Determination of functional domains in polypyrimidine-tract-binding protein

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Polypyrimidine-tract-binding protein (PTB) is involved in pre-mRNA splicing and internal-ribosomal-entry-site-dependent translation. The biochemical properties of various segments of PTB were analysed in order to understand the molecular basis of the PTB functions. The protein exists in oligomeric as well as monomeric form. The central part of PTB (amino acids 169–293)

plays a major role in the oligomerization. PTB contains several RNA-binding motifs. Among them, the C-terminal part of PTB (amino acids 329–530) exhibited the strongest RNA-binding activity. The N-terminal part of PTB is responsible for the enhancement of RNA binding by HeLa cell cytoplasmic factor(s).

INTRODUCTION

Polypyrimidine-tract-binding protein [PTB; also known as p57 and heterogeneous nuclear ribonucleoprotein I (hnRNPI)] is a member of the hnRNP family. PTB shuttles between nucleus and cytoplasm in a transcription-sensitive manner [1]. Different functions for PTB have been suggested relative to different subcellular localizations. PTB was identified originally as a protein binding to the polypyrimidine tracts (Py-tracts) of adenoviral major-late- and α -tropomyosin pre-mRNAs. On this basis, it was proposed that PTB is a splicing factor [2,3]. Binding of PTB to the Py-tract was shown recently to inhibit splicing of alternatively spliced introns [4,5]. Independently, a cellular protein with an apparent molecular mass of 57 kDa (p57) was shown to interact specifically with internal ribosome entry sites (IRES) of encephalomyocarditis virus (EMCV) [6], poliovirus [7] and foot and mouth disease virus (FMDV) [8], which command translation of the downstream genes [9–11]. The binding of PTB to the IRES was proposed to be guiding translation [6,12 and references therein]. PTB and p57 were subsequently identified as the same protein [13]. The presence of other cellular factor(s), which increase RNA-binding specificity of PTB, was proposed by Witherell et al. [14]. Therefore it is possible that PTB could regulate alternative splicing in the nucleus, and enhance translation via IRES in the cytoplasm, by associating with different factors.

Three isoforms of PTB have been reported [2,3,15]. The prototype of PTB (PTB1) consists of 531 amino acids, with a molecular mass of 57 kDa. PTB2 and hnRNP I have insertions of 19 and 26 amino acids respectively after the amino acid at position 291 of PTB1 [3,15]. Four loosely conserved RNA-recognition motifs (RRMs) are distributed throughout the PTB molecule (see Figure 1A). RRM in some RNA-binding proteins are sufficient for specific RNA binding, whereas other RRM require other parts of the protein for their RNA-binding activity [16–18]. Some RRM are known to be involved in protein–protein interactions between certain RNA-binding proteins [16,19]. The

putative RNA-binding and protein–protein-interaction activities of PTB may mediate both its nuclear and cytoplasmic functions. In this study, we determined the functional domains of PTB. First, PTB is found to exist in oligomeric as well as monomeric form. The central part of PTB (amino acids 169–293) plays a major role in the oligomerization, according to results obtained from an *in vivo* yeast two-hybrid system method [20] and an *in vitro* chemical cross-linking method. Secondly, PTB contains several RNA-binding domains, among which the C-terminal part of PTB (amino acids 329–530) shows the strongest RNA-binding activity. Thirdly, the N-terminal part of PTB is responsible for the enhancement of RNA binding by HeLa cell cytoplasmic factor(s).

