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Coiled-Coil Tag—Probe System for Quick Labeling of Membrane Receptors in Living Cells

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ABSTRACT The specific labeling of proteins in living cells using a genetically encodable tag and a small synthetic probe targeting the tag has been craved as an alternative to widely used larger fluorescent proteins. We describe a rapid method with a small tag (21 amino acids) for the fluorescence labeling of cell-surface receptors using a high affinity coiled-coil formation without metals or enzymes. The peptide probes K3 $(KIAALKE)_3$ and K4 $(KIAALKE)_4$ labeled with a fluorophore specifically stained the surface-exposed tag sequence E3 $(EIAALEK)_3$ attached to the N-terminus of the mouse-derived prostaglandin EP3β receptor in living cells (K_d = 64 and 6 nM for K3 and K4, respectively). The labeling was quick (<1 min), nontoxic, and available even in culture medium without affecting receptor function. As an application of this tractable method, the agonist-induced internalization of the human-derived β_2-adrenergic receptor and epidermal growth factor receptor was successfully visualized.

Genetic fusion of a fluorescent protein to a target protein for specific labeling in living cells has been widely used to investigate the intracellular trafficking, conformational change, and oligomerization of proteins (*1*). However, the use of fluorescent proteins with a considerable size (~27 kDa for GFP) may give artifactual results, for example, formation of aggregates or impairment of protein function (*2, 3*). In addition, for measurements of the oligomerization of cell surface proteins, for example, receptors, by nonradiative energy transfer between fluorophores, post-translational labeling methods with smaller fluorophores are superior to fluorescent proteins because of facile control of the donor−acceptor ratio (*4, 5*). Another advantage of post-translational labeling is that cell-surface-specific labeling is possible by use of membrane-impermeable fluorescent probes to study, for example, the membrane trafficking of receptors (*6*). Therefore, an increasing number of methods using pairs of much smaller genetically encodable tags and synthetic probes targeting the tags have emerged to specifically label proteins in living cells (see reviews (*7, 8*)). Most of these tag−probe labeling methods are based on well-known specific interactions or reactions, such as the formation of complexes between peptides and metal ions (*9–11*) or formation of covalent bonds by enzymatic reactions (*4, 5, 12, 13*), although tag sequences have been identified or optimized by screening (*14–16*). Despite an expanding repertoire of labeling techniques, each method has limitations, such as a long labeling time, low labeling specificity, little color variation, and high toxicity. For example, the HisZiFit probe and hexahistidine tag (*10*) combine to make a satisfactory small tag−probe system (~1.6 kDa), but currently only one fluorophore is available and the presence of free zinc ions (1−10 μM) is required. On the other hand, for labeling with the ACP tag (*4, 5*), several fluorophores with different colors are available, but the labeling time for the enzymatic reaction is relatively long (typically 20−40 min) and large excess amounts of substrates (5 μM) are required. In addition, the development of new labeling methods orthogonal to preexisting principles is important for multicolor labeling of different proteins.

Here we show that a heterodimeric coiled coil is useful for the rapid, nontoxic, and specific labeling of cell-surface proteins in living cells without the need for particular metals or enzymatic reactions. The heterodimeric coiled-coil peptides K3 $(KIAALKE)_3$, K4 $(KIAALKE)_4$, E3 $(EIAALEK)_3$, and E4 $(EIAALEK)_4$ originally designed by Litowski and Hodges (*17*), were tested as tag and probe. These peptides have net positive (+3 for K3 and +4 for K4) or negative (−3 for E3 and −4 for E4) charges and

