J|A|C|S COMMUNICATIONS

Published on Web 07/11/2002

Protein Splicing Triggered by a Small Molecule

Henning D. Mootz and Tom W. Muir*

Laboratory of Synthetic Protein Chemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10021

Received May 2, 2002

The use of small molecules that turn specific proteins on or off provides a level of temporal control that is difficult to achieve using standard genetic approaches. Consequently, the development of small molecule switches of protein function is a very active area of chemical biology, sometimes referred to as chemical genetics.[1] Most studies in this area rely on the identification of small molecules that bind directly to the active site of a target protein, thereby acting as agonists or antagonists of function. Strategies have also been described in which the small molecule triggers a change in the secondary, tertiary, or ternary structure of the protein, in so doing changing the functional state of the molecule.[2] Another approach to this problem would be to alter the primary structure of a target protein in response to a small-molecule trigger; a dramatic change in primary sequence would be directly coupled to function. In principle, this can be achieved by harnessing protein splicing, a posttranslational editing process that results in the precise removal of an internal domain (termed an intein) from two flanking sequences termed the N- and C-exteins.[3] In this communication we introduce a technique that allows protein splicing to occur only in the presence of the small molecule, rapamycin. This approach is expected to be independent of the nature of the two exteins and so should provide a general vehicle for controlling protein function using small molecules.

The principle of our conditional protein splicing system is outlined in Figure 1A. In essence, the approach integrates two established technologies; protein trans-splicing[4−7] and small-molecule-induced protein heterodimerization.[8] Trans-splicing refers to the observation that protein splicing can be triggered by reconstituting inactive N- and C-terminal halves of an intein. Both artificial[4−6] and natural[7] split intein systems are available, and these have facilitated a variety of protein engineering[6] and two-hybrid[9] strategies. The FKBP12/FRB heterodimerization system[1,8] might provide a mechanism by which to control protein trans-splicing through the addition of the bidentate ligand, rapamycin, which binds to the FKBP and FRB protein domains simultaneously. Accordingly, fusing each half of a split intein to either FKBP or FRB will allow the two intein fragments to be brought together in response to the dimerizer molecule. Provided the juxtaposition of the intein fragments in the resulting heterodimer is compatible with functional complementation, this should lead to the splicing together of the flanking extein sequences (Figure 1A). This strategy requires that the individual intein fragments have a low affinity for one another, a criterion that should be met by using an artificially split *Saccharomyces cerevisiae* VMA intein.[10]

A series of model proteins were designed to test the conditional protein splicing system. Each was composed of one-half of a split VMA intein (VMA^N or VMA^C),[11] either FKBP or FRB and, as model N- and C-exteins, either maltose binding protein (MBP) or

* To whom correspondence should be addressed. E-mail: muirt@rockvax.rockefeller.edu.

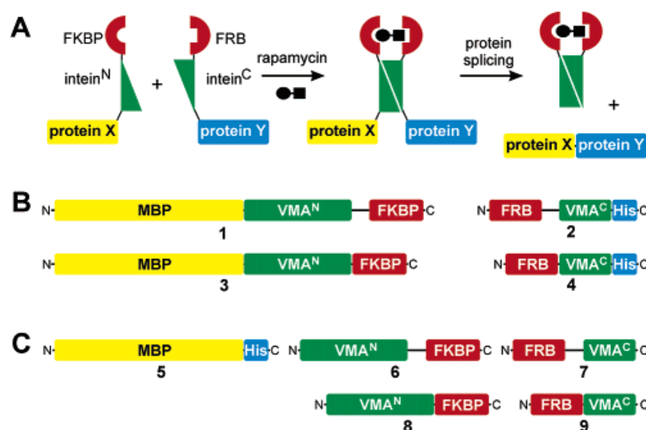

Figure 1. (A) Principle of the approach. (B) Constructs used in this study. (C) Products of the conditional trans-splicing reaction. Calculated molecular masses are 45.2 kDa (**5**), 34.2 kDa (**6**), 20.0 kDa (**7**), 33.3 kDa (**8**), and 19.1 kDa (**9**).

a polyhistidine-containing sequence (His), respectively. Included in one pair of constructs was a peptide-linker sequence between the intein fragment and the corresponding dimerization domain. This linker was inserted to explore the steric constraints associated with intein complementation. The domain architectures of the four fusion proteins, **1**−**4**, are shown in Figure 1B. Each of the fusion proteins was overexpressed in *Escherichia coli* and purified from the soluble fraction by a combination of affinity and ion-exchange chromatography.[12]

