REVIEW

U2AF homology motifs: protein recognition in the RRM world

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Recent structures of the heterodimeric splicing factor U2 snRNP auxiliary factor (U2AF) have revealed two unexpected examples of RNA recognition motif (RRM)-like domains with specialized features for protein recognition. These unusual RRM, called U2AF homology motifs (UHMs), represent a novel class of protein recognition motifs. Defining a set of rules to distinguish traditional RRM from UHMs is key to identifying novel UHM family members. Here we review the critical sequence features necessary to mediate protein–UHM interactions, and perform comprehensive database searches to identify new members of the UHM family. The resulting implications for the functional and evolutionary relationships among candidate UHM family members are discussed.

The processes of RNA splicing, transport, capping, editing, and polyadenylation are heavily dependent on protein factors that recognize the pre-mRNA and assemble the appropriate pre-mRNA processing complexes. Surprisingly, the many different protein factors that guide pre-mRNA modification pathways are composed of a limited number of conserved, modular RNA-binding domains (Burd and Dreyfuss 1994). Of these, the RNA recognition motif (RRM) domain is by far the most abundant type of eukaryotic RNA-binding motif. In addition to associations between protein and RNA, protein–protein interactions are essential to recruit catalytic components to sites of RNA modification and to coordinate pre-mRNA processing with other cellular pathways. Interestingly, traditional protein interaction domains, such as SH2, SH3, and WW motifs, are rarely observed in pre-mRNA processing factors (e.g., see Shatkin and Manley 2000; Zhou et al. 2002), implying that the ability to interact with other proteins may reside in the sequences previously thought to be involved in RNA binding. Con-

sistent with this idea, recent structures of the heterodimeric splicing factor U2 snRNP auxiliary factor (U2AF) have revealed two unexpected examples of RRM-like domains with specialized features for protein recognition (Kielkopf et al. 2001; Selenko et al. 2003). In light of this structural information, we call these unusual RRM U2AF homology motifs (UHMs) to reflect their distinct role in protein recognition. Here, the critical sequence features necessary to mediate protein–UHM interactions are reviewed and formulated in a manner that has permitted a comprehensive database search designed to identify members of the UHM family. The resulting implications for the functional and evolutionary relationships among candidate members of the UHM family are discussed. This review represents a first step toward distinguishing canonical RRM from UHMs, and thereby contributes toward a major goal of the postgenomic era (Thornton et al. 2000): to convert genomic sequences into testable functional hypotheses.

Structural features of RNA recognition by canonical RRM

The RNA-binding function of the canonical RRM domain has been extensively investigated over the last two decades. The most conserved RRM signature sequence is an eight-residue motif called ribonucleoprotein 1 (RNP1; Adam et al. 1986; Sachs et al. 1986), which has the consensus [RK]-G-[FY]-[GA]-[FY]-[ILV]-X-[FY] (where X is any amino acid). A second six-residue region of homology, called RNP2, is typically located ~30 residues N-terminal to RNP1 (Lahiri and Thomas 1986; Dreyfuss et al. 1988), and has the consensus [ILV]-[FY]-[ILV]-X-N-L. Additional conserved amino acids define an ~80-residue domain that encompasses the RNA-binding function (Query et al. 1989; Scherly et al. 1989; Birney et al. 1993).

The three-dimensional structure of the canonical RRM domain was first determined for the RRM of U1A (Nagai et al. 1990; Hoffman et al. 1991). The RRM fold is composed of two α -helices packed against four antiparallel β -strands with topology $\beta\alpha\beta\beta\alpha\beta$, which form an α/β sandwich (Fig. 1). The RNP consensus motifs form two central β -strands, with RNP1 in β_3 and RNP2 in β_1 . Because of the alternating side-chain conformations of

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Figure 1. Representative canonical RRM fold, from the structure of the U1A/RNA complex (PDB code 1URN). The N- and C-terminal ends of the RRM are indicated. The position and orientation of the RNA are represented with a ribbon diagram.

