Structural basis for recognition of 2',5'-linked oligoadenylates by human ribonuclease L

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An interferon-induced endoribonuclease, ribonuclease L (RNase L), is implicated in both the molecular mechanism of action of interferon and the fundamental control of RNA stability in mammalian cells. RNase L is catalytically active only after binding to an unusual activator molecule containing a 5'-phosphorylated 2',5'-linked oligoadenylate (2-5A), in the N-terminal half. Here, we report the crystal structure of the N-terminal ankyrin repeat domain (ANK) of human RNase L complexed with the activator 2-5A. This is the first structural view of an ankyrin repeat structure directly interacting with a nucleic acid, rather than with a protein. The ANK domain folds into eight ankyrin repeat elements and forms an extended curved structure with a concave surface. The 2-5A molecule is accommodated at a concave site and directly interacts with ankyrin repeats 2–4. Interestingly, two structurally equivalent 2-5A binding motifs are found at repeats 2 and 4. The structural basis for 2-5A recognition by ANK is essential for designing stable 2-5As with a high likelihood of activating RNase L.

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Introduction

In mammals, viral infections initiate an innate immune response predominantly mediated by type I interferons (IFNs) (Sen, 2001). Type I IFNs regulate the transcription of a number of genes that inhibit or block viral replication by diverse mechanisms (Stark *et al.*, 1998). There are three well-established antiviral pathways for IFN action: these three pathways are associated with the double-stranded RNA (dsRNA)-dependent protein kinase PKR (Williams, 1995), the Mx proteins (Pavlovic and Staeheli, 1991), and the 5'-triphosphorylated, 2',5'-phosphodiester-linked oligoadenylate (2-5A) system (Player and Torrence, 1998).

In the 2-5A system, treatment of cells with IFN activates genes encoding several 2',5'-linked oligoadenylate synthe-

tases (OASs) (Chebath *et al.*, 1987) and a single gene encoding ribonuclease L (RNase L) (Zhou *et al.*, 1993). The OASs are activated by binding to dsRNA (Hovanessian *et al.*, 1977), a frequent byproduct of viral infection. The activated OASs generate 2-5A from ATP. The crystal structure of porcine OAS1 was recently reported (Hartmann *et al.*, 2003). The mechanisms of its activation induced by dsRNA and its synthesis of 2-5A were discussed based on its structure and structure-based mutagenesis studies, although the structure contained neither activator nor substrate analogs. RNase L is known to be activated by binding to 2-5A, changing from an inactive monomer to a catalytically active homodimer (Dong and Silverman, 1995). The activated RNase L cleaves RNA containing dyads of UU, UA, AU, AA, and UG (Floyd-Smith *et al.*, 1981; Wreschner *et al.*, 1981). The RNA degradation inhibits protein synthesis and thus inhibits viral replication.

RNase L is an unusual ribonuclease because it requires the activator molecule 2-5A to catalyze the hydrolysis of single-stranded RNA. 2-5A is itself very unusual, consisting of a type of oligoadenylates with 2',5' internucleotide linkages, in contrast to the typical 3',5' linkages found in RNA and DNA. 2-5A is unstable in cells due to the activities of phosphodiesterases and phosphatases. Early studies revealed the requirement of at least two 5'-phosphoryl groups and at least three 2',5'-linked adenylyl residues for the efficient activation of murine RNase L (Kerr and Brown, 1978). Thereafter, it was shown that only a single 5'-phosphoryl group on the trimer of 2',5'-linked oligoadenylate was required for the full activation of human RNase L (Haugh *et al.*, 1983; Yoshimura *et al.*, 2002).

The human form of RNase L is a 741-amino-acid protein with a molecular mass of 83 543 Da (Zhou *et al.*, 1993). RNase L consists of three domains, namely the N-terminal ankyrin repeat domain, the protein kinase homology domain, and the C-terminal ribonuclease domain. The N-terminal ankyrin repeat domain, a region containing nine ankyrin-like macromolecular recognition repeats (the ninth ankyrin repeat is incomplete), is responsible for 2-5A binding, and the C-terminal domain is responsible for catalytic activity (Dong and Silverman, 1997; Nakanishi *et al.*, 2004). The binding of 2-5A to RNase L is thought to induce a conformation change in the enzyme or result in the unmasking of an interaction domain, permitting dimerization and activation of RNase L.

Recently, the enzyme was proposed as a candidate risk factor for hereditary prostate cancer (Casey *et al.*, 2002; Xiang *et al.*, 2003). Among the naturally occurring mutants of RNase L that have been examined, only the Arg462Gln variant showed low RNase activity, and this variant was also shown to be significantly associated with prostate cancer. Therefore, elucidation of all of the amino-acid residues that might influence RNase L activity (i.e., 2-5A binding, dimerization, and catalysis) remains necessary.

Here, we present the crystal structure at 1.8 Å resolution of the N-terminal ankyrin repeat domain of human RNase L complexed with 2-5A. To our knowledge, this is the first

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structural view of an ankyrin repeat structure directly interacting with a nucleic acid, rather than with a protein. The crystal structure of the ANK/2-5A complex clearly shows that the bound 2-5A molecule directly interacts with ankyrin repeats 2–4. We have re-evaluated the structure–function relationship studies of RNase L and 2-5A in terms of the present crystal structure analysis. The structural basis for 2-5A recognition by ANK is essential for designing stable 2-5As with a high likelihood of activating RNase L.

