The double-stranded RNA-binding domains of *Xenopus laevis* ADAR1 exhibit different RNA-binding behaviors

Roman Brooks[1], Christian R. Eckmann[1], Michael F. Jantsch*

Department of Cytology and Genetics, Institute of Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria

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Abstract We have cloned cDNAs encoding two versions of *Xenopus* double-stranded RNA adenosine deaminase (ADAR1). Like ADAR1 proteins from other species *Xenopus* ADAR1 contains three double-stranded RNA-binding domains (dsRBDs) which are most likely required for substrate binding and recognition of this RNA-editing enzyme. Analysis of mammalian ADAR1 identified the third dsRBD in this enzyme as most important for RNA binding. Here we analyzed the three dsRBDs of *Xenopus* ADAR1 for their in vitro RNA-binding behavior using two different assays. Northwestern assays identified the second dsRBD in the *Xenopus* protein as most important for RNA binding while in-solution assays demonstrated the importance of the third dsRBD for RNA binding. The differences between these two assays are discussed and we suggest that both the second and third dsRBD of *Xenopus* ADAR1 are important for RNA binding in vivo. We show further that all three dsRBDs can contribute to a cooperative binding effect.

Key words: Double-stranded RNA-binding domain; RNA binding; RNA editing; *Xenopus laevis*

1. Introduction

Double-stranded RNA adenosine deaminase (dsRAD, ADAR1 or DRADA) converts adenosines to inosines in double-stranded RNAs by deamination [1,2]. The conversion of an adenosine to inosine in the coding region of an mRNA can lead to the alteration of a codon as inosines are recognized as guanosines and hence basepair with a cytosine residue present in the anticodon of tRNA [1,3]. The enzymatic activity responsible for this base conversion was first described in *Xenopus* embryos as an RNA-modifying and unwinding activity, but has since been found in all metazoans tested so far [4,5]. In the last few years ADAR1 has been isolated and cloned from rat, human, and – more recently – *Xenopus* [6–8].

In all cases, ADAR1 cDNAs encode proteins in the range of 120–150 kDa. The putative translation products contain a C-terminal deamination domain, three double-stranded RNA-binding domains (dsRBDs) located at the center of the protein and a relatively long N-terminal region containing a putative Z-DNA-binding motif [9]. In addition to full length ADAR1 two shorter variants of the enzyme, termed RED-1 and RED-2, have been isolated and cloned from rat and human [10–12]. In agreement with a new nomenclature for all adenosine deaminases that act on RNA, RED-1 has recently been renamed to ADAR2 [13]. Like ADAR1, ADAR2 and RED-2 cDNAs encode a putative deaminase region at their C-termini. However, they only contain two dsRBDs and lack most of the N-terminal residues found in ADAR1. It also seems possible that further members of this family of RNA adenosine deaminases will be isolated in the future.

ADAR1 can convert adenosines to inosines within any double-stranded substrate but shows a 5′ next-neighbor preference. Additionally, ADAR1 activity is low at the 3′ end of an RNA [14]. Nonetheless, despite the low substrate specificity ADAR1 exhibits in vitro or under experimental conditions, only a few in vivo candidate editing substrates for ADAR1 are known. Potential editing substrates are the genomes of several RNA viruses which show biased hypermutation most likely caused by ADAR1-mediated base conversion, the kainate and AMPA glutamate-gated (GluR) ion channel subunits and the serotonin 2C receptor [1,3,14–19]. GluR subunits are edited at several sites. The best studied example, GluR-B subunit, is modified at three sites, the so called Q/R site, the R/G site and a cryptic site which alters an intron-encoded site and thus does not lead to a change in the coding potential of the mature mRNA. Editing at the Q/R site, however, alters the permeability of the receptor dramatically [20]. Most interestingly, not all three sites within GluR-B mRNA seem to be edited by the same enzyme. In vitro, ADAR1 can only edit the R/G site and the cryptic intronic site while ADAR2 seems responsible for the efficient editing of the Q/R site but can also edit the R/G site [10,17,21]. Similarly, serotonin 2C receptor can be edited at a total of four sites, each of which can be specifically modified in vitro by either ADAR1 or ADAR2 [19].

