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Short Peptide Tag for Covalent Protein Labeling Based on Coiled Coils

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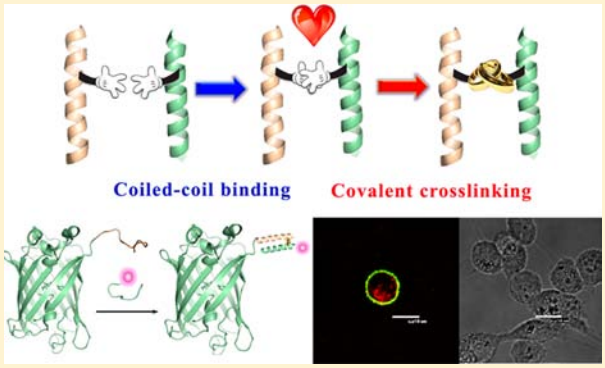
Supporting Information

ABSTRACT: To label proteins covalently, one faces a trade-off between labeling a protein specifically and using a small tag. Often one must compromise one parameter for the other or use additional components, such as an enzyme, to satisfy both requirements. Here, we report a new reaction that covalently labels proteins by using engineered coiled-coil peptides. Harnessing the concept of "proximity-induced reactivity", the 21-amino-acid three-heptad peptides CCE/CCK were modified with a nucleophilic cysteine and an α-chloroacetyl group at selected positions. When pairs of coiled coils associated, an irreversible covalent bond spontaneously formed between the peptides. The specificity of the cross-linking reaction was characterized, the probes were improved by making them bivalent, and the system was used to label a protein *in vitro* and receptors on the surface of mammalian cells.



INTRODUCTION

Genetic fusion of a reporter such as a fluorescent protein or an enzyme stamps a permanent mark on a protein of interest (POI) that imaging techniques can trace and visualize. Although widely utilized, the cumbersome size of the tags (for example, the green fluorescent protein is a 238-amino-acid polypeptide with a molecular weight of 26.9 kDa) can interfere with the intracellular transport or activity of the target protein.[1,2] In contrast, covalent labeling based on chemical reactions at amino acids with specific chemical reactivities exhibits a variety of virtues, such as smaller tag sizes, a versatile choice of labels, and temporospatial control of the labeling event.[3]

The most common covalent labeling reactions harness the chemical reactivities of natural amino acids.[4] Many chemical reactions that modify a single amino acid, such as cysteine,[4,5] lysine,[4] tyrosine,[6,7] or tryptophan,[8,9] several consecutive amino acids in a motif,[10,11] or one of the two termini of a POI,[12−16] have been developed. Because of the multitude of chemical moieties with similar reactivities in a cellular environment, labeling reactions solely driven by chemical reactivity often face the challenge of selectivity. One tactic to overcome this hurdle is to introduce bioorthogonal chemical moieties by genetically incorporating unnatural amino acids.[17−20] Chemical biologists seek help from enzymes, which have unparalleled specificity in recognizing and converting substrates. Covalent labels have been developed based on suicide inhibitors, cofactors, and substrates of various enzymes; examples include activity-based protein probes,[21,22] CoA-affinity-based kinase tags,[23] the enzyme−suicide substrate-based SNAP/CLIP-tags,[24,25] the Halo-tag,[26] the lactam-based β-lactamase-tag,[27] and the small molecule inhibitor-based TMP tag.[28,29] However, despite their outstanding specificity, the enzyme tags are still larger than 100 amino acids.

A third strategy uses short peptide sequences from natural substrates of ligases or transferases and converts them into covalent tags. Examples in this category include a 15-amino-acid receptor peptide AP (the substrate of the biotin ligase BirA),[30] a 13-amino-acid sequence LAP (the substrate of a mutant lipoic acid ligase),[31] a 7-residue Q-tag (the substrate of a transglutaminase),[32] the small sortagging motif LPXTG (the substrate of a sortase),[33] a LCTPSR formyl glycine tag (the substrate of a formyl glycine generating enzyme),[34] and the peptides A1 and S6 (the substrates of different PPTases).[35] These labeling reactions, however, require the addition or coexpression of external enzymes.

A fourth type of covalent labeling, affinity labeling, does not require enzymes.[36−41] In the process of perfecting "pretargeting", Meares and co-workers pioneered the development of antibodies with infinite affinity based on the principle of effective local concentration.[42] Two complementary reactive groups were brought into proximity in an antibody−antigen complex and subsequently formed a covalent conjugate to permanently attach metal−EDTA or metal−DOTA chelates to their engineered monoclonal antibodies through nucleophilic reaction of thiol moiety of cysteines.[42−45] The same group

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recently surpassed the limitation of thiol chemistry and developed a 19-mer peptide ligand, carrying a lysine-tagged dinitrofluorobenzene group, which can covalently attach to a unique lysine residue on human vascular endothelial growth factor, even in the presence of serum proteins.[46] Hamachi and co-workers reported a spontaneous nucleophilic S_N2 reaction between a peptide tag, an N-terminal CA6D4 tag (11 amino acids), and a label, an *N*-α-chloroacetyl Zn(II)-DpaTyr molecule, when the two bound *in vitro*.[47] By increasing the valency using an N-terminal CA6D4×2 tag (16 amino acids) and a tetranuclear Zn(II)-DpaTyr molecule carrying the α-chloroacetyl moiety, the labeling efficiency can be greatly improved to achieve covalent labeling of cell surface proteins.[48] A second example is the Spytag-Spycatcher system reported by Howarth and others.[49−51] Through domain spliting and rational engineering, a 13-residue Spytag peptide was found to spontaneously and rapidly form an isopeptide bond with the Spycatcher-tagged proteins.[49] The scarcity of more examples of this strategy reflects the difficulty in satisfying the trade-off between labeling specificity and tag size, in particular in the context of covalent labeling. However, Matsuzaki and co-workers pioneered the use of coiled-coil interaction as a specific labeling strategy for membrane receptors; notwithstanding its noncovalent nature, this tag has found great success.[52−54] Their rapid and stable interaction allowed for an antibody-like labeling of membrane receptors.[52]

Inspired by these approaches, we sought to combine the specificity of noncovalent peptide−peptide interaction and the stability of a covalent linkage to develop a short peptide tag for the covalent labeling reaction, which is nonenzymatic, spontaneous, site-specific, and fully biocompatible (Figure 1). By screening specific positions in two paired coiled-coil peptides, we designed a covalent (irreversible) labeling reaction that is nonenzymatic, spontaneous, and site-specific, and it requires a small tag of only 21 proteinaceous amino acids.

