Comparative Mutational Analysis of the Double-stranded RNA Binding Domains of Xenopus laevis RNA-binding Protein A*

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Xenopus laevis RNA-binding protein A is a ubiquitously expressed, double-stranded RNA-binding protein that is associated with the majority of cellular RNAs, ribosomal RNAs, and hnRNAs. X. laevis RNA-binding protein A contains three copies of the double-stranded RNA-binding domain (dsRBD) in tandem arrangement. Two of them, x11 and x12, belong to the type A group of dsRBDs that show strong homologies to the entire length of a defined consensus sequence. The x13 domain, in contrast, is a type B dsRBD which only matches the basic C-terminal end of the dsRBD consensus sequence. Here we show that only x12 but neither x11 nor x13 are able to bind double-stranded RNA substrates in vitro, suggesting that different dsRBD copies have varying RNA binding activities. By fine mapping mutagenesis of the isolated x11 domain, we identified at least two central aromatic amino acids and a C-terminal α-helix that are indispensable for dsRNA binding. Furthermore, we show that different charge distributions within the C-terminal α-helices of x11 and x12 seem responsible for the different RNA binding behaviors of these two dsRBDs. Analyses of the RNA binding properties of constructs containing various combinations of different dsRBDs reveal that type A dsRBDs exhibit a cooperative binding effect, whereas type B dsRBDs show a rather low binding activity, thus contributing only to a minor extent to a stable RNA-protein interaction.

RNA-binding proteins are required for a wide variety of important cellular and developmental functions ranging from RNA processing and editing to RNA transport, localization, stabilization, and translational control of certain mRNAs (reviewed in Refs. 1–7). Binding of target RNAs by these proteins is frequently mediated by specific RNA-binding domains (RBDs).1 Amino acid homologies and structural similarities have allowed the definition of several conserved RNA binding motifs, including the RNP motif, also referred to as the RNA recognition motif, the RGG box, the arginine-rich motif, the zinc-finger motif, the Y-box, the KH motif, and the double-stranded RNA-binding domain (dsRBD). Typically, these motifs are characterized by specific consensus sequences (reviewed in Ref. 8). Detailed structural information is only available for a limited number of RBDs but, where available, has allowed a more detailed insight into the mechanism of RNA-protein interaction (8, 9).

An RNA-binding domain that exclusively binds double-stranded RNA or RNA-DNA is the so-called double-stranded RNA-binding domain (dsRBD). The dsRBD is approximately 70 amino acids in length and can be found in at least 20 known or putative RNA-binding proteins from different organisms (Fig. 1) (10–12). Two types of dsRBDs can be distinguished. Type A dsRBDs show a strong homology to the entire length of a defined consensus sequence, whereas type B dsRBDs are highly conserved at their basic C terminus but fit the overall consensus at their N terminus only poorly (Fig. 1) (12).

Interestingly, the dsRBD fails to bind single-stranded nucleic acids (neither RNA nor DNA) or double-stranded DNA in vitro (10, 12). This unique binding characteristic could possibly be explained by the fact that double-stranded RNA molecules occur exclusively as A-form helices where the minor groove is shallow and broad, thus providing access to hydrogen bonding atoms (reviewed in Ref. 13). A 20-base pair duplex RNA has originally been described as a sufficient binding site for dsRBDs (10). Nonetheless, other data indicate that the minimal binding site for dsRBDs could be as short as 11 base pairs of a duplex RNA (14, 15).2 Several proteins contain multiple copies of dsRBDs (Fig. 1). It has been shown, however, that not all copies present in a given protein exhibit identical RNA binding abilities and functions. The double-stranded RNA-activated protein kinase (PKR), for instance, contains one type A and one type B dsRBD. Mutational analyses suggest that the type A dsRBD is more important for dsRNA binding and activation of the kinase than the type B dsRBD (11, 15–19). Similarly, Drosophila Staufen, a protein required for the proper localization of maternal RNAs in the developing embryo, contains three type A and two type B domains. Here, only the third dsRBD is capable to bind dsRNA in vitro.3 Furthermore, human TAR-binding protein (TRBP), a protein originally identified by its ability to bind the stem loop in HIV-1 TAR transcripts, contains two type A and one type B dsRBDs (20). Deletion data demonstrated that the second type A dsRBD but not the type B copy is needed for dsRNA binding in vitro (20, 21).