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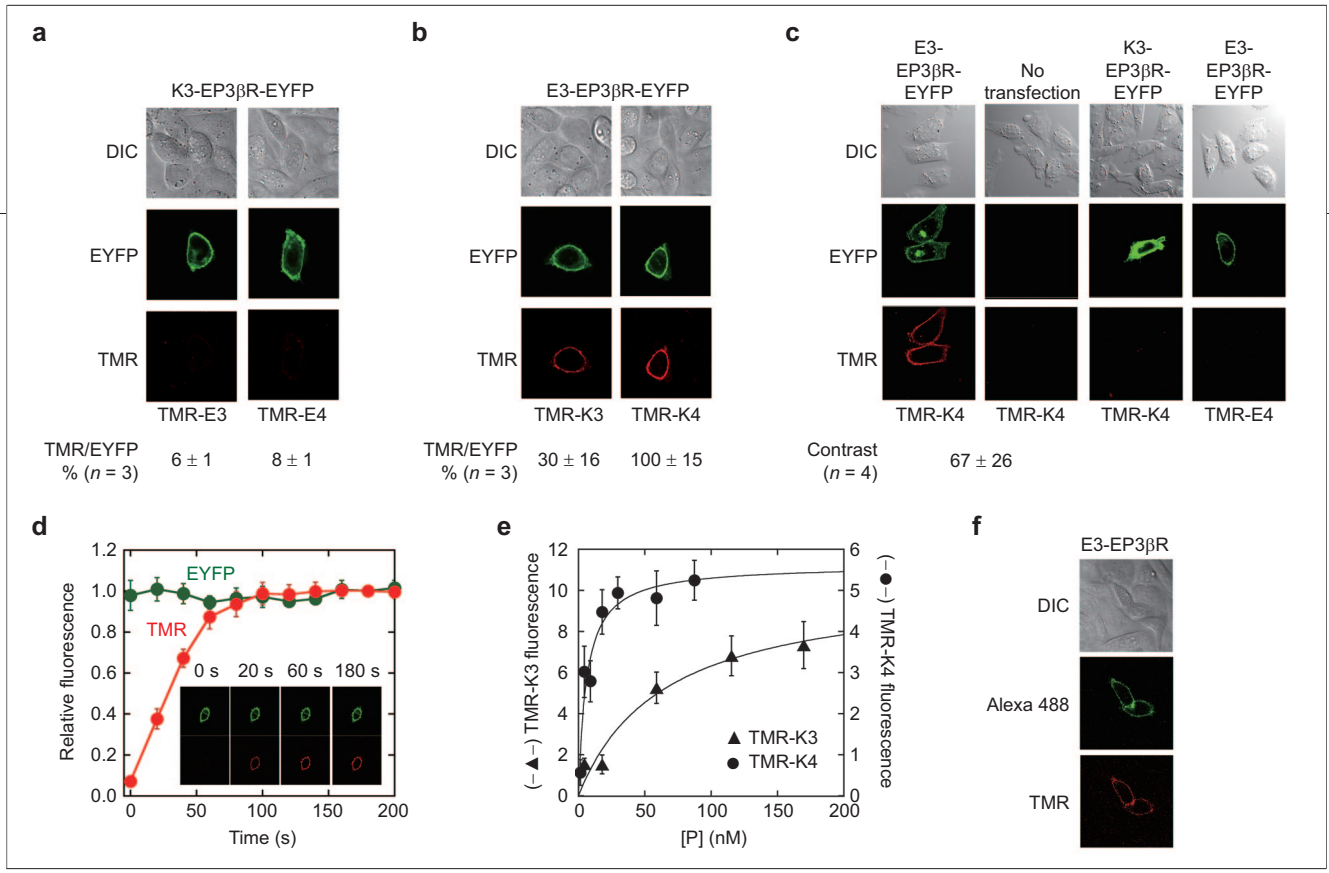

Figure 1. Labeling of membrane receptors in living cells by the coiled-coil tag. a)–c) Examination of tag–probe combinations. a) Tetramethylrhodamine-$(EIAALEK)_3$ (TMR-E3) or tetramethylrhodamine-$(EIAALEK)_4$ (TMR-E4) added to cells expressing $(KIAALKE)_3$-prostaglandin E2 receptor EP3β subtype-EYFP (K3-EP3βR-EYFP). b) Tetramethylrhodamine-$(KIAALKE)_3$ (TMR-K3) or tetramethylrhodamine-$(KIAALKE)_3$ (TMR-K4) added to Chinese hamster ovary (CHO) cells expressing $(EIAALEK)_3$-prostaglandin E2 receptor EP3β subtype-EYFP (E3-EP3βR-EYFP). Relative fluorescence intensity of TMR normalized to that of EYFP is shown below. The intensity for the TMR-K4/E3-EP3βR-EYFP combination was set to 100%. c) Labeling specificity using E3-tagged receptors and the TMR-K4 probe. Fluorescence intensity of TMR relative to that of untransfected cells (background) is shown below (contrast). The TMR-probes (20 nM) dissolved in culture medium containing 10% serum were incubated with cells for 5 min. The cells were rinsed once with the medium and observed by confocal microscopy. Differential interference contrast (DIC) images are also shown. d) Labeling kinetics for the TMR-K4 (20 nM)-E3-EP3βR-EYFP pair. Relative fluorescence intensities of EYFP and TMR on cell membranes after addition of the probe (0 s) are shown ($n = 5$). Inset: confocal images for EYFP (upper) and TMR (lower). e) Increase in TMR fluorescence intensity (FI) for TMR-K3 (▲) and TMR-K4 (●) on cell membranes as a function of probe concentration [P]. Error bars indicate standard error of the mean ($n = 10$). The dissociation constant K_d (±standard error of the mean) was obtained from the fitting, $FI = k[P]/([P] + K_d)$, where k is a constant proportional to the density of labeled receptor. f) Colabeling of $(EIAALEK)_3$-prostaglandin E2 receptor EP3β subtype (E3-EP3βR) by TMR-K4 and Alexa Fluor 488-K4. TMR-K4 and Alexa Fluor 488-K4 (25 nM each) were coincubated in the medium with the CHO cells. The cells were rinsed and observed.