EXPERIMENTAL

Construction of plasmids

cDNA encoding PTB1 was amplified from RNA of HeLa cells using the reverse transcriptase–polymerase chain reaction method. The PTB-coding sequence was cloned between the *Bam*HI and *Sal*I sites of the pT7-7 vector [pT7-7/PTB(1–530)]. *Escherichia coli* expression vectors pRSETc/PTB(1–327) and pRSETc/PTB(1–169) were constructed by inserting DNA fragments of pT7-7/PTB(1–530) treated with *Eco*RI/*Sma*I/Klenow and *Eco*RI/*Eag*I/Klenow respectively into the pRSETc vector treated with *Bam*HI/*Nhe*I/Klenow. pRSETc/PTB(169–293) was constructed by inserting PTB DNA treated with *Sma*I/*Eag*I/Klenow into the pRSETc vector treated with *Nhe*I/*Hind*III/Klenow. pRSETb/PTB(329–530) was constructed by inserting the DNA fragment from pGBT/PTB(329–530) treated with *Sal*I/Klenow/*Eco*RI into the pRSETb vector treated with *Hind*III/Klenow/*Eco*RI. For the yeast two-hybrid system, plasmids pGBT9 and pGAD424 were used as sources of DNA-binding and transcription activation domains respectively. For insertion of full-length PTB1, plasmid pT7-7/PTB1 was

Abbreviations used: DEAE, diethylaminoethyl; DTT, dithiothreitol; EMCV, encephalomyocarditis virus; FMDV, foot and mouth disease virus; hnRNP, heterogeneous nuclear ribonucleoprotein; IRES, internal ribosomal entry site; PTB, polypyrimidine-tract-binding protein; Py-tract, polypyrimidine tract; RRM, RNA-recognition motif.

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digested with *EcoRI*, filled in with Klenow fragment, and then digested with *SalI*. A DNA fragment of 1.6 kb was isolated. Vectors pGBT9 and pGAD424 were digested with *EcoRI*, filled in with Klenow, and then digested with *SalI*. Fragments of 5.4 kb and 6.6 kb were isolated, which were then ligated with the PTB insert resulting in constructs pGBT/PTB(1–530) and pGAD424/PTB(1–530). Deletion mutants of PTB were made from pGBT9 and pT7-7/PTB1, and used to determine the domain responsible for protein–protein interactions. Plasmid pGBT9 was digested with *EcoRI*, filled in, and then digested with *SmaI* for the vector portion. Inserts for pGBT9/PTB(1–327), pGBT9/PTB(1–293), pGBT9/PTB(1–189), pGBT9/PTB(1–169) and pGBT9(329–530) were prepared from pTM1H/PTB1 by enzymic treatments with *EcoRI*/Klenow/*SmaI*, *EcoRI*/Klenow/*SacII* T4 DNA polymerase, *XmnI*/*EcoRI*/Klenow, *EcoRI*/Klenow/*EagI* and *SmaI*/*SalI* respectively. The insert of pPTB9(109–293) was prepared from pGBT9/PTB(1–293) by treatment with *NcoI*/*EcoRI*/Klenow.

Protein purification

E. coli strain BL21(DE3)pLysS was used to produce PTB(1–530), PTB(1–327), PTB(1–169), PTB(169–293) and PTB(329–530) from plasmids pRSET/PTB(1–530), pRSET/PTB(1–327), pRSET/PTB(1–169), pRSET/PTB(169–293) and pRSET/PTB(329–530) respectively. After lysis of transformed *E. coli* cells, the cell extracts were loaded on to a diethylaminoethyl (DEAE)-Sepharose column (Sigma). The flow-through fraction was loaded on to a phosphocellulose column (Whatman) and eluted with an NaCl gradient. Bound PTB was eluted around 0.6 M NaCl. Peak fractions were loaded on to a poly(U)-Sepharose column from which the PTB was eluted around 0.4 M NaCl. The PTB deletion mutant PTB(1–327) was also purified by this scheme. Other deletion mutants were purified by one-step affinity chromatography with Ni-nitrilotriacetic acid-agarose (Qiagen). The PTBs were eluted between 80 and 100 mM imidazole.

Protein chemical cross-linking with glutaraldehyde

Glutaraldehyde was used to make chemical bridges between proteins interacting with each other. PTB and its derivatives (0.5 µg) were incubated at room temperature for 5 min in the presence of 0.006% or 0.012% glutaraldehyde. The samples were then boiled for 3 min after addition of 2 × sample buffer and analysed by SDS/PAGE (10% or 15% gels), followed by silver staining.

