Boosting of activity enhancement of K(+) responsive quadruplex hammerhead ribozyme.

Yamaoki, Yudai; Mashima, Tsukasa; Nagata, Takashi; Katahira, Masato

Chemical communications (2015), 51(27): 5898-5901

This journal is © The Royal Society of Chemistry 2015.; The full-text file will be made open to the public on 13 Feb 2016 in accordance with publisher's 'Terms and Conditions for Self-Archiving'.; This is not the published version. Please cite only the published version. この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。
Two second-generation Quadruplex Hammerhead Ribozymes, whose activity enhance in response to K+ via quadruplex formation of embedded r(GGA)3GG, were developed. Different strategies were applied to suppress basal activity when K+ is absent. As a result, the activity enhancement upon the addition of K+ has reached as high as 21-fold.

Nucleic acids that are rich in guanosine residues can take on a compact quadruplex structure in the presence of K+. However, in the absence of K+, the 3′-HR domain (5′-GAAACAGG-3′), are linked via a central stem, and developed an 'intelligent' ribozyme, named here Quadruplex Hammerhead (QHR), whose activity is exerted in response to K+. In a hammerhead ribozyme (HR), two portions of the catalytic core, the 5′-HR domain (5′-GCGGUCUGAUGA-3′) and the 3′-HR domain (5′-GAAACAGG-3′), are linked via a central stem, thereby being brought into close proximity and exerting activity (Fig. S1c). In detail, we suggested that the enzymatic activity of QHR through quadruplex formation in response to K+, and is approximately 2.5-fold for QHR (Fig. S2). It has been noticed that QHR exhibits weak residual activity even in the absence of K+. The presence of such basal activity of QHR at the concentration of 0 mM K+ is unfavorable, because it may cause a destructive attack on target and/or non-target molecules before the switch is turned ON. Suppression of the basal activity is important not only for this reason, but also to realize higher enhancement of the enzymatic activity of QHR in response to K+, namely higher switching efficiency.

We hypothesized the origin of the above mentioned basal activity of QHR even in the absence of K+ was partial and/or transient formation of the active core structure. In detail, we suggested that due to joining of the 5′-HR and 3′-HR domains through R11 together with some linker residues, fluctuation of the structure of QHR in solution or binding of QHR to the substrate may bring the 5′-HR and 3′-HR domains into close proximity, even without quadruplex formation. This may cause the basal activity. Along these lines, here we adopt two strategies to inhibit the formation of the partial and/or transient structure of the active core and suppress the basal activity of QHR in the absence of K+. For the first strategy, we split QHR in the middle of the R11 region; and for the second strategy, we introduced partially complementary RNA sequences into QHR. These second-generation QHRs not only suppressed the basal activity in the absence of K+ but also resulted in improvement of the activity enhancement in response to K+.

In the first strategy, to keep the 5′-HR and 3′-HR domains away from each other in the absence of K+, we split QHR in the middle of the R11 region (Fig. 1a). We named the resulting subunits 5′-spQHR (5′-GCGGUCUGAUGAUUGGAGGA-3′) and 3′-spQHR (5′-GGAGGUUGAACAAGG-3′), respectively. Since these 5′- and 3′-spQHRs are no longer covalently connected, we thought formation of the catalytic core would be prevented, expecting the basal activity...
The enzymatic activities of a mixture of the 5'- and 3'-spQHRs in the absence of 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f. The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of K+ and 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f.

The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f. The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of K+ and 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f.

The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f. The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of K+ and 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f. The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f.

The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of K+ and 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f. The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of K+ and 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f. The enzymatic activities of QHR with each CS (CS1 – CS6) in the absence of 100 mM K+ were analyzed by gel electrophoresis (Fig. 3c). The first-order rate constants for all combinations of QHR with CSs were calculated and are shown in Fig. 2f.
enhancement of the activity of QHR with CS1 by K⁺ (\(k_{\text{obs}}^{100 \text{ mM}} / k_{\text{obs}}^{0 \text{ mM}}\)) was 5.9-fold. This is an improvement as compared with that of free QHR (2.3-fold), having a comparable \(k_{\text{obs}}^{100 \text{ mM}}\) value (just ca. 10 % reduction) to free QHR (Table S1 and Fig. 2f).

