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Quenched Ligand-Directed Tosylate Reagents for One-Step Construction of Turn-On Fluorescent Biosensors

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Abstract: Semisynthetic fluorescent biosensors consisting of a protein framework and a synthetic fluorophore are powerful analytical tools for specific detection of biologically relevant molecules. We report herein a novel method that allows for the construction of turn-on fluorescent semisynthetic biosensors in a one-step manner. The strategy is based on the ligand-directed tosyl (LDT) chemistry, a new type of affinity-guided protein labeling scheme which can site-specifically introduce synthetic probes to the surface of proteins with concomitant release of the affinity ligands. Novel quenched ligand-directed tosylate (Q-LDT) reagents were designed by connecting an organic dye to a conjugate of a protein ligand and a fluorescence quencher through a tosyl linker. The Q-LDT-mediated labeling directly converts a natural protein to a fluorescently labeled protein that remains noncovalently complexed with the cleaved ligand-tethered quencher. The fluorescence of this labeled protein is initially quenched and only in the presence of specific analytes is the fluorescence enhanced (turned on) due to the expulsion of the ligand-quencher fragment. Using a single labeling step, this approach was successfully applied to carbonic anhydrase II (CAII) and a Src homology 2 (SH2) domain to generate turn-on fluorescent biosensors toward CAII inhibitors and phosphotyrosine peptides, respectively. Detailed investigations revealed that the obtained biosensors exhibit their natural ligand selectivity. The high target-specificity of the LDT chemistry also allowed us to prepare the SH2 domain-based biosensor not only in a purified form but also in a bacterial cell lysate. These results demonstrate the utility of the Q-LDT-based approach to expand the applications of semisynthetic biosensors.

Introduction

Protein-based fluorescent biosensors are now recognized as indispensable tools in many areas of biological science and biomedical research because they allow the monitoring and quantification of specific biological substances both in vitro and in vivo.[1,2] In particular, with the advent of chemical biology, the need for biosensors for investigating diverse biological processes has gained considerable momentum.[3] Fluorescent biosensors have also attracted much attention as powerful platforms for drug screening.[4] It is fortunate that a variety of proteins and protein domains are currently available as scaffold building blocks (analyte-binding motifs) for the preparation of biosensors.[5] However, the creation of biosensors is still not straightforward due to the paucity of general methodologies to construct such molecules.

The past decade has witnessed significant advances in the development of genetically encoded biosensors using fluorescent proteins (FPs).[1,6] Several elegant FP-fusion biosensors have been produced by fusing two distinct FPs, such as cyan and yellow FPs, to sandwich a protein scaffold so that the analyte can be detected through the change of fluorescence resonance energy transfer (FRET) efficiency. This type of biosensor is undoubtedly powerful, especially for use in living cells. However, the large size of FPs (27 kDa) and the constraints placed on the fusion

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site (i.e., in most cases, restricted to tagging at the N- and C-terminus of proteins) severely limit the range of scaffold candidates that the FP-fusion strategy can be applied to. The construction of FP-based biosensors has so far been successful only when the fusion constructs induce a large conformational change upon binding to the analyte. Such cases are unfortunately rather rare.

Site-specific chemical modification of proteins with synthetic fluorescent dyes is another important means to afford semisynthetic, fluorescent biosensors.[2,7,8] The major strength of this chemistry-driven approach is the flexibility in terms of the choice of fluorophore and the attachment site (not restricted at the termini of proteins). Consequently, this approach essentially permits various fluorescence transduction mechanisms to be integrated into protein frameworks in a tailor-made manner. During the past decades, a number of semisynthetic biosensors have been constructed, particularly by installing an environment-sensitive dye into a protein scaffold at a specific site such as the periphery of the analyte-binding pocket.[7,8] These biosensors have been shown to transduce the analyte-binding event into a change in the fluorescence intensity and/or wavelength by sensing a subtle change of the microenvironment surrounding the fluorophore, such as pH and solvent polarity. Importantly, these examples have clearly demonstrated that the semisynthetic strategy is powerful and has the potential to be applicable in the conversion of nearly all possible protein scaffolds to corresponding biosensors. However, there are challenges to be addressed. First, it remains difficult to rationally predict the appropriate site and type of fluorophore to be introduced to achieve the optimal fluorescence response. In this regard, fluorescence enhancement, rather than reduction, is favorable for more sensitive and accurate detection. Second, even after the optimization through a laborious trial-and-error process, in many cases, the signal change is small to moderate. Third, as a more fundamental problem, methods for site-specific protein modification are limited. Most of the semisynthetic biosensors previously reported were prepared though genetic incorporation of a cysteine residue, followed by the use of thiol chemistry to attach an organic dye.[2,7] However, many proteins are not amenable to this strategy because of the presence of an inherent cysteine residue(s). The acquisition of mutant proteins which retain natural ligand-binding properties (affinity and specificity) is not always successful. Although we have developed the post(photo)affinity labeling modification methods as tools for introducing fluorescent molecules into natural proteins without genetic manipulation, these approaches require multiple chemical reactions to be performed on the protein surfaces.[8] In addition, all the prevalent protein modification procedures[9] require a careful purification step after the labeling reaction and cannot be applied to crude protein mixtures. Clearly, the establishment of a new methodology that can overcome the aforementioned limitations is of considerable importance for the progression of semisynthetic biosensors.

Here we describe a novel, rational protein engineering strategy that allows one-step construction of turn-on fluorescent semisynthetic biosensors. The method is based on the recently developed ligand-directed tosyl (LDT) chemistry, which is capable of labeling specific proteins with diverse synthetic probes with high site-specificity and target-selectivity.[10] By designing a new class of labeling reagents, quenched ligand-directed tosylate (Q-LDT) compounds, it was feasible to convert natural proteins to fluorescently labeled proteins that can be directly used as biosensors based on the bimolecular fluorescence quenching and recovery (BFQR) mechanism (Figure 1).[11] We successfully applied the strategy to two proteins, carbonic anhydrase II (CAII) and the Src homology 2 (SH2) domain, to generate turn-on fluorescent biosensors toward the inhibitors and phosphorylated peptides, respectively, by a single labeling step not only in a purified form but also in a bacterial cell lysate.