In initial proof-of-principle studies, the complementary fusion proteins, **1** and **2**, were combined under physiological conditions in the presence or absence of a slight molar excess of rapamycin.[13] In the absence of the small molecule, no reaction was observed after 2.5 h at 25 °C (Figure 2A, lane a). In contrast, addition of the dimerizer to the mixture resulted in depletion of the starting materials and the generation of three new components whose migration pattern on SDS-PAGE was consistent with the expected reaction products (Figure 1C), MBP−His (**5**), VMA^N−FKBP (**6**) and FRB−VMA^C (**7**) (Figure 2, lane b). Further analysis of the reaction mixture by MALDI-MS[14] and Western blotting using an anti-His-tag antibody (Figure 2B, lanes e and f) confirmed the identity of the products. Similar results were obtained using constructs **3** and **4** that lack the linker regions between the intein and dimerizer domains (Figure 2A, lanes c and d, and Figure 2B, lanes g and h). The observation that efficient intein complementation can occur in the absence of the peptide linkers is somewhat surprising given that the N- and C-termini of FKBP and FRB are ~35 Å apart in the complex.[15] The most likely explanation for this finding is that the first several residues in the VMA^C fragment, which lie out with the core intein domain,[16] are unstructured in the

10.1021/ja026769o CCC: $22.00 © 2002 American Chemical Society

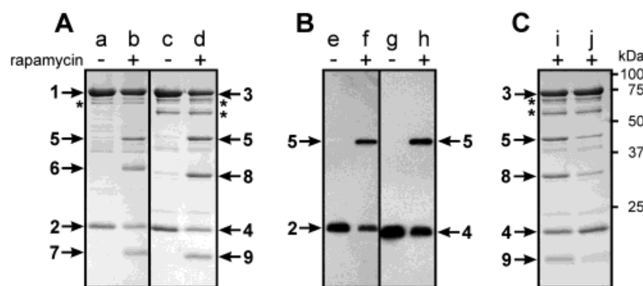

Figure 2. Analysis of reactions of **1** and **2** (lanes a,b and e, f) and **3** and **4** (lanes c, d and g, h) by Coomassie-stained SDS-PAGE (A), and Western blotting using an anti-His-tag antibody (B). Panel (C) shows a competition experiment with **3** and **4** in the absence (lane i) or presence (j) of a 10-fold excess of ascomycin. Arrows indicate the assignment of the bands. (* = contaminants)

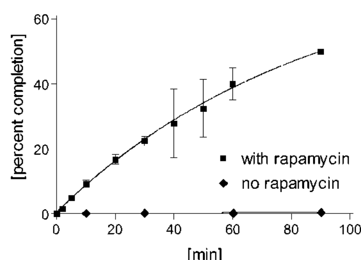

Figure 3. Time-course of the reaction of **3** and **4** with and without rapamycin. Formation of the product **5** was determined from Western blotting analysis using the program Scion Image.

trans-intein complex and are thus able to bridge the distance between the FKBP and FRB termini.

To confirm that rapamycin elicits its effect on splicing by inducing the heterodimerization of FKBP and FRB, the trans-splicing experiments were repeated in the presence of the FK506 analogue, ascomycin, which binds tightly to the same pocket in FKBP as rapamycin, but does not interact with FRB.[17] Inclusion of a 10-fold molar excess of ascomycin compared to rapamycin was found to reduce the level of protein splicing by about 3−4-fold using constructs **1** and **2** or **3** and **4** (Figure 2C, lanes i and j and data not shown). This result provides compelling evidence that the dimerizer is acting at the level of FKBP/FRB and not through some other mechanism.

Kinetic experiments were conducted to determine the rate of product formation in the presence or absence of dimerizer. Constructs **3** and **4** were combined with or without rapamycin, and the trans-splicing reaction was monitored over time by Western blotting. As shown in Figure 3, the level of product (**5**) formation in the absence of rapamycin was extremely small (estimated from Western blotting to be below 0.2% after 1.5 h). In contrast, the reaction went to ca. 50% completion in the presence of the dimerizer at the same time-point. Thus, inclusion of the dimerizer results in a ≥250-fold induction of splicing over background. Consistent with previous kinetic studies on protein trans-splicing,[18] the data for the rapamycin-induced reaction could be fit to a simple first-order function to give a k_{obs} of 1.9×10^{-4} s^{-1}. This rate constant is comparable to the reported protein trans-splicing reactions observed for the Ssp DnaE intein (k_{obs} of 6.6×10^{-5} s^{-1}),[18] where the ligation step is rate determining. Importantly, the half-life of the trans-splicing reaction in the presence of rapamycin was on the order of 60 min. This suggests that our conditional trans-splicing strategy will be a practical tool for triggering changes in protein structure and function on a biologically useful time-scale.

