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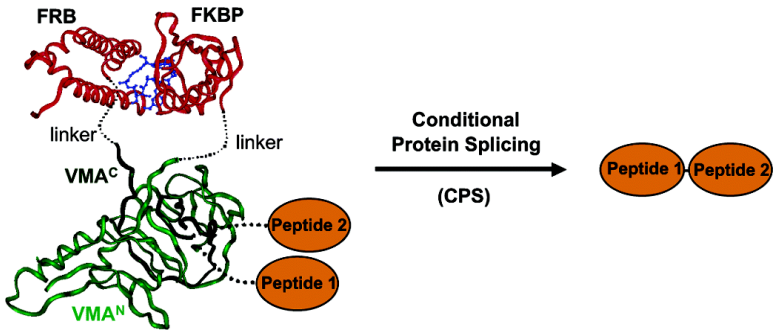
J. Am. Chem. Soc., **2003**, 125 (35), 10561-10569• DOI: 10.1021/ja0362813 • Publication Date (Web): 08 August 2003



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Conditional Protein Splicing: A New Tool to Control Protein Structure and Function in Vitro and in Vivo

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Abstract: Protein splicing is a naturally occurring process in which an intervening intein domain excises itself out of a precursor polypeptide in an autocatalytic fashion with concomitant linkage of the two flanking extein sequences by a native peptide bond. We have recently reported an engineered split VMA intein whose splicing activity *in trans* between two polypeptides can be triggered by the small molecule rapamycin. In this report, we show that this conditional protein splicing (CPS) system can be used in mammalian cells. Two model constructs harboring maltose-binding protein (MBP) and a His-tag as exteins were expressed from a constitutive promoter after transient transfection. The splicing product MBP−His was detected by Western blotting and immunoprecipitation in cells treated with rapamycin or a nontoxic analogue thereof. No background splicing in the absence of the small-molecule inducer was observed over a 24-h time course. Product formation could be detected within 10 min of addition of rapamycin, indicating the advantage of the posttranslational nature of CPS for quick responses. The level of protein splicing was dose dependent and could be competitively attenuated with the small molecule ascomycin. In related studies, the geometric flexibility of the CPS components was investigated with a series of purified proteins. The FKBP and FRB domains, which are dimerized by rapamycin and thereby induce the reconstitution of the split intein, were fused to the extein sequences of the split intein halves. CPS was still triggered by rapamycin when FKBP and FRB occupied one or both of the extein positions. This finding suggests yet further applications of CPS in the area of proteomics. In summary, CPS holds great promise to become a powerful new tool to control protein structure and function in vitro and in living cells.

Introduction

Protein function is directly correlated to the amino acid sequence and chemical structure of the polypeptide chain. Posttranslational modification or processing of the primary structure of a protein bypass transcription and translation and can therefore result in an immediate change of the properties of the protein. These strategies are widely used in nature when quick responses to a changing environment are necessary. Examples are the regulation of protein activity by phosphorylation in signal transduction cascades and the activation of zymogens by specific proteases. Consequently, to fully understand many biological processes, research tools are required that act on a similar time scale. By analogy with natural regulatory mechanisms, this temporal resolution can only be achieved on the posttranslational level, by exerting control directly on the gene products. Approaches based on genetic methods, such as gene knockouts or conditional expression systems, are hampered by their slow response time and by compensating effects that can occur while the phenotype is built up.[1] The most common way to directly and quickly perturb the function of a target protein is by employing specific small-molecule agonists or antagonists. Often referred to as Chemical Genetics,[2] this pharmacology-based approach usually requires significant efforts in chemical synthesis of structurally diverse libraries of small-molecule candidates, which in turn requires the technical infrastructure to screen these libraries against target proteins.[3,4] In an alternative strategy, structure−activity information on a target system is used to design specific mutations in the gene of interest, which then allows "allele-specific" inhibitors or activators of the mutant version of the protein to be prepared; this approach is often referred to as "bump-and-hole" strategy since the small molecule often binds in the engineered vacant space in the protein scaffold.[5−11] Due to the high-affinity binding of such small-molecule effectors, these approaches in some

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respects can be regarded as the equivalent of covalent post-translational modifications.

We have recently developed a new technique, termed conditional protein splicing (CPS),[12] to posttranslationally change the primary structure of a protein by using an engineered protein trans-splicing element that is dependent on the presence of a small molecule.[13] Protein splicing is an autocatalytic process in which an intervening sequence, termed the intein, excises itself out of a precursor protein with concomitant linking of the flanking sequences, termed the exteins, by a native peptide bond.[14] In protein trans-splicing the intein domain is split into two pieces and the splicing occurs only when the two polypeptides are reconstituted. The DnaE intein from *Synechocystis* sp. is the only naturally occurring split intein thus far characterized.[15] In the CPS approach,[13] which is outlined in Figure 1, the cis-splicing VMA intein from *Saccharomyces cerevisiae* was artificially split into N- and C-terminal halves, VMA^N and VMA^C, that have little or no affinity for each other. These pieces were fused to a pair of heterodimerization domains, FKBP and FRB, which form a tight ternary complex in the presence of the small molecule, rapamycin.[16] Upon addition of this dimerizer molecule, the intein was reconstituted, and protein trans-splicing was triggered. The newly ligated exteins thus constitute a new polypeptide with potentially new function. Due to the promiscuity of inteins in terms of their extein sequences,[14,17] CPS should be generally applicable. Moreover, the favorable reaction kinetics, on the order of minutes, suggests CPS to be a powerful tool to study biological processes with high temporal resolution.

In this work, we have explored the potential of CPS for applications in living cells. We also further investigated the topologic tolerance of the system toward different arrangements of the dimerization domains relative to the intein parts, which can be useful for various application of CPS.

Results and Discussion

General Considerations. It was not clear at the outset whether CPS would work in the complex chemical environment within a cell. Rapamycin-induced heterodimerization of FKBP and FRB is well established in mammalian cell culture and even whole organisms such as mice.[18,19] Protein trans-splicing using the naturally occurring split DnaE intein has been shown to occur in *Escherichia coli*, mammalian and plant cells.[15,20−27] Moreover, the artificially split DnaB and RecA inteins have been reported to trans-splice when coexpressed in *E. coli*,[28,29] although they required a refolding step when purified separately and reconstituted in vitro. Thus, we were concerned that our constructs, when coexpressed in cells, might exhibit background splicing activity in the absence of the inducer rapamycin. Previous work by Ozawa et al. on comparable constructs of the artificially split VMA intein fused to calmodulin and its M13 recognition peptide suggested extremely low splicing activity in *E. coli*.[21−23] Because of the artificial nature of the split VMA intein, protein stability was another issue to be examined in this study.

