Enzyme Activity

Activation of an Autoregulated Protein Kinase by Conditional Protein Splicing**

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The temporal and spatial control of protein function is of fundamental importance in biology. Most cellular processes require that a small subset of the proteome be active at a particular time and place, and that this activity have a defined duration. To probe the role of a protein in a biological system, one must be able to control these parameters as precisely as possible.[1,2] We recently developed a new tool, termed conditional protein splicing (CPS), to control the primary structure, and hence function, potentially of any protein by using a small molecule.[3,4] The basic principle of CPS is illustrated in Figure 1a. Exploitation of a split intein that is only active in the presence of the small molecule rapamycin allows proteins or polypeptides to be linked by a peptide bond through protein splicing. The sequences of interest are expressed as recombinant fusions to the N- and C-terminal halves of the intein, which are themselves linked to FKBP and FRB domains. These domains form a high-affinity ternary complex with rapamycin.[5] The induced proximity of the intein halves in this complex mediates the reconstitution of the active intein. Since CPS acts at the posttranslational level, it has the advantage of a short response time (as little as 10 min), which allows high temporal resolution.

There are many conceivable strategies for specifically altering the function of a protein by CPS. The most obvious method is to reassemble the protein from two inactive pieces and thereby switch on its activity. This strategy exploits the bond-making feature of CPS and requires that the newly spliced polypeptide spontaneously adopts an active structure. We decided to take advantage of another feature of the CPS reaction, the peptide-bond-breaking steps. The CPS system depicted in Figure 1a can be regarded not only as a conditional protein ligase that forms the peptide bond between the two extein sequences, but also as a conditional protease that breaks the peptide bonds between the intein and extein sequences. We conceived a strategy in which the protein of interest and a peptide sequence that acts as an inhibitor are fused to opposite ends of one of the CPS constructs such that they are cleaved from one another in the course of the protein splicing reaction. This cleavage should result in a relative increase in the activity of the protein because the inhibitor should display a lower potency when free than while fused to the protein as a result of its higher local concentration in the intramolecular arrangement. This design borrows from the principles often used by nature to control the activity of enzymes. So-called active-site-directed intrasteric autoregulation[6] has been observed for many proteins, for example, zymogens are kept in an autoinhibited state until posttranslational processing reveals the active protease.

Protein kinases are key players in a myriad of important processes in the cell and are thus attractive targets upon which to test our idea. We chose the cAMP-dependent protein kinase (PKA) for our investigations because it is probably one of the best-understood kinases in terms of its structure, regulation, and function.[7] PKA is the major mediator of cAMP responses in mammalian cells. The PKA holoenzyme is an inactive tetramer composed of two catalytic subunits and two regulatory subunits. The regulatory subunits each contain a pseudosubstrate sequence that competitively inhibits the catalytic subunit by binding to the substrate recognition pocket. Binding of cAMP to the regulatory subunit results in release of the active catalytic subunit, which is then able to phosphorylate Ser/Thr-containing consensus sequences in substrate proteins such as the cyclic AMP response element binding protein. Peptide inhibitors of the catalytic subunit with inhibition constants (K_I) ranging from low nanomolar to micromolar values have been described.[8,9] These peptides are derived from the heat-stable protein kinase inhibitor (PKI), a 75-residue protein that, like the regulatory subunit of PKA, contains a pseudosubstrate sequence. Biochemical[8,9] and structural[10] studies have allowed the key residues in PKI that interact with the kinase to be defined. The 20-mer peptide PKI(5–24) has the sequence TTYAD-FIASGRTGRRNAIHD (key residues underlined) and a K_I value of 2.3 nM.[8] Lawrence and co-workers exploited these structure–activity data to design and prepare a caged version of the catalytic subunit of PKA in which a low-affinity peptide inhibitor is chemically linked to an active-site Cys residue.[11] The caged kinase was found to be autoinhibited until the peptide was released by photolysis, which led to kinase activation. This important study suggests that it should be feasible to design an autoinhibited PKA by using the general CPS-based strategy outlined in Figure 1b.