To our knowledge, this work represents the first time that protein splicing has been controlled by using a small molecule. This "conditional" protein splicing is made possible by fusing each half of an artificially split intein to the complementary components of a protein heterodimerization system. The current strategy employs a split VMA intein and the FKBP/FRB heterodimerization pair; however, other protein trans-splicing[4−6] and three-hybrid systems[19] could, in principle, be used. While the present study involves model exteins, there is every reason to expect, on the basis of the known promiscuity of inteins,[3] that the approach will be quite general. It is also important to note that our conditional protein splicing system results in both a ligaton step (i.e., between the two exteins) and two cleavage steps (i.e., between the exteins and the intein fragments). In principle, any one of these events could be used to modify or regulate the function of a protein of interest either in vitro or in vivo. Studies along these lines are currently underway in our laboratories.

Acknowledgment. We are grateful to M. Trester-Zedlitz for generous help with mass spectrometry measurements and R. Hofmann for helpful discussions. We thank Ariad Pharmaceuticals for providing DNA templates of the FKBP and FRB encoding genes. Support was provided by the National Institutes of Health (GM59908, T.W.M).

Supporting Information Available: Full experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Schreiber, S. L. *Bioorg. Med. Chem.* **1998**, *6*, 1127−1152.

(2) for example: Foster, B. A.; Coffey, H. A.; Morin, M. J.; Rastinejad, F. *Science* **1999**, *286*, 2507−2510.

(3) Giriat, I.; Muir, T. W.; Perler, F. B. *Genet. Eng.* **2001**, *23*, 171−199.

(4) Mills, K. V.; Lew, B. M.; Jiang, S.; Paulus, H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3543−3548.

(5) Southworth, M. W.; Adam, E.; Panne, D.; Byer, R.; Kautz, R.; Perler, F. B. *EMBO J.* **1998**, *17*, 918−926.

(6) Yamazaki, T.; Otomo, T.; Oda, N.; Kyogoku, Y.; Uegaki, K.; Ito, N.; Ishino, Y.; Nakamura, H. *J. Am. Chem. Soc.* **1998**, *120*, 5591−5592.

(7) Wu, H.; Hu, Z.; Liu, X. Q. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9226−9231.

(8) Ho, S. N.; Biggar, S. R.; Spencer, D. M.; Schreiber, S. L.; Crabtree, G. R. *Nature* **1996**, *382*, 822−826.

(9) Ozawa, T.; Takeuchi, T. M.; Kaihara, A.; Sato, M.; Umezawa, Y. *Anal. Chem.* **2001**, *73*, 5866−5874.

(10) Artificially split inteins typically require a refolding step before trans-splicing occurs (see ref 3−6).

(11) VMA^N corresponded to residues 1−184 (VMA intein numbering) and VMA^C comprised residues 390−454. The central endonuclease domain, which is not necessary for intein catalysis, has been removed (see ref 9).

(12) Proteins were expressed in *E. coli*. **1** and **3** were affinity purified using an amylose column. **2** and **4** were purified using Ni^{2+}−NTA affinity chromatography. All proteins were further purified by anion-exchange chromatography with 2 mM DTT present.

(13) The reactions were carried out at 25 °C in 50 mM Tris/HCl pH 7.0, 300 mM NaCl, 1 mM EDTA, 2 mM DTT. Protein concentrations were 2.5 μM in each, rapamycin was added to a concentration of 10 μM.

(14) MALDI-TOF MS analysis of the crude reaction mixtures: **5**, obs = 45175, calc = 45186; **6**, obs = 34231, calc = 34234; **7**, obs = 20001, calc = 20005, for the reaction of **1** and **2**; **5**, obs = 45186, calc = 45186; **8**, obs = 33343, calc = 33349; **9**, obs = 19106, calc = 19120, for the reaction of **3** and **4**.

(15) Choi, J.; Chen, J.; Schreiber, S. L.; Clardy, J. *Science* **1996**, *273*, 239−242.

(16) Duan, X.; Gimble, F. S.; Quiocho, F. A. *Cell* **1997**, *89*, 555−564.

(17) Kawai, M.; Lane, B. C.; Hsieh, G. C.; Mollison, K. W.; Carter, G. W.; Luly, J. R. *FEBS Lett.* **1993**, *316*, 107−113.

(18) Martin, D. D.; Xu, M. Q.; Evans, T. C., Jr. *Biochemistry* **2001**, *40*, 1393−1402.

(19) Licitra, E. J.; Liu, J. O. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12817−12821.

JA026769O