DOI: 10.1002/anie.201007078

Protein Activity

Photocontrol of Protein Activity Mediated by the Cleavage Reaction of a Split Intein**

*Jens Binschik, Joachim Zettler, and Henning D. Mootz**

Photocontrol of proteins is an exquisite goal for the investigation of biological systems, for example to modulate conformational changes in proteins or to manipulate protein activity in living cells.[1] Light can be applied with high temporal and spatial precision to biological samples and is furthermore orthogonal to most proteins and cells. However, there is no general blueprint of how to convert a non-photoresponsive protein of interest into a variant that can be specifically regulated by light. This is because of our limited capability in protein design, that is, how a photoreactive synthetic group or genetically encoded photoreceptor domain can be functionally coupled to the protein's activity. Moreover, incorporation of a synthetic group into any given protein may pose a significant technical challenge, although the use of various photolabile protecting groups and reversible photoswitches has been reported.

We were interested in developing a photoactivatable split intein, because these autocatalytic polypeptide sequences can be linked to the activity of fused proteins in potentially quite general ways through protein *trans*-splicing and *trans*-cleavage reactions.[2] Split inteins have been previously rendered photoactivatable by genetic fusion to a phytochrome domain,[3a] as well as by synthetic incorporation of a photoremovable protection group on an *O*-acyl isomer[3b] or in the backbone sequence of a synthetic split intein half.[3c] However, these systems have so far only been used for proof-of-principle studies using model proteins and unrelated tag sequences or short peptide sequences. They have not yet been applied to manipulate the function of a protein of interest. Herein, we report a novel light-induced split intein obtained by synthetic manipulation of both side chain and backbone structures in the synthetic fragment. The protein of interest is expressed as a fully recombinant protein fused to the complementary intein fragment. We apply this system to regulate native prothrombin by light.

We envisaged a novel strategy for protein functional control that, instead of the protein *trans*-splicing reaction, exploits the C-terminal cleavage reaction of the intein to generate a protein of interest with a new N terminus (Figure 1 A) in a light-induced manner. In C-terminal cleavage the peptide bond at the C-terminal splicing junction is cleaved

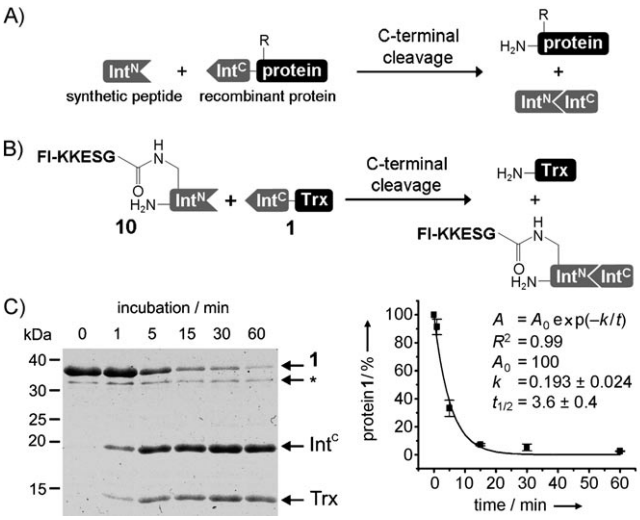

Figure 1. Intein-mediated C-terminal cleavage. A) Int^N peptides lacking the N-extein efficiently induce C-terminal cleavage. Various side chains R are tolerated at the N terminus of the cleaved protein. B) C-terminal cleavage using the intermediate analogue peptide **10**. C) Analysis of the cleavage reaction of **10** and **1** (100 and 20 μM, respectively) on a coomassie-stained SDS-PAGE gel. Fl = 5(6)-carboxyfluorescein; Int^N = CISGDSLISLA; Trx = thioredoxin; * = protein impurity band; A_0 = initial concentration; k = rate constant.

without previous joining of the flanking sequences and therefore results in liberation of the C-terminally fused polypeptide. We hypothesized this to be a potentially powerful approach because the N terminus is a crucial determinant for many proteins and polypeptides.