Next, to achieve further repression of the basal activity, we constructed CS2, which is complementary to the whole sequence of the R11 region. The \(k_{\text{obs}}^{0 \text{ mM}}\) value of QHR with CS2, however, did not show further improvement as compared with that with CS1, exhibiting a similar residual activity value (Table S1 and Fig. 2e). This suggests that covering of only the R11 region and inhibition of its quadruplex formation are not sufficient to eliminate the basal activity, probably due to partial and/or transient formation of the active core structure in the HR domains, even though the quadruplex structure is not formed. On the other hand, the \(k_{\text{obs}}^{100 \text{ mM}} / k_{\text{obs}}^{0 \text{ mM}}\) value of QHR with CS2 turned out to be 0.92-fold, indicating that there was no enhancement of the activity by K⁺ (Table S1 and Fig. 2f). It is assumed that the double-strand formed with CS2 is more stable than the quadruplex structure in the R11 region even in the presence of K⁺, and thereby activity switching by K⁺ is restrained. Therefore, keeping an appropriate length for binding to the R11 region, while searching for an appropriate complementary sequence to other regions, such as the HR domains, may lead to the finding of a suitable CS.

In CS3, CS4, and CS5, the sequence that is complementary to the R11 region is short (4-nt long, which is one nucleotide shorter than CS1) and identical, while the sequences that are complementary to 5'-HR domain are varied (lengthened by one base in CS3, CS4, and CS5 in that order) (Fig. 2b). As we have expected, these three CSs repressed the basal activity of QHR significantly, the \(k_{\text{obs}}^{0 \text{ mM}}\) values being in the range of one-fourtieth to one two-hundredth of the value of free QHR (Table S1 and Fig. 2e). This indicates that connection of complementary sequences to parts of the 5'-HR domain and that to the R11 region are highly effective for suppressing the basal activity of QHR. A probable explanation for this suppression of the basal activity is that complementary sequences in CS3, CS4, and CS5 as to parts of the 5'-HR domain of QHR have formed a double-strand and thereby inhibited the partial and/or transient formation of the active core structure in the HR domains of QHR, which would otherwise exhibit basal activity even in the absence of the quadruplex structure.

We then determined the \(k_{\text{obs}}^{100 \text{ mM}} / k_{\text{obs}}^{0 \text{ mM}}\) values of QHR with CS3, CS4, and CS5, all of which improved in that order (9.3-, 11.8-, and 17.9-fold), and were much larger than that of free QHR, 2.3-fold (Table S1 and Fig. 2f). However, the \(k_{\text{obs}}^{100 \text{ mM}}\) values of QHR with CS3, CS4, and CS5 decreased in that order, and more importantly, all had much smaller values than that of free QHR (Table S1 and Fig. 2e). A probable reason for the latter point is that each of CS3, CS4, and CS5 forms a double-strand with QHR through a rather long stretch (regions through a part of 5'-HR, a uridine-linker, and a part of the R11 region), thereby dissociation of CSs, formation of the quadruplex structure in the R11 region, and activity switching in the presence of K⁺ are strongly restricted. Although the further improvement of suppression of the basal activity, \(k_{\text{obs}}^{0 \text{ mM}}\) and activity enhancement, \(k_{\text{obs}}^{100 \text{ mM}} / k_{\text{obs}}^{0 \text{ mM}}\), for CS3, CS4, and CS5, as compared with CS1, is encouraging, \(k_{\text{obs}}^{100 \text{ mM}}\) needs to be increased.

Finally, CS6 targets exactly the same region of QHR as CS5, except that CS6 has a three base-pair mismatch as to QHR (Fig. 2b). This base-pair mismatch was introduced to make an adjustment to obtain a sequence that retains a similar level of basal activity suppression as CS5 but is capable of dissociating from QHR upon...
quadruplex structure formation by R11 in the presence of K’. Although the $k_{\text{obs}}^0$ of QHR with CS6 was not as low as that of QHR with CS5, QHR with CS6 exhibited effective suppression of the basal activity, the $k_{\text{obs}}^{100\text{mM}}$ value being $4.77 \times 10^{-3}$ min$^{-1}$, which is one-eighth of the value of free QHR (Table S1 and Fig. 2e). Importantly, the $k_{\text{obs}}^{100\text{mM}}$ value was $8.57 \times 10^{-3}$ min$^{-1}$, which is just a minor decrease from the $k_{\text{obs}}^{100\text{mM}}$ value of free QHR (only ca. 5 % reduction), and thereby the $k_{\text{obs}}^{100\text{mM}} / k_{\text{obs}}^{0}$ value turned out to be as high as 21.4-fold (Table S1 and Fig. 2f). We suggest that the three base-pair mismatch between CS6 and QHR was suitable for CS6 to dissociate from QHR upon formation of the quadruplex of R11, thereby the active structure of QHR being induced in the presence of K’.

We attempted to design a CS that suppresses the basal activity of QHR in the absence of K’ and also leads to QHR high activity enhancement ($k_{\text{obs}}^{100\text{mM}} / k_{\text{obs}}^{0}$ value) through quadruplex formation in response to K’. In total, we examined the effects of six CSs on the activity of QHR (Fig. 2b). Among them, CS6 exhibited good results in leaving QHR with highly suppressed basal activity in the absence of K’, and causing high activity enhancement for QHR in response to K’. Thus, we have successfully developed a second-generation QHR.