Secondary structure predictions of the dsRBDs found in PKR kinase suggest the formation of two α-helices on either end of the domain (17). Mutational analyses of these two dsRBDs further demonstrated the importance of the basic C-terminal α-helix for dsRNA binding (11, 19). Lately, NMR data of the third dsRBD of Drosophila Staufen and the dsRBD of Escherichia coli RNase III were published

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1 The abbreviations used are: RBD, RNA-binding domain; dsRBD, double-stranded RBD; PKR, double-stranded RNA-activated protein kinase; TRBP, TAR-RNA-binding protein; NMR, nuclear magnetic resonance; Xlrpa, X. laevis RNA-binding protein A; PCR, polymerase chain reaction.
2 M. Jantsch, unpublished data.
3 D. St. Johnston, personal communication.

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Mutational Analysis of the dsRNA Binding Domain

(14, 22). Both studies determined an α-β-β-α secondary structure element arrangement in which the two terminally positioned α-helices lie on one face of the three-stranded anti-parallel β-sheet region. These NMR studies also indicate that several conserved aromatic and basic residues interspersed throughout the whole sequence are packed toward one side of the domains analyzed to form a hydrophobic surface, possibly contacting RNA (14, 22). The mechanism of RNA binding by dsRBDDs, however, remains unknown to this point.

Here we report an extensive mutagenesis study of the dsRBDDs of Xenopus laevis RNA-binding protein A (Xlrba). Xlrba is a 33-kDa protein isolated from an ovary-specific cDNA library by its ability to bind double-stranded RNA (12). In vitro Xlrba is associated with the majority of cellular RNAs, ribosomal RNAs, and hnRNAs, possibly acting as a general ribonucleoprotein for double-stranded RNAs.4 Sequence homologies identify Xlrba as the Xenopus homologue of human TRBP. Like human TRBP, Xlrba exhibits two type A dsRBDDs and one type B dsRBD in tandem arrangement (Fig. 1).

Our mutagenesis study allowed the identification of specific amino acids and structures within the dsRBD required for dsRNA binding in vitro. The use of three different double-stranded RNA substrates, namely poly(I·C), U1 snRNA, and 18S rRNA (Pharmacia Biotech Inc.) were partially hydrolyzed in 100 mM sodium carbonate buffer, pH 10.2, at 70°C for 40 min to get an average fragment size of 50 nucleotides. Using T4 polynucleotide kinase, 10 µg of RI and 10 µg of RI-C were each end-labeled with [γ-32P]ATP (6000 Ci/µmol, 10 µCi/µl). Labeled RNAs were annealed by heating to 70°C and subsequent slow cooling; then labeled RNAs were finally purified over a Sephadex G-10 column. The entire 20 µg of annealed dsRNA (specific activity, approximately 3 × 106 cpm/µg) were incubated with the blot. Incubation conditions and washes were as described (12). To determine the amount of RNA bound to certain constructs, areas of interest were cut out from the blot and subjected to scintillation counting. The obtained values were used to determine the molar ratio of bound RNA to protein on the blot.

Radiolabeled U1 snRNA or 18 S rRNA were produced by in vitro transcription of the cloned genes in the presence of [α-32P]ATP using T7 RNA polymerase. Template for the U1 snRNA was a cloned chicken U1 gene (kindly provided by Iain Mattaj). As a template for the 18 S RNA, we used a cloned 18 S RNA gene from Arabidopsis thaliana (kindly provided by A. Bachmair). Blots were incubated with 10 µg of radiolabeled RNA with a specific activity of 2 × 106 cpm/µg. To determine the amounts of bound RNA, regions of interest were cut out from the blot and assayed for scintillation counting.

