Oligomerization activity of a double-stranded RNA-binding domain

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Abstract ***Xenopus laevis*** **RNA-binding protein A (Xlrbpa) is a highly conserved, ubiquitously expressed hnRNP- and ribosome-associated RNA-binding protein that contains three double stranded RNA-binding domains (dsRBDs) in tandem arrangement. A two-hybrid screen with Xlrbpa as a bait recovered Xlrbpa itself as the strongest interaction partner, indicating multimerization of this protein. To search for regions responsible for the observed interaction, we conducted two-hybrid assays with Xlrbpa deletion constructs and identified the third dsRBD of Xlrbpa as the exclusive interacting domain. Additionally, these results were confirmed by coimmunoprecipitation experiments with truncated proteins expressed both in yeast and *Xenopus* oocytes. In PACT, the human homologue of Xlrbpa, we could demonstrate that the third dsRBD displays the same multimerization activity. Interestingly, this domain is essential for the activation of the dsRNA-activated protein kinase PKR. Addition of RNAses to coimmunoprecipitation experiments did not affect the dimerization, suggesting that the interaction is independent of RNA-binding. We report here a homomultimerization activity of a type B dsRBD and suggest possible implications that include a model for PKR activation by PACT.**

Keywords: *Xenopus laevis* RNA-binding protein A; PACT; Double-stranded RNA-binding domain; Protein–protein interaction

1. Introduction

The double-stranded RNA-binding domain (dsRBD) is found in more than 100 proteins from different organisms ranging from *Escherichia coli* to humans. dsRBDs are approximately 70 amino acids in length and can bind double-stranded RNA (dsRNA) but neither single-stranded RNA (ssRNA) nor DNA [1,2]. By sequence comparison, dsRBDs can be divided into two types: type-A dsRBDs are conserved over the entire length of a defined consensus sequence, whereas type-B dsRBDs fit the consensus only at the C-terminal end [1]. In vitro RNA-binding experiments demonstrated that only type-A dsRBDs are competent in dsRNA binding, while type-B domains fail to bind RNA by themselves. However, the presence of multiple domains in tandem arrangement enhances their ability to bind dsRNA dramatically [3,4]. NMR and crystal structures of several type-A dsRBDs have been solved [5–8] all showing a general αββββα structure of the domain in which the two α-helices lie on one side of a three stranded antiparallel β-sheet. Furthermore, the NMR structure of the second dsRBD of PKR, which is of type-B, shows the same general architecture suggesting that at least some type-B domains show the same structure as type-A domains [7].

Xlrbpa is a 33 kDa, ubiquitously expressed, double-stranded RNA-binding protein that is associated with the majority of cellular RNAs, ribosomal RNAs and hnRNAs [9]. Xlrbpa is composed of three dsRBDs in tandem arrangement, the first two are of type-A whereas the third is type-B. The second but neither the first nor the third dsRBD of Xlrbpa is able to bind dsRNA as an isolated domain in vitro. Xlrbpa is highly homologous to human PACT and mouse RAX proteins, respectively. Both of these mammalian proteins were shown to be cellular activators of the interferon induced, dsRNA-activated protein kinase PKR [10,11]. Additional work on PACT and PKR indicated that the first and second domains of PACT are responsible for a physical interaction with PKR, whereas the third domain of PACT is responsible for activation.

Several proteins can be coimmunoprecipitated with Xlrbpa, including hnRNPs and ribosomal components [9]. Therefore, to search for interacting partners, we performed a yeast two-hybrid screen of an embryonic *Xenopus laevis* cDNA library using Xlrbpa as a bait. Amongst the positives recovered from the screen, Xlrbpa itself turned out to be the strongest interaction partner. To determine the minimal region of Xlrbpa required for multimerization we performed two-hybrid assays and coimmunoprecipitation experiments of tagged Xlrbpa deletion constructs. These experiments identified the highly conserved third, type-B dsRBDs of Xlrbpa and PACT as the sole regions within these homologues that show multimerization activity.