Results and Discussion

General Design of Q-LDT Compounds. Our idea to construct turn-on fluorescent biosensors is illustrated in Figure 1. The concept is based on the LDT chemistry that we recently developed and is a new type of affinity-guided protein surface labeling scheme.[10] In contrast to existing affinity labeling reactions,[12,13] the LDT-based approach is unique in that the ligand moiety is cleaved concomitantly with the labeling process, thus enabling covalent, selective attachment of probes of interest to specific target proteins without irreversibly abolishing the function of the labeled proteins. By taking advantage of this strategy, we designed a new class of protein labeling reagents, Q-LDT compounds, which contain a fluorescent dye and its quencher together so that the labeling reagent itself is only weakly fluorescent. The quencher is introduced as a part of the leaving group. Upon binding of the ligand moiety to the target

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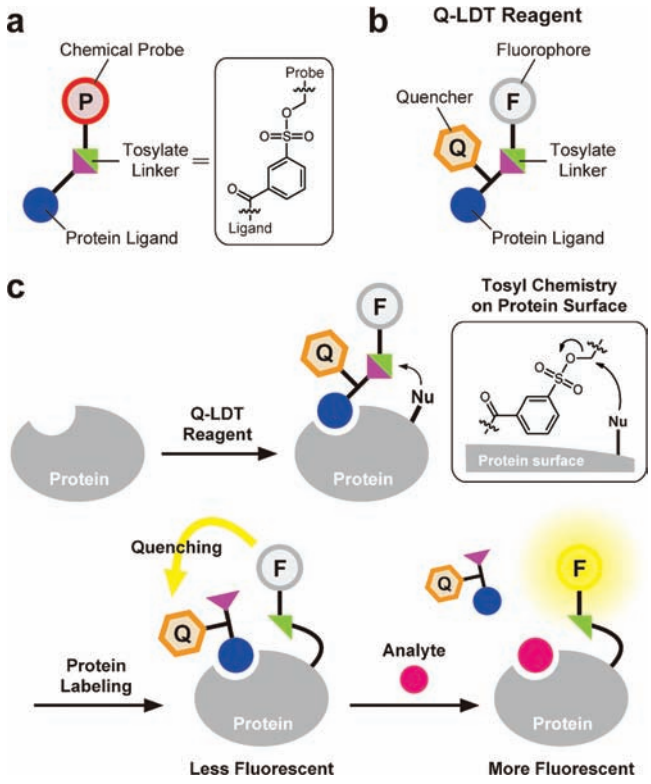

Figure 1. Molecular configurations of (a) original ligand-directed tosylate (LDT) reagent and (b) quenched ligand-directed tosylate (Q-LDT) reagent. (c) Schematic illustration of the strategy for the Q-LDT-mediated construction of turn-on fluorescent biosensors.

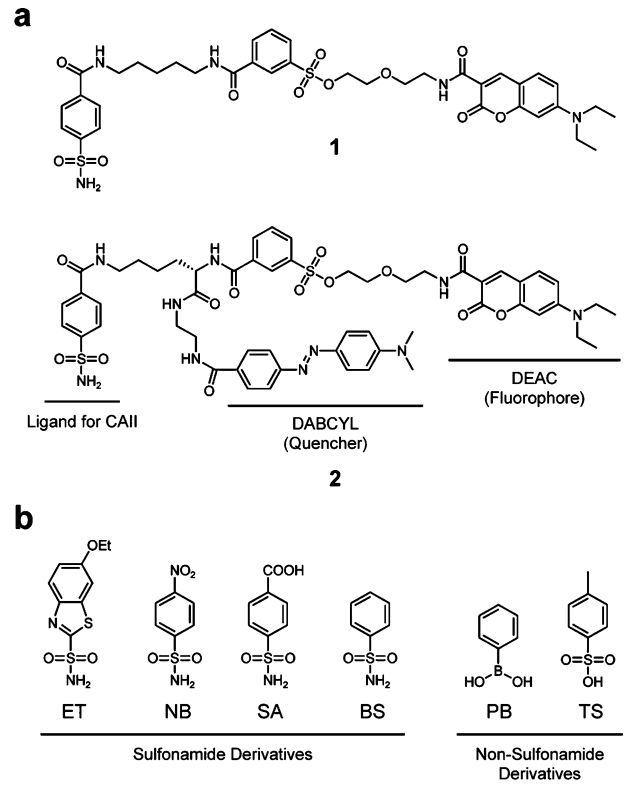

Figure 2. (a) Molecular structures of LDT reagent **1** and Q-LDT reagent **2** designed for CAII labeling. (b) Sulfonamide and nonsulfonamide derivatives used in this study. ET, ethoxzolamide; NB, *p*-nitro-benzenesulfonamide; SA, 4-sulfamoylbenzoic acid; BS, benzenesulfonamide; PB, phenylboronic acid; TS, *p*-toluenesulfonic acid.

protein, the fluorescent dye is covalently transferred to a nucleophilic amino acid located on the protein surface through the proximity-induced S_N2-type tosyl chemistry. Concomitantly with this process, the affinity ligand is cleaved off but remains noncovalently bound to the ligand binding pocket of the protein, thereby still allowing the efficient quenching of the fluorescence. The fluorescence can then be efficiently recovered (turned on) in the presence of specific analytes by expelling the quencher-tethered ligand from the binding pocket of the labeled protein. Therefore, the Q-LDT reagents are expected to serve as a unique tool to label and convert target proteins in one step to generate semisynthetic, turn-on fluorescent biosensors which function on the basis of the BFQR scheme.[11]

One-Step Conversion of CAII to a Turn-On Fluorescent Biosensor for Sulfonamide Derivatives. We initially tested the above strategy using human carbonic anhydrase II (CAII) as a model protein scaffold and sought to convert this protein to a fluorescent biosensor toward its inhibitors. Carbonic anhydrases are a group of metalloenzymes involved in numerous physiological and pathological processes such as gluconeogenesis, ureagenesis, and tumorigenicity.[14] We have previously shown that CAII can be site-specifically labeled with the 7-diethylaminocoumarin (DEAC) fluorophore using the LDT reagent **1** (Figure 2a).[10] **1** contains benzenesulfonamide as a specific ligand for CAII,[15] which is connected with the DEAC dye (probe) through an electrophilic phenylsulfonate (tosylate) ester bond.[13] On the basis of this, Q-LDT reagent **2** was designed. To the core structure of **1**, the 4-dimethylaminophenylazobenzene-4-carboxylic acid (DABCYL) quencher[16] was tethered through a lysine spacer in a way that the ligand−DABCYL conjugate is cleaved as a leaving group upon the labeling reaction. The synthesis of **2** was carried out as shown in Scheme S1.