the pleated β -sheet, some of the consensus residues maintain the core fold, whereas others are displayed on the surface for nucleic acid recognition. Structures of single RRM complexes with RNA have been determined for the U1A-RRM bound to a hairpin loop of U1 snRNA (Oubridge et al. 1994; Price et al. 1998; Deo et al. 1999; Handa et al. 1999; Allain et al. 2000; Wang and Tanaka Hall 2001), and for a ternary complex of the U2 snRNP proteins U2B'-RRM/U2A' with a U2 snRNA hairpin loop (Price et al. 1998). In contrast to the isolated RRM of U1A, in most cases multiple RRM domains are observed within a single polypeptide, with an average of two RRM domains per protein (Letunic et al. 2004). The structures of several proteins composed of two tandem RRM domains complexed with single-stranded RNA oligonucleotides have been determined, including the alternative splicing factor Sxl (Handa et al. 1999), PAB (Deo et al. 1999), pre-rRNA packaging protein nucleolin (Allain et al. 2000), and translation regulatory protein HuD (Wang and Tanaka Hall 2001). A comparison of these six different structures has revealed some common themes, as well as differences, in the mode of canonical RRM/RNA recognition. When all the structures are superimposed, structural equivalent hydrogen-bonds or stacking interactions are observed between single-stranded RNA and residues in the RNP1 and RNP2 motifs. A variety of sequences and RNA conformations are recognized by a variety of complementary hydrogen bonds with specific bases and differing arrangements of single or multiple RRM domains.

U2AF, the UHM prototype

During pre-mRNA splicing, U2AF and other essential factors facilitate sequential association of small nuclear RNP particles (snRNPs), including U1, U2, U4, U5, and U6 snRNPs, with the borders of intervening pre-mRNA sequences (for review, see Brow 2002). Following assembly of the functional spliceosome, the intron is excised as a branched lariat by two catalytic steps, and adjacent exons are joined together to form the spliced mRNA.

U2AF was identified as a factor that binds to pre-mRNA consensus sequences at the 3' splice site (3'SS), and is required for stable association of the U2 snRNP core spliceosome particle with the pre-mRNA branch point sequence (BPS) during the first ATP-dependent step of the splicing process (Complex A; Ruskin et al. 1988; Zamore and Green 1989). The importance of U2AF *in vitro* was soon corroborated by the discovery that both subunits are essential in *Drosophila melanogaster* (Kanaar et al. 1993; Rudner et al. 1996, 1998b) and *Caenorhabditis elegans* (Zorio and Blumenthal 1999b). Moreover, U2AF⁶⁵ is an essential protein in *Schizosaccharomyces pombe* (Potashkin et al. 1993), and U2AF³⁵ is necessary for vertebrate development (Golling et al. 2002). Because U2AF commits the pre-mRNA to the first critical ATP-dependent step of splicing, its binding is often regulated during alternative splicing (Smith and Valcarcel 2000). In humans, the products of five U2AF³⁵-like open reading frames and the single U2AF⁶⁵ subunit may form distinct heterodimers with different functional activities (Tupler et al. 2001; Shepard et al. 2002). In addition to U2AF, other non-snRNP protein factors are required for formation of Complex A, including Splicing Factor 1 (SF1) and Splicing Factor 3b (SF3b), a multisubunit component of the U2 snRNP (Kramer and Utans 1991).

To perform its role in RNA splicing, two central canonical RRM domains of U2AF⁶⁵ recognize the polypyrimidine tract (Py-tract) in the pre-mRNA (Fig. 2). Binding of U2AF⁶⁵ to the Py-tract is strengthened by cooperative protein-protein interactions with SF1 at the upstream BPS (Berglund et al. 1998; Rain et al. 1998) and with U2AF³⁵, which contacts the downstream 3'SS con-

Figure 2. Diagram of protein-protein interactions mediated by the U2AF heterodimer during the initial stages of pre-mRNA splicing. The U2AF heterodimer (mediated by the U2AF³⁵-UHM/U2AF⁶⁵-ligand interaction) binding to the polypyrimidine tract (Py-tract) and 3'-splice site (3'SS) is facilitated by cooperative interactions between the U2AF⁶⁵-UHM and SF1 at the branchpoint sequence (BPS). Subsequently, SF1 interactions with the U2AF⁶⁵-UHM are replaced by SAP155. The N- and C-terminal ends of the proteins are indicated; the conserved ligand Trp residue (W) is also shown (discussed further in the text).