Our intein of choice was the *Ssp* DnaB intein split into a short synthetic Int^N (11 amino acids (aa)) and a longer Int^C fragment (143 aa), because the Int^N piece is readily accessible by solid-phase peptide synthesis (SPPS).[4] Incubation of a recombinant model protein Int^C–thioredoxin (Int^C-Trx; **1**) with fluorescein-labeled peptide **2** containing the Int^N frag-

[*] J. Binschik,[+] Dr. J. Zettler, Prof. Dr. H. D. Mootz[+]
Fakultät Chemie—Chemische Biologie, TU Dortmund
Otto-Hahn-Strasse 6, 44227 Dortmund (Germany)
E-mail: henning.mootz@uni-muenster.de
Dr. J. Zettler
Institute of Clinical Neuroimmunology
University Hospital Grosshadern
Ludwig Maximilians University, Munich (Germany)
and
Department of Neuroimmunology
Max-Planck-Institute of Neurobiology, Martinsried (Germany)
[+] Present address: Institute of Biochemistry
University of Muenster (Germany)

[**] We thank Markus Kaiser for discussions and Paul Bock for kindly providing SC protein and plasmid. We are grateful to Dirk Schwarzer for experimental support. Financial support was provided by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201007078.

Table 1: $Int^{N(1-11)}$ peptides used in this study.

Peptide[a]	Rate of C-terminal cleavage [$M^{-1}s^{-1}$][b]
2 Fl-KKESG-*CISGDSLISLA*-OH	0.8 ± 0.1[c]
3 Fl-KKESG-*AISGDSLISLA*-OH	0.2 ± 0.1
4 H-*CISGDSLISLA*-SKK(Fl)A-OH	11.8 ± 1.5
5 H-*C*(DMNB)*ISGDSLISLA*-SKK(Fl)A-OH	3.3 ± 0.3
6 DMNB-*CISGDSLISLA*-SKK(Fl)A-OH	0.4 ± 0.1
7 H-*KISGDSLISLA*-SKK(Fl)A-OH	3.7 ± 0.3
8 H-*LISGDSLISLA*-SKK(Fl)A-OH	3.6 ± 0.3
9 Nvoc-*LISGDSLISLA*-SKK(Fl)A-OH	0.3 ± 0.1
10 H-*Dap*(Fl-KKESG)*ISGDSLISLA*-OH	31.3 ± 1.3
11 Nvoc-*Dap*(Fl-KKESG)*ISGDSLISLA*-OH	none detected (< 0.01)

[a] Int^N in italics. [b] Determined with protein **1**. [c] For splicing. Fl: 5(6)-carboxyfluorescein.

ment (Table 1) results in efficient protein *trans* splicing ($k_{splicing} = (0.8 \pm 0.1)\,M^{-1}s^{-1}$).[4b]

To selectively induce only C-terminal cleavage, the substitution of the catalytically essential Cys1 to Ala (peptide **3**) was known to be sufficient;[4b] however, this reaction proved to be very slow ($k_{cleavage} = (0.2 \pm 0.1)\,M^{-1}s^{-1}$; see Figure S2 in the Supporting Information). In search for a better solution, we realized that the *trans*-cleavage reaction with an Int^N peptide lacking any N-extein sequence is significantly faster (peptide **4**; Figure S3 in the Supporting Information; Table 1; $k_{cleavage} = (11.8 \pm 1.5)\,M^{-1}s^{-1}$), in agreement with a recent report by Liu and co-workers.[5] For rendering this reaction photoactivatable we then sought to attach a photolabile group at the sequence of **4**, such that cleavage activity is blocked before irradiation by light through steric hindrance of the intein fragments. This task turned out to be more complicated than expected.

We first protected the thiol side chain and the α-amino group of the Cys1 in peptide **4** with the 4,5-dimethoxy-2-nitrobenzyl (DMNB) group to give peptides **5** and **6**, respectively. However, these still showed considerable C-terminal cleavage when incubated with protein **1** (see Figures S4 and S5 in the Supporting Information; Table 1). Obviously, the steric demand of a single DMNB group at either the α-amino or the thiol side chain moiety of the Cys1 did not sufficiently distort the interaction between the Int^N and Int^C pieces. To further increase the steric demand, we substituted the Cys1 residue by an amino acid with a bulky side chain and changed the DMNB moiety at the α-amino group to a 4,5-dimethoxy-2-nitrobenzyl carbamate group (Nvoc; peptides **7**–**9**; Table 1). However, the level of suppression in the case of the protected peptide **9** was not improved over the previous attempts (see Figures S6–S8 in the Supporting Information), which indicated that these straightforward approaches were inappropriate to achieve a significant switch in activity.

