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Generation of Active Protease Depending on Peptide-Protein Interactions Using Interaction-Dependent Native Chemical Ligation and Protein Trans-Splicing

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Abstract

An artificial signal transduction system has been constructed by employing engineered human immunodeficiency type-1 (HIV-1) protease and *Nostoc punctiforme* PCC73102 (*Npu*) DnaE intein. While the truncation of four amino acid residues at N-terminus of HIV-1 protease diminished its activity, the attachment of the PQIT sequence into the truncated protease by protein trans-splicing (PTS) reconstituted the enzymatic activity. By combining interaction-dependent native chemical ligation (IDNCL) with the PTS reaction, the peptide-protein interaction was clearly detected by measuring HIV-1 protease activity. Src homology domain 2 (SH2) of c-Src (SrcSH2) and phosphopeptides were used as model binding pairs. HIV-1 protease activities were dose-dependently increased after the IDNCL-PTS reaction when the peptides containing pYEEI (pY = phosohotyrosine) and pYEE sequences were used as the input peptides. HIV-1 protease activity generated by IDNCL-PTS might activate several enzymes, and therefore, the artificial signal transduction system might be available in synthetic biology.

Keywords: peptide-protein interaction, protein trans-splicing, HIV-1 protease, native chemical ligation, signal transduction

1. Introduction

In nature, several signal transduction systems, such as kinase cascades and ubiquitin signaling cascades, are used to maintain higher-order functions in bio-organisms, especially in eukaryotes.[1,2] In these signal transductions, specific interactions between receptor proteins and extracellular ligands usually work as input signals. For instance, ligand binding to a death receptor that induces apoptosis of the cell can activate an initiator caspase; it then cleaves effector caspases that mediate several proteolytic events of apoptosis.[3,4] Thus, construction of a mimetic system in which binding events between a synthetic ligand and a target protein is transduced as the generation of an active protease is valuable.

To generate active enzymes and reporter proteins that depend on input signals, such as binding events, protein-fragment complementation assay has been widely employed.[5-9] Sakamoto and Kudo noted active green fluorescent protein (GFP) reporter responses to host-guest interactions by employing split-GFP.[7] Moreover, combining protein trans-splicing (PTS), that is self-catalyzed by native and engineered split inteins,[10-15] expands its availability owing to the covalent bond formation between two fragments (N-extein and C-extein) of the protein. Detection methods for protein-protein interactions and small molecule-protein interactions have been developed using split inteins with several enzymes and reporter proteins.[16-19] Sonntag and Mootz reported the generation of active tobacco etch virus (TEV) protease by conditional protein splicing that depends on the interaction of rapamycin with FK506 binding protein (FKBP) and FKBP-rapamycin binding.[19] In this system, intracellular proteins can be manipulated through proteolysis by the active TEV protease. Sonntag and Mootz used split VMA intein from *Saccharomyces cerevisiae* in which two fragments (N-intein and C-intein) were comprised of 184 and 64 amino acid residues, respectively. TEV protease was also split into two pieces with more than 37 amino acid residues. Therefore, it has been difficult to apply this method to the development of a signal transduction system that depends on the binding event between a synthetic ligand and a target protein, because the synthetic ligand must be chemically conjugated to the fusion protein comprising the TEV protease fragment and VMA N-intein or C-intein.

On the other hand, I have constructed a detection method for ligand-protein interactions using interaction-dependent native chemical ligation and PTS (IDNCL-PTS).[20] In this method, IDNCL reaction occurs between the short peptide (residues 24–34 of β-galactosidase; βGal) bearing a ligand, such as maltose, via a thioester linkage and N-intein of *Ssp* DnaB (residues 1–11) bearing a target protein, such as maltose binding protein, when the ligand binds to the target protein. Using this IDNCL product, active bGal is generated by PTS with a fusion protein of C-intein of *Ssp* DnaB M86 variant[21] and C-terminal fragment of bGal (residues 35–1024). Thus, the binding events were successfully transduced into the βGal activity.

