Recent advances in RNA–protein recognition

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The past few years have witnessed remarkable progress in knowledge of the structure and function of RNA-binding proteins and their RNA complexes. X-ray crystallography and NMR spectroscopy have provided structures for all major classes of RNA-binding proteins, both alone and complexed with RNA. New computational and experimental tools have provided unprecedented insight into the molecular basis of RNA recognition.

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Abbreviations

dsRBD	double stranded RNA binding domain
KH	K-homology
Pab	poly(A)-binding protein
PIE	polyadenylation inhibition element
RNP	ribonucleoprotein
RRM	RNA-recognition motif

Introduction

Eukaryotic mRNAs are almost always associated with RNA-binding proteins that control each and every aspect of RNA metabolism, including the formation of its mature ends, splicing, transport, localisation, stability and the efficiency with which protein synthesis is initiated. Understanding how RNA-binding proteins and RNA interact with each other is therefore central to understanding basal gene expression and its regulation.

RNA-binding proteins have a modular structure and contain RNA-binding domains of 70–150 amino acids that mediate RNA recognition [1,2]. The past two years have seen spectacular progress in understanding structure/function relationships for each of the three major classes of eukaryotic RNA-binding protein domains: the RNA-recognition motif (RRM), the double stranded RNA binding domain (dsRBD) and the K-homology (KH) domain. In addition, computational and NMR studies of protein dynamics have provided insight into energetic and dynamics aspects of molecular recognition in both RNA and RNA–protein complexes. These studies are summarised here and have remarkably improved our understanding of how RNA-binding proteins bind RNA and function in the regulation of eukaryotic gene expression.

Strands and helices at the end of RNA-recognition motifs

The RRM or ribonucleoprotein (RNP) domain is by far the best-characterised RNA-binding domain and is also the most widespread [3,4]. All RRMs studied to date share the same topology and three-dimensional structure [3,5], but a recently determined structure [6•] has revealed a new feature of a divergent member of the superfamily. RRM proteins have a four-stranded β sheet packed against two α helices, but the third RRM of the polypyrimidine-tract-binding protein (PTB) contains a fifth strand in the β sheet [6•]. Human U1A was the first protein shown to contain an addendum to the canonical RRM fold, an α helix immediately C-terminal to the domain [7–9]. Structurally equivalent but shorter helices have also been found recently in the interdomain linkers of multidomain RRM–RNA complexes [10••–13••]. In each case, the helix is either less well ordered or not present at all in the free protein, but becomes well defined and plays a crucial role in RNA recognition. What appeared to be a feature specific to U1A is, instead, a widespread structural feature of the RRM.