At this point it is not known how substrate and site specificity by ADAR1 and related enzymes is achieved. It seems likely, however, that the dsRBDs are involved in the specific recognition of substrates. Specific RNA recognition has also been observed for the dsRBD containing *Drosophila* Staufen protein which can recognize the 3′ UTR of *bicoid* mRNA [22,23]. ADAR1, like many other dsRBD proteins, contains multiple copies of this RNA-binding motif. Interestingly, isolated dsRBDs apparently fail to exhibit sequence-specific binding in vitro [24,25]. Also, data from other dsRBD proteins indicate that not all dsRBDs in a given protein can bind RNA as an isolated domain but rather contribute to a cooperative binding effect [25,26].

It is thus possible that specific RNA recognition is mediated by these seemingly inactive dsRBDs which might require specific sequences for proper RNA binding. Alternatively, RNA recognition might be achieved by the cooperative binding and spatial distribution of several dsRBDs within a given protein. The dsRBDs within a protein might have to align with the

*Corresponding author. Fax: (43) (1) 79 794 131.
E-mail: jantsch@s1.botanik.univie.ac.at

[1]The first two authors contributed equally to this work.

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position of double-stranded structures in a specific substrate RNA.

Analysis of human ADAR1 has identified the third dsRBD in this protein as most important for RNA binding and enzymatic activity while the second dsRBD seems dispensable for both functions [27,28]. However, alternatively spliced variants of human ADAR1 that show minor variations in the spacer between dsRBD-2 and 3 as well as at the end of dsRBD-3 have been identified. Mutational analyses of the dsRBDs found in these splice variants indicate that the first and second dsRBDs can gain importance in the context of the alternatively spliced protein [29].

Similarly, an amino-terminal deletion in *Xenopus* ADAR1.2, an isoform of *Xenopus* ADAR1, that deletes the first dsRBD still shows enzymatic activity. However, no further analysis of the dsRBDs of *Xenopus* ADAR1 has been performed to this point [8].

We have recently cloned two cDNAs encoding two variants of *Xenopus* ADAR1 which are virtually identical with the cDNAs previously published [8]. Here we report the analysis of the three dsRBDs found in this protein. Using two different methods to detect RNA binding we demonstrate that both the second and third dsRBDs of *Xenopus* ADAR1 are important for proper RNA binding.

2. Materials and methods

2.1. Cloning of two isoforms of Xenopus ADAR1

A partial cDNA encoding *Xenopus* ADAR1 had been isolated from an expression screen for snRNA binding proteins [22,30]. The partial cDNA was used for hybridization of the cDNA library resulting in the isolation of two different cDNA clones both encoding a variant of *Xenopus* ADAR1 [8]. Additionally, 5′ ends of the two ADAR1 isoforms were isolated using the 5′ RACE technique [31]. The isolated clones were also tested by Northern blotting. Sequencing of our cDNAs revealed that they are virtually identical to the ones previously published [8].

2.2. Cloning of dsRBDs into Escherichia coli expression vectors

To allow the expression of individual dsRBDs of *Xenopus* ADAR1-1 the regions encoding single domains were amplified by PCR using suitable primers. 5′ primers carried a *Bam*HI site while 3′ primers had a *Kpn*I restriction site. Primer sequences were as follows: dsRBD-1, 5′: TGT GGT ACC CTG CTC TTC GCG G, 3′: ATA GGA TCC TGT CGT GCC AAG AG; dsRBD-2, 5′: AGA AGG GAT CCA TGT GCC AGC ACA GCC AT, 3′: TTG GAG GTA CCT TCT CAG GCT GCA GAG C; dsRBD-3, 5′: AGC GGT ACC CTC TGC TTC ACC GAT TA, 3′: GGG GGA TCC TTA AAT ATC TGA ATG CCA A. The PCR-amplified DNA was gel-purified, cut with the two enzymes and ligated into either pRSET C (Invitrogen, La Jolla, CA, USA) cut with *Bam*HI and *Kpn*I or into pGEX-3 (Pharmacia, Sweden), into which a linker carrying the appropriate restriction enzyme sites had been cloned [22].

Constructs expressing dsRBDs-1 and 2 (dsRBD-1-2), dsRBDs-2 and 3 (dsRBD-2-3) and all three dsRBDs (dsRBD-1-2-3) were also amplified by PCR using suitable combinations of the same primers used for cloning of the individual domains. For construct dsRBD-1-2 the 5′ primer was located at the beginning of dsRBD-1 while the 3′ primer was located at the end of dsRBD-2. Construct dsRBD-2-3 was amplified using primers located at the beginning of dsRBD-2 (5′ primer) and at the end of dsRBD-3 (3′ primer), while dsRBD-1-2-3 was amplified with a primer at the 5′ end of dsRBD-1 and one at the end of dsRBD-3 (3′ primer). PCR products were also cut with *Bam*HI and *Kpn*I and ligated into pRSET C and the modified pGEX-3 vector.