In the present study, a signal transduction system that

activates a protease enzyme depending on the binding events has been constructed. To do this, human immunodeficiency virus type 1 (HIV-1) protease was employed[22] because it is a small protein made up of two identical 99-amino acid polypeptide monomers and it has catalytic activity when it forms a dimer.[23,24] Split HIV-1 protease was designed and conjugated with an engineered split intein, and the binding events between a ligand and a target protein were transduced into active HIV-1 protease generated by IDNCL-PTS.

2. Experimental

Preparation of IntC-prC: The gene encoding HIV-1 protease (5–99) was constructed by ligation between two double-stranded DNA fragments, which were developed by overlapping polymerase chain reaction (PCR) using synthetic oligonucleotides. The gene encoding DnaE intein (36–137) was amplified by PCR using pSKDuet16 plasmid (Addgene) as a template.[25] Two DNAs encoding HIV-1 protease and DnaE intein were treated with MfeI/XhoI and KpnI/MfeI, respectively. The product DNA was ligated into a pET28a-based vector (Novagen) having a gene encoding YFP as a fluorescent tag to give a plasmid, named pET28-intc-prc. For the production of IntC-prC, BL21(DE3)pG·KJE8 was transformed by pET22-intc-prc. The cells were grown in a Luria-Bertani medium containing ampicillin as an antibiotic at 37°C to an optical density at 600 nm around 0.6. Expression of the protein was induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside for 16 hours at 18°C. The cells were harvested, and the expressed protein was extracted by sonication. The protein was purified by Ni-NTA resin (QIAGEN), and further purified by size-exclusion chromatography using HiLoad 16/600 Superdex 75 column (GE Healthcare) with a splicing buffer [50 mM Tris·HCl (pH 7.0), 300 mM NaCl, 1 mM EDTA, 2 mM DTT, 5% (v/v) glycerol].

Preparation of IntN-SrcSH2: The gene encoding SrcSH2 was amplified from a human placenta cDNA library (TaKaRa BIO) by PCR. The product DNA was restricted with NdeI and XhoI and ligated into a vector encoding Ssp DnaB intein (1–11) and $(Gly\text{-}Gly\text{-}Ser)_{12}$ linker. The region of the gene encoding DnaB was replaced by double-stranded synthetic DNA encoding *Npu* DnaE (1-35) to give a plasmid, named pET22-intn-sh2. The production of IntN-SrcSH2 was performed using BL21(DE3)pG·KJE8 as a host strain. After induction of protein expression and harvesting cells, the protein was extracted from the periplasmic space by osmotic shock using the TES buffer [200 mM Tris·HCl (pH 8.0), 0.5 mM EDTA, 0.5 M sucrose]. The protein was purified by Ni-NTA resin, and the buffer was exchanged using a desalting column (PD10, GE Healthcare) to an assay buffer [100 mM Tris·HCl (pH 7.4), 100 mM NaCl].

Syntheses of PrN-intN and Rpep-intN: Peptide synthesis was performed using the Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase method using *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate as a coupling reagent. PrN-intN peptide was elongated on an Fmoc-NH-SAL-PEG resin (Watanabe Chemical Industry; 12.5 μmol). After elongation, peptidyl resin was treated with trifluoroacetic acid (TFA) with 2.5% (v/v) ethanedithiol, 2.5% (v/v) water, and 1% (v/v) triisopropyl silane as scavengers. To synthesize Rpep-intN, two peptide fragments were synthesized and conjugated by NCL. The N-terminal peptide (EELIKENMHMK) was elongated on a 2-chlorotrityl chloride resin (Watanabe Chemical Industry; 36 μmol after incorporation of 1st Lys residue). A side-chain protected peptide was obtained by a treatment with mixed solvent of hexafluoro-2-propanol (HFIP) : dichloromethane = 1 : 4 and reacted with thiophenol (10 eq.) using 1-hydroxy-7-azabenzotriazole (3 eq.) and diisopropylcarbodiimide (3 eq.). The protecting groups were removed with TFA to give a peptide thioester (EELIKENMHMK-S-phenyl). A C-terminal peptide corresponding to the sequence of DnaE (1–35) was synthesized on Fmoc-NH-SAL-PEG resin, and reacted by NCL with the N-terminal peptide thioester to give Rpep-intN. The crude peptides were purified by reversed-phase HPLC using a linear gradient of acetonitrile/0.1% TFA. The peptides were identified by their molecular ion peaks $(M+H)^+$ by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOFMS; Shimadzu, AXIMA Performance): m/z found (calcd): PrN-intN, 4428.32 (4428.31); Rpep-intN, 5371.50 (5371.73). The substrate peptide (Fsbst), Abz-Thr-Ile-Nle-Phe(pNO_2)-Gln-Arg-NH_2, was also synthesized on Rink Amide MBHA resin and identified by RP-HPLC compared with that of the authentic sample,[27] and MALDI–TOFMS; Fsbst, m/z found (calcd): 940.64 (940.50) $[(M+H)^+]$.