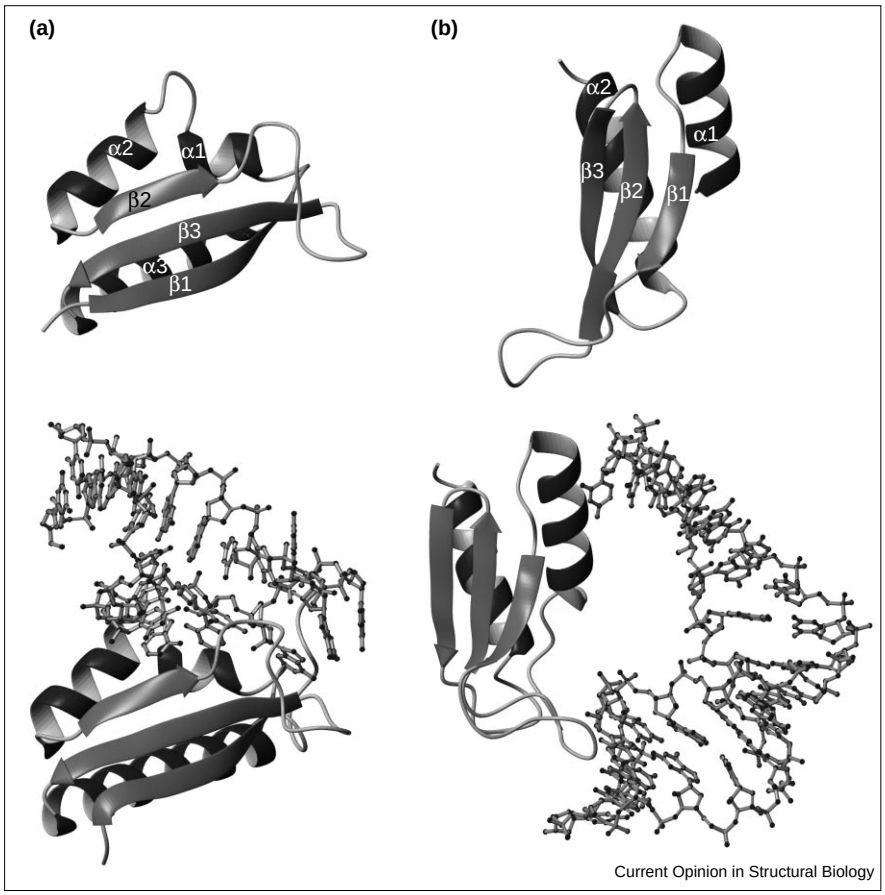
K-homology domain

First identified in the human hnRNP K protein, the KH domain is another common RNA-recognition unit. It has been associated with a variety of cellular functions and implicated in several genetic diseases; for example, fragile-X syndrome can be caused either by reduced expression or by a single amino acid mutation that unfolds a KH domain within the FMR1 protein [14].

NMR spectroscopy [15,16] and, more recently, X-ray crystallography studies [17•] revealed that KH domains share the same αβ structure as the dsRBD (see below) and RRM, with an antiparallel β sheet packed against an α-helical surface (Figure 1a). The structure exposes the invariant Gly-X-X-Gly segment (where X represents lysine, arginine or glycine) in the loop connecting helices 1 and 2, and a more variable region in the loop connecting the second and third strands of the β sheet. These two important loops were predicted to participate in RNA recognition and were shown by NMR spectroscopy to be conformationally flexible in domains derived from FMR1 [16] and hnRNP K.

In contrast to expectations, KH proteins bind RNA in a completely different manner to both RRMs and dsRBDs, suggesting that the convergence of RNA-binding protein domains to the common αβ structural theme has little to do with RNA recognition. The crystal structure of Nova-2 KH3 bound to a stem-loop RNA [18••] revealed, in fact, a new RNA-recognition theme. In the complex, the single-stranded tetranucleotide sequence (5′ UCAC 3′) lies on a hydrophobic α/β platform formed by helices H1 and H2 and strand S1, and is gripped by the Gly-X-X-Gly segment and the variable loop (Figure 1a). The contacts between the protein loops and the backbone phosphates and sugars of the tetranucleotide play a central role in RNA recognition.

Figure 1



RNA recognition by KH and dsRBD proteins. **(a)** Crystal structure of the third KH domain from Nova protein, both free (top) [17•] and in complex with an RNA stem-loop (bottom) [18••]. **(b)** NMR structure of the third dsRBD from *Drosophila* Staufen protein, both free (top) [40] and in complex with an RNA stem-loop (bottom) [21••].

Neither intermolecular stacking interactions (as observed in the RMM) nor specific contacts to the 2′-OH (as seen with the dsRBD) were reported in the KH domain structure, highlighting another important difference between these three protein families.