sensus (Merendino et al. 1999; Wu et al. 1999; Zorio and Blumenthal 1999a). The C-terminal UHM domain of U2AF⁶⁵ interacts with the N-terminal domain of SF1 (U2AF⁶⁵-UHM/SF1-ligand; Rain et al. 1998). At the opposite end of the large subunit, the N-terminal domain of U2AF⁶⁵ provides a ligand that interacts with the central UHM domain of U2AF³⁵ (U2AF³⁵-UHM/U2AF⁶⁵-ligand; Zhang et al. 1992; Rudner et al. 1998b). Subsequently, entry of the U2 snRNP displaces SF1 by interacting with the BPS via the U2 snRNA (Nelson and Green 1989; Wu and Manley 1989; Zhuang and Weiner 1989; Query et al. 1994), and with the U2AF⁶⁵ C-terminal domain via the SF3b subunit, SAP155 (Gozani et al. 1998; Habara et al. 1998). Once the U2 snRNP has contacted the pre-mRNA, U2AF is dissociated by conformational rearrangements of the spliceosome components (Bennett et al. 1992; Chiara et al. 1997). In summary, key protein-protein interactions are mediated by the U2AF⁶⁵-UHM, which interacts with SF1 and subsequently SAP155, and by the U2AF³⁵-UHM, which interacts with the U2AF⁶⁵ N terminus.

Structural features of protein-protein interactions by UHMs

Based on primary sequence analysis, both the U2AF⁶⁵ C-terminal domain and the central domain of U2AF³⁵ were suspected to contain unusual variations of the RRM fold (Birney et al. 1993). However, the borders of the U2AF-UHM domains could not be assigned accurately because of sequence insertions in the first helix of the fold (Helix A) and the absence of aromatic amino acids in the RNP-like motifs that are normally critical for RNA recognition. The independent determination of the X-ray structure of the U2AF³⁵-UHM/U2AF⁶⁵-ligand complex (Kielkopf et al. 2001) and NMR structure of the U2AF⁶⁵-UHM/SF1-ligand complex (Selenko et al. 2003) confirmed that both the C-terminal U2AF⁶⁵ and central U2AF³⁵ protein interaction domains adopt the $\beta\alpha\beta\alpha\beta$ RRM-fold topology. Within the RRM-like fold, the sequence insertions separating the RNP-like motifs increase the length of Helix A from three turns observed among canonical RRMs to five or eight turns for U2AF⁶⁵ and U2AF³⁵, respectively; the functional role of these sequence insertions, if any, is unclear. The parallel use of an RRM-like fold to recognize similar peptide ligands implies that the U2AF³⁵-UHM and U2AF⁶⁵-UHM domains represent a new type of protein-protein interaction motif, hitherto undetected amid the many canonical RRMs of pre-mRNA processing factors.

Three-dimensional structural information revealed unanticipated sequence features of U2AF³⁵-UHM and U2AF⁶⁵-UHM domains that enable interaction with short protein ligands. Despite low primary sequence identity (23%), ligand recognition by the different UHM domains is very similar (Fig. 3). In both the U2AF³⁵-UHM/U2AF⁶⁵-ligand and U2AF⁶⁵-UHM/SF1-ligand structures, a critical Trp residue in the ligand sequence inserts into a tight hydrophobic pocket between the α -helices and the RNP1- and RNP2-like motifs (Kielkopf