Syntheses of PrN-pYEEI and PrN-pYEE: To prepare the thioester linkage, *N*-(2,2-dimethoxyethyl)-2-[4-(mercaptomethyl)phenyl]acetamide (compound **1**) was synthesized according to Scheme S1. Potassium thioacetate (2.05 g, 18.0 mmol) was added to a solution of 4-(bromomethyl)phenylacetic acid (1.97 g, 8.60 mmol) in dimethylformamide (DMF; 25 mL). The resulting solution was stirred at room temperature for one hour. DMF was removed by evaporation, and the mixture was extracted with ethyl acetate and 10% citric acid in water (x 1) and water (x 2). The organic layer was dried over Na_2SO_4, concentrated in vacuo, and recrystallized with ethyl acetate and hexane to give compound **2**. ^{1}H NMR (400 MHz, $CDCl_3$): d = 7.28 – 7.18 (m, 4 H), 4.09 (s, 2 H), 3.61 (s, 2 H), 2.33 (s, 3 H). Dicyclohexylcarbodiimide (1.21 g, 5.89 mmol) was added to a solution of compound **2** (1.20 g, 5.35 mmol) in tetrahydrofuran (25 mL) on ice. After five minutes, aminoacetaldehyde dimethyl acetal (0.58 mL, 5.35 mmol) was added and stirred for one hour at 4°C, and for five hours at room temperature. After filtration, the solvent was removed and the mixture was extracted with ethyl acetate and 10% citric acid in water (x 2), 4% $NaHCO_3$ (x 2), and brine (x 1). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. Silica gel chromatography was performed with hexane and ethyl acetate (2 : 3) to give compound **3** (0.97 g, 59%). Compound **3** was treated with K_2CO_3 (1.3 eq.) in MeOH (30 mL) and water (10 mL) for one-and-a-half hours at room temperature. After evaporation, ethyl acetate was added and extracted with 10% citric acid in water (x 1), water (x 3), and brine (x 1). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. Hexane was added, and the precipitate was collected by filtration to give compound **1** (587 mg, 70%). ^{1}H NMR (400 MHz, $CDCl_3$): d = 8.13 (t, *J* = 5.3 Hz, 1H), 7.24-7.11 (m, 4H), 4.29 (t, *J* = 5.5 Hz, 1H), 3.65 (s, 2H), 3.20 (s, 6H), 3.11 (t, *J* = 5.5 Hz, 2H), 2.78 (s, 1H).

A peptide thioester (PQIT-S-phenyl; 30 mg, 54.6 μmol) and compound **1** (36 mg, 134 μmol) were dissolved in trifluoroethanol (0.5 mL) and then 200 mM Tris·HCl buffer (pH 8.0; 0.5 mL) was added and stirred for one-and-a-half hours at room temperature (Scheme S2). Water (50 mL) was added and evaporated. The crude mixture was purified by RP-HPLC to afford PrN-CHO (9.1 mg, 21%). The peptide was identified by MALDI–TOFMS; m/z found (calcd): 663.19

(663.32) [(M+H)$^+$]. Aoa-βA-pYEEI-NH_2 and Aoa-βA-pYEE-NH_2 (Aoa = aminooxyacetyl) were synthesized on Rink Amide MBHA resin (30 μmol). Ten μL of PrN-CHO (10 mM in DMSO) and 10 μL of Aoa-βA-pYEEI-NH_2 or Aoa-βA-pYEE-NH_2 (10 mM in DMSO) were mixed with 20 μL of 20 mM acetic acid in water. The reaction completion was checked by RP-HPLC (Figure S1). MALDI–TOFMS; PrN-pYEEI, m/z found (calcd): 1420.57 (1420.58) [(M+H)$^+$]; PrN-pYEE, m/z found (calcd): 1307.72 (1307.50) [(M+H)$^+$].