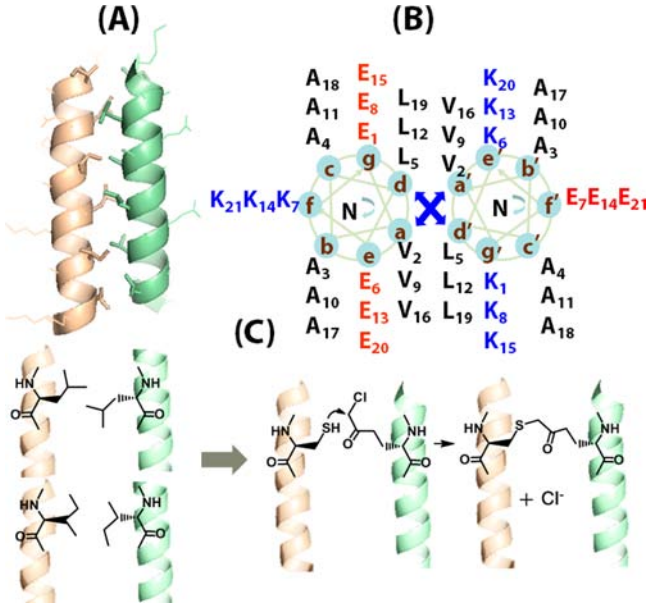

Figure 1. Design of a cross-linking reaction induced by coiled-coil binding. (A) Coiled-coil structure of the CCE/CCK heterodimer (PDB ID 1U0I). (B) Helical wheel representation of the parallel CCE/CCK heterodimer. The coiled-coil is viewed in cross-section, with both peptide chains propagating into the page from the NH2 to the COOH terminus. Blue arrows denote the interhelical hydrophobic interactions at the *a*-*a*′ and *d*-*d*′ positions of the heptads. (C) The principle of a cross-linking reaction induced by coiled-coil binding. Replacing one pair of Leu-Leu residues at *a*-*a*′ or *d*-*d*′ positions with Cys and an unnatural amino acid **X** generates coiled-coil derivatives that can still form heterodimers. The local proximity of Cys and **X** in the heterodimer induces the formation of an interstrand covalent bond. **X** denotes (2*S*)-2-amino-3-[(2-chloroacetyl)amino]propanoic acid.

MATERIALS AND METHODS

Peptide Cross-Linking Reaction and Kinetics Measurement. The synthetic peptide was dissolved in HEPES buffer (50 mM HEPES and150 mM NaCl, pH 7.4), and the concentration was measured by a UV−vis spectrometer (Varian, Cary 5G, USA) based on the absorption coefficient constants of the dyes (ε_{487} = 75090 cm^{-1} M^{-1} for *fl*, and ε_{555} = 91000 cm^{-1} M^{-1} for *tmr*). *fl* represents the green fluorescent dye 5(6)-carboxyfluorescein, and *tmr* represents the red fluorescent dye 5(6)-tetramethylrhodamine. Peptide pairs (100 μM) were mixed in phosphate buffered saline (PBS) containing 1 mM *tris*(2-carboxyethyl)phosphine (TCEP) and incubated in the dark at RT. Aliquots were taken at different time points and injected into RP-HPLC and monitored at 215, 448, 565, and 650 nm simultaneously. The kinetics of the reaction was analyzed according to the integrated peak areas.

***In Vitro* Protein Labeling and Kinetics Measurement.** *EGFP-CCE-1* protein (or wild type EGFP) (EGFP represents green enhanced fluorescent protein) was mixed with fluorescently labeled CCK-based probes in HEPES buffer in the presence of 1 mM TCEP and incubated in the dark at RT. Aliquots taken at different time points were mixed with 5× protein loading buffer (0.313 M Tris-HCl (pH 6.8), 10% SDS (w/v), 0.05% bromophenol blue (w/v), 0.5 M DTT, and 5% glycerol (v/v)) and denatured at 95 °C for 10 min. Samples were then loaded to denaturing SDS−PAGE and the protein gel was applied to Typhoon TRIO+ Variable Mode Imager (GE Healthcare, USA) for in-gel fluorescence scanning. Fluorescent images were acquired with the TRITC channel ($\lambda_{ex}/\lambda_{em}$, 532 nm/580 nm) for *tmr* labeled peptides. The fluorescent protein bands were quantified by pixel numbers using Image J.

For labeling on resin, 20 μL of protein solutions were immobilized on Ni-NTA resins and labeled with fluorescent peptide probes (100 μM) in HEPES buffer for 100 min. The beads were washed with PBS buffer, precipitated through centrifugation, and redispersed in PBS buffer. After each washing trial, 2 μL of beads were loaded on a glass slide and imaged by a confocal fluorescent microscope (Nikon eclipse T*i*, Nikon Instrument Inc., USA) at the TRITC channel (G-2A, excitation, 510−560 nm; dichroic mirror, 575 nm; and barrier filter, 590 nm). The fluorescence intensity was measured using Nikon software associated with a T*i* microscope.

Cell Culture, Transfection, and Labeling. Chinese Hamster Ovary (CHO) cells or Human Embryonic Kidney 293 (HEK293) cells were grown in DMEM/F12 medium (Dulbecco's modified Eagle's medium/nutrient mixture F-12, Life technology, USA) supplied with 10% fetal bovine serum (FBS, Life technology, USA) and antibiotics (penicillin/streptomycin, Life technology, USA) in a 10 cm culture dish (Corning, USA) and maintained at 37 °C in a humidified incubator supplied with 5% CO_2.

For cell labeling, 1.5 × 10^5 cells were seeded in a 35 mm confocal dish (ibidi, Germany) one day prior to transfection. At ∼40% confluence, cells were transfected with a DNA (0.8 μg)/

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Table 1. List of Coiled-Coil Peptide Derivatives

name	peptide sequence[a]	name	peptide sequence[a]	$t_{1/2}$(min)
CCK-1	*fl*-GGGK **X**AALKEK VAALKEK VAALKE	*CCE-1*	*tmr*-GGGE **C**AALEKE VAALEKE VAALEK	13.8
		CCE-1′	*tmr*-KSEESYE**C**AALEKEVAALEKEVAALEK	16.9
CCK-2	*fl*-GGGK VAA**X**KEK VAALKEK VAALKE	*CCE-2*	*tmr*-GGGE VAA**C**EKE VAALEKE VAALEK	>1500
CCK-3	*fl*-GGK VAALKEK **X**AALKEK VAALKE	*CCE-3*	*tmr*-GGE VAALEKE **C**AALEKE VAALEK	920
CCK-4	*fl*-GGK VAALKEK VAA**X**KEK VAALKE	*CCE-4*	*tmr*-GGE VAALEKE VAA**C**EKE VAALEK	720
CCK-5	*fl*-GGGK VAALKEK **X**AALKEK VAALKEK VAALKE	*CCE-5*	*tmr*-GGGE VAALEKE **C**AALEKE VAALEKE VAALEK	>2000
CCK-6	*fl*-GGGK VAALKEK VAA**X**KEK VAALKEK VAALKE	*CCE-6*	*tmr*-GGGE VAALEKE VAA**C**EKE VAALEKE VAALEK	770
CCK-7	*fl*-GGK VAALKEK VAALKEK **X**AALKEK VAALKE	*CCE-7*	*tmr*-GGE VAALEKE VAALEKE **C**AALEKE VAALEK	>4000
CCK-8	*fl*-GGK VAALKEK VAALKEK VAA**X**KEK VAALKE	*CCE-8*	*tmr*-GGE VAALEKE VAALEKE VAA**C**EKE VAALEK	>4000

[a] *fl* denotes 5(6)-carboxyfluorescein; and *tmr* denotes 5(6)-tetramethylrhodamine.