therefore are expected to be membrane-impermeable. Electrostatic attraction between K and E coils drives the formation of a heterodimer whereas K–K or E–E repulsion inhibits the formation of homodimers (Supplementary Figure 1). The heterodimer composed of E3 and K3 is completely α-helical and assumes a compact coiled-coil structure, as revealed by NMR spectroscopy (*18*). The size of the heterodimers (K3–E3, K3–E4, E3–K3, and E3–K4) labeled by a fluorophore is 5–6 kDa, which is significantly smaller than that of fluorescent proteins (~27 kDa). K3 and E3 were selected for tag sequences because the longer K4 and E4 are known to self-associate at concentrations of 300–400 μM in aqueous solution (*17*) and possibly promote self-association of the tagged proteins on cell membranes where the proteins are locally concentrated. A concentration of 300 μM corresponds to a high expression level of ~1000 receptors/μm^2, assuming a local thickness of 5 Å on the membrane surface. In contrast, K3 and E3 do not form homodimers even at 300 μM (*17*). E3, E4, K3, and K4 were labeled at the N-terminus with the fluorophore tetramethylrhodamine (TMR), Alexa Fluor 488, or fluorescein (FL). As target proteins, we used the mouse-derived prostaglandin E2 receptor EP3β subtype (EP3βR) (*19*), the human-derived β_2-adrenergic receptor (β_2AR) (*20*), and the rat-derived epidermal growth factor receptor (EGFR) (*21*) as typical receptors.

The K3 or E3 tag sequence was attached to the N-terminus of EP3βR, which is exposed to the extracellular side, and to monitor the expression and localization of the receptor, EYFP was fused to the C-terminus (K3-EP3βR-EYFP and E3-EP3βR-EYFP). The tagged receptors were transiently expressed in Chinese hamster ovary (CHO) cells. TMR-labeled probes (20 nM) dissolved in the culture medium containing 10% serum were added (the TMR-E3 and TMR-E4 probes for the K3-tagged receptor and the TMR-K3 and TMR-K4 probes for the E3-tagged receptor).

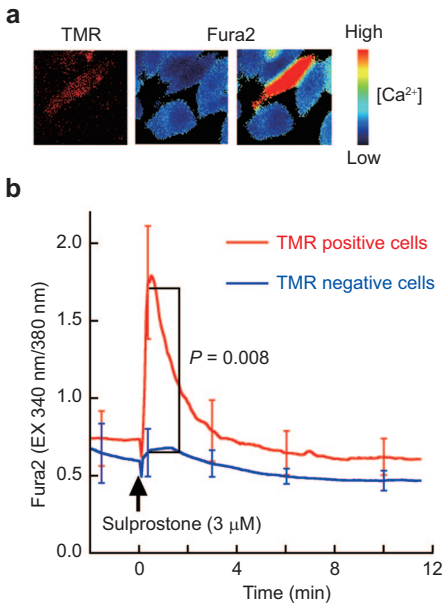

Figure 2. Ca^{2+} imaging of CHO cells expressing E3-EP3βR labeled with TMR-K4 (60 nM). a) Epifluorescence images for TMR-K4 and Fura2 (ratio excited at 340/380 nm). Local Ca^{2+} concentrations are represented by red (high) and blue (low) colors. b) Time course of change in the Fura2 fluorescence ratio for TMR-K4-positive cells (red line) and TMR-K4-negative cells (blue line). Error bars indicate the range ($n = 5$). The EP3 agonist sulprostone (final concentration, 3 μM) was added at the time shown by the arrow. A statistical analysis of the ratios at 30 s after addition of the agonist was performed using the Wilcoxon rank sum test (two-tailed).