Yeast two-hybrid system

A yeast genetic system called a ‘two-hybrid system’ is often used for detection of protein–protein interactions [20]. We adapted the two-hybrid system in order to confirm the PTB–PTB interaction, and to determine which PTB region is responsible for homomeric interactions. Two kinds of fusion genes encoding hybrid proteins were constructed: one that expresses PTB connecting to the DNA-binding domain of GAL4 protein, and another that expresses PTB connecting to the transcriptional domain of GAL4. After co-transformation of the plasmid pair, yeast transformants containing both of the plasmids were selected by cultivating the yeast cells (Leu[−], Trp[−]) on Leu/Trp-deficient media. PTB–PTB interactions were monitored by measuring the expression level of the reporter gene β-galactosidase. Transformants were grown on synthetic dropout Leu/Trp-deficient plates, and then transferred to filter paper. Liquid N₂ was applied

to the filter to break open the yeast cells. The filter was then laid on top of another filter paper pre-soaked with Z-buffer (60 mM Na₂HPO₄/40 mM NaH₂PO₄/10 mM KCl/1 mM MgSO₄) containing 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside (X-Gal) (20 ng/ml). The filters were kept at 30 °C for the β-galactosidase reaction to proceed. The colour change in the yeast colonies was monitored for up to 30 h of reaction time.

In vitro transcription

Transcription reactions were carried out with T7 RNA polymerase, as described by Van der Werf et al. [22]. Plasmid pBS-ECAT (393–488) was linearized with *HindIII* completely, and the EMCV(393–488) RNA was transcribed with T7 RNA polymerase (Boehringer–Mannheim). In order to obtain ³²P-labelled RNAs, 1 µl of [α-³²P]UTP (3000 Ci/mmol) was added to the transcription reaction mixture.

RNA gel mobility-shift assay

To obtain the probe, ³²P-labelled RNA was isolated with the help of a Push column (Stratagene). EMCV 5′ non-translated region (NTR) corresponding to nucleotides 393–488 was used as probe. RNA (3 × 10⁴ c.p.m.) was incubated with purified PTB or its derivatives. The reaction was carried out in binding buffer [10 mM Hepes (pH 7.4)/50 mM KCl/80 mM potassium acetate/0.6 mM magnesium acetate/1 mM MgCl₂/0.05 mM EDTA/0.8 mM dithiothreitol (DTT)/1.4 mM 2-mercaptoethanol] with 0.3–1.0 µM purified proteins. Samples were incubated at 30 °C for 10 min, and then 2 µl of 0.35 mg/ml heparin was added. Samples were incubated for a further 10 min at 30 °C, and then 2 µl of 10 × sample buffer (50% glycerol/1 mg/ml Bromophenol Blue/1 mg/ml Xylene Cyanol) was added. Samples were loaded on to 4% non-denaturing polyacrylamide gels, and electrophoresed for 2 h at 13 V/cm. Gels were then dried and exposed to film for autoradiography.

UV cross-linking

To obtain a probe, ³²P-labelled RNA was isolated using a Push column. RNA (2 × 10⁶ c.p.m.) was incubated with 0.5 µg of purified proteins, which were dialysed against translation buffer [16 mM Hepes (pH 7.5)/36 mM KCl/169 mM potassium acetate/1.2 mM magnesium acetate/1.6 mM DTT/2.8 mM 2-mercaptoethanol]. The 30 µl reaction mixture also included 3 µl of 5 mM DTT, 5 × binding buffer [25 mM Hepes (pH 7.6)/125 mM KCl/10 mM MgCl₂/0.5 mM EDTA/19% (v/v) glycerol], 20 units of RNasin and 3 µg of rRNA. After 20 min incubation at 30 °C, the samples were transferred to ELISA plates, where they were irradiated with UV light in the UV Stratalinker (Stratagene) for 30 min. RNase cocktail [2 µl of RNase A (10 mg/ml)/2 µl of RNase T1 (100 units/ml)/1 µl of RNase V1 (700 units/ml)] was added (5 µl), and the sample was incubated at 37 °C for 30 min and subsequently analysed by SDS/PAGE. In order to investigate the effect of HeLa cell extract, 35 µg of cytoplasmic extract was added to some of the UV cross-linking reactions.