PLUS reagent (0.8 μL)/lipofectamine LTX (2 μL) mixture at a ratio of 1:1:2.5 with the final DNA concentration set to be 1 μg/mL in Opti-mem I (Life technology, USA). Cell medium was changed back to DMEM/F12 5 h post-transfection. After 48−50 h, the cells were pretreated with HEPES buffer containing 0.5 mM TCEP for 10 min at RT and then incubated with fluorescent peptide probes for 20 min. The cells were then washed, fixed by 4% paraformaldehyde in PBS (w/v), and then incubated with anti-HA-FITC antibody (in 1:300 dilution) (Sigma-Aldrich, USA) at RT for 1 h. The cells were washed at least 3 times with PBS (10 min each) and imaged by confocal fluorescent microscope.

RESULTS AND DISSCUSSION

Design of the System. Formed by repeats of heptads $[abcdefg]_n$, individual coiled-coil peptides adopt random coiled structures in solution. When specific pairs are mixed, hydrophobic interactions between the *a* to *a′* and *d* to *d′* positions together with electronic attractions between the *e* to *g′* and *g* to *e′* positions drive the formation of a parallel coiled-coil heterodimer.[55,56] For example, the *CCE3* and *CCK3* peptides (*CCE3*: E VAALEKE VAALEKE VAALEK and *CCK3*: K VAALKEK VAALKEK VAALKE; 3 denotes three heptads) are a pair of parallel coiled-coil peptides (Figure 1A and B) with a dissociation constant of about 64 nM and whose interaction reaches equilibrium within 1 min.[52]

A nucleophilic reaction between cysteine and the α-chloroacetyl moiety can spontaneously introduce a covalent thioether bond between a small molecule and a cysteine (Figure 1C). The reaction rate becomes appreciable only when the two moieties are in close proximity; this feature renders the reaction particularly suitable for proximity-induced reactions.[47,48] We envisioned that parallel coiled-coil interactions between CCK and CCE derivatives would juxtapose the two carefully positioned groups: the thiol group of the cysteine residue in one peptide and the α-chloroacetyl moiety in another, into close proximity to induce an interstrand thioether bond. We used the α-chloroacetyl-containing unnatural amino acid (2*S*)-2-amino-3-[(2-chloroacetyl)amino]propanoic acid (denoted **X** here) because the length of the resultant thioether bond would match the distance between the *a* and *a′* residues. For example, the distance between the α-carbons of the residues at *a* or *d* positions and those at corresponding positions is 5.6 ± 0.2 Å in the crystal structure of the CCE/CCK heterodimer (PDB ID 1U0I).[56] Containing 7 single bonds between the two α-carbons, the resultant thioether linkage mimics this distance (Figure S1, Supporting Information). Thus, the coiled-coil structure should not be disturbed.

Screening a Cross-Link Library. We first screened the *a* or *d* positions of the CCK and CCE peptides for an optimal cross-linking position. A set of three-heptad *CCK3* derivatives, with **X** at the first *a* (*CCK-1*), first *d* (*CCK-2*), second *a* (*CCK-3*), and second *d* positions (*CCK-4*) were synthesized (Table 1). Synthesized as well were a set of four-heptad *CCK4* derivatives, with **X** placed at the second *a* (*CCK-5*), second *d* (*CCK-6*), third *a* (*CCK-7*), and third *d* positions (*CCK-8*). A corresponding set of CCE derivatives, with cysteine replacing the *a* or *d* amino acid at the matching positions of CCK derivatives, was also synthesized. To monitor the cross-linking reaction by chromatographic techniques, all of the CCK derivatives were labeled with the green fluorescent dye 5(6)-carboxyfluorescein (*fl*), and the CCE derivatives were labeled with the red fluorescent dye 5(6)-tetramethylrhodamine (*tmr*). Both dyes have distinct absorption spectra in a diode array, so we could specifically monitor the reaction progress by the chromatographic method.

Incubation of equimolar amounts of a CCK derivative with its corresponding CCE derivative at room temperature (RT) and PBS at pH 7.4 spontaneously formed a covalent conjugate (Figure 2A). TCEP was included in the solution to rule out disulfide formation between CCE peptides. Covalent heterodimeric conjugates can be observed by high performance liquid chromatography (HPLC) (solvent A, 0.1% trifluoroacetic acid (TFA) in water; solvent B, 0.1% TFA in acetonitrile). The denaturing condition of HPLC ensured that only covalently bonded heterodimers were observed.[57] The covalent heterodimer *CCK-1-CCE-1* rapidly emerged, with 50% conversion achieved in 13.8 min ($t_{1/2}$) or a reaction rate of 24 $M^{-1}\cdot s^{-1}$ at 25 °C (Figure 2B). The fluorescent tags did not affect the reactivity compared to that when we swapped fluorophors (*fl* labeled *CCE-1* and *tmr* labeled *CCK-1*); similar kinetics was observed (data not shown). In contrast, incubation of *CCK-1* peptide in 500 μM glutathione (10-fold excess) resulted in only 16% of *CCK-1*-glutathione conjugate after 10 h (Figure S2, Supporting Information). The α-chloroacetyl moiety thereby has rather low reactivity toward general thiols but reacts very rapidly with a thiol moiety that is brought into close proximity through peptide−peptide binding. We should note that only cross-linked heterodimers could be probed, although other assembly states might also exist between the pair.[58]

Other pairs of CCK/CCE derivatives showed very different reaction kinetics (Figure S3, Supporting Information). On the basis of comparisons of the reaction time to form 50% of the heterodipeptide conjugate ($t_{1/2}$), the best efficiency was observed for peptides with cross-linking sites at their termini, whereas cross-linking reactions between sites in the middle of peptides were slower (Table 1). The positional effect is

