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COMMUNICATION

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Atom-specific mutagenesis reveals structural and catalytic roles for active site–adenosine and hydrated Mg^{2+} in pistol ribozyme

Sandro Neuner,[+] Christoph Falschlunger,[+] Elisabeth Fuchs, Maximilian Himmelstoss, Aiming Ren, Dinshaw J. Patel, and Ronald Micura*

Abstract: The pistol RNA motif represents a new class of self-cleaving ribozymes of yet unknown biological function. Our recent crystal structure of a pre-catalytic state of this RNA shows guanosine G40 and adenosine A32 close to the G53-U54 cleavage step. While the N1 of G40 is in 3.4 Å distance to the modeled G53 2'-OH group that attacks the scissile phosphate and hence suggests its direct role in general acid–base catalysis, the function of A32 is not clear. We present evidence by atom-specific mutagenesis that neither N1 nor N3 base positions of A32 are involved in catalysis. By contrast, the ribose 2'-OH of A32 seems crucial for proper positioning of G40 via H-bond networks that involve G42 as a bridging unit between A32 and G40. We also found that disruption of the inner-sphere coordination of the active site–Mg^{2+} cation to N7 of G33 makes the ribozyme drastically slower. A mechanistic proposal is suggested with A32 playing a structural and hydrated Mg^{2+} playing a catalytic role in cleavage.

The discovery of twister, twister sister, pistol and hatchet self-cleaving ribozymes by comparative genomic analysis has reinvigorated research to explore the chemical strategies RNA utilizes to catalyze chemical reactions.[1,2] To this end, the three-dimensional architectures of twister,[3-6] twister-sister,[7,8] and pistol ribozymes[9,10] in their pre-catalytic states have been recently revealed by crystallography; these provide a solid foundation for mechanistic proposals that await their experimental evaluation in solution by structure-function analysis and targeted atomic mutagenesis. While for twister ribozymes, chemical,[4,6,11,12] biochemical,[1-3,13] and computational[14] studies have already provided valuable insights into the molecular mechanism of this ribozyme,[15,16] such studies lag behind for the three other new ribozyme classes.