et al. 2001; Selenko et al. 2003). In addition to aliphatic residues, a conserved Arg-X-Phe motif (where X is any amino acid; see below) on the loop connecting the last α -helix (Helix B) and β -strand of the UHM fold contributes to the Trp-binding pocket. The Arg residue in the loop (U2AF³⁵-Arg 133 or U2AF⁶⁵-Arg 452) forms an intramolecular salt bridge with the last Glu residue of Helix A (U2AF³⁵-Glu 88 or U2AF⁶⁵-Glu 405) that shields one face of the ligand-Trp, whereas the Phe residue (U2AF³⁵-Phe 135 or U2AF⁶⁵-Phe 454) encloses the opposite Trp face. In addition to the extensive interface with the ligand-Trp, a series of acidic residues in Helix A of the UHM interacts with basic residues at the N terminus of the protein ligand. Specifically, electrostatic interactions between U2AF³⁵-Glu 84 and U2AF⁶⁵-Lys 90 as well as U2AF⁶⁵-Asp 401 and SF1-Arg 21 are observed at similar positions for both structures. The essential nature of acidic residues within Helix A, Phe 454, and the Trp-binding pocket was confirmed for the U2AF⁶⁵-UHM/SF1-ligand complex by site-directed mutagenesis of the U2AF⁶⁵-UHM or SF1-ligand followed by pull-down assays (Selenko et al. 2003). Likewise, the U2AF⁶⁵-ligand-Trp 92 was found to contribute two orders of magnitude to the affinity of the U2AF³⁵-UHM/U2AF⁶⁵-ligand complex by isothermal titration calorimetry.

In the U2AF³⁵-UHM, a distinctive Trp residue (U2AF³⁵-Trp 134) is observed at the X position of the Arg-X-Phe motif on the last loop of the UHM domain. Bulky aromatic residues such as Trp at this solvent-exposed position are especially rare among canonical RRM domains (1% of 676 annotated RRM domains in the SWISS-PROT database). The most frequently observed residues at the corresponding position of canonical RRMs are highly charged, including Glu (16%) and Lys (15%), as is also observed for the U2AF⁶⁵-UHM (Arg-Lys-Phe). The unusual U2AF³⁵-Trp 134 inserts between a series of unique Pro residues at the C terminus of the U2AF⁶⁵-ligand, which are completely absent from the SF1-ligand of the U2AF⁶⁵-UHM. The additional Trp/Pro interaction significantly contributes to the high affinity of the U2AF heterodimer (1.7 nM K_d; Kielkopf et al. 2001). Because the U2AF⁶⁵-UHM/SF1-ligand complex lacks the corresponding Trp/Pro interaction, the affinity is relatively weak (~100 nM K_d; Selenko et al. 2003). The sequence differences in the ligands recognized by the U2AF³⁵-UHM and U2AF⁶⁵-UHM domains reflect the different functional roles of the complexes, which, respectively, maintain the constitutive U2AF³⁵-UHM/U2AF⁶⁵ heterodimer (Zhang et al. 1992; Rudner et al. 1998b) or form a transient U2AF⁶⁵/SF1 intermediate during spliceosome assembly (Rutz and Seraphin 1999).

The structures of the U2AF³⁵-UHM/U2AF⁶⁵-ligand and U2AF⁶⁵-UHM/SF1-ligand complexes revealed several sequence features that distinguish UHMs from canonical RRM domains. One striking feature of UHM domains is their atypical RNP-like motifs. The first residue of the RNP1-like motif and the second residue of the RNP2-like motif are unusual in that they are exposed on the β -sheet surface rather than directly involved in RNA binding. Residues in these positions consist of aliphatic

Figure 3. Structures of U2AF-UHM/ligand complexes. The UHM is shown in blue, the protein ligand is shown in yellow. Key interacting side chains are drawn in ball-and-stick representation. (A) The U2AF³⁵-UHM bound to the U2AF⁶⁵-ligand. (B) The U2AF⁶⁵-UHM bound to the SF1-ligand. (C) Schematic representation of signature UHM protein-protein interactions shared by the two complexes.