The reactivity of **2** with CAII was investigated by incubating the two molecules in a buffer solution. As shown by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence gel imaging (Figure 3a), CAII was covalently modified with the DEAC fluorophore in a time-dependent manner (lanes 2, 3, and 4). The labeling was completely abolished in the presence of the strong inhibitor ethoxzolamide[15] (ET, Figure 2b) (lane 5), indicating that the labeling occurs by an affinity-driven proximity effect. Consistent with our previous results,[10] no noticeable decrease in the labeling yield was observed even under a high concentration of reduced glutathione (GSH, lane 6). Using an independently prepared DEAC−CAII (1:1) conjugate[10] as a standard marker (lane 7), the labeling yield after 48 h of incubation was estimated to be ~50%. We also confirmed the site-specificity of this modification by subjecting the DEAC-labeled CAII to conventional peptide mapping (Figure S1). Lysyl endopeptidase digestion followed by reversed-phase HPLC and MALDI-TOF mass spectrometry analysis revealed that the N-terminal L1 fragment corresponding to Ser2−Lys9 was selectively modified in accordance with our previous report.[10] This result suggests that the labeling site is His3. Interestingly, despite the attachment of the relatively large

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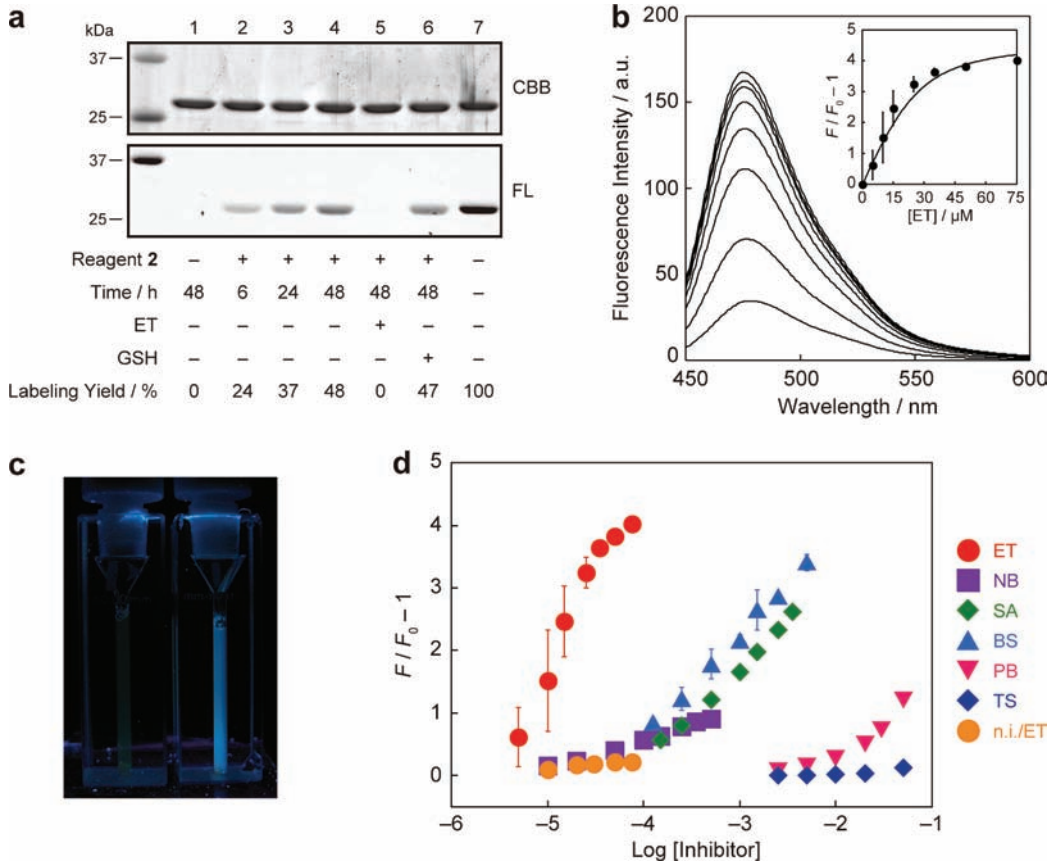

Figure 3. Construction of the CAII-based turn-on fluorescent biosensor for sulfonamide derivatives. (a) SDS-PAGE analysis of the covalent labeling of CAII with **2**. Reaction conditions: 10 μM CAII, 20 μM **2**, 100 μM ET (lane 5), 10 mM GSH (lane 6), 50 mM HEPES, pH 7.2, and 37 °C. In lane 7, an independently prepared DEAC−CAII (1:1) conjugate was used as a standard marker for the determination of the labeling yields. The gel was analyzed by *in-gel* fluorescence imaging (FL, lower), and stained with Coomassie brilliant blue (CBB, upper). (b) Fluorescence spectral change of **2**-treated CAII upon the addition of ET. Labeling conditions: 30 μM CAII, 30 μM **2**, 50 mM HEPES, pH 7.2, 37 °C, and 48 h. Measurement conditions: 5 μM **2**-treated CAII (the postlabeling sample was 6-fold diluted with no purification, i.e., a mixture of labeled (48%) and native CAII), 0−75 μM ET, 50 mM HEPES, pH 7.2, 25 °C, and λ_{ex} = 427 nm. (Inset) Fluorescence titration curve. λ_{em} = 473 nm. All data points are mean ± SD obtained by three independent experiments. (c) Photograph of the **2**-treated CAII in the absence (left) and presence (right) of ET. Measurement conditions: 25 μM **2**-treated CAII, 175 μM ET, 50 mM HEPES, pH 7.2, 25 °C, and λ_{ex} = 365 nm. (d) Fluorescence titration profiles of the **2**-treated CAII at the emission wavelength of 473 nm with various sulfonamide and nonsulfonamide derivatives. Experiments were performed in triplicate under the same conditions as (b). n.i./ET = the ET titration with no incubation for the labeling.

DABCYL group, the labeling yield was not significantly reduced compared with that with **1** and the labeling site was also identical.

The postlabeling solution exhibited an emission with a wavelength maximum at 473 nm upon excitation at 427 nm and this emission was ascribed to the DEAC fluorophore. Given the binding affinity of sulfonamide derivatives (typically $>10^6$ M^{-1}),[15] the cleaved sulfonamide−DABCYL conjugate should remain bound in the ligand-binding pocket of the DEAC-labeled CAII, which may cause considerable fluorescence quenching.[17] The formation of this noncovalent complex was confirmed by simple purification of the labeled CAII by size-exclusion chromatography. The UV−visible spectrum of the CAII-containing fraction clearly showed two absorption bands at 427 and 474 nm (broad) due to the DEAC and the DABCYL groups, respectively (Figure S2).[18] More importantly, as shown in Figure 3b, the fluorescence was significantly recovered (by 5-fold) when ET was added to the postlabeling solution (without purification). The fluorescence recovery was so efficient that the brighter emission in the presence of ET was easily detectable by the naked eye (Figure 3c). In contrast, almost no fluorescence change was observed by the addition of *p*-toluenesulfonic acid (TS, Figure 2b) which has no affinity toward CAII. Also, a simple mixture of CAII and **2** without incubation did not induce a significant fluorescence increase upon addition of ET (Figure 3d). These results are fully consistent with the proposed BFQR scheme of Figure 1c, in which the fluorescence recovery was due to the expulsion of the noncovalently bound DABCYL-containing fragment from the labeled CAII by ET.