The cells were rinsed with the medium and imaged by confocal microscopy. When the TMR-E probes were added to cells expressing the K3-tagged receptors, no significant labeling was observed (Figure 1, panel a). Even at a higher concentration of the TMR-E probes (300 nM), no improvement in labeling was observed (data not shown), indicating that the poor labeling was not due to a reduced dissociation constant caused by the membrane environment of the K3 peptide. All of the K3-tagged N-termini of the receptors might not assume the correct membrane topology. In contrast, addition of the TMR-K probes to cells expressing the E3-tagged receptors stained receptors only on cell membranes because of the membrane impermeability of the probes, although the receptors were also present in the cell interior (Figure 1, panels b and c). Compared to the K-tag/E-probe combinations, the relative fluorescence intensity of TMR was increased by 5- and 12.5-fold for the K3 and K4 probes, respectively. Several control experiments to demonstrate the specificity were carried out under the same experimental settings (Figure 1, panel c). No significant nonspecific staining for TMR-K4 or TMR-K3 (data not shown) was present in untransfected cells and cells expressing K3-tagged receptors. The latter observation indicates (1) that the binding of the K4 probe to the cells expressing the E3-tagged receptors was not because an increase in overall protein density by transfection increased nonspecific probe stickiness to the cell membranes and (2) that the heterodimer formation is sequence-specific. Similary, TMR-E4 or TMR-E3 (data not shown) did not bind to cells expressing E3-tagged receptors. From these control experiments, the signal-to-background contrast was estimated to be 40−90.

Kinetic and equilibrium studies demonstrated that the binding of the K probes to the E3-tagged receptor was rapid and strong. Fluorescence intensities of EYFP and TMR on cell membranes were monitored as a function of time after addition of the TMR-K4 probe (Figure 1, panel d). The labeling was completed within 1 min. Next, to evaluate the strength of the binding between the tag and the probe, cells expressing the E3-tagged receptors were titrated with TMR-K4 (Supplementary Figure 2) and TMR-K3 (data not shown) in the concentration range 1.5−200 nM. At higher probe concentrations, high background fluorescence hampered a precise determination of fluorescence intensity on cell membranes. Apparent dissociation constants were determined from concentration-dependent increases in TMR fluorescence intensity on cell membranes (Figure 1, panel e, $K_d = 64 \pm 31$ nM for TMR-K3; $K_d = 6 \pm 2$ nM for TMR-K4). The dissociation constant for TMR-K3 and the E3 tag was similar to that for the corresponding K3 and E3 peptides measured in buffer (~70 nM) (*17*). The stronger labeling with the TMR-K4 probe enabled efficient visualization of the receptor even at a low concentration of 20 nM (Figure 1, panel b). After the titration, the probes were continuously washed out with the medium at a flow rate of 1 mL min^{-1} (Supplementary Figure 3). Even after a 50-mL washout, ~80% of TMR-K4 remained attached to the E3 tag. On the other hand, significant amounts of TMR-K3 could be washed out from the tag, suggesting that the probe is suitable for reversible labeling (*vide infra*).

To confirm the versatileness of this labeling method, different cells and fluorescent probes were also used. The E3-tagged receptors on cell membranes could be specifically colabeled with TMR-K4 and Alexa Fluor 488-labeled K4 (Figure 1, panel f). E3-tagged receptors expressed in PC12 cells were also specifically labeled by TMR-K4 (Supplementary Figure 4). Thus, our simple method with a high sensitivity is superior to reported labeling methods that typically require combinations of probes at much higher concentrations, metals, enzymes, and/or washing reagents (*15*).