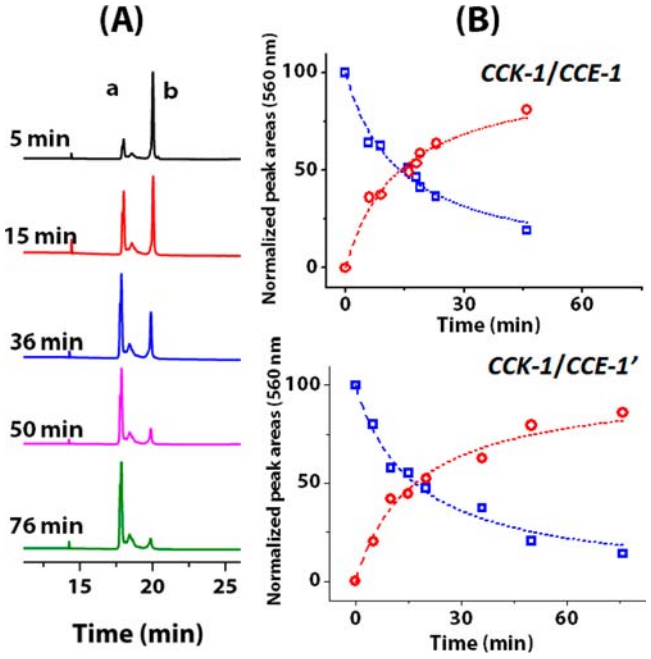

Figure 2. Covalent cross-linking of coiled-coil peptides. (A) Reaction progress of *CCE-1* and *CCK-1* monitored by HPLC traces at 560 nm. Peak a, the heterodimer *CCK-1-CCE-1*; b, *CCE-1*. The small peak at 18 min is an impurity. (B) Reaction kinetics of the *CCK-1*/*CCE-1* pair and the *CCK-1*/*CCE-1′* pair. The dashed lines show curve fits to second-order reaction kinetics. [Peptide] = 50 μM. Red circles represent the heterodimer, and blue squares represent *CCE-1* or *CCE-1′*.

consistent with a previous report about disulfide linking of a homodimeric coiled-coil,[59] and it might be explained by several reasons. First, mutation at the terminal *a-a′* or *d-d′* position causes less disturbance to the stability of the coiled-coil structure than mutations in the internal hydrophobic cores. Second, structural flexibility of the peptide termini will facilitate reaction. Third, the nucleophilic S_N2 reaction is driven by the departure of a Cl^- ion (Figure 1C). Because the termini are more flexible, the chloride ion can be more easily hydrated and leave, driving the reaction forward. This notion is supported by an out-of-heptad cross-linking reaction (Figure S4, Supporting Information). Notably, *CCE-1′*, which has an additional sequence of charged and polar amino acids at the N-terminus relative to the *CCE-1* peptide (*CCE-1′* was designed to examine whether any additional N-terminal sequence was tolerated in the conjugation reaction), reacted with *CCK-1* with a similarly fast reaction rate (Figure 2B), the $t_{1/2}$ being 16.9 min. Thus, a peripheral sequence appended to the coiled-coil structure does not seem to affect the cross-linking reaction, suggesting that CCE-based tagging does not require being at the N terminus of the protein.

Orthogonality of Reacting Pairs. The beauty of the coiled-coil interaction lies not only in its simplicity, but also in its extendability to heterospecific modules of multiple assembling components in synthetic biology.[60] On the basis of an algorithm to search the specificity of coiled-coils, Bromley et al. designed coiled-coil tectons: three pairs of coiled-coil peptides that have maximal specificity toward their binding partners (Table S1, Supporting Information).[61] Covalent cross-linking reactions based on tecton pairs should exhibit orthogonality. (Orthogonality here means that peptide A only reacts with its partner peptide A′ and peptide B with B′ to form the specific covalent conjugates A-A′ and B-B′ but not the heterodimers A-B′ or B-A′.)

Having the tecton peptides p_1–p_6 as parents, we synthesized six derivatives p_{1X}, p_{2C}, p_{3X}, p_{4C}, p_{5C}, and p_{6X} (Table 2). In all three pairs, the Cys/**X** mutation was introduced at the N-terminal *a-a′* position. To differentiate the three sets of cross-linking reactions, the six peptides were labeled with three different dyes, *fl*, *tmr*, and *cy5*, respectively, and monitored at the wavelengths of *fl* (448 nm), *tmr* (520 nm), and *cy5* (650 nm) in a single HPLC run. By comparing the retention times of the cross-linked heterodimers with single-pair reaction systems as standards, we could assign each peak to monomers or heterodimers (Figure S5, Supporting Information). The cross-linking reactions were found to proceed faithfully within the specific pairs. Namely, a p_{1X}–p_{2C} heterodimer (peaks **a**–**b**), a p_{3X}–p_{4C} heterodimer (peaks **c**–**d**), and a p_{5C}–p_{6X} heterodimer (peaks **e**–**f**), but not nonspecific heterodimers could be observed (Figure 3A).

Table 2. List of Tecton Peptide Derivatives

p_{1X}	*tmr*-GGE **X**AALKQE NQALEQK IAALKGY
p_{2C}	*tmr*-GGE **C**AALKQK NKYLKQE IQQLE
p_{3X}	*fl*-GGK **X**QALQQK IKQLKQK IAALKGY
p_{4C}	*fl*-GGQ **C**AALEQE IAALEQE IAALE
p_{5C}	*cy5*-GGE **C**AALEQQ NKYLKQE IAALKGK
p_{6X}	*cy5*-GGK **X**KALKQE NAYLQQE IQALK

The four-peptide system containing the p_{1X}/p_{2C} pair and the p_{5C}/p_{6X} pair shows a clearer picture of the orthogonality of the reactions; *fl*-labeled peptides were excluded to obtain clearer spectra. Clearly, only the specific heterodimers p_{1X}–p_{2C} (peaks **a**–**b**, with a retention time of 33.64 min) and p_{5C}–p_{6X} (peak **e**–**f**, with a retention time of 32.33 min) were detected (Figure 3B). The UV–vis spectra of the two heterodimer peaks were also indicative (Figure 3C). Peak **a**–**b** showed the characteristic spectrum of *tmr* (the small peak at 650 nm is caused by spectral leaking of *cy5*), while peak **e**–**f** showed the characteristic spectrum of *cy5*. Taken together, within the detection limit of our assay, the cross-linking reactions all happened within specific pairs and did not occur randomly. The orthogonality demonstrated here is consistent with the previous report that cysteine-labeled tecton peptides p_1 to p_6 could specifically cross-link with each partner.[61] Aside from marked orthogonality, the reaction rates of the three pairs in a mixture significantly differed. The p_{1X}/p_{2C} pair proceeded the most rapidly, while the p_{3X}/p_{4C} pair occurred the most slowly. (The cause of this discrepancy is unknown to us at present.) Through the reserved specificity in tecton derivatives, we further demonstrated that affinity-induced proximity is a *sine qua non* of the S_N2 reaction. In other words, the covalent cross-linking reaction is driven by local proximity, instead of general chemical reactivity.

