**Functional Characterization of 2',5'-Linked Oligoadenylate Binding Determinant of Human RNase L**

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RNase L is activated by the binding of unusual 2',5'-linked oligoadenylates (2–5A) and acts as the effector enzyme of the 2–5A system, an interferon-induced anti-virus mechanism. Efforts have been made to understand the 2–5A binding mechanism, not only for scientific interests but also for the prospects that the understanding of such mechanisms lead to new remedies for viral diseases. We have recently elucidated the crystal structure of the 2–5A binding ankyrin repeat domain of human RNase L complexed with 2–5A. To determine the contributions of amino acid residues surrounding the 2–5A binding site, point mutants and a deletion mutant were designed based on the crystal structure. These mutant proteins were analyzed for their interaction with 2–5A using a steady-state fluorescence technique. In addition, full-length RNase L mutants were tested for their activation by 2–5A. The results reveal that π–π stacking interactions of Trp and Phe, electrostatic interactions of Lys and Arg, and hydrogen bonding by Glu make crucial contributions to 2–5A binding. It was also found that the crystal structure of the ankyrin repeat domain L:2–5A complex accurately portrays the 2–5A binding mode in full-length RNase L.

Interferons are immunomodulatory cytokines that trigger anti-pathogenic and anti-proliferative mechanisms in the cells (1). The 2',5'-linked oligoadenylate pathway (2–5A) system is a controlled RNA decay pathway comprising 2–5A synthetases and RNase L, which constitutes a steady-state configuration; i.e. each repeat consists of pairs of anti-parallel α-helices stacked side by side that are connected by a series of intervening β-hairpin motifs (10). The ankyrin domain of RNase L is characterized by a distinctive curved structure and the existence of an extra helix between repeats 4 and 5. The ligand 2–5A is bound by the second to the fourth ankyrin repeats and interacts with the RNase L residues Trp, Asn, Gin, Lys, Phe, Glu, Tyr, and Arg by forming hydrogen bonds, salt bridges, or stacking interactions (12). In addition, the extra helix has been found to constitute the bottom of the 2–5A binding site. To determine the contribution of these residues and the extra helix to the binding of 2–5A, we have now designed nine point mutants and a deletion mutant of the ankyrin repeat domain protein and full-length RNase L. Biochemical characterization of these mutants allowed us to determine the 2–5A binding determinants of human RNase L.

**MATERIALS AND METHODS**

**Expression Constructs**—For the expression of the ankyrin repeat domain of human RNase L with an N-terminal His tag (hANK), we used the pQEHisXaANK plasmid (12). The QuikChange method was used to introduce site-directed mutations on the plasmid, using the pairs of complementary oligonucleotide primers listed in **TABLE 1**. The plasmids encoding human RNase L and including the same mutations as the hANK mutants were generated from pEGXRN(L) (15) by substituting an equivalent 0.6-kb PstI-Ncol fragment of pQEHisXaANK. This 0.6-kb fragment encodes residues 22–228 of RNase L. All constructs were verified by sequencing the entire hANK-coding region of isolated plasmids.
Protein Preparation—The wild-type and mutant hANK proteins with N-terminal His tags were expressed in *Escherichia coli* strain JM109 and purified by SP-Sepharose FF (Amersham Biosciences AB), TALON affinity resin (Clontech, Palo Alto, CA), and Sephacryl S200HR (Amersham Biosciences) column chromatographies (12). The RNase L mutants were expressed as N-terminal glutathione S-transferase (GST) fusion enzymes in *E. coli* strain JM109 and purified by chromatography on a glutathione-Sepharose 4B (Amersham Biosciences) column and gel filtration on a Sephacryl S200HR column, as described previously (16).

