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DNA aptamer-mediated regulation of the hairpin ribozyme by human α-thrombin

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Abstract

The combination of specific ligand-binding aptamers with hairpin ribozyme catalysis generates molecules that can be controlled by external factors. Here we have generated hairpin ribozymes that can be regulated by a short DNA aptamer specific for human α-thrombin. This was achieved by constructing a ribozyme variant harboring an RNA sequence complementary to the aptamer, to which the aptamer can hybridize forming a heteroduplex. In this way, the DNA aptamer completely abolishes the catalytic activity of the ribozyme, due to the formation of an inactive ribozyme conformation. However, in the presence of the aptamer's target protein human α-thrombin, the inhibitory effect of the DNA aptamer is competitively neutralized and the ribozyme is activated in a highly specific fashion. Protein-responsive allosteric ribozymes are proposed to act as tools with potential applications in medicine where fast detection of clinically relevant targets is required.

Keywords: Competitive regulation; Hairpin ribozyme; Aptazyme; Allosteric ribozyme; Ribozyme engineering

Introduction

The catalytic mechanism of the hairpin ribozyme relies on reversible conformational changes between extended and docked conformations. The secondary structure of the hairpin ribozyme comprises two internal loop domains, A and B, each of them flanked by two helices, that must interact with one another to permit site-specific cleavage of the substrate oligonucleotide, generating 5′-hydroxyl and 2′,3′-cyclic phosphate termini [1–4]. The catalytic mechanism of the hairpin ribozyme relies on a reaction pathway in which binding of the substrate oligonucleotide forms domain A which is first aligned with domain B in a linear extended conformation [5,6]. This conformer then folds into a docked structure in which loops A and B contact each other to form the catalytic center, stabilized by non-canonical base pairs. The docked conformation sharply bents at the so-called hinge region [7–10], a flexible linker that mediates the docking process [11,12]. Following cleavage, the docked complex unfolds back into the extended structure and the cleaved products dissociate (Fig. 1).

The hinge region therefore constitutes a flexible joint that mediates the docking process of domains A and B by facilitating the tertiary contacts that define the active site within the interface between the internal loops and by stabilizing the active conformation of the ribozyme [11,12]. Consequently, this motif was used as a target domain for controlling the structure and folding pathway of the hairpin ribozyme by potentially binding effector oligonucleotides. In this way, hairpin ribozyme variants that could either be induced or repressed by external oligonucleotide effectors were engineered by introducing a third regulatory domain C at the junction between helix 2 and helix 3 [13]. This new domain confers precise control over the formation of distinct structural motifs mediated by adaptive binding to defined effector RNA sequences like mRNAs, miRNAs, or aptamers (Fig. 2). In prototype approaches, we have used aptamers [14,15], mRNAs [13], or different microRNAs [16] as effector molecules. Other reporter-ribozymes that are regulated by external oligonucleotide

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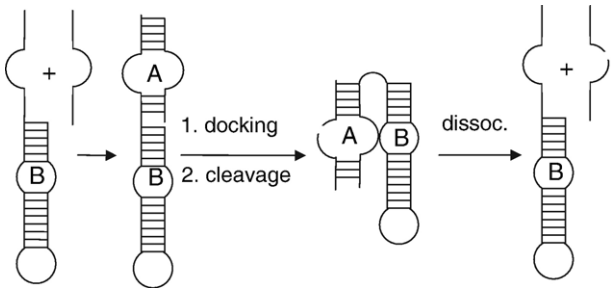

Fig. 1. Architecture and mechanism of the hairpin ribozyme. The substrate is bound by the ribozyme in an extended conformation (left). This conformer folds into a docked structure, in which loops A and B interact. Subsequently, site-specific cleavage occurs (middle). The complex unfolds and the cleavage products dissociate (right). Scheme modified after [9].

effectors have been engineered by exploiting different regulatory mechanisms [17–21].