Preparation of HeLa cell cytoplasmic extract

HeLa S3 cells (approx. 5 × 10⁸) were cultured in a suspension incubator and then harvested. After washing with isotonic buffer [35 mM Hepes (pH 7.5)/150 mM NaCl/10 mM glucose], the cell paste was resuspended in 1.5 vol. hypotonic buffer [10 mM Hepes (pH 7.5)/10 mM potassium acetate/1.5 mM magnesium acetate/2.5 mM DTT] and then Dounce-homogenized. The sample was centrifuged at 30000 g for 20 min. The supernatant

was dialysed against buffer [10 mM Hepes (pH 7.5)/90 mM potassium acetate/1.5 mM magnesium acetate/ 2.5 mM DTT], and treated with micrococcal nuclease to remove endogenous nucleic acids.

RESULTS

Expression and purification of PTB and its deletion mutants

In order to determine the functional domains of PTB, full-length PTB and truncated versions of PTB were expressed in *E. coli* and purified by column chromatography (Figure 1B). We dissected PTB gene into several pieces and purified polypeptides expressed from the segmented genes. PTB was dissected arbitrarily into two parts [PTB(1–327) and PTB(329–530)] bounded by an alanine stretch (8 alanine residues, commencing from the amino acid at

position 316), which is often found at the hinge region of functional domains in a protein (Figure 1B). Since the N-terminal part of PTB(1–293) is essential for PTB–PTB interactions in the yeast two-hybrid system (Figure 4), the N-terminal part of PTB was further divided into two parts for a more precise mapping of the functional domains: one part contains RRM1 [PTB(1–169)], and the other contains RRM2 [PTB(169–293)]. PTB was purified using DEAE-Sepharose, phosphocellulose, poly(U)-Sepharose and/or heparin-Sepharose, consecutively. It was enriched by flow-through fractionation through a DEAE-Sepharose column. Our PTB preparation exhibited high affinity towards phosphocellulose, poly(U)- and the heparin-Sepharose resin. The purified PTB and its truncated forms are shown in Figure 1(B). Most polypeptides were of high purity, as is indicated by the single bands in the SDS gel (Figure 1B). One exception

A

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RRM1 (60–132)  VIHIRKLPIDVTEGE – VISLGLPFGKVTNLLMLKG – KNQAFIEM NTEEAANTMVNYTSTVTPVLRGQPIYIQFSN
RRM2 (183–259) VLRIIVENLFYPVTLDLVHQIFSGFGTIVLKIITFTKNN QFQALLQY ADPVSAQHAKLSLDGQNIYNACCTLRIDFSK
RRM3 (338–410) VLLVSNLNPervTPQS – LFILFGVYGDVQVRVILFNK – KENALVQM ADGNQAQLAMSHLNGHKLHGK – PIRITLSK
RRM4 (456–528) LHLNIPPSVSEEDLK – VLFSSNGGVVGKGFQFKD RKMALIQM GSVEEAVQALIDLHNHDLGNGHH – LRVSFPSK

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RNP1

B

Figure 1 Comparison of RRM1 to RRM4 in PTB and purification of PTB and its derivatives

(A) Four repeated motifs in PTB designated as RRM1 to RRM4 are depicted. Amino acid residues may be classified to be either identical or conserved [conserved is defined by Leu(L)=Val(V)=Ile(I), Phe(F)=Tyr(Y), Ser(S)=Thr(T), Lys(K)=Arg(R), and Asp(D)=Glu(E)]. Where at least three of the four residues are conserved, these are indicated in bold-type and by asterisks above the specified residues. The region with similarity to RNP1, which is conserved in other RNP proteins, is boxed. Schematic diagrams of PTB and its derivatives are shown in (B). The polypeptides expressed in *E. coli* and purified through a series of chromatographic columns are shown in the lower panel.