(17) As described in the caption of Figure 3b, we performed the labeling reaction and fluorescence titration under the conditions in which the concentrations of the LDT reagent (1 equiv of CAII) and CAII are sufficiently higher than the dissociation constant of the sulfonamide ligand to maintain the complex formation. We also confirmed that when the labeling is carried out in the presence of an excess amount of CAII, the cleaved sulfonamide−DABCYL conjugate is transferred from the DEAC-labeled CAII to unmodified CAII (data not shown).

(18) After the labeling reaction (30 μM CAII, 30 μM **2**), ~50% of the total LDT reagent **2** was used for the labeling, 20% of that was hydrolyzed, and 30% remained intact. Since the affinity of the sulfonamide ligand is high, only the free and small DEAC fragment generated by hydrolysis can be removed by a short gel column we used (that is, other sulfonamide-containing components including the cleaved sulfonamide−DABCYL conjugate and intact reagent **2** remains bound to CAII). In agreement with this, the absorbance ratio at 427 and 474 nm (427/474) was decreased from 2.00 to 1.76 after the gel filtration, as shown in Figure S2.

Table 1. Apparent Binding Constants (K_{app}, M^{-1}) of the CAII-Based Biosensor to Various Inhibitors and the Relative Fluorescence Intensities (F/F_0) against the Initial State[a]

inhibitors[b]	K_{app} (M^{-1})[c]	K_a for native CAII (M^{-1})[e]	F/F_0[g]
ET	8.0×10^5	1.3×10^8	5.0 (75 μM)
NB	8.2×10^4	1.6×10^7	1.9[h] (0.5 mM)
SA	6.5×10^3	3.7×10^6	3.6 (3.5 mM)
BS	9.9×10^3	6.5×10^5	4.4 (5 mM)
PB	n.d.[d]	9.4×10^1	2.2 (50 mM)
TS	n.d.[d]	n.a.[f]	1.1 (50 mM)

[a] Measurement conditions: 5 μM **2**-treated CAII (a mixture of labeled (48%) and native CAII) in 50 mM HEPES, pH 7.2, $\lambda_{ex} = 427$ nm, and 25 °C. [b] The structures of the inhibitors are shown in Figure 2b. [c] Determined by a nonlinear curve-fitting analysis. [d] Not determined due to the low affinity. [e] Reported values.[15] [f] No literature value is available. [g] F/F_0 indicates the relative fluorescence intensities (F) at 473 nm in the presence of the inhibitor at the highest concentration used in the assay, which is shown in parentheses, against those of the initial state (F_0). [h] The relatively low F/F_0 value is due to the nitrobenzene compound which also has fluorescence-quenching properties.[21]

The analyte-induced fluorescence increase (turn-on mode) is a beneficial feature of the present fluorescent biosensor. We next investigated in more detail the fluorescence response of the **2**-treated CAII toward various sulfonamide and nonsulfonamide derivatives with different binding affinities. As shown in the inset of Figure 3b, the fluorescence titration curve for ET showed the typical saturation behavior with an apparent binding constant of 8.0×10^5 M^{-1}. The titration curves and the apparent binding affinities are summarized in Figure 3d and Table 1. The relative order of the affinity of the tested compounds for the semisynthetic biosensor was clearly shown to be practically identical with that for native CAII. This demonstrated that the natural ligand specificity is retained even after the labeling. In addition, the obtained apparent affinity values for each of the sulfonamide inhibitors are ~2 orders of magnitude smaller than those reported for native CAII. This is because the present system is a competitive assay. In other words, the present system can set an appropriate threshold to eliminate weak binding ligands, which is generally advantageous for the screening of more potent binders.[19] On the basis of the competitive inhibition model, the net affinities of each tested CAII inhibitor were estimated to be 3.3×10^8 M^{-1} for ET, 3.4×10^7 M^{-1} for NB, 2.7×10^6 M^{-1} for SA, and 4.1×10^6 M^{-1} for BS.[20] These values are almost identical to the reported binding constants of the inhibitors to native CAII (Table 1), indicating that the original molecular recognition ability of CAII is fully retained.

Overall, the intact CAII was converted in one-step to a turn-on fluorescent biosensor for its inhibitors (sulfonamide derivatives) by the Q-LDT-mediated labeling. Notably, following the incubation of the protein and the Q-LDT reagent, the resulting solution could be used directly for the fluorescence assay without the need of any additional step. Since carbonic anhydrases are recognized as important therapeutic targets to treat a range of disorders such as obesity, cancer, and glaucoma,[14] the present biosensor platform might be useful for the future development of new potent CA inhibitors.

Construction of a SH2 Domain-Based Biosensor for Phosphotyrosine Peptides. Due to the valuable modular feature of the (Q-)LDT chemistry, it is rationally expected that the present strategy can be applied to other proteins or protein domains by changing the affinity ligand. The construction of a fluorescent biosensor toward phosphorylated peptides by exploiting a phosphopeptide-recognition domain was our next target. Since protein phosphorylation plays a central role in the regulation of cell functions, the development of fluorescent (bio)sensors for specific phosphorylated peptides/proteins represents an important area of research.[22,23] Here, we chose an N-terminal SH2 domain of the p85α subunit of human phosphatidylinositol-3-kinase (PI3K) (p85-nSH2) as a scaffold,[24] which recognizes peptides containing a pYZXM (pY, phosphotyrosine; Z, Met or Val; X, any amino acid) motif.[25] Accordingly, by adopting a pYVPM sequence derived from the platelet-derived growth factor receptor (PDGFR) as an affinity ligand,[25] LDT reagent **3** and its quenched version **4** were designed (Figure 4a). The syntheses were conducted as shown in Scheme S2 by using thiol-maleimide ligation to incorporate the DEAC fluorophore via a tosylate linker to peptides prepared by solid-phase peptide synthesis. The p85-nSH2 domain corresponding to residues 328−435 of the human p85α was overexpressed in bacteria as a fusion construct with a glutathione *S*-transferase (GST). After purification by glutathione-Sepharose chromatography, the GST tag was removed by thrombin proteolytic digestion.