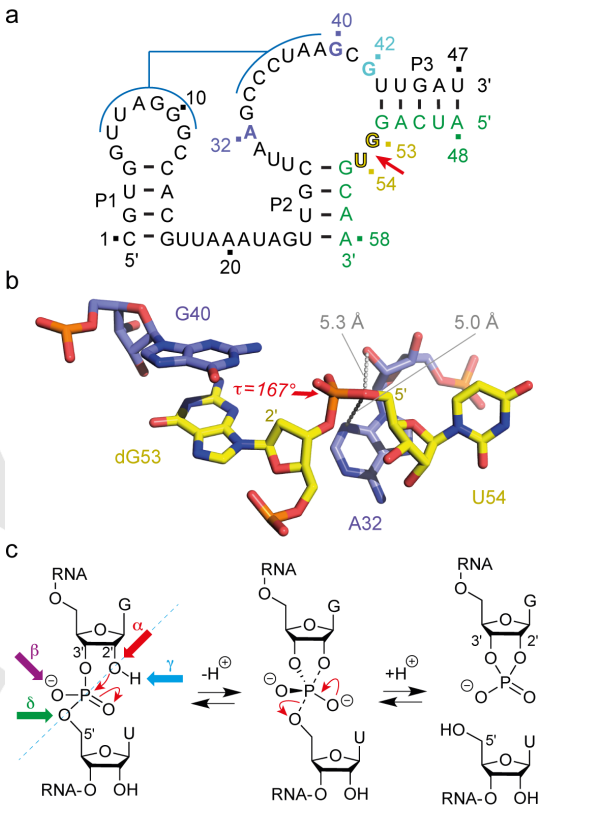

Figure 1. Secondary structure of the *env25* pistol ribozyme (**a**) and crystal structure of the active site showing the dG53-U54 cleavage step (yellow) and the closest neighbors G40 and A32 (pdb code 5K7C)[9] (**b**). Numbers in grey indicate distances; red arrow indicates the direction of nucleophilic attack on the scissile phosphate (from the 2'-O of G53 in the native ribozyme); for ideal in-line alignment of the 2'-O relative to the to-be-cleaved P–5'O bond, the angle τ is 180°. (**c**) RNA phosphodiester cleavage: The internucleotide linkage ('scissile' phosphate) passes through a pentacoordinate transition state that results in two cleavage products carrying either a 2',3'-cyclic phosphate terminus and a 5'-hydroxyl terminus, respectively. The four catalytic strategies that impact on the reaction are:[23] α, in-line nucleophilic attack, S_N2-type (blue); β, neutralization of the (developing) negative charge on nonbridging phosphate oxygens (purple); γ, deprotonation of the 2'-hydroxyl group (red); and δ, neutralization of negative charge on the 5'-oxygen atom by protonation (green).

Thus far, pre-catalytic structures of the pistol ribozyme have been reported from two groups,[9,10] with good consensus on the overall folding topology and the alignment of catalytic residues at the cleavage site (Figure 1). Both pyrimidine and purine at the pyrimidine-purine cleavage step of the pistol ribozyme are anchored in a splayed-apart conformation with in-line alignment of the modeled 2′-O of the purine nucleoside properly located for attack on the adjacent to-be-cleaved P-O5′

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bond (Figure 1b,c). Importantly, highly conserved residues, a guanosine (G40; N1 position)[9,10] and a purine nucleoside (either A[9] or G[10]) (position 32; N3 and 2′-OH) are aligned to potentially act as general base and general acid, respectively (Figure 1b). A first set of cleavage assays with ribozyme mutants was supportive for these putative roles although an evaluation *via* deazapurine *ribo*nucleosides has not yet been provided.[9]

Interestingly, NMR spectroscopy revealed only a slightly increased pK_a of 4.7 for A32 for a non-cleavable (dG53 modified) pistol variant.[9] Furthermore, our previous studies demonstrated that the deletion of the 2'-OH alone (2'-deoxyadenosine) significantly reduced the rate of cleavage while simultaneous deletion of N3 and 2'-OH (3-deaza-2'-deoxyadenosine, c^3dA32) abolished activity.[9] The precise roles of these functional groups, however, remained unclear, not least because the key mutations of 1-deazaadenosine (c^1A) and 3-deazaadenosine (c^3A) both comprising the native 2'-OH group were not included in the previous studies.[9,10]

We have therefore set out to shed light on the putative mechanistic role of A32 by applying an atomic mutagenesis approach that comprehensively utilizes deazapurine ribonucleosides. Because the imino *N*-hydrogen donor/acceptor capabilities become lost in site-specific manner if c^1A and c^3A, respectively, were substituted for A32 in pistol ribozymes, their cleavage activities constitute a reliable measure for the verification/elimination of this nucleobase to participate in general acid–base catalysis for phosphodiester cleavage.