Construct dsRBD-1-3, only expressing the first and third dsRBDs of *Xenopus* ADAR1.1, was made by digesting construct dsRBD-2-3 with *Sac*I which cuts after dsRBD-2 at the beginning of the spacer located between dsRBD-2 and dsRBD-3. The *Sac*I site was polished using T4 DNA-polymerase and dsRBD-2 was subsequently removed by digestion with *Bam*HI which cuts at the beginning of dsRBD-2. The first dsRBD was isolated from construct dsRBD-1 by digesting the plasmid with *Asp*718. The resulting cleaved ends were blunt-ended using Klenow polymerase and dsRBD-1 was released by a second digest with *Bam*HI. dsRBD-1 was gel-purified and ligated into the remaining dsRBD-3 containing vector from construct dsRBD-2-3. After transformation candidate clones were verified by sequencing.

2.3. Northwestern assays

Northwestern assays were performed as described previously [22,25]. Northwestern assays were quantified as described [25].

2.4. In-solution RNA-binding assays

For in-solution binding assays pRSET fusion proteins were expressed in *E. coli* BL21 (DE3). 50 ml cultures were inoculated with 1 ml of an overnight culture. When cultures reached an OD_{600}=0.6 protein production was induced by addition of IPTG to 1 mM final concentration. After 3 h of induction cells were harvested and lysed in 1×PBS, 0.1% Tween by sonication. The soluble fraction was purified over a Ni-NTA column (Qiagen, Germany) following the manufacturer's protocol. Purified protein was dialyzed against H_2O containing 0.05% trifluoroacetic acid and lyophilized.

For the binding assay 3 μg of protein was mixed with 10 μg of in vitro transcribed, radiolabeled U1 snRNA in 100 μl binding buffer (50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 10 mM HEPES, pH 7.8, 3 mg/ml BSA). The mixture was incubated for 30 min at 4°C then shifted for 5 min to 37°C before being filtered over nitrocellulose (BA85, Schleicher and Schuell, Germany). Filters were washed with 5 ml of 1×binding buffer without BSA, air-dried and subjected to liquid scintillation counting. An aliquot of the in vitro transcribed U1 snRNA was counted for comparison allowing the precise quantification of the amount of bound RNA.

3. Results

3.1. Cloning of two variants of Xenopus ADAR1

From an expression screen for cDNAs that encode proteins that bind RNA we have isolated several cDNAs encoding a total of five different *Xenopus* RNA-binding proteins. We have previously reported the characterization of one double-stranded RNA-binding protein isolated from this screen, called Xlrbpa [22,30]. Another group of cDNAs showed a high degree of homology to the dsRBDs and C-terminal end of human ADAR1 suggesting that they would encode the *Xenopus* homologue of this enzyme [8].

Closer analysis of our primary cDNA clones revealed that none of the clones contained a complete 5′ end. In order to isolate the missing 5′ end of *Xenopus* ADAR1 we followed two strategies. On the one hand we rescreened our library by hybridization with probes located relatively 5′ within our partial cDNA clones, on the other hand we tried to obtain the missing 5′ ends following the protocol for the rapid amplification of cDNA ends (5′ RACE) [31]. Both strategies resulted in the isolation of two groups of cDNAs both seemingly encoding *Xenopus* homologues of human ADAR1. The 5′ ends obtained through 5′ RACE were virtually identical with those encoded by the cDNA clones isolated from the rescreen of the library. Since the rescreen resulted in the isolation of cDNAs containing complete open reading frames we continued our work with the longest clones obtained from the cDNAs screen each encoding a variant a *Xenopus* ADAR1.

In the meantime two cDNAs encoding *Xenopus* ADAR1.1 and ADAR1.2 were published by B. Bass and R. Hough [8] and it turned out that our two cDNAs were almost identical to the cDNAs isolated by this group. We therefore decided to follow the previously introduced nomenclature and termed the cDNAs isolated by us ADAR1.1 and ADAR1.2.