Here, we used a domain C that is complementary to a functional DNA rather than the previously used functional RNAs [13–16]. We describe an engineered hairpin ribozyme variant harboring the complementary RNA sequence of the 15 nt-long α-thrombin-binding DNA aptamer (Fig. 3). The presence of this aptamer results in allosteric repression of substrate cleavage [22] in a regulatory mechanism that relies on hybridization of the thrombin aptamer DNA to its complementary RNA sequence in the ribozyme (domain C) which leads to complete inhibition of catalysis by enforcing an extended conformation that precludes stable docking of domains A and B. However, this inhibitory effect can be neutralized by the aptamer's target protein α-thrombin, presumably by competitively recruiting the DNA aptamer, which in turn shifts the equilibrium into a catalytically active conformation (Fig. 5). The ribozymes described here represent an example of allosteric hairpin ribozymes regulated by a protein via a DNA aptamer sequence.

Materials and methods

Oligonucleotides

Both 14 nt-long hairpin ribozyme substrates used in this study were purchased from Eurogentec, Belgium (non-labeled) and from IBA (FRET-labeled). The applied DNA-oligonucleotides were either purchased from MWG (Ebersberg, Germany), IBA (Göttingen, Germany), or Metabion (Martinsried, Germany) or were prepared by standard solid-phase methods using CPG-columns (controlled pore glass). After synthesis, the columns resin was resuspended in 1 ml 32% ammonia solution to allow deprotection of DNA-oligonucleotides by an overnight incubation at 55°C. Finally, the resulting solution was concentrated in a vacuum apparatus, redissolved in 200 μl of water, ethanol precipitated and collected by centrifugation. This precipitated RNA was further purified by PAA-gel electrophoresis.

FRET-based assays

All reactions were carried out under multiple turnover conditions (MTO) with excess substrate (1 μM) over ribozyme (50 nM) in Tris reaction buffer (50 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$) at 32°C in 96-well plates (Corning Inc.). Ribozymes were pre-incubated with anti-thrombin aptamer in the Ascent Fluoroscan apparatus at 32°C for 15 min. The cleavage reaction was then initiated upon addition of the FRET-labeled substrate (5 μl of 10 μM stock solution). Changes in FRET decay were demonstrated through a variable emission scan (500–650 nm) at a fixed excitation wavelength (492 nm). Dissociation of the 5′- and 3′-cleavage products from the ribozyme resulted in a loss of FRET and a shift in dominant emission spectrum from TAMRA (585 nm) to FAM fluorescein (520 nm). The cleavage reaction was monitored in real time by measuring FAM-dye fluorescence for 20 min in 30 s intervals. The initial velocity (fluorescence/min) was determined by plotting the fluorescence intensity (y), multiplied by factor 10,000, versus time (x) by linear regression analysis fitting to the following equation using the Microsoft EXEL software, where n = number of data points:

$$k(\text{fluorescence/min}) = \left[n\sum xy - \left(\sum x\right)\left(\sum y\right)\right] \times \left[n\sum x^2 - \left(\sum x\right)^2\right]^{-1} \times 10.000$$

The initial fluorescence/min values were corrected by subtracting values derived from reactions lacking ribozyme.

Thrombin-dependent ribozyme assay

All reactions were carried out under multiple turnover conditions as described above. However, rHP-thrombin was pre-incubated in HP-buffer lacking $MgCl_2$ for 5 min at 32°C. Effectors (50 nM aptamer and 1 μM proteins) were then added, and the reaction was further incubated for 10 min at 32°C. The reaction was initiated by adding $MgCl_2$ to a final concentration of 20 mM.

Results

Rationale for the regulation mechanism

As the basis for the design of new hairpin ribozyme variants, a kinetically well characterized version of the hairpin ribozyme was used [3,23,24]. Briefly, helix H4 in this construct is extended by three base pairs and a stable GUAA tetraloop is used to stabilize the loop B domain. Furthermore, this construct contains a rate-enhancing U47C mutation. Three base pairs, one in H1 and two in H2, were changed to minimize self-complementarities of the substrate. The rest of the ribozyme is identical to the wild-type ribozyme [25]. This variant, which is hereafter designated as the "construct 1 hairpin (C1H)", possesses a preferred cleavage activity compared to that of the wild-type hairpin ribozyme [3].