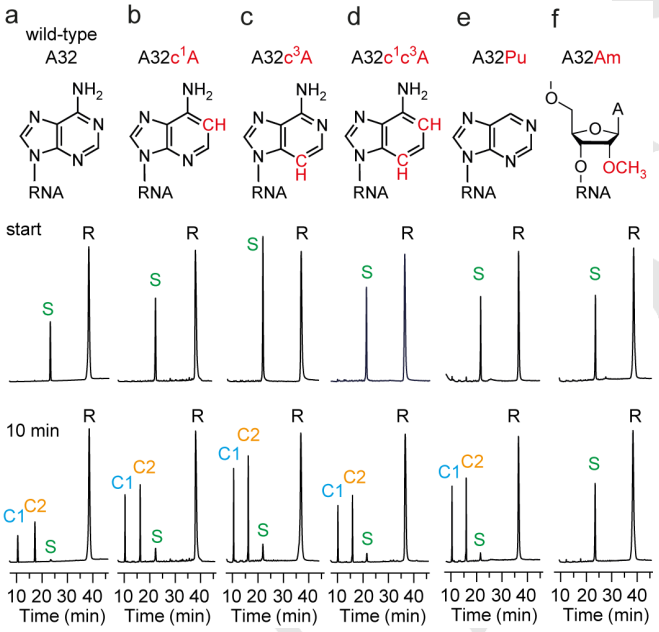

Figure 2. Self-cleavage of the *env25* pistol ribozyme (a) and mutants (b-f). Cleavage analysed at 55 µM RNA each strand; 2 mM $MgCl_2$, 100 mM KCl, 30 mM HEPES, pH 7.5, 23 °C. R 47-nt ribozyme, S 11-nt substrate; C1 and C2, 5-nt and 6-nt cleavage products. HPLC conditions: Dionex DNAPac column (4x250 mm), 80 °C, 1 ml min^{-1}, 0–60% buffer B in 45 min. Buffer A: Tris–HCl (25 mM), urea (6 M), pH 8.0. Buffer B: Tris–HCl (25 mM), urea (6 M), $NaClO_4$ (0.5 M), pH 8.0.

The above mentioned deazaadenosine mutations require phosphoramidite building blocks for RNA solid-phase synthesis that are not commercially available. Therefore, the c^1A phosphoramidite was synthesized in eleven steps in analogy to previously published procedures (Supporting Information),[17] and c^3A phosphoramidite was synthesized in five steps according to our recent protocol.[18] With these phosphoramidites in hand, we synthesized the corresponding two-stranded pistol ribozymes with c^1A and c^3A mutations in position 32. Next, we performed cleavage assays of these two ribozyme variants using anion-exchange high-performance liquid chromatography (HPLC; Figure 2). Under single-turnover conditions, wild-type *env25* pistol ribozyme cleaves its substrate to completeness in less than 10 min in the presence of 2 mM Mg^{2+} and 100 mM K^+ at pH 7.5 (30 mM HEPES buffer) and at 23°C (Figure 2a). When A32 was mutated to c^1A, cleavage appeared as fast as for the wild-type ribozyme (Figure 2b); this was surprising because N1 is the preferred site of protonation of adenosine and the prime candidate for general acid catalysis to donate a proton to the 5'-O leaving group.[24] However, we remembered that our studies on twister ribozymes showed that only the c^3A but not the c^1A twister variant was deleterious for cleavage.[15] Therefore, we decided to next analyze the c^3A32 pistol variant. Even more surprising, we observed that for this c^3A32 variant, cleavage was absolutely comparable in activity to the wild-type ribozyme (Figure 2a,c).

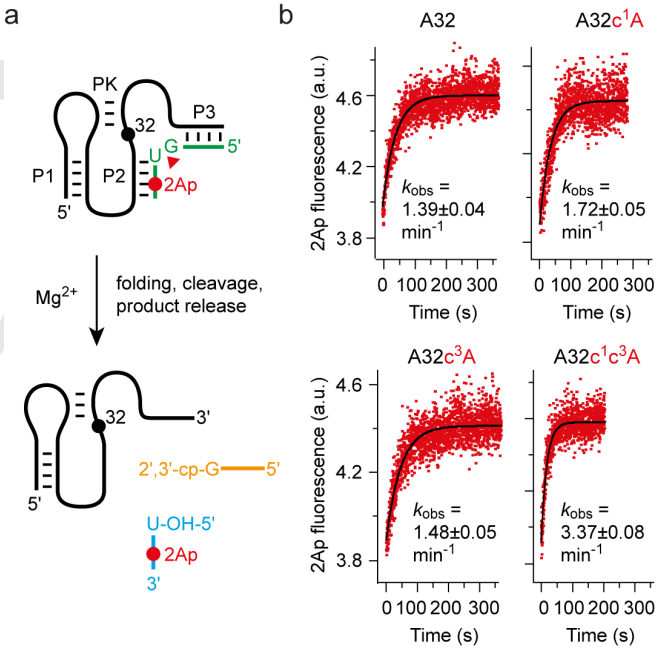

Figure 3. Self-cleavage of the *env25* pistol ribozyme and mutants. (**a**) 2-Aminopurine fluorescence assays using a A57Ap variant. (**b**) Exemplary fluorescence time courses for rate determination at 15 °C; arbitrary units (a.u); $MgCl_2$ was added at t = 0; conditions: c(RNA) = 0.5 µM (1:1 ratio), 50 mM KMOPS, 100 mM KCl, 15°C, pH 7.5; mixing was performed manually in less than 2 s resulting in 10 mM Mg^{2+} concentration (each k_{obs} obtained from three independent experiments).