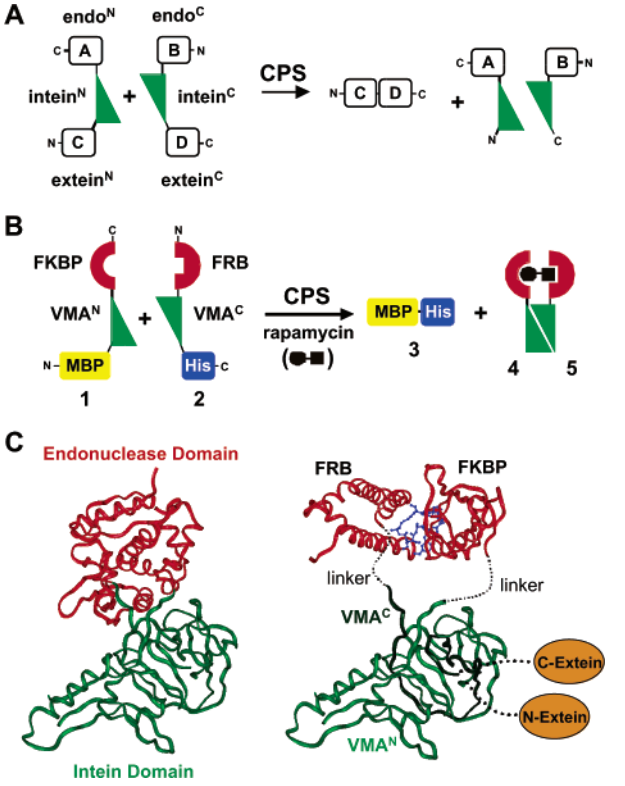

Figure 1. Principle of conditional protein splicing (CPS). (A) General nomenclature for the constructs used. The extein sequences are located at the C and D positions, whereas the positions A and B on the opposite ends of the split intein halves are termed the endo sequences. The CPS reaction between the two complementary intein halves can be promoted by interactions of different sequences at the positions A−D and results in formation of a native peptide bond between the exteins C and D. (B) In the original CPS approach, the active intein is reconstituted by rapamycin, which heterodimerizes the FKBP and FRB domains located at the endo positions A and B. When using the model exteins MBP and His-tag, protein splicing results in formation of MBP−His. Calculated molecular masses are: 77.5 kDa (**1**), 21.2 kDa (**2**), 45.2 kDa (**3**), 34.2 kDa (**4**), and 19.1 kDa (**5**). (C) Crystal structure of the wild-type VMA protein (ref 34) which is composed of the catalytic intein domain (green) and the intervening endonuclease domains (red). The modularity between the two domains should be noted (left). Model showing the arrangement of the VMA intein domain and the FKBP−rapamycin−FRB complex (ref 16), indicating fusion sites as employed in the constructs **1** and **2** illustrated above (right). Rapamycin is shown in ball-and-stick representation (blue).

(12) The abbreviations used in this paper are: CPS, conditional protein splicing; FKBP, FK506 binding protein; FRB, FKBP−rapamycin-binding; MALDI, matrix-assisted laser desorption ionization; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; VMA, vacuolar ATPase subunit. Standard single and triple letter amino acid codes were used.

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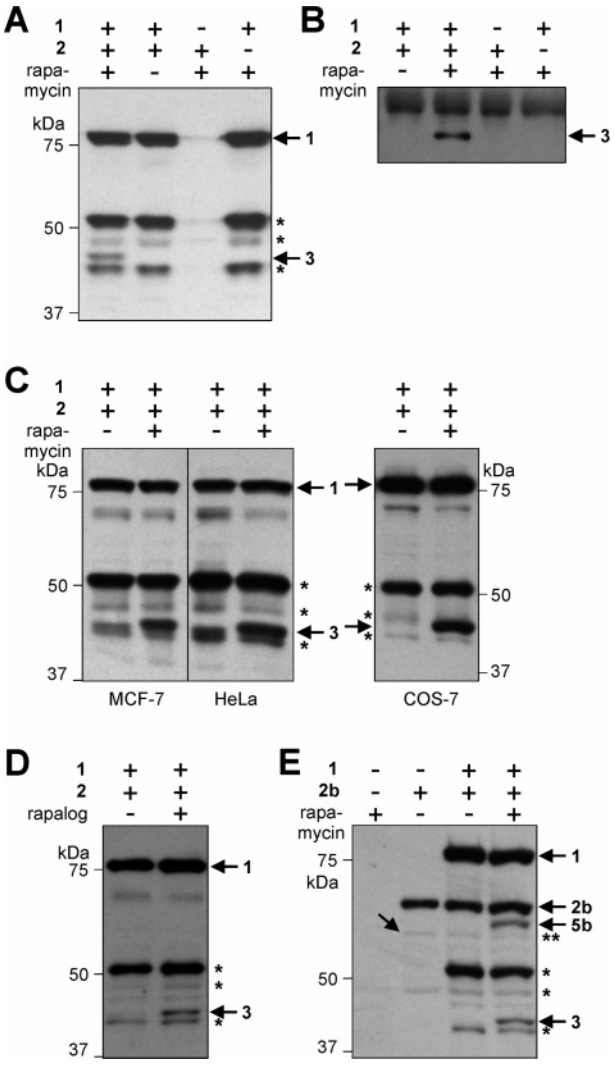

Figure 2. CPS in mammalian cells transiently transfected with plasmids encoding constructs **1** and **2** or **2b** under the CMV promoter. Cells were incubated for 24 h after transfection and then for further 2 h with or without the dimerizer molecule. Asterisks indicate degradation products of **1**. (A) Anti-MBP Western blot of HEK293 cell extracts. (B) Anti-His-tag Western blot of anti-MBP immunoprecipitate from HEK293 cell extracts. (C) Anti-MBP Western blot of MCF-7, HeLa, and COS-7 cell extracts. (D) Induction of CPS using the nontoxic analogue of rapamycin, AP21967 (rapalog). Anti-MBP Western blot of HEK293 cells extracts. (E) Anti-MBP Western blot of HEK293 cell extracts. Double-asterisk and arrow indicate degradation product of **2b**.

Establishing CPS inside Mammalian Cells. For expression in mammalian cells, the gene fragments encoding the model constructs **1** and **2** (Figure 1B) were cloned into the vector pcDNA3 under the control of the strong and constitutive CMV promoter. HEK293 cells were then transiently transfected with each of the plasmids. After allowing expression for 24 h at 37 °C, cells were washed, and cell lysates were prepared using a passive lysis buffer, separated on SDS-PAGE, and examined by Western blotting. Using an anti-MBP antibody to detect expression of construct **1**, a protein band of the right molecular mass (calc 77.5 kDa) was identified (Figure 2A, fourth lane). Three additional proteins, all of lower molecular weight, were also recognized by the anti-MBP antibody. These presumably corresponded to degradation products of **1** (see below). Detection of construct **2** using an anti-His-tag antibody failed. We reasoned that this finding was due to the lower sensitivity of this latter antibody and proceeded to the actual splicing experiments. To this end, cells were transfected with both plasmids and incubated for 24 h as described above, followed by addition of rapamycin at a concentration of 1 μM to the growth medium. After an additional incubation for 2 h, cells were washed, lysed, and examined by Western blotting for the formation of the splicing product MBP−His **3**, which should be recognized by both the anti-MBP and the anti-His antibodies. A new protein was detected with the anti-MBP antibody, and importantly this was not present in the control experiment without rapamycin (Figure 2A). This protein comigrated with a standard of **3** (not shown), prepared from purified proteins, at the calculated mass of 45.2 kDa. Further controls, in which one or both of the plasmids were omitted in the transfection, showed that the expression of both **1** and **2** was required for the formation of the product (Figure 2A). However, as no signals were obtained once more by Western blotting with the anti-His antibody, the presence of the His-tag and thus the identity as the predicted splicing product could not be verified unambiguously. We therefore enriched the protein by immunoprecipitation of the cell lysate with the anti-MBP antibody. Subsequent blotting against the His-tag antibody showed that the new protein indeed carried the His-tag, which was thus confirmed as the correct splicing product. By contrast, no product could be detected in the samples without rapamycin or without one of the two plasmids, suggesting that no significant background splicing occurred (see Figure 2B).