After contemplating the mechanism of protein splicing, we resorted to peptides **10** and **11** containing the cysteine isoster diamino propionic acid (Dap) at position 1 (Figure 1B and Table 1). These peptides represent stable analogues of the thioester intermediate in the first step of the protein splicing reaction, as they contain the N-extein sequence Fl-KKESG in an isopeptide bond on the Dap side chain. Interestingly, the unprotected peptide **10** induced C-terminal cleavage of **1** at an even increased rate. Under pseudo-first-order conditions using a fivefold excess of peptide **10** a $t_{1/2}$ value of 3.6 min was determined, which represents one of the fastest reactions measured for inteins so far (Table 1, Figure 1C, and Figure S9 in the Supporting Information). These findings may be explained by folding of the intein's active site into a conformation that closely resembles the 3D arrangement adopted for transesterification and/or asparagine cyclization and will require further investigation.

More importantly for this study, when using the Nvoc-protected peptide **11** shown in Figure 2 in a 1:1 ratio with protein **1** (20 μM each) no cleavage activity could be detected

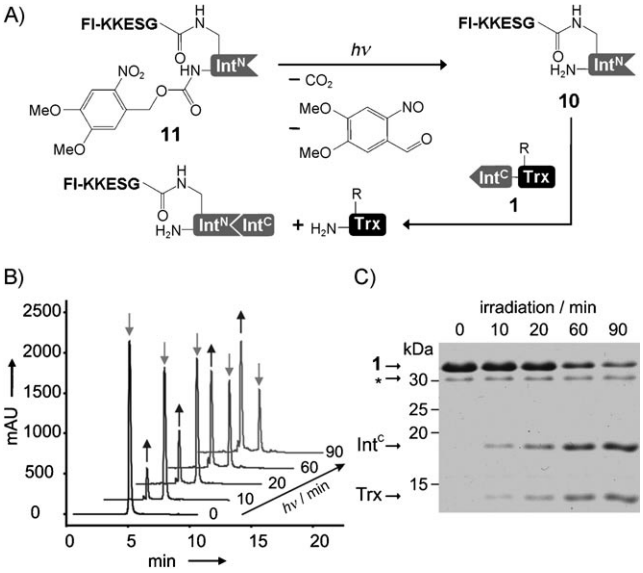

Figure 2. Light-triggered C-terminal cleavage. A) Scheme of the reaction. B) HPLC analysis of the peptide deprotection reaction upon irradiation at 366 nm. The newly formed peak corresponds to the deprotected peptide. C) Analysis of reactions of protein **1** with peptide **11** (20 μM each) that were irradiated for the indicated time periods and then incubated for 24 h. Shown is a coomassie-stained SDS-PAGE gel. * = protein impurity band.

(Figure 2C, left lane), whereas with a fivefold excess of peptide **11** only trace amounts of cleavage product just above the detection limit were observed after 24 h (see Figure S10 in the Supporting Information). Irradiation with light removed the Nvoc group and restored cleavage activity (Figure 2B and C). Thus, peptide **11** can be used to efficiently control C-terminal cleavage in Int^C–Xaa–protein constructs (Xaa = any amino acid). In agreement with previous studies,[5] we found a broad tolerance towards the nature of the first amino acid C-terminal to the intein (the +1 position), that is, Ser, Ala, Arg, Asp, Pro (Figure S11 in the Supporting Information, and data not shown).