The rationale for the design strategy was to exploit and modify the structural dynamics of CH1 so that a *trans*-cleaving ribozyme construct arises, which can conformationally respond to the binding states of an aptamer sequence without being

covalently linked to its sequence. This was accomplished by the insertion of a hybridization site into the CH1 to which the aptamer can bind sequence specifically and enforce a conformational switch towards an extended, thus inactive, ribozyme structure. The mechanism of allosteric regulation is schematically shown in Fig. 2. The aptamer binding domain is positioned in a manner that is distal from the catalytic core of the ribozyme. In this arrangement, the new site serves as a structurally independent binding site for the aptamer, thereby replacing the H2–H3 interface. Notably, the resulting new domain, termed C-domain, is linked via a pseudo-three-way junction to enable catalysis to occur in a *trans*-cleaving mode, as well as to selectively favor the active bent conformation. The aptamer antisense region is appended to the terminal stem element of domain C, termed as "terminal connecting domain" (TCD). The sequence of the TCD can be rationally designed in order to minimize the potential of domain C for disruptive interactions during catalysis by stabilizing the stem–loop formation and to provide sufficient flexibility permitting a functional interaction between the domain A and B.

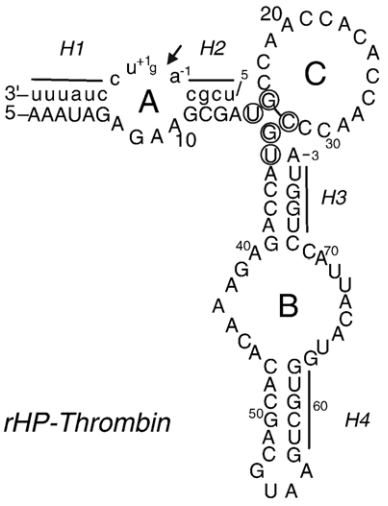

Fig. 3. Secondary structure of rHP-thrombin with the integrated aptamer receptor domain C. The aptamer antisense sequence is shown in bold letters. The TCD (circled) acts as a module to connect both catalytic domains A and B via a pseudo-three-way junction, positioned as a new interface at the hinge region. The secondary structure prediction was performed using the mfold software program.

A DNA aptamer regulated hairpin ribozyme (rHP-thrombin)

For the initial construct design, domain C was made complementary to a fifteen nucleotide-long anti-thrombin DNA aptamer. This antisense RNA sequence was inserted via an asymmetric stem in between residues A14 and A15 at the connections of helices H2 and H3 (Fig. 3). While the residue U34 at the 3′-terminus of domain C is expected to mask the 3′-dangling nucleotide A77, a weak G•U wobble pair is anticipated to form at the ends of domain C, providing the necessary flexibility at the junction. This is followed by a single G–C base pair which is expected to be sufficiently strong to facilitate a stable loop formation. Terminal G–C base pairing is known to be the most efficient interaction for the closure of an unpaired loop.