Covalent Labeling of Peptide-Tagged Proteins *in Vitro*. We then examined the covalent labeling of CCE-1 tagged protein *in vitro*. A *CCE-1* tag was fused to the C-terminus of an enhanced green fluorescent protein (EGFP) to yield the protein *EGFP-CCE-1*. We observed that *EGFP-CCE-1* could be labeled in a nondissociable manner by *tmr-CCK-1* but not by the parental *CCK3* peptide that lacked the α-chloroacetyl group. Furthermore, although containing a solvent-exposed Cys48, wild-type EGFP (wt EGFP) did not react with *tmr-CCK-1* (Figure 4). We also conducted the labeling reaction in a lysate of mouse brain containing a total

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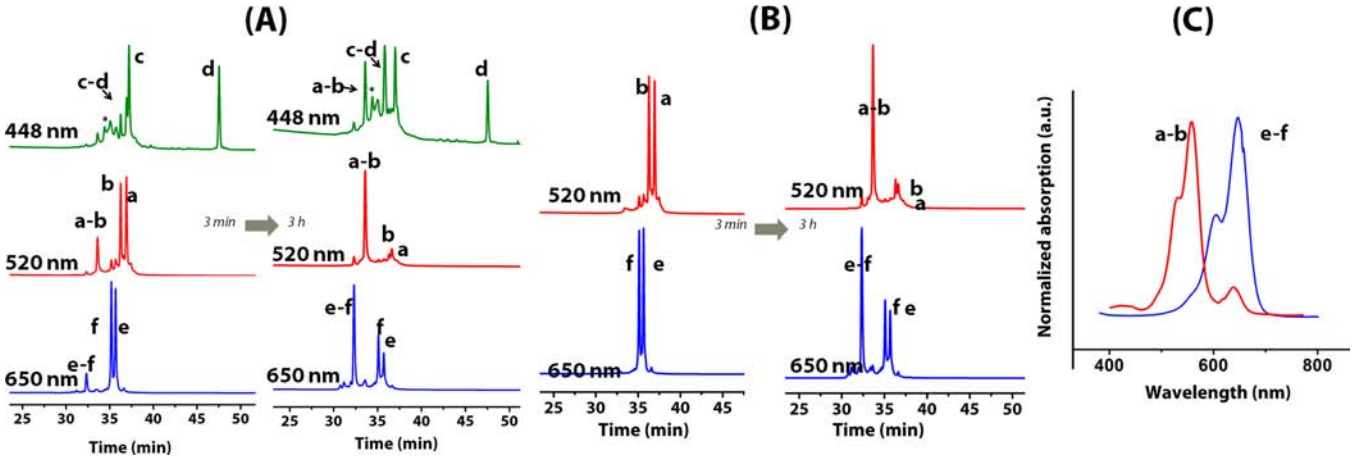

Figure 3. Covalent cross-linking of coiled-coil peptides. (A) Progress of a reaction with equimolar amounts of six peptides, p_{1X} (peak a), p_{2C} (peak b), p_{3X} (peak c), p_{4C} (peak d), p_{5C} (peak e), and p_{6X} (peak f), after 3 h of incubation at RT and pH 7.4. The reaction was monitored by HPLC at 448 nm (green trace), 520 nm (red trace), and 650 nm (blue trace). Peaks **a**–**b**, **c**–**d**, and **e**–**f** were assigned as the heterodimers p_{1X}–p_{2C}, p_{3X}–p_{4C}, and p_{5C}–p_{6X}, respectively. The unassigned peaks at 448 nm were caused by "spectral leaking" of *tmr* and *cy5*-labeled peptides. * indicates a minor impurity. (B) Progress of a reaction with equimolar amounts of four peptides, p_{1X} (peak **a**), p_{2C} (peak **b**), p_{5C} (peak **e**), and p_{6X} (peak **f**). (C) UV–vis spectra of peaks **a**–**b** (retention time 33.64 min) and peaks **e**–**f** (retention time 32.33 min) in B.

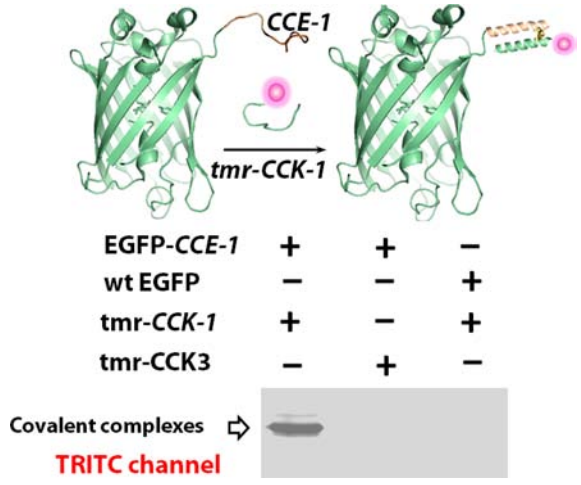

Figure 4. Covalent labeling of *EGFP-CCE-1* in solution. The protein/peptide solution was thermally denatured and resolved by reducing SDS–PAGE. The gel was imaged with a Typhoon Imager at the TRITC channel. *CCK3*, the parental peptide without the *α*-chloroacetyl moiety, was included as a control.

protein up to 7.5 mg/mL. Only one band corresponding to the molecular weight of *EGFP-CCE-1* was detected by the Typhoon imager (Figure S6, Supporting Information), showing that covalent labeling of the *CCE-1* tagged protein *in vitro* is site-specific.