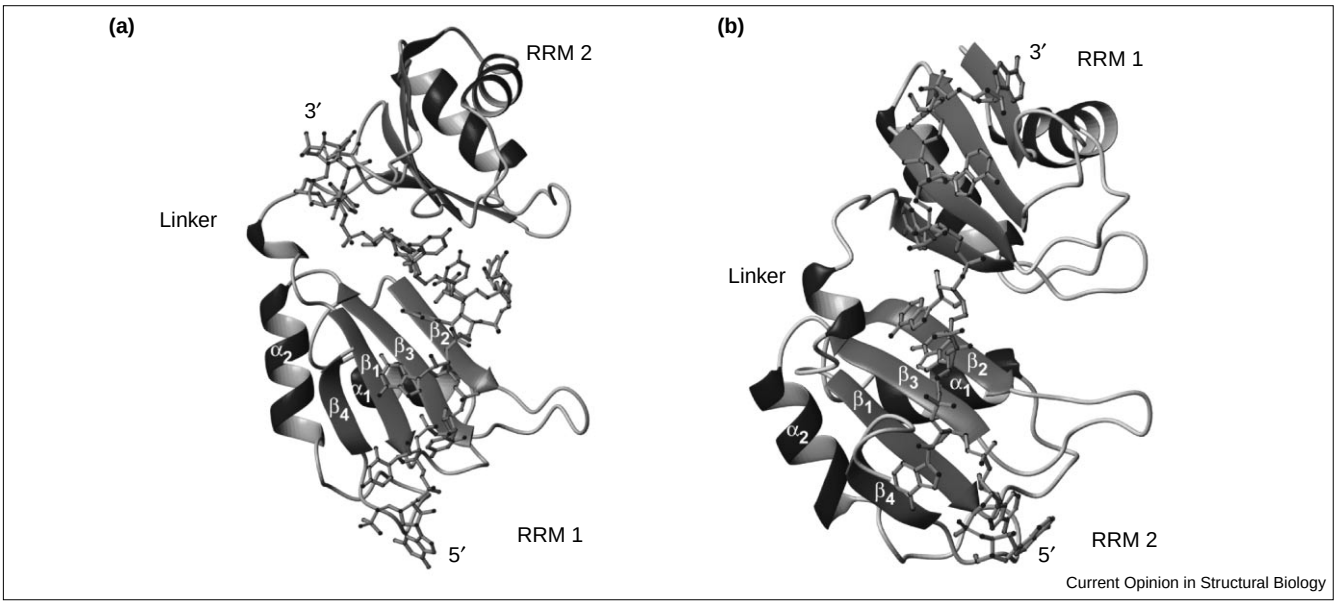
Double stranded RNA binding domain

Two recently determined structures clarified how the dsRBD — the third most common RNA-binding domain [19] — can bind dsRNA selectively, but can bind neither dsDNA nor DNA–RNA hybrids, and displays no sequence specificity. These were the crystal structure of the second dsRBD of *Xenopus laevis* RNA-binding protein A (Xlrbpa) bound to dsRNA [20] and the NMR structure of the third dsRBD of *Drosophila* Staufen protein bound to an RNA hairpin [21••].

Surprisingly, there was an absence of significant conformational rearrangements in the dsRBD complexes, the first time this was observed in the context of RNA recognition. The structures confirmed the essential role of two highly conserved basic loops. These loops were found to be disordered in the free protein and only partially ordered in the Staufen–RNA complex [21••]. Contacts mediated by loop 2 and loop 4 allow recognition of dsRNA and discrimination against dsDNA (Figure 1b). Loop 2 interacts with several 2′-OH groups in the minor groove of the dsRNA type-A helix and loop 4 interacts with the phosphodiester backbone across the major groove from the sites of loop 2 contacts. The third recognition site, helix H1, binds differently to the RNA in the two structures. In the NMR structure [21••], helix H1 recognises a hairpin loop in a non-sequence-specific fashion; in the crystal structure [20], helix H1 interacts instead with the minor groove of the second RNA duplex of a pseudo-continuous double helix formed by crystal packing.

Both structures have provided intriguing (if somewhat conflicting) suggestions concerning how the dsRBD works in a biological context. Staufen (and other dsRBD-containing proteins) recognises a specific set of RNAs in the cell, none of which contains a *bona fide* binding site for its dsRBDs as defined by *in vitro* studies with isolated domains. Many dsRBD proteins, including Staufen, contain multiple domains and these could define its RNA-binding activity cooperatively. Both the different binding abilities of helix H1 and the high levels of sequence conservation within it [21••,22] suggest that this helix plays a central role in the organisation of RNP complexes formed by multidomain dsRBD proteins; however, this intriguing suggestion remains to be tested experimentally.

Figure 2



How two RRMs bind RNA. (a) Crystal structure of the complex between the *Drosophila* Sxl protein and a U-rich sequence derived from the *tra* pre-mRNA [10••]. (b) Crystal structure of the complex between the two N-terminal RRMs of human poly(A)-binding protein and poly(A) [11••].