Figure 2 Oligomerization pattern of PTB

(A) The PTB oligomerization pattern, as analysed in a non-denaturing polyacrylamide gel, is shown. (B) shows the oligomerization pattern of PTB analysed by SDS/PAGE, followed by silver staining before (lane 1) and after (lane 2) chemical cross-linking with glutaraldehyde. (C) The effect of RNase on PTB oligomerization. Either 0.1 μ g (+) or 1 μ g (++) of RNase was included in the reaction mixtures.

Figure 3 Oligomerization pattern of PTB deletion mutants

PTB(1–169), PTB(169–293) and PTB(329–530) were treated with glutaraldehyde and analysed by SDS/PAGE followed by silver staining. Samples in lanes 2, 5 and 8 were treated with 0.006% glutaraldehyde. Samples in lanes 3, 6, and 9 were treated with 0.012% glutaraldehyde.

was PTB(169–293), which also had a smaller-sized band. This smaller protein is most likely a cleavage product of PTB(169–293), because no similar-sized protein was detected in other PTB construct preparations purified by the same procedure.

Self-association activity of purified PTB and its deletion mutants

Protein–protein interactions were tested with a biochemical method. When full-length PTB was electrophoresed on a non-denaturing gel, several species of protein complexes were detected, as seen in Figure 2(A), whereas a single species of PTB

was detected on an SDS gel (see Figure 2B, lane 1). This indicates that full-length PTB may exist in different oligomeric forms, in addition to its monomeric form. In order to determine the molecular mass of the oligomeric forms of PTB, a chemical cross-linking experiment with glutaraldehyde was performed using the full-length PTB. Dimer-, trimer- and tetramer-sized bands, as well as a monomer-sized band, were detected in the presence of glutaraldehyde (Figure 2B). The identity of oligomeric forms of PTB was confirmed by immunoblot analysis using anti-PTB antibodies (results not shown). The oligomerization pattern did not change with the addition of RNase (Figure 2C, lanes 5 and 8). This indicates that oligomerization of PTB *in vitro* is not mediated by RNA–protein interactions, which might have occurred owing to contaminating RNA in the protein source, but rather by protein–protein interactions. Under the same cross-linking conditions, the deletion mutants showed different oligomerization patterns. A monomer- and a faint dimer-sized band were detected for the N-terminal domain PTB(1–169) and the C-terminal domain PTB(329–530) (Figure 3, lanes 2, 3, 8 and 9). In contrast, monomer-, dimer- and trimer-sized bands were detected with the central domain of PTB(169–293) (Figure 3, lanes 5 and 6). This indicates that the central domain may have the major role in PTB–PTB interactions.

Determination of the domain responsible for protein–protein interactions by use of the yeast two-hybrid system

The PTB–PTB interaction was confirmed using the yeast two-hybrid system. Full-length PTB was fused to the GAL4 transcription–activation domain as one part of the two-hybrid system. For its counterpart, either full-length PTB or truncated PTBs were fused to the GAL4 DNA-binding domain, as shown in the

Figure 4 *In vivo* analysis of PTB–PTB interaction using the yeast two-hybrid system

The left panel shows a schematic diagram of the plasmid pairs used in the two-hybrid analysis. The right panel shows β -galactosidase activity in the yeast cells containing the plasmids shown in the left panel. The β -galactosidase activity was tested by filter paper assay.

left panel of Figure 4. When two fusion proteins containing full-length PTB were expressed in yeast cells, β -galactosidase activity was detected (Figure 4, right panel, filter 1). This suggests that PTB forms either a homodimer or a multimer in yeast cells. Both PTB(1–327) and PTB(1–293) demonstrated levels of protein–protein interaction similar to that found for full-length PTB. In contrast, the mutant PTBs with further deletions [PTB(1–189), PTB(1–169), PTB(109–293), PTB(169–293) and PTB(329–530)] did not induce strong β -galactosidase activity (Figure 4, filters 6–10). These data indicate that the domain responsible for the PTB–PTB interaction resides within the N-terminal 293 amino acids of PTB.