To additionally verify our findings, we developed a 8-step synthesis for 1,3-dideazaadenosine (c^1c^3A) amidite (Supporting Information; in analogy to a report by Müller et al. for c^1c^3dA),[19]

synthesized the corresponding c^1c^3A32 modified pistol ribozyme and tested its activity. We again found unhindered specific phosphodiester cleavage (Figure 2d). This confirms that neither N1 nor N3 can pitch in for its counterpart and that indeed none of these imino functionalities is involved in general acid base catalysis.

In this context, the precise rates k_{obs} of A32, c^1A32, c^3A32 and c^1c^3A32 modified pistol ribozymes were determined in comparative manner utilizing a previously established 2-aminopurine fluorescence assay (Figure 3a).[25] Indeed, the rates of the deazaadenosine modified ribozymes are similar (or even slightly faster) compared to the wild-type ribozyme (Figure 3b-d, Supporting Table 1).

Because of these unanticipated results, we reanalyzed the active site residues and focused on the shortest (and most likely strongest) H-bond interaction of A32, formed between its 2'-OH to the exocyclic NH_2 group of G42 (Figure 4). Equally important, the O6 of G42 is in 2.7 Å H-bond distance to the NH_2 of G53 (of the cleavage step) and in 2.8 Å H-bond distance to the NH_2 of G40, the latter assigned to take the role as general base in pistol ribozyme cleavage. It seems that G42 is the structural 'glue' between G40 (general base) and A32 (putative general acid) and thereby generates a cleft to accommodate the scissile phosphate of the splayed-apart G53-U54 cleavage step.

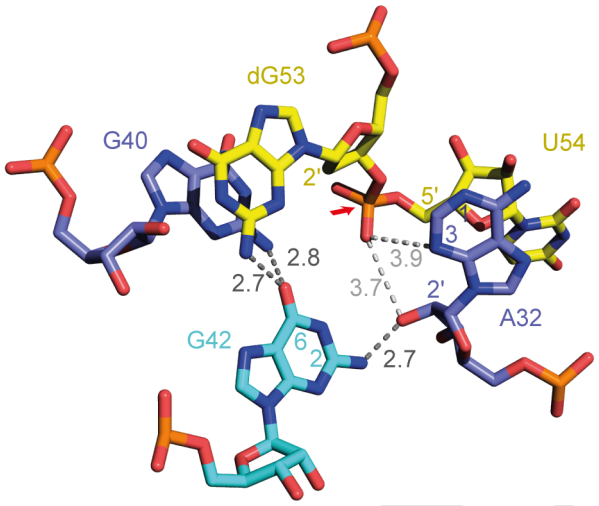

Figure 4. Active site of the *env25* pistol ribozyme with G42 interactions highlighted (pdb code 5K7C)[9] Nucleoside positions are color-coded: cleavage step dG53–U54 (yellow); numbers in grey indicate distances in Å; red arrow indicates the scissile phosphate.

From our previous study, we knew that the A32dA modified pistol ribozyme showed only about 25% cleavage yield after 10 min and about 90% after 45 min.[9] In the light of the new analysis, we additionally tested a A32Am variant and observed hardly any cleavage after 10 min (Figure 2f) and only miniscule amounts of cleavage products (about 10%) after 45 min (Supporting Figure 1a). This is consistent with the loss of the specific H-bond that otherwise helps to keep G42 and G40 in place, thereby supporting the architecture for proper accommodation of the G53-U54 cleavage step. Additionally, the 2'-OH of A32 with most likely lowered pK_a (because of the H-bond to NH_2 of G42) might be involved in proton transfer to the U54 5'O leaving group, although further conformational changes were needed to bring these groups closer together. At this point we mention that we also tested A32G,[9] A32dG and A32Gm (Supporting Figure 1b-c, Supporting Figure 2), resulting in the same reactivity profiles as observed for A32, A32dA and A32Am, highlighting the consistency of our model.