How two RNA-recognition motifs bind RNA

Many proteins containing RNA-binding domains have multiple copies of the RRM, dsRBD or KH domain. In numerous cases, the RNA-binding activity is determined cooperatively by two or, occasionally, more domain. Four recently determined structures have provided considerable insight into how multiple RRMs bind single-stranded RNA sequences. These are the structures of *Drosophila* Sxl, a developmental regulator of alternative splicing, bound to a U-rich sequence [10••]; the closely related neuronal HuD protein bound to two different AU-rich elements [12••], RNA sequences that control mRNA stability; the two N-terminal RRMs of human poly(A)-binding protein (Pab) bound to poly(A) [11••]; and the complex between the nucleolar protein nucleolin and an RNA hairpin [13••]. In addition, the complex between hnRNP A1 and a telomeric DNA sequence was also reported [23•]. Similar studies with dsRBD or KH proteins containing more than one domain have not yet been described.

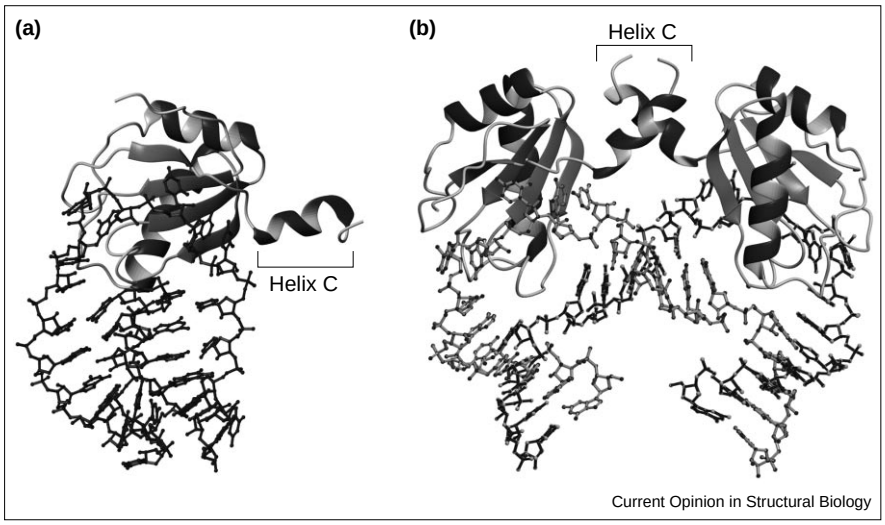
Several common features have emerged by the comparison of these structures (Figure 2). Each of these proteins contains short (8–11 amino acids) interdomain linkers. Although these regions are highly mobile in the free protein [24•,25•], making the two domains structurally independent, in each complex the linker forms a short helix that makes crucial contacts to the RNA. These contacts drive the formation of a well-defined RNA-binding platform by fixing the relative orientation of the two domains.

Two additional common features suggest a common evolutionary origin for the RRM tandem arrangement. The path of the RNA along the protein surface is similar in each complex (Figure 2). The 5′ and 3′ ends of the RNA interact with the C-terminal and N-terminal domains, respectively. Furthermore, the highly conserved aromatic residues within RNP-1 and RNP-2 provide similar stacking interactions in each of these structures; strikingly, each of these interactions is very similar to those observed for the U1A complex containing a single RRM [8,26].

The most striking difference between the four structures is the grossly different RNA-binding surface formed by the β sheet and the linker regions. In the Sxl and HuD complexes [10••] (Figure 2a), it is closed and V-shaped, whereas it is open and planar in the Pab complex [11••] (Figure 2b). In the Pab–poly(A) complex, the two β-sheet surfaces of each RRM sit side-by-side, defining, in essence, a single eight-stranded antiparallel β sheet for recognition of polyadenylate RNA. Nucleolin is different still and it binds a tightly folded RNA hairpin.

These gross topological differences are, undoubtedly, a primary feature of the ability of these proteins to discriminate between different RNA sequences, a concept that has been reinforced by the consideration of the differences in RNA structure. Most adenosine residues in poly(A) are involved in intramolecular stacking interactions in the Pab complex [11••], whereas a single RNA–RNA interaction is observed in the pyrimidine-rich sequences recognised by both HuD and Sxl [10••]. As direct protein–protein interdomain interactions are minimal in each case, it appears that the RNA conformational preferences and, of course, the different size of pyrimidine and purine residues are

Figure 3



Induced fit in RNA recognition is important for biological regulation. (a) NMR structure of the complex between human U1A protein and the half-site PIE RNA [26]. (b) NMR structure of the functional complex of two U1A proteins bound to the complete PIE RNA [27••].

critical determinants of how the two domains are arranged when in complex with RNA.

