analyzed using a confocal laser scanning microscope (CLSM; Olympus, FLUOVIEW FV1000) equipped with a ×100 lens and the appropriate emission filters for Oregongreen488, EGFP, TAMRA, and Cy5. In the case of the imaging study using Streptavidin(SA)-HiLyte647, the cells labeled with **5**−4Zn(II) (1.0 μM) were further treated with SA-HiLyte647 (5 μg in 1 mL PBS) and subjected to the imaging analysis.

Blotting Analysis of B2R Labeling. HEK293 cells expressing the tag fused B2R were labeled with **5**−4Zn(II) by the procedure described above. The cells were collected by cell scraper and lysed with 200 μL of RIPA lysis buffer (pH 7.6 25 mM Tris·HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% Deoxycholic acid) containing 1% protease inhibitor cocktail set III (Novagen) at 4 °C for 60 min. The insoluble material was removed by centrifugation at 12 000 rpm for 10 min. The supernatant was mixed with 2× sample buffer for membrane protein (pH 6.8 125 mM Tris·HCl, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 100 mM DTT) and subjected to SDS-PAGE (10% T, 0.33% C) followed by electrotransfer onto an Immun-Blot PVDF membrane (Bio-Rad). The immunodetection of the B2R was performed with anti-B2R antibody (1:200 dilution, Santa Cruz Biotechnology) and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:5000 dilution, Santa Cruz Biotechnology). The chemiluminescence signal using Chemi-Lumi One (Nacalai Tesque) was detected with ChemiDoc XRS (BIO-RAD). After removing anti-B2R antibody by washing with the stripping buffer (pH 2.0 2.5 mM glycine, 1% SDS), the membrane was treated with HRP-conjugated streptavidin (1: 5000 dilution; Invitrogen) and subjected to the chemiluminescence detection.

Pulse-Chase Labeling of B2R Internalization. The tag-fused B2R expressing on HEK293 cell surface was labeled by the treatment with 1 mL of HBS (pH 7.4) containing 1.0 μM **2**−4Zn(II) and 2.0 μM **5**−4Zn(II). The dish was incubated for 10 min at room temperature. After removing the probe solution, the cells were washed with 1.0 mL of PBS containing 1 mM PPi. For the first internalization, the cells were stimulated with 200 nM bradykinin for 60 min at 25 °C. After washing the bradykinin solution, the cells were treated with 1 mL of PBS containing 5 μg of Streptavidin (SA)-HiLyte647. For the second internalization, the cells were stimulated by 200 nM bradykinin for 30 min at 25 °C. The CLSM analysis was performed with a ×100 lens and appropriate emission filter for TAMRA and SA-HiLyte647.

Sensing of pH Change in Endosomes Containing GPCR. The tag-fused B2R expressing on HEK293 cell surface was labeled by the treatment with 1 mL of HBS (pH 7.4) containing 1.0 μM **5**−4Zn(II). The dish was incubated for 20 min at room temperature. After removing the probe solution, the cells were washed with 1.0 mL of PBS containing 1 mM PPi and treated with 1 mL of PBS containing 2 μg of SA-HiLyte488 and 5 μg of SA-CypHer5E. After washing with PBS, the cells were stimulated with 200 nM bradykinin for 60 min at 25 °C. The CLSM analysis was performed with a ×100 lens and appropriate emission filter for TAMRA and SA-HiLyte647.

Supporting Information Available: Detailed synthetic procedures and compound characterizations, plasmid constructions, evaluation of cell toxicity and expression number of receptor protein, functional analysis of the labeled receptor, and the complete ref 3g. This material is available free of charge via the Internet at http://pubs.acs.org.

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