We then performed several control experiments to ensure that protein splicing indeed took place in vivo and not in vitro after cell lysis. First, we switched to denaturating conditions for cell lysis by directly using SDS-PAGE loading buffer (containing 4% SDS and 200 mM β-mercaptoethanol). This buffer was used previously to stop the rapamycin-triggered protein splicing reaction of purified constructs **1** and **2** in vitro.[13] Under these conditions, the same amount of product **3** could still be detected by Western blotting against the anti-MBP antibody (data not shown). Furthermore, two aliquots of HEK293 cells were transfected with each of the two plasmids individually, treated with rapamycin, washed, and combined immediately after adding the lysis buffer. In this case, the splicing product **3** could not be detected by Western blotting with the anti-MBP antibody, suggesting that protein splicing did not occur under the conditions of cell lysis (data not shown). Both these findings prove that protein splicing indeed must have occurred in vivo before cell lysis. We continued to lyse the cells under denaturing conditions in all the following experiments.

To investigate the generality of CPS in terms of amenable cell lines, a few widely used mammalian cell lines were tested. HeLa, MCF-7, and COS-7 cells were transfected with plasmids encoding **1** and **2** and exposed to rapamycin as described above. As shown in Figure 2C, the splicing product **3** was formed in all cases. Degradation products of **1** were found to be comparable to those observed in HEK293 cells.

For some applications, the use of rapamycin may be precluded by its cell cycle inhibitory effects. These result from the inhibition of the FRAP kinase through binding of the FKBP−rapamycin complex in a region on the enzyme from which the FRB domain of the heterodimerization system was derived. However, the FRB domain used here contains a single amino acid change (T2098L according to FRAP numbering), which allows the use of a structural analogue of rapamycin, AP21967,

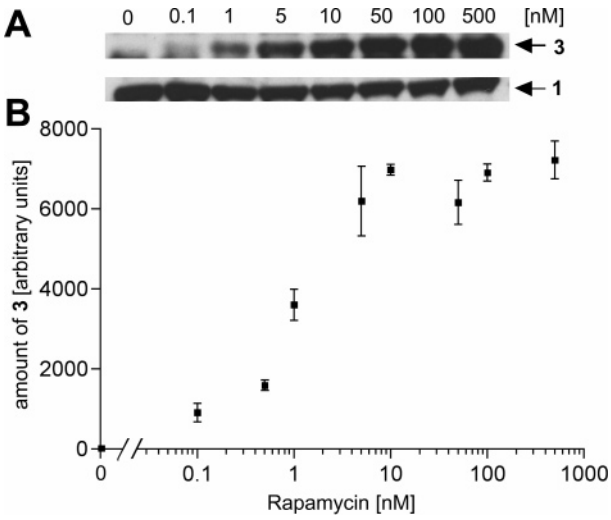

Figure 3. CPS is dependent on the concentration of rapamycin. The dimerizer drug was added at various concentrations to HEK293 cells coexpressing constructs **1** and **2**. After exposure for 2 h, cell lysates were prepared, and the amount of the splicing product MBP−His (**3**) was determined from immunoblotting using an anti-MBP antibody. (A) Representative immunoblotting data showing the product **3** and starting material **1** for different concentrations of rapamycin. (B) Dose-response curve calculated by quantifying the gel bands in (A) using a densitometer. Each data point was normalized to the amount of starting material **1** and represents the average of three independent experiments.

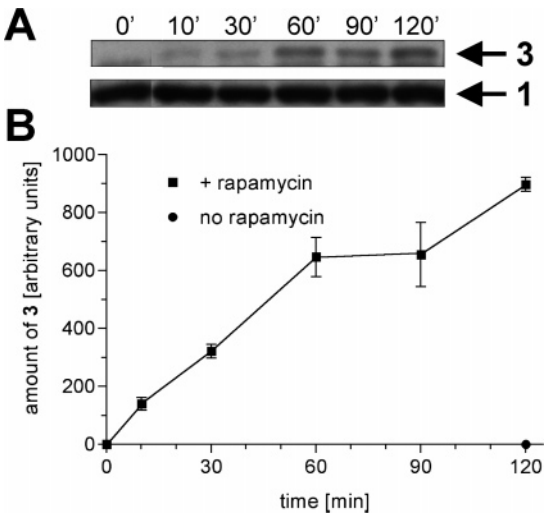

Figure 4. Time-dependence of CPS over a 2-h time course in HEK293 cells. Cells were allowed to express the proteins for 24 h after transfection before adding rapamycin, the zero-time point is defined as the point at which the drug was added. (A) Representative Western blotting data showing the product **3** and starting material **1**. (B) Time-course curve obtained by densitometric quantification of the gel bands in (A). Each data point was normalized to the amount of starting material **1** and represents the average of three independent experiments.

as a chemical inducer of dimerization.[30] This molecule is inert to the endogenous FRAP kinase and therefore not toxic to the cells. We confirmed that AP21967 also triggers CPS in cells (see Figure 2D).

CPS Is Dosable, Quickly Induced, and Can Be Turned Off Reversibly. We next characterized the induction conditions and kinetics of CPS in the HEK293 cells. Figure 3 shows the dose−response curve determined by measuring product formation as a function of rapamycin concentration. A good dynamic range is observed between 0.5 and 8 nM rapamycin, and saturation is reached at 10 nM with a half-maximal effective concentration (EC_{50}) of approximately 1−2 nM. These concentrations are in agreement with the dissociation constant of the FKBP−rapamycin−FRB complex (K_d = 2 nM)[16] and previously reported EC_{50} values for in vivo applications of the FKBP/FRB system.[5,18] Thus, the amount of splicing product obtained is dosable by the concentration of the dimerizer added. This control over product concentration on the protein level represents an important advantage over transcription-based inducible systems that at best allow dose-dependent control at the pre-mRNA level.