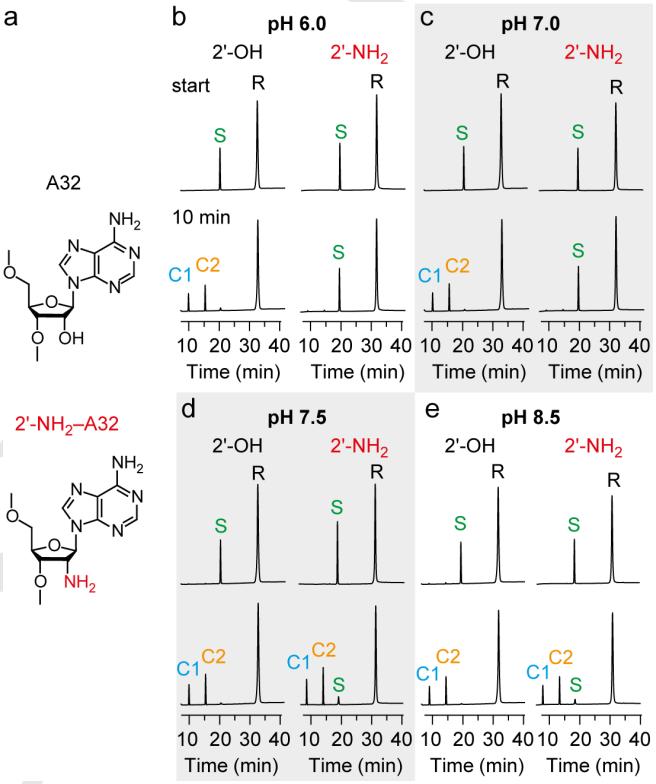

Figure 5. Self-cleavage of 2'-OH versus 2'-NH_2 A32 modified *env25* pistol ribozyme. Cleavage analysed at 55 µM RNA each strand; 2 mM $MgCl_2$, 100 mM KCl, 30 mM HEPES, pH 7.0 or 7.5 or 8.5 (c-d), 23 °C. For pH 6.0 (b), MES buffer (30 mM) was used; R 47-nt ribozyme, S 11-nt substrate; C1 and C2, 5-nt and 6-nt cleavage products. HPLC conditions as in Figure 2, except 60°C column oven temperature.

To learn more about the role of the 2'-OH of A32, we were wondering how its substitution to an amino group would affect cleavage activity. We synthesized the corresponding 2'-amino-2'-deoxyadenosine phosphoramidite,[26] prepared the 2'-NH_2–A32 modified pistol ribozyme and performed cleavage experiments. It was reported previously that the cleavage rate k_{obs} of the pistol ribozyme increases with increasing pH (albeit measured at suboptimal 25 µM Mg^{2+} concentrations).[20] This trend was also observed in our experimental set-up at saturating 2 mM Mg^{2+} concentrations (Supporting Figure 3). In direct comparison of 2'-OH versus 2'-NH_2–A32 pistol ribozymes (Figure 5), we found that the native ribozyme cleaves readily at pH 6.0 and 7.0 while for its 2'-NH_2 counterpart, hardly any cleavage occurs after 10 min (Figure 5b,c). Only at higher pH values (7.5 and 8.5), the 2'-NH_2 modified ribozymes are able to cleave their substrates almost as efficiently (Figure 5d,e).

The pK_a of a 2'-OH group in RNA is assumed to be approximately 12,[21] while for 2'-NH_2 modified pyrimidine nucleosides[22] in RNA the pK_a was determined to be 6 (this value refers to the corresponding 2'-NH_3^+ moiety). Although the pK_a can be shifted to some extent by the nature of the nucleobase and its specific RNA micro-environment, it is reasonable to assume that at pH 6.0 a significant portion of the ribose (sp^3 hybridized) 2'-NH_2 is protonated. In its protonated form, however, it cannot function as H-bond acceptor and hydrogen bond formation to the 2-NH_2 of G42 (sp^2 hybridized; H-bond donor) becomes impaired. As a consequence, proper formation of the G40–G42–A32 scaffold, which is required to optimally mould the active site architecture, is compromised (Figure 4). This explanation is consistent with the observation that no cleavage was observed at pH 6.0 and 7.0 (2'-NH_3^+ A32) while at higher pH values (>7.0; 2'-NH_2 A32) cleavage is restored (Figure 5).