Splicing: Splicing reaction between Rpep-intN and IntC-prC was performed in a splicing buffer at 25°C. [Rpep-intN] = 25 μM, [IntC-prC] = 5 μM. The assay was triplicated. The reaction solution was analyzed by SDS-PAGE (15% acrylamide), and image analysis was performed with the Image J analysis software.

Protease activity measurement: PrN-intN and IntC-prC were reacted in a splicing buffer at 25°C. [PrN-intN] = 25 μM, [IntC-prC] = 5 μM, [Fsbst] = 100 μM. Fluorescence spectra were recorded on HITACHI F-2500 spectrofluorometer (λex = 335 nm). Fluorescence intensities at 440 nm were plotted. The assay was triplicated.

IDNCL-PTS: In time-course experiments, PrN-pYEEI and IntN-SrcSH2 were incubated in an assay buffer containing tris(2-carboxyethyl)phosphine (TCEP) at 37°C for zero, two, four, six, and eight hours. [PrN-pYEEI] = 10 μM, [IntN-SrcSH2] = 4 μM, [TCEP] = 2.5 mM. After incubation, 20 μL of each sample solution was mixed with 1 μL of Fsbst (final conc. = 100 μM) and 21 μL of IntC-prC (final conc. = 2.6 μM). These were incubated at 25°C for eight hours. Then, 2 μL of 3 M NaOAc (pH 5.2) was added to the sample solution to acidify, and the apparent dissociation constants of PrN-pYEEI and PrN-pYEE with IntN-SrcSH2 were calculated using equation (1) and a KaleidaGraph (Synergy Software), in which a 1:1 binding stoichiometry was assumed.

$$\Delta F = \Delta F_{max} ([P]_0 + [S]_0 + K_d - (([P]_0 + [S]_0 + K_d)^2 - 4([P]_0[S]_0)^{1/2})/(2[S]_0) \quad (1)$$

where $[P]_0$ and $[S]_0$ represent the initial concentrations of PrN-pYEEI or PrN-pYEE and IntN-SrcSH2, respectively; ΔF is the difference between the value of the fluorescence increase per hour in test solutions and in the absence of the peptide; and ΔF_{max} is the difference between the value of fluorescence increase per hour when IntN-SrcSH2 is completely complexed with PrN-pYEEI or PrN-pYEE and in the absence of the peptide.

3. Results and Discussion

Design of split HIV-1 protease

In HIV-1 protease, N-terminal and C-terminal sequences, residues 1–4 and 96–99, are crucial for dimer formation and enzymatic activity because these residues of the two monomers associate to form a four-stranded antiparallel b-sheet structure.[24] Therefore, N- or C-terminal deletion variants are unable to form active dimeric structures. On this basis, I have designed an engineered protein in which residues 1–4 of HIV-1 protease were replaced by a C-terminal fragment of *Nostoc punctiforme* PCC73102 (*Npu*) DnaE (36–137)[26] (see Figure 1). *Npu* DnaE intein is a naturally occurring split intein[11] that is separated between residues 1–102 and 103–137. In my signal transduction system, a shorter peptide sequence of the N-terminal fragment of the intein (N-intein) is better because it needs to be placed at the N-terminus of the protein of interest. Iwai and colleagues reported that an engineered *Npu* DnaE intein whose sequence is divided between 1–35 and 36–137 was active.[23] On this basis, I designed two engineered molecules: IntC-prC protein having 36–137 residues of *Npu* DnaE intein and 5–99 residues of HIV-1 protease, and PrN-intN peptide having 1–4 residues of HIV-1 protease and 1–35 residues of *Npu* DnaE intein. The leucine residue at position 5 in the HIV-1 protease was substituted by cysteine because the cysteine residue is necessary at the +1 position of the extein sequence in the *Npu* DnaE intein. A yellow fluorescent protein (YFP) tag was also incorporated at the N-terminus of IntC-prC.[20] The IntC-prC protein was expressed by an *Escherichia coli* (*E. coli*) strain, BL21(DE3)pG·KJE8, and purified by size exclusion chromatography. The peptide PrN-intN was synthesized using a solid-phase method and purified by reversed-phase high performance liquid chromatography (RP-HPLC). A peptide, Rpep-intN, for checking the splicing activity was also designed and synthesized (Figure 1).