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dsRBD-1    568      lscqeKNPVSGLLEfthycsqqCdFaLlNQsGPsHDPrFkiQAvIdGRrFPvaeANSKKtAKkdAAalALRiLLrEeq  645
dsRBD-2    668 vpaqpsfpcNKNPiSiLmEhgqksGnmCEFqLVsQeGPPHDPKFtytvKIGnqtFPpVvANnKKmAKhlAAeAAvReLLGEgalqpe  754
dsRBD-3    791       kylNaNPVSGLLEyarakGfaaEFkmVNQtGPPHDPKFvfQAKvGGRwFPaVsA SnKqAKaeAAdAALRvLiGEae  867

Consensus                NPV  LNEY QKRG  L Y LI   GP H   F F V V G  FP G G SKK AK  AA  AL  L
                           L  V  #  RK   # F #V           M # I                      ##  L
                           I  I              L           #   L
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Fig. 1. Alignment of the three dsRBDs found in *Xenopus* ADAR1.1. Amino acid sequence of the tree dsRBDs is given in single-letter code. Numbers refer to the position of the first amino acid in *Xenopus* ADAR1.1. Capital letters mark amino acids found in at least two of the three dsRBDs while bold letters indicate amino acids found in the dsRBD consensus sequence. The dsRBD consensus sequence is given below. # marks hydrophobic residues.

Like the published *Xenopus* ADAR1.1 and ADAR1.2 sequences the putative proteins encoded by both of our cDNAs show a high degree of homology to each other in their central region and at their C-terminal ends but differ considerably at their amino-terminal ends. Similarly, the central and C-terminal ends of both putative proteins are highly homologous to human and rat ADAR1 while the amino-terminal ends show marked differences. Also Northern blots with probes specific for our ADAR1.1 and ADAR1.2 cDNAs show signals comparable to those published (data not shown) [8]. It is interesting to note, however, that our ADAR1.2 cDNA is lacking a proper 5′ AUG codon, just like the previously published ADAR1.2 sequence.

3.2. Northwestern RNA-binding analysis of dsRBDs

RNA substrate recognition by ADARs is most likely mediated by the three conserved dsRBDs found in this protein. Mutagenesis studies on human ADAR1 have identified the third dsRBD as most important for proper RNA binding while the first dsRBD seems to contribute to RNA binding. The second dsRBD, in contrast, seems dispensable [11,28]. In *Xenopus* only a single deletion on ADAR1.2 has been analyzed which showed that the first dsRBD in this protein is not required for enzyme function [8]. We therefore set out to study the RNA-binding behaviors of the three dsRBDs of *Xenopus* ADAR1.1 by Northwestern RNA-binding assays. Using this assay we have previously shown that only the second dsRBD in the *Xenopus* protein Xlrbpa can bind RNA as an isolated domain while dsRBDs-1 and 3 contribute to an overall binding effect [25]. We therefore isolated the three dsRBDs of ADAR1.1 by PCR and cloned them into the protein expression vectors pRSET and pGEX which allow the expression of the fusion protein as 6×His fusions or glutathione *S*-transferase fusions, respectively. Fig. 1 shows the predicted amino acid sequence of the expressed individual dsRBDs. In addition, constructs expressing dsRBD-1-2, dsRBD-2-3, dsRBD-1-3 and dsRBD1-2-3 of ADAR1.1 were cloned into these protein expression vectors.

All proteins were expressed in *E. coli*, crude lysates were separated by SDS-PAGE, blotted and tested for RNA-binding activity on a Northwestern assay [22,25]. As a positive control for RNA binding we included the second dsRBD of Xlrbpa. Since no specific RNA substrate for *Xenopus* ADAR1 is known at this point Northwestern assays were performed in parallel with two different substrates: annealed homopolymeric rI/rC, and U1 snRNA. Both substrates had been used previously to determine the RNA-binding properties of Xlrbpa [25]. Also, to determine the amount of dsRBD containing protein in the extracts a third gel was stained with Coomassie brilliant blue (Fig. 2).