The predicted secondary structure of the resulting ribozyme construct, designated as repressible HP-thrombin (rHP-thrombin), is shown in Fig. 3. In this configuration, the aptamer sequence serves as the inhibiting effector molecule that can be added externally. The tertiary structure of this aptamer is known to adopt a compact G-quartet structure, comprising fifteen nucleotides [22]. This aptamer binds to the exosite 1 of the human α-thrombin with a dissociation constant (K_d) of 20 nM. However, when this aptamer anneals to its corresponding antisense sequence, domain C is converted into a stable DNA/RNA hybrid which then triggers a disruptive conformation in favor of the undocked, inactive rHP-thrombin ribozyme (Fig. 4a). To examine the proposed ribozyme regulation, the engineered ribozyme construct rHP-thrombin was assayed for RNA-cleavage activity in the absence or presence of the anti-thrombin DNA aptamer. A qualitative assessment of the function of rHP-thrombin is depicted in Fig. 4b. The activity of rHP-thrombin remains unaffected relative to a control hairpin ribozyme without domain C inserted, when tested in the absence of the aptamer. rHP-thrombin operates with a rate constant k_{obs} of 0.11 min^{-1}, which is within the same range of that of CH1. Notably, this finding emphasizes an advantage compared to other design strategies by which modifications of ribozyme constructs often result in significant loss of activity of up to 100-fold. However, in the presence of the DNA aptamer the catalytic activity of the rHP-thrombin ribozyme is strongly reduced, indicating that this construct responds in accordance to the allosteric mechanism proposed in Fig. 2. Maximum inhibition of rHP-thrombin can be readily obtained by an

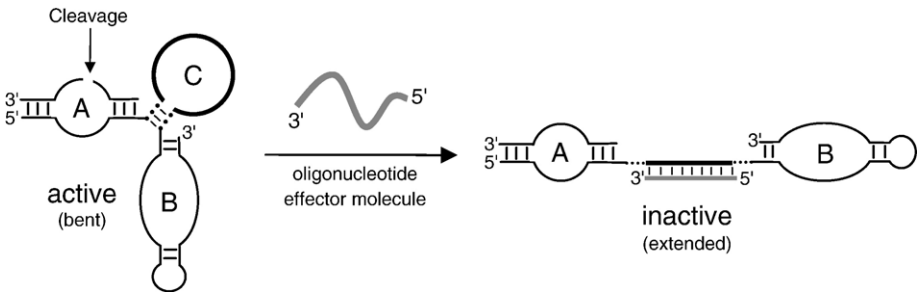

Fig. 2. Schematic representation of the allosteric mechanism exhibited by the thrombin-dependent ribozyme. Domain C serves as an allosteric binding site for the oligonucleotide effector molecule, the thrombin-binding DNA aptamer (gray).

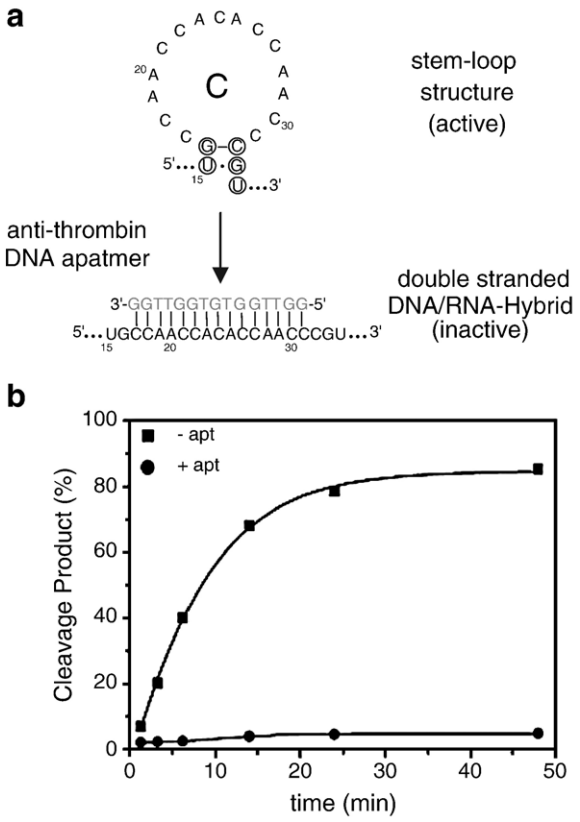

Fig. 4. Inhibition of rHP-thrombin ribozyme (50 nM) by anti-thrombin DNA aptamer (50 nM). (a) Aptamer hybridization is expected to lead to a breakup of TCD (circled) with subsequent inhibition of ribozyme activity. This is assumed to convert the stem–loop structure into a double stranded helix. (b) Plot of rHP-thrombin kinetics in the absence (squares) and presence (circles) of aptamer effector molecule. Reaction conditions were as described in Materials and methods.

aptamer effector concentration of 50 nM which corresponds to a 1:1 aptamer–ribozyme ratio as displayed in Fig. 4b.