Results

Structure determination

The crystallization trials have to date not successfully obtained the crystals of full-length human RNase L, as only a minute amount of the full-length recombinant human RNase L has been obtainable; however, crystals of the N-terminal ankyrin repeat domain (ANK) of human RNase L (Figure 1A) were successfully obtained. Addition of 2-5A to the ANK solution was essential for obtaining crystals. For this study, a 2-5A trimer with 5'-monophosphate (p5'(A2'p5')₂A) was used for crystallization (Figure 1B). We determined the crystal structure of the ANK/2-5A complex by the molecular replacement (MR) method using the coordinate of a 'consensus ankyrin repeat protein' (Kohl *et al*, 2003) (PDB code: 1MJ0) as the template for constructing a search model, and we refined the resulting model to an *R*-factor of 0.202 (*R*_{free} of 0.230) at 1.8 Å resolution. The final model consisted of residues 21–305, 220 water molecules, and one 2-5A molecule. The refinement statistics are summarized in Table I.

Overall fold of ANK

ANK folds into eight ankyrin repeat elements and forms an extended curved structure with a groove running across the long concave surface (Figure 1C and D). Previous primary structure analysis suggested that RNase L had nine ankyrin repeats, but the ninth ankyrin repeat is incomplete (Hassel *et al*, 1993). However, this prediction for RNase L differs from the present crystal structure of ANK, which consists of eight ankyrin repeats (R1–R8 in Figure 1C and D). Residues 306–333, corresponding to the incomplete ninth repeat (Hassel *et al*, 1993), are disordered. As in other ankyrin repeat proteins (Sedwick and Smerdon, 1999), each repeat is formed by ~33 amino-acid residues and consists of pairs of antiparallel α -helices stacked side by side and which are connected by a series of intervening β -hairpin motifs. In general, the structure is shaped similar to a cupped hand (Jacobs and Harrison, 1998). There is a noticeable curvature across the 'palm', such that the surface created by the β -hairpins (fingers) and the α 1 'inner' helices is concave, whereas that formed by the α 2 'outer' helices, that is, the back of the cupped hand, is convex (Figure 1D). The 2-5A molecule fits in the concavity and directly interacts with ankyrin repeats 2–4.

Ankyrin repeats of RNase L

A structure-based sequence alignment of the eight ankyrin repeats of RNase L is shown in Figure 2. Definition of the ankyrin repeat consensus is based on the exon boundaries of the *ankyrin* gene (Lux *et al*, 1990). This definition separates the two β -strands of the β -hairpin structure and incorporates the most commonly occurring nonconserved elements within

Figure 1 Crystal structure of ANK complexed with 2-5A. (A) Structural and functional domains of RNase L. Ankyrin repeats are shown starting with blue at repeat 1 and ending with red at repeat 8. (B) Structure of the predominant trimeric species of 2-5A ((pp)p(A2'p5')₂A) (Kerr and Brown, 1978). (C, D) Surface (top) and ribbon (bottom) representations of the ANK/2-5A complex. Ankyrin repeats (R1–R8) are shown as in (A). The bound 2-5A molecule is shown as a ball-and-stick model. The view in (D) was obtained by rotating the view in (C) by 90°.

Table 1 Data collection and refinement statistics of ANK

<i>Data collection statistics</i>	
X-ray source	PF-AR NW12
Wavelength (Å)	0.978
Resolution range (outer shell) (Å)	40–1.8 (1.9–1.8)
Observed reflections (no sigma cutoff)	154 126
Unique reflections	35 922
Multiplicity	4.3 (3.7)
Mean $\langle I/\sigma(I) \rangle$	5.5 (2.1)
B-factor (Wilson plot) (Å ²)	27.3
R _{sym} (%)	8.4 (33.8)
Completeness (%)	99.7 (99.5)
<i>Refinement statistics</i>	
Resolution range (Å)	40–1.8
No. of reflections	
Working set	34 084
Test set	1791
R-factor	0.202
Free R-factor	0.230
No. of protein atoms ^a (average B-factors (Å ²))	2184 (28.3)
No. of water molecules (average B-factors (Å ²))	220 (37.0)
No. of 2-5A atoms (average B-factors (Å ²))	67 (23.5)
R.m.s. deviations	
Bond distances (Å)	0.008
Bond angles (deg)	1.119
B-factors related by main-chain bonds (Å ²)	0.669
B-factors related by side-chain bonds (Å ²)	1.825
Ramachandran plot	
Most favored (%)	92.6
Additional allowed (%)	7.4

^aHis tag, N-terminal residues (1–20), and C-terminal residues (306–333) are disordered.

the repeating unit (Sedwick and Smerdon, 1999); however, the insertion helix (α I: residues 159–164) of ANK is incorporated between R4 and R5 (Figures 1C, D, and 2). We therefore consider the ankyrin repeat motif of RNase L to be defined as a strand–helix–loop–helix–loop–strand ($\beta\alpha\alpha\beta$) structure.