2. Materials and methods

2.1. Two-hybrid-screen, expression vectors and antibodies

The two-hybrid screen with Xlrbpa as bait was performed according to the user manual of MATCHMAKER LexA two-hybrid system from Clontech (Palo Alto, CA) using *Saccharomyces cerevisiae* strain EGY48^{C} (***MATα***, *his2*, *trp1*, *ura3*, *LexAop(x6)-LEU2*) that was co-transformed with the bait vectors pLexA expressing Xlrbpa and the prey vector pB42AD that expressed the *Xenopus* cDNA library. The EGY48^{C} strains carried plasmid p8op-lacZ containing the LacZ reporter gene under control of the LexA operator. To measure the strength of the two-hybrid interaction, β-galactosidase liquid culture assays using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as

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substrate were performed according to the manufacturer's instructions (Clontech). For coimmunoprecipitation experiments of yeast expressed proteins, we used EGY48^{C} strains that were transformed with vectors expressing myc-tagged Xlrbpa under the control of the Gal promoter. HA-tagged wild type Xlrbpa was expresssed from vector pCM252 under the Tet promoter [12,13].

The third dsRBD of Xlrbpa (Xl3) was cloned into pB42-AD and expressed as a fusion protein with the Gal4 activation domain and an HA tag under the control of the Gal promoter. Monoclonal mouse IgG1/k antibody 16B12, which recognizes the HA-tag, was purchased from BABCO (Berkeley, USA). For Western blots a 1:1000 dilution was used. 5 μl of this antibody was used for a single coimmunoprecipitation experiment. 9E10 is a mouse monoclonal antibody that recognizes the myc-tag [14].

2.2. Cloning of deletion constructs

PACT was amplified from a human cDNA library using the 5′ primer CGCTGATCAGTCCCTTCTCGCCATGTC and the 3′ primer TTTAAGTGGATCCAGATTTACTTTCTTTC, was tagged with a single myc at the N-terminus and subcloned into vector pLexA. The third dsRBD of PACT (pt3), the construct consisting of the first and second dsRBDs of PACT (pt1:2) and deletion constructs of the third dsRBD of Xlrbpa (xl3) were amplified by PCR using appropriate 5′ primers that contained *Eco*RI sites and 3′ primers that contained XhoI sites. The PCR products were cloned into the same sites of vectors pLexA or pB42AD from Clontech and sequenced prior to testing for two-hybrid interactions.

2.3. Yeast protein extracts and coimmunoprecipitation experiments

A 2 ml preculture of yeast cells was grown overnight in synthetic medium lacking the amino acids tryptophan and leucine and containing 10% galactose and 1% raffinose as a carbone source (SC–Trp-Leu 10% Gal 1% Raff) at 30 °C. The following day, a 10 ml culture was inoculated and grown for 5 h. For cells expressing HA-tagged Xlrbpa from the pCM252 vector, 1 μg/ml doxycycline was added [12,13]. The cells were pelleted and washed three times with water. The following steps were performed on ice. The cell pellet was resuspended in 4–5 times its volume of cold RIPA buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, and 50 mM Tris–HCl, pH 8.0). An equal volume of acid washed glass beads was added and the mixture was vortexed at high speed for 30 min at 4 °C. The mixture was centrifuged at 14 000 rpm for 10 min and the protein extract in the supernatant was transferred to a new Eppendorf tube and used for coimmunoprecipitation. Typically for one experiment, 25 μl protein G-coupled Sepharose beads and 5 μl 16B12 (anti-HA) antibody were used. The antibody was coupled to beads in RIPA buffer overnight at 4 °C. Next day, the beads were washed twice with 500 μl RIPA, resuspended in 200 μl RIPA and 200 μl yeast extract (in RIPA) was added. The mixture was turned on a wheel for 90 min at 4 °C. For coimmunoprecipitations with RNAses, 500 units of RNAseT1 and 50 μg of RNAseA were added and the mixture was left at room temperature for 10 min before turning at 4 °C. The beads were washed four times with cold RIPA buffer. To extract the proteins from the beads, 40 μl SDS loading buffer was added and incubated in boiling water for 5 min. The beads were centrifuged 5 min at full speed in an Eppendorf centrifuge and the supernatant was directly loaded onto two small Laemmli 10% SDS protein gels. The gels were blotted onto nitrocellulose membranes. One membrane was developed with the anti-HA 16B12 antibody and the other with the anti-myc 9E10 antibody.

2.4. Oocyte expression and coimmunoprecipitations

For expression of Xlrbpa and constructs containing different dsRBD deletions, the corresponding fragments were amplified from an Xlrbpa cDNA using primers that allowed an in-frame cloning downstream of either a single myc-tag in pBluescript KS (Stratagene, LaJolla, CA) or a 6xHis tag in pRSET [3] (Amersham Pharmacia, Uppsala, Sweden). The single third dsRBD of Xlrbpa (Xl3) was cloned downstream of a single HA tag in pBluescript. For RNA stability, the 3′ UTR of the NO38 cDNA was added at the 3′ end of all constructs [15].