We first carried out the labeling of the recombinant SH2 domain. As shown in Figure 5a, by incubating the SH2 domain with **3** or **4** for 44 h, the protein domain was labeled by the DEAC dye with an approximate yield of 20% or 30%, respectively (lane 3 and 8). In the presence of an excess amount of phosphopeptide ligand **5** (Figure 4b), almost no fluorescence band was observed. This indicated that the labeling was driven by the proximity effect induced by the recognition of the phosphopeptide moiety of the reagents by the SH2 domain. The DEAC-labeled SH2 domain was also characterized by MALDI-TOF mass analysis. In addition to a peak at 13131.4 Da corresponding to the intact SH2 domain, a peak at 13460.2 Da was detected (data not shown). This peak corresponds to the molecular weight of the singly DEAC-labeled SH2 domain.[26] Conventional peptide mapping using **3** and subsequent tandem mass analysis showed that the solvent exposed His407 located

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(26) The calculated molecular weights of the p85-nSH2 domain and its singly DEAC-labeled derivative are 13141.68 and 13472.06, respectively.
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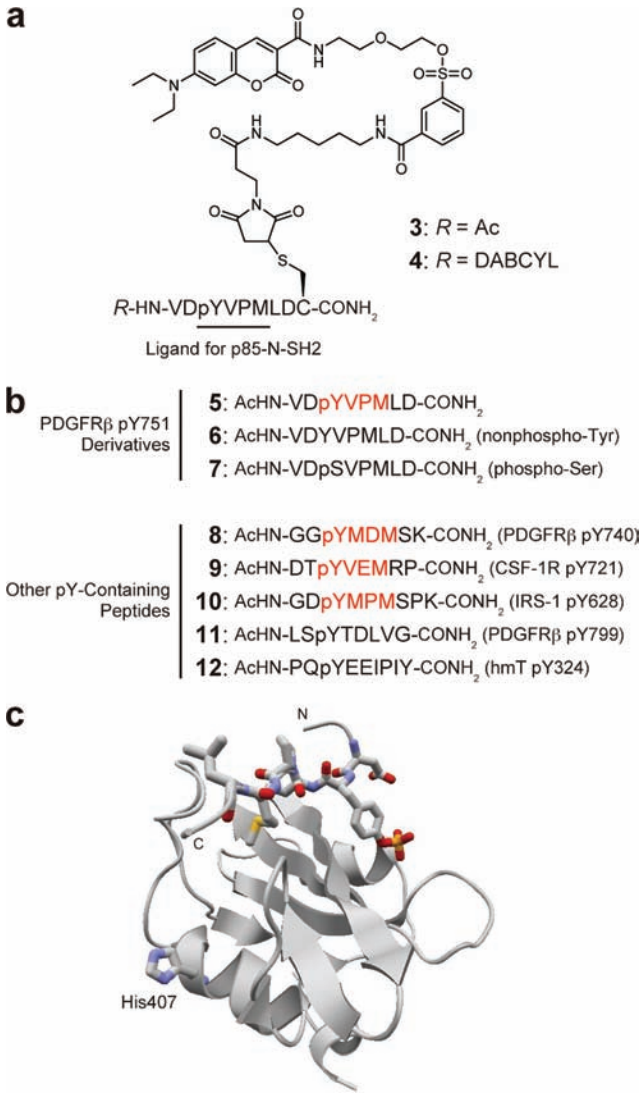

Figure 4. (a) Molecular structures of LDT reagent **3** and Q-LDT reagent **4** designed for p85-N-SH2 domain labeling. (b) Sequences of peptide ligands used in this study. pYZXM (Z = Met or Val, X = any amino acid) motifs are shown in red. CSF-1R, colony-stimulating factor 1 receptor; IRS-1, insulin receptor substrate 1; hmT, hamster polyoma middle tumor antigen. (c) Crystal structure of p85-nSH2 complexed with its phosphopeptide ligand (the DpYVPML region is presented as a stick model).[27]

in the αB helix of the SH2 domain was predominantly modified (Figure 4c and Figure S3).[27,28]

The reaction mixture after the labeling using **4** was passed through a short gel filtration column, and the eluent was used for evaluating the function of the fluorescent biosensor.[29] This brief purification procedure removes only molecules unbound on the SH2 domain, i.e., the eluted SH2 domain still complexes with the DABCYL-carrying fragment.[30] Addition of phosphorylated peptide **5**, a natural ligand for the SH2 domain, to this

