Origin of Higher Affinity to RNA of the N-terminal RNA-binding Domain than That of the C-terminal One of a Mouse Neural Protein, Musashi1, as Revealed by Comparison of Their Structures, Modes of Interaction, Surface Electrostatic Potentials, and Backbone Dynamics

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Musashi1 is an RNA-binding protein abundantly expressed in the developing mouse central nervous system. Its restricted expression in neural precursor cells suggests that it is involved in maintenance of the character of progenitor cells. Musashi1 contains two ribonucleoprotein-type RNA-binding domains (RBDs), RBD1 and RBD2, the affinity to RNA of RBD1 being much higher than that of RBD2. We previously reported the structure and mode of interaction with RNA of RBD2. Here, we have determined the structure and mode of interaction with RNA of RBD1. We have also analyzed the surface electrostatic potential and backbone dynamics of both RBDs. The two RBDs exhibit the same ribonucleoprotein-type fold and commonly make contact with RNA on the β-sheet side. On the other hand, there is a remarkable difference in surface electrostatic potential, the β-sheet of RBD1 being positively charged, which is favorable for binding negatively charged RNA, but that of RBD2 being almost neutral. There is also a difference in backbone dynamics, the central portion of the β-sheet of RBD1 being flexible, but that of RBD2 not being flexible. The flexibility of RBD1 may be utilized in the recognition process to facilitate an induced fit. Thus, comparative studies have revealed the origin of the higher affinity of RBD1 than that of RBD2 and indicated that the affinity of an RBD to RNA is not governed by its fold alone but is also determined by its surface electrostatic potential and/or backbone dynamics. The biological role of RBD2 with lower affinity is also discussed.

In Drosophila, during sensory organ development, Notch signaling directs the asymmetry between neuronal and non-neuronal lineages (1), and a zinc finger transcriptional repressor, Tramtrack69, acts downstream of Notch as a determinant of non-neuronal identity (2, 3). Drosophila musashi was identified as a gene whose mutation causes a severe defect in the development of external sensory organs (4). It was demonstrated that the Drosophila Musashi protein represses the expression of the Tramtrack69 protein translationally in a neuronal lineage by binding to a cis-acting element in the 3′-untranslated region of tramtrack69 messenger RNA (5, 6).

Also, in mammalian neural development, neural RNA-binding proteins play important roles by regulating gene expression post-transcriptionally. During mammalian central nervous system development, neurons and glial cells are thought to be generated from common neural precursor cells located in the periventricular area (7). Musashi1, a mammalian homologue of Drosophila Musashi, is expressed in the neural precursor cells that exist in the ventricular area of the developing mouse neural tube (6, 8), and it is expressed complementarily to another neural RNA-binding protein, Hu, a member of the Elav subfamily localized in differentiated neurons in the central nervous system (11–13). This combination of neural RNA-binding proteins might be important for cell fate determination in the developing central nervous system (14). Musashi1, which is evolutionally conserved in different species, may be required for the self-renewal and neuronal potential retention of neural stem cells in the developing mouse nervous system (6, 15), as judged from the analogy to its Drosophila homologue. In fact, it was demonstrated that Musashi1 translationally regulates the expression of a mammalian numb gene, which encodes a membrane-associated antagonist of Notch signaling, by interacting with numb mRNA (16).

Mouse Musashi1 consists of 362 residues and has two ribonucleoprotein (RNP)1-type RNA-binding domains (RBDs), RBD1 and RBD2 (8, 17). The RNP-type RBD is one of the most common eukaryotic protein sequence motifs (18), being found in hundreds of proteins (19–22). 46% of the amino acid sequence is identical between RBD1 and RBD2. When similar amino acids are taken into account, the homology reaches as high as 75%. Previously, we reported that RBD1 binds to poly(U) and poly(G), whereas the binding of RBD2 to RNA homopolymers was not detected under the same conditions (17). Then, the target sequences of mouse Musashi1 were de-
terminated by the in vitro selection method (23, 24). (G/A)U_AGU
(n = 1–3) sequences were identified as consensus ones (16).
Here, we report the gel retardation experiments indicating that
RBD1 alone specifically binds to one of the target sequences,
r(GUUAGUAGGUAGUU) (T4-3), whereas such binding is
not detected for RBD2 under the same conditions.