The half-life of the protein trans-splicing reaction was previously determined with purified proteins to be about 60 min.[13] Figure 4 shows the time-dependence of the reaction in vivo over a 2-h time range. Within 10 min of adding rapamycin at 10 nM, a significant amount of product **3** could be detected by Western blotting with the anti-MBP antibody, and it further increased with time. However, due to the continuous expression of the starting materials, no kinetic constant could be calculated. Thus, response with CPS can be achieved on the order of minutes after induction instead of hours, as is the case for transcription-based approaches such as the tet-promoter system.[31] This finding underlines the advantage of the posttranslational nature of CPS. By acting directly on the gene products, the time-consuming processes of transcription and translation are bypassed.

In additional experiments, we examined ways to stop the splicing reaction after a defined time period. After induction with 10 nM rapamycin for 2 h, the small molecule ascomycin was added at a concentration of 5 μM (500-fold excess). Ascomycin, a structural analogue of FK506, is a competitor of rapamycin with similar affinity for the FKBP binding site.[32] However, it does not promote formation of the ternary complex involving FRB. Figure 5 shows the effect of ascomycin on product formation. After an initial further increase over a time period of about 4 h, the concentration of the splicing product **3** remained roughly constant over time. Almost identical results were obtained when an additional washing step before the addition of ascomycin was carried out (data not shown). In contrast, in cell samples that were permanently exposed to rapamycin, the concentration of **3** continuously increased over 24 h. Importantly, in the uninduced control experiment no background splicing could be detected even over this longer time course, underlining the excellent induction-to-background ratio of CPS. The initial further product formation after adding ascomycin is most likely attributable to already formed active intein complexes that continue to splice. Competition with ascomycin can only effectively prevent the formation of new complexes. Thus, ascomycin or similar competitive inhibitors of rapamycin on the FKBP or FRB binding sites provide a means to turn off CPS in a reversible fashion for experiments when product formation is desired only over a certain time period.

Stability of the CPS Components. One concern at the beginning of this work related to the stability of the CPS

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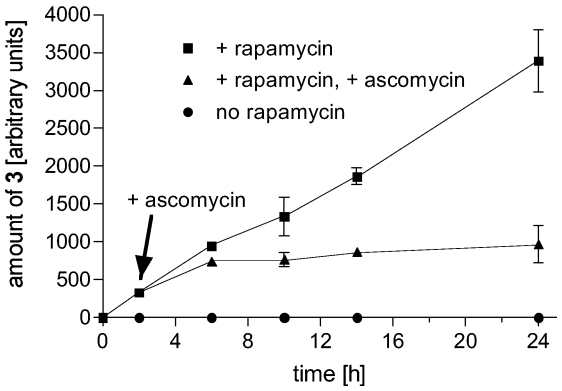

Figure 5. CPS can be stopped with ascomycin. Cells were allowed to express the proteins for 24 h after transfection before adding rapamycin, the zero-time point is defined as the point at which the drug was added. Following exposure to rapamycin (10 nM) for 2 h, ascomycin (5 μM) was added to the growth media of HEK293 cells. Ascomycin is a competitor of rapamycin for the binding site on FKBP; however, it does not induce heterodimerization with FRB. The time-course curves were calculated by quantifying gel bands of product **3** in anti-MBP Western blots using a densitometer. Each data point was normalized to a tubulin loading control (see Materials and Methods). The graphs represent averages from three independent experiments.

components in the cell. As a consequence of the artificial engineering of the VMA intein, the VMA^N (184 residues) and VMA^C (64 residues) parts likely cannot adopt their native structure (see Figure 1C), which probably explains the little or no intrinsic affinity observed for the individual pieces. However, exposure of unfolded regions might cause (partial) degradation in the cell. Indeed, two major and one minor byproducts of about 53, 42, and 48 kDa were observed for construct **1** by Western blotting against MBP (see Figure 2A, marked with an asterisk). The relative amounts of these fragments varied somewhat between different sets of experiments. The sizes of the fragments are consistent with proteolytic cleavage in the middle of the VMA^N part and close to its N-terminal end (or in the long linker region between MBP and VMA^N). Indeed, these fragments did not appear to participate in the CPS reaction (to give smaller-molecular weight products), consistent with proteolysis occurring within the VMA^N−FKBP component of the system rather than the N-extein. Examination of the stability of construct **2** containing the VMA^C part was not possible by Western blotting against the His-tag, since we failed to detect any protein expression with the anti-His antibody. We therefore fused MBP to the N terminus of **2** to give construct **2b** and monitored expression, stability, and protein splicing by Western blotting against MBP. As shown in Figure 2E, a protein migrating in SDS-PAGE at the calculated size of 64.1 kDa was expressed and underwent protein splicing in the presence of **1** and rapamycin. Only a very minor side-product (<3%) of about 60 kDa was detected which would be attributable to proteolysis within the VMA^C sequence (marked with two asterisks in Figure 2D), indicating that this part of the intein is significantly more stable than VMA^N. Importantly, partial proteolysis within either of the intein halves inevitably leads to inactive fragments that cannot participate in the CPS reaction. The proteolysis sites within the VMA^N component, in particular, might be identified and mutated in future studies for applications in which partial degradation of this component is problematic.

Exploring the Geometric Flexibility of the CPS System. In constructs **1** and **2** used in the above-described studies, the arrangement of the FKBP and FRB domains relative to the two intein halves can be thought of as a replacement for the endonuclease domain of the wild-type VMA intein.[33] The crystal structure of the VMA intein revealed that the endonuclease forms a separate structural domain, which is inserted into a loop region of the intein (see Figure 1C).[34−36] This structural modularity probably explains why the endonuclease domain could be exchanged for the dimerization domains in our constructs. As illustrated in Figure 1A, we refer to these localizations as the endo positions A and B relative to the two intein halves. According to this nomenclature, the two exteins are located at positions C and D on the other side of the intein halves.

We were interested in whether CPS involving the split VMA intein could also be induced with one or both of the FKBP and FRB domains representing the extein sequences at the positions C and D. This would provide information about the geometric and structural tolerance of the system and expand the scope of potential applications of CPS. To this end, the new proteins depicted in Figure 6 were prepared. The two constructs shown in Figure 6A correspond to slightly modified versions of the aforementioned proteins **1** and **2**. To facilitate easier protein purification, a His-tag was fused to construct **1** to give protein **1b**. Similarly, to improve protein expression yields of construct **2**, an N-terminal MBP domain was added to give protein **2b** (which was already introduced above for the in vivo experiments). Neither of these changes affect the relative position of the heterodimerization domains at the endo positions A and B. In contrast, two conceptually new constructs, **6** and **7**, were prepared in which the FKBP and FRB domains are located at the N-terminal end of VMA^N and C-terminal end of VMA^C, respectively, and are therefore part of the extein sequences (see Figure 6B). A splicing reaction between these two constructs would give rise to product **8** in which the two heterodimerization domains are ligated together. Finally, with the combinations of the complementary constructs **6** and **2b** as well as **1b** and **7**, reconstitution of the intein by domains interacting between either of the two extein and the endo positions could be explored (see Figure 6, C and D). The respective protein splicing products **11** and **12** would contain one of the two domains that facilitated the interaction.