As can be seen in Fig. 2a all constructs expressed well allowing the clear identification of the recombinant protein band. Of the individual domains only dsRBD-2 showed clear binding to both radiolabeled rI/rC and U1 snRNA. Only on longer exposures weak binding of dsRBD-3 could be detected (data not shown). Consistent with this finding was the RNA-binding behavior of constructs expressing combinations of dsRBDs. Here, only constructs containing dsRBD-2 showed significant RNA binding, while the construct solely expressing

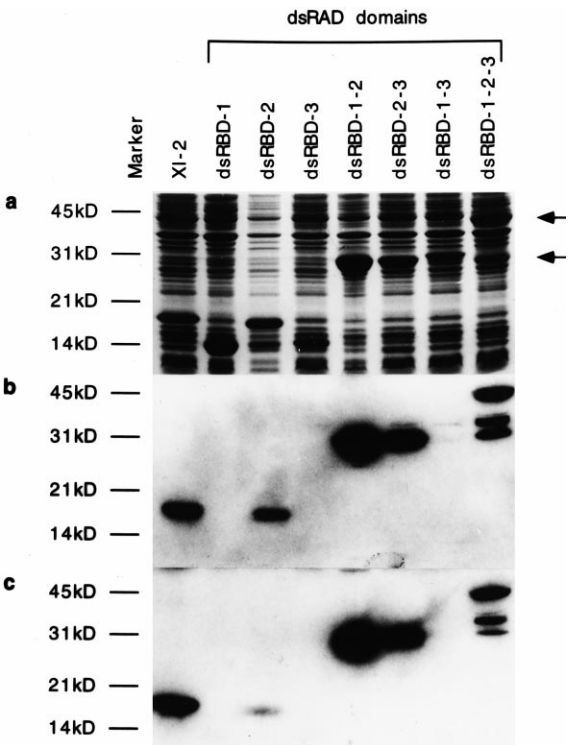

Fig. 2. Northwestern analysis of individual dsRBDs and combinatorial constructs. Coomassie-stained gel (a) and RNA-binding assay performed with U1 snRNA (b) or rI/rC-RNA (c) of dsRBD constructs. Xl2 expresses the second dsRBD of *Xenopus* Xlrbpa as a positive control. Constructs dsRBD-1, dsRBD-2 and dsRBD-3 express only single dsRBDs while constructs dsRBD-1-2, dsRBD-2-3 and dsRBD-1-3 express two dsRBDs. Construct dsRBD-1-2-3 expresses all three dsRBDs. Arrows mark the position of proteins containing two or all three dsRBDs in the Coomassie-stained gel. Only constructs expressing dsRBD-2 alone or in combination with other dsRBDs exhibit strong RNA binding. A faint signal, indicating weak RNA binding, can be observed for construct dsRBD-1-3. Construct 1-2-3 shows reduced RNA binding when compared to construct dsRBD-1-2, indicating that the third dsRBD can negatively interfere with RNA binding. Also, several lower molecular weight bands can be observed in lane dsRBD-1-2-3 which are breakdown products of the full-length protein.

dsRBDs-1 and 3 showed almost no RNA binding (Fig. 2). Also, constructs expressing combinations of active and inactive dsRBDs (dsRBD-1-2 and dsRBD-2-3) showed stronger binding than the isolated dsRBD-2 alone indicating that the inactive dsRBDs can contribute to overall RNA binding and thus exhibit a cooperative binding effect. It is also interesting to note that dsRBD-2 of ADAR1.1 shows stronger binding to U1 snRNA than to rI/rC when compared to the binding of Xl2 which showed comparable binding of both substrates.

To get a clearer view of the subtle differences exhibited by the various constructs the autoradiograms were quantified using a Phosphoimager and the amounts of overexpressed proteins were determined by laser densitometry. Furthermore, to determine the molar amount of RNA bound per mol of protein some reference bands from the Northwestern blot were cut out and quantified by scintillation counting. Using these data we calculated the molar amounts of each RNA substrate bound per mol of each protein (Fig. 3).

As can be seen RNA binding of all constructs is relatively weak as only micromolar amounts of RNA are bound per mol of protein. Furthermore, binding of U1 was generally weaker than binding of rI/rC, with the exception of dsRBD-2 which showed slightly stronger binding to U1 snRNA. The generally stronger binding of rI/rC can most likely be explained by the perfect double-stranded nature of this annealed, homopolymeric RNA. Binding of hybrid constructs