We reported the structure and qualitative analysis of the backbone dynamics of RBD2 (25). Additionally, we succeeded in
detecting the binding of RBD2 to the target RNA and in charac-
terizing the mode of interaction by NMR (25). Here, to eluci-
date the origin of the much higher affinity of RBD1 to RNA
than that of RBD2 and the basis of the recognition of the target
RNA, we have determined the structure and mode of interac-
tion with RNA of RBD1. We have also compared the surface
electrostatic potentials of the interactive surface as to RNA for
the two RBDs. Moreover, we have compared the backbone
dynamics of the two RBDs by means of quantitative model-free
analysis. These studies have indicated that the higher affinity
to RNA is brought about by the positive surface potential of the
interactive surface electrostatic potentials of RBD1 and RBD2 were calculated with
Musashi1 RBD1-(20–103) was grown in M9 minimal medium with
amines and Gln residues, and H
1 of a Trp residue.

EXPERIMENTAL PROCEDURES

Gel Retardation Experiments—Gel retardation experiments were
performed as described previously (16). 32P-labeled r(GUUAGU-
AGUUAGUU) (T4-3) was incubated with various amounts of RBD1,
RBD2, or their tandem link (RBD1-RBD2) at room temperature for
30 min. The complexes were run on a 10% polyacrylamide gel containing
0.5× Tris borate buffer and EDTA. The gels were then dried and
exposed to XAR autoradiography film (Eastman Kodak Co.).

Sample Preparation for NMR—Escherichia coli B/L21(DE3), trans-
formed with a plasmid, pMMA, containing DNA encoding mouse
Musashi1 RBD1-(20–103) was grown in M9 minimal medium with
15NH4Cl as a sole nitrogen source and then harvested. The cells were
lysed by sonication. After purification with ammonium sulfate, RBD1
was loaded onto the DE52 anion-exchange column (Whatman), and then
the flow-through fraction was loaded onto a HiTrap-SP cation-exchange
column (Amersham Biosciences). The fraction eluted with NaCl was
loaded onto a HiTrap-Q anion-exchange column (Amersham Biosciences), and the flow-through fraction was collected. RBD1 was dia-
lyzed against 20 mM sodium phosphate (pH 6.0), 10 mM
[3H]dithiothreitol, and 1 mM Na3C3O7, and then concentrated to 2 mM
using Centricon-3 (Amicon). RNA oligomers were prepared as described
previously (25).

NMR Spectroscopy—NMR spectra were recorded with a Bruker
DRX600 spectrometer equipped with a quadruple resonance probe with
X, Y, and Z gradients. 2,2-Dimethyl-2-silapentane-5-sulfonate was
used as an internal chemical shift reference. The following NMR exper-
iments were performed to assign the resonances of RBD1 and RBD2 and to obtain
distance and dihedral angle constraints: NOESY, TOCSY, double quan-
tum filtered COSY, 15N-edited NOESY-HSQC, 15N-edited TOCSY-
HSQC, and HNHA experiments. For titration experiments, a concen-
trated RNA solution was added step by step to the RBD1 solution,
and the 1H-15N HSQC spectra were recorded at each step. Spectra were pro-
cessed with XWIN-NMR (Bruker), Felix (MSI), NMRPipe (26), and
Capp/Pipp/Stapp (27).

15N T1, T2, and NOE spectra of RBD1 and RBD2 were recorded and
processed as described previously (28). T1 spectra were recorded at
various protein concentrations, 2.0, 0.4, and 0.25 mM for RBD1 and 2,
0.6, and 0.3 mM for RBD2.

Structure Calculations—870 distance constraints (230 intraresidue,
254 sequential, 130 medium range, and 283 long range distance con-
straints) were obtained for RBD1 from NOESY and 15N-edited NOESY-
HSQC spectra, as described previously (25, 28, 29). Thirty-seven dihe-
dral angle constraints for ψ were obtained from an INHHA experiment.
Eight dihedral angle constraints for χ1 were also obtained, together
with stereospecific assignment of the β-methylene protons of AMX
and the γ-methyl protons of valine residues. Additionally, in the later stage
of the calculations, 42 distance constraints for 21 hydrogen bonds were
included for slowly exchanging amide protons within the identified
secondary structure elements.

Structure calculations were carried out using distance and dihedral
angle constraints with a simulated annealing protocol supplied with
X-PLOR v. 3.8 (30). A final set of 15 structures was selected from 100
calculations on the basis of the criteria of the smallest residual energy
values. None of them violated the distance constraints by more than 0.4
Å or dihedral angle constraints by more than 4°. A mean structure was
obtained by averaging the coordinates of the 15 structures, and a
restrained energy minimized mean structure was obtained by energy
minimization of the mean structure under the constraints. The quality
of the structures was evaluated with PROCHECK (31).

Surface Electrostatic Potentials and Backbone Dynamics—The sur-
face electrostatic potentials of RBD1 and RBD2 were calculated with
GRASP (32). The backbone dynamics of RBD1 and RBD2 were quanti-
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RESULTS

Difference in RNA Binding Activity among RBD1, RBD2, and RBD1-RBD2—RNA binding activities of RBD1, RBD2, and their tandem link with a native linker (RBD1-RBD2) were examined by gel retardation experiments. r(GUUAGUUAGUUAGUU) (T4-3), which was obtained as one of the target sequences by means of the in vitro selection method (16), was used for the examination. T4-3 comprises at least two consensus sequences, (G/A)U (n = 1–3), tandemly, and even three if overlapping is allowed. T4-3 was incubated with RBD1, RBD2, or RBD1-RBD2 and run on the gel. Binding of RBD1 to T4-3 was observed, whereas that of RBD2 was not detected under the experimental conditions applied (Fig. 1A). From the densitometric analysis of the results, the dissociation constant for the RBD1-RNA complex was determined to be 3–9 μM. Binding of RBD1-RBD2 to T4-3 was also observed (Fig. 1B), and the dissociation constant was determined to be 40–88 nM.

Resonance Assignments and Structure Calculations for RBD1—Sequential assignments of the main chain and side chain 1H and 15N resonances of RBD1 were made in the same way as reported for RBD2 (25). The assignments of the main chain H8 and N resonances, together with those of H2 and N2 of Gln residues and H1 and N1 of a Trp residue, are presented in Fig. 2.

The structures of RBD1 were calculated on the basis of distance and dihedral angle constraints. The structural statistics are shown in Table I. The backbone root mean square deviation of the 15 final structures versus the mean structure was 0.49 ± 0.10 Å for the 21–95 region, excluding less well defined loop 3 (53–62) and loop 4 (69–71). The backbone atoms (Å) for 21–95 region excluding loop 3 (53–62) and loop 4 (69–71) and all heavy atoms (Å) are 0.49 ± 0.10 and 0.95 ± 0.11, respectively.

Although the binding of RBD2 to RNA was not detected in gel retardation experiments (Fig. 1A), it was detected with NMR (25). This is explained by the fact that higher RBD2 and RNA concentrations and the absence of salts and a detergent for NMR experiments facilitate the formation of the complex (25). In this sense, NMR is a suitable method for the characterization of a weak interaction that is hard to detect by means of biochemical methods.

Comparison of Surface Electrostatic Potentials between RBD1 and RBD2—The surface electrostatic potential of the β-sheet side, which interacts with RNA, was calculated for RBD1 and RBD2 and compared (Fig. 5). Notably, the β-sheet surface of RBD1 is entirely positively charged, whereas that of
RBD2 is largely neutral. It was confirmed that these characters are seen for all final structures of RBD1 and RBD2. RNA is negatively charged due to phosphate groups. Thus, from the viewpoint of electrostatic interactions, RBD1 is expected to bind RNA more strongly than RBD2.

**Backbone Dynamics of RBD1 and RBD2**—To shed light from a dynamical point of view on the recognition of RNA by Musashi1, the backbone dynamics of RBD1 and RBD2 were analyzed quantitatively, in the same way as described in detail for another RNP-type RBD (28). Examination of the concentration-dependence of the $15N\ T_2$ value confirmed that RBD1 and RBD2 are in a monomeric form at the protein concentrations of 0.4 and 0.6 mM, respectively. Model-free analysis was performed for relaxation data recorded at these concentrations.

First, it was found that an axially symmetric diffusion model can well describe the overall rotational diffusion of both RBD1 and RBD2. Three relaxation data, $R_1(=1/T_1)$, $R_2(=1/T_2)$, and NOE, were fitted for each RBD with five models consisting of the following subsets of extended model-free parameters: 1) $S^2$; 2) $S^2$ and $\tau_e$; 3) $S^2$ and $R_{ex}$; 4) $S^2$, $\tau_e$, and $R_{ex}$; and 5) $S^2$, $\tau_e$, and $\tau_v$, where $S^2$ is the square of a generalized order parameter, $\tau_e$ an effective correlation time, $R_{ex}$ is a chemical exchange term,
and $S^2$ is the square of an order parameter for an internal motion on a fast time scale (28, 33–36). The numbers of residues fitting each model were as follows for RBD1: 1) 10, 2) 5, 3) 12, 4) 25, and 5) 13 residues. Those for RBD2 were as follows: 1) 15, 2) 5, 3) 5, 4) 28, and 5) 10 residues.

The results of analyses of the backbone dynamics are summarized in Fig. 6, residues being mapped that require either a chemical exchange term, $R_{ex}$, greater than 1 s$^{-1}$, or a two-time scale spectral density function with a correlation time for slow internal motion, $\tau_c$. $R_{ex}$ and $\tau_c$ represent the flexibility on the milli- and pico- to nanosecond time scales, respectively. It is notable that the two central $\beta$-strands, $\beta_1$ and $\beta_3$, of RBD1 exhibit flexibility on either the milli- or pico- to nanosecond time scale, whereas those of RBD2 do not.

**DISCUSSION**

The mouse Musashi1 protein is drawing great attention because of its possible dominant role in regulation of the self-renewal and neuronal potential retention of neural stem cells in the developing nervous system (16). It is supposed that Musashi1 translationally regulates the expression of a certain gene such as numb, which is involved in Notch signaling, through interaction with mRNA of that gene (16). Musashi1 possesses two RBDs, RBD1 and RBD2. Despite the high sequence homology between them, RBD1 binds to target RNA much more strongly than RBD2. Here, we have elucidated the mechanism underlying the higher affinity of RBD1 to RNA. The study also suggests how Musashi1 is able to bind to the target RNA effectively.

Although a short antiparallel $\beta$-sheet has been detected exclusively for RBD1, a similar $\beta$-sheet-like structure has also been found for RBD2 (25). Thus, the fold is essentially identical for RBD1 and RBD2, a four-stranded antiparallel $\beta$-sheet being packed against two $\alpha$-helices (Fig. 3, B and C). Either the Phe or Tyr residue is conserved at the positions of Phe-23 and Phe-65 of RBD1 (Phe-112 and Phe-154 of RBD2) in RNP-type RBDs (20, 21, 37). The stacking interactions of these residues of RNP-type RBDs with the bases of the target RNA are one of the major forces for the stabilization of a protein-RNA complex. In fact, mouse Musashi1 and Drosophila Musashi with the mutation of these residues cannot bind to RNA (5, 16). Although the orientations of the aromatic rings of Phe-23 and Phe-65 of RBD1 are somehow different from those of Phe-112 and Phe-154 of RBD2, all of these rings are exposed to the solvent for the achievement of the stacking interactions with bases on the $\beta$-sheet.

A slightly greater right-handed twist of the $\beta_1$- and $\beta_3$-strands was observed for RBD1 in comparison with that of RBD2. We noticed a similar difference between RBD1 and RBD2 of an RNP-type U1A protein on inspection of their structures deposited in the Protein Data Bank. It is interesting that in the cases of both proteins, RBD1, which exhibits higher affinity to RNA, exhibits a slightly greater right-handed twist of the $\beta_1$- and $\beta_3$-strands. The greater twist of these $\beta$-strands may be favorable for amino acid residues to interact with RNA.

The mode of interaction with the target RNA is also identical for RBD1 and RBD2 of Musashi1 in the sense that the $\beta$-sheet...
is the interactive surface (Fig. 4, A and B). Thus, studies on the protein fold and mode of interaction with RNA have left the origin of the higher affinity of RBD1 to RNA to be answered largely from different viewpoints.

It is notable that the surface electrostatic potential of the \( \beta \)-sheet, which interacts with RNA, differs drastically between RBD1 and RBD2, despite the essentially identical fold (Fig. 5, A and B). The positive surface potential is prevailing for the \( \beta \)-sheet side of RBD1. On the other hand, the surface potential of the \( \beta \)-sheet side of RBD2 is largely neutral, and even the negative surface potential has been found for some portions. Apparently, the electrostatic character of RBD1 is favorable for the binding of negatively charged RNA as compared with that of RBD2. Thus, the higher affinity of RBD1 to RNA can be accounted for from the electrostatic viewpoint of the interactive surface.

The amino acid residues that contribute to the formation of the positive surface for the \( \beta \)-sheet side of RBD1 are mostly conserved in RBD2. In the case of RBD1, the side chains of basic residues, Arg and Lys, are located on the \( \beta \)-sheet, where they form a large positive surface area, those of acidic residues, Asp and Glu, being spread away from the \( \beta \)-sheet without disturbing the positive surface. In the case of RBD2, on the other hand, the side chains of basic residues are spread away from the \( \beta \)-sheet, and those of some acidic residues are located on the \( \beta \)-sheet, which results in the mostly neutral and even partially negative surface of the \( \beta \)-sheet. In this way, the drastic difference in the surface electrostatic potential arises.