Determination of the RNA-binding domain of PTB

RNA-binding activity of PTB and its derivatives was tested by RNA gel-shift analysis using purified PTB and its derivatives, and ^{32} P-labelled EMCV 5'NTR (393–488), which is known to bind to PTB. Full-length PTB showed strong RNA-binding activity (Figure 5, lanes 4 and 5). A smaller PTB–RNA complex, which migrated faster, appeared when a lower concentration of PTB was used (results not shown). The N-terminal domain (amino acids 1–169) showed moderate RNA-binding activity (Figure 5, lanes 6 and 7). Weak RNA-binding activity, shown as a smear on lane 9 in Figure 5, was detected. RNA binding was clearly observed when higher amounts of PTB (169–293) were used in an RNA gel-shift assay (results not shown), but the C-terminal domain (amino acids 329–530) was strong in RNA-binding activity (Figure 5, lanes 10 and 11).

Alternatively, a UV cross-linking method was used to confirm PTB–RNA interactions with both ^{32} P-labelled EMCV 5'NTR and purified PTB and its derivatives as protein sources (Figure 6). Deletion mutants of PTB showed various degrees of RNA binding in the UV cross-linking experiment (compare Figure 6, lanes 3, 5, 7 and 9). The C-terminal domain (329–530) of PTB cross-linked to the stem-loop E much more strongly than either the central domain (169–293) or the N-terminal domain (1–169) (compare lane 9 with lanes 3, 5 and 7 in Figure 6). The order of the relative affinity of the PTB derivatives for RNA, as determined by UV cross-linking, correlates well with the order determined by the gel mobility-shift method described above. Interestingly, PTB(1–327), containing RRM1 and RRM2, showed a much weaker signal in the UV cross-linking experiments than PTB(329–530) containing RRM3 and RRM4 (compare Figure 6, lanes 3 and 9). This might suggest that PTB(329–530), rather than PTB(1–327), features the major RNA-binding domain. This supports a previous report by Kaminski et al. [12], which also showed strong RNA-binding activity by the C-terminal part of PTB. Interestingly, PTB(1–169) polypeptide showed weak RNA binding at 0.3 μ M concentration (Figure 5, lane 6) but rather strong binding at 1.0 μ M (Figure 5, lane 7). This may suggest that PTB(1–169) has co-operative RNA-binding properties.

HeLa extract enhances RNA-binding activity of PTB

The affinity of PTB for RNA increases after the addition of HeLa cell extract [14]. In this study, we determined the regions

Figure 5 RNA binding of PTB and its derivatives

Affinity of PTB and its derivatives for RNA was tested by RNA gel-shift assay. ^{32} P-labelled RNA corresponding to the EMCV 5'NTR (nts 393–488) was used as a probe. Samples in lanes 2, 4, 6, 8 and 10 were incubated with polypeptides at $0.3 \mu\text{M}$. Samples in lanes 3, 5, 7, 9 and 11 were incubated with polypeptides at $1.0 \mu\text{M}$. The polypeptides used in the test are indicated at the top of the gel.

in PTB required for the enhancement of the RNA-binding by HeLa cell cytoplasmic factor(s). RNA-binding activities of the polypeptides containing the N-terminal domain of PTB [PTB(1–530), PTB(1–327), and PTB(1–169)] were enhanced by the addition of HeLa extract (compare lanes 1, 3 and 5 with lanes 2, 4, and 6 in Figure 6 respectively). The enhancing effect of HeLa extract in the UV cross-linking experiment measured by densitometry was two- to four-fold, depending on the deleted regions in PTB. Addition of the HeLa extract did not influence the degree of cross-linking of either the central domain (169–293) or the C-terminal domain (329–530) of PTB (compare lanes 7 and 9 with lanes 8 and 10 in Figure 6 respectively). This suggests that the N-terminal domain (1–169) of PTB is the part involved in the enhancement of RNA-binding by HeLa cell extract.