All constructs were expressed in *E. coli*, purified, and mixed in the four possible combinations depicted in Figure 6. The reactions were started with the addition of rapamycin at a slight molar excess, allowed to proceed for 2 h at 25 °C, and compared to control reactions without rapamycin. The formation of new products was monitored and analyzed by SDS-PAGE as shown in Figure 7A. Strikingly, protein splicing was triggered by rapamycin in all cases, indicating a significant flexibility toward the spatial arrangement of domains that can induce reconstitution of the active intein. The combination shown in Figure 6D, with FKBP in the $endo^N$ and FRB in the $extein^C$ positions, resulted in lower product yields as compared to those for the other three combinations. To test whether this outcome might be caused

(33) The endonuclease domain comprises amino acids 183−410 of the VMA intein, whereas the intein domain comprises amino acids 1−182 and 411−454 (see refs 34 and 35). The modularity of the VMA intein was previously demonstrated by deletion (ref 36) of the endonuclease domain and by its replacement with calmodulin and its M13 recognition peptide (ref 21).

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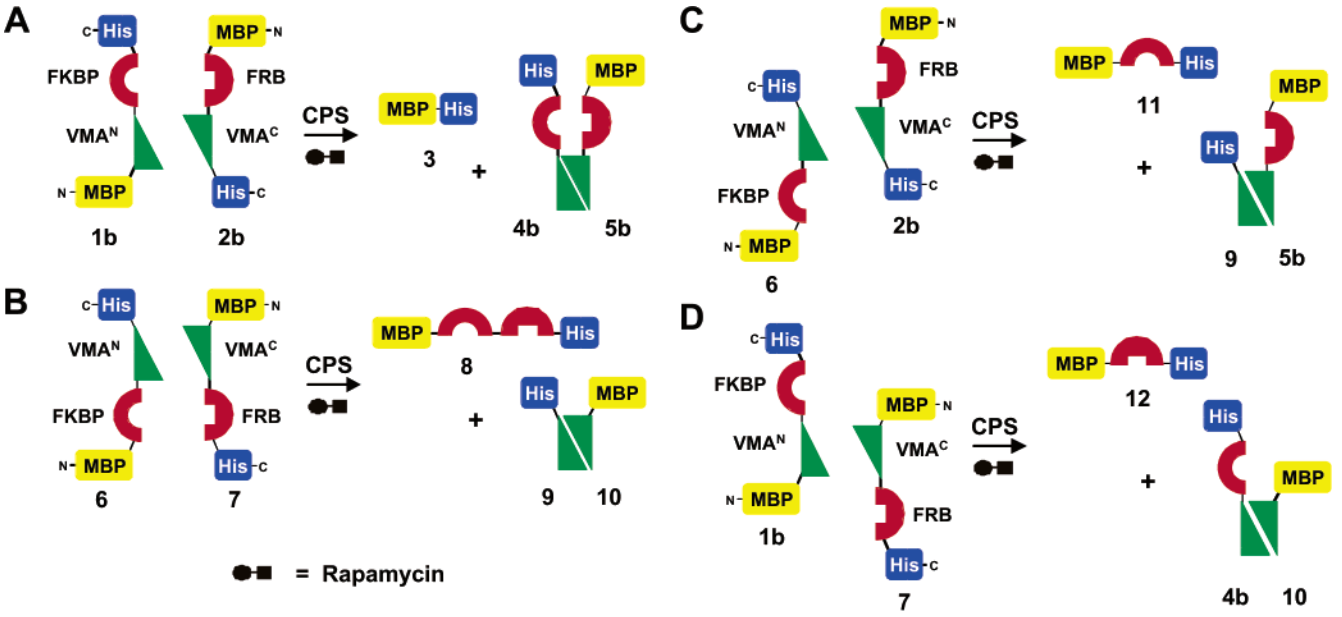

Figure 6. Different arrangements of the heterodimerization domains FKBP and FRB relative to the intein parts VMA^N and VMA^C were tested to promote protein splicing of the exteins in positions C and D (see Figure 1A for the general nomenclature used). (A) In the original CPS approach, both the FKBP and FRB domains are located in the endo positions A and B. For reasons of clarity, rapamycin is not shown with the reaction products, but would be complexed to the FKBP and FRB domains in all cases. (B) Localization of both the FKBP and FRB domains in the extein sequences C and D leads to their fusion in the splicing reaction. (C and D) When one of the heterodimerization domains is in an extein position and one is at the complementary endo position, just one heterodimerization domain is incorporated into the splicing product.

by spatial constraints, flexible linkers of 9 and 18 amino acids were inserted between the VMA^C and FRB domains of construct **7** to give constructs **7b** and **7c**, respectively. Indeed, in both cases this strategy led to significant improvements in product yield such that the splicing reaction went equally well, to about 40−50% completion after 2 h at 25 °C, as in the other three combinations (see Figure 7B). Western blotting using anti-MBP and anti-His antibodies further supported the assigned identities of all formed products (data not shown). The predicted molecular masses of all splicing products were further confirmed by MALDI-TOF mass spectrometry of the crude reaction mixtures (see Materials and Methods).

For all four in vitro CPS combinations shown in Figures 6 and 7, we could clearly observe some splicing background in the absence of rapamycin, which was higher than that found in our original work[13] using slightly different constructs **1** and **2**. In general, the level of background always varied to some degree between different experiments. Further studies will be necessary to better address this issue. Importantly, however, background splicing was not observed in vivo (see also conclusions).

Zinc Ions Inhibit Protein Splicing of the VMA Intein. A recent crystallographic study of the VMA intein revealed a Zn^{2+} ion coordinated to key residues at the C-terminal splice junction of the protein.[37] This structural work employed a mutant VMA intein unable to undergo splicing with the purpose of capturing a presplicing state containing the extein sequences. Thus, it was unclear whether the chelated Zn^{2+} ion played a role in the structure or function of the intein, or if it was just an artifact of the protein purification procedure. Subsequent work on the RecA and DnaE inteins showed that the presence of Zn^{2+} in low concentrations effectively inhibits the protein splicing activity of these proteins.[38−40] However, the effect of Zn^{2+} on the VMA intein remained to be tested experimentally. Therefore, constructs **1b** and **2b** were incubated with 2 mM $ZnCl_2$ (a 1 mM excess over EDTA which was present at 1 mM in all buffers) and rapamycin. As shown in Figure 8, no splicing could be detected in the presence of this divalent ion, whereas $MgCl_2$ at 10 mM was found to have no effect. These findings underline the hypothesis[38,39] that Zn^{2+} represents a general inhibitor of protein splicing. Furthermore, it is likely that the crystal structure of the VMA intein complexed with Zn^{2+} captures an inhibited rather than an active state of the intein, and conclusions drawn from these data[37] on the mechanism of splicing should be interpreted accordingly.

The above-described experiment also demonstrates an important advantage of our artificially split VMA intein over naturally occurring inteins for various mechanistic studies. Both halves of the intein can be purified separately and mixed under native conditions without triggering the splicing reaction. This allows for experimental designs that are problematic or impossible with *in cis* inteins or artificially split *in trans* inteins that require a refolding step due to their spontaneous splicing activity during the protein expression, purification, or refolding steps.

Conclusions

In this work, we have shown that CPS can be used inside living cells to control the primary structure of a protein. Rapamycin, or a nontoxic analogue thereof, was used as the trigger molecule to heterodimerize the FKBP and FRB domains fused to the split VMA intein sequences at the endo positions and thereby reconstitute protein splicing activity (as shown in Figure 1B). The quick response time, on the order of minutes, the dose dependency, and the attenuation of active intein formation by addition of ascomycin are all favorable features

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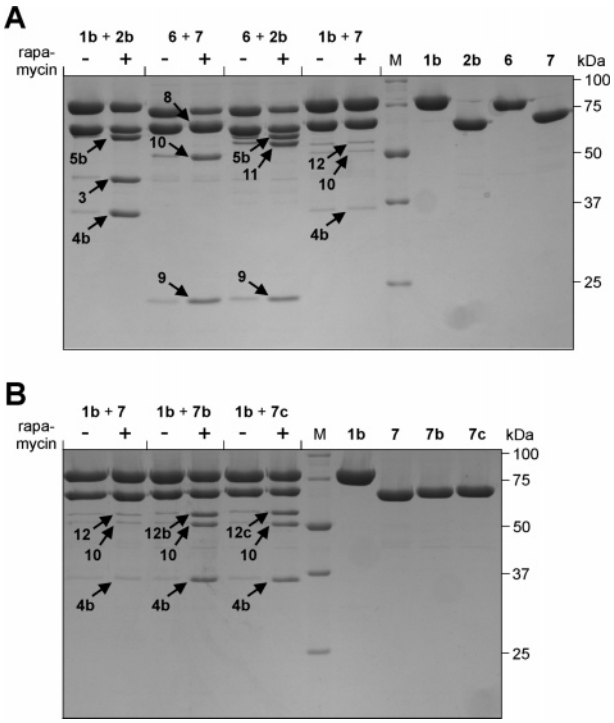

Figure 7. Analysis of the different CPS reactions shown in Figure 6 by Coomassie-stained SDS-PAGE. Reactions were performed for 2 h at 25 °C. Products are indicated with arrows. In each case, the purified starting materials are shown to the right of each panel. (A) Combinations **1b** and **2b**, **6** and **7**, **6** and **2b**, and **1b** and **7**. Product **8** could only be sufficiently resolved from starting material **7** on a lower percentage SDS-PAGE gel (not shown). (B) A flexible linker of 9 and 18 amino acids in constructs **7b** and **7c** inserted between the VMA^C and FRB domains of construct **7** significantly improves the splicing reaction. Calculated molecular masses are: 78.7 kDa (**1b**), 64.1 kDa (**2b**), 45.2 kDa (**3**), 35.4 kDa (**4b**), 62.1 kDa (**5b**), 77.6 kDa (**6**), 63.9 kDa (**7**), 68.9 kDa (**8**), 22.2 kDa (**9**), 50.5 kDa (**10**), 57.4 kDa (**11**), and 56.7 kDa (**12**). Splicing of constructs **7b** (64.8 kDa) and **7c** (65.7 kDa), which are not shown in Figure 6, with **1b** results in formation of **12b** (57.6 kDa) and **12c** (58.5), respectively. All experimentally determined masses are listed in Materials and Methods. M = molecular weight marker.

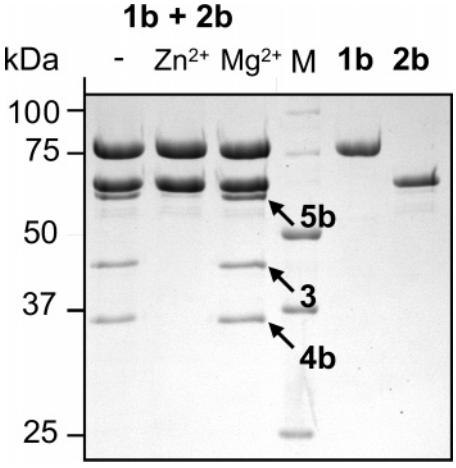

Figure 8. The VMA intein is inhibited by zinc ions. Coomassie-stained SDS-PAGE gel of rapamycin-induced CPS reactions of constructs **1b** and **2b** carried out for 2 h at 25 °C in the presence of 2 mM $ZnCl_2$ or 10 mM $MgCl_2$. The assay buffer contained 1 mM EDTA. M = molecular weight marker.

of the CPS technology. Most importantly, we could not detect any background splicing activity in any of our in vivo experiments, even after prolonged incubations in the absence of rapamycin. Indeed, the reaction in vivo was much cleaner than when performed in vitro with separately expressed and purified proteins. In the latter case, traces of background product formation could be observed (see Figure 7). There are several possible explanations for this phenomenon. One is a simple concentration effect caused by lower concentrations of the two protein constructs inside the cells than those used in the in vitro experiments (2 μM in each). However, we estimate that the cellular concentrations of proteins **1** and **2b** are in the high nanomolar to low micromolar range.[41] Thus, it seems likely that other or additional factors must come into play. One of these could be a temperature effect, taking into account that in vitro reactions were carried out at 25 °C as opposed to 37 °C for the mammalian cell culture experiments. Preliminary studies with the purified proteins **1** and **2** revealed that splicing activity decreases at higher temperature, whereas background splicing increases at lower temperature (Mootz and Muir, unpublished results). This could explain the lower level of background splicing observed under the in vivo conditions.

Due to the promiscuity of inteins in general[14] and the VMA intein in particular,[42] CPS is expected to be generally applicable. In fact, the only strict extein sequence requirement is the cysteine residue (or serine/threonine for some other inteins) at the N-terminal end of the C-extein. A number of principal applications for the original CPS setup, i.e., with FKBP and FRB at the endo positions, are conceivable. In the most general form, a polypeptide with a novel function could be obtained by splicing together two fragments that lack that function when expressed individually. The two fragments could be two proteins or domains thereof, or a protein and a peptide, or just two peptides. For example, two domains of a protein that are inactive while separated could be spliced together to give the active protein. Alternatively, a peptide localization sequence could be created by the splicing event, leading to the relocalization of one component upon addition of rapamycin. Likewise, the peptide-bond-breaking steps that occur between the intein halves and the exteins could be exploited, for example in schemes similar to the proteolytic processes observed for the activation of zymogens. In this strategy, the two polypeptides to be cleaved from each other would be fused to opposite sides of one intein half, e.g. similar to MBP and FKBP in construct **1**.