Residue Gly(2) in β 1 is invariant across all repeats (Figure 2), except for R1. Hereafter, the consensus ankyrin repeat numbering will be shown in italics and parentheses. This glycine residue allows a sharp turn within the β -hairpin structure. Since R1 lacks β 1, Gly(2) is not conserved in R1. Highly conserved hydrophobic residues (*Leu*(6) and *Ala*(9) from the inner helix α 1, and *Leu*(21) and *Leu*(22) from the outer helix α 2) are involved in packing interactions within and between repeats, giving rise to a continuous hydrophobic core. As previously noted (Jacobs and Harrison, 1998), the inner helix α 1 contains hydrophobic residues (*Ala*(9) and *Ala/Val*(10)) smaller than those in the outer helix α 2 (*Leu*(21) and *Leu*(22)), causing the stack of ankyrin repeats to curve toward the inner helix α 1. The signature *Thr*(4)–*Ala/Pro*(5)–*Leu*(6)–*X*(7) motif characteristic of ankyrin repeats forms a tight turn that initiates the inner helix α 1. Because the inner helix α 1 starts at four residues upstream from *Thr*(4) in R1, *Thr*(4) is not conserved in R1. Residues Gly(25) and Ala(26) allow a sharp turn at the end of the outer helix α 2. The side chain of the highly conserved polar residue *Asn*(29) is involved in hydrogen bonds with the carbonyl oxygen atom of Gly(25) and the imino nitrogen atom of X(27) from the next repeat.

Several insertions are found within and between repeats. Met191 and Glu262 are inserted after the outer helix α 2 within R5 and R7, respectively. Each of these insertions allows for a substitution of the Gly(25)–Ala(26) residues in

R5 or R7, which enable a sharp turn at the end of α 2. Four residues (Ser213–Asp214–Asp215–Ser216) are inserted after the inner helix α 1 within R6. The insertion of two acidic residues would be expected to affect the electrostatic properties of the surface. Glu57 is inserted between R1 and R2. A total of 10 residues (Thr157 to Lys166), including the insertion helix α I (residues 159–164), are inserted between R4 and R5. These insertions also exist for mouse and rat RNase Ls.

2-5A binding mode

ANK is the first representative of an ankyrin repeat protein for which the structure has been solved in the form of a nucleotide (rather than a protein) complex. Here, the electron density, shown at 1.8 Å resolution, corresponding to the bound 2-5A molecule is very well defined (Figure 3A). Thus, we were able to unequivocally determine the conformations of 2-5A and the surrounding amino-acid residues, as well as the location of water molecules. As shown in Figure 1C and D, only one 2-5A molecule is bound to the concavity of ANK. Since ANK is responsible for 2-5A binding in RNase L (Dong and Silverman, 1997), the present crystal structure is consistent with previous observations demonstrating that the dimerization of RNase L requires the binding of one 2-5A molecule per RNase L monomer and that the enzymatic activity of RNase L is also maximized at a 1:1 2-5A:RNase L stoichiometry (Cole *et al*, 1996). As shown in Figure 3A, the bound 2-5A molecule adopts an extended conformation in which the first and second adenine rings (Ade1 and Ade2, respectively) are in an *anti* conformation, whereas the third adenine ring (Ade3) is in a *syn* conformation. The first and second ribose rings have ³E (C3'-*endo*) puckering, whereas the third ribose has ⁴E (C4'-*exo*) puckering (IUPAC-IUB, 1983). The 2-5A molecule is accommodated in the concavity and directly interacts with ankyrin repeats 2–4 (Figure 1C and D). In the present crystal structure analysis, we used a 2-5A trimer with 5'-monophosphate (p5'(A2'p5')₂A) for crystallization (Figure 1B). The 2-5A molecule is thus considered to be a 2',5'-linked trimer of 5'-AMP.

The first AMP moiety of the 2-5A directly interacts with the fourth repeat (R4) of ANK (Figure 3A and B). The 5'-phosphate group of the first AMP (Phos1) forms bifurcated salt bridges with the side chain of Arg155. The side chain of Arg155 is fixed by bifurcated salt bridges with the side chain of Asp174 (Figure 3A). The adenine ring of the first AMP (Ade1) is stacked between the side chain of Phe126 and the adenine ring of the second AMP (Ade2), and is fixed by bifurcated hydrogen bonds with the side chain of Glu131, that is, OE1(Glu131)—N6(Ade1) and OE2(Glu131)—N1(Ade1). The side chain of Phe126 also stacks with the guanidino group of the side chain of Arg155, forming a quadruplex (Arg155–Phe126–Ade1–Ade2) of stacking interactions (Figure 3B).

The second AMP moiety of 2-5A interacts only slightly with ANK. The 5'-phosphate group of the second AMP (Phos2) is exposed to solvent, and no direct interactions are found between Phos2 and the surface of ANK. The adenine ring of the second AMP (Ade2) is stacked with Ade1 as described above, and is fixed by a single hydrogen bond with the side chain of Tyr135, that is, OH(Tyr135)—N1(Ade2). The 3'-OH group of the second AMP is involved in a hydrogen bond network and is fixed on the protein

Figure 2 Structure-based sequence alignment of the eight ankyrin repeats (R1–R8) of human, mouse, and rat RNase Ls. Secondary structural elements are shown on top and are colored as in Figure 1. The 2-5A binding motifs, equivalently found in repeats 2 and 4, are boxed in pink. The two GKT motifs are boxed in gray.

surface via water molecules. The second AMP appears to be rather weakly fixed on the ANK surface relative to the two ends of the 2-5A molecule.