Several ribozymes were reportedly conjoined with aptamers, by which the activities were altered in response to small compounds. These ribozymes that are activated by small compounds are used as biosensors and gene regulators. Another activity switching ribozyme, that was reported, was composed of a ribozyme portion and a conjoined inhibitor portion, which is an antisense RNA against the ribozyme portion. This ribozyme-inhibitor system is activated upon a presence of a complementary RNA strand (named activator), by which the inhibitor portion is displaced. This system, which employs cis-acting RNA duplex is somewhat similar to our QHR/CS system in the sense that it uses inhibitor of ribozyme. To our knowledge, only two groups have reported ribozymes connected with quadruplexes instead of an aptamer. Hartig and coworkers replaced the stem of a hammerhead ribozyme with a guanosine-rich RNA fragment, r(GGGGUGGGUGGGUGGG), with which the activity was enhanced by the addition of meso-5,10,15,20-tetrakis-(N-methyl-4-pyridyl) porphine (TMPyP4), a known quadruplex stabilizer. The activity enhancement of their conjoined ribozyme upon addition of TMPyP4 was about 8-fold. Perreault and coworkers incorporated two guanosine-rich RNA fragments into hepatitis D virus ribozyme. It is thought that one of the incorporated fragments forms a series of hydrogen bonds with part of the original hepatitis D virus ribozyme in the absence of K’, resulting in a misfolded ribozyme with no activity. In the presence of K’, however, the two fragments are suggested to fold into a quadruplex and thereby the active catalytic structure is restored. In the latter two cases, the precise structures of the quadruplexes are not fully understood.

In our case, we have precise, at atomic resolution, structural knowledge of the quadruplex that we have used, which provides us a guide to obtain the ability of activity switching to other ribozymes and aptamers. Our strategy enabling induction of the activity of a ribozyme / aptamer in response to K’ is rather straightforward. Firstly, the ribozyme or aptamer is divided into two parts, and each part is linked to one end of R11 through some linker residues. At this stage, we only need to focus on obtaining such a ribozyme or aptamer conjoined with R11 that exerts as high activity as possible in the presence of K’. This can be accomplished by finding an appropriate position to divide the ribozyme or aptamer, and adjusting the length of the linker residues. At the next stage, we use two different strategies, both of which are fruits of the present study, to suppress the basal activity in the absence of K’ and to achieve highly efficient enhancement of the activity in response to K’: splitting the R11 region in the middle or introducing partially complementary RNA sequences. In summary, we have shown two methods, which might be applicable to other ribozymes and aptamers, endowing them with activity switching capabilities that can be controlled.

We thank Professors T. Morii and E. Nakata of Kyoto University for their technical support with the fluorescence gel imaging systems, and Professor Y. Tanaka of Tohoku University for the valuable discussion on the idea of splitting QHR. We are grateful to the Ministry of Education, Science, Sports and Culture of Japan for Grants-in-Aid for Scientific Research (23570146 and 26440026 to T.N.; 25115507, 25291013, 26104520, and 26650014 to M.K.).

Notes and references

Institute of Advanced Energy, Graduate School of Energy Science, Kyoto University, Gokasho, Uji, Kyoto 611-0011 (Japan). E-mail: katahira.masato.6u@kyoto-u.ac.jp, nagata.takashi.6w@kyoto-u.ac.jp.

† Electronic Supplementary Information (ESI) available: Materials and experimental details. See DOI: 10.1039/c000000x/


Boosting of Activity Enhancement of K⁺-Responsive Quadruplex Hammerhead Ribozyme

Yudai Yamaoki, Tsukasa Mashima, Takashi Nagata* and Masato Katahira*

Institute of Advanced Energy, Graduate School of Energy Science, Kyoto University, Gokasho, Uji, Kyoto 611-0011 (Japan)

Supplementary Information

Materials

QHR (5'-GCGGUCUGAUUUGAGGA GGAGGUUGAAACAGG-3'); subunits of split QHR, 5'-spQHR (5'-GCGGUCUGAUUUGAGGA-3') and 3'-spQHR (5'-GGAGGUUGAAACAGG-3'); various complementary RNAs (CSs); and substrate RNA (5'-CCUGUCACCGC-3'), labelled with FITC, were synthesized, purified, and de-salted by FASMAC Co., Ltd. (Kanagawa, Japan), were purchased.