Expression of Fusion Proteins and Northwestern Filter Binding Assays—Protein expression and Northwestern RNA binding assays were performed as reported previously (12). For the double-stranded RNA substrates, poly(I·C) and poly(rI·rC) were each end-labeled with [γ-32P]ATP (6000 Ci/µmol, 10 µCi/µl). Labeled RNAs were annealed by heating to 70°C and subsequent slow cooling; then labeled RNAs were finally purified over a Sephadex G-10 column. The entire 20 µg of annealed dsRNA (specific activity, approximately 3 × 106 cpm/µg) were incubated with the blot. The SSU snRNA was a clone of the chicken U1 gene. As a template for the 18 S RNA, we used a cloned 18 S RNA gene from Arabidopsis thaliana (kindly provided by A. Bachmair). Blots were incubated with 10 µg of radiolabeled RNA with a specific activity of 2 × 106 cpm/µg. To determine the amounts of bound RNA, regions of interest were cut out from the blot and assayed for scintillation counting.

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Quantification of Northwestern Assays—Equal amounts of E. coli lysates containing overexpressed proteins were loaded on four identical SDS gels. While one gel was used for staining with Coomassie, the other three were blotted and used for Northwestern probing with radiolabeled poly(I·C), U1 snRNA, and 18 S rRNA. The amount of overexpressed protein was determined by densitometric scanning of Coomassie-stained gels. The corresponding Northwestern signals were quantified using a PhosphorImager (Molecular Dynamics). Northwestern signals were subsequently normalized to the amount of overexpressed protein loaded in each lane. As a reference for RNA binding, construct X2 wild-type Xlrbpa (protein 1:2:3) was used, which was assumed to exhibit 100% binding efficiency.

Secondary Structure Predictions—Secondary structure predictions for x1, x2, x3, and an alignment of over 20 dsRBDDs were made using the PHD server and the PHD program at EMBL (Heidelberg) (25).

Identification of dsRBDDs and Multiple Sequence Alignments—dsRBDDs were identified by Blastsearch using the x2 protein sequence as an input against a translation of the GenBank and EMBL nucleotide data bases as well as the Swissprot and PIR protein data bases (26).

4 C. Eckmann and M. F. Jantsch, manuscript in preparation.
Alignments were further analyzed by eye and optimized manually. Accession numbers of sequences are indicated before (12) or as follows: DmMALE, P24785; HsHELIC, Q08211; Xl4f1, U07155; RrCOOF, P31894; HsDRADA, U10493; HsNF90, U10324; MmSPNR, X84692; CeT20G5, Z30423; RnDEAM, U18942; RnTIK, L29281; HsCDNA, R59760; VaE3L, X69198; CeYM68, X69198; ScMITO, P36516.

**RESULTS**

Various Binding Activities of Different dsRBDS—Like its human homologue TRBP, Xlrbpa contains two type A dsRBDS (designated as xl1 and xl2) and one type B dsRBD (designated as xl3) in tandem arrangement (Figs. 1 and 2). To determine the functions fulfilled by multiple dsRBDS in one protein, we first wanted to investigate whether all three dsRBDS of Xlrbpa show identical affinities to dsRNA in vitro. We, therefore, cloned the individual dsRBDS of Xlrbpa (xl1, xl2, and xl3) into the protein-expression vector pRSET, from where they were expressed as 6xHis fusion proteins. RNA binding of the fusion proteins was then tested in Northwestern assays. To assess potential substrate preferences of the domains, three different radiolabeled dsRNA substrates, namely poly(rI/rC), U1 snRNA, and 18 S rRNA, were used in this and all subsequent Northwestern assays. Interestingly, only xl2 but not xl1 or xl3 showed dsRNA binding activity, irrespective of the substrate used, indicating that the three dsRBDS of Xlrbpa
have different RNA binding activities in vitro (Fig. 2).