Splicing activity of the designed intein

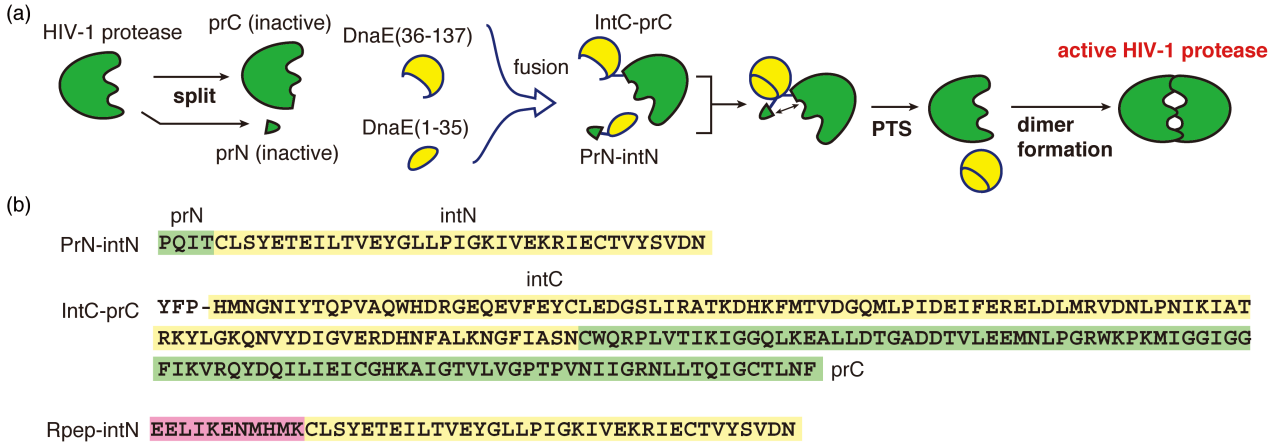

Figure 1. Design of split HIV-1 protease for generating active protease by PTS. (a) Schematic representation of the split HIV-1 protease conjugated with the DnaE split intein.(b) Amino acid sequences of PrN-intN, IntC-prC, and Rpep-intN. The sequences derived from HIV-1 protease and DnaE intein are colored with green and yellow, respectively.

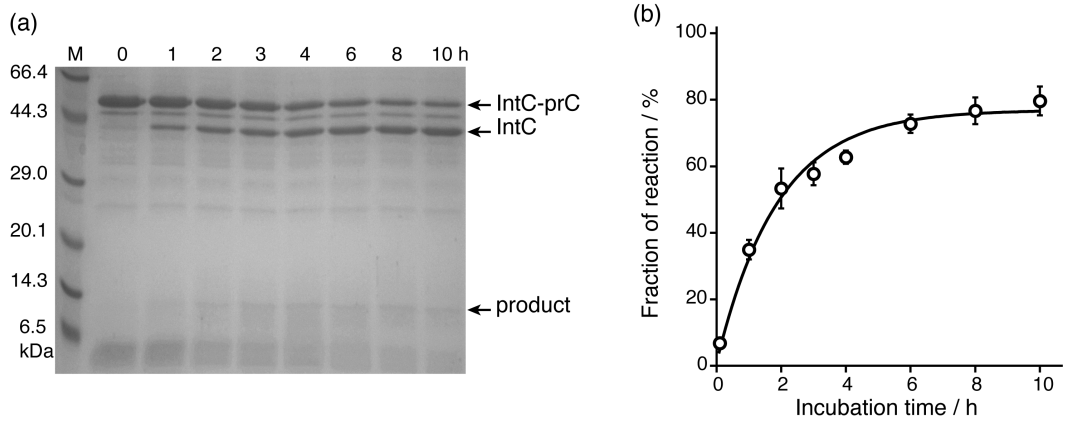

Figure 2. PTS reaction between Rpep-intN and IntC-prC. (a) SDS-PAGE (15% acrylamide) analysis of the time course of the PTS reaction. (b) Kinetic analysis of producing IntC determined by densitometric analysis of the SDS-PAGE. All experiments were performed in triplicate, and error bars represent standard deviation.