2–5A Binding Assay—The 2–5A binding ability of hANK was assayed by static fluorescence spectroscopy (13). Briefly, the fluorescence emission spectra of hANK were measured from 295 to 385 nm, with the excitation wavelength set at 280 nm. The dissociation constant ($K_d$) was determined from the relationship between the 2–5A concentration and the fluorescence quenching at 340 nm, upon the addition of 5′-O-monophosphoryladenylyl(2′→5′)adenylyl(2′→5′)adenosine (pApApA) to the solution of ANK, according to the following equation,

$$\alpha = \frac{(L_0 - E_0\alpha)^n}{K_d + (L_0 - E_0\alpha)^n}$$  (Eq. 1)

where $\alpha$ is the ratio of quenched fluorescence intensity at a specified pApApA concentration ($\Delta F$) to the fully quenched fluorescence intensity ($\Delta F_{max}$), $L_0$ is the total concentration of pApApA, $E_0$ is the total concentration of hANK, and $n$ is the Hill coefficient. The $n$ values ranged from 0.8 to 1.2.

RNase Activity Assay—5′-Labeled RNA substrate (5′-fluorescein-C$_{11}$U$_2$C$_7$-3′) was synthesized with an Applied Biosystems DNA/RNA synthesizer (Model Expedite) using phosphoroamidite units from GLEN Research (Sterling, VA). The oligonucleotide (1 μM) was digested.
in buffer A (20 mM Tris-HCl (pH 7.5), 0.1 M KCl, 10 mM Mg(CH$_3$COO)$_2$, 1 mM ATP, and 8 mM 2-mercaptoethanol) containing 0–2 μM pApApA and 20 nM GST-RNase L for 30 min at 30 °C. An aliquot (10 μl) of the reaction mixture was electrophoresed on a 20% polyacrylamide/7 M urea/1× Tris borate-EDTA gel. The labeled RNA in the gel was quantified using a Typhoon system (Amersham Biosciences). The activity of RNase L was expressed as a percentage of the cleaved product.

Molecular Modeling—The three-dimensional structure of the ankyrin repeat domain of mouse RNase L (mANK) was modeled from the crystal structures of human ANK (Protein Data Bank accession code 1WDY) at the SWISS-MODEL Internet server (www.expasy.org/swissmod/) (17-18, 19). The structure was displayed using the CueMol program (R. Ishitani, CueMol: Molecular Visualization Framework; cuemol.sourceforge.jp). A solvent-excluded surface of hANK was calculated using the program MSMS (20).

RESULTS

Scheme of Site-directed Mutagenesis—Wild-type hANK is composed of eight ankyrin repeat elements, designated 1–8 from the N terminus, forming a significantly curved structure (12). The ligand 2–5A fits into the concave side of hANK and interacts with residues from repeats 2 and 4. Repeat 2 interacts with the third AMP unit of pApApA (Fig. 1). Trp$_{60}$ in repeat 2 is responsible for the π-π stacking interaction with the adenine ring of the third AMP unit (Ade$_3$), whereas Asn$_{65}$ and Gln$_{68}$ in repeat 2 fix the same adenine ring by a hydrogen bond network (OD1(Asn$_{65}$)-N6(Ade$_3$) and ND2(Asn$_{65}$)-O(Wat)-N7(Ade$_3$)) and (OE1(Gln$_{68}$)-N6(Ade$_3$) and NE2(Gln$_{68}$)-N1(Ade$_3$)), respectively. In addition, Lys$_{89}$ electrostatically interacts with the phosphoryl residue of the third AMP moiety (Phos$_3$), and Phe$_{126}$ in repeat 4 is responsible for the π-π stacking interaction with the adenine ring of the first AMP moiety (Ade$_1$). Glu$_{131}$ in repeat 4 fixes Ade$_1$ by bifurcated hydrogen bonds (OE1(Glu$_{131}$)-N6(Ade$_1$) and OE2(Glu$_{131}$)-N1(Ade$_1$), whereas Tyr$_{135}$ in repeat 4 binds to the adenine ring of the second AMP moiety (Ade$_2$) by a single hydrogen bond (OH(Tyr$_{135}$)-N1(Ade$_2$)), and Arg$_{155}$ forms a salt bridge with the S'-monophosphoryl group of the first AMP moiety (Phos$_1$). In addition, an insertion helix αI (residues 159–164) constitutes a bottom of the first AMP moiety binding site.