For RNA synthesis, plasmids were linearized immediately downstream of the NO38 poly(A) tail and capped in vitro transcripts were synthesized using bacteriophage T7 or T3 RNA polymerases as described [16]. 50 ng of capped RNA was injected per oocyte prior to overnight culture in OR-2 to allow protein synthesis to occur. For double injections oocytes were cultured for 12 h after the first injection, followed by a second round of injection with an RNA encoding the second construct. After the second injection, oocytes were cultured for an additional 12 h to allow protein expression to occur.

Expression of proteins was monitored by Western blotting of homogenized oocytes. For immunoprecipitations, antibodies were coupled to protein A or protein-G Sepharose in NET-2 [17]. Oocytes were also homogenized in NET-2 cleared of insoluble proteins by centrifugation and 3 oocyte equivalents were added per immunoprecipitation.

Washed precipitates and aliquots of the crude oocyte lysates were loaded on SDS gels. After electrophoresis, gels were blotted and proteins detected using the appropriate antibodies directed against the epitope tags.

3. Results

3.1. The third dsRBD of Xlrbpa is sufficient for multimerization of Xlrbpa

From a two-hybrid screen with Xlrbpa as a bait, Xlrbpa itself was recovered as a strong interacting partner suggesting multimerization of the protein. The smallest Xlrbpa prey cDNAs consisted of the C-terminal end starting slightly upstream of the third type-B dsRBD (xl3). To find the minimal region of interaction, constructs with deletions from both the N-terminal and C-terminal ends were generated by PCR, cloned into the pB42AD prey vector, and tested for interaction with Xlrbpa expressed from the bait plasmid. The strength of the interaction was measured by quantitative β-galactosidase liquid assays using ONPG as substrate. Fig. 1 shows the different deletion constructs and the strength of their interaction with Xlrbpa. The results of these experiments identify the entire third, type-B dsRBD of Xlrbpa (xl3) as a protein–protein interaction domain (Fig. 1). A construct with a single amino acid deletion from the N-terminus of xl3 lost most of its interaction ability (construct number 3, Fig. 1(a) and (b)). A second construct with a two-amino acid deletion from the same terminus failed to interact (construct number 2, Fig. 1(a) and (b)). The last five C-terminal residues of Xlrbpa do not belong to the consensus of xl3 and were thus dispensable for interaction (construct 10, Fig. 1(a) and (b)). However, deletion of one additional residue (six residues from C-terminus of Xlrbpa, one residue from C-terminus of the xl3 consensus) led to loss of the interaction (construct number 9, Fig. 1(a) and (b)). Expression of the HA-tagged prey constructs was monitored by Western blot analysis (Fig. 1(c)). We observed that constructs with amino acid deletions from the C-terminal end (constructs 5, 6, 7, 8, 9 and 10) were either weaker expressed or less stable than constructs with deletions from the N-terminal end (constructs 2, 3 and 4) (Fig. 1(c)). Taken together, our two-hybrid results indicate that the third domain of Xlrbpa is sufficient for the self-interaction of Xlrbpa (Fig. 1).

The constructs described so far were always cloned in the prey plasmid and tested for an interaction with full-length Xlrbpa in the bait plasmid. To test if xl3 can interact with itself, it was cloned in the bait plasmid and tested with the same domain in the prey plasmid (Fig. 1). Xl3 proved to be sufficient for a strong interaction with itself in the two-hybrid assay (Fig. 1(b), sample 4*).