DISCUSSION

It has been suggested that PTB performs at least two unrelated functions: pre-mRNA maturation and 5'-cap-independent translation. Two different sets of factors may be required for the distinctive functions in the nucleus and in the cytoplasm. For the different functions of PTB, specific RNA-protein and protein-protein interactions are likely to be involved. We investigated the biochemical properties of PTB in order to understand the molecular mechanism of PTB function. PTB exists in monomeric and oligomeric forms, as shown by both biochemical and genetic methods. The central domain of PTB plays a major role in PTB-PTB interaction, and the N- and C-terminal parts of PTB

Figure 6 Effect of HeLa cell extract on RNA binding of PTB and its derivatives

The RNA-binding activity of the proteins was analysed by a UV cross-linking technique. ^{32} P-labelled RNA corresponding to the EMCV 5'NTR (nts 393–488) was used as a probe. Affinities of PTB and its derivatives for RNA were analysed in the presence (+) or absence (–) of HeLa cell extracts. Samples in lanes 1, 3, 5, 7 and 9 were incubated in the absence of HeLa cell extract, whereas those in lanes 2, 4, 6, 8 and 10 were incubated with $35 \mu\text{g}$ of HeLa cell extract.

serve to augment this interaction. Full-length PTB exists as a trimer, or even larger complexes, rather than as a dimer (Figure 2B, lane 2). In contrast, truncated PTB(169–293) forms dimers rather than larger complexes (Figure 3, lanes 5 and 6). Very small amounts of PTB(1–169) and PTB(329–530) exist in dimeric form, and no larger complexes were detected for these truncated PTBs. This might indicate that full-length PTB is required for the complete PTB-PTB interaction. The oligomerization of PTB may play an important role in translational activation. EMCV [7] and poliovirus [14] mRNAs contain at least three PTB-binding sites in each IRES element. Luz and Beck [8] reported that the binding of PTB to the FMDV IRES is co-operative. This co-operative binding of PTB to the IRES element is likely to be mediated by a PTB-PTB interaction, and this interaction may assist in the formation of a conformation of the IRES suitable for translation to proceed.

The RNA-binding domain(s) in PTB were analysed by both RNA mobility-shift assay and UV cross-linking. The C-terminal domain of PTB [PTB(329–530)] exhibited similar RNA-binding affinity to the full-length PTB, on the basis of the UV cross-linking experiment (Figure 6; compare lane 1 with lane 9), but the N-terminus of PTB [PTB(1–327)] containing RRM1 and RRM2 exhibited only weak binding to the RNA (Figure 6, lane 3). The weak RNA binding of the N-terminal and the central domain of PTB might contribute towards the RNA-binding specificity of PTB.

HeLa cell extract enhanced dramatically the RNA-binding activity of PTB and its derivatives containing the N-terminal part of PTB (amino acids 1–169), regardless of the presence of other parts of PTB. This suggests that a factor(s) in the HeLa extract facilitates interaction between PTB and RNA via the N-terminal part of PTB. The putative enhancing factor may exert

Figure 7 Schematic diagram of the functional domains in PTB

an allosteric effect on PTB because no specific RNA-binding protein interacting stoichiometrically with both PTB and the RNA probe was detected in the UV cross-linking experiments (Figure 6; compare lanes 1 and 2). The factor may catalyse the RNA-protein interaction in an enzyme-like mode by interacting transiently with both PTB and RNA. Alternatively, the putative factor might induce a conformational change in the PTB protein for enhanced RNA binding. The enhancement of RNA binding by HeLa cell extract does not depend on the specific RNA structure, because an enhancing effect of the same order of magnitude was observed with RNA molecules with different secondary structures (results not shown). The identity of the cellular factor remains to be disclosed.

The functional domains of PTB analysed in this study are presented schematically in Figure 7. Alanine stretches are often present between functional domains in proteins [15], and therefore the stretch of eight alanine residues in the spacer region between RRM2 and RRM3 is likely to serve as a hinge region between the N-terminal protein-protein interactive domain and the C-terminal RNA-protein interactive domain. The analyses of functional domains in PTB led us to speculate on the molecular evolution of a PTB with multiple functions. PTB might have started out as a simple protein with a single RNA-binding domain (RRM). Consecutive duplications of the RRM motif then resulted subsequently in a quadripartite molecule containing four RRMs. Some of the RRMs may have acquired other function(s), such as protein-protein interaction, while still retaining some RNA-binding activity that could contribute towards the specificity of the RNA binding.

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