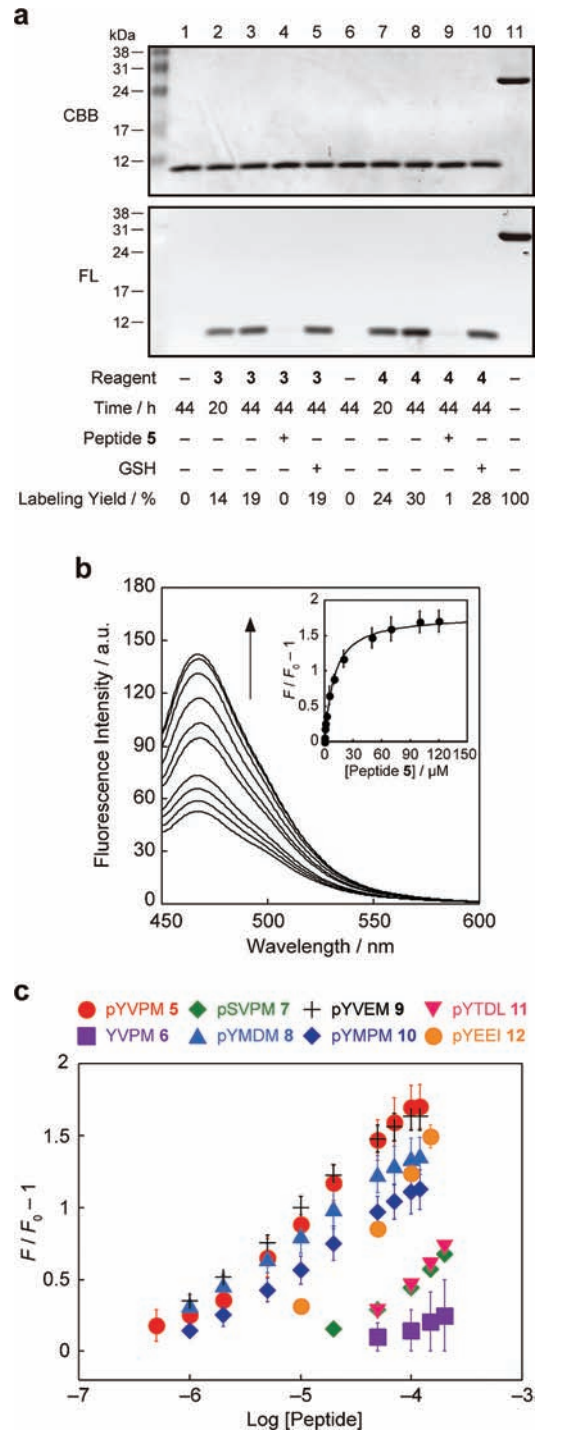

Figure 5. Construction of the SH2 domain-based turn-on fluorescent biosensor for specific phosphotyrosine peptides. (a) SDS-PAGE analysis of the covalent labeling of the SH2 domain with **3** and **4**. Reaction conditions: 5 μM p85-N-SH2, 10 μM **3** or **4**, 1 mM peptide **5** (lane 4 and 9, Figure 4b), 10 mM GSH (lane 5 and 10), 50 mM HEPES, pH 8.0, and 37 °C. Lane 11, DEAC−CAII (1:1) conjugate. (b) Fluorescence spectral change of **4**-treated p85-N-SH2 upon the addition of phosphotyrosine peptide **5**. Measurement conditions: 1 μM **4**-treated SH2 domain (the labeled sample shown in lane 8 of (a) was passed through a gel column and diluted, i.e., a mixture of labeled (30%) and unlabeled SH2 domain), 0−120 μM **5**, 50 mM HEPES, pH 7.4, 25 °C, and λ_{ex} = 427 nm. (Inset) Fluorescence titration curve, λ_{em} = 473 nm. All data points are mean ± SD obtained by three independent experiments. (c) Fluorescence titration profiles of the **4**-treated p85-N-SH2 at the emission wavelength of 473 nm with various peptides. Experiments were performed in triplicate under the same conditions as (b).

(28) According to the sequence alignment of various SH2 domains, many SH2 domains possess a His residue at the same (or almost same) location with the His407 of the p85-nSH2 domain. Thus, it is reasonable to expect that the present strategy can be efficiently applied to the labeling of other SH2 domains by simply modifying the recognition sequence. Kuriyan, J.; Cowburn, D. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 259.

(29) This purification step is not essential. However, we have found that it can reduce the initial background fluorescence caused by partial hydrolysis of the tosyl linkage of **4** and increase the relative fluorescence intensity (F/F_0).

Table 2. Apparent Binding Constants (K_{app}, M^{-1}) of the SH2 Domain-Based Biosensor to Various Peptide Ligands and the Relative Fluorescence Intensity (F/F_0) against the Initial State[a]

peptides[b]	pYZXM[c]	K_{app} ($\times 10^5$ M^{-1})[d]	ID_{50} (μM) for native SH2[e]	F/F_0[h]
5	+	1.1	1.8 (0.47)[f]	2.7 (120 μM)
6	−	0.02	n.a.[g]	1.2 (200 μM)
7	−	0.06	n.a.[g]	1.7 (200 μM)
8	+	2.0	1.1	2.4 (120 μM)
9	+	1.9	0.5	2.6 (120 μM)
10	+	1.1	1.1	2.1 (120 μM)
11	−	0.08	n.a.[g]	1.7 (200 μM)
12	−	0.13	n.a.[g]	2.5 (150 μM)

[a] Measurement conditions: 1 μM **4**-treated SH2 domain (a mixture of labeled (30%) and unlabeled p85-nSH2) in 50 mM HEPES, pH 7.4, λ_{ex} = 427 nm, and 25 °C. [b] The sequences of the peptide ligands are shown in Figure 4b. [c] This indicates whether the peptide contains the pYZXM motif. [d] Determined by the nonlinear curve-fitting analysis. [e] Reported half-maximal inhibition values determined by the competition experiments using a resin-immobilized GST-fusion p85-nSH2 and peptides shown.[25b] [f] The value in parentheses is the actual dissociation constant (μM) of a phosphotyrosine peptide (H_2N-SVDpYVDMSK-CO_2H) to p85-nSH2, which was determined by isothermal titration calorimetry (ITC).[25c] [g] No literature value is available. [h] F/F_0 indicates the relative fluorescence intensities (F) at 473 nm in the presence of the peptide at the highest concentration used in the assay, which is shown in parentheses, against those of the initial state (F_0).

solution induced an increase in the intensity of the fluorescence by ca. 3-fold with a typical saturation profile. In contrast, the fluorescence scarcely changed upon the addition of the nonphosphorylated peptide **6** (Figure 4b) because this peptide has no affinity to the SH2 domain. The apparent binding affinity of peptide **5** to the SH2 domain was determined to be 1.1×10^5 M^{-1} from the titration curve. This value is smaller than that reported for intact p85-nSH2. This is because the remaining cleaved quencher-ligand conjugate acts as a competitive binder for the labeled SH2 domain. These observations are in good agreement with the sensing mechanism (BFQR) proposed in the case of CAII discussed above.

The SH2 domain-based fluorescent biosensor thus prepared was further tested for its ability to distinguish specific phosphorylated sequences from other derivatives. Figure 5c and Table 2 summarize the titration curves and the apparent binding affinities for various peptides. Clearly, peptides **5**, **8**, **9**, and **10** bearing the pYZXM motif, which is essential for the high-affinity interaction with p85-nSH2,[25] can be fluorescently detected in a concentration range of 1−100 μM. In contrast, replacement of the pY residue in **5** with phosphoserine (peptide **7**) greatly reduced the sensing response. More importantly, neither phosphotyrosine peptide **11** nor **12** containing pYTDL and pYEEI sequences, respectively, were detected efficiently. Both these peptides are natural ligands for other members of the SH2 domain family.[25a] The observed sensing selectivity agrees well with the affinity of natural p85-nSH2. Overall, these results indicate that the SH2 domain was successfully converted to a biosensor equipped with a turn-on fluorescent switch that retains the natural recognition selectivity for phosphopeptides.

Generation of a Turn-On Biosensor in a Crude Bacterial Cell Lysate. The Q-LDT strategy was finally applied to construct a turn-on fluorescent biosensor for direct use in bacterial cell lysates. After expression of the GST-fused p85-nSH2 (GST-SH2) in *Escherichia coli*, **4** was incubated with the crude lysate

(30) We also confirmed that the UV−visible spectrum of the fraction containing the SH2 domain after the gel filtration showed absorption bands of both the DEAC and DABCYL groups as with the case of the CAII experiments.