We then aimed to use our light-triggered cleavage system to regulate the activity of an ambitious protein target through liberation of a new N terminus that is important for function. Prothrombin is a member of the blood-clotting cascade, in

which this inactive zymogen is proteolytically activated by the upstream protease factor Xa. An alternative pathway for prothrombin activation is mediated by the protein staphylocoagulase (SC) through an allosteric mechanism. SC is secreted by *Staphylococcus aureus* as a part of this organism's pathogenic program. It binds human prothrombin through multiple contacts. A crucial part of this interaction is the N-terminal sequence (IVTKDY) of SC, which inserts into the activation pocket of prothrombin and thereby induces a rearrangement of the catalytic site to the active conformation. Previous studies by Friedrich et al. showed the importance of this native N terminus, as slight modifications, including the presence of an N-terminal methionine as a single extra amino acid, dramatically reduced the efficiency of the allosteric mechanism.[6]

As illustrated in Figure 3A, we thus wondered whether prothrombin could be artificially regulated by releasing an active SC from an inactive Int^C–SC fusion protein, in which the α-amino group is "masked". His_6–Int^C–SC was expressed in *Escherichia coli* and purified by Ni-NTA chromatography (construct **12**). We first verified that the intein was still active in this sequence context (Figure 3B). Using a photometric assay for thrombin, we also showed that the ability of **12** to allosterically induce prothrombin activity was reduced to 0.6% relative to the unmodified SC control protein (Figure 3C, columns 1 and 3). Importantly, preincubation of the masked construct **12** with unprotected Int^N peptide **10** restored prothrombin stimulation back to 36.3%, which indicates that the SC released by intein cleavage is able to bind and activate the zymogen (Figure 3C, column 5). We then integrated the light trigger into the system by using the Nvoc-protected peptide **11**. Addition of this peptide in the dark resulted in 1.3% activity, whereas irradiation for 20 min restored 26.2% activity (Figure 3, columns 7 and 8) relative to controls using the unmodified SC. Photoactivation of the intein thus resulted in a 20-fold increase of activity by this coupled mechanism.

Furthermore, we carried out the assay as a one-pot experiment with no prior incubation to allow C-terminal cleavage to occur. Following irradiation with light for 60 min, we observed a 13-fold increase in activity. This result is particularly noteworthy, because under these conditions the polypeptides containing the Int^N and Int^C fragments were present at only 500 and 100 nM concentrations, respectively, which is below the K_D value of (1.1 ± 0.2) μM (K_D = dissociation constant).[4b]

To show the potential of our approach for protein functional control in complex biological mixtures, we finally aimed to induce prothrombin in human blood plasma. Plasma contains prothrombin at approximately 1–2 μM[7] concentration and had a total protein concentration of 62 mg mL^{-1} as determined by the Bradford assay. Thrombin activity results in the coagulation of the plasma, which can be easily observed in the test tube by its transition from liquid to gel-like. We mixed all intein components with undiluted plasma (final total protein concentration 40 mg mL^{-1}) and then irradiated the samples with light. The observed time periods required for coagulation reflected our results obtained with the purified proteins (Figure 4). These findings suggest that the prothrombin in the plasma could be regulated by the same artificial mechanism.