The third AMP moiety of 2-5A directly interacts with the second repeat (R2) of ANK (Figure 3A and C). The 5'-phosphate group of the third AMP (Phos3) forms a salt bridge with the side chain of Lys89. The adenine ring of the third AMP (Ade3) is stacked with the side chain of Trp60, and is fixed by a hydrogen bond network involving the side chains of Gln68 (OE1(Gln68)—N6(Ade3) and NE2(Gln68)—N1(Ade3)) and Asn65 (OD1(Asn65)—N6(Ade3)), as well as a water molecule (O(Wat)—N7(Ade3) and O(Wat)—ND2(Asn65)). The side chain of Trp60 also stacks with the CD–CE–NZ bonds of the side chain of Lys89, forming a triplex (Lys89–Trp60–Ade3) of stacking interactions (Figure 3C). Unlike Ade1 and Ade2, Ade3 adopts a *syn* conformation. The 2'- and 3'-OH groups of the third AMP do not interact with either the protein surface or with water molecules.

Interestingly, the 2-5A binding residues in the R4 and R2 of ANK are located at the structurally equivalent position of the ankyrin repeat (Figure 2, boxed in pink) and these residues play a functionally equivalent role. The side chains of Arg155 in R4 and Lys89 in R2 form salt bridges with Phos1 and Phos3, respectively (Figure 3B and C). The side chains of

Phe126 in R4 and Trp60 in R2 stack with Ade1 and Ade3, respectively. Furthermore, a quadruplex (Arg155–Phe126–Ade1–Ade2) and a triplex (Lys89–Trp60–Ade3) of stacking interactions are observed at R4 and R2, respectively. The side chains of Glu131 in R4 and Asn65 in R2 form hydrogen bonds with Ade1 and Ade3, respectively. It should be noted here that these 2-5A binding residues are completely conserved among human, mouse, and rat RNase Ls (Figure 2).

Two insertions are found around the 2-5A binding region of ANK. One of these insertions is Glu57, which is inserted between R1 and R2. This insertion extends a finger between R1 and R2, which constitutes a wall of the third AMP binding site (Figure 1D). The other insertion is the insertion helix $\alpha 1$ (residues 159–164), which is inserted between R4 and R5. This helix constitutes the bottom of the first AMP binding site (Figure 1D).

The eight ankyrin repeats of ANK are kinked at the middle

As shown in Figure 1C and D, the eight ankyrin repeats of ANK adopt an extremely curved structure. To obtain insight into the curved structure of ANK, we superposed repeats 1 and 2 of other ankyrin repeat proteins on those of ANK (Figure 4). In general, a longer ankyrin repeat protein has a more concave structure than a shorter ankyrin repeat protein

Figure 3 Recognition of 2-5A by ANK. (A) Stereodiagram showing the mode of 2-5A binding to ANK. The carbon atoms of ANK are colored as in Figure 1, and those of the bound 2-5A molecule are shown in white. Possible hydrogen bonds or salt bridges are indicated by dashed lines, and distances (in Å) are given. The refined model is superimposed on the weighted $2|F_o| - |F_c|$ map (1.5σ , yellow) and the $|F_o| - |F_c|$ omit map of 2-5A (4.5σ , orange), calculated at 1.8 Å resolution. Bound water molecules are shown as spheres (cyan). (B, C) Recognition of the first and third AMP moieties of 2-5A by repeats 4 and 2, respectively, of ANK.

does; for example, the structure of ankyrin (Michaely *et al*, 2002), which consists of 12 ankyrin repeats, is more concave than those of Bcl-3 (Michel *et al*, 2001) and gankyrin (Krzywda *et al*, 2004), which each consist of seven ankyrin repeats (Figure 4). However, it is of note that the degree of curvature across the eight ankyrin repeats of ANK is more remarkable than that of the 12 ankyrin repeats of ankyrin in two directions (Figure 4).

Figure 4 The kinked structure of ANK. (A, B) Superposition of repeats 1 and 2 of human gankyrin (cyan, PDB ID: 1UOH), human Bcl-3 (yellow, 1K1A), and human ankyrin (green, 1N11) on those of ANK (magenta, 1WDY). The views in (A) and (B) are the same as those shown in Figure 1C and D, respectively.

As shown in Figure 4, the eight ankyrin repeats of ANK are clearly kinked at R4 and R5 (15° and 11° in Figure 4A and B, respectively, as compared with ankyrin). A total of 10 residues (Thr157 to Lys166), including the insertion helix $\alpha 1$ (residues 159–164), are inserted between R4 and R5 (Figure 2). This insertion of a longer polypeptide chain enables more bending. In addition, the more bulky side chains of Phe143 and Tyr145, instead of Leu residues, are found in the outer helix $\alpha 2$ of R4 (Figure 2). These arrangements appear to result in the wider separation of the outer helices $\alpha 2$ of R3, R4, and R5.

The relationship between the higher degree of curvature of ANK and the 2-5A binding is still unclear, as we have not yet obtained the structure of ANK in the absence of 2-5A. However, the results of time-resolved fluorescence measurements and protease protection assays have indicated that the conformation of ANK does indeed change upon 2-5A binding (M Nakanishi *et al*, unpublished data). Therefore, we suggest here that the higher degree of curvature of ANK is due to 2-5A binding.