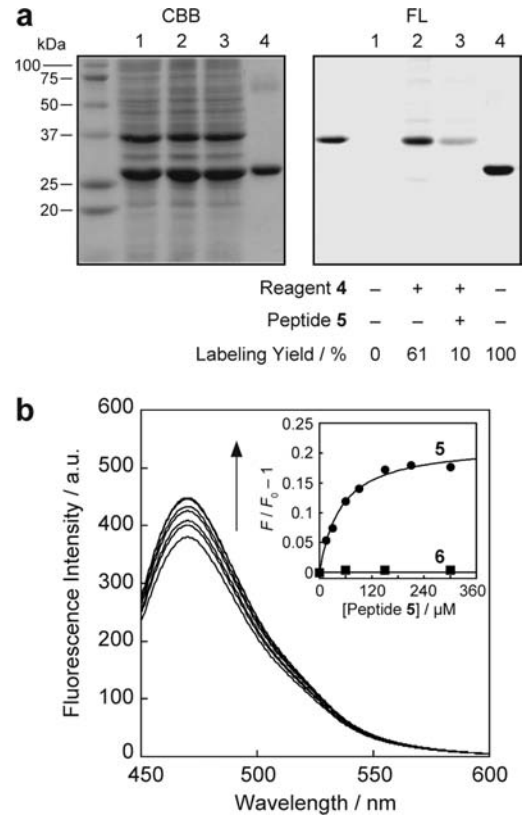

Figure 6. One-pot construction of a SH2 domain-based turn-on fluorescent biosensor in a bacterial lysate. (a) SDS-PAGE analysis of the labeling of GST-SH2 in the bacterial lysates with **4**. Reaction conditions: 40 μM **4**, 4 mM **5** (lane 3), lysate of GST-SH2-expressing *E. coli* containing ~8 μM GST-SH2 (50 mM HEPES, pH 8.0), 37 °C, and 40 h. Lane 4, DEAC−CAII (1:1) conjugate. (b) Fluorescence spectral change of the **4**-treated bacterial lysate upon the addition of peptide **5**. Measurement conditions: **4**-treated bacterial lysate (the postlabeling sample was diluted with no purification such that the concentration of the GST-SH2 is ~3 μM), 0−300 μM **5** or **6**, 50 mM HEPES, pH 8.0, 25 °C, and λ_{ex} = 427 nm. (Inset) Fluorescence titration curve. λ_{em} = 473 nm, peptide **5** (circle), **6** (square).

of the cells. *In-gel* fluorescence analysis of the reaction mixture showed a single fluorescent band corresponding to the DEAC-labeled GST-SH2, which was significantly diminished by the excess amount of phosphopeptide ligand **5** (Figure 6a). These results confirm the high target-selectivity of the **4**-mediated affinity labeling even in the presence of many other contaminating proteins in the lysate. More remarkably, the fluorescence at 473 nm was intensified in a typical saturation manner when phosphotyrosine peptide **5** was added to the postlabeling lysate without any purification step (Figure 6b). In contrast, no change in fluorescence occurred when the nonphosphorylated peptide **6** was added. This clearly demonstrates that the Q-LDT strategy is widely applicable to convert an intact protein to a turn-on fluorescent biosensor even under crude heterogeneous conditions.

Conclusions

While there is a growing recognition of the importance of semisynthetic fluorescent biosensors in biological research and drug discovery, engineering strategies for generating such functionalized proteins remain very limited. In this work, we have developed a new methodology using modular, Q-LDT reagents to convert target proteins to 'turn-on' fluorescent biosensors in a one-step labeling procedure. The approach was successfully applied to two different protein scaffolds, CAII and an SH2 domain, without genetic mutations, by adopting

appropriate affinity ligands. We have also demonstrated that the high target-selectivity of the Q-LDT-mediated labeling allows us to prepare biosensors not only in test tubes using purified proteins but also in a cell lysate. To our knowledge, this work represents the first demonstration of the construction of a semisynthetic biosensor in a crude mixture. Since we have previously proved that the LDT chemistry is applicable to the labeling of specific proteins in living cells or tissues,[10] the present strategy may be extended to create fluorescent biosensors directly in such environments. Moreover, it is envisioned that new LDT reagents containing a FRET pair of fluorophores (donor and acceptor) can be designed along the same lines to provide an approach to a one-pot construction of FRET-based ratiometric biosensors.[22a] Such efforts will significantly enhance the prospect of semisynthetic biosensors and are currently underway in our laboratory.

Experimental Section

Synthesis. All synthetic procedures and compound characterizations are described in the Supporting Information.

General Materials and Methods. Unless otherwise noted, all proteins/enzymes and reagents were obtained from commercial suppliers (Sigma, Aldrich, Tokyo Chemical Industry (TCI), or Wako Pure Chemical Industries) and used without further purification. UV−visible spectra were recorded on a Shimadzu UV-2550 spectrophotometer. Fluorescence spectra were recorded on a PerkinElmer LS 55 fluorescence spectrometer. SDS-PAGE was carried out using a Bio-Rad Mini-Protean III electrophoresis apparatus. Fluorescence gel images were acquired using a Bio-Rad ChemiDoc XRS system with a 480BP70 filter, and analyzed with Quantity One 1-D Analysis Software (Bio-Rad Laboratories). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi LaChrom L-7100 system equipped with LaChrom L-7400 UV and L-7485 fluorescence detectors. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra were recorded on an Applied Biosystems Voyager Elite mass spectrometer using α-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA) as the matrix. Matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry/mass spectrometry (MALDI-QIT-TOF MS/MS) analysis was performed by Dr. Masaki Yamada (Shimadzu Corporation) with a Shimadzu Biotech AXIMA QIT mass spectrometer.

Evaluation of the CAII Labeling. The concentration of human CAII (Sigma) was determined by measuring the absorbance at 280 nm using a molar absorption coefficient of 54 000 M^{-1} cm^{-1}.[31] CAII (10 μM) was incubated with **2** (20 μM) in HEPES buffer (50 mM, pH 7.2) at 37 °C. Control reactions with ethoxzolamide (ET, 100 μM) or reduced glutathione (GSH, 10 mM) were also included. Aliquots were taken at 6, 24, and 48 h and mixed with an equal volume of 2× SDS-PAGE loading buffer. The samples were applied to a 12.5% SDS-PAGE and the labeled CAII was detected by *in-gel* fluorescence imaging. The 1:1 DEAC−CAII conjugate was prepared as previously described[10] and used as a standard marker to determine the labeling yields on the basis of the relative fluorescence intensities. After fluorescence imaging, the gel was stained with CBB.