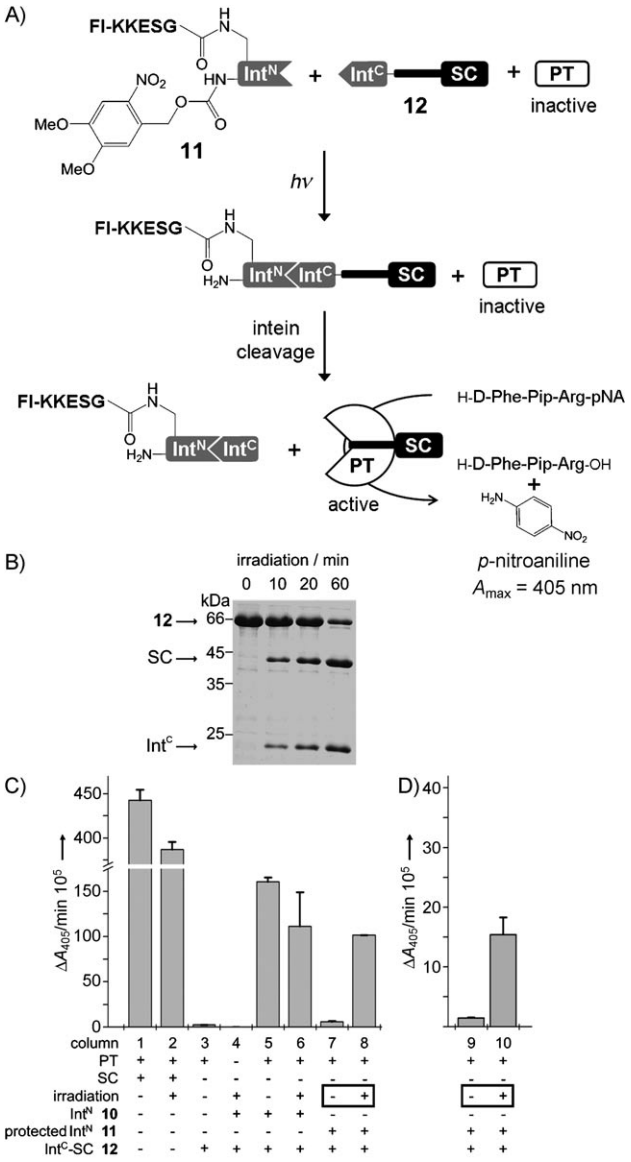

Figure 3. Coupled allosteric activation of prothrombin (PT). A) Irradiation induces intein-mediated liberation of active staphylocoagulase SC(1–325) by C-terminal cleavage. SC(1–325) binds and allosterically activates prothrombin, the activity of which is monitored photometrically. B) Analysis of the light-induced liberation of SC(1–325). Peptide **11** and protein **12**, each at 20 μM, were irradiated as indicated and incubated in the dark for 24 h. Shown is a coomassie-stained SDS-PAGE gel. C) Rates of *p*-nitroaniline formation. Cleavage assays of peptides **10**/**11** and protein **12** were irradiated for 20 min as indicated, subsequently preincubated for 2.5 h, and then diluted and mixed with prothrombin and its substrate for photometric measurements. D) One-pot experiment with all components (see text for lower concentrations of the intein fragments). Irradiation was carried out for 60 min.

In conclusion, we have developed and optimized a new light-triggered split intein for protein *trans*-cleavage reactions. For the first time, we show that such an intein can be used to regulate the activity of another protein in a light-dependent fashion. It should be underlined that in the

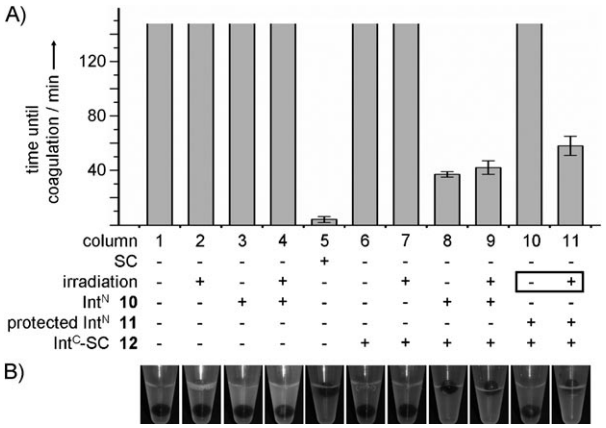

Figure 4. Light-induced coagulation of blood plasma. A) The intein components were mixed with undiluted human blood plasma as indicated and the time period measured until coagulation occurred. In experiments shown in columns 1–4, 6, 7, and 10 no coagulation was observed up to 180 min, which was the latest time point taken. B) A glass bead added to the sample after coagulation confirmed the gel-like texture of the clotted plasma.

coupled assay based on the C-terminal cleavage reaction we were able to manipulate the native prothrombin zymogen. Moreover, the protein that was directly controlled by the intein, in this case the SC, was fully genetically encoded. This is in contrast to other reports that required installation of the photoresponsive group on the protein of interest by chemical means, and is therefore expected to facilitate the extension of the described approach to other proteins. We demonstrated that the light-triggered intein can be applied in complex mixtures, and envision its utilization even inside living cells.