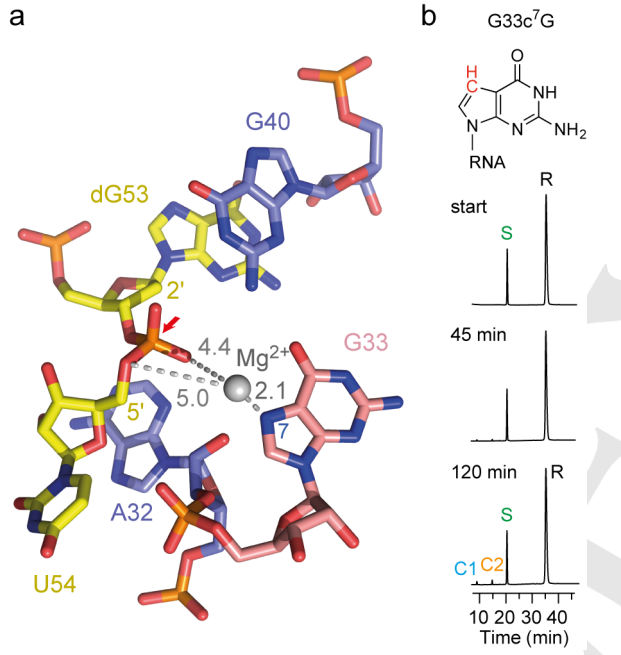

Figure 6. Active site of the *env25* pistol ribozyme with the Mg^{2+}–N7-G33 interaction highlighted (a);[9] note that the hydrated Mg^{2+} ion is in proper distance to potentially form a water-mediated hydrogen bond to the non-bridging oxygen of the scissile phosphate; nucleoside positions are color-coded as in Figure 1; numbers in grey indicate distances in Å; red arrow indicates the scissile phosphate; (b) Cleavage of the c^7G33 modified pistol ribozyme is almost completely abolished; setup of cleavage assays and HPLC conditions as in Figure 5.

What can we learn from the current set of experiments about the mechanism of phosphodiester cleavage for the pistol ribozyme? In our opinion, the major insight is that neither N1 nor N3 of the highly conserved purine base (A or G) in position 32 is directly involved in catalysis in the native RNA. Second, the structure–sculpting role of A32 has been revealed: A32 participates through its 2'-O···H_2N-G42 hydrogen bond in the H-bond network between G40, G42 and A32 to generate the cleft for accommodation of the G53-U54 cleavage step; in other words, A32 assists to position the general base G40. What is more difficult to verify or nullify (because of the structural entanglement described above) is the role or requirement of A32 in proton transfer to the 5'-O leaving group (δ catalysis; see also Figure 1c and caption). In its absence (dA32 mutant), the ribozyme can still cleave, albeit with a significantly reduced rate (more than 100-fold).[9] It could be concluded that for dA32 modified ribozymes, the purine N3 might in part take over this role, because only the double deletion of 2'-OH and N3 abolishes cleavage completely.[9] Alternatively, the H^+ needed for protonation of the 5'-O leaving group may stem from the hydrated Mg^{2+} that is inner sphere coordinated to N7 of G33 and in about 5 Å distance to the 5'-O atom. This hydrated Mg^{2+} is a candidate for proton transfer given that the 2'-OH of purine-32 is also not ideally positioned (in ~5.5 Å distance), and additional conformational changes from the pre-catalytic conformation to the transition state are required in any case. To finally shed light on the putative involvement of this hydrated Mg^{2+} ion, we synthesized a 7-deazaguanosine–33 (c^7G33) pistol ribozyme that lacks the ability to coordinate to Mg^{2+} because of the N7-to-C7 replacement. Strikingly, this modified ribozyme had hardly any cleavage activity (Figure 6, Supporting Figure 4; k_{obs} = 4.27± 0.4 x 10^{-3} min^{-1}). This finding suggests a crucial role for the active site–hydrated Mg^{2+} that is seen in both crystal structures.[9,10] Hence, taken all our atomic-mutagenesis experiments together, this leads to the mechanistic proposal illustrated in Figure 7.