amino acids (U2AF³⁵-Ala 47, Val 110, or U2AF⁶⁵-Cys 379, Cys 429) as opposed to the basic and aromatic residues used for RNA recognition by canonical RRM domains. Other prominent distinguishing sequences include the Arg-X-Phe motif and acidic residues in Helix A (especially U2AF³⁵-Glu 84/Glu 88 and U2AF⁶⁵-Asp 401/Glu 405). As a consequence of the acidic nature of Helix A and lack of a basic RNP1 residue that usually contacts the RNA, the isoelectric points of UHM domains are remarkably low (pI 4.1 for the U2AF³⁵-UHM and pI 4.3 for the U2AF⁶⁵-UHM) compared with the typically basic character of canonical RRMs (pI >9) that function to bind anionic RNA ligands. The majority of the aliphatic residues lining the Trp-binding pocket (including U2AF³⁵-UHM Leu 48, Val 85, Leu 130, and Ile 140 and their U2AF⁶⁵-UHM counterparts Leu 380, Val 402, Leu 449, and Val 459), however, cannot be used to dis-

tinguish UHM from canonical RRM domains, because they also serve to preserve the RRM fold (Birney et al. 1993). One exception is the last aliphatic residue of the RNP2 motif (U2AF³⁵-Ile 51 or U2AF⁶⁵-Met 383), which contributes to the Trp-binding pocket and consequently differs from the conserved RNP2-Leu residue within the hydrophobic core of canonical RRM domains. Thus, at least three major sequence differences required for UHM-protein interactions distinguish UHMs from canonical RRM domains: (1) atypical RNP-like motifs, (2) an Arg-X-Phe motif in the last loop, and (3) an acidic character of Helix A.

Identifying novel UHM family members

The discovery of two examples of RRM-like domains with specialized sequence characteristics for protein-

protein interactions raised the question of whether the U2AF³⁵-UHM and U2AF⁶⁵-UHM domains represented a larger family of modular protein interaction domains. Examples of proteins with domains similar to the U2AF⁶⁵ or U2AF³⁵-UHM had been previously noted (Kielkopf et al. 2001; Selenko et al. 2003), including the C-terminal homodimerization domain of PUF60, which has previously been referred to as the PUF60, U2AF⁶⁵, MUD2 protein-protein interaction (PUMP) domain (Page-McCaw et al. 1999). Several search strategies were used to further extend the UHM family. An initial consensus pattern for the U2AF⁶⁵, U2AF³⁵, PUF60, and Tat-SF1 UHM domains, defined automatically using the program PRATT (Brazma et al. 1996), proved too stringent as it only matched homologs of these proteins in a ScanProSite search of the SWISS-PROT/TrEMBL databases (Gattiker et al. 2002). Therefore, a target pattern ([ILM-VFC]-X-[LIFV]-X-[NSHT]-[ILMVC]-X(6,40)-[VLIT]-X(2)-[ED]-X(4,5)-G-X-[IVA]-X(4)-[VIL]-X(4,25)-[GV]-X-[VIAL]-[FY]-[VIL]-X-[FYC]-X(6,12)-[AC]-[LVMIC]-X-X-[LMIF]-X-[NG]-R-[WYKM]-[FY]-X-G-X(4,8)-[IVL]) was defined manually based upon conserved residues that either maintain the RRM-like fold (Birney et al. 1993) or mediate protein-protein interactions in the structures of U2AF³⁵-UHM/U2AF⁶⁵-ligand and U2AF⁶⁵-UHM/SF1-ligand (Kielkopf et al. 2001; Selenko et al. 2003). A search of the SWISS-PROT/TrEMBL databases with this target pattern identified several novel UHM candidates. The UHM family was further extended by manually inspecting RRM family alignments (Prosite PS50102) and the results of iterative PHI-PSI BLAST searches (Altschul et al. 1997) for similarities to the signature Arg-X-Phe motif observed in the last loop of the prototype U2AF-UHM domains.

Sequence comparisons revealed that the principal features that distinguish UHM candidates from canonical RRMs are conserved among 12 novel UHM candidates (Table 1; Fig. 4A), including (1) poor conservation of amino acids in the RNP1- and RNP2-like consensus motifs that would normally bind RNA (first/third and second positions, respectively); (2) an Arg-X-Phe motif in the last loop of the RRM-like fold; and (3) conserved acidic residues in the predicted Helix A and a low isoelectric point (average pI ~4.5). Seven additional UHM candidates displayed a subset of the UHM characteristics. To further investigate the evolutionary relationship among members of the UHM and RRM families, a phylogenetic tree of the candidates was constructed using neighbor joining with correction for multiple substitutions (Fig. 4B; Thompson et al. 1997). A comparison with canonical RRMs whose role in RNA recognition has been established by structure determination (including U1A, SXL, PAB, HuD, and nucleolin) revealed that the 12 convincing UHM candidates occupy a phylogenetic branch distinct from canonical RRMs that diverged from a common ancestral domain. The dendrogram also confirms that several of the putative UHM candidates (i.e., those that displayed only a subset of the UHM characteristics) are more closely related to canonical RRM domains than to U2AF or other UHM candidates, indicat-

ing that these proteins may have independently evolved UHM-like sequence motifs. Additional proteins from diverse eukaryotes displayed UHM signature sequences, but were considered homologs of other UHM candidates based on high sequence identity (Table 2). Given the difficulty of distinguishing UHMs from canonical RRM domains based on primary sequence comparisons alone, additional UHM protein interaction domains may be hidden within the RRM superfamily.

In a few cases, UHM candidates that share a similar domain organization may represent homologs despite low sequence identities and/or a lack of consistent functional data, including SPF45 and DRT111; HCC1 and PAD1; TAT-SF1, UAP2, and CUS2; and MUD2 and U2AF⁶⁵. In a well-studied example, MUD2 is the *S. cerevisiae* homolog of U2AF⁶⁵ based on similar functional interactions with the Py-tract, U2 snRNP, and SF1 (Abovich et al. 1994; Rain et al. 1998). Despite low sequence identity (16%), heterologous complexes between MUD2 and human SF1, or between *S. cerevisiae* SF1 and human U2AF⁶⁵ have not been observed (Rain et al. 1998) indicating that the ligand specificity of the human U2AF⁶⁵-UHM has diverged from the MUD2-UHM. These differences in protein-protein interaction specificity are consistent with functional divergence of MUD2 from other U2AF large subunits. For example, MUD2 is dispensable for viability in *S. cerevisiae* (Abovich et al. 1994), whereas the UHM domain of the *S. pombe* U2AF⁶⁵ homolog (which shares 31% sequence identity with human U2AF⁶⁵) is required in vivo (Banerjee et al. 2004). The U2AF⁶⁵-UHM interacts with an N-terminal domain of the SAP155 subunit of the U2 snRNP that is absent from the *S. cerevisiae* homolog of SAP155 (Gozani et al. 1998). Moreover, *S. cerevisiae* lacks an ortholog of the U2AF small subunit, indicating that MUD2 functions in the absence of the heterodimeric partner. These differences between *S. cerevisiae* and other U2AF homologs, coupled with the identification of eight human UHM candidates and 35 more homologs in a variety of higher eukaryotes compared with only three convincing yeast UHM candidates, suggests that the UHM diverged from the canonical RRM late in the evolutionary timeframe to serve the complicated pre-mRNA processing requirements of multicellular organisms.

The 12 candidate UHM domains are found in the context of a variety of domain arrangements within their protein sequences; a subset is detailed in Table 3. With the exception of the central URP-UHM, the UHM domains often occur near the C terminus of the candidate proteins, providing an exposed position to facilitate molecular recognition. Many of the UHM candidates also contain motifs frequently observed in splicing factors, such as canonical RRMs, arginine-serine (RS) domains, zinc fingers, and Gly-rich regions. Additional unexpected domains are also observed, including the LAP2-Emerin-Man1 (LEM) protein-protein interaction domain of MAN1 and kinase domain of KIS.

The diverse functional domains of the UHM candidates are accompanied by an array of different biological functions (Table 3). Like U2AF⁶⁵ and U2AF³⁵, many of

Table 1. Signature sequences of UHM candidates compared with representative canonical RRM

UHM candidate	Accession number	RNP2	RNP1	RXF motif	Acidic residues ^a	pI
U2AF³⁵	Q01081	IALLNI	VGNVYVKF	RWF	y/y	4.1
U2AF⁶⁵	P26368	LCLNMN	CGKIFVEF	RKF	y/y	4.3
UHM candidates closely related to U2AF						
URP	BAA08533	LLIKSM	RGNVYVQY	RWY	y/y	4.3
KIS	Q8TAