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Rapid Covalent Fluorescence Labeling of Membrane Proteins on Live Cells via Coiled-Coil Templated Acyl Transfer

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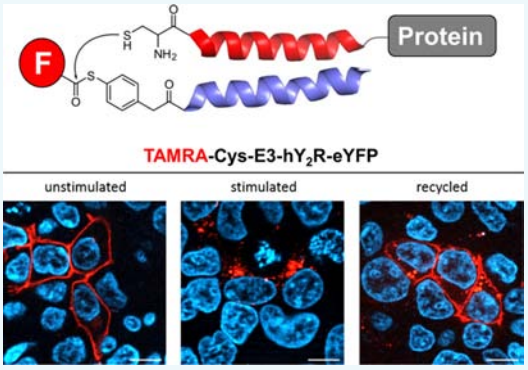
Supporting Information

ABSTRACT: Fluorescently labeled proteins enable the microscopic imaging of protein localization and function in live cells. In labeling reactions targeted against specific tag sequences, the size of the fluorophore-tag is of major concern. The tag should be small to prevent interference with protein function. Furthermore, rapid and covalent labeling methods are desired to enable the analysis of fast biological processes. Herein, we describe the development of a method in which the formation of a parallel coiled coil triggers the transfer of a fluorescence dye from a thioester-linked coil peptide conjugate onto a cysteine-modified coil peptide. This labeling method requires only small tag sequences (max 23 aa) and occurs with high tag specificity. We show that size matching of the coil peptides and a suitable thioester reactivity allow the acyl transfer reaction to proceed within minutes (rather than hours). We demonstrate the versatility of this method by applying it to the labeling of different G-protein coupled membrane receptors including the human neuropeptide Y receptors 1, 2, 4, 5, the neuropeptide FF receptors 1 and 2, and the dopamine receptor 1. The labeled receptors are fully functional and able to bind the respective ligand with high affinity. Activity is not impaired as demonstrated by activation, internalization, and recycling experiments.



INTRODUCTION

The ability to visualize proteins in a complex biological environment is of great interest and facilitates investigations of the localization and function of proteins. This calls for methods that allow the introduction of fluorescent labels into the protein of interest (POI). The genetically encodable autofluorescent proteins are the workhorses for live cell studies.[1,2] These reporters do not require an actual labeling reaction because the fluorophore is generated from native amino acids. However, the demand for smaller labels and fluorophores with improved photophysical properties, such as long-term photostability, greater brightness, and near-infrared emission, has fueled the development of alternative protein labeling methods. To visualize fast biological processes, rapid and covalent labeling methods are desired.

Self-modifying enzyme-based tags such as Snap,[3] Clip,[4] or Halo[5] allow efficient, fast, and robust labeling reactions. Yet, the size of these tags (≥180 amino acids) is almost in the range of autofluorescent proteins. Recently, Kikuchi et al. reported smaller enzyme tags based on the β-lactamase[6] (29 kDa) and the photoactive yellow protein[7] (PYP, 14k Da), which is capable of binding fluorogenic fluorescein and coumarin moieties. The use of enzymatic labeling methods enabled a further reduction of tag size. Lactamase,[8] formyl-generating enzyme (FGE),[9] and sortase[10] require rather high substrate concentrations (μM for FGE and sortase) and/or long reaction times (1 h for lactamase) for labeling of membrane proteins.

Small tag sizes are also offered by tetracysteine[11] or tetraserine[12] peptides that undergo covalent conjugation reactions with organobisarsenic acid thioesters or bisboronic acid compounds, yet special precautions are required to maintain the selectivity of the labeling reaction. Split-intein mediated protein trans-splicing has been fashioned to allow protein labeling.[13−15] The N-intein sequence fused to the POI cleaves itself off during the labeling reaction, so the peptide tag remaining on the target protein can be kept as short as three amino acids. However, the protein-based labeling agent is rather large. By using amber (STOP)[16,17] and expanded size codon[18−20] technologies, "bioorthogonal" functional groups[21−34] can be introduced in specific cellular proteins. While very successful in many cases, technologies based on non-natural amino acids are still an expert's science and currently far from routine.

We[35] and others[36−38] reported that the formation of coiled coils enables the covalent labeling of proteins. Xia and co-workers equipped a pair of coiled-coil peptides with a central cysteine and a chloroacetyl group.[38] A chemical cross-link was established upon formation of the coiled coil. Best results were obtained with a bidentate 53-amino-acid-long labeling agent. At 20 μM concentration ligation led to the formation of a 79-aa-

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long protein label within 4 h. Avoiding mass increases by ligation of peptidic fragment, Ball and co-workers connected a rhodium catalyst to the central position of one coil peptide to catalyze, after coiled-coil formation and addition of diazo agents, the alkylation of a tryptophane within the other coil peptide.[36,37] The labeling of tagged recombinant proteins in cell lysate was achieved within 16 h reaction time.

Given our experience with nucleic acid triggered reactions[39−43] we assumed that carefully designed templates should provide the opportunity to combine desirable labeling features, i.e., small mass increase, short labeling time, and high specificity. We took inspiration from Matsuzaki et al.[44] who showed that the 21-amino-acid-long, 3-heptad repeat peptides E3 ($(EIAALEK)_3$) and K3 ($(KIAALKE)_3$) form coiled-coil structures on the surface of living cells. We introduced an end-of-coiled-coil templated reaction.[35] In parallel coiled coils the N-terminal end of one coil peptide is proximal to the N-terminus of the second coil peptide. Therefore, the N-terminal, thioester-linked acyl group in coil peptide **2** should come into reach of a cysteine protruding from the N-terminus of coil peptide **1** (Figure 1). We demonstrated that the reaction

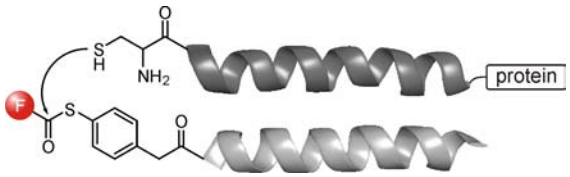


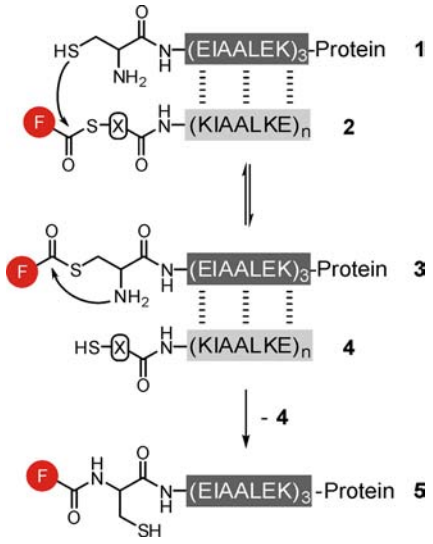
Figure 1. Structure of a parallel coiled coil with N-terminal cysteine and thioester-linked fluorescence label (red).

between thioester-linked tetramethyl-rhodamine (TAMRA)-K3 peptide conjugates and Cys-E3-tagged membrane proteins leads to transfer of the TAMRA dye.[35] Herein, we extend our labeling technique and show live cell labeling with a second fluorophore. The yields of our previously published labeling reactions were quite low. In this report we describe the improvement of dye transfer yields. In addition, we report details on the synthesis of thioester-linked dye−peptide conjugates, describe reactivity studies, and point out the important role of thioester reactivity, size-matching of coiled-coil structures, and stoichiometry. Furthermore, we addressed potential concerns about the negative effect of the cysteinyl-coiled-coil-based tags and demonstrate the general applicability by expanding the repertoire of labeled GPCRs and evaluate their full biological function in living cells.

RESULTS AND DISCUSSION

Based on the reports from Matsuzaki et al., the artificial heterodimeric coiled-coil sequences K3 $(KIAALKE)_3$, K4 $(KIAALKE)_4$ and E3 $(EIAALEK)_3$, E4 $(EIAALEK)_4$ are appropriate as peptide templates since the excess of positive charge (K peptides) and negative charge (E peptides) favors the heterodimeric E-K-coils.[45] Previous studies revealed that K-peptides are unsuitable as protein tags.[44] Coiled-coil formation was not observed on living cells. Rather, it was required that the E-peptide was fused to the protein of interest. To avoid potential homodimerization at higher expression levels of the protein of interest and in order to minimize the mass increase on the target, we envisioned the E3-peptide **1** rather than the E4-peptide to serve as the acceptor of the fluorophore (Scheme 1). The cysteine-E3-peptide **1** was envisioned to react with

Scheme 1. Mechanism of the Coiled-Coil Templated Acyl Transfer (F = Fluorophore)



thioester-linked fluorophore-K-peptide conjugates **2**. The formation of the coiled coil will trigger an acyl transfer reaction via thiol exchange. The rate of the fluorophore migration will depend on the nature of the thiol component in the starting thioester conjugate **2**. This reaction is, in principle, reversible. However, the subsequent S−N-acyl transfer within the formed thioester intermediate **3** traps the fluorophore as amide in **5**. Of note, the reaction does not occur through ligation of the K-peptide with the E-peptide and, therefore, avoids large increases of the tag size. The mass increase upon labeling is minimal.

Solid-Phase Synthesis of Thioester-Linked Dye−K-Peptide Conjugates. In the pursuit of a modular synthesis scheme that enables facile diversification of the thioester structures by variations of both the dye-acyl component and the thiol leaving group, we favored an approach in which all steps can be performed on the solid phase. Reactive K3 and K4 conjugates (**9** and **10**) were prepared on the TentaGel resin by using a Rink amide anchor (Scheme 2). The coil peptide part was assembled by using Fmoc-protected amino acid building blocks and HCTU as coupling reagent. At the N-terminal end, the thiol component was introduced in *S*-Mmt-protected form by using, again, HCTU activation. We selected mercaptophenylacetic acid (MPAA), mercaptopropionic acid (MPA), mercaptoacetic acid (MA), and N-acetyl cysteine (Ac-Cys). The thiol function was liberated upon treatment with 2% TFA in dichloromethane. To establish the thioester bond, *N*-Mmt-glycine was coupled. After removal of the Mmt protecting group, the conjugate **7** was charged with a fluorophore such as carboxytetramethylrhodamine (TAMRA), alexa fluor 350 (AF350), or ATTO488 by means of a conventional amide bond forming reaction. The final treatment of the full-length conjugates **8** with TFA/TIS/H_2O (96:2:2) provided the desired products **9**−**15**, which were purified to homogeneity by reverse-phase HPLC.

Influence of Thioester Length on the Acyl Transfer. For achieving a high selectivity of the labeling reaction, a high mutual affinity for the tag−probe pair is important. Both combinations, E3/K3 and E3/K4, offer K_D values in the nanomolar range.[44] In the initial reactivity studies (Figure 2), the MPAA-thioester-linked TAMRA-K-peptide conjugates **9** or **10** were incubated with the Cys-E3-peptide **16** or a control

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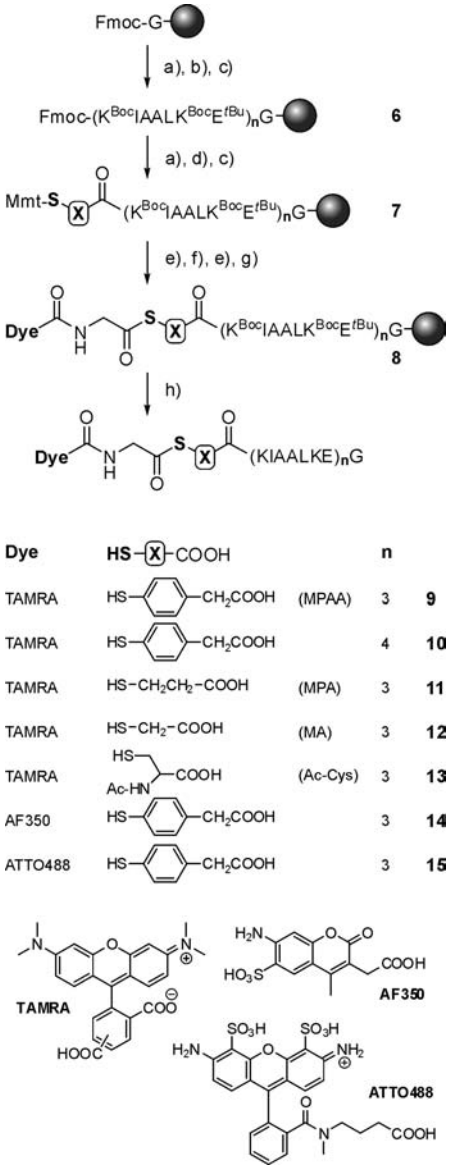
Scheme 2. Solid-Phase Synthesis of Thioester-Linked Dye–K-Peptide Conjugates[a]



[a](a) 20% piperidine, DMF; (b) Fmoc-amino acid, HCTU, NMM, DMF; (c) 5% Ac_2O, 6% lutidine; DMF; (d) Mmt-X-COOH (Mmt-MPAA, Mmt-MPA, Mmt-MA, Ac-Cys(Mmt)), HCTU, DIPEA, DMF; (e) 2% TFA, 2% TIS, CH_2Cl_2; (f) N-Mmt-Gly, HCTU, DIPEA, DMF; (g) Dye-COOH, HCTU, DIPEA, DMF; (h) TFA, 2% TIS, 2% H_2O. Overall yields 1–8%.

peptide **20** (H-CRAESYK) at 5 μM concentration in phosphate buffer (100 mM NaH_2PO_4, 1 mM TCEP, pH 7). After 30 min the reactions were stopped by addition of 2 vol % trifluoroacetic acid (TFA) and the transfer of TAMRA was analyzed via fluorescence-detected HPLC. Labeling did not proceed when the thioester probes **9** or **10** were incubated with the control cysteinyl peptide **20** (background, Figure 2b,c). By contrast, the reaction between the K peptide conjugates and the Cys-E3 peptide furnished new fluorescent products which based on fluorescence properties and MS-analysis were assigned to TAMRA-labeled Cys-E3 conjugate **17**. A comparison of the HPLC traces revealed marked reactivity differences. The reaction between the length-matched E3 and K3 conjugates

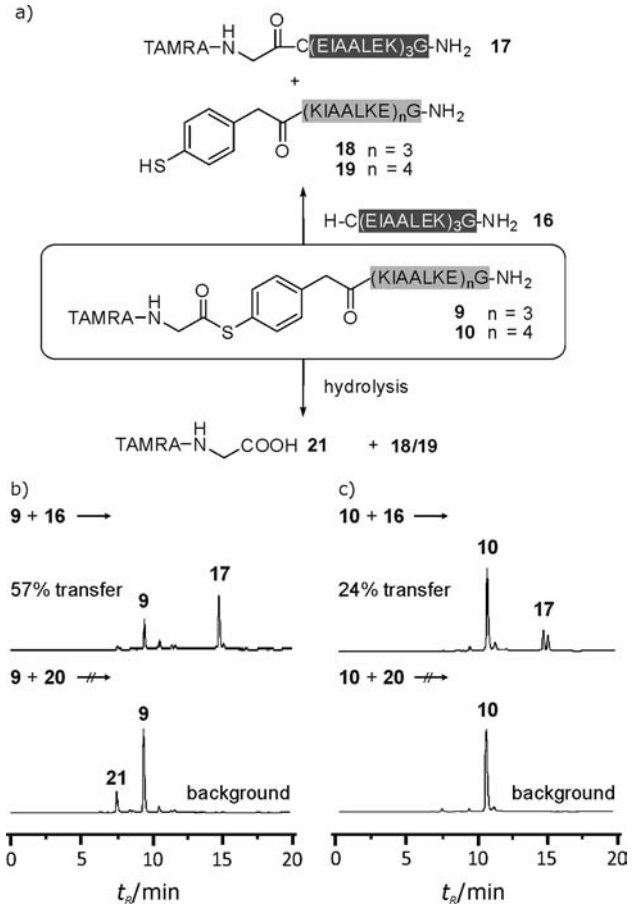


Figure 2. (a) Peptide-templated acyl transfer reactions involving the donating thioester peptides **9** or **10** and the acceptor peptide tag **16** or the cysteinyl peptide **20** (= specificity control). Fluorescence-detected HPLC analysis after 30 min reactions between cysteinyl peptides **16** (top) or **20** (bottom) and (b) thioester **9** or (c) thioester **10**. Conditions: 5 μM peptides, 100 mM NaH_2PO_4, 1 mM TCEP, pH 7; λ_{ex} = 555 nm, λ_{em} = 584 nm. Ligation product **17** appears as double peak due to simultaneous N- and S-acylation.

(**16** and **9**, respectively) provided 57% labeled product after 30 min reaction time. Of note, only 24% labeling was obtained when the Cys-E3 was allowed to react with the reactive TAMRA-K4 conjugate **10**. Furthermore, this reaction yielded double, N- and S-acylated products (see **17** in Figure 2c). At a first glance, the reactivity differences seem surprising. The K4 conjugate is expected to have a higher affinity for E3 than the K3 conjugate (K_D(E3/K4) = 6 nM; K_D(E3/K3) = 67 nM).[44] However, formation of the coiled coil was probably not rate-limiting under the chosen conditions. Rather, it is likely that the structure of the formed coiled-coil complex plays an important role. In the E3/K3 pair, the highest stability should be obtained when all heptad repeats are involved in the coiled coil. This leads to a blunt-end complex, in which the effective molarity of the reactive groups at the N-terminal end should be high. By contrast, the structure of the E3/K4 coiled coil probably is less defined. The N-terminal heptad of the K4 conjugate may not be involved in the coiled-coil structure. This N-terminal offset reduces the effective molarity of the N-terminal reactive groups and template-triggered rate enhancements would be lower than for the blunt-ended E3/K3 pair. Based on the higher transfer yields the combination of E3 and K3 peptide was selected for further investigations.

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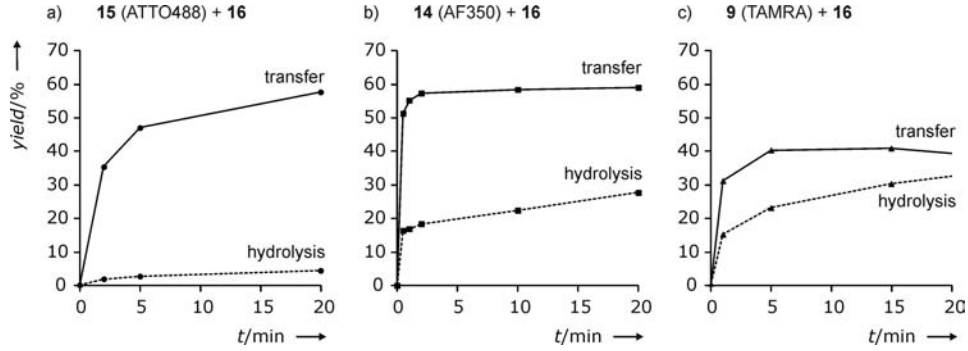

Figure 3. Time course of transfer and competing hydrolysis during labeling of Cys-E3 conjugate **16** with (a) ATTO488 conjugate **15**, (b) AF350 conjugate 14, and (c) TAMRA conjugate **9**. Conditions: 0.5 μM peptides, 100 mM NaH_2PO_4, 1 mM TCEP, pH 7.

Expanding the Color Repertoire. Previously, we had demonstrated the coiled-coil templated transfer of the TAMRA dye and a blue light emitting AF350 dye.[35] To assess the generality of the coiled-coil templated labeling reaction and to expand the repertoire of available colors to the green region, we compared dye transfer reactions involving MPAA-K3 conjugates charged with TAMRA (**9**) or AF350 (**14**) with reactions of the green emitting ATTO488 conjugate **15** (Figure 3). Please note that the attachment of the reporter group is the last step during solid-phase synthesis of the labeling agents (Scheme 2). The modular design facilitates variations of the reporter group.

The thioester-linked dye−peptide conjugates were allowed to react with the Cys-E3 peptide in phosphate buffer at 500 nM concentration. Under these conditions, the transfer of ATTO488 required 10 min to reach 50% labeling yield (Figure 3a). By comparison, the reaction of the AF350 thioester **14** provided 58% labeling yield within 2 min only (Figure 3b), while the reaction with TAMRA agent **9** (Figure 3c) also showed high initial rates of labeling but a plateau was reached at 41% yield after 5 min. The HPLC analyses revealed competing hydrolysis reactions which limited the achievable labeling yields. This effect was more pronounced in the TAMRA and AF350 transfer reactions than in the ATTO488 transfer. Obviously, the structure of the dye significantly affected the reactivity of the thioester linkage. Though the underlying causes for the reactivity differences are unknown to us at this stage, we note that among the three dyes studied the ATTO488 was linked via the longest alkyl chain.

Reactivity Tuning. The comparative dye transfer studies showed that TAMRA labeling was substantially affected by competing hydrolysis. Though rapid depletion of excess thioester reagent may actually help improve the selectivity of the labeling reaction in live cell studies, we sought for a means to improve the labeling yields. In native chemical ligation reactions, the thioester reactivity is determined, among other influences, by the leaving group character of the thiol component.[46] In order to improve the resistance against hydrolysis, we connected the TAMRA group via different thiol components to the K3 peptide. Indeed, the half live of the alkylthioester reagents in phosphate buffer was considerably higher than the half live of the arylthioester **9** (Figure S2). However, the transfer reactions of the alkylthioesters **11**−**13** were too slow to enable effective labeling in less than 10 min (Figure 4a). For the envisaged application, i.e., rapid live cell labeling of GPCRs, arylthioester reagents such as **9** seemed more appropriate.

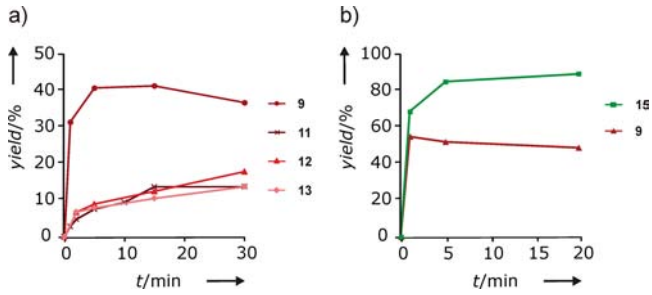

Figure 4. Time course of labeling of cysteinyl peptide **16** by reactions with (a) stoichiometric amounts of TAMRA thioester peptides **9**, **11**−**13**, and (b) excess TAMRA donor **9** (red) or ATTO488 donor **15** (green). Conditions: 0.5 μM donor conjugate, 0.5 μM (a)) or 0.25 μM (b)) Cys-E3 acceptor, 100 mM NaH_2PO_4, 1 mM TCEP, pH 7.

In labeling reactions of proteins on live cells, the thioester reagent will most likely be applied in excess of the Cys-E3-tagged protein target. Under these conditions, the labeling yield may be higher than in reactions involving stoichiometric ratios of reactions partners. The K3-linked TAMRA and ATTO488 labeling agents **9** and **15**, respectively, were allowed to react with the Cys-E3 conjugate **16** in 2-fold excess (Figure 4b). Of note, the ATTO488-labeled product was formed in 85% yield after 5 min reaction time. In the reaction of the TAMRA-agent **9**, the use of excess donor was combined with a small decrease in pH from 7.0 to 6.5 (to reduce hydrolysis). After 1 min reaction time, only the TAMRA transfer succeeded in 55% yield.

Live Cell Labeling of G-Protein Coupled Receptors. We have previously shown that the peptide templated acyl transfer enables the selective and rapid TAMRA labeling of Cys-E3-tagged constructs of the human neuropeptide Y_2 receptors (hY_2R) and neuropeptide Y_5 receptors (hY_5R) in living cells.[35] To evaluate the general applicability of the labeling protocol we extended the range of proteins and cloned another five N-terminally Cys-E3-tagged constructs including the human neuropeptide Y_1 and Y_4 receptor (hY_1R, **22**, and hY_4R, **23**, respectively), human neuropeptide FF_1 and FF_2 receptors ($hNPFF_1R$, **24**, $hNPFF_2R$, **25**) as well as the human dopamine 1 receptor (hD_1R, **26**) and expressed them in human cells. For control, the autofluorescent eYFP was fused to the C-terminus of the receptors in the first set of experiments. The receptor constructs were transiently expressed in HEK293 cells. Measurements of the eYFP emission confirmed the expression in the cells and microscopic analysis revealed the correct integration in the cell membrane (Figure 5).