3.2. The third dsRBD of Xlrbpa is necessary for multimerization

Having shown that xl3 can self-interact in a two-hybrid assay, we wanted to support this finding by biochemical analysis. We also wanted to test whether xl3 was the only dsRBD within Xlrbpa capable of mediating homomultimerization. Therefore, *S. cerevisiae* was cotransformed with vectors expressing either myc-tagged Xlrbpa (myc-1:2:3) or a myc tagged deletion construct lacking the third dsRBD (myc-1:2) together with a plasmid expressing HA-tagged xl3 as a fusion with the Gal 4 activation domain (HA-xl3). Coimmunoprecipitation (coIP) experiments were performed from cell lysates using anti-HA antibody coupled to protein-G Sepharose (Fig. 2(a)). In these experiments, myc-tagged Xlrbpa (myc-1:2:3) could be efficiently coimmunoprecipitated with HA-xl3 (Fig. 2(a). However, a construct lacking the third dsRBD (myc-1:2) failed to coimmunoprecipitate with HA-xl3, indicating that xl3 fails to associate with either the first (xl1) or with the second (xl2) dsRBD of Xlrbpa (Fig. 2(a)). To test if xl1 and xl2 are capable to self-interact or to interact with each other, we made coimmunoprecipitation experiments from two yeast strains coexpressing HA-tagged Xlrbpa (HA-1:2:3) together with either myc-tagged Xlrbpa (myc-1:2:3) or with a myc-tagged deletion of Xlrbpa lacking the third dsRBD (myc-1:2) (Fig. 2(b)). As expected, full length Xlrbpa constructs could coprecipitate each other, while no interaction was observed between myc-1:2 and HA-1:2:3. Taken together, these results indicate that the first two domains of Xlrbpa xl1 and xl2 are incapable to multimerize, leaving xl3 as the sole domain that is necessary and sufficient for the oligomerization of Xlrbpa.

3.3. Multimerization of xl3 is independent of RNA binding

In vivo Xlrbpa associates with hnRNPs and ribosomes and in vitro it is a strong dsRNA binder [1,3,18]. To investigate if RNA-binding has an effect on the oligomerization via xl3, we performed coIP experiments from yeast extracts expressing HA-xl3 and construct myc-1:2^{-}:3 which contains the previously reported histidine to lysine (H141K) point mutation at position 141 in the second dsRBD that weakens the RNA-binding ability of the whole protein [3,8]. Myc-1:2^{-}:3 interacts as strongly as wild type Xlrbpa with xl3, suggesting that the interaction is not affected by RNA binding (Fig. 2(a)). Moreover, addition of RNAseT1 and RNAseA showed no effect on the coimmunoprecipitation of HA-xl3 with myc-tagged wild type Xlrbpa (myc-1:2:3) (Fig. 2(a)). These findings together with the previously reported inability of xl3 to bind dsRNA suggest that the interaction via xl3 was not dependent on RNA-binding [3].

3.4. Multimerization of Xlrbpa also occurs in Xenopus cells

To determine whether the observed interaction of xl3 with itself would also occur in *Xenopus*, we expressed either myc-tagged full length Xlrbpa (123-myc) or a deletion construct lacking the third dsRBD (12-myc) together with the HA-tagged third dsRBD of this protein (3-HA) in *Xenopus* oocytes. Oocyte extracts were tested by immunoprecipitation for an