Compared with the reaction of synthetic coiled-coil peptides, the covalent labeling of the *EGFP-CCE-1* fusion occurred more slowly, possibly because the *CCE-1* tag in the fusion was sterically hindered or had decreased rotational freedom. The same effect was observed in the labeling reaction with the CA6D4 tag.[47] To improve the labeling efficiency, we followed the strategy of Hamachi and co-workers that used a bivalent interaction to more efficiently label cell surface receptors.[48] A bivalent *CCK-1-dimer* was synthesized using a lysine residue as the branching point (Figures 5A and S7 (Supporting Information)). *CCK-1-dimer* reacted with *EGFP-CCE-1* roughly 5 times faster than the monovalent *CCK-1* probe (38 min vs 150 min by $t_{1/2}$) (Figures 5B and C, and S8 (Supporting Information)). Interestingly, after *EGFP-CCE-1* was covalently linked to *CCK-1* or the *CCK-1-dimer*, its mobility in denaturing SDS–PAGE increased (Figure 5B). As shown in the gel image stained by Coomassie blue, both the covalent conjugates (*EGFP-CCE-1*)–(*tmr-CCK-1*) and (*EGFP-CCE-1*)–(*tmr-CCK-1-dimer*) migrated faster than *EGFP-CCE-1*, despite their higher molecular weights. It could likely be explained by the postulation that the covalently cross-linked *CCE-1*–*CCK-1* heterodimer has a more compact structure than the uncomplexed *CCE-1* under denaturing conditions.

Still one question remained: what percentage of the complex formed between *EGFP-CCE-1* and *CCK-1* is linked through covalent linkage under native conditions? To better estimate this number, we conducted the labeling reaction on solid beads to exert a washing protocol and to mimic the labeling of cell surface receptors. Surface-immobilized *EGFP-CCE-1* was labeled by *tmr-CCK-1* or *tmr*-CCK3 on Ni-NTA resins. The resins were washed by PBS, and the fluorescence of the resins was quantified immediately with the TRITC channel of a fluorescent microscope. We then washed the labeled particles again and took fluorescent images (Figure 6A). This procedure was reiterated. After three repetitive washing steps, the fluorescent signal of noncovalent labeling by *tmr-CCK3* decreased to the basal level. About 65% of the fluorescent signal still remained after five washings of resins labeled by *tmr-CCK-1* (Figure 6B). This result suggests that before extensive washings were exerted under native conditions, possibly two-thirds of the *EGFP-CCE-1/CCK-1* complex were linked through a covalent bond, whereas the remaining one-third of labeling was through noncovalent coiled-coil interaction. In addition, three rounds of rigorous washing were found to be sufficient to remove the noncovalently associated *CCK-1* probe under this setting. Therefore, we deliberately utilized a rigorous washing protocol below to probe covalent labeling of cell surface receptors.

Labeling Peptide-Tagged Cell Surface Receptors. The CCE-based peptide tag was used to label receptors on the surface of mammalian cells. The first receptor we cloned was a modified epidermal growth factor receptor (EGFR).[62,63] Futaki and co-workers reported that replacing the extracellular

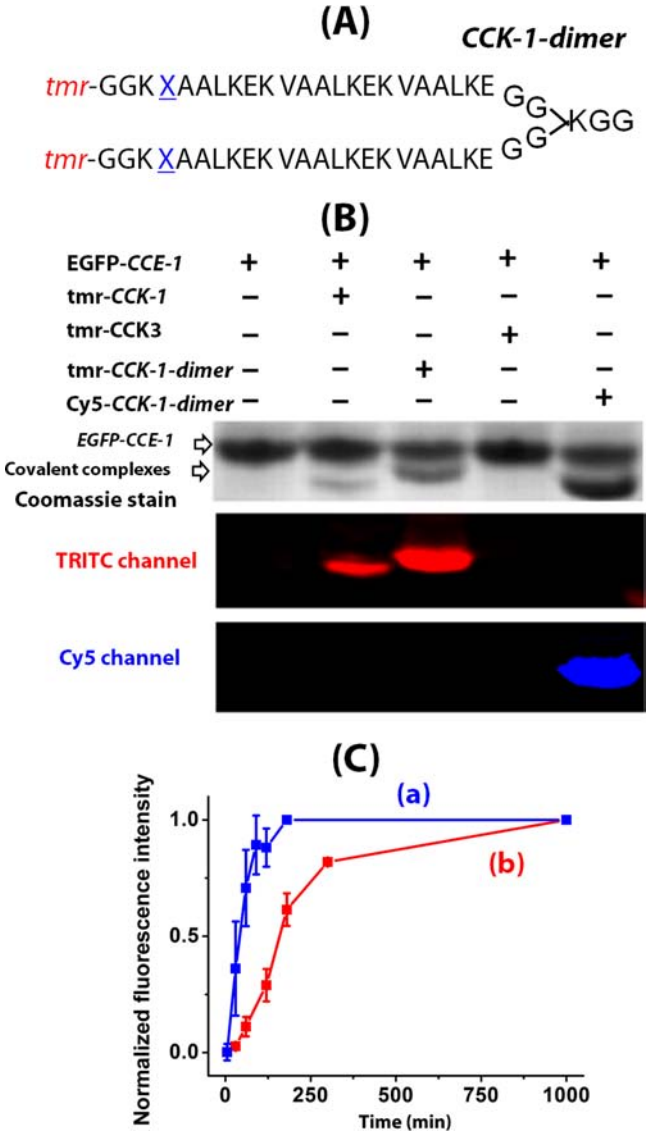

Figure 5. *CCK-1-dimer* probe labeled *EGFP-CCE-1* more efficiently. (A) Structure of the *CCK-1-dimer*. (B) Covalent labeling of *EGFP-CCE-1* by monomeric and dimeric peptides. The gel was imaged by a Typhoon Imager at the TRITC or cy5 fluorescent channels, after which the gel was stained by Coomassie blue. [Protein] = 40 μM, [peptide] = 20 μM, 4 °C overnight. (C) The reaction kinetics of covalent labeling of *EGFP-CCE-1* by the *CCK-1-dimer* (a) or *CCK-1* (b). [Protein] = [peptide] = 20 μM at RT.

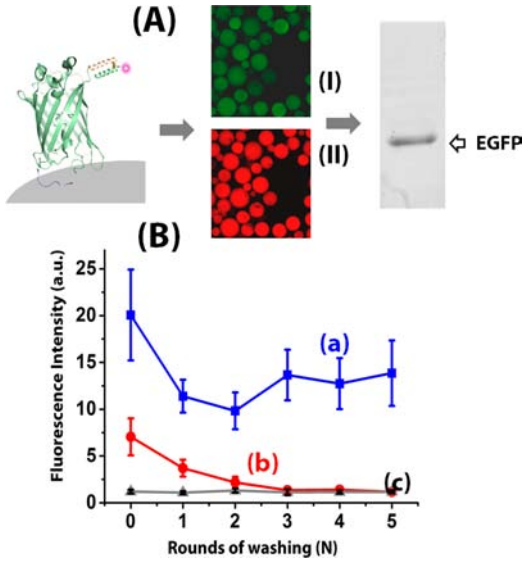

Figure 6. Covalent labeling of immobilized *EGFP-CCE-1*. (A) *EGFP-CCE-1* was immobilized on Ni-NTA resins. *Tmr-CCK-1* peptide was then added to label the protein-covered resins at RT. The resins were then washed and imaged by a fluorescent microscope at the FITC (I) and TRITC (II) channels. Pixels in the image from the TRITC channel were quantified as a measure of the fluorescent signal on the resins. The protein was last eluted by a solution of 500 mM imidazole and analyzed by denaturing SDS−PAGE. The gel was scanned by a Typhoon Imager at the TRITC channel. (B) Fluorescence intensity of the protein-loaded beads after each round of washing. (a) *EGFP-CCE-1*, labeled with *tmr-CCK-1*; (b) *EGFP-CCE-1*, labeled with *tmr-CCK3*; (c) wt EGFP, labeled with *tmr-CCK-1*.

domains I, II, and III, and a part of domain IV of EGFR, with the *CCE3′* sequence EIAALEKEIAALEKEIAALEK and an HA tag did not affect the function of the intracellular domain of the receptor.[62] Beginning with this EGFR construct, we mutated the first Ile to Cys (converting *CCE3′* to the *CCE-9* tag, E**C**AALEKEIAALEKEIAALEK) to yield a construct *CCE-9-EGFR*. As in the original construct, the N-terminus of the *CCE-9* tag had an HA tag appended to be labeled by the anti-HA antibody (Figure S9A, Supporting Information). The pDisplay vector was used to express *CCE-9-EGFR* in the plasma membrane of Chinese hamster ovary (CHO) cells. A *tmr*-labeled *CCK-9-dimer*, (*tmr*-GGK **X**AALKEK IAALKEK IAALKEGG)$_2$-KGG, was used for the labeling reaction.

CHO cells expressing *CCE-9-EGFR* were briefly treated with 0.5 mM TCEP for 10 min in cell culture dishes (the same as in ref 48) and then were labeled with 1 μM or 0.2 μM *CCK-9-dimer* in HEPES buffer at pH 7.4. After the peptide solution was aspirated, the cells were extensively washed with HEPES buffer, fixed by paraformaldehyde, and stained with FITC-labeled anti-HA antibody. Finally, the dishes were extensively washed with PBS buffer and imaged by a confocal microscope. CHO cells expressing *CCE-9-EGFR* exhibited both green fluorescence (FITC-anti-HA antibody) and red fluorescence (*tmr* from the *CCK-9-dimer*) around the plasma membrane, indicating that the *CCE-9* tag on EGFR was successfully labeled (Figure 7A). Some *tmr* signal was found in the cytosol. It is likely caused by receptor-mediated internalization during peptide labeling on the live cells.[62] The fluorescent signal along the transection of a labeled cell also confirmed that the FITC signal and the *tmr* signal colocalized at the plasma membrane (Figure S9B, Supporting Information). Notably, labeling by the *CCK-9-dimer* persisted after 10 washing steps.

To confirm the covalent nature of the linkage between the label *CCK-9-dimer* and the tag *CCE-9* on EGFR, surface-labeled CHO cells were harvested and lysed. At the molecular weight of ~145 kDa, a fluorescent band was clearly visible in the lane for pDisplay-CCE-9-EGFR transfected cells but not in control lanes for pDisplay-CCE3′-EGFR or pDisplay transfected cells (Figure 7B). The *CCK-9-dimer* thereby "locked" surface-expressed *CCE-9-EGFR* in dimeric form. The covalent linkage showed marked advantage here as it allowed us to visualize the probe−tag complex under denaturing conditions.

We then labeled a full-length receptor. We chose to clone a prostacyclin receptor (hIP) that belongs to the seven-transmembrane G protein-coupled receptor (GPCR) superfamily.[64] GPCRs including the C-X-C chemokine receptor type

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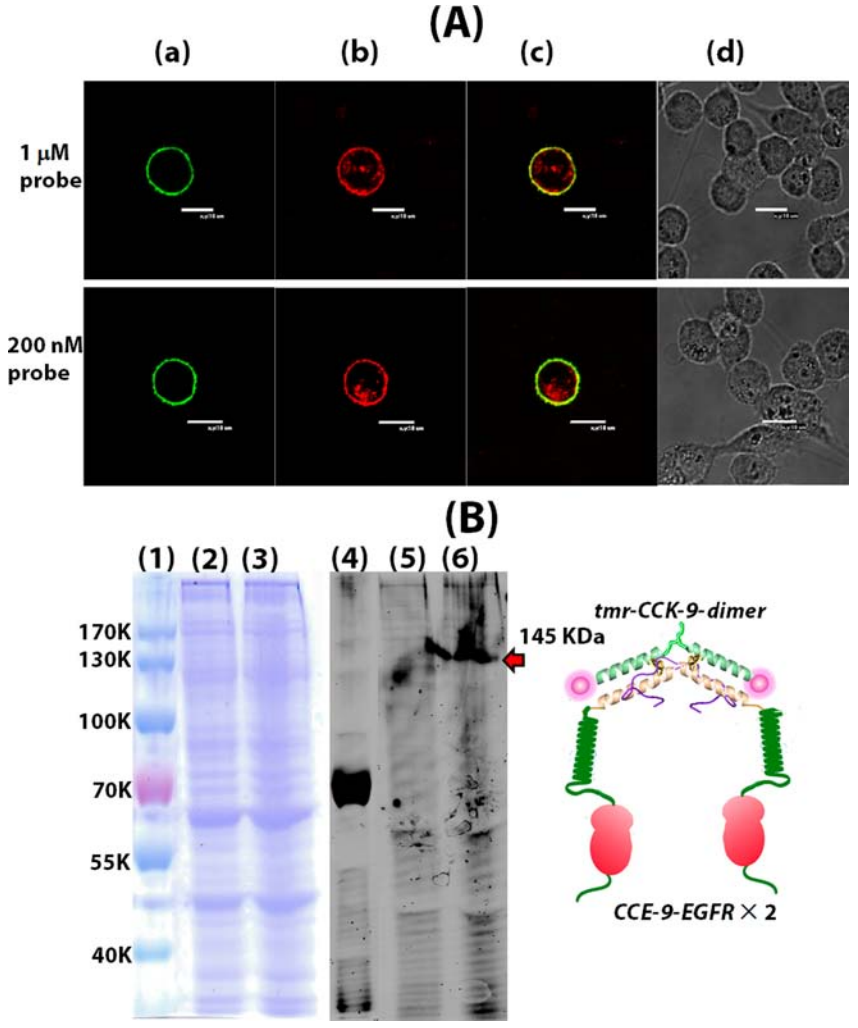

Figure 7. Covalent labeling of *CCE-9-EGFR* on a cell surface. (A) Confocal fluorescent images of CHO cells expressing *CCE-9-EGFR* and treated with 1 μM or 200 nM *tmr-CCK-9-dimer* and FITC-anti-HA antibody. Scale bar = 10 μm. (a) FITC channel; (b) TRITC channel; (c) overlay of a and b; (d) bright field. (B) Covalently labeled *CCE-9-EGFR* in the membrane fraction by *tmr-CCK-9-dimer*. Lanes 1−3 show the gel image stained by Coomassie blue; lanes 4−6 show the corresponding fluorescent image acquired at the TRITC channel of a Typhoon imager. Lanes 1 and 4, molecular weight markers; lane 2 and 5, cells transfected by pDisplay control; lane 3 and 6, cells transfected by pDisplay-CCE-9-EGFR.