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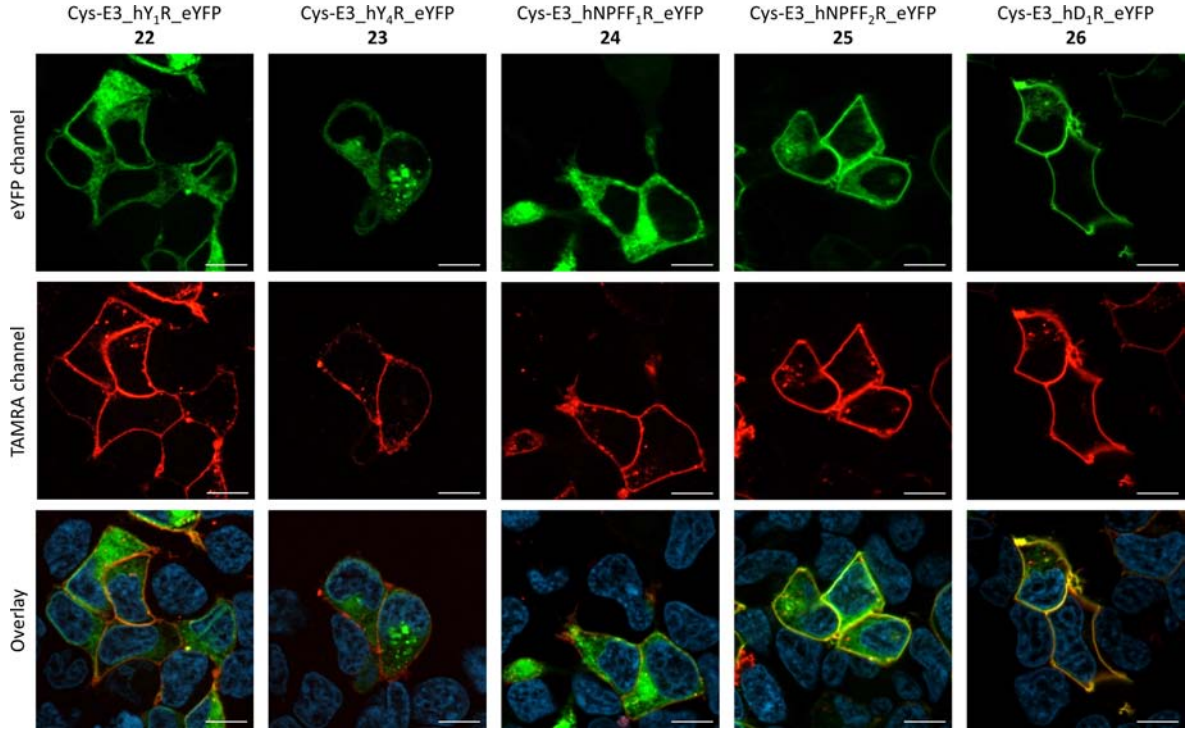

Figure 5. Live cell fluorescence microscopy images of TAMRA labeling. HEK293 cells were transiently transfected with Cys-E3_hY$_1$R_eYFP, Cys-E3_hY$_4$R_eYFP, Cys-E3_hNPFF$_1$R_eYFP, Cys-E3_hNPFF$_2$R_eYFP, or Cys-E3_hD$_1$R_eYFP. Cells were treated with 0.5 mM TCEP in 20 mM HEPES/HBSS for 10 min followed by immediate labeling with 100 nM TAMRA-K3 conjugate (**9**) in 1% BSA/HBSS for 2 min. Cell nuclei were stained with Hoechst33342. eYFP channel: excitation 488−512 nm; emission 520−550 nm; TAMRA channel: excitation 550−580 nm; emission 590−650 nm and Hoechst33342 channel: excitation 335−383 nm; emission 420−470 nm. Scale bar equals 10 μm.

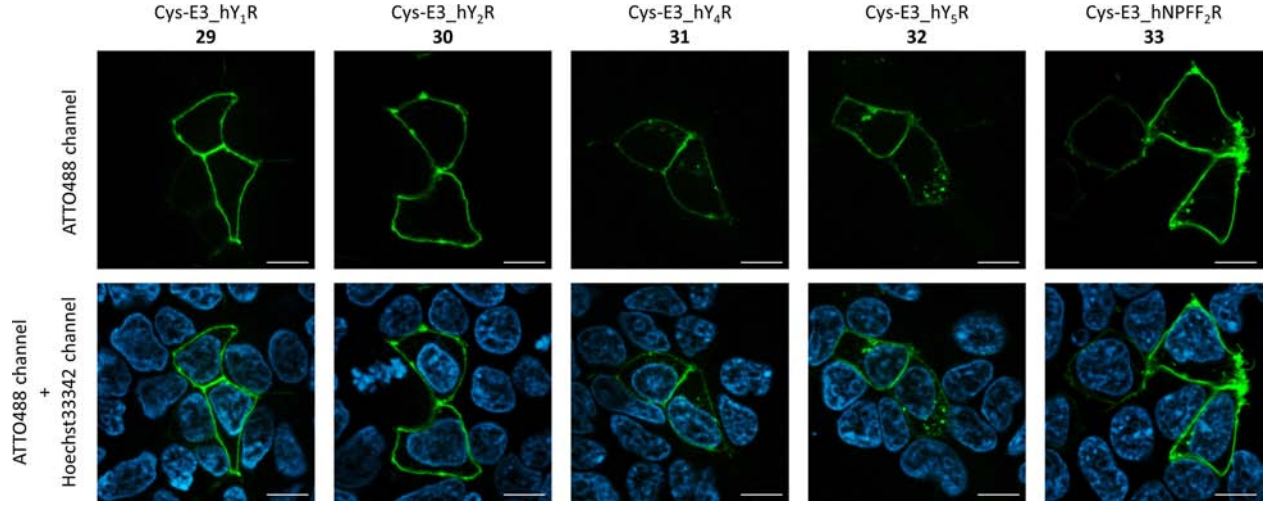

Figure 6. Live cell fluorescence microscopy images of ATTO488 labeling. HEK293 cells were transiently transfected with Cys-E3_hY$_1$R, Cys-E3_hY$_2$R, Cys-E3_hY$_4$R, Cys-E3_hY$_5$R, or Cys-E3_hNPFF$_2$R. Fluorescent images were taken after labeling with 100 nM ATTO488-K3 conjugate (**15**) in 0.1 mM TCEP/20 mM HEPES/HBSS buffer for 5 min and subsequent short basic wash with 150 mM NaHCO$_3$/HBSS pH = 8.6. Atto488 channel: excitation 488−512 nm, emission 520−550 nm, and Hoechst33342 channel: excitation 335−383 nm, emission 420−470 nm Scale bar equals 10 μm.

Next, cells transfected with the constructs **22**−**26** were treated with 0.5 mM TCEP in 20 mM HEPES/HBSS for 10 min and, subsequently, with 100 nM of TAMRA-K3 conjugate **9** in 1% BSA/HBSS. After 2 min only, the solution was exchanged for HBSS and the cells were analyzed by fluorescence microscopy. The TAMRA emission nicely colocalized with the eYFP emission signals. For the hY$_4$R and the hNPFF$_1$R we observed weak staining in both eYFP and TAMRA channels, due to overall low expression levels of these constructs. Furthermore, the microscopy images show no TAMRA fluorescence for intracellularly localized receptor constructs. This is an indication that the TAMRA-K3 conjugate **9** is not able to penetrate the cell membrane and therefore only allows a coiled-coil-mediated acyl transfer reaction at the cell surface. This can be nicely confirmed for the hY$_1$R. Owing to the high expression, some of the receptors are not yet localized

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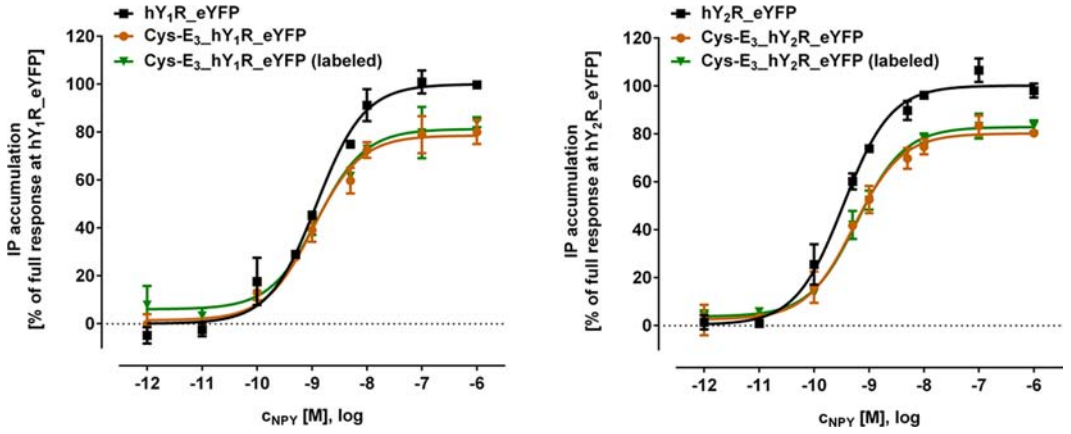

Figure 7. IP accumulation assay. COS-7 cells were transiently cotransfected with one of the described receptor constructs and the chimeric G protein $G\alpha_{\Delta qi4myr}$. Cells were incubated with increasing concentrations of pNPY. IP accumulation was normalized to either hY_1R_eYFP or hY_2R_eYFP. Data is given as means ± SEM of two independent experiments, each performed in duplicate.

in the membrane as seen by the fluorescence of the eYFP (Figure 5, left row). However, only surface localized receptors are labeled with the TAMRA-K3 conjugate. Therefore, a clear discrimination of receptors imbedded in the membrane and trafficking receptors can be performed with this labeling technique. This was observed also for hY_4R, $NPFF_1R$, and $NPFF_2R$ receptors, however, with a reduced amount of still trafficking receptors. By comparison, all hD_1Rs are well integrated in the membrane (Figure 5, right row).

A key feature of the new peptide templated acyl transfer labeling is to avoid the fusion of eYFP or any other large fluorescent protein to the receptor. Therefore, in the next set of experiments receptor constructs devoid of the C-terminal fused eYFP were cloned, expressed, and subsequently labeled. We chose the human neuropeptide Y receptors (**29**−**32**) and the human neuropeptide FF_2 receptor (**33**) as targets. In addition, we selected the ATTO488 dye for labeling in these studies to demonstrate the flexibility of the labeling design. This dye is less hydrophobic and brighter than TAMRA. Furthermore, a time optimized labeling protocol was applied. Transiently transfected HEK293 cells were treated with 100 nM ATTO488-K3 conjugate **15** in 0.1 mM TCEP/20 mM HEPES/HBSS buffer for 5 min. After a short basic wash with 150 mM $NaHCO_3$/HBSS to remove nontransferred fluorescent moieties and exchange to 20 mM HEPES/HBSS buffer the cells were analyzed (Figure 6). Fluorescence microscopy confirmed successful labeling for all receptor constructs at the cell surface. For the hY_4R and hY_5R we sometimes detected fluorescence signals inside the cells, which arise naturally from constitutively internalized receptors of the cell surface.

To characterize the biological activity of the Cys-E3-tagged receptors signal transduction assays were performed. In our previous work we showed that the Cys-E3 tag had no impact on the activity of hY_2R and hY_5R receptors.[35] The activity after dye labeling was not studied. Here we applied the full labeling procedure to the hY_1R and hY_2R, which are G_i protein-coupled receptors and measured the accumulation of inositol phosphates (IP) using coexpression of chimeric $G\alpha_{qi}$ protein, which depends on the G protein activation. The response of the receptors lacking the Cys-E3 tag to the native peptide ligand pNPY was compared to the response of receptors carrying the Cys-E3 tag with and without labeling (Figure 7). The determined EC_{50} values confirmed that the biological activity in signal transduction studies was not affected; neither by the tag nor by the labeling procedure (Table 1). The EC_{50} and pEC_{50} values are also in agreement with already published data.[47,48] The slightly decreased maximal efficacy is caused by the slightly reduced expression level of the Cys-E3-tagged constructs.

Table 1. Functional Characterization of hY_1R and hY_2R with and without Cys-E3 tag[a]

receptor	EC_{50} [nM]	pEC_{50} ± SEM	*n*
hY_1R_eYFP	1.2	−8.9 ± 0.08	2
Cys-E3_hY_1R_eYFP	1.0	−9.0 ± 0.08	2
Cys-E3_hY_1R_eYFP (labeled)	1.2	−8.9 ± 0.09	2
hY_2R_eYFP	0.3	−9.4 ± 0.06	2
Cys-E3_hY_2R_eYFP	0.5	−9.2 ± 0.07	2
Cys-E3_hY_2R_eYFP (labeled)	0.6	−9.2 ± 0.05	2

[a]EC_{50} values as well as the pEC_{50} (negative logarithm of EC_{50}) ± SEM were determined by IP accumulation assays. The data of two independent experiments is shown, each was performed in duplicate.

For further characterization of the Cys-E3-tagged receptors, internalization and recycling studies were performed, which are shown for the Cys-E3_hY_2R_eYFP (Figure 8). The cells were transiently cotransfected with Rab4a-CFP as a marker for the early endosome compartment.[49] The receptors were labeled with 100 nM TAMRA-K3 conjugate **9** in the same manner as described for the treatment with ATTO488-K3 conjugate **15**. The TAMRA-labeled hY_2R were localized at the cell membrane. After 60 min incubation with the pNPY ligand the receptor was internalized and transferred from the cell membrane into endosomal vesicles, which was verified by partial colocalization with Rab4a. Exchange of the medium and thus removal of the ligand allowed the receptors to traffic back to the cell surface. This is in agreement with the literature and demonstrates the full biological activity for the Cys-E3_hY_2R_eYFP.[50]

Previously, we have shown that the mutation of *N*-terminal cysteine to alanine prevents the acyl transfer, yet still allows the coiled-coil formation.[35] To demonstrate the importance of the covalent nature of the labeling for biological issues, the internalization and recycling experiments were performed with the Ala-E3_hY_2R_eYFP. The expression and correct localization of the Ala-E3-tagged hY_2R_eYFP in the cell membrane was observed by fluorescence microscopy as seen

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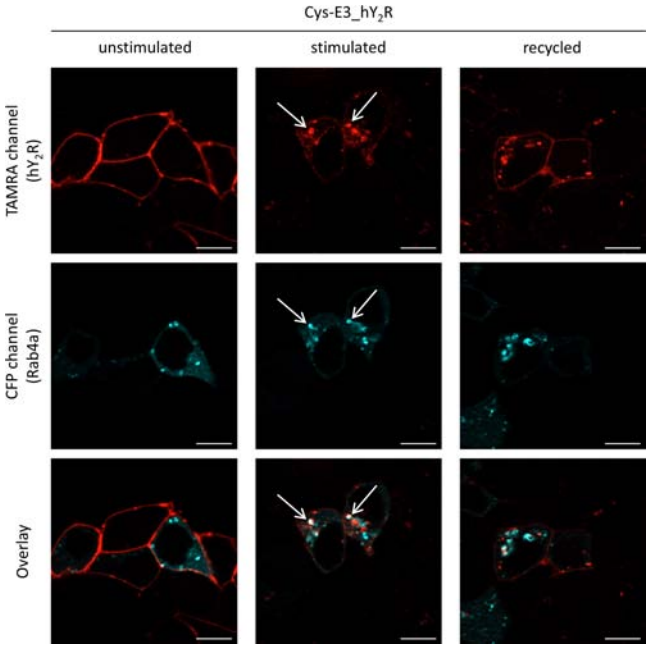

Figure 8. Fluorescence microscopy images of internalization and recycling studies with living HEK293 cells. Cells were transiently cotransfected with Cys-E3_hY$_2$R and Rab4a_CFP. The receptor was fluorescently labeled with 100 nM TAMRA-K$_3$ conjugate **9** in 0.1 mM TCEP/20 mM HEPES/HBSS buffer for 5 min, followed by a short basic wash with 150 mM $NaHCO_3$/HBSS pH = 8.6 and neutralization with 20 mM HEPES/HBSS buffer. For stimulation the cells were incubated with 1 μM pNPY in OptiMEM for 60 min at 37 °C. The recycling was performed by removal of the pNPY containing medium with OptiMEM for another 60 min at 37 °C. Cell nuclei were stained with Hoechst33342. Scale bar equals 10 μm.