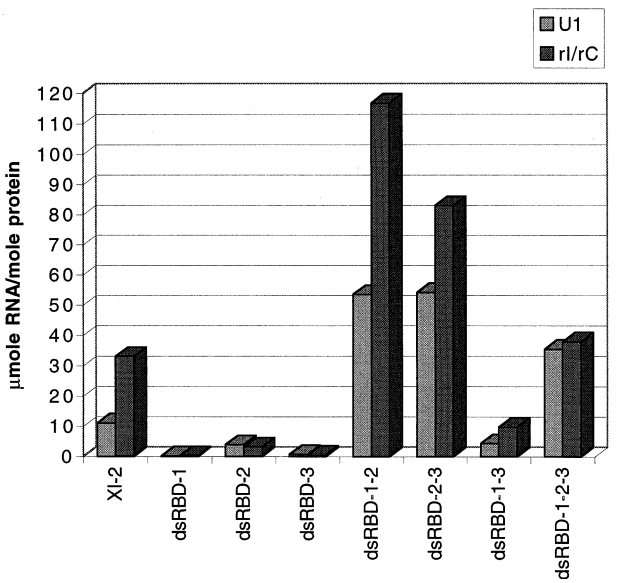

Fig. 3. Quantitation of RNA-binding activity of dsRBD-expressing constructs by Northwestern assays. Overexpressed proteins were quantified by laser densitometry on Coomassie-stained gels and RNA binding was measured on a Phosphoimager and by liquid scintillation counting. The molar amounts of RNA bound were normalized to the molar concentration of each dsRBD construct. Light gray bars indicate amount of U1 snRNA bound while dark gray bars represent rI/rC binding. Hybrid constructs dsRBD-1-2 and dsRBD-2-3 show 10–40-fold increased RNA binding when compared to dsRBD-2 alone, indicating a strong cooperative binding effect. In contrast, construct dsRBD-1-3 shows only a moderate increase in RNA binding demonstrating the importance of dsRBD-2 for RNA binding. A construct containing all three dsRBDs of *Xenopus* ADAR1.1 (dsRBD-1-2-3) shows reduced RNA binding when compared to the two-domain constructs containing dsRBD-2. In most cases RNA-binding to rI/rC is stronger than binding to U1 snRNA which is probably due to the perfect double-stranded nature of this homopolymeric synthetic substrate.

dsRBD-1-2 and dsRBD-2-3 was 14–40-fold stronger than that of dsRBD-2 alone depending on the substrate used. However, binding of construct dsRBD-2-3 was weaker than binding of construct dsRBD-1-2 when rI/rC was used as a substrate while almost identical binding values were obtained for both constructs with U1. The Phosphoimager data also revealed some minor binding of dsRBD-3 alone which was not detected on the autoradiograms. Similarly, combination of domains 1 and 3 (dsRBD-1-3) revealed binding comparable to that of domain 2 (in molar terms) suggesting that both domains can contribute to RNA binding when expressed in combination with another dsRBD. Interestingly, expression of all three domains (dsRBD-1-2-3) showed weaker binding than either constructs dsRBD-1-2 or dsRBD-2-3 indicating that dsRBD-3 can also negatively influence RNA binding. Taken together, the Northwestern analysis identified the second dsRBD of ADAR1.1 as most important for RNA binding.

3.3. In-solution binding of Xenopus ADAR1.1 dsRBDs

Previous studies on human ADAR1 have identified the third dsRBD in this protein as most important for RNA binding and enzymatic activity [11,28]. Additionally, the first dsRBD has been shown to contribute to both processes while the second dsRBD seemed almost dispensable [11,28]. This is in clear contrast with our observation that identified the second dsRBD of *Xenopus* ADAR1.1 as most important for RNA binding. However, not only the proteins investigated in these studies differed but also the assays employed to determine RNA binding. While our study relied on a Northwestern assay where immobilized protein is refolded by treatment with chaotropic agents and subsequently incubated with substrate RNA, analyses of human ADAR1 were done by a solution binding assay where either overexpressed or in vitro translated protein is incubated in solution with substrate RNA and subsequently filtered over nitrocellulose to determine the amount of bound RNA.

We therefore repeated our analysis of individual dsRBDs of *Xenopus* ADAR1 using an in-solution RNA-binding assay. To do this, the individual domains of *Xenopus* ADAR1.1 and the second dsRBD of Xlrbpa were expressed in *E. coli* as 6×His fusion proteins and purified by Ni-chelating affinity chromatography. When the purified proteins were used for in-solution binding assays a completely different picture emerged (Fig. 4). The in-solution assay identified the third dsRBD of *Xenopus* ADAR1.1 as the most actively RNA-binding domain while both dsRBD-1 and dsRBD-2 showed almost no RNA binding. However, RNA binding of dsRBD-2 was generally somewhat stronger than that of dsRBD-1. Also, RNA binding of all constructs was stronger in solution than in Northwestern assays as millimolar rather than micromolar amounts of RNA were bound per mol of protein in this assay.