Discussion

2-5A binding domain

Extensive efforts employing various techniques have been directed toward functional analyses of RNase L. As summarized in Figure 5A, we can re-evaluate these results in terms of the newly established crystal structure of the ANK/2-5A complex. The results of the N-terminal truncations of RNase L (Dong and Silverman, 1997) are completely consistent with the present crystal structure analysis showing that the 2-5A molecule directly interacts with ankyrin repeats 2–4 (Figure 1C and D). Truncation of the 23 N-terminal amino acids preceding the first ankyrin repeat (R1), which are disordered in the present crystal structure and are not

Figure 5 Re-evaluation of previous structure-function relationship studies of ANK and 2-5As in terms of the present crystal structure analysis. (A) Biochemical studies of ANK truncation mutants (Dong and Silverman, 1997). (B) The two GKT motifs in repeats 7 and 8. The side chains of Lys240 and Lys274 are involved in an intrarepeat salt bridge (dashed lines) with the side chains of Glu248 and Glu282, respectively, rather than in 2-5A binding. (C) Schematic representation of the 2-5A binding mode. The side chains of ANK are colored as in Figure 1. Hydrogen bonds or salt bridges and a hydrogen bond network of water molecules are indicated by red and gray dashed lines, respectively, and distances (in Å) are given. Aromatic residues, Trp60 and Phe126, involved in the stacking interactions with 2-5A (see Figure 3B and C) have been omitted for clarity. Water molecules are shown as 'Ow'. The [n] indicates important interactions for oligonucleotide [n] in (D). (D) Chemical studies of 2-5A and its analogs (Player and Torrence, 1998). The relative activities of 2-5A analogs, as compared with canonical 2-5A [4], for RNase L activation are shown as '+ + +' (1/2~), '+ + ' (1/20~1/2), '+ ' (1/200~1/20), and '-' (~1/200).

involved in interactions with 2-5A, does not result in the loss of 2-5A binding activity (NΔN(24-741)). Other N-terminal truncations, that is, mutants NΔR1(57-741), NΔR1-2(91-741), NΔR1-3(124-741), and NΔR1-6(238-741), lacking 1, 2, 3, and 6 ankyrin repeats, respectively, do show a loss of 2-5A binding activity. Although R1 is not directly involved in 2-5A binding, it constitutes a wall of the third AMP moiety of the 2-5A binding site (Figure 1D). Moreover, results showing the C-terminal truncation of RNase L (CΔC(1-335)) (Dong and Silverman, 1997) are also consistent with the present crystal structure analysis demonstrating that the N-terminal ankyrin repeat domain of RNase L is sufficient for 2-5A binding activity.

Before the present crystal structure analysis was conducted, two GKT motifs, Gly239-Lys240-Thr241 in R7 and Gly273-Lys274-Thr275 in R8 (Figure 2, boxed in gray), had been postulated as the 2-5A binding motifs (Zhou *et al*, 1993; Diaz-Guerra *et al*, 1999). Substitution of the two lysines with asparagines greatly reduced the affinity for 2-5A (Zhou *et al*, 1993). Similarly, one mutant (NΔN/CΔR7-9C(24-237)) (Dong and Silverman, 1997), which is truncated at both termini and lacks R7-R9, with which Lys240 and Lys274 are involved, was shown to lose 2-5A binding activity (Figure 5A). These observations, together with the lack of structural information about ANK at the time the observations were made, led to the assumption that the two GKT motifs were the 2-5A binding motifs. However, the present crystal structure has clearly revealed that the side chains of Lys240 and Lys274 are involved in an intrarepeat salt bridge with the side chains of Glu248 and Glu282, respectively (Figure 5B), rather than in 2-5A binding.

Although the structural role of the two GKT motifs remains unclear without additional structural studies, one possible explanation for their role would be that the salt bridges in R7 and R8 contribute to maintaining the folding of the ankyrin repeat domain, and hence to its integrity. Consequently, structural perturbation at R7 and R8 greatly diminishes the 2-5A binding ability of RNase L, even though these regions are not directly involved in 2-5A binding.

Structure-activity relationship of 2-5A and its analogs

Several chemical approaches have produced a considerable amount of information about the atoms or groups of atoms in 2-5A that interact with RNase L. Similar to the re-evaluation of previous functional studies of RNase L described above, we can now re-evaluate previous studies of the structure-activity relationship of 2-5A and its analogs (Torrence *et al*, 1994; Player and Torrence, 1998) in terms of the present crystal structure (Figure 5C and D).

The most intriguing feature of the 2-5A molecule is the presence of the 2',5'-linked phosphodiester bonds (Figures 1B and 5C). Lesiak *et al* (1983) showed that replacing either of the 2',5'-linkages in a 2-5A trimer, [1] and [2] in Figure 5D, decreased binding by 1 and 2 orders of magnitude; however, substituting both linkages [3] reduced binding drastically. The present structure thus accounts for the structural significance of the two 2',5'-linkages, based on the assumption that the interactions between R4 and R2 of ANK and the first and third AMP, respectively, of 2-5A (Figures 3B, C, and 5C) must be maintained. Replacing the first 2',5'-linkage with a 3',5'-linkage [1] would drastically shift the adenosine moiety of the second AMP and the phosphate of the third AMP outwardly, and then the stacking interaction between Ade1 and Ade2 and the salt bridge between Phos3 and Lys89 would be weakened. Replacing the second 2',5'-linkage [2] would shift the adenosine moiety of the second AMP inward (or drastically shift Phos3 outward), resulting in a weakening of Ade1-Ade2 stacking and possibly the steric hindrance of Ade2 at the protein surface (or the Phos3-Lys89 salt bridge might be weakened). Replacing both the 2',5'-linkages [3] additively was found to weaken both the Ade1-Ade2 stacking and the Phos3-Lys89 salt bridge. Since the 2',5'-linkages orient backbone-inwards toward the bases (Figure 1B), rather than away from the bases as in 3',5'-linkages, 2-5A with both

the 2',5'-linkages enabled all of the essential interactions between 2-5A and ANK (Figure 5C).