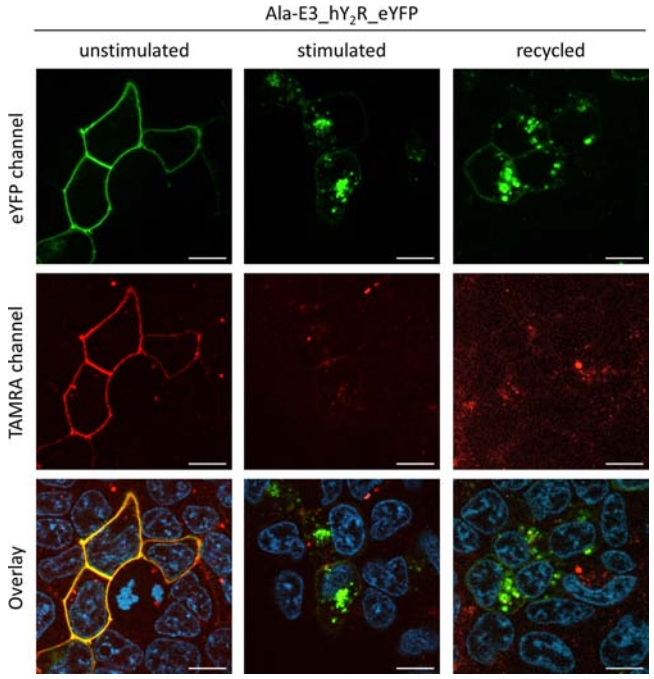

Figure 9. Fluorescence microscopy images of internalization and recycling studies with living HEK293 cells. Cells were transiently transfected with Ala-E3_hY$_2$R_eYFP. Fluorescent labeling was attempted with 100 nM TAMRA-K$_3$ conjugate **9** in HBSS buffer for 2 min, followed by exchange to HBSS buffer. Stimulation was performed with 100 nM pNPY in OptiMEM for 60 min at 37 °C. Recycling was realized by exchanging the pNPY containing medium by OptiMEM and further incubation for 60 min at 37 °C. Cell nuclei were stained with Hoechst33342. Scale bar equals 10 μm.

in the eYFP channel (Figure 9). After treatment with 100 nM TAMRA-K3 conjugate **9** the receptors at the cell surface were also seen in the TAMRA channel. Stimulation with the pNPY ligand for 60 min induced internalization, which was clearly detected in the eYFP channel. Yet, the TAMRA channel revealed a different picture. The receptors were stained poorly and receptor internalization was hard to follow. After removal of the ligand, the receptors recycled back to the cell surface as seen in the eYFP channel. However, the TAMRA labeling was completely lost. As a result, the colocalization of TAMRA signals and eYFP signals vanished and recycled receptors at the cell surface could not be detected in the TAMRA channel anymore.

These experiments point to the covalent nature of the labeling as important characteristic to study the receptor biology. Without the covalent transfer of the fluorophore, rapid hydrolysis and dissociation occurs as the coiled-coil complex undergoes endosomal trafficking as seen for the Ala-E3-tagged hY$_2$R, due to the reactivity of the TAMRA-K3 conjugate **9**. Please note that HPLC analyses confirmed that Ala-E3 cannot be labeled with 100 nM **9** (Figure S3). In contrast, the TAMRA labeling was still visible with Cys-E3-tagged hY$_2$R after the recycling (Figure 8). Therefore, we infer that the treatment with the thioester-linked TAMRA-K3 conjugate **9** indeed installed a covalent bond between TAMRA and the Cys-E3-tagged GPCR (which is not possible with Ala-E3-tagged GPCR), and once labeling had been achieved, GPCR imaging does not require the integrity of the coiled coil. This data is in agreement with previous studies, in which we submitted labeled receptors at the cell surface to conditions, which allowed hydrolysis of thioesters and dissociation of coiled coils.[35]

CONCLUSION

The work described here offers a potentially general approach to selectively label the extracellular *N*-terminus of tagged G-protein coupled receptors (GPCRs) on live cells. The method relies on the formation of a parallel coiled-coil structure which serves the purpose to align an *N*-terminal cysteine and a thioester structure in close proximity. Our results show that the template effect increases the speed of a native chemical ligation-like reaction to enable rapid labeling within minutes (2−5 min) despite low concentration (100 nM) of the labeling agent.

To put the templated labeling reaction into practice the targeted GPCR is equipped with a genetically encoded, 22-amino-acid-long tag sequence, which comprises the three heptad repeats long E3 coil peptide and an *N*-terminal cysteine. According to the signal transduction assays the added *N*-terminal sequence did not perturb the functional properties of the GPCRs studied. The labeling reagent involves a reactive thioester structure which links a reporter group via a carboxyl group to a mercaptan-functionalized K-peptide. Owing to the reaction design, the peptide templated native chemical coupling occurs through transfer of the reporter group rather than ligation of the coil peptides. As a result, the mass increase that is conferred to the tagged protein remains low. The experiments with synthetic peptides showed that size-matched coiled coils (E3/K3) provided higher labeling yields than coiled coils in which the donating K peptide was longer than the accepting E peptide. Furthermore, a modular solid-phase synthesis

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approach facilitates variations of both dyes and thiol leaving groups in the reactive dye−K peptide conjugates. The evaluation of four different thioester structures unraveled a high reactivity of mercaptophenylacetyl-based thioesters. Though hydrolysis of such reactive thioesters occurs with half life $t_{1/2}$ = 60 min in phosphate buffer (Figure S2a), the rate of the templated reaction is sufficiently high ($t_{1/2}$ = 1−2 min) to enable efficient protein labeling. The labeling agents can be stored in lyophilized form or as frozen aqueous solution. At ambient temperature aqueous solution of the labeling agents should be used immediately. Reactions with two different rhodamine dyes (TAMRA, ATTO488) and a coumarin dye (AF350) provided proof of the flexibility of the labeling method.