A remarkable difference between RBD1 and RBD2 of Musashi1 was also noticed in dynamics. In particular, the central two \( \beta \)-strands, \( \beta 1 \) and \( \beta 3 \), of RBD1 exhibit flexibility on the milli- or pico- to nanosecond time scale, whereas those of RBD2 do not (Fig. 6, A and B). The two Phe residues (Phe-23 and Phe-65 of RBD1 and Phe-112 and Phe-154 of RBD2) located in \( \beta 1 \) and \( \beta 3 \), respectively, undergo key stacking interactions with RNA bases, as described earlier. Thus, the flexibility of RBD1 may be utilized in the recognition process because it allows different conformational states to be accessed and facilitates an induced fit. In fact, the utilization of flexibility in recognition has been reported for other RNP-type RBDs, U1A and hnRNP D (28, 38). Thus, the flexibility of Musashi1 RBD1 may be favorable for the induction of ideal mutual positioning for strong stacking between the aromatic rings of the Phe residues and RNA bases. The flexibility of RBD1 may also be utilized by other residues to interact with RNA. On the other hand, the lack of flexibility of the \( \beta 1 \)- and \( \beta 3 \)-strands of Musashi1 RBD2 may restrict the induction of ideal mutual positioning of the Phe rings and RNA bases, which may result in weaker binding. The chemical shift perturbations of Phe-23 and Phe-65 of RBD1 are large, −80 and 110 Hz, respectively. Although the perturbation of Phe-112 of RBD2 is relatively large, −75 Hz, that of Phe-154 of RBD2 is rather small, −30 Hz. These results seem to be consistent with the idea of the better stacking for RBD1, although the backbone NH group, which is located relatively far from the side chain aromatic ring, is occasionally not a good indicator as to stacking interactions. Thus, the higher affinity of RBD1 to RNA can also be rationalized from a dynamical viewpoint.

The \( \beta 1 \)- and \( \beta 3 \)-strands approximately correspond to the RNP2 hexamer and RNP1 octamer sequences, respectively, both of which are highly conserved in RNP-type RBDs. Therefore, the difference in flexibility of the \( \beta 1 \)- and \( \beta 3 \)-strands between the two RBDs is not brought about by the sequence difference, but is supposed to originate from the global character of the proteins.

r(GUUAGUAGUAGU) (T4-3) comprises at least two consensus sequences, G/AU_\text{AGU} (n = 1–3), tandemly, and even three consensus sequences tandemly if overlapping is allowed. The chemical shift perturbation experiment to detect the interactions was also carried out with r(GUUAGU), which only possesses a single consensus sequence. The perturbations obtained with r(GUUAGU) were almost the same as those with r(GUUAGUAGUAGU) (data not shown). This suggests that the binding of a single RBD can be achieved at a single consensus sequence.

Musashi1 binds to numb mRNA and translationally regulates the expression of the numb gene (16). The putative binding sequence of Musashi1 present in numb mRNA,
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t(GUAGUAGU), is composed of two consensus sequences arranged tandemly in an overlapped way (16). When Musashi1 comprising both RBD1 and RBD2 binds to numb mRNA, it is supposed that RBD1 and RBD2 bind in two consensuses sequences, respectively. In this case, RBD1, the affinity of which to RNA is much higher than that of RBD2, may play a primary role in the binding. However, although the affinity of RBD2 to RNA is much lower, RBD2 may still be able to play a certain role. For example, even if the dissociation constant of an RBD2-RNA complex is 10−3 M, the joining of RBD2 to RBD1 theoretically could increase the affinity by 1000 times maximally. Actually, it was shown experimentally that the joining of RBD2 to RBD1 increased the affinity to T4-3 by ~100-fold (dissociation constants: 3–9 μM to 40–88 nM), as described previously. This increase in affinity may be necessary for the regulation of the numb gene by Musashi1. Thus, the contribution of RBD2 could potentially be critical. There is another possibility that if the affinity of RBD2 to RNA is as high as that of RBD1, Musashi1 may bind to numb mRNA too strongly for turn over. Thus, the lower affinity of RBD2 may be suitable for Musashi1 to exert a biological function. In this context, it was interesting to find that the affinity of RBD to RNA can be easily tuned by changing the surface electrostatic potential and/or the backbone dynamics, the fold itself remaining unchanged.

The assumption of a structural genomics approach is that the function of a protein can be deduced from its structure. This reminds us of the necessity of being aware that although the fold of RBD1 and RBD2 is essentially identical, their affinity to RNA is drastically different. To deduce the function of a protein, the surface electrostatic potential and backbone dynamics should also be considered, together with fold.

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