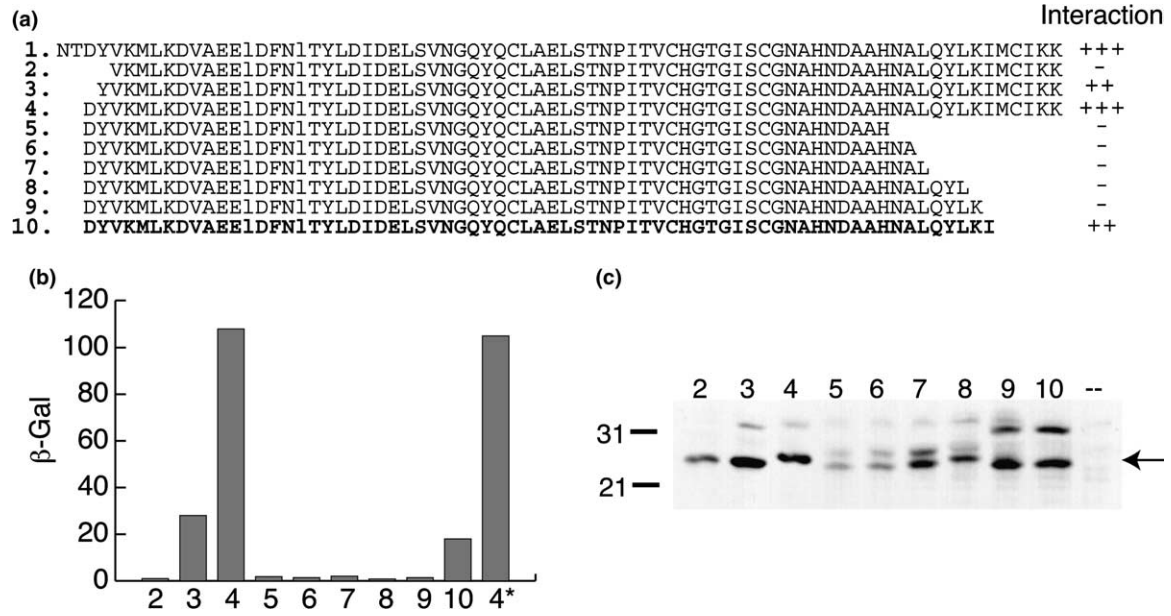

Fig. 1. The entire third dsRBD of Xlrbpa is required for protein–protein interaction. The third domain of Xlrbpa (xl3) and xl3-deletion constructs were cloned into the prey vector and tested for interaction with Xlrbpa that was cloned in the bait vector. ONPG β-galactosidase assays were performed to measure the strength of interaction. (a) Alignment of the different constructs of (xl3) tested by two-hybrid assays. The xl3 deletion constructs are numbered from 1 to 10. Number 1 represents the shortest interacting clone recovered from the two-hybrid screen, while number 10 is the minimal deletion-construct still able to interact with Xlrbpa. It exactly spans the minimal dsRBD consensus sequence. The strength of interaction is indicated by (+) and (−). (b) Graph of β-galactosidase units obtained by the constructs shown in (a). 4* indicates the β-galactosidase units when construct 4 is expressed both in the bait and prey vectors. (c) Western blot confirming the expression of constructs used in the two-hybrid assays. Constructs are HA-tagged, therefore the blot was developed with anti-HA antibody. Numbers refer to the constructs in (a). In "–" protein extracts from a strain without a bait vector were loaded. The expected size of the Gal4 AD-HA fusions is indicated by an arrow. In some lanes bands of higher molecular weight (~30 kDa) can be observed. The presence of these bands varies amongst different gels and may represent protein conglomerates.

interaction of these constructs. Again, the third dsRBDs (xl3) was capable to coprecipitate full length myc-tagged Xlrbpa (123-myc), while no interaction was observed with 12-myc, a construct lacking the third dsRBD (Fig. 3(a)). To confirm that the first and second dsRBDs of Xlrbpa were incapable to interact with each other in oocytes, his-tagged full length Xlrbpa (123-his) was coexpressed with either myc-tagged full length Xlrbpa (123-myc) or a deletion construct lacking the third dsRBD (12-myc). As observed in yeast cells, his-tagged Xlrbpa was only able to coprecipitate full length myc-tagged Xlrbpa but failed to show an interaction with the deletion construct 12-myc, lacking the third dsRBD (Fig. 3(b)). Taken together, these results clearly demonstrate that only the third dsRBD of Xlrbpa is both necessary and sufficient for multimerization of Xlrbpa. At the same time, these experiments indicate that endogenous Xlrbpa might exist as a dimer/multimer in vivo.