A C-terminal α-Helix and Aromatic Residues Are Required for dsRNA Binding—NMR data of the third dsRBD in Staufen and the one dsRBD of E. coli RNase III illustrated an α-β-β-α structure of the domain. According to these NMR studies, RNA-protein contact could be mediated through aromatic and basic residues, mostly located in looped regions throughout the domain (14, 22). However, mutational analyses of other dsRBDs demonstrated the importance of the highly conserved, potential α-helix at the C-terminal end of the domain for the interaction with RNA. It was also suggested that basic amino acids within this α-helical region might be important for the contact with RNA (11, 16, 17, 21, 27).

Projection of available NMR data on the xl2 domain of Xrbpa as well as secondary structure predictions using the PHD server suggest the formation of an α-helix between residues Gln-164 and Lys-179 in the xl2 domain (Fig. 3, top). To determine the role of this potential C-terminal α-helix and to identify amino acids essential for dsRNA binding, we performed site-directed mutagenesis studies on this domain (Fig. 3A).

One set of mutations introduced α-helix-breaking prolines or a single glycine shortly upstream of and within the potential C-terminal α-helical region of xl2 (T161P, V165P, R168P, A171P, A171G, and L175P) (Figs. 3A and 4A). Two conserved aromatic residues His-141 and Phe-145 are also essential for full dsRNA binding. In B, chimeric construct 1/2, fusing the N-terminal end of xl1 with the C-terminal end of xl2, does not bind dsRNA as do several mutants exchanging residues in the xl1 part of this construct with the corresponding amino acids found in the xl2 domain. The reciprocal chimeric construct 2/1 shows reduced dsRNA binding. In several mutants replacing amino acids in the xl1 part of this construct with the corresponding residues of the xl2 domain, RNA binding is restored or even stronger than that of xl2. In C, mutants restoring dsRNA binding in construct 2/1 were introduced into the xl1 domain and tested for their influence on dsRNA binding. Although most mutants showed no effect, mutant xl1-R,K introducing two basic residues in α-helix 2 confers dsRNA binding activity to domain xl1. The single mutant xl1-R shows weak dsRNA binding but not xl1-K. Because of relatively high background arising from E. coli proteins specifically binding 18 S rRNA mutants, xl1-R and xl1-K could not be quantitated appropriately (n.d.).

FIG. 3. dsRNA binding properties of various mutants of xl1, xl2, and chimeric constructs. Top, alignment of xl1, xl2, and xl3. Amino acids conserved in the dsRBD consensus sequence are shown in boldface. Secondary structure elements, as predicted by computer analysis and by comparison with available NMR data, are shown on top. Helix 2 is predicted to extend more N-terminal in xl2. A, single amino acid exchanges in xl2 and their influence on dsRNA binding. Relative RNA binding of all constructs to rI/rC, U1 snRNA, and 18 S rRNA compared with xl2 binding (100%) are given in percentages on the right. The summarized average data are indicated by “+++” (100% and more), “++” (60–90%), “+” (15–60%), and “–” (no RNA binding). 33 mM of rI/rC, 11 μM of U1 snRNA, and 265 nmol of 18 S rRNA were bound per mole of xl2 protein. Introduction of α-helix-breaking residues in helix 2 reduces dsRNA binding clearly. Two conserved aromatic residues His-141 and Phe-145 are also essential for full dsRNA binding. In C, mutants restoring dsRNA binding in construct 2/1 were introduced into the xl1 domain and tested for their influence on dsRNA binding. Although most mutants showed no effect, mutant xl1-R,K introducing two basic residues in α-helix 2 confers dsRNA binding activity to domain xl1. The single mutant xl1-R shows weak dsRNA binding but not xl1-K. Because of relatively high background arising from E. coli proteins specifically binding 18 S rRNA mutants, xl1-R and xl1-K could not be quantitated appropriately (n.d.).
are responsible for the different binding abilities exhibited by these two dsRBDs. Both x1 and x2 harbor the conserved aromatic residues required for RNA binding. However, the predicted C-terminal α-helix is slightly longer in x2 than in x1 (Fig. 3). Therefore, we wanted to investigate whether differences in length and/or charge of the C-terminal α-helix could influence binding activity in vitro or, alternatively, whether other essential differences crucial for RNA binding exist between the two domains.

Initially, we created chimeric domains consisting of the N terminus of x1 and the C terminus of x2 (construct 1/2) or the N terminus of x2 and the C terminus of x1 (construct 2/1), respectively. When tested in Northwestern assays, construct 1/2 was unable to bind any dsRNA in vitro, and construct 2/1 showed greatly reduced RNA binding to all three substrates, indicating that amino acids and structures essential for dsRNA binding are distributed throughout the whole sequence of the x2 domain (Figs. 3B and 4B).

To identify single amino acid residues that contribute to a stable protein-RNA contact, we performed fine mapping mutagenesis of the chimeric constructs. Therefore, amino acids within the x1 parts of the chimeric domains were changed to the corresponding residues of the x2 domain. The resulting constructs were then tested for their dsRNA binding activity.

Interestingly, none of the exchanges within construct 1/2 (1/2-L,Q; 1/2-V,E; and 1/2-W,R,E) could lead to dsRNA binding activity in vitro (Figs. 3B and 4B). In contrast, three different mutants within construct 2/1 increased or fully restored RNA binding, depending on the RNA substrate used (Figs. 3B and 4B). 2/1-C, replacing a conserved leucine in the middle of the domain by a cysteine, 2/1-C,N, additionally replacing aspartic acid by asparagine at the very end of the domain, and 2/1-R,K, replacing glutamine and phenylalanine in the α-helical region by the two basic residues arginine and lysine, all showed RNA binding activities comparable or stronger than that of x2 when tested with any of the three substrate RNAs. Two other mutant constructs, 2/1-T, replacing a proline by a threonine at the N terminus of the predicted α-helix, and 2/1-R,T,I, replacing isoleucine, glycine, and aspartic acid at the very end of the domain by lysine, threonine, and isoleucine, respectively, did not increase the RNA binding activity of chimeric construct 2/1 (Figs. 3B and 4B).

To test whether those x2-specific residues that are able to induce dsRNA binding in the chimeric 2/1 domain are sufficient to convert the otherwise inactive x1 domain into an active one, the same amino acid exchanges were tested in the context of the wild-type x1 domain (Figs. 3C and 4C). Of these, the double exchange x1-R,K, introducing two basic residues into the α-helical region of the x1 domain, resulted in good binding ability to all three substrate RNAs. Of the corresponding single amino acid substitution, only x1-R led to weak RNA binding, whereas mutation x1-K failed to bind dsRNA completely. Also, all other mutations leading to RNA binding in the chimeric construct 2/1 did not confer RNA binding ability to x1 (Figs. 3C and 4C).

These data indicate the importance of basic amino acids in the putative α-helical region of the domain for efficient RNA binding. To get a clearer view of the distribution of residues located on the surface of the predicted α-helices of x1 and x2, we established helical wheel plots and horizontal projections of these helices (Fig. 5). On the helix of x2, basic residues Arg-168 and Lys-179 are located on one surface, while Lys-173 and Lys-177 are located on the other side of the helix in a hydrophobic surrounding. In contrast, a clear basic surface is missing on the α-helix of x1. The helical wheel plot of the binding-competent x1-R,K shows that the introduced lysine and arginine lead to a charge distribution efficiencies of U1 snRNA and led to a moderate decrease or increase of rI/rC or 18 S rRNA binding, respectively (Figs. 3A and 4A). Cys-148S is thought to contribute to the formation of the hydrophobic core of the dsRBD (14, 22). Since exchange C148S does not alter the charge density at this position and only introduces a side chain of similar shape, it is understandable that this mutation does not alter RNA binding dramatically.

Chimeric Domains: Charge Distribution within the α-Helix—x1 and x2, the two type A dsRBDs within Xlrbpa, share 34% identity and 40% similarity at the amino acid level. Still only x2 is able to bind dsRNA in vitro. We, therefore, focused on the identification of essential differences between x1 and x2 that
similar to the one present in xl2.

Taken together, the mutational analyses of the chimeric domains and xl1 suggest that the highly basic charge of a putative C-terminal α-helix seems to be a striking criterion for proper and stable RNA-protein contact. However, other residues crucial for efficient interaction of the binding motif and its dsRNA target seem distributed throughout the entire length of the domain.

Similar results were obtained for all three substrate RNAs used. 18 S rRNA, however, showed generally stronger binding to the binding-positive revertants of the chimerical constructs than either rI/rC or U1 snRNA. Nonetheless, this seems to be a consequence of the relatively weak binding of this RNA to the xl2 domain that was used as a reference sample to calculate binding efficiencies.

Cooperative Binding of Type A and Type B dsRBDs—It was demonstrated for the double-stranded RNA-activated, interferon-dependent kinase (PKR) that the N-terminal type A dsRBD, known to be more critical for RNA binding, can substitute the type B dsRBD, whereas replacement of the type A dsRBD by a second type B dsRBD results in a binding-deficient and enzymatically inactive protein both in vitro and in vivo (16, 19). This result indicates that the two dsRBDs of PKR fulfill different functions and seemingly act together in a cooperative manner.

Similarly, our analyses of the three isolated dsRBDs of Xlrbpa demonstrated that only xl2 but neither xl1 nor xl3 can bind a dsRNA substrate in vitro. We thus decided to investigate the contribution of individual dsRBDs of Xlrbpa in the context of the entire protein and in artificial constructs to overall RNA binding. To do this, various combinations of different dsRBD copies were expressed and tested for RNA binding to rI/rC, U1 snRNA, or 18 S rRNA on Northwestern assays (Figs. 6 and 7).

Protein 1:1, a construct with a duplicated xl1 domain (xl1 fails to bind dsRNA when expressed as an isolated domain) exhibited clearly detectable RNA binding, at least 5-fold stronger than that of the binding-competent xl2 domain. Combination of xl1 and xl2 (protein 1:2) resulted in an RNA binding activity comparable to that of wild-type Xlrbpa. Protein 1:2 consists of one xl1 domain and a xl2 domain carrying the mutation H141K that abolishes RNA binding of xl2. This construct showed stronger RNA binding activity than protein 1:1 but reduced binding activity when compared to wild-type RNA binding to rI/rC RNA, U1 snRNA, and 18 S rRNA of all constructs is compared with the binding level of Xlrbpa (100%). A summary of the data is indicated by up to four “+”s or “-”s. 1.6 mmol of rI/rC, 104 μmol of U1 snRNA, and 3.2 μmol of 18 S rRNA were bound per mole of full-length Xlrbpa.

![Helical wheel representations and horizontal projection of predicted C-terminal α-helices in xl2, xl1, and xl1-R,K.](image)

**FIG. 5.** Helical wheel representations and horizontal projection of predicted C-terminal α-helices in xl2, xl1, and xl1-R,K. The predicted α-helix 2 in xl2 has two basic surfaces that are absent in xl1. Mutation xl1-R,K introduces a charge distribution in the α-helix 2 of xl1 similar to the one in xl2. Because xl1 contains a proline at position 73, parts of this putative α-helix are drawn in brackets. Gray circles, basic; open circles, acidic; squares, hydrophobic; triangles, polar. Exchanges in xl1-R,K are indicated by dark gray circles.
binding.

Taken together, these results demonstrate that type A dsRBDS incapable to bind dsRNA by themselves gain RNA binding ability when combined with another inactive type A domain. Similarly, inactive type A dsRBDS, like xl1, seem to enhance the overall binding activity of a given protein, suggesting that multiple type A motifs present in a given protein exhibit a cooperative binding effect, as already suggested by others (15, 16).

In contrast, the RNA binding potential of type B dsRBDS, like xl3, seems very low. However, comparison of xl2 with construct 2:3 indicates that also type B dsRBDS can contribute to RNA binding when expressed in combination with an active type A domain.

**DISCUSSION**

The double-stranded RNA-binding domain (dsRBD), thus far found in about 20 different proteins in different organisms, binds exclusively to double-stranded RNA and RNA-DNA hybrids (10, 12). It was the aim of this study to characterize the dsRBD in more detail and to analyze the mechanisms of RNA binding and kinase activation that the corresponding changes in the type B dsRBD. Moreover, the type B dsRBD could be replaced by a second type A dsRBD without altering the RNA binding ability and kinase activation, whereas duplication of the type B dsRBD diminished binding ability and enzyme activity to a negligible level both in vitro and in vivo (16, 19).

Furthermore, it has been reported that the isolated type A dsRBD of PKR but not the type B dsRBD expressed as a fusion protein is sufficient to bind dsRNA in vitro (15, 17, 18). Similarly, all five dsRBD copies (three type A and two type B) of the Drosophila Staufen protein were tested individually for their dsRNA binding ability in vitro. Notably, only sta3, the third dsRBD within this protein, is capable to bind dsRNA when isolated from the entire protein.5, 6

Furthermore, human TRBP, the human homologue of Xlrbpa, contains two type A and one type B dsRBDS in tandem arrangement. Deletion of the second type A dsRBD or even a part of it abolishes RNA binding activity of the whole protein, whereas deletion of the C-terminal half of the first type A dsRBD does not affect RNA binding in vitro. Similar to Xlrbpa, the first type A dsRBD of human TRBP is not able to bind dsRNA in vitro but can do so when joined with the basic C terminus of the second type A dsRBD (21).

Mutagenesis of the xl2 domain identified at least two essential regions critical for RNA binding in vitro.

(a) Two conserved amino acids, histidine 141 and phenylalanine 145, in the middle of the motif appear to be indispensable for dsRNA binding in vitro (Figs. 3A and 4A). According to NMR data of other dsRBDS, both residues are exposed on one side of the domain and are thought to form part of an RNA recognition surface. The histidine is located in the loop connecting the second and the third β-strand, and the phenylalanine is situated within the third β-strand. Both are likely to make direct contact to the dsRNA substrate, although substitution of this central histidine by an alanine does not reduce dsRNA binding ability in the sta3 domain (14, 22). Both histidine and phenylalanine, when situated on a protein surface, have previously been reported to make direct contact to nucleic acids (reviewed in Ref. 8).

(b) A putative C-terminal α-helix seems to be of striking importance for a stable RNA-protein contact. Several α-helix-breaking residues introduced in the C terminus of xl2 abolish RNA binding completely, consistent with the existence of an α-helix in this region of the motif and indicating the need of this structural element for dsRNA binding in vitro (Figs. 3A and 4A). Similarly, analyses of other dsRBDS have suggested the existence of a basic α-helix at the C-terminal end of the domain required for proper RNA binding (16, 17).

xl1 and xl2 share a high degree of sequence similarity with an amino acid identity of 34%. More importantly, both domains are conserved at those positions believed to be required for RNA binding. Both xl1 and xl2 contain the two conserved aromatic residues His and Phe and several basic residues shortly before and at the beginning of the predicted C-terminal