During site-directed mutagenesis, each of the eight residues mentioned above was replaced by Ala, and the insertion αI-helix (residues 157–166) was deleted. All of the mutants were expressed in E. coli as His-tagged proteins and purified. Because the deletion mutant aggregated during purification, it was not further characterized. Although all of the substitution mutants were purified, the yield of the R155A mutant was low because of its tendency to precipitate.

2–5A Binding Ability of the hANK Mutants—The ability of purified wild-type and mutant hANKs to bind 2–5A was assayed using a static fluorescence technique. The fluorescence spectrum of hANK showed a peak at 340 nm, which was due to its single and solvent-exposed Trp residue (Trp$_{60}$) (Fig. 2A). Upon the addition of 2–5A, the fluorescence intensity of hANK was attenuated as the concentration of 2–5A was increased. Because these attenuations reflect changes in the environment of Trp$_{60}$ caused by 2–5A binding, these correlations allowed us to estimate the 2–5A binding ability of hANK. For wild-type hANK, the degree of fluorescence attenuation (ΔF) followed saturation kinetics relative to pApApA concentration (Fig. 2B). The $K_d$ value of wild-type hANK for pApApA was calculated as 0.10 μM from the equation shown under “Materials and Methods.”

The mutation of Gln$_{68}$ by Ala (Q68A) resulted in a saturation kinetics similar to that of wild-type and a slightly increased $K_d$ value (0.27 μM), indicating a minor contribution of the hydrogen bond formed between Glu$_{131}$ and Ade$_3$ for pApApA binding (Fig. 2 and TABLE TWO). The disruption of the hydrophobic bonds formed by Asn$_{65}$ (N65A) with Ade$_3$ affected more than just the case of Q68A. The simultaneous mutations of Asn$_{65}$ and Gln$_{68}$ (NAQA) resulted in an additive effect for impairing its affinity to 2–5A but still maintained a low affinity for the ligand. The single mutation of Tyr$_{135}$ (Y135A), which hydrogen bonded with Ade$_2$, also moderately reduced the affinity, as was the case with N65A. In contrast, the substitution of Ala for Glu$_{131}$ (E131A) resulted in a linear relationship of ΔF (rather than saturation kinetics) to pApApA, implying impaired ability of the mutant to bind 2–5A. In this case, we were not able to estimate a reliable $K_d$ value under the experimental conditions. The mutation of the residues involved in the salt bridge formations (K89A and R155A) gave results similar to those observed for E131A, indicating that these residues provide critical contributions to 2–5A binding. Two residues (Trp$_{60}$ and Phe$_{126}$) interact with 2–5A through π-π stacking effects. Because the F126A mutant also showed the linear relationship, the critical role of the residue in 2–5A binding was suggested. On the other hand, substitution of Trp$_{60}$ by Ala (W60A) resulted in the disappearance of the 340-nm fluorescence peak, because Trp$_{60}$ is the responsible fluorophore. Therefore, this method could not be applied to the analysis of the W60A mutant. The contribution of Trp$_{60}$ to 2–5A binding and RNase L function will be described in the next section.

Effects of Mutations on Full-length RNase L Activity—Because hANK is part of RNase L and does not exist independently in nature, we could not rule out the possibility that the effects of point mutations on hANK/pApApA binding were artifacts. To determine the effects of these mutations on overall RNase L activity, we constructed full-length RNase L protein with the various mutations.

FIGURE 2. 2–5A-dependent quenching of hANK fluorescence. A, a solvent-excluded surface of hANK was calculated using the program MSMS (20) as described under “Materials and Methods.” The red area indicates the location of Trp$_{60}$. B, the degree of fluorescence quenching (ΔF) was plotted against 2–5A concentration (0, 1.5, 3, 4.5, 6, 7.5, 9, 10.5, 12, 13.5, and 15 μM). Representative data of triplicate experiments are plotted. WT, wild type.
mutants as GST fusion forms (9, 11, 16). Because 2–5A activated the enzyme, RNase L mutants lacking 2–5A binding activity should be enzymatically inactive. Moreover, this assay used synthetic oligo(RNA)-bearing 5'-fluorescein (5'-fluorescein rC11UUC7–3') for better quantitative results. The molecular species of full-length RNase L are described with the prefix "RNL" to discriminate them from those of RNase L with deletion mutants.