Altering rHP-thrombin for real time signal generation

The transformation of molecular recognition directly into catalysis opens a variety of opportunities for signal generation. A simple assay was designed to detect the presence of the anti-thrombin DNA aptamer effector molecule by monitoring the rHP-thrombin activation in real time as a function of fluorescence signal amplification. To achieve this, we labeled the substrate with a fluorescence molecule at its 3′-end and a fluorescence quencher at its 5′-end. In this format, substrate cleavage can be followed in real time by fluorescence resonance energy transfer (FRET) because substrate cleavage results in fluorescence dequenching, causing a red to green shift in the respective emission spectra (Fig. 5). This signal is amplified during the course of the cleavage reaction in which fluorescence intensity is proportional to the substrate cleavage turnover. Thus, to achieve high signal amplification, ribozyme reactions were conducted in a multiple turnover kinetic assay. In this way we examined the concentration-dependent effects of the anti-thrombin aptamer approaching 50 nM (Fig. 6).

As shown in Fig. 6a, rHP-thrombin inhibition occurs in a concentration-dependent manner. A semi-log plot of the initial cleavage rate for rHP-thrombin obtained in the presence of various concentrations of aptamer revealed a sigmoidal curve (Fig. 6b). The apparent K_i for rHP-thrombin was estimated to be 14 nM, which was the aptamer effector concentration required to achieve half-maximal inhibition.

The rHP-thrombin ribozyme detects DNA aptamer binding in real time

We next tested whether the FRET-equipped ribozyme construct responds to the binding status of the DNA aptamer by performing the same experiment in the presence of the aptamer's cognate protein target human α-thrombin. In the presence of α-thrombin, a positive signal is expected, resulting from disruption of the aptamer–ribozyme complex, ultimately rendering the ribozyme active for cleaving the FRET-labeled substrate. In this competition assay, the signal intensity directly reports aptamer binding; it shows whether the aptamer is bound by the ribozyme, resulting in a negative signal, or by the thrombin protein, in which case a positive signal is obtained (Fig. 5).

All assay components were added in parallel to allow α-thrombin to compete with the ribozyme for aptamer binding. In the presence of 20-fold excess of α-thrombin, a 60–70% restoration of the cleavage activity was observed, indicating a shift in the favor of α-thrombin–aptamer complex. In addition, a collection of 13 different proteins were investigated to probe rHP-thrombin specificity for α-thrombin. The remarkable specificity is summarized in Fig. 7. Notably, γ-thrombin, which is identical to α-thrombin but lacks exosite 1, the domain recognized by the aptamer, was unable to affect the ribozyme–aptamer complex formation. Human factor Xa, the active form of the blood clotting factor X exposing macromolecular recognition exosites related to α-thrombin [26], caused only a ribozyme activity to a level of 10% of uninhibited rHP-thrombin. In summary, only α-thrombin was able to generate a fluorescent signal, further establishing the specificity of the observable response.

Discussion

The precise understanding of the hairpin ribozyme's cleavage mechanism and reaction pathway permits the targeted design of new hairpin ribozyme variants with particular characteristics. In the work described here and in previous studies [13,15,16], we have used the hinge region as a target domain to confer precise control over the structure and folding pathway of the hairpin ribozyme by binding effector oligonucleotides. Specific regulation was achieved by converting this motif into a regulatory RNA domain whose binding status triggers distinct conformational changes influencing the docking process. A novel allosteric effector binding region followed as a new junction between helix 2 and helix 3, called domain C.