Identification of the third dsRBD in *Xenopus* ADAR1 is in good agreement with studies on human ADAR1 which too identified the third domain in this protein as most important for RNA binding. However, the in-solution assay is in clear contrast to our Northwestern studies where the second dsRBD seemed most important for RNA binding.

Northwestern assays rely on the proper refolding of proteins that have been blotted onto membranes after separation on an SDS-PAGE. To facilitate refolding, the membranes are treated with chaotropic agents such as urea or guanidinium hydrochloride and subsequently incubated in a stepwise dilu-

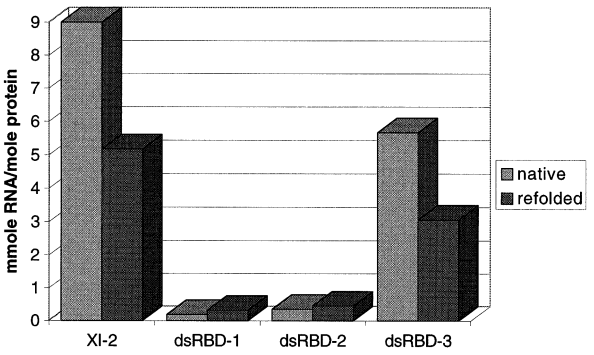

Fig. 4. In-solution RNA binding of individual dsRBDs. Individual dsRBDs of ADAR1.1 were expressed as His-fusion protein and purified by Ni-chelating chromatography. Purified proteins were allowed to bind to radiolabeled U1 snRNA in solution and filtered over nitrocellulose disks. The amount of bound RNA was determined by liquid scintillation counting and normalized to the molar amount of protein. Light gray bars are the results of binding to 'native' *E. coli*-produced proteins. Dark gray bars indicate binding of U1 snRNA to refolded *E. coli*-produced proteins. Only the third dsRBD of *Xenopus* ADAR1.1 shows significant RNA binding while dsRBDs-1 and 2 show binding values comparable to background binding to the filter. Refolding of the protein does not influence the in-solution binding behavior of the constructs although binding of refolded proteins was generally reduced when compared with native proteins.

tion series of the chaotropic agent in a physiological buffer. The slow renaturation has been shown to facilitate correct folding of several proteins [32,33]. In contrast, the in-solution assay uses proteins overexpressed and purified from various sources or in vitro translated proteins and does not employ any refolding step. As *E. coli*-expressed proteins also tend to misfold it seemed conceivable that the differences observed in the two assays resulted from misfolding of various constructs in either assay.

To test this possibility we subjected the purified, *E. coli*-produced proteins used in the in-solution assay to a denaturation and refolding step. Urea was added to the purified proteins to a final concentration of 8 M. After denaturation, the proteins were subjected to slow dialysis for 24 h against stepwise dilutions of urea in TBS. This treatment should also lead to a slow renaturation of the purified proteins thus allowing their proper folding. However, RNA binding of the refolded proteins was generally comparable to that of the untreated ones. Again, dsRBD-3 and Xl2 showed the strongest RNA binding while dsRBDs-1 and 2 were almost inactive (Fig. 4). RNA binding of refolded Xl2 and dsRBD-3 was somewhat weaker than that of the corresponding native proteins. Taken together these data suggest that the observed differences in RNA binding of constructs tested in the two assays employed are not simply caused by the refolding of proteins. Instead, other factors such as the interaction of proteins with the membrane might influence folding.

4. Discussion

Double-stranded RNA adenosine deaminase (dsRAD, DRADA-1 or ADAR-1) converts adenosines to inosines in double-stranded RNA by hydrolytic deamination [1,2]. The enzyme has been implemented in the editing of certain subunits of glutamate-gated ion channels and serotonin receptor 2C in mammalian brain. Also some RNA viruses seem suitable substrates for ADAR1 [1,3]. cDNAs encoding ADAR1 have been cloned from mammals and, more recently, *Xenopus* [6–8]. The proteins encoded by these cDNAs all have a conserved deaminase domain at their C-terminus and three double-stranded RNA-binding domains in their central region. However, considerable variability is observed at the amino-terminal ends of the putative proteins.

Substrate recognition by ADAR1 is most likely achieved through the three centrally located dsRBDs. Studies on human ADAR1 have identified the third dsRBD in this protein as most important for RNA binding and enzyme function [27,28].