Cleavage reaction

Firstly, spQHRs (final concentration of 10 μM for both 5'- and 3'-spQHRs) or QHR (10 μM), either alone or mixed with various CSs (30 μM), were dissolved in a solution comprising 50 mM Tris-Cl buffer (pH 8.0) and either 0 or 100 mM KCl. Each obtained solution was heated at 95 °C for 5 min and then gradually cooled to 25 °C. Next, MgCl₂ was added to the final concentration of 50 mM. Then, the cleavage reaction was started by adding substrate RNA, which was labeled with fluorescein-5-isothiocyanate (FITC) (1 μM). The temperature of the solution was kept at 25 °C throughout the reaction. A small aliquot was taken from the reaction solution at various time points, and the reaction was stopped by adding EDTA and urea to final concentrations of 83 mM and 7.5 M, respectively. These small aliquots were applied to a denaturing 20% polyacrylamide gel, uncleaved and cleaved substrates being separated during electrophoresis. The amounts of FITC-labeled substrate RNAs were determined by means of fluorescence using a Pharos FX™ Molecular Imager (BIO-RAD).

The cleaved percentage was defined as the amount of cleaved substrate divided by the total amount of the substrate, namely the sum of the amounts of uncleaved and cleaved substrates. The first-order rate constant, \( k_{obs} \), was determined by fitting the time-course experiment data to the equation:

\[
P(t) = P_{\max} - (P_{\max} - P_0) \exp (-k_{obs}t)
\]

where \( P(t) \) is the cleavage percentage at time \( t \), \( P_{\max} \) the cleavage percentage at infinite time, and \( P_0 \) the extrapolated cleavage percentage at \( t = 0 \).\(^{1,2}\) Error bars are standard deviation calculated from 3 trials.
Fig. S1 Architecture of Quadruplex Hammerhead Ribozyme (QHR) and the concept of its activity switching in response to K⁺. (a) The sequence of R12 and its quadruplex structure in the presence of K⁺. (b) The sequences of Hammerhead Ribozyme (HR) (green), in which the 5'-HR and 3'-HR domains (shaded), and stem regions (outlined) are highlighted, and substrate RNA (purple). (c) The sequence of QHR and its expected structure in the presence of K⁺. In QHR, the stem of HR is replaced by the R11 sequence, r(GGAGGAGGAGG) (red), and uridine-linker sequences (yellow).
Table S1.

<table>
<thead>
<tr>
<th></th>
<th>$k_{obs}^{0 \text{mM}} \text{[min}^{-1}]$</th>
<th>$k_{obs}^{100 \text{mM}} \text{[min}^{-1}]$</th>
<th>$\frac{k_{obs}^{100 \text{mM}}}{k_{obs}^{0 \text{mM}}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>QHR without CS</td>
<td>$3.91 \times 10^{-2}$</td>
<td>$9.05 \times 10^{-2}$</td>
<td>2.3</td>
</tr>
<tr>
<td>QHR with CS1</td>
<td>$1.38 \times 10^{-2}$</td>
<td>$8.10 \times 10^{-2}$</td>
<td>5.9</td>
</tr>
<tr>
<td>QHR with CS2</td>
<td>$1.66 \times 10^{-2}$</td>
<td>$1.53 \times 10^{-2}$</td>
<td>0.9</td>
</tr>
<tr>
<td>QHR with CS3</td>
<td>$2.46 \times 10^{-3}$</td>
<td>$1.82 \times 10^{-2}$</td>
<td>9.3</td>
</tr>
<tr>
<td>QHR with CS4</td>
<td>$9.52 \times 10^{-4}$</td>
<td>$1.06 \times 10^{-2}$</td>
<td>11.8</td>
</tr>
<tr>
<td>QHR with CS5</td>
<td>$3.39 \times 10^{-4}$</td>
<td>$4.47 \times 10^{-3}$</td>
<td>17.9</td>
</tr>
<tr>
<td>QHR with CS6</td>
<td>$4.77 \times 10^{-3}$</td>
<td>$8.57 \times 10^{-2}$</td>
<td>21.4</td>
</tr>
</tbody>
</table>

Fig. S2 Activity of Quadruplex Hammerhead Ribozyme (QHR). Polyacrylamide gel electrophoresis of the products produced on cleavage of the substrate with QHR. The top and bottom panels show cleavage in the absence of $K^+$ and presence of 100 mM $K^+$, respectively. (b) Time course of the increase in cleaved substrate with QHR in the absence of $K^+$ (open diamonds, dashed line) or presence of 100 mM $K^+$ (filled diamonds, solid line). (c) The magnification of the first 35 minutes.
Fig. S3 (a) The magnification of the first 35 minutes of Fig. 1b. Time course of the increase in cleaved substrate with QHR in the absence of K⁺ (open diamonds, dashed line) or presence of 100 mM K⁺ (filled diamonds, solid line). (b) The magnification of the first 35 minutes of Fig. 2d. Time course of the increase in cleaved substrate with QHR in the absence of K⁺ (open circles, dashed line) or presence of 100 mM K⁺ (filled circles, solid line); and with QHR and CS6 in the absence of K⁺ (open diamonds, dashed line) or presence of 100 mM K⁺ (filled diamonds, solid line).