By introducing sequence changes within this region, we designed a constitutively active ribozyme that can be inactivated

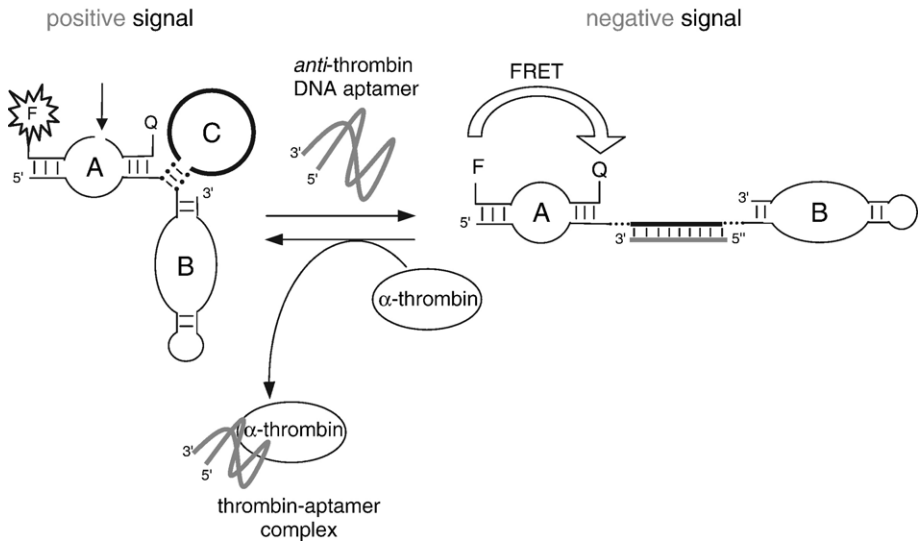

Fig. 5. Schematic for the anti-thrombin aptamer regulated hairpin ribozyme and its response to aptamer-specific human α-thrombin protein. Specific aptamer–protein interaction decoys the ribozyme from the aptamer (gray line) shifting the equilibrium towards the active ribozyme construct. Substrate cleavage generates a positive fluorescence signal. F: fluorescein-label (FAM); Q: fluorescence quencher-label (TAMRA).

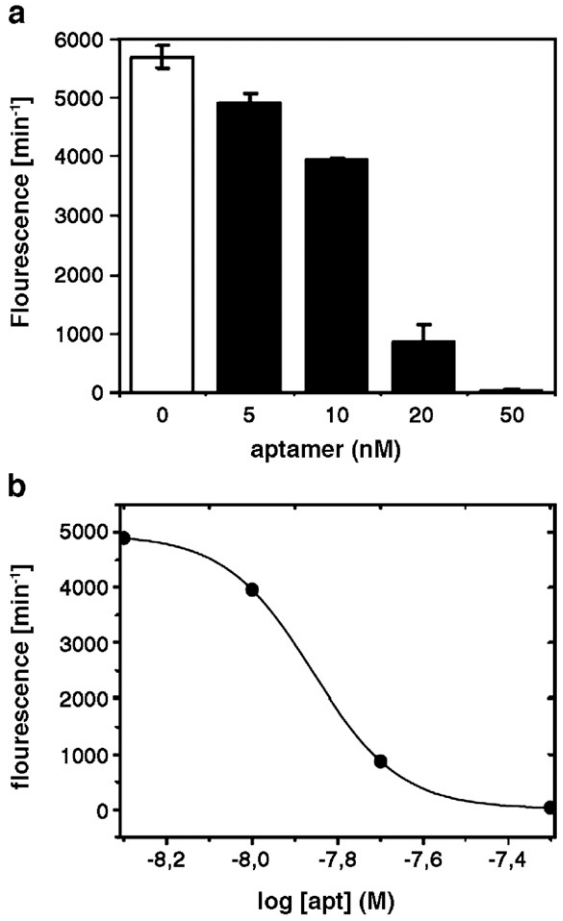

Fig. 6. Real-time detection of anti-thrombin aptamer concentration by rHP-thrombin by fluorescence signal generation. (a) Inhibition of rHP-thrombin by increasing aptamer concentrations. Maximal cleavage reduction occurs at a ratio of 1:1 of aptamer and rHP-thrombin ribozyme. (b) Plot showing the dependence of cleavage activity on aptamer concentration to determine the K_i value.