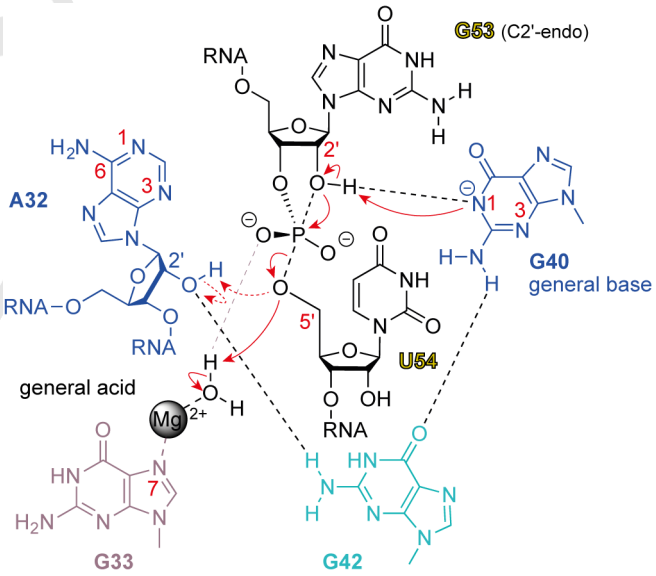

Figure 7. Mechanistic proposal for phosphodiester cleavage in the *env25* pistol ribozyme.

In summary, our experiments shed light on the structural role of the 2'-OH of the purine nucleoside–32 and of the catalytic role of the hydrated Mg^{2+} coordinated to G33 of the pistol ribozyme. Our preferred view for pistol ribozyme phosphodiester cleavage draws a mechanistic scenario where a combination of catalytic strategies is employed. Both available crystal structures (from independent groups)[9,10] suggest that in the precatalytic state, the attacking 2'-OH of G53 is orientated nearly ideal for in-line attack at the scissile phosphate (α catalysis). Likewise, in

both structures, G40 finds itself in ideal distance to activate the attacking 2'-OH of G53 (γ catalysis). With respect to protonation of the 5'-O leaving group (δ catalysis), we can now exclude H^+ donation from the purine-32 base. The corresponding ribose 2'-OH which is structurally tightly engaged to build the active site, may fulfill this role, however more likely, H^+ donation to the 5'-O leaving group originates from one of the water molecules of the hydrated Mg^{2+} cation that is inner sphere coordinated to N7 of G33. We note that this Mg^{2+} ion also allows for water-mediated hydrogen bond formation to the non-bridging oxygen of the scissile phosphate. Such an arrangement may contribute to the stabilization of the transition state (ß catalysis). Further dedicated investigations and well-designed experiments will be needed to fully explore and understand this mechanistic aspect of the pistol ribozyme.

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Keywords: nucleosides • oligonucleotides • RNA modification • catalytic RNA • structure-function relationship

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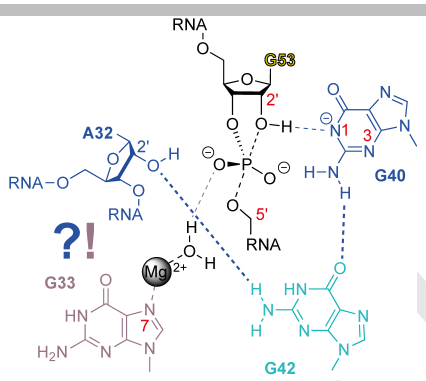
Entry for the Table of Contents

COMMUNICATION

The ribose of a conserved adenosine and a hydrated Mg^{2+} ion in the active site of the pistol ribozyme contribute the activity-determining functional groups. Their potential roles in general acid-base catalysis versus active site structuring is discussed.



*S. Neuner, C. Falschlunger, E. Fuchs, M. Himmelstoss, A. Ren, D. J. Patel, and R. Micura**

Page No. – Page No.

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