To check the splicing activity of the engineered *Npu* intein in vitro, IntC-prC protein and Ppep-intN, not having the HIV-1 protease sequence, were reacted. IntC-prC (5.0 μM) was mixed with Rpep-intN (25 μM) in a splicing buffer (pH 7.0) at 25°C, incubated for zero hours, and analyzed by SDS-PAGE (Figure 2). Increasing the incubation period, IntC-prC, shown at around 50 kDa (calcd = 49.7 kDa), was gradually decreased. The new proteins were detected at around 10 kDa and 40 kDa, corresponding to the splicing product (calcd = 11.7 kDa) and IntC fragment (calcd = 39.4 kDa), respectively. By quantifying the bands corresponding to the IntC fragment, the kinetic parameter was estimated as an observed rate constant (k_{obs}) of (1.3 ± 0.2) x 10^{-4} s^{-1}. The activity of the engineered *Npu* DnaE intein was weaker than those of the native form *Npu* DnaE (k_{obs} = 3.5 x 10^{-3} s^{-1})[11] and the M86 variant of *Ssp* DnaB intein,[21] but it was similar or slightly higher than those of the wt *Ssp* DnaB (k_{obs} = 4.1 x 10^{-5} s^{-1})[12] and *Ssp* DnaE (k_{obs} = 8.0 x 10^{-5} s^{-1})[11] inteins. Although the designed intein has moderate activity, it can be applicable for developing the detection system.

Generation of active HIV-1 protease by PTS

Next, splicing reaction between IntC-prC protein and PrN-intN, having N-terminal residues (1–4) of HIV-1 protease, was examined to assess whether the splicing product can work as an active enzyme. The protease activity was evaluated using a fluorogenic substrate (Fsbst) having the sequence of Abz-Thr-Ile-Nle-Phe(pNO_2)-Gln-Arg-NH_2 (Abz = 2-aminobenzoyl).[27,28] While the fluorescence of the Abz group can be quenched by the nitrophenyl moiety via Förster resonance energy transfer, it can increase by the proteolytic cleavage between Nle and Phe(pNO_2). The IntC-prC protein (5.0 μM) was reacted with PrN-intN (25 μM) in the presence of Fsbst (100 μM) in a splicing buffer (pH 7.0) at 25°C, and the fluorescence spectra were measured (Figure 3A). The fluorescence intensities at 420 nm were increased as a sigmoid function of time (Figure 3B). This result suggests that the product protein generated by PTS can readily form the active structure and cleave the substrate peptide. Moreover, the enzyme activity was clearly observedeven when incubated for two hours. After incubation of eight hours, the fluorescence signal at 420 nm was almost saturated, indicating that the substrate was largely cleaved by the generated enzyme. This kinetic behavior of generated HIV-1 protease shows good correlation with that for the PTS reaction between Rpep-intN peptide and IntC-prC. In contrast, when IntC-prC was incubated alone, fluorescence was not changed even after eight hours of incubation (Figure 3B). This finding suggests that the precursor protein, IntC-prC, is completely inactive. Since the N-terminal four amino acid residues are critical to form the dimeric structure of the protease, N-terminal truncation of HIV-1 protease by four amino acids completely abolished the enzymatic activity

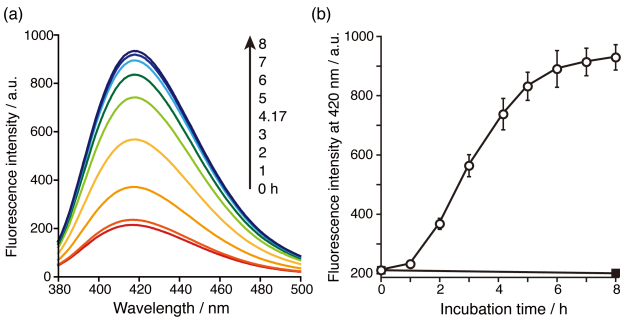

Figure 3. Generation of active HIV-1 protease by PTS reaction between PrN-intN and IntC-prC. (a) Time course of fluorescence emission spectra of Fsbst (100 μM) incubated with PrN-intN (25 μM) and IntC-prC (5 μM) at 25°C. λex = 380 nm. (b) Fluorescence intensity at 420 nm as a function of incubation time in the presence (open circle) and absence (closed square) of PrN-intN. All experiments were performed in triplicate, and error bars represent standard deviation.