Although for each artificially controlled protein unique design aspects will come into play, we believe that the presented approach, in essence the unmasking of a polypeptide sequence in a proteolytic fashion, will be applicable in a variety of ways. First of all, a number of protein–protein interactions and enzymatic activities require a specific amino terminus. For example, the N-terminal tetrapeptide sequence of Smac/DIABLO is crucial for binding and inhibiting the apoptosis inhibitor XIAP in the regulation of caspase 9,[8a] and the phospholipase A2 requires a free amino terminus to be catalytically active.[8b] Furthermore, other and more general variations are conceivable for how the photoinduced cleavage reaction could be exploited, for example by cleaving off a localization tag or an autoinhibitory peptide,[8c] or by unmasking a new N terminus to subject the cleaved protein to proteasomal degradation according to the N-end rule.[8d]

Received: November 10, 2010
Revised: December 16, 2010
Published online: March 7, 2011

Keywords: allosterism · biological activity · cleavage reactions · photochemistry · protein modifications

[1] a) H. M. Lee, D. R. Larson, D. S. Lawrence, *ACS Chem. Biol.* **2009**, *4*, 409; b) E. A. Lemke, *ChemBioChem* **2010**, *11*, 1825; c) G. Mayer, A. Heckel, *Angew. Chem.* **2006**, *118*, 5020; *Angew. Chem. Int. Ed.* **2006**, *45*, 4900; d) A. Levskaya, O. D. Weiner, W. A. Lim, C. A. Voigt, *Nature* **2009**, *461*, 997; e) S. Shimizu-Sato, E. Huq, J. M. Tepperman, P. H. Quail, *Nat. Biotechnol.* **2002**, *20*, 1041; f) Y. I. Wu, D. Frey, O. I. Lungu, A. Jaehrig, I. Schlichting, B. Kuhlman, K. M. Hahn, *Nature* **2009**, *461*, 104.

[2] a) H. D. Mootz, *ChemBioChem* **2009**, *10*, 2579; b) V. Muralidharan, T. W. Muir, *Nat. Methods* **2006**, *3*, 429; c) D. W. Wood, W. Wu, G. Belfort, V. Derbyshire, M. Belfort, *Nat. Biotechnol.* **1999**, *17*, 889; d) S. N. Cook, W. E. Jack, X. Xiong, C. J. Noren, L. E. Danley, J. A. Ellmann, P. G. Schultz, *Angew. Chem.* **1995**, *107*, 1736; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1629.

[3] a) A. B. Tyszkiewicz, T. W. Muir, *Nat. Methods* **2008**, *5*, 303; b) M. Vila-Perelló, Y. Hori, M. Ribo, T. W. Muir, *Angew. Chem.* **2008**, *120*, 7878; *Angew. Chem. Int. Ed.* **2008**, *47*, 7764; c) L. Berrade, Y. Kwon, J. A. Camarero, *ChemBioChem* **2010**, *11*, 1368.

[4] a) C. Ludwig, M. Pfeiff, U. Linne, H. D. Mootz, *Angew. Chem.* **2006**, *118*, 5343; *Angew. Chem. Int. Ed.* **2006**, *45*, 5218; b) C. Ludwig, D. Schwarzer, H. D. Mootz, *J. Biol. Chem.* **2008**, *283*, 25264.

[5] G. Volkmann, W. Sun, X. Q. Liu, *Protein Sci.* **2009**, *18*, 2393.

[6] R. Friedrich, P. Panizzi, P. Fuentes-Prior, K. Richter, I. Verhamme, P. J. Anderson, S. Kawabata, R. Huber, W. Bode, P. E. Bock, *Nature* **2003**, *425*, 535.

[7] A. M. Smiles, E. M. Macy, P. A. Sakkinen, E. G. Bovill, K. G. Mann, R. P. Tracy, *Blood Coagulation Fibrinolysis* **1998**, *9*, 525.

[8] a) S. M. Srinivasula, R. Hegde, A. Saleh, P. Datta, E. Shiozaki, J. Chai, R. A. Lee, P. D. Robbins, T. Fernandes-Alnemri, Y. Shi, E. S. Alnemri, *Nature* **2001**, *410*, 112; b) B. W. Dijkstra, J. Drenth, K. H. Kalk, *Nature* **1981**, *289*, 604; c) H. D. Mootz, E. S. Blum, T. W. Muir, *Angew. Chem.* **2004**, *116*, 5301; *Angew. Chem. Int. Ed.* **2004**, *43*, 5189; d) A. Varshavsky, *J. Biol. Chem.* **2008**, *283*, 34469.