For the practical use of this labeling method, the following points should be checked. First, TMR-K-labeled E3-EP3βR should maintain receptor activity. Second, the probes should be nontoxic. Third, the probes should be chemically stable in solution. The activity of the labeled receptor was examined based on the agonist-dependent influx of Ca^{2+} using the Ca^{2+}-sensitive probe Fura2, imaged by an epifluorescence microscope. The E3-tagged EP3β receptor without EYFP (E3-EP3β) was transiently ex-

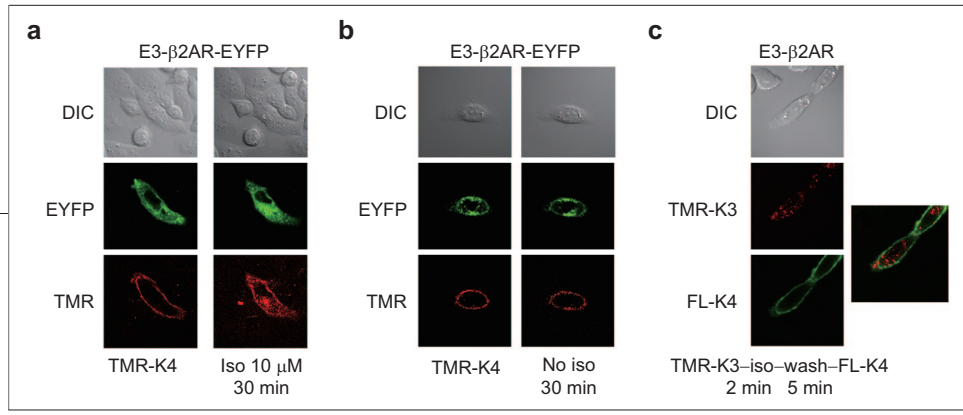

Figure 3. Internalization of β_2-adrenergic receptor (β_2AR) in response to receptor stimulation. a) Confocal images of CHO cells transiently expressing E3-β_2AR-EYFP. The cells were labeled with 20 nM TMR-K4 (left) and then incubated with 10 μM isoproterenol for 30 min (right). b) Negative control in the absence of isoproterenol. c) Pulse-chase experiment. CHO cells expressing E3-β_2AR were labeled with TMR-K3 (60 nM) for 2 min, then incubated with isoproterenol (10 μM) for 5 min. After the cells were washed with PBS, 20 nM fluorescein-K4 (FL-K4) was added and the cells were observed. The TMR and FL images are merged in the lower right panel.

pressed in CHO cells and labeled with the TMR-K4 probe at 60 nM, a concentration at which more than 90% of the tagged receptors at the cell surface should be labeled by the probe (Figure 1, panel e). Only in cells expressing E3-EP3βR that had been labeled by TMR-K4 did the intracellular Ca^{2+} level increase after addition of the EP3R agonist sulprostone (Figure 2), verifying that the labeling did not impair the function of EP3βR. The toxicity of the probes was examined by the WST-1 assay. Excess concentrations of TMR-K3 and TMR-K4 (10 μM) showed no significant toxicity against CHO cells even after a 20 h incubation (Supplementary Figure 5). The stability of the probes was also examined. In aqueous solution at 4 °C, no degradation of TMR-K3 and TMR-K4 was observed for at least 1 month (Supplementary Figure 6). Thus, we found that the E3 tag and the K probes are a suitable combination for the specific labeling of cell-surface receptors. Notably, labeling using the K4 probe is quick (<1 min), sensitive (tens of nanomolars), and does not impair receptor function.

As an application of this labeling method, the internalization of β_2AR in response to receptor stimulation (*20*) was visualized. The E3 tag sequence and EYFP were attached to the N-terminus and the C-terminus of the receptor, respectively (E3-β_2AR-EYFP). It was confirmed that the TMR-K4 probe selectively stained receptors on cell membranes (Figure 3, panel a). After addition of the β_2AR agonist isoproterenol, the internalization of cell-surface receptors was clearly visualized in the TMR image, indicating that the labeling, again, did not affect receptor activity (Figure 3, panel a), whereas in the absence of isoproterenol, no significant internalization was observed (Figure 3, panel b). It was difficult to distinguish the internalized receptors from the original intracellular receptors in the EYFP image (Figure 3, panel a), demonstrating an advantage of the coiled-coil labeling over fluorescent proteins. Furthermore, the quickness and reversibility of the coiled-coil labeling enables a pulse-chase labeling (Figure 3, panel c). After the agonist stimulation of the TMR-K3-labeled E3-β_2AR, the receptors on the cell surface (receptors that had not been internalized and newly externalized receptors) could be labeled by the second probe FL-K4 after the washout of TMR-K3. The absence of TMR fluorescence on the cell surface indicates the reversibility of the labeling. The ligand-induced internalization could be also visualized for the E3-tagged epidermal growth factor receptor (EGFR), indicating the versatility of this labeling method (Supplementary Figure 7). Thus, coiled-coil labeling is an attractive tool for screening of ligands based on receptor internalization.