Detection of phosphopeptide and SrcSH2 protein using HIV-1 protease reconstitution system

Finally, a signal transduction system that depends on ligand-protein interactions has been constructed based on generation of active HIV-1 protease by PTS. In this system, IDNCL was employed to convert the interaction event between a ligand and a target protein into the production of the peptide having the PrN-intN sequence. To this aim, Src homology domain 2 of c-Src (SrcSH2) and its ligand, pTyr-Glu-Glu-Ile (pYEEI; pY = phosphorylated tyrosine) peptide, were used as a model binding pair (Figure 4A).[29-31] SH2 proteins can recognize the phosphorylation sites with the specific peptide sequences. SrcSH2 binds to the Ac-pYEEI-NH_2 peptide with a dissociation constant of K_d = 0.5 μM.[31] It has been questioned

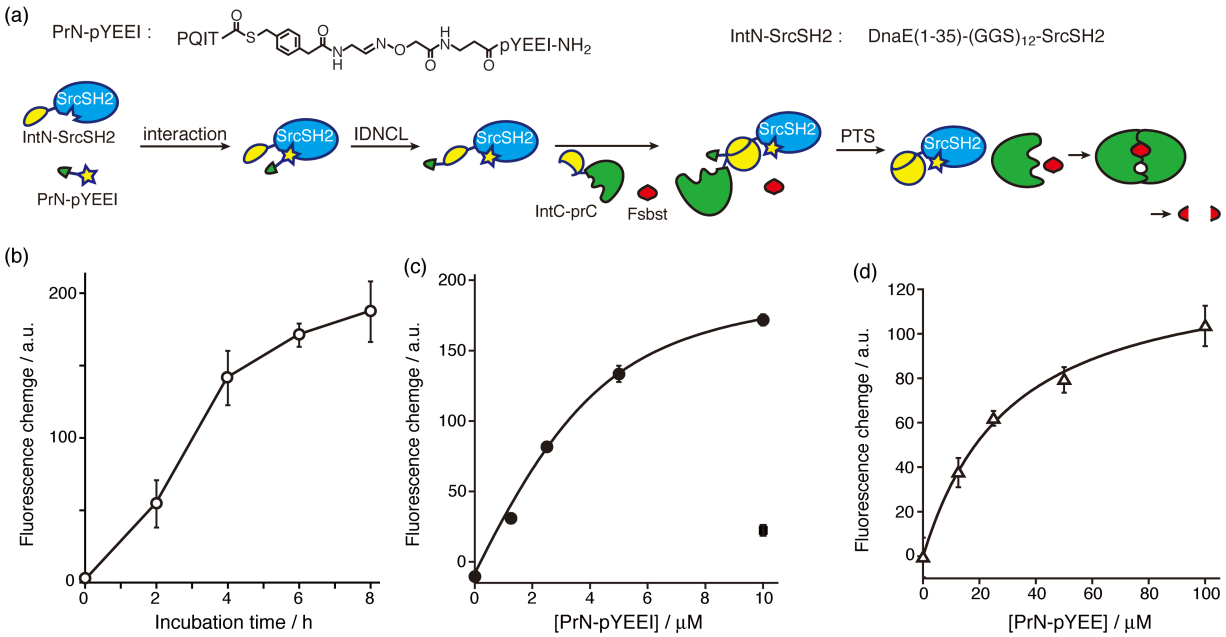

Figure 4. Signal transduction depending on the interaction of pYEEI and SrcSH2. (a) Structures of PrN-pYEEI and IntN-SrcSH2, and schematic representation of the signal transduction system. (b) Fluorescence change of Fsbst as a function of IDNCL reaction time. (c) Fluorescence change of Fsbst as a function of PrN-pYEEI in the absence (closed circle) and presence (closed square) of Ac-pYEEI-NH_2 (50 μM). (d) Fluorescence change of Fsbst as a function of PrN-pYEE (open triangle) concentrations. All experiments were performed in triplicate, and error bars represent standard deviation.

whether the input signals from the moderate binding pair can be discriminated by IDNCL-PTS. The input peptide, named PrN-pYEEI, which contains PrN and pYEEI sequences via a thioester linkage, was designed and synthesized. Moreover, the PrN-pYEE peptide, with a lack of the isoleucine residue of PrN-pYEEI, was also synthesized to compare whether the difference of binding affinities affects the output signal. The input protein, named IntN-SrcSH2, which contains intN and SrcSH2 sequences via a flexible $(Gly\text{-}Gly\text{-}Ser)_{12}$ linker, was also designed and expressed in *E. coli*. When the pYEEI sequence of input peptide can interact with SrcSH2 of the input protein, IDNCL occurs between the peptide and protein and can provide the PrN-intN sequence at the N-terminal region of SrcSH2.

IDNCL between PrN-pYEEI (10 μM) ligand and IntN-SrcSH2 (4 μM) protein was performed for zero, two, four, six, and eight hours of incubation. The reaction was directly confirmed by RP-HPLC, but the product was almost undetectable due to the low reaction yield (≤ 1%). After the IDNCL reaction, IntC-prC (2.6 μM) and Fsbst (100 μM) were added and incubated at room temperature for eight hours to promote PTS at pH 7.4. To increase the enzyme activity, pH was shifted at around 4.5,[26] and fluorescence spectra were recorded before and after incubation at 25°C for several hours. Figure 4B shows the fluorescence change at 420 nm as a function of incubation time for IDNCL. Fluorescence changes clearly appeared after the increased reaction time of IDNCL between PrN-pYEEI and IntN-SrcSH2. Thus, the interaction events between pYEEI and SrcSH2 were successfully transduced into the protease activity. Under the eight hours incubation condition, dose dependency of PrN-pYEEI was examined. Figure 4C shows the IDNCL-PTS results using various concentrations of PrN-pYEEI (0, 1.25, 2.5, 5, and 10 μM) and IntN-SrcSH2 (4 μM). The fluorescence changes reflecting the enzyme activity were dose-dependently increased. When assuming that the fluorescence change is correlated with the complex formation between pYEEI and SrcSH2, the binding affinity of PrN-pYEEI for IntN-SrcSH2 was estimated as K_d = 1.1 μM, which is a similar value of the reported one (0.5 μM). Moreover, the weak interaction between PrN-pYEE and IntN-SrcSH2 was also assessed by IDNCL-PTS, and the affinity was estimated as K_d = 28 μM (Figure 4D). In contrast, incubation of PrN-pYEEI (10 μM) with IntN-SrcSH2 (4 μM) in the presence of Ac-pYEEI-NH_2 (50 μM) as a competitor caused a slight increase in fluorescence (Figure 4C). These findings indicate that the specific interaction between pYEEI peptide and SrcSH2 promotes an NCL reaction for generating the PrN-intN sequence at the N-terminal site upon SrcSH2. Thus, binding events between a ligand and a protein, such as pYEEI and SrcSH2, were successfully transduced into the protease activity. This can be used for generating a variety of functional proteins, such as several enzymes and recognition modules.

4. Conclusion

In this study, an artificial signal transduction system based on IDNCL-PTS has been successfully developed using an engineered split intein, derived from *Npu* DnaE, and HIV-1 protease. Truncation of only four amino acid residues at N-terminus of HIV-1 protease completely abolished the protease activity due to lack of the active dimer structure. The attachment of the N-terminal residues into the truncated HIV-1 protease by PTS using the engineered *Npu* DnaE intein generated the active enzyme that cleaves the fluorogenic substrate. By combining IDNCL with the PTS reaction, the signal transduction system was successfully constructed. The protease activity was clearly observed depending on the interactions of input PrN-pYEEI and PrN-pYEE with input IntN-SrcSH2 protein. The HIV-1 protease activity generated by IDNCL-PTS might activate several enzymes working in naturally occurring signal cascades. Indeed, the low reaction yield of IDNCL has to be improved by changing the chemical

structure of the thioester linkage to increase the detection limit. While the improvement of the IDNCL efficiency is necessary, the artificial signal transduction system might be available in synthetic biology in the future.

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