We designed a protein construct (**2**) in which a pseudosubstrate peptide sequence, TGRRNAI ($K_I \approx 1$ μM, extrapolated from data given in ref. [8]; key residues underlined), and the PKA catalytic subunit were genetically fused to the N and C termini, respectively, of the FRB-intein^C (FRB-I^C) component of the CPS system (Figure 1b, see the Supporting Information for full experimental protocols). In this arrangement, the catalytic subunit represents the C-terminal extein, which following the protein splicing reaction ends up on a different protein fragment from the peptide inhibitor. The catalytic subunit was incorporated as the C-terminal, rather than the N-terminal, extein because the N terminus of the

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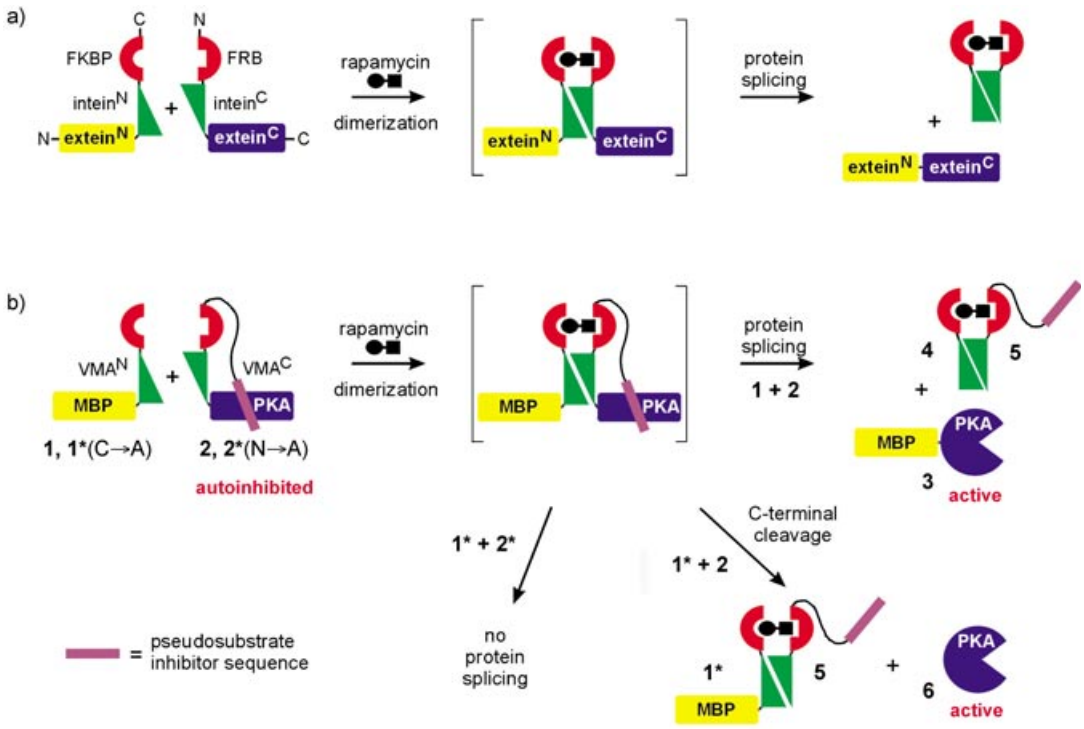

Figure 1. Activation of an enzyme by conditional protein splicing. a) The principle of CPS. The complementary halves of the PI-*Sce* vacuolar ATPase (VMA) intein (ATP, adenosine triphosphate) are marked intein^N and intein^C.[3,21,22] FKBP and FRB are the rapamycin interaction domains. b) Scheme for controlling the kinase activity of the catalytic subunit of cAMP-dependent protein kinase (PKA; AMP, adenosine monophosphate) by CPS. Constructs **1*** and **2*** contain inactivating mutations within intein^N and intein^C, respectively. The His tags present at the N and C termini of constructs **2** and **1**, respectively, are not shown. MBP maltose-binding protein.