4 (CXCR4), prostaglandin E2 receptor (EP3βR), and β-2 adrenergic receptor (β2AR) have been labeled through noncovalent coiled-coils[52−54] but not prostacyclin receptors. We fused the *CCE-9* sequence at the N-terminus of the full-length hIP protein followed by 3 HA tags, producing a CCE-9−3HA-hIP construct. The pcDNA3.1 vector was used to express hIP proteins on the surface of CHO cells. Following the same procedure of Figure 7, we successfully labeled *CCE-9−3HA-hIP* receptors on the surface of CHO cells with the *tmr-CCK-9-dimer* probe and the anti-HA antibody (Figure 8A). In contrast, an EGFP-tagged hIP failed to be displayed on the plasma membrane, neither could the receptor be labeled by the probes (Figure S10, Supporting Information), indicating that EGFP tagging affected the function of the receptor.

We then investigated whether the *CCE-9* tag on hIP might adversely affect the function of the receptor. Transfected cells were stimulated with 10 μM forskolin, and the intracellular level of cAMP was measured by a [^{3}H]cAMP assay. Forskolin is known to stimulate resensitized cell receptors by activating the enzyme adenylyl cyclase and increasing intracellular levels of cAMP.[65,66] Cells expressing hIP receptors produced levels of cAMP similar to those of the control (Figure 8B). This result indicated that the expression of the *CCE-9−3HA-hIP* receptor did not alter normal signal transduction within the cells. The function of the hIP receptors was also examined by using cicaprost, a prostacyclin analogue and an agonist of hIP.[67−70] Ten micromolar cicaprost stimulated similar levels of cAMP production in cells transfected with pcDNA3.1−3HA-hIP and pcDNA3.1-CCE-9−3HA-hIP but not in cells without the hIP receptors, indicating that the constructs were functional. This result shows that the peptide tag, owing to its small size, caused negligible disturbance to the function of prostacyclin receptors under this condition.

■ CONCLUSIONS

Covalently cross-linking coiled-coil peptides by disulfide bonds has been an instrumental technique for analyzing the orientation and stability of this simplest protein folding system.[57,58] Native coiled-coil interactions have also been widely applied to studies of protein assembly, affinity purification, surface tethering, and Western blotting.[52−54,62,63,71−74] Our combination of these two foci and the upgrading of the disulfide bond to an irreversible thioether

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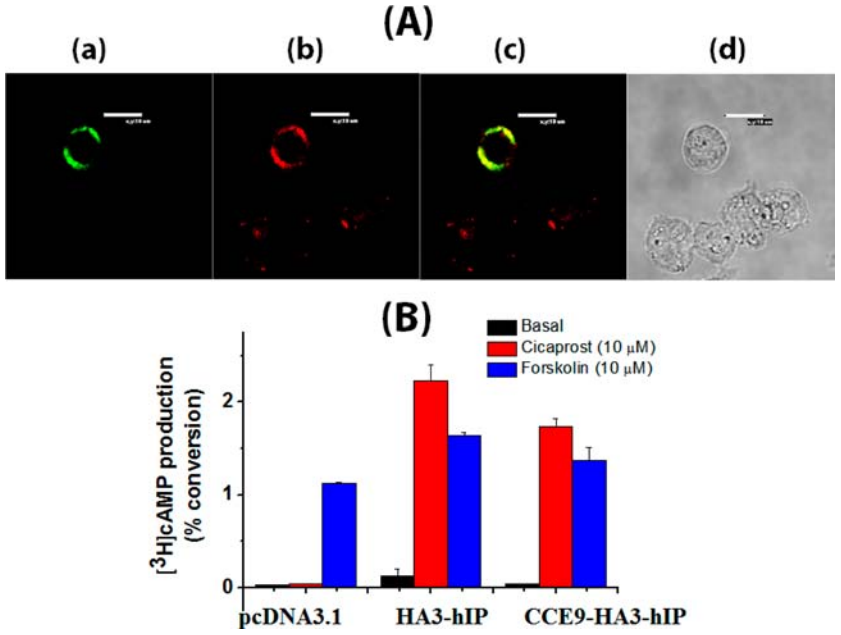

Figure 8. CCE-tagging on hIP. (A) Labeling of *CCE-9-hIP* on the cell surface by the *tmr-CCK-9-dimer* and the FITC-anti-HA antibody. (a) FITC channel; (b) TRITC channel; (c) overlay of a and b; (d) bright field. Scale bar: 10 μm. (B) cAMP response to drug treatments in hIP transfected cells.

bond has produced a new bioconjugation strategy, a cross-linking reaction induced by coiled-coil binding.

By systematically screening the position of cross-linking, examining the orthogonality of multipair tectons, and accelerating the reaction rate by bivalent peptides, we have demonstrated the versatility of our cross-linking reaction. The specificity of the strategy harnesses the secondary structure of a protein (the coiled coil), rather than primary (chemical reactivity) or three-dimensional (enzymes or other sophisticated protein structures) folds. We successfully addressed the trade-off between the size of the tag and the selectivity of the reaction: the binding of a coiled-coil interaction provides the specificity and biocompatibility of the covalent reaction, and the Cys−chloroacetyl nucleophilic S_N2 reaction driven by enhanced local reactivity permanently and spontaneously "freezes" the interaction. One limitation of our strategy, which similar approaches will share, is the impermeability of the probe, which limits the labeling to cell surface receptors.[24] However, the combined use of surface-specific probes and cell-permeable probes has the advantage of differentiating surface-presented versus intracellular proteins. Having demonstrated our cross-linking strategy, we are now engineering other short peptide tags in order to simultaneously label multiple proteins of interest. Multiple orthogonal pairs of covalent probes might allow sequential labeling of cell surface receptors to track the fate of surface-presented receptors during internalization, intracellular targeting, and recycling.

■ ASSOCIATED CONTENT

Supporting Information

Materials and instruments, peptide synthesis, purification and characterization, plasmid information, protein expression and purification, covalent labeled EGFR for gel electrophoresis, cAMP assay, and additional characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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