The coiled-coil-triggered reporter group transfer was applied to live cell labeling with extended range of target proteins from previously two to now seven different GPCR (hY_1R, hY_2R, hY_4R, hY_5R, $hNPFF_1R$, $hNPFF_2R$, hD_1R). The breadth of the investigated GPCRs suggests a general applicability of our covalent labeling strategy. Fluorescence microscopic analysis revealed the specificity of the labeling. Cells that expressed GPCR constructs containing a C-terminal eYFP and the N-terminal Cys-E3 tag showed, after coiled-coil-triggered TAMRA labeling, colocalization of eYFP and TAMRA signals. By contrast, bystander cells which did not express the construct were devoid of both eYFP and TAMRA. The fluorescence microscopy experiments suggested that the thioester-linked dye−K3 conjugates did not penetrate the cell membrane. Therefore, the coiled-coil-triggered reporter group transfer is confined to the extracellular parts of membrane proteins. We consider this an advantage for the envisaged application, i.e., monitoring GPCR trafficking after treatment with agonists because it will be possible to distinguish between kinetics of receptor recycling and kinetics of trafficking of de novo produced receptors. The investigation of the biological activity of the Cys-E3-tagged GPCR by internalization and recycling experiments as well as signal transduction assays provided evidence that neither the tag nor the labeling procedure interferes with function of the GPCR in their native environment. Even the required *N*-terminal cysteine, which could be suspected to form undesired disulfide bridges or cause receptor dimerization, showed no negative influence in our studies. This demonstrates that the coiled-coil-triggered labeling has no influence on the biology of the tested GPCR at any stage. The extremely fast and selective labeling chemistry will be an advantage to monitor GPCR trafficking in finer detail by pulse chase type experiments.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. The peptides were synthesized in 1−2 μmol scale on a glycine-loaded TentaGel R RAM resin from Rapp Polymere (Tuebingen, Germany) by Fmoc-based manual solid-phase synthesis and employing 2 mL polypropylene syringe reactors from Multisyntech (Witten, Germany) or by employing a ResPep synthesizer from Intavis (Koeln, Germany). Amino acids were purchased from Iris Biotech (Marktredwitz, Germany). HCTU was purchased from Carl Roth (Karlsruhe, Germany). Alexa Fluor 350 succinimid ester was purchased from Life Technologies (Darmstadt, Germany), Atto488 carboxylic acid was purchased from ATTO TEC (Siegen, Germany). Dry DMF (H_2O < 150 ppm) was purchased from VWR (Darmstadt, Germany). The *S*-Mmt-protected mercaptophenyl acetic acid,[51] *S*-Mmt-protected mercaptopropionic acid,[51] *S*-Mmt-protected mercaptoacetic acid,[51] *S*-Mmt-protected N-acetyl cysteine,[51] and *N*-Mmt-protected glycine[52] building blocks were prepared as described. The peptide stems $(KIAALKE)_nG$, n = 3, 4) were assembled via automated synthesis. For Fmoc removal the resin was treated 2 × 2 min with DMF/piperidine (4:1, 200 μL) and washed (3 × 200 μL DMF). The coupling reaction was prepared by charging a preactivation vessel with a 0.6 M HCTU solution in NMP (5.4 equiv), a 4 M NMM solution in DMF (12 equiv) and a 0.3 M amino acid solution in NMP (6 equiv). After 8 min, the preactivation solution was transferred to the resin. After 45 min, the resin was washed (2 × 200 μL DMF). Capping was achieved by treating the resin with Ac_2O/2,6-lutidine/DMF (5:6:89, 200 μL) for 5 min followed by washing (2 × 200 μL DMF). Manual solid-phase synthesis was performed for the introduction of the S-Mmt-protected thiol handles and the dye coupling. The manual synthesis was continued with a 1 μmol aliquot of the resin, which was treated with DMF/piperidine (4:1, 2 × 2 min, 0.5 mL) and washed (5 × DMF, 5 × CH_2Cl_2, 5 × DMF) prior to the coupling with the S-Mmt-protected thiol handle (4.5 equiv Mmt-MPAA-OH (final concentration approximately 0.05 M in DMF), 4 equiv HCTU, and 8 equiv NMM). After 45 min the coupling was repeated once for further 45 min. The resin was washed (5 × DMF, 5 × CH_2Cl_2, 5 × DMF). Subsequently, the resin was treated with DMF/2,6-lutidine/Ac_2O (89:6:5, 10 min) and washed (5 × DMF, 10 × CH_2Cl_2). After Mmt-deprotection with CH_2Cl_2/TFA/TIS (96:2:2, 2 × 1 min), 4 equiv Mmt-Gly-OH (final concentration approximately 0.05 M in DMF), 3.6 equiv HCTU and 8 equiv NMM (45 min) were added. The resin was washed (5 × DMF, 10 × CH_2Cl_2). Subsequently, the Mmt group was removed upon treatment with CH_2Cl_2/TFA/TIS (96:2:2, 2 × 1 min). The introduction of the dyes was performed by using dye acids (4 equiv TAMRA-OH, purchased from ChemPep Inc. (Wellington, USA), final concentration approximately 0.05 M in DMF or 1.2 equiv Atto488-COOH, purchased from ATTO TEC (Siegen, Germany), final concentration approximately 0.02 M in DMF), HCTU (3.6 equiv), and NMM (8 equiv) in DMF or by means of a dye *N*-hydroxysuccinimide ester (4 equiv. AF 350 succinimide ester, purchased from Life Technologies (Darmstadt, Germany), final concentration approximately 0.05 M in DMF and 4 equiv NMM. After 45 min the resin was washed (5 × DMF, 10 × CH_2Cl_2). For liberation of the product, the resin was treated with TFA/H_2O/TIS (96:2:2, 0.6 mL) for 1.5 h and subsequently washed with 0.3 mL neat TFA. Both TFA eluates were added to cooled diethyl ether. The precipitate was collected by centrifugation and decantation. The crude was dissolved in water and purified by semi preparative HPLC with an Agilent 1100 series instrument (column: Varian Polaris C18 A 5 μ 250 × 100, pore size 220 Å) by using eluents A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in a linear gradient (20% B − 60% B in 30 min) at a flow rate of 6 mL/min. Fractions containing product were combined and the volatiles removed in vacuo. The product was dissolved in degassed H_2O + 0.1% TFA and characterized by means of analytical HPLC measurements on a Merck-Hitachi Elite LaChrom chromatograph (column: Varian Polaris C18 A 5 μ 250 × 46, pore size 220 Å) using eluents A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in a linear gradient at a flow rate of 1 mL/min. Detection of the signals was achieved with a photodiode array detector. ESI masses were recorded with an Agilent 1100 series

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LC/MSD mass spectrometer and deconvoluted to molecular masses. MALDI-TOF mass spectra were recorded with a Voyager-DE Pro Biospectrometry Workstation from PerSeptive Biosystems (matrix: DHB). Yields and characterization data are listed in the Supporting Information section.

Templated Acyl Transfer Reactions in Test Tubes. The reactions were performed in buffer (100 mM NaH_2PO_4, 1 mM TCEP) which was degassed and the pH adjusted to 7.0 by using a 2 M NaOH solution. Aliquots of peptides were withdrawn from aqueous stock solutions and were added to the buffer at room temperature to give a final volume of 100 μL and the stated final concentrations (**9**: c_{stock} = 126.3 μM, 3.96 μL added to give 5 μM final concentration or c_{Stock} = 183.8 μM, 0.272 μL added to give 0.5 μM final concentration (or c_{Stock} = 183.8 μM, 0.27 μL added to give 0.5 μM final concentration for Figure 4b); **10**: c_{stock} = 33.9 μM, 14.75 μL added to give 5 μM final concentration; **11**: c_{stock} = 65.2 μM, 0.765 μL added to give 0.5 μM final concentration; **12**: c_{stock} = 61.2 μM, 0.817 μL added to give 0.5 μM final concentration; **13**: c_{stock} = 37.2 μM, 1.344 μL added to give 0.5 μM final concentration; **14**: c_{Stock} = 71.1 μM, 0.704 μL added to give 0.5 μM final concentration; **15**: c_{stock} = 102.6 μM, 0.487 μL added to give 0.5 μM final concentration (or c_{Stock} = 31.8 μM, 1.572 μL added to give 0.5 μM final concentration for Figure 4b); **16**: c_{stock} = 59.3 μM, 8.43 μL added to give 5 μM final concentration, 0.84 μL added to give 0.5 μM final concentration, 0.42 μL added to give 0.25 μM final concentration; **20**: c_{stock} = 42.6 μM, 11.75 μL added to give 5 μM final concentration). For a homogeneous probe distribution the reaction mixture was collected and dispensed by a pipet two times. For the HPLC analysis the reactions were quenched by adding 2% TFA after the indicated reaction time. Aliquots containing 50 pmol (or 10 pmol for Figure 4b) of peptide were analyzed by analytical HPLC using eluent A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and eluent B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in a linear gradient (20% A − 80% B in 20 min at 55 °C) at a flow rate of 1 mL/min (or 0.8 mL/min for Figure 4b). Detection of the signals was achieved with a fluorescence detector (TAMRA: λ_{ex} = 555 nm, λ_{em} = 584 nm; AF 350: λ_{ex} = 346 nm, λ_{em} = 442 nm; Atto488: λ_{ex} = 501 nm, λ_{em} = 523 nm).

Plasmid Construction. The cDNA of hY_1R, hY_2R, hY_4R, $hNPFF_1R$, $hNPFF_2R$, and hD_1R was C-terminally fused to the cDNA of the enhanced yellow fluorescent protein (eYFP) and cloned into the eukaryotic expression vector pVitro2-hygro-mcs (InvivoGen Europe, Toulouse, France). Fusion of the Cys-E3 tag was achieved by overlap-extension PCR with PCRBIOHifi-Polymerase (PCRBIOSYSTEMS, London, United Kingdom). The forward primer with a *MluI* restriction site at the 5′ end and the genetically encoded Cys-E3 tag with a short double glycine linker was designed. The 3′ end contained a 16−18 base pair long overlapping sequence with the N-terminal site of the receptor. In case for the hY_2R, hY_4R, hY_5R, and $hNPFF_1R$ the 3′ end overlapped with the genetically encoded sequence for the hemagglutinin tag (YPYDVPDYA), which was still fused to the receptor N-terminus. Cys-E3_hY_1R: 5′-AAACCACGCGTACCATGTGCGAGATCGCCGCCCTGGAGAAGGAGATCGCCGCCCTGGAGAAGGAGATCGCCGCCCTGGAGAAGGGCGGCAATTCAACATTATTTTCCC-3′; Cys-E3_hemaglutinin for hY_2R, hY_4R, hY_5R, and $hNPFF_1R$: 5′-AAACCACGCGTACCATGTGCGAGATCGCCGCCCTGGAGAAGGAGATCGCCGCCCTGGAGAAGGAGATCGCCGCCCTGGAGAAGGGCGGCTACCCATACGACGTGCCC-3′; Cys-E3_$hNPFF_2R$: 5′-AAACCCGCGTACCATGTGCGAGATCGCCGCCCTGGAGAAGGAGATCGCCGCCCTGGAGAAGGAGATCGCCGCCCTGGAGAAGGGCGGCAATGAGAAATGGGACAC-3′; Cys-hD_1R: 5′-AAACCACGCGTACCATGTGCGAGATCGCCGCCCTGGAGAAGGAGATCGCCGCCCTGGAGAAGGAGATCGCCGCCCTGGAGAAGGGCGGCAGGACTCTGAACACCTC-3′. The reverse primer overlapped with the pVitro2-hygro-mcs vector after the stop codon of the eYFP sequence. The obtained PCR products were digested with *MluI* and *Bsp1407I* and ligated in an equally digested pVitro2-hygro-mcs vector. Deletion of the eYFP for the Cys-E3-tagged hY_1R, hY_2R, hY_4R, hY_5R, and $hNPFF_2R$ was achieved by overlap-extension PCR. The forward primer overlapped with the pVitro2-hygro-mcs vector before the already introduced *MluI* restriction site and the reverse primer contained an 18-base-pair-long sequence of the respective receptor C-terminus followed by a stop codon and a *BSP1407I* restriction site. After digestion of the PCR products with *MluI* and *Bsp1407I*, ligation in the equally digested pVitro2-hygro-mcs vector was performed. The mutation of the *N*-terminal cysteine to alanine to obtain the Ala-E3_hY_2R_eYFP_pVitro2-hygro-mcs was achieved by QuikChange site-directed mutagenesis (Stratagene, LaJolla, USA) as recommended by the manufacturer. The already cloned Cys-E3_hY_2R_eYFP_pVitro2-hygro-mcs was used together with the following primer: Cys-E3_Y2_C2A_forward: 5′-CCACGCGTACCATGGCCGAGATCGCCGCCC-3′; Cys-E3_Y2_C2A_reverse: 5′-GGGCGGCGATCTCGGCCATGGTACGCGTGG-3′. All constructs were verified by DNA sequencing.

The cDNA of the human Rab4a was generously provided by M. Zerial (MPI of Molecular Cell Biology and Genetics, Dresden, Germany) and cloned into the pECFP-C1 (Clontech, Heidelberg, Germany) using *Bgl*II and *Sal*I restriction sites.

Cell Culture and Live Cell Fluorescence Microscopy. Cell culture materials were supplied by LONZA (Lonza Group Ltd., Basel, Switzerland), except of fetal calf serum (FCS) which was supplied by Biochrom (Biochrom AG, Berlin, Germany). Cells were grown as monolayers at 37 °C, 5% CO_2, and 95% humidity.

Cells for Live Cell Fluorescence Microscopy Studies. HEK293 cells (human embryonic kidney cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and HAMsF12 containing L-glutamine (1:1 v/v), supplied with 15% (v/v) heat-inactivated FCS. HEK293 cells were seeded out into 8-well μ-slides (ibid GmbH, Martinsried, Germany), which were prior coated with poly-D-lysine (0.01% w/v in PBS) and grown to 70% confluence overnight. Subsequently the cells were transiently transfected with the respective plasmid DNA and Lipofectamin 2000 (Invitrogen GmbH, Karlsruhe, Germany), as recommended by the manufacturer. For single transfection 800 ng receptor DNA with 1 μL Lipofectamin 2000 per well was applied. For cotransfection 800 ng receptor DNA and 80 ng of Rab4a-CFP-N1 with 1 μL Lipofectamin 2000 per well was used.

Cells for IP Accumulation Assays. COS-7 cells (African green monkey) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose supplied with 10% (v/v) heat-inactivated FCS. COS-7 cells were grown in 48-well plates to 70% confluence overnight. Afterward the cells were transiently transfected with 320 ng receptor DNA, 80 ng G$\alpha\Delta$qi4myr-pcDNA3 and 0.8 μL Metafecten Pro (Biontex Laboratories GmbH, Munich, Germany) per well in DMEM overnight, as recommended by the manufacturer.

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Live Cell Fluorescence Microscopy with Peptide-Templated Acyl Transfer in Living Cells. Hanks balanced salt solution (HBSS) was purchased by LONZA (Lonza Group Ltd., Basel, Switzerland). Transiently transfected HEK239 cells with Cys-E3-tagged receptors fused to eYFP were labeled with TAMRA-K3 conjugate **9**. The cells were washed with 5% BSA (w/v) in HBSS buffer pH = 7, followed by incubation with 0.5 mM TCEP in 20 mM HEPES/HBSS buffer pH = 7 for 10 min at 37 °C to reduce the *N*-terminal cysteine. Labeling was applied with 100 nM TAMRA-K3 conjugate **9** in 1% BSA (w/v) in HBSS pH = 7 for 2 min at room temperature. Subsequently the cells were washed with HBSS pH = 7 and analyzed under the microscope. Transiently transfected HEK293 cells with Cys-E3-tagged receptors without fusion to eYFP were labeled either with TAMRA-K3 conjugate **9** or ATTO488-K3 conjugate **15**. The cells were washed with 20 mM HEPES/HBSS buffer pH = 7. Reduction of the *N*-terminal cysteine and labeling with 100 nM of respective K3 conjugate was applied in 0.1 mM TCEP in 20 mM HEPES/HBSS buffer pH = 7 for 5 min at room temperature. After a subsequent basic wash with 150 mM $NaHCO_3$ in HBSS buffer pH = 8.6 for 1.5 min and exchange to 20 mM HEPES/HBSS buffer pH = 7 the cells were analyzed under the microscope.

For internalization and recycling studies Rab4a-CFP-N1 was cotransfected. Transiently transfected HEK293 cells with Ala-E3-tagged hY_2R fused to eYFP were labeled with TAMRA-K3 conjugate **9**. The cells were directly incubated with 100 nM TAMRA-K3 conjugate **9** in HBSS buffer pH = 7 for 2 min at room temperature. TCEP addition was not necessary because of the *N*-terminal cysteine to alanine exchange. After subsequent exchange to HBSS buffer pH = 7 the labeled cells were analyzed under the microscope. A basic wash as described before was not performed to prevent the early hydrolysis of the thioester.

Cell nuclei were visualized with Hoechst33342 (0.5 mg/mL, Sigma-Aldrich, Taufkirchen, Germany) prior to labeling. Stimulation of transient transfected cells was performed with 1 μM *porcine* NPY (pNPY) in OptiMEM (Life Technologies GmbH, Darmstadt, Germany) for the indicated time. The stimulation was stopped by an acidic wash (50 mM glycine, 180 mM NaCl, pH = 3.1) for 30 s and exchange to OptiMEM.

Fluorescent images were taken by using a Zeiss AxioObserver.Z1 microscope with ApoTome Imaging System and a C-Apochromat 63×/1.20 W objective, with an eYFP/ATTO488 filter (excitation 488−512 nm; emission 520−550 nm), TAMRA filter (excitation 550−580 nm; emission 590−650 nm), CFP filter (excitation 426−446 nm; emission 460−500 nm) and DAPI filter (excitation 335−383 nm; emission 420−470 nm). Images were processed with Zeiss ZEN2012 software.

IP Accumulation Assays with Peptide-Templated Acyl Transfer in Living Cells. Transient transfected COS-7 cells in 48-well plates radioactive labeled and stimulated based on a published protocol.[53,54] Overnight transfection was stopped by exchanging the medium to DMEM with 4.5 g/L glucose supplied 10% FCS (v/v) and further cultivation for 6−8 h. COS-7 cells were radioactive labeled with 2 μCi/ml myo-[2-^{3}H]-inositol (PerkinElmer, Waltham, Massachusetss, USA) in culture medium for at least 16 h. For Cys-E3-tagged receptors, labeling with ATTO488-K3 conjugate (**15**) was performed as described for live cell fluorescence microscopy. As controls one Cys-E3-tagged and one untagged receptor were labeled by the same protocol but without ATTO488-K3 conjugate. Afterward cells were washed and stimulated with pNPY at concentrations from 10^{-12} to 10^{-6} M for 1 h at 37 °C. Radioactive phosphoinositides were isolated and measured by a scintillation counter as described by Hofmann et al. and Findeisen et al.[53,54] Concentration−response curves and EC_{50} as well as pEC_{50} values were calculated by nonlinear regression using GraphPad Prism 5.0 software. Assays were performed in duplicate and two independent experiments were performed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00387.

Characterization data, HPLC analyses of labeling reactions, and stability tests (PDF)

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Notes

The authors declare no competing financial interest.

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