The biological consequence of induced fit

An important question raised by the nearly universal observation of induced fit in RNA–protein recognition is whether the conformational changes are mechanistically important but biologically irrelevant features of RNA recognition or whether they are central aspects of biological regulation. The complex of human U1A with the polyadenylation inhibition element (PIE) RNA [26] provided perhaps the best example of the importance of induced fit in RNA recognition, with both RNA and protein undergoing significant order-disorder transitions and conformational changes during complex formation (Figure 3a). The recently determined structure of the functional homodimeric U1A complex [27••] has provided a very convincing case that conformational rearrangement and biological function are intimately connected (Figure 3b).

As originally described [26], the conformational change of helix C upon RNA binding repositions both the helix and the effector region [28] of U1A that immediately follows the helix. In the functional complex of two U1A proteins bound to the complete regulatory element, protein–protein interactions that provide cooperativity are mediated by helix C [27••]. Coupled with the structural analysis, elegant biochemical experiments [28] have suggested that the sole purpose of the RNA–protein complex is to present the effector region of U1A to the enzyme that is regulated, poly(A) polymerase, in a conformation suitable for binding and that this cannot occur in the RNA-free conformation of the protein.

In the U1A case, the conformational changes have a clear biological role in controlling features of the protein surface that are essential for its function. Another example is provided by Pab. The surface of Pab involved in interaction with the translation initiation factor eIF-4G spans both RRM domains at the N terminus of the protein opposite to the RNA-binding surface [29]. The Pab–poly(A) structure [11••] reveals that the binding surface for eIF-4G is created by the RNA-bound conformation of the protein construct and, indeed, binding to RNA is required for full biological activity of Pab in translation initiation, at least in yeast [29,30].

Molecular recognition

Even now with numerous high-resolution crystal structures of RNA complexes of RRM proteins, it is very difficult to understand the different specificity observed for distinct RRM proteins. Recent computational and experimental studies have, nonetheless, improved our understanding of how the RRM specifically recognises RNA.

A first important suggestion has been provided by computational studies [31••,32••], which showed that the energetic penalties associated with the conformational changes observed in U1A–RNA binding are large, comparable in fact to the total binding energy [33••]. Among other important observations, this work and systematic investigation of the thermodynamics of binding [34,35•,36] have suggested that it might be impossible to understand RRM–RNA recognition by simply considering the interactions formed in the protein–RNA complex. Changes in energy that accompany binding depend on the conformation of the free components, which, in turn, depends on RNA and protein sequence in a way that cannot be easily understood at present.

A second important suggestion was provided by studies of protein dynamics based on NMR relaxation methods and by a computational study [37••]. Protein backbone dynamics studies suggested that changes in protein sequence that affect binding also affect specific aspects of protein

dynamics [35•,38] in regions of the interface that are essential for RNA recognition. A second study, extending the investigation to protein sidechain dynamics, revealed a complex distribution of conformational freedom in the free and bound proteins [39••]. Parts of the interface appeared to be rigidly ordered, whereas others retained conformational flexibility, even when bound to RNA. Remarkably, this distribution correlated well with what is known about specificity in U1A recognition. The 'rigid' sites corresponded well with regions of the interface that are critical for specificity, whereas more flexible regions coincided with parts of the interface where even gross mutations of RNA or protein residues are tolerated.

Conclusions

The past two years have seen a true explosion in the number of structures of RNA–protein complexes. Structures are now available not only for all of the most common RNA-recognition units, but also for their RNA complexes. The availability of structures for several members of the RRM superfamily has allowed a thorough understanding of the basic principles of RNA–RRM recognition. Comparable knowledge is still missing for KH and dsRBD proteins, as we have determined just one or two structures in each case and we still lack any structures of multidomain proteins for both of these two families. In addition, what determines specificity is still not well understood, even for closely related proteins such as Sxl and HuD. It is likely that further progress in characterising protein and RNA dynamics, and a further integration of computational and experimental tools will generate this knowledge in the near future. It will also be important to address how RNA-binding proteins function as part of multimolecular assemblies that constitute the structural and functional units of gene expression. Clearly, we still need to understand how RNA-binding proteins interact with other factors when bound to RNA and how, through these interactions, they participate in directing the fate of specific RNAs in the cell.

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