Peptide Mapping of the Labeled CAII. Labeling was performed under the following conditions: 50 μM CAII, 50 μM **2**, 50 mM HEPES, pH 7.2, and 37 °C. After 72 h of incubation, the labeled CAII was purified by size-exclusion chromatography using a TOYOPEARL HW-40F (Tosoh Corporation) and dialyzed against HEPES buffer (50 mM, pH 8.5) with a Spectra/Por dialysis membrane (MWCO: 10 000) (Spectrum Laboratories). The resulting solution was concentrated using a Centricon Ultracel YM-10 (Millipore). To this solution, urea (at a final concentration of 2 M) and lysyl endopeptidase (LEP) (LEP/substrate ratio = 1:50 (w/w)) were added. Native (unlabeled) CAII was also subjected to LEP digestion. After incubation at 37 °C for 48 h, the digested peptides were separated by RP-HPLC using an YMC-Pack ODS-A column (5 μm, 4.6ϕ × 250 mm). The peaks were monitored by UV absorbance at 220 nm and fluorescence at 473 nm (λ_{ex} = 427 nm). The collected fractions were analyzed by MALDI-TOF MS (CHCA).

Fluorescence Titration of the CAII-Based Biosensor. Labeling was performed under the following conditions: 30 μM CAII, 30 μM **2**, 50 mM HEPES, pH 7.2, and 37 °C. After 48 h of incubation, the labeling solution was diluted 6-fold with no purification. Titration experiments with various sulfonamide and nonsulfonamide derivatives were carried out using the solution containing a mixture of labeled (48%) and native CAII (the total concentration of CAII = 5 μM, 50 mM HEPES, pH 7.2) in a quartz cell (500 μL) at 25 °C. The fluorescence spectral change (λ_{ex} = 427 nm) was monitored upon addition of a solution of the analyte with a microsyringe. All the titration experiments were performed in triplicate on each analyte to obtain mean and standard deviation values of the relative fluorescence intensities (F/F_0). Fluorescence titration curves at 473 nm were analyzed with the nonlinear least-squares curve-fitting method to evaluate the apparent binding constants (K_{app}, M^{-1}).

Evaluation of the SH2 Domain Labeling. Recombinant human p85-nSH2 was obtained by bacterial expression as a fusion protein with GST, followed by cleavage of the GST tag using thrombin (the plasmid encoding GST-p85-nSH2 was kindly provided by Prof. Teruyuki Nagamune). The concentration of p85-nSH2 was determined by measuring the absorbance at 280 nm using a molar absorption coefficient of 21 300 M^{-1} cm^{-1}. Purified p85-nSH2 (5 μM) was incubated with **3** or **4** (10 μM) in HEPES buffer (50 mM, pH 8.0) at 37 °C. Control reactions with **5** (1 mM) or GSH (10 mM) were also included. Aliquots were taken at 20 and 44 h and mixed with an equal volume of 2× SDS-PAGE loading buffer. The samples were applied to a 15% SDS-PAGE and the labeled p85-nSH2 was detected by *in-gel* fluorescence imaging. The 1:1 DEAC−CAII conjugate was used as a standard marker to determine the labeling yields on the basis of the relative fluorescence intensities. After fluorescence imaging, the gel was stained with CBB.

Peptide Mapping of the Labeled SH2 Domain. Labeling was performed under the following conditions: 22 μM p85-nSH2, 110 μM **3**, 50 mM HEPES, pH 8.0, and 37 °C. After 40 h of incubation, the labeled p85-nSH2 was purified by semipreparative RP-HPLC using an YMC-Pack ODS-A column (5 μm, 10ϕ × 250 mm). The peaks were monitored by UV absorbance at 220 nm and fluorescence at 473 nm (λ_{ex} = 427 nm). The fraction containing the labeled p85-nSH2 was collected and dialyzed against HEPES buffer (50 mM, pH 8.0) with a Spectra/Por dialysis membrane (MWCO: 3000). The resulting solution was concentrated using a Centricon Ultracel YM-3 (Millipore). To this solution, urea (at a final concentration of 2 M) and trypsin (trypsin/substrate ratio = 1:10 (w/w)) were added. Unlabeled, intact p85-nSH2 was also subjected to trypsin digestion. After incubation at 37 °C for 40 h, the digested peptides were separated by RP-HPLC using an YMC-Pack ODS-A column (5 μm, 4.6ϕ × 250 mm). The collected fractions were analyzed by MALDI-TOF MS (CHCA) and the labeled fragment was further characterized by MALDI-QIT-TOF MS/MS analysis.

Fluorescence Measurement of the SH2 Domain-Based Biosensor. Labeling was performed under the conditions mentioned in the Evaluation of the SH2 Domain Labeling section. After 44 h of incubation, the labeling solution was briefly purified by size-exclusion chromatography using a PD-10 column (GE Healthcare) (Note that, after this procedure, the eluted SH2 domain still complexes with the DABCYL-tethered ligand). Titration experiments with various peptides were carried out using the solution

(31) Supuran, C. T.; Briganti, F.; Tilli, S.; Chegwidden, W. R.; Scozzafava, A. *Bioorg. Med. Chem.* **2001**, *9*, 703.

containing a mixture of labeled (30%) and unmodified p85-nSH2 (the total concentration of SH2 domain = 1 μM, 50 mM HEPES, pH 7.4) in a quartz cell (500 μL) at 25 °C. The fluorescence spectral change (λ_{ex} = 427 nm) was monitored upon addition of a solution of the analyte with a microsyringe. All the titration experiments were performed in triplicate on each analyte to obtain mean and standard deviation values of the relative fluorescence intensities (F/F_0). Fluorescence titration curves at 473 nm were analyzed with the nonlinear least-squares curve-fitting method to extract the apparent binding constants (K_{app}, M^{-1}).

Generation of a SH2 Domain-Based Biosensor in a Bacterial Cell Lysate. Bacterial cells (*E. coli* BL21(DE3)) transformed with the plasmid encoding GST-p85-nSH2 were grown at 37 °C in TB medium containing ampicillin to an O.D. (600 nm) of 0.8, at which time expression of the protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were further grown at 25 °C overnight. The cells were harvested and lysed by sonication in Tris buffer (100 mM, 50 mM EDTA, 0.2 M NaCl, pH 8.0). The lysate was dialyzed against HEPES buffer (50 mM, pH 8.0) with a Spectra/Por dialysis membrane (MWCO: 3000) and used for the following experiments. The labeling was performed by incubating the lysate with **4** (40 μM) in the absence or presence of **5** (4 mM) at 37 °C for 40 h. The labeling was analyzed by SDS-PAGE followed by *in-gel* fluorescence imaging as described above. The concentration of the GST-p85-nSH2 in the lysate was also roughly estimated by SDS-PAGE/CBB staining using purified and quantified GST-p85-nSH2 as a standard. The fluorescence titration experiments with **5** and **6** were carried out as described above with a lysate solution containing ~3 μM of total GST-p85-nSH2.

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Supporting Information Available: Figures S1−S3, Schemes S1−S2, and experimental details of the synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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