In this study, we have explored the tolerance of the CPS reaction to changes in the relative locations of the interaction domains, FKBP and FRB, and the intein halves. We find that FKBP and FRB can be located not only at the endo positions A and B but also at the extein positions C and D and even in the two mixed combinations (see Figure 6). In other words, the splicing of C and D can be triggered by the interaction of domains at positions A and B, C and D, A and D, or C and B. These findings suggest a wide range of applications in which FKBP and FRB are replaced by two polypeptides of interest, whose interaction then activates the splicing process. Indeed, Ozawa et al. have already shown that split inteins can be used in a two-hybrid approach in which the interaction of two proteins, at the endo positions A and B, triggers the splicing of

(41) To calculate protein concentrations of construct **2b** in vivo, extracts of HEK293 cells expressing **2b** were immunoblotted against the anti-MBP antibody along with the purified protein **2b** of known concentration. Quantification of the protein bands was performed by densitometric analysis, which allowed an estimate of the protein concentration in the extract to be determined, assuming that each cell has as volume of 1 pL.

(42) Chong, S.; Williams, K. S.; Wotkowicz, C.; Xu, M. Q. *J. Biol. Chem.* **1998**, *273*, 10567−10577.

a reporter protein such as GFP or firefly luciferase.[21−23] An interesting variation of this strategy, which exploits the topological robustness noted above, would be to place one or both of the interacting proteins at the extein positions. Thus, an interaction could be read out by covalently fusing the protein "bait" to the "fish", i.e. the interaction partner to be identified, or alternatively, by covalently modifying the "fish" with a small peptide tag suitable for subsequent affinity purification. This tagging procedure could be useful for studying transient protein−protein interactions, which are difficult to detect using standard biochemical or two-hybrid approaches. In a more general sense, various logical operations could be performed depending on the input, i.e. the trigger of the interaction. Systems reminiscent of simple signal transduction pathways could be devised in which, for example, an input signal promotes interaction of C and B, which in turn results in the splicing of C and D. The splicing product C−D could then represent the output signal.

In summary, CPS is a new and unique technique to post-translationally manipulate the primary structure of peptides and proteins in vitro and in vivo. This approach will be useful whenever the function of a polypeptide, or a process in a cell, can be coupled to the linkage of two otherwise separate polypeptides by a native peptide bond. Therefore, CPS holds great potential as a powerful new research tool.

Materials and Methods

General. Rapamycin and ascomycin were purchased from Sigma-Aldrich Co. (St. Louis, Missouri). AP21967 was a gift from ARIAD Pharmaceuticals (Cambridge, Massachusetts). All other chemical reagents were purchased from Sigma or Fisher (Pittsburgh, Pennsylvania). Restriction enzymes and amylose resin were purchased from New England Biolabs (Beverly, Massachusetts). Ni^{2+}-NTA resin was bought from Novagen (Madison, Wisconsin). Cell culture reagents were purchased from Gibco BRL (Carlsbad, California). FuGene transfection reagent was bought from Roche (Indianapolis, Indiana). Antibodies were purchased from New England Biolabs (anti-MBP), Covance (Princeton, New Jersey; anti-His, clone MMS-156R), and Sigma (anti-tubulin, clone DM1A). DNA primers were ordered from Invitrogen (Carlsbad, California) and IDT (Coralville, Iowa). MALDI-mass spectrometry was performed on a MALDI time-of-flight mass spectrometer Voyager-DE STR (PE Biosystems, Foster City, California), and 4-hydroxy-α-cyanocinnamic acid was used as the ionization matrix. SDS-PAGE and Western blotting analyses were performed using standard protocols.

Plasmid Construction. DNA cloning was carried out according to standard protocols. PCR was performed with PfuTurbo polymerase (Stratagene, La Jolla, California). For the expression of **1**, **2**, and **2b** in mammalian cells, the encoding gene fragments were cloned into pcDNA3 (Invitrogen) to give plasmids pEB3, pEB4, and pHM52, respectively. The template plasmids pHM24 and pHM27 for expression of **1** and **2** in *E. coli* were described previously.[13] For the construction of pEB3, the insert of plasmid pHM24 was amplified by PCR using oligonucleotides 5′-ATAAAGCTTACCATGGCTTCTAGAA-TCCTC-3′ and 5′-ATACTCGAGTTAGTGATGGTGATGGT-GATGAG-3′ (restriction sites underlined) and ligated into *Hin*dIII- and *Xho*I-treated pcDNA3. pEB4 was obtained by cloning the PCR product amplified from pHM27 using oligonucleotides 5′-ATAGGATCCACCATGAAAATCGAAGAAG-GTAAACTG-3′ and 5′-ATACTCGAGTTAATAACTAGTTTC-CAGTTTTAGAAG-3′ into *Bam*HI- and *Xho*I-treated pcDNA3. pHM52 was constructed by cloning the insert from pHM45 (see below), PCR amplified using oligonucleotides 5′-ATAAAGCT-TACCATGAAAATCGAAGAAGGTAAACTGG-3′ and 5′-ATACTCGAGTTAGTGATGGTGATGGTGATGAG-3′, into *Hin*dIII- and *Xho*I-treated pcDNA3. Plasmids for protein expression in *E. coli* were constructed as follows: To give plasmid pHM41 for expression of **1b**, the insert of pHM27 was amplified by PCR using oligonucleotides 5′-ATACCATG-GAAATCGAAGAAGGTAAACTGGTAATC-3′ and 5′-ATA-GGATCCATAACTAGTTTCCAGTTTTAGAAGC-3′ and cloned into the vector fragment obtained from treatment of pHM24 with *Nco*I and *Bam*HI. Plasmid pHM45 for expression of **2b** in *E. coli* was obtained by cloning the *Xba*I−*Hin*dIII insert of pHM24 into pMAL-c2X (New England Biolabs). Plasmids pAT1 and pAT2 for the expression of **6** and **7** were constructed from pHM45 and pHM41 by stepwise fragment exchange of the FRB−VMA^C coding regions for an FKBP−VMA^N coding fragment and of the fragment encoding VMA^N−FKBP for a VMA^C−FRB coding piece, respectively. For this purpose, the new DNA fragments were PCR amplified from pHM41 and pHM45 using 5′-ATAGAATTCTCTAGAGGAGTGCAGGTG-GAAAC-3′ and TCTGGATCCATAACTAGTTTCCAG-3′ for the FKBP region, 5′-ATAACTAGTCTTAAGGGGTGCTTTGC-CAAGGG-3′ and 5′-ATAGGATCCATAAAGAATTGGAGCG-TAAGTCTG-3′ for the VMA^N part, 5′-ATAGAATTCACTA-GTGTTTTGCTTAACGTTCTTTC-3′ and 5′-ATATCTAGA-GTCCTCCTTCTCGTCGCAATTGTG-3′ for the VMA^C fragment, and 5′-TTCGGATCCTCTAGAATCCTCTGG-3′ and 5′-ATAGGATCCCTTTGAGATTCGTCGGAACACATG-3′ for the FRB encoding region, and cloned using the underlined restriction sites. Finally, to insert a flexible linker in construct **7** as described in the Results section, plasmid pAT2 was linearized and dephosphorylated at its *Xba*I site, and ligated with the two complementary, phosphorylated, and annealed oligonucleotides 5′-CTAGAAACAACGGCAACGGGAACGGCA-3′ and 5′-CTAGTGCCGTTCCCGTTGCCGTTGTTT-3′ to give pHM58 (one linker) and pHM64 (two linkers). Each linker codes for the amino acid sequence RNNGNGNGT.

Mammalian Cell Culture Experiments. HEK293, HeLa, COS-7, and MCF-7 cell lines were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum at 37 °C and 5% CO_2 according to standard procedures. Transient DNA transfection of equal amounts of the appropriate plasmids from the set, pEB3, pEB4, pHM52, and pcDNA3 (500 ng each for cells in 2 mL six-well plates) was performed using the FuGene 6 transfection reagent and following the manufacturer's protocol. Heterologous gene expression was allowed to proceed at 37 °C for 24 h, at which point protein splicing experiments were started. Both rapamycin and ascomycin were administered to transfected cell cultures using stock solutions of the drugs in DMSO (2 μL, 1000-fold dilution). The compound AP21967 was similarly diluted from a stock solution in ethanol. The same volume of DMSO or ethanol was added to the noninduced control samples. Prior to harvesting, cells were washed once with phosphate-buffered saline (PBS) buffer (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3). Cell

lysis was performed either with passive lysis buffer (Promega, Madison, Wisconsin) or by adding 2× SDS PAGE loading buffer (100 mM Tris/HCl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol, 200 mM β-mercaptoethanol) directly onto the cells. For immunoprecipitation experiments, cells were washed twice with PBS, scraped off the Petri dish in 1 mL of PBS, collected by microcentrifugation at 3000 rpm, and lysed for 1 h at 4 °C in 1 mL of PBS with 1% *n*-dodecylmaltoside, 2 mM *N*-ethylmaleimide, 10 mM iodoacetamide, and a protease inhibitor cocktail (Roche). Following centrifugation to remove insoluble material, the supernatant was precleared by incubation with Protein A-Sepharose beads for 1 h at 4 °C. The beads were then removed by microcentrifugation at 13000 rpm, and the supernatant was mixed with 70 μL of anti-MBP−Protein-A-sepharose preblocked with bovine serum albumin. Immunoprecipitation was allowed to take place overnight at 4 °C, and the beads were then washed three times with 1 mL of PBS buffer before being resuspended in SDS sample buffer for analysis by Western blotting. For kinetic and dose−response experiments, relative band intensities of the Western blotting data were quantified by densitometry using the program Scion Image (http://www.scioncorp.com) and normalized against a loading control. For the dose−response measurement and the 2-h time course, the band of the starting material **1** was used as the loading control, whereas for the 24-h time points in the ascomycin inhibition experiments, the signal of an anti-tubulin Western blot was used as a loading control after stripping the membranes and reblotting with anti-tubulin antibody. All kinetic and dose−response experiments were performed at least in triplicate, and the data from independent experiments were normalized by multiplication with the average coefficient of each of the comparable data points.

Protein Expression and Purification. Proteins were expressed and purified essentially as previously described.[13] Briefly, *E. coli* BL21 cells were transformed with each of the expression plasmids. The expression strains were grown in 1-L flasks at 37 °C to an OD (600 nm) of 0.5−0.7, at which point the temperature was lowered to 25 °C, and expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM. After 3−5 h, the cells were harvested by centrifugation, and the cell pellets were resuspended in buffer A (Tris/HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) and frozen at −80 °C. For protein purification, each cell suspension was thawed on ice and lysed by three passages through a French press. Insoluble material was pelleted by centrifugation at 30000*g* for 15 min, and the supernatant was applied to an amylose column. After washing the column with buffer A, the proteins were eluted with buffer A containing 10 mM maltose. Subsequently, the protein solutions were dialyzed against buffer B (50 mM Tris/HCl pH 8.0, 300 mM NaCl) and applied to an Ni^{2+}-NTA column. Following washing of this column with buffer B containing 20 to 40 mM imidazole, the proteins were eluted with buffer B containing 250 mM imidazole. Finally, the proteins were dialyzed against assay buffer (50 mM Tris/HCl, 300 mM NaCl, 1 mM EDTA, 5 mM DTT) and kept on ice or frozen at −80 °C with 10% (v/v) glycerol for prolonged storage.

In Vitro Protein Splicing Assays. Purified protein constructs (**1b**, **2b**, **6**, **7**, **7b**, and **7c**) were mixed in complementary combinations in 100 μL of assay buffer to a final concentration of 2 μM in each protein. For the Zn^{2+} inhibition studies, tris(2-carboxyethyl)phosphine (100 μm) was used instead of DTT in the buffer. Rapamycin was then added to a final concentration of 10 μM, and the reactions were allowed to proceed at 25 °C for 2 h. Reactions were quenched by adding 0.5 volumes of 2× SDS-PAGE loading buffer and analyzed by SDS-PAGE with Coomassie staining, Western blotting using anti-MBP and anti-His probes. In addition, all reaction mixtures were analyzed by MALDI time-of-flight mass spectroscopy: **1b**, obs = 78650.1, calc = 78684.1; **2b**, obs = 64033.3, calc = 64063.3; **3**, obs = 45156.4, calc = 45186.8; **4b**, obs = 35429.9, calc = 35444.3; **5b**, obs = 62082.1, calc = 62133.4, for the reaction of **1b** and **2b**; **6**, obs = 77619.2, calc = 77635.1; **7**, obs = 63920.3, calc = 63920.1; **8**, obs = 68940.3, calc = 68930.1; **9**, obs = 22165.1, calc = 22163.3; **10**, obs = 50487.1, calc = 50478.9, for the reaction of **6** and **7**; **6**, obs = 77632.0, calc = 77635.1; **2b**, obs = 64072.8, calc = 64036.3; **11**, obs = 57418.6, calc = 57418.8; **9**, obs = 22159.4, calc = 22163.3; **5b**, obs = 62130.6, calc = 62133.4, for the reaction of **6** and **2b**; **1b**, obs = 78635.8, calc = 78684.1; **7b**, obs = 64764.2, calc = 64805.0; **12b**, obs = 57579.3, calc = 57582.9; **4b**, obs = 35434.5, calc = 35444.3; **10**, obs = 50437.6, calc = 50478.9, for the reaction of **1b** and **7b**; **1b**, obs = 78678.2, calc = 78684.1; **7c**, obs = 65679.0, calc = 65689.9; **12c**, obs = 58458.3, calc = 58467.8; **4b**, obs = 35423.9, calc = 35444.3; **10**, obs = 50431.3, calc = 50478.9, for the reaction of **1b** and **7c**.

Acknowledgment. We are indebted to Jörn Lausen for advice on cell culture experiments. We thank Martine Cadene for help with mass spectrometry measurements and ARIAD Pharmaceuticals for providing compound AP21967. This work was supported by the Rockefeller University's Women and Science Program and by NIH Grants (GM59908 and GM55843, T.W.M.).

JA0362813