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5 B. Krovat, unpublished data.
6 D. St Johnston, unpublished data.
\(\alpha\)-helix. Finally, computer predictions indicate that both domains contain the same secondary structure elements. Still, only xl2 but not xl1 is able to bind dsRNA \textit{in vitro}. The fact that neither of the two chimeric constructs 1/2 and 2/1, in which either the N terminus of xl1 had been fused to the C-terminal end of xl2 or vice versa, shows significant RNA binding activity indicates that rather subtle differences between xl1 and xl2 are responsible for their different RNA binding behaviors. NM-R studies on two different dsRBDSs have shown that three basic residues immediately preceding and at the beginning of the C-terminal \(\alpha\)-helix are exposed and accessible for a potential RNA interaction (14, 22). However, the corresponding residues Lys-70, Lys-71, and Lys-74, as well as Lys-163, Gln-164, and Lys-167, in xl1 and xl2, respectively, are, with the exception of Gln-164 in xl2, also basic in nature and thus most likely not responsible for different RNA binding behaviors of these two domains. Instead, the two nonconserved residues Arg-168 and Lys-173 of xl2 can convert the inactive xl1 domain into an active one when inserted at the corresponding positions in this domain. Arg-168 and Lys-173 are located at the beginning and in the center of the predicted \(\alpha\)-helical region. Helical wheel plots indicate that Arg-168 and Lys-173 are located on almost juxtaposed positions of the putative \(\alpha\)-helix and both could contribute to the formation of a basic hydrophobic surface. However, Arg-168 but not Lys-173 can lead to weak but detectable RNA binding when introduced alone at the corresponding position (Arg-75) in xl1. Additionally, according to the NMR-based secondary structure models of two dsRBDSs, Arg-168 in x12 would appear on the exposed surface of the domain, while Lys-173 would be directed toward its center, making an interaction of the exposed amino acid Arg-168 and Lys-173 of xl2 into the minor groove of duplex RNA (28, 29). Nonetheless, other structures and specific residues essential for a stable dsRBDS-RNA contact seem to be interspersed throughout the entire length of the motif since reciprocal chimeric domains of xl1 and xl2 exhibit none or strongly reduced RNA binding ability.

It should also be noted that RNA binding of all constructs was tested in Northwestern assays. This technique relies on the refolding of denatured proteins on blotting membranes after treatment with chaotropic agents. Although our data are in good agreement with similar studies, it has to be considered that some mutant constructs might fail to refold properly, leading to loss of RNA binding. However, it is likely that constructs unable to fold properly in our assay will also fail to do so in solution, thus also leading to a loss of RNA binding in a cellular environment. To study the function and interaction of dsRBDSs in proteins carrying multiple copies of the motif, we determined the binding activities of constructs containing various combinations of dsRBDSs. This study revealed that type A dsRBDSs (xl1 and xl2) can act together in a cooperative manner. Two type A dsRBDSs, for instance, that are not capable to bind RNA as isolated domains can exhibit RNA binding when joined in one protein. In general, duplications of type A dsRBDSs lead to a stronger RNA binding activity of the resulting construct than each of the individual domains.

Type B dsRBDSs, on the other hand, appear to be of minor importance for stable protein-RNA interaction. xl3, for instance, when expressed in combination with an inactive type A dsRBDS, does not promote RNA binding. Only in combination with an already binding-competent dsRBDS, xl3 seems to enhance the binding activity of the resulting construct. Similarly, it has been demonstrated for PKR that the type B dsRBDS in this protein is less important for RNA binding and can be substituted by a type A dsRBDS, whereas two type B dsRBDSs could not fulfill type A-like functions (16, 19).

Our data show that the three dsRBDSs of Xhbpa exhibit different RNA binding activities but can act in a cooperative manner, at least \textit{in vitro}. In vivo, Xhbpa is associated with both ribosomal RNAs and hnRNAs and does not exhibit any apparent sequence-specific binding. This finding is further supported by our current study, which shows that isolated dsRBDSs, chimeric constructs, and mutated dsRBDSs show strikingly similar binding to different RNA substrates. To this point, no sequence-specific binding has been reported for any isolated dsRBDS \textit{in vitro}. In vivo, however, several dsRBDS-containing proteins seem to exhibit sequence-specific interaction (1, 30). Unraveling the mechanism of sequence-specific binding of dsRBDS-containing proteins will require future experiments.

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