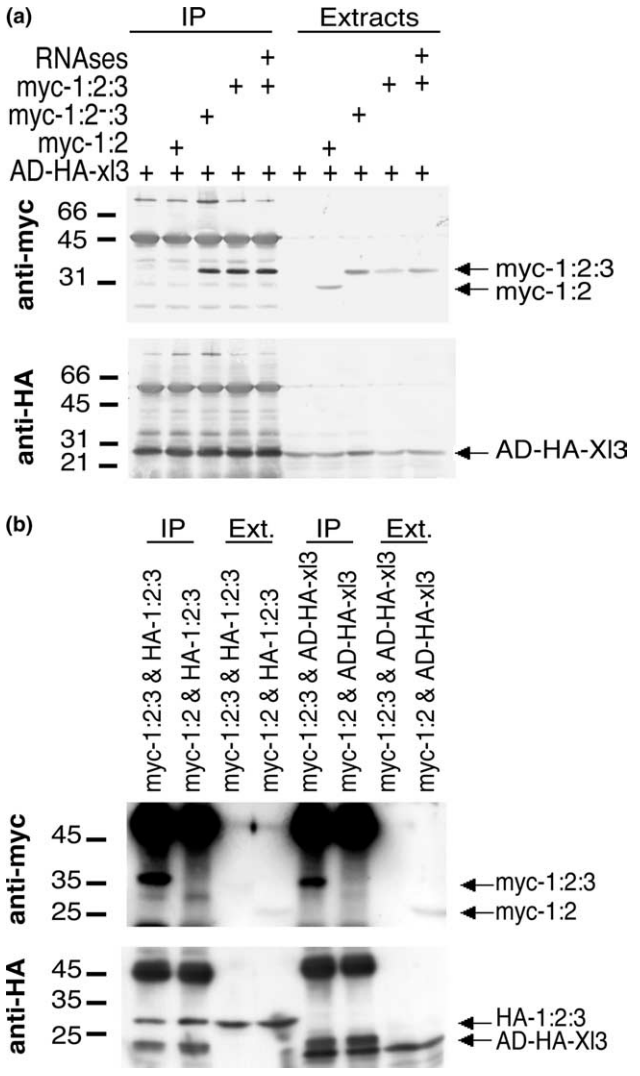

Fig. 2. Coimmunoprecipitation experiments of myc- and HA-tagged Xlrbpa constructs. myc- and HA-tagged Xlrbpa derivatives were expressed in yeast under the control of Gal4 or tetracyclin inducible promoters. Protein-A sepharose coupled with anti-HA antibody was used for immunoprecipitation. Myc-1:2:3 designates myc-tagged wild type Xlrbpa, myc-1:2^{-}:3 carries an H141K mutation that decreases RNA-binding and construct myc-1:2 has the third dsRBD deleted. AD-HA-xl3 expresses the third domain of Xlrbpa as a Gal4 AD + HA-fusion, while HA-1:2:3 expresses HA-tagged full length Xlrbpa (without Gal4 AD). (a) xl3 is sufficient for protein–protein interaction. "+" signs indicate the constructs expressed. "IP" indicates precipitated proteins, while in "Extracts" an aliquot of the total cell lysate was loaded. A strong interaction of xl3 (AD-HA-xl3) with Xlrbpa (myc-1:2:3) can be detected, while construct myc-1:2 completely fails to interact with AD-HA-xl3. Construct myc-1:2^{-}:3 interacts as efficiently as wild type Xlrbpa with AD-HA-xl3, suggesting that RNA-binding has no effect on interaction. Addition of RNAseT1 and RNAseA (indicated by +) had no effect on the coIP of AD-HA-xl3 with myc-1:2:3. (b) xl3 is essential for protein–protein interaction. coIPs of cell extracts expressing the indicated constructs demonstrate that only constructs containing xl3 can interact, while others fail to do so. IP: immunoprecipitation, Ext.: protein extracts.

3.5. *The multimerization behavior of Xlrbpa is conserved in the human homologue PACT*

PACT, the human homologue of Xlrbpa, is a cellular activator of the dsRNA-activated protein kinase PKR which is a key mediator of the antiviral effects of interferons and has functions in the regulation of cell proliferation, apoptosis and signal transduction [19]. It was also shown that the first two dsRBDs of this protein are responsible for a direct protein interaction with PKR, whereas the third domain (pt3) is the PKR-activating domain [20,21]. Xl3 and pt3 are highly homologous to each other. Therefore, it appeared especially interesting whether pt3 retained the ability to homomultimerize. We tested this possibility by two-hybrid assays and, as expected, pt3 like xl3 turned out to be a strong protein–protein interaction domain. In this assay, pt3 interacts efficiently with wild type PACT, while a construct that consists of the first and second domains of PACT (pt1:2) fails to do so (Fig. 4). We could even show that xl3 strongly interacts with pt3 (Fig. 4). These results suggest that both in PACT and Xlrbpa, the third domains are essential for homomultimerization.

4. Discussion

In this work, we provide evidence for the oligomerization of a highly conserved RNA-binding protein. We could show that the homologous proteins Xlrbpa and PACT have a multimerization domain that consists of their third, type-B double stranded RNA-binding domain (dsRBD).

Type-B dsRBDs fail to bind RNA as isolated domains. However, multimerization of the type-B dsRBD xl3 could explain how a domain that is unable to bind RNA by itself can contribute to the overall RNA binding ability of a protein containing additional type-A dsRBDs. Tandem arrangement of dsRBDs enhances their RNA-binding capabilities dramatically [3,4]. The first type-A domain of Xlrbpa, for instance, shows no detectable interaction with RNA in vitro. However, constructs consisting of either two first domains or a first and a second domain (Type-A) become potent dsRNA binders [3]. Addition of xl3 to the first or second domains also increases their RNA-binding ability. It appears plausible that in the context of wild type Xlrbpa xl3 can bring two pairs of type-A dsRBDs into close proximity, thus enhancing the RNA binding ability of the resulting complex.