The requirement for the 5'-phosphoryl group is an interesting feature of the 2-5A molecule. It was previously demonstrated that only a single 5'-phosphoryl group on the trimer of a 2',5'-linked oligoadenylate, [4], [5], [6], and [7] in Figure 5D, is required for the full activation of human RNase L (Kerr and Brown, 1978; Haugh *et al*, 1983; Yoshimura *et al*, 2002). The present structure reveals that the 5'-monophosphoryl group is tightly fixed by bifurcated salt bridges with Arg155 (Figures 3B and 5C), which is consistent with the finding that the 5'-monophosphoryl group is sufficient for binding to ANK. It is also of note that there is an extra space in the concavity of ANK (Figure 1D, top) to accommodate the 5'-di or -triphosphoryl group of 2-5A. Since [6], which was used for the present crystal structure analysis to prepare ANK/2-5A complex, was about 40% less effective than [4] (Yoshimura *et al*, 2002), additional interactions between the β - or γ -phosphate of [4] and ANK may exist.

Studies on the ribose moiety of 2-5A showed that only the 3'-OH of the second AMP moiety of 2-5A is required for the efficient activation of RNase L (Torrence *et al*, 1988). The present structure demonstrates that the 3'-OH group of the second AMP is involved in the hydrogen bond network and is fixed on the protein surface via water molecules (Figure 5C). Our observations are consistent with those of a previous study suggesting that the 3'-OH group of the central adenosine of trimer 2-5A may form a hydrogen bond with an acceptor in RNase L (Torrence *et al*, 1994).

The length of the 2-5A species is critical for the activation of RNase L. While the dimer, [8] in Figure 5D, failed to activate RNase L, the longer 2-5A oligomer [9], a 5'-monophosphate type of [9], or the canonical 2-5A trimer [4] maximally activated purified recombinant RNase L at 1 nM in the poly-U degradation assay (Dong *et al*, 1994). Thus, the trimeric 2-5A and tetrameric 2-5As maximally activated RNase L at the same concentration. This finding is consistent with our obtained structure showing that the Phos3 and Ade3 of the third AMP of 2-5A are involved in binding with ANK (Figures 3C and 5C) and that the dimeric form of 2-5A lacking the third AMP is clearly insufficient for binding. On the other hand, the longer 2-5A oligomers appear to be harmless for interactions with ANK. If there were strong interactions between the fourth AMP of a longer 2-5A and the R1 of ANK (Figure 1C and D), the tetrameric 2-5As described above could be more effective than the trimeric 2-5A. Thus, we assume that the fourth or longer AMP moieties of 2-5A would extend out of ANK and would therefore have no or only limited possibility of interacting with ANK.

As regard the role of the adenine rings in 2-5A, it has been reported that RNase L has drastically lower affinity for cytidine, uridine, or inosine analogs, [10] in Figure 5D, compared to its adenine counterpart [6] (Torrence *et al*, 1984). Similarly, [11] failed to activate RNase L (Drocourt *et al*, 1982). Torrence *et al* (1984) suggested that RNase L interacts with N1 and/or N6 of the adenine ring of 2-5A. As suggested by Torrence *et al*, the present structure shows an adenine ring-specific recognition that includes both N1 and N6 atoms of the adenine ring at the first and the third AMP moiety of 2-5A by R4 and R2 of ANK, respectively (Figures 3B, C, and 5C). Interestingly, the second adenine ring was

less important for the activation of RNase L ([12] and [13]) (Imai *et al*, 1985). Use of uridine analogs of 2-5A also produced similar results (Kitade *et al*, 1991a). These results are consistent with our structure showing that the second adenine ring (Ade2) is recognized by only a single hydrogen bond with the nonconserved side chain of Tyr135 (Figures 3A and 5C).

We have previously shown that 2-5A molecules modified at the 8-position (8-bromo or 8-methyl) of Ade3, which is thereby forced into a *syn* conformation, are more effective activators of RNase L, whereas those modified at the 2-position of Ade3, which is thereby forced into an *anti* conformation, are less effective ([14] and [15]) (Kitade *et al*, 1991b, 1998, 2000; Yoshimura *et al*, 2002). In accord with these results, we demonstrated in the present study that the third adenine ring (Ade3) is in a *syn* conformation, whereas the first and second adenine rings (Ade1 and Ade2, respectively) of 2-5A are in an *anti* conformation (Figures 3A and 5C). The *syn* conformation of the Ade3 of 2-5A is stabilized by a hydrogen bond network involving the side chains of Asn65 and Gln68, and a water molecule (Figures 3C and 5C).

In summary, we concluded that the Phos1 and Ade1 of the first AMP moiety, the 3'-OH of the second AMP moiety, and the Phos3 and Ade3 of the third AMP moiety of 2-5A are essential for achieving interactions with ANK. In other words, 2-5A is not a perfect molecule for interacting with RNase L; for example, the 3'-OH group of the first AMP, the Phos2 and Ade2 of the second AMP, and the 2'- and 3'-OH groups of the third AMP could be modified for optimal binding, as described in previous structure-function relationship studies on 2-5A analogs (Torrence *et al*, 1994; Player and Torrence, 1998). Since our obtained structure revealed that these groups are solvent-exposed, the introduction of a functional group at these positions may enable direct or water molecule-mediated interactions between the group and the protein surface, and may thereby enhance binding and/or activation ability with respect to RNase L.