In this study we have analyzed the three dsRBDs of *Xenopus* ADAR1.1 by Northwestern and in-solution assays for their dsRNA-binding behavior. Our Northwestern analysis revealed that the second dsRBD is most important for RNA binding both when expressed as an isolated domain and in combination with other dsRBDs. We could show further that domains showing little or no RNA binding by themselves can contribute to overall RNA binding when expressed in combination with another dsRBD. Similar effects have also been observed for PKR, *Xenopus* Xlrbpa and human ADAR1 where domains incapable of RNA binding could exhibit a cooperative binding effect [24,25,27,28,34].

In-solution assays, on the other hand, identified the third dsRBD of ADAR1.1 as the most active one while dsRBD-2 seemed almost completely inactive in this assay. This latter datum is in good agreement with studies on human ADAR1 which also identified the third dsRBD as most important for RNA binding and enzyme activity. However, all studies on mammalian ADAR1 were also performed by in-solution assays.

While it is possible that both Northwestern and in-solution binding assays lead to false-positive signals, several arguments make us believe that the opposite is the case, namely that some domains fail to show RNA binding in either assay.

First, the second dsRBD of Xlrbpa (Xl2) shows RNA binding in both assays (Figs. 2 and 4) [25] indicating that the two assays can give identical results. Second, studies performed on the double-stranded RNA-dependent, interferon-activated kinase PKR have identified the first dsRBD in this protein as most important for RNA binding both by Northwestern and in-solution assays, demonstrating again that both assays can give comparable results [35,26,36]. Third, studies on human ADAR1 expressed and purified from COS cells identified all three domains as important for RNA- diting, indicating that all domains are important at least for enzyme activity [29]. Furthermore, Northwestern assays have been used to identify actively binding dsRBDs in several other proteins. Finally, the data obtained from the Northwestern assays identify the second dsRBD as RNA binding not only when expressed as an isolated domain but also when in context with other dsRBDs, indicating that the context of the domain which can also influence folding does not influence the RNA-binding activity of dsRBD-2 in this assay. Similarly, identical results were obtained when proteins were expressed as 6×His or GST fusions, again indicating that the context of the protein did not influence the outcome of the experiment.

The differences observed in the two assays are thus most likely the result of different folding of individual constructs in the two assays. All in vitro RNA-binding assays rely on the proper folding of proteins. It has been shown that *E. coli*-

produced proteins tend to misfold [32,37]. It is therefore possible that the second dsRBD which does not show any activity in solution but shows considerable RNA-binding activity in Northwestern assays fails to fold properly when expressed in *E. coli*. Similarly, all proteins tested by Northwestern are denatured during SDS-PAGE. Proper folding of the proteins is believed to be achieved by denaturation of membrane-bound proteins following slow renaturation which should allow refolding of the proteins [32]. This treatment in turn might lead to misfolding of dsRBD-3 while facilitating proper folding of dsRBD-2 which proved to be RNA binding in our Northwestern experiments. Interestingly, refolding by itself does not lead to activation of dsRBD-2 as urea treatment and subsequent dialysis of purified dsRBD-2 does not lead to RNA-binding activity of this domain in solution. We therefore believe that interaction of the protein with the membrane is required for RNA binding. Ionic interactions between the membrane and protein might alter folding thus helping to expose certain amino acids which are required for RNA binding. In solution, certain amino acids or regions within dsRBD-2 might be buried at least in some cases, thus leading to false inactivity of an otherwise active domain.

The importance of the second dsRBD in ADAR1 is also underscored by a study on alternatively spliced variants of human ADAR1 which showed that mutations in the second dsRBD of this protein can have different effects on enzyme activity in alternatively spliced versions of the enzyme [29]. Similarly, we could show recently that the second dsRBD of ADAR1.1 is required for proper localization of the protein on nascent transcripts on *Xenopus* lampbrush chromosomes, again indicating the importance of this dsRBD (Eckmann and Jantsch, in preparation). In vivo, interactions with other proteins particularly chaperones might help to fold all RNA-binding domains into an actively binding form. This view is also supported by the finding that all three dsRBDs contribute to maximum enzyme activity of human ADAR1 overexpressed in COS cells [29]. Taken together, we therefore believe that both domains identified in our study are required for proper RNA binding. In fact, given the high degree of conservation of all dsRBDs amongst the ADAR1 homologues identified thus far, it appears very likely that all three dsRBDs play a role in active RNA binding in vivo. However, further in vivo studies will be required to clarify this point.

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