by a DNA effector. The allosteric regulatory mechanism described here relies on direct control of the hairpin ribozyme structural dynamics rather than a direct distortion of its catalytic domain. This is achieved by the adaptive binding of the effector molecule to domain C inducing an inactive conformational isomer by the formation of an alternate stable RNA/DNA hybrid motif at the hinge region. Using the anti-thrombin DNA aptamer,

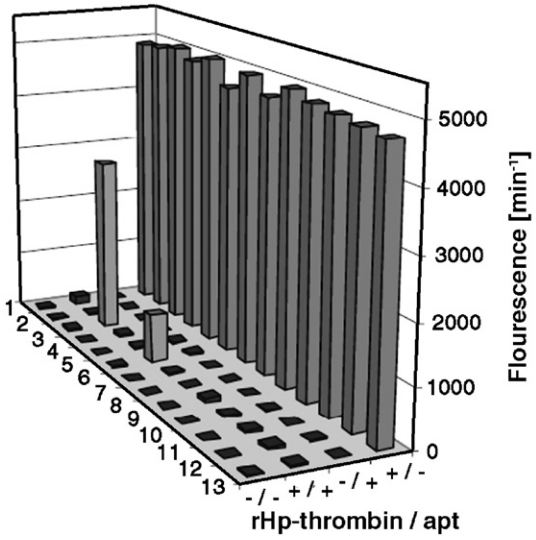

Fig. 7. rHP-thrombin specifically reports the anti-thrombin–aptamer interaction with the exosite 1 of α-thrombin. From the randomly selected control proteins, only α-thrombin activated the aptamer-inhibited AHP-Thr. None of the other proteins affected the activity of the ribozyme in the absence of the aptamer. (1) human γ-thrombin; (2) NFκB transcription factor p52; (3) human α-thrombin; (4) Bcl-3, a member of the IkB protein family; (5) cytohesin-1, a cycloplasmic regulatory protein with guanine nucleotide exchange factor function; (6) human factor Xa; (7) Rev-protein of HIV-1; (8) bovine serum albumin; (9) papain; (10) hen egg white lysozyme; (11) ADH, Alcohol dehydrogenase; (12) hirudin; (13) anti-thrombin III.

the presence of the cognate protein was identified and directly measured in a FRET or fluorescence-dequenching assay.

As an advantage, this ribozyme based assay can be used with minimal modifications as it does not require labeling of compounds or proteins, and uses only small amounts of analytes because positive signals are catalytically amplified. Notably, we have shown in another study that ribozyme activity, controlled by a regulatory protein, can be reverted in the presence of a second interaction partner that complexes the regulatory protein [15]. In a different format, we have monitored complex regulatory pathways of biological relevance. As a prototype, the L-tryptophan regulation pathway involving TRAP and *trp* leader mRNA was explored by *trp*-mRNA dependent ribozymes [13].

The outcome of the present study and previous works show that allosteric ribozymes allow sensitive analysis of metabolic networks consisting of small molecule metabolites, mRNAs, and proteins. These studies represent first examples of hairpin RNA catalysts regulated through specific interactions *in trans*, a prerequisite for the evolution of complex protein–RNA based machines such as the ribosome or the spliceosome that are thought to be ultimately derived from catalytic RNAs.

Taken together, it is feasible that such allosteric hairpin ribozymes can be used as biosensor components, precise switches in nanotechnology, or genetic control elements that that can be regulated by combinations of effector molecules and can respond to the presence of almost any effector molecule.

Acknowledgments

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