protein is less structured than the C terminus.[12] The complementary construct **1** contains the FKBP domain with the counterpart intein fragment intein^N and carries a maltose-binding protein as the N-terminal extein (Figure 1b). This construct was described previously.[4]

Constructs **1** and **2** were overexpressed separately in *Escherichia coli* and purified from the soluble fractions after cell lysis. Protein splicing reactions were initiated by mixing the purified proteins together, either with or without rapamycin. After incubation at 25 °C for 2 h, product formation was analyzed by SDS-PAGE and Western blotting with anti-MBP and anti-PKA antibodies. Formation of the expected splicing product, MBP–PKA (**3**), was observed in the presence of rapamycin but not in its absence (Figure 2a, lanes denoted **1**+**2**). The cleaved intein fragments **4** and **5** were also observed in the reaction mixture containing rapamycin (data not shown). We investigated the kinase activity of the catalytic subunit in these splicing reactions by measuring enzyme-catalyzed phosphoryl group transfer from γ-[^{32}P]-ATP to the serine residue of the short "Kemptide" peptide substrate LRRASLG.[8] As shown in Figure 2b, a 4- to 5-fold increase in kinase activity was observed for the reaction mixture containing both protein constructs and rapamycin compared to the reaction without the small molecule inducer. A control sample containing only construct **2** displayed even lower kinase activity than the mixture containing **1** and **2** (about 7-fold lower than that of the rapamycin-induced sample). The increased kinase activity of **2** in the presence of **1** is possibly the result of a small amount of background splicing that occurs in the absence of rapamycin. This phenomenon has been observed previously for in vitro reactions with similar intein constructs containing model extein sequences.[4] Other control reactions in which either construct **2** or the substrate was omitted from the mixture resulted in background levels of activity (Figure 2b). Addition of a PKI-derived peptide inhibitor, the 18-mer "Wiptide" peptide (TTYADFIASGRTGRRNAI), which has a reported K_I value of 3.1 nM for the catalytic subunit,[8] reduced the kinase activity of the autoinhibited construct **2** to background levels. This result suggests that autoinhibition with the TGRRNAI sequence is not as efficient as inhibition with the native inhibitor PKI.

We conducted a series of control experiments to verify that the observed rapamycin-dependent induction of PKA activity indeed occurs by the proposed mechanism, that is, cleavage of the inhibitor sequence during protein splicing. To rule out the effects of possible conformational changes or steric clashes caused by the simple dimerization of constructs **1** and **2**, we sought to uncouple the rapamycin-induced protein dimerization from the protein splicing event. Mutations were introduced at key intein residues to abrogate protein splicing without affecting the dimerization domains FKBP and FRB (Figure 1b).

We prepared a mutant version of construct **1** in which the catalytic cysteine residue in the I^N fragment was changed to an alanine residue (construct **1***). Incubation of the mutant with construct **2** resulted in rapamycin-dependent peptide bond cleavage at the intein-C-extein junction, that is, between PKA