We observed RNA degradation in RNL wild type, RNL N65A, RNL Q68A, RNL NAQA, and RNL Y135A (Fig. 3B), with $K_m$ for 2–5A of 4, 64, 23, 168, and 131 nM, respectively (TABLE THREE). These values could be correlated with the $K_d$ data obtained by analyzing the hANK species. That is, the mutations at Asn65 and Tyr135 affected the affinity for 2–5A more than that at Gln68, and the affinity of the Gln68 mutant for 2–5A was comparable with that of wild type. The combined double mutations, NAQA, additively reduced the RNase activity. The decrease of the $V_{\text{max}}$ value may represent that proper fitting of 2–5A through hydrogen bond interactions is required, not only for activation of RNase L, but also for efficient catalysis. As for the other mutants, no significant degradation was detected.

**Homology of the Ankyrin Repeat Domain of Human and Mouse RNase L**—The genes encoding RNase L are found in mice (21), rats (GenBank accession number NM182693) and red jungle fowl (chicken) (GenBank accession number NM001031267) as well as in humans. Because the functions of the mouse enzyme have been studied extensively (5, 22, 23), we compared the structures of mouse RNase L and hANK. We hypothesized that residues important for 2–5A recognition and binding should be conserved. Homology modeling of the ankyrin repeat domain from mANK was performed by taking advantage of a high sequence homology with human RNase L, with residues 21–305 of human RNase L and 21–304 of mouse RNase L having 65.6% sequence identity, allowing fairly accurate prediction of the structure of mANK (Fig. 4). The backbone of mANK could be superimposed nearly completely on that of hANK (Fig. 4A). According to this model, the positions and orientations of the side chains of the target residues identified in this study were highly conserved between the human and mouse proteins, with the exception of residue 135, which was Tyr in hANK and Arg in mANK (Fig. 4B). The 135th residue was not conserved, but the residues occupied a similar position enabling them to be able to form a hydrogen bond with Ade2. The model supported the result that the mutation of Tyr135 had a moderate effect on 2–5A binding, although the residue was not conserved. Mutagenesis at Gln68 of the human enzyme was less effective than that at Tyr135, despite this residue being conserved among all four species. This suggests that Gln68 shares 2–5A binding with Asn65 by constituting a hydrogen bond network.

## DISCUSSION

We have used structure-based site-directed mutagenesis to identify the human RNase L residues crucial for the recognition and binding of 2–5A. The contributions of each residue were verified using two different methods, fluorescence spectrometry and RNase L activation assay. Furthermore, the results of the RNase L activation assay indicate the validity of the hANK/2–5A interaction mode that was previously revealed by crystal structure analysis.

Substitution for either Trp60 or Phe126 significantly hampered the 2–5A binding ability of RNase L, as well as inactivating 2–5A-dependent RNase activity, indicating that the $\pi-\pi$ stacking interactions Trp60–Ade3 and Phe126–Ade1 are critical for 2–5A binding. In general, stacking effects are fundamental non-covalent interactions that are more common in the recognition of single-stranded nucleic acids. For example, stacking interactions are important for single-stranded nucleic acid
binding by the oligonucleotide/oligosaccharide binding fold (24, 25), single-stranded DNA-binding proteins (26, 27), and other RNA-binding proteins (28). They are also used by mRNA cap-binding proteins to recognize methylated guanosine (29). Thus, although 2–5A is an unusual nucleic acid, its recognition by RNase L is based on a relatively general mechanism.

Mutations of residues Lys\textsuperscript{89} and Arg\textsuperscript{155} also led to inactivation of RNase L, indicating the importance of the electrostatic interactions Lys\textsuperscript{89}, Phos\textsuperscript{3} and Arg\textsuperscript{155}, Phos\textsuperscript{1} for 2–5A binding. The positions and orientations of these residues were conserved in the structure of mANK, confirming that at least a single 5′-phosphoryl group is required for the efficient activation of human RNase L (30). Compared with the 2–5A trimer with the 5′-triphosphoryl group (pppApApA), the 2–5A trimer without the 5′-phosphoryl group (ApApApA, known as the “core” species) has only ~0.1% the ability to activate human RNase L. In addition, the tetramer or pentamer 2–5A core (ApApApA or ApApApApA) activates RNase L ~25-fold more efficiently than the trimer core. This improvement could be explained by the formation of a salt bridge between Arg\textsuperscript{155} and the phosphoryl group between the first and second AMP moiety of the 2–5A tetramer core, thus increasing the affinity of the latter to RNase L. In addition, Arg\textsuperscript{155} may stabilize the ankyrin repeat structure by forming a salt bridge with the side chain of Asp\textsuperscript{174} at the third ankyrin repeat (Fig. 5). As mentioned previously under “Results,” R155A mutant was prone to precipitation. If this precipitation were caused by the lack of a salt bridge between Arg\textsuperscript{155} and Asp\textsuperscript{174} leading to structural disorder, this structural stabilization would be another important role of Arg\textsuperscript{155}. The importance of Lys\textsuperscript{89} and Trp\textsuperscript{60} may be due to the inability of 2–5A dimers to activate RNase L. It has been reported that 2–5A dimers with 5′-triphosphoryl groups, at concentrations up to 1 mM, do not activate human RNase L (30).

The contribution of the four hydrogen bonds depended on their positions. Substitution of Glu\textsuperscript{68} or Asn\textsuperscript{65} by Ala had a limited effect on hANK and full-length RNase L. Even the double mutant of the residues still maintains the 2–5A binding and RNase activity. Accordingly, Ade\textsubscript{3} is fixed by the π–π stacking interactions with Trp\textsuperscript{60} primarily and by the hydrogen bondings secondarily. This suggests that N1 of Ade\textsubscript{3} can be substituted by other atoms when artificial 2–5A is designed. N6 of Ade\textsubscript{3} is, however, important for interaction with RNase L, because the position is recognized by both Asp\textsuperscript{65} and Gln\textsuperscript{68}. In practice, pppApApG, which possesses O6 instead of N6, showed no activation of RNase L (31).

Deletion of the region containing the α-helix also increased the inactivation of RNase L. This may have been due to the improper placement of Arg\textsuperscript{155} rather than the absence of the bottom framework of the 2–5A binding site. Fig. 5 shows the structures of wild-type hANK and Δα1, which were built by homology modeling. This model shows that Arg\textsuperscript{155} was relocated as a result of the deletion. This relocation results in a steric clash between Arg\textsuperscript{155} and Phos\textsuperscript{1}, and with a spoilage of the electrostatic interaction between Arg\textsuperscript{155} and Asp\textsuperscript{174}. Therefore, these mutants are likely to cause structural disorders. As mentioned under “Results,” both the Δα1 mutant and R155A were easy to aggregate, suggesting that the role of the extra α-helix may be to set Arg\textsuperscript{155} into its proper position.

There are numerous ankyrin repeat proteins, which share a common three-dimensional structure, i.e. helix-loop-helix structures with a β-hairpin/loop region projecting outward from the helices at a 90° angle stack side by side. Nevertheless, hANK is the sole molecule to bind 2–5A, due, at least in part, to the five interactions shown here. Optimal binding requires two π–π stacking interactions by Trp\textsuperscript{60} and Phe\textsuperscript{126}, one hydrogen bond by Glu\textsuperscript{131} and two salt bridges by Lys\textsuperscript{69} and Arg\textsuperscript{155}. All of these interactions should be taken into account when developing new ligands that activate RNase L. Conversely, it may be possible to use other ankyrin repeat proteins to bind 2–5A.

In conclusion, we have identified the crucial residues of human RNase L for the binding of its essential activator, 2–5A. The information is valuable for designing new anti-viral drugs based on the 2–5A antivirus system. Determination of the three-dimensional structure of the RNase L-2–5A-RNA ternary complex will provide further insight into the enzymatic mechanism of this unique protein.
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