A major bottleneck to the development of a practical tag–probe labeling method is intractability, for example, complicated labeling procedures and considerable nonspecific labeling. Our coiled-coil tag–probe labeling system for surface receptors in living cells is unique in using simple peptide–peptide interaction without additional components such as metals or enzymes. Notably, the combination of the E3 tag and the K4 probe was found to be highly tractable: quick, nontoxic, sensitive, and available even in culture medium. Furthermore, the reversibility of the labeling using the K3 probe enabled discrimination between the internalized and the cell-surface receptors, whereas the K4 probe is suitable for long-time observation. Another benefit is that diverse fluorophores or other synthetic molecules can be easily attached to the probe by conventional peptide synthesis procedures. This promising method should have diverse applications, such as in the detection of receptor internalization and oligomerization.

METHODS

Cell Culture and Transient Expression of EP3βR Mutants. CHO cells were maintained in alpha modification Eagle's medium (αMEM) with 10% heat-inactivated fetal bovine serum in 5% CO_2 at 37 °C. For transfection using LipofectAMINE and PLUS reagents (Invitrogen), 1×10^5 cells in a 35 mm glass bottom dish were incubated with a transfection mixture composed of 0.3 μg of DNA, 2 μL of LipofectAMINE, and 3 μL of PLUS reagent for 3 h.

Examination of Tag–Probe Combination. Cells expressing the tag-labeled EP3βR were imaged 24–48 h after transfection. Five minutes after the incubation with probes in 1 mL of αMEM (pH 7.4) with 10% serum and 10 mM 2-[4-(2-hydroxyethyl)-1-piperadinyl] ethansulfonic acid (HEPES), the cells were rinsed once with the medium and observed by confocal microscopy (Zeiss LSM Pascal). Green (EYFP and Alexa 488) and red (TMR) channels were images excited by 488 and 543 nm lasers, and detected through BP 505–530 nm and LP 560 emission filters, respectively. For the measurement of labeling kinetics, probes dissolved in medium (1 mL) were added to cells immersed in medium (1 mL) to give a concentration of 20 nM. In the titration experiments, a Nikon C1 confocal microscope equipped with a temperature and CO_2 controlled stage was used (green and red channels were excited by 488 and 561 nm lasers, detected through BP 505–530 nm and BP 560–640 emission filters, respectively).

Intracellular Ca^{2+} Imaging. Cells expressing the tag-labeled EP3βR were rinsed with αMEM with

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10% serum containing 0.5 μM indomethacin and 0.5 mM probenecid (pH 7.4) and then incubated for 30 min with 10 mM Fura 2 a.m. (Dojindo) at 37 °C. After the incubation, the medium was replaced with 1 mL of physiological saline solution (135 mM NaCl, 10 mM HEPES, 5.5 mM glucose, 5 mM KCl, and 1 mM $MgCl_2$, pH7.4) containing 2 mM Ca^{2+} and 60 nM TMR-K4 probe. Fluorescence of Fura 2 was obtained at 510 nm with excitation wavelengths of 340 nm/380 nm. Sulprostone (6 μM, 1 mL) was added to give a final concentration of 3 μM.

Internalization of Receptors. Cells expressing E3-β_2AR-EYFP were incubated with 1 mL of αMEM (containing 1% 1 M HEPES, pH7.4) with 10% serum containing 20 nM TMR-K4. Isoproterenol (20 μM, 1 mL) was added to give a final concentration of 10 μM. After incubation for 30 min at RT, the confocal image was obtained. In the pulse-chase experiment, cells expressing E3-β_2AR were labeled with TMR-K3 (60 nM) in F-12 medium (pH 7.4) for 2 min, then incubated with isoproterenol (10 μM) for 5 min at 37 °C. After the cells were washed with PBS (10 times), FL-K3 (20 nM) was added and the cells were observed.

Peptide Synthesis and Construction of Plasmids. See Supporting Information for details.

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Supporting Information Available: This material is available free of charge *via* the Internet.

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