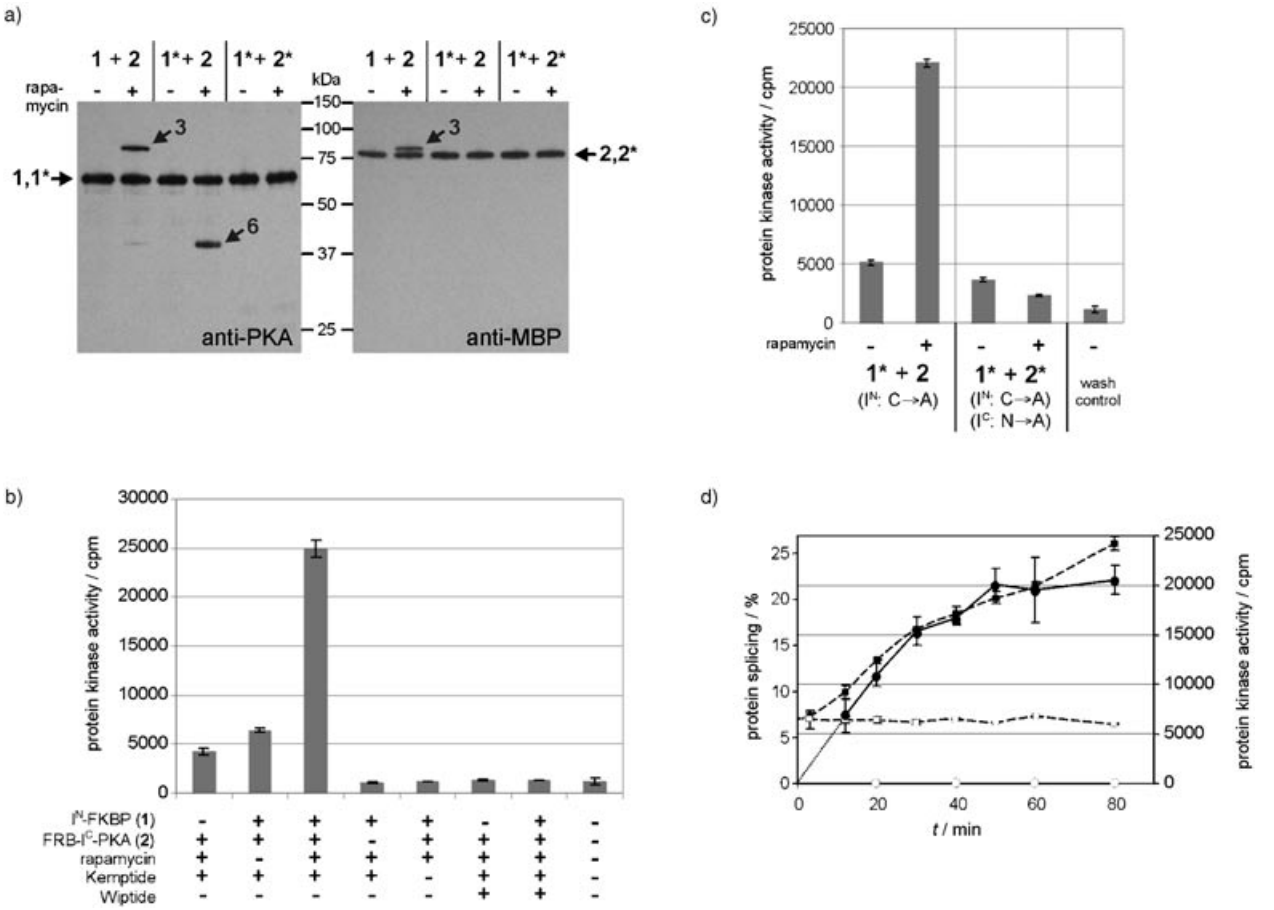

Figure 2. In vitro CPS and kinase activities. a) Western blot of CPS reaction products treated either with anti-PKA or with anti-MBP antibodies. Splicing reactions were carried out by mixing complementary proteins (1 μM each) in assay buffer (50 mM tris(hydroxymethyl)aminomethane/HCl, 300 mM NaCl, 1 mM ethylenediaminetetraacetate, 5 mM 1,4-dithiothreitol, pH 8.0) and adding rapamycin from a stock solution (1 mM) in dimethylsulfoxide (DMSO) to a final concentration of 10 μM. For reactions without rapamycin, the equivalent volume of DMSO was added. The reactions were allowed to run for 2 h at 25 °C. b, c) In vitro phosphorylation of the Kemptide substrate peptide by the indicated CPS mixtures. CPS reaction mixtures were incubated for 2 h and the kinase activity was measured by using a radioactive filter-binding assay (see the Supporting Information for details). Each bar represents the average result of three experiments ± standard deviation (SD). d) Time courses of protein splicing (solid lines) and kinase activity (dashed lines). Rapamycin addition was carried out at $t = 0$ (filled circles and squares) and was omitted in negative controls (empty circles and squares). Splicing efficiency was determined by quantification of the bands in the Western blots and kinase activity was monitored as described in (B). Each data point is the average result of two experiments ± SD.

and the I^C fragment (Figure 2a, lanes **1*** + **2**). Mutant inteins that support cleavage at the intein-C-extein junction have been reported previously[13,14] but this is the first example of a pH/temperature-independent inducible cleavage system. This mutant CPS system acts as a specific conditional protease and could thus be of general utility. As illustrated in Figure 1b, this cleavage process also unleashed the protein kinase from its fused inhibitory peptide, which explains the observed induction of kinase activity at similar levels to those observed with **1** and **2** (Figure 2c).

To block the C-terminal cleavage reaction, the catalytic asparagine residue in the intein^C fragment of **2** was mutated to alanine to give construct **2***. No protein splicing or C-terminal cleavage was observed when mutated constructs **1*** and **2*** were mixed with rapamycin (Figure 2a, lanes **1*** + **2***) and we observed no increase in kinase activity during incubation with the small molecule (Figure 2c). These results show that formation of the dimeric complex does not induce PKA activation. We also performed a time-course experiment with constructs **1** and **2**, which revealed that the level of PKA activation is correlated with the degree of protein splicing (Figure 2d). These data suggest that kinase activity would increase by around 25-fold at 100% splicing efficiency. Together, our results demonstrate that the kinase is switched from an autoinhibited to an active state by the proposed mechanism, which involves unleashing the enzyme from its inhibitor.

Optimization of kinase autoinhibition and activity induction was achieved by replacing the TGRRNAI sequence with a series of peptide sequences exhibiting gradually increasing affinities for the active site of PKA (see the Supporting Information for details). Insertion of the entire high-affinity "Wiptide" sequence (see above) or the sequence TGAANAI in which the key recognition arginine residues were mutated provided further controls. In these systems, the kinase was either fully inhibited irrespective of the progress of protein

splicing, or almost completely lacked autoinhibition (see the Supporting Information).

In conclusion, we have prepared an autoregulated version of a protein kinase that is activated by cleavage of a specific peptide bond, much like a zymogen. Protein design principles have previously been used to generate artificially autoregulated enzymes[11,15–17] but our design is the first in which allosteric ligand binding and concomitant processing are integrated into the same autoregulation system. Our studies also demonstrate that kinase activation depends upon the extent of protein splicing and the nature of the autoinhibitor (Figure 2 and Supporting Information). Both these parameters can be controlled; the level of protein splicing depends on the amount of rapamycin added to the system and the duration of the reaction,[3,4] whilst the autoinhibitor sequence is defined at the genetic level. In effect, the system acts like a protein rheostat that can be adjusted in one of several ways. It should be possible to translate the autoregulation scheme described herein to other protein kinases, and perhaps other classes of enzymes for which peptidic inhibitors are available.

We demonstrated in previous studies that CPS works in mammalian cells.[4] Therefore, the autoregulation strategy described herein should work in an appropriate cellular context (e.g. where the endogenous enzyme has been knocked out or knocked down) and could allow the timing and level of enzyme activity to be controlled. Our system is also one of the few examples[18,19] of activation of an enzyme by the addition of a small molecule; most chemical genetic studies result in the design or discovery of small-molecule inhibitors.[2] In principle, it should be possible to switch an enzyme on with rapamycin and then turn it off again with a second specific small molecule, for example with an allele-specific inhibitor.[20] Thus, the use of CPS to control the activation of an autoregulated enzyme therefore holds great potential as a way to probe the function of proteins.

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