Surface properties of ANK and the implications for inter- and intramolecular interactions

Several X-ray structures of protein-protein complexes involving ankyrin repeats have been determined (Sedwick and Smerdon, 1999). Although the mechanisms of binding between target molecules and ankyrin repeats are known to vary considerably (aromatic, hydrophobic, hydrophilic, or electrostatic), the binding to target molecules commonly involves contacts with β -hairpin fingers and the surface of the inner helices α 1 facing into the concave region of the ankyrin repeat domain. The residues involved in the contact are not conserved in the consensus sequence and are not structurally constrained, and therefore they can be ideally located in order to carry out binding functions (Sedwick and Smerdon, 1999).

Here, we provide the first structural view of the binding of an ankyrin repeat protein to a nonprotein partner. Similar to protein-protein complexes involving ankyrin repeats, the concave region of ANK is involved in 2-5A binding. Since the various structures of nucleic acid binding proteins have shown that the basic surface patch of the proteins is responsible for binding to the exposed phosphate moiety of the target nucleic acid, it is not unreasonable to expect that ANK must possess a basic surface patch for 2-5A binding.

Figure 6 Electrostatic surface potential of ANK. The surfaces are shown in blue and red to indicate positive and negative charges, respectively, from 20 to -20 kT. The view at top is the same as the view provided in Figure 1C. (A) Surface potential of ANK without 2-5A. The bound 2-5A is shown as a ball-and-stick model and has been omitted from the potential calculation. The phosphate binding sites are indicated by yellow arrows. A remarkable basic patch near repeats 3 and 4 is indicated within a green circle. (B) Surface potential of the ANK/2-5A complex. The negative patch arising from bound 2-5A is indicated in a yellow ellipsoid.

Although local basic patches have been found for the binding sites for Phos1 and Phos3 of 2-5A, the majority of the concave region of ANK is covered by acidic patches (Figure 6A). Since the binding mechanism of 2-5A to ANK not only involves electrostatic interactions but also aromatic and hydrophilic interactions (Figure 3), it is not surprising that only a small portion of the concave surface is positively charged. As observed in the ankyrin repeat–protein complexes described above, the residues involved in 2-5A binding are not conserved in the consensus ankyrin repeat sequence (Figure 2, boxed in pink), thus enabling unique 2-5A binding roles.

The electrostatic surface properties of ANK suggest the potential interactions with the kinase and RNase domains of the full-length RNase L. In the amino-acid sequence of human RNase L, there is a group of negatively charged residues at 451–459 (EDVENEDE) in the kinase domain, and positively charged residues at 677–684 (KHKKMKLK) in the RNase domain (Zhou *et al*, 1993). The overall acidic feature of ANK (Figure 6) appears to be favorable for interactions with a basic patch on the other parts of the full-length molecule, and thus acidity may be directly involved in the inhibition/activation mechanism of the positively charged RNase domain. This acidic feature of ANK is furthermore enhanced by 2-5A binding (Figure 6B). The enhancement of the negative charge may be a trigger of dimerization and activation of the full-length RNase L. Although the basic patch around R3 and R4 at the convex region is remarkable (Figure 6), the basic residues in the $\alpha 2$ helices in R3 and R4 are not well conserved among mammalian RNase Ls (Figure 2).

Conclusions

Here, we present the crystal structure of the N-terminal ankyrin repeat domain of human RNase L complexed with activator 2-5A containing unusual 2',5' internucleotide linkages. Because the catalytically active form of RNase L is a potent antiviral and anticellular protein, stable derivatives of 2-5A able to penetrate cell membranes might eventually provide novel therapeutic agents for viral infection and tumor development. The structural basis for 2-5A recognition by ANK is essential for designing stable 2-5As with a high likelihood of activating RNase L. Based on the structural information obtained about the ANK/2-5A complex discussed here, the design and synthesis of new 2-5A analogs are currently underway in our laboratory.

It has been reported that the binding of 2-5A to RNase L converts RNase L from its inactive monomer to its catalytically active homodimer. However, the following issues regarding the molecular mechanism of RNase L activity remain unexplained: (1) it is unclear why RNase L is inactive in the absence of 2-5A, and (2) it is unclear how the binding of 2-5A enables RNase L to dimerize and act on target RNA. Although the crystal structure of the ANK/2-5A complex did not provide any definitive answers to the above questions, it clearly revealed the 2-5A binding site of RNase L. Thus, the present structure serves as a starting point for further structure–function relationship studies on RNase L.

Materials and methods

Expression and purification of HisXaANK

A 1.0-kb cDNA encoding residues 1–333 of the N-terminal ankyrin repeat domain of human RNase L (ANK) was PCR-amplified from pKRRNL (Yoshimura *et al*, 2002) with primers 5'-cgcaggcctatgga gacaggatcataac-3' and 5'-gcgaagctttaagcaggagggtg-3' (underlinings indicate added *StuI* and *HindIII* sites, respectively), digested with *StuI* and *HindIII*, and then ligated into the corresponding sites of a pQE-30Xa expression vector (pQEHsXaANK). *Escherichia coli* JM109 harboring pQEHsXaANK was grown in 4 l of LB medium at 303 K. When OD₆₀₀ reached 0.6, expression was induced with 0.5 mM IPTG. The bacterial cells were collected after an additional 7 h cultivation by centrifugation at 5000 g for 10 min. The cell pellet was resuspended in buffer A (25 mM Tris–HCl (pH 7.5) and 10% glycerol), and then homogenized by sonication disruption on ice. Cell debris was removed from the homogenate by centrifugation at 12 000 g for 15 min at 277 K. The supernatant was loaded onto a SP-Sepharose FF column (bed volume: 40 ml) pre-equilibrated with buffer A. After an extensive wash with buffer A, HisXaANK was eluted from the column with buffer A containing a linear gradient of NaCl, from 0 to 0.8 M, and the fractions containing HisXaANK were pooled. The pooled fractions were combined and then loaded onto a TALON metal affinity column (bed volume: 2 ml) pre-equilibrated with buffer A. Elution was carried out with buffer A containing 150 mM imidazole. The fractions containing HisXaANK were then subjected to gel filtration on a Sephacryl S200 column (bed volume: 180 ml) pre-equilibrated with buffer A. The elution containing HisXaANK was pooled and concentrated with YM-10 ultrafiltration membrane, and stored at 253 K.