Most interestingly, our observations could provide an explanation for how PACT mediates PKR activation. PKR is a ubiquitously expressed serine/threonine kinase that is induced by interferon and activated by dsRNA. When activated by

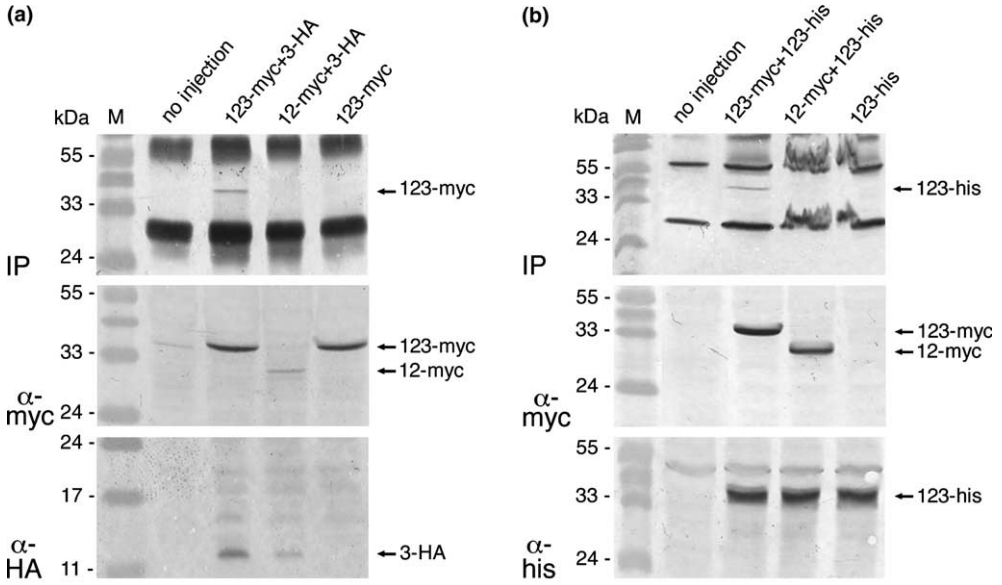

Fig. 3. Multimerization in *Xenopus* oocytes. To determine whether the third dsRBD of Xlrbpa is able to promote multimerization of Xlrbpa in *Xenopus* oocytes, epitope-tagged full length Xlrbpa and various deletion constructs were coexpressed in oocytes and cell lysates were used for immunoprecipitation experiments. (a) Full length Xlrbpa (123-myc) can be precipipitated by a single HA-tagged third dsRBD of Xlrbpa (3-HA). A construct lacking the third dsRBD (12-myc), in contrast, cannot be precipitated by 3-HA. HA-tagged proteins were precipitated with beads to which anti-HA antibody was coupled. IP shows the coprecipitated proteins detected with the anti-myc antibody. α-myc demonstrates expression of myc-tagged proteins by Western blotting, while the α-HA Western blot proves expression of 3-HA. (b) Only the third dsRBD of Xlrbpa can promote multimerization. Full length myc tagged Xlrbpa (123-myc) can be coprecipitated by full length his-tagged Xlrbpa (123-his). While a construct lacking the third dsRBD (12-myc) fails to interact with 123-his. (IP) Proteins were precipitated with anti-his antibody and coprecipitated myc-tagged proteins were detected by an anti-myc antibody. (α-myc) and (α-his) demonstrate expression of the corresponding epitope-tagged proteins by Western blotting.

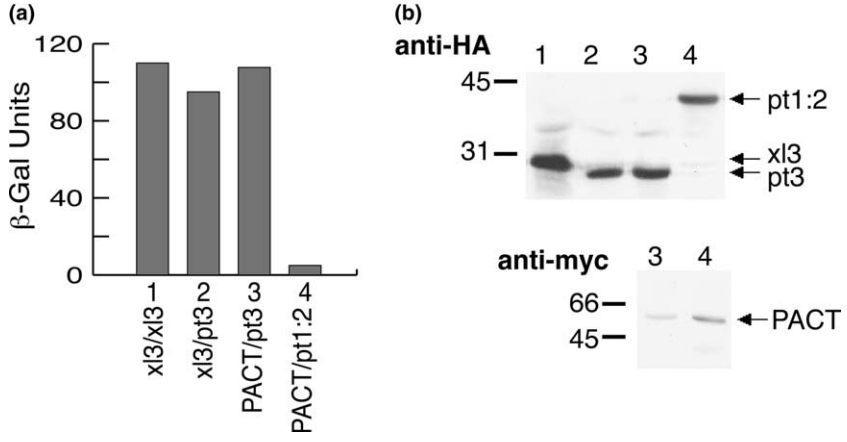

Fig. 4. The third dsRBD of PACT is a protein–protein interaction domain. (a) Graph showing the β-galactosidase unit score of two-hybrid interactions. Interaction scores of (1) the third domain of Xlrbpa (xl3) expressed as bait with xl3 expressed as prey, (2) xl3 as bait with the third domain of PACT (pt3) as prey, (3) Full length PACT as bait with pt3 as prey, and (4) PACT as bait with a construct that contains the first and second domain of PACT (pt1:2) as prey. (b) Two Western blots were made to confirm expression of the bait and prey constructs; numbers above the blots refer to the strains in (a). The first blot was developed with anti-HA antibody that recognizes the constructs expressed in the prey vector, the second was developed with antibody 9E10 that recognizes the myc-tag of PACT. Wild type PACT interacts with pt3 but not with pt1:2, indicating that pt3 is the only domain that is necessary and sufficient for the multimerization of PACT.

viral infections, PKR inhibits translation by phosphorylating the translation initiation factor subunit eIF2α [18]. The regulatory N-terminal region of PKR consists of two dsRNA-binding domains (dsRBDs) in tandem arrangement, whereas its kinase catalytic domain lies at the C-terminal end. Upon binding RNA, PKR undergoes dimerization and *trans*autophosphorylation [22–28]. It was shown that the first two domains of PACT are responsible for a direct protein interaction with PKR, whereas the third domain (pt3) is the PKR-activating domain [20,21]. Our observation could provide a model for PKR activation by PACT. PACT might facilitate the dimerization of PKR by binding PKR via its first and second dsRBDs like that reported previously [20,21] while dimerizing via its third domain. This would help bringing two PKR molecules into close proximity and thereby facilitating their dimerization. Most interestingly, it was recently shown that the

third dsRBD of PACT is capable to stimulate PKR activity, while the third dsRBD of its human paralogue TRBP failed to do so [29]. PACT and TRBP are paralogous proteins showing an almost identical molecular architecture. However, minor differences can be observed both within dsRBDs and in the spacer regions between them. In light of our findings, it thus appears likely that the different effects these domains exert on PKR activity are mediated by different multimerization abilities.

Finally, protein–protein interaction mediated by type B-dsRBDs might also play a role in particle formation of the *Drosophila* Staufen protein. It has been shown that Staufen forms larger complexes that are required to transport maternal RNAs in the early embryo. Type B dsRBDs in this protein might therefore also be required for the observed complex formation [30,31].

When the double-stranded RNA-binding domain was first discovered, it was generally believed that it exclusively functions in binding to dsRNA molecules, while other parts of the proteins would be responsible for other activities. However, it is becoming clear that some dsRBDs have additional or more specialized functions. The *Drosophila* Staufen protein, for instance, has five dsRBDs, only dsRBD 1, 3, and 4 can bind dsRNA in vitro whereas dsRBD 2 is required for the microtubule-dependent localization of *oskar* mRNA and dsRBD5 for the derepression of *oskar* mRNA translation [32]. An extensive search for the nuclear localization signal (NLS) of the RNA-editing enzyme ADAR1 identified a dsRBD as the NLS [33]. Brownawell and Macara, in turn, identified a novel human karyopherin, exportin-5, that binds dsRBDs of several proteins in a RanGTP dependent manner, indicating that dsRBDs can also function as nuclear export signals [34]. Also, dsRBDs were reported to be involved in the dimerization of PKR and ADARs [35–40]. In these studies, however, both RNA-binding and protein dimerization were mediated by the same domains, while in this work we demonstrate oligomerization activity of a type B dsRBD that is incapable to bind RNA by itself. Finally, it should be noted that while our preliminary crosslinking experiments suggest that Xlrbpa predominantly forms dimers in solution, there is also some evidence for the formation of larger complexes. Thus, further work will be required to address the question whether dsRBD3 in Xlrbpa and PACT leads to dimer or multimer formation.

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