Crystallization of ANK

We used purified HisXaANK (hereafter simply ANK), containing a factor Xa-digestion site between His tag and ANK, for crystallization without removal of the His tag. 5'-O-monophosphoryl-adenylyl(2' → 5')adenylyl(2' → 5')adenosine (p5'(A2'p5'))₂A, 2-5A trimer with 5'-monophosphate) was synthesized in our laboratory as described previously (Kitade *et al*, 1998). Crystallization of ANK was carried out in a way similar to the method described for non-tag ANK (Tanaka *et al*, in preparation). Briefly, a stock solution of 0.5 mg/ml ANK was concentrated using a Centricon-10 (Millipore), yielding a working solution of 3 mg/ml ANK. The protein solution was mixed

with 20 mM 2-5A trimer with 5'-monophosphate dissolved in pure water at a volume ratio of 9:1. Crystallization was carried out at 293 K by the hanging-drop vapor diffusion method. In the best case, a droplet was prepared by mixing equal volumes (1.5 + 1.5 μ l) of the protein solution (2.7 mg/ml ANK and 2 mM 2-5A) described above and a reservoir solution (500 μ l) containing 15% (w/v) PEG8000, 0.2 M sodium acetate, and 20% (v/v) glycerol in 0.1 M Hepes buffer at pH 7.5. Rod-shaped crystals with typical dimensions of approximately $0.02 \times 0.02 \times 0.25$ mm³ could be grown in 2 weeks.

Data collection

Since the crystallization conditions of ANK described above contained 20% (v/v) glycerol in reservoir solutions, X-ray data collections could be performed at cryogenic conditions without further addition of a cryoprotectant. The data collection was performed by rotation method at 100 K using an ADSC Q210 CCD detector with synchrotron radiation ($\lambda = 0.978$ Å at beam line NW12 of the PF-AR). The crystals belong to an orthorhombic space group $P2_12_12_1$ with cell dimensions of $a = 63.20$ Å, $b = 72.83$ Å, and $c = 82.63$ Å ($\alpha = \beta = \gamma = 90^\circ$). An assumption of one molecule per asymmetric unit leads to an empirically acceptable V_M value of 2.38 Å³/Da, corresponding to a solvent content of 48%. The best diffraction data from a native crystal were collected up to 1.8 Å resolution and processed with the program packages DPS (Ross-mann and van Beek, 1999) and CCP4 (Collaborative computational project, number 4, 1994). The data collection statistics are summarized in Table I.

Structure determination

Initial phase determination was performed by the MR technique. The coordinate set of 'consensus ankyrin repeat protein' (Kohl *et al*, 2003) (PDB code: 1MJ0), having an approximately 15% sequence identity with ANK and consisting of five consensus ankyrin repeats, was used as a template of the search model. The search model was constructed as follows: the ankyrin repeats 1–4 of one coordinate set of 1MJ0 were superimposed on the ankyrin repeats 2–5 of the other coordinate set of 1MJ0. The fifth ankyrin repeat of the former coordinate set is regarded as the additional sixth ankyrin repeat of the latter coordinate set. Thus, a coordinate set of consensus ankyrin repeat protein consisting of six ankyrin repeats was constructed. Crossrotation and translation functions were calculated using the program AMoRe (Navaza, 1994) in the CCP4 suite. After one cycle of simulated-annealing refinement using the

program CNS (Brunger *et al*, 1998) (R -factor of 0.468 and free R -factor of 0.517 in the resolution range 40–1.8 Å), initial model building was automatically carried out using the program ARP/wARP (Lamzin and Wilson, 1993). Without the simulated-annealing refinement before the ARP/wARP model building, the auto model building failed. Manual rebuilding was carried out using the program XtalView (McRee, 1999). Difference electron density maps clearly showed density corresponding to the bound 2-5A trimer with 5'-monophosphate. Crystallographic refinement was performed with the maximum-likelihood procedure of the program REFMAC (Murshudov *et al*, 1997) in the resolution range of 40–1.8 Å. The stereochemistry of the model was verified using the program PROCHECK (Laskowski *et al*, 1993). The final model consists of residues 21–305, 220 water molecules, and one 2-5A molecule. His tag, N-terminal residues (1–20), and C-terminal residues (306–333) are disordered. The refinement statistics are summarized in Table I.

Structure analysis

Least-squares comparisons of the molecular models were carried out using the program MATRAS (Kawabata, 2003). Figures were produced using the program ViewerPro (Figures 1C, D, 3B, C, 4, and 5B), the two programs Raster3D (Merritt and Murphy, 1994) and XtalView (McRee, 1999) (Figure 3A), and the program SPOCK (Figure 6).

Accession number

The atomic coordinates have been deposited in the Protein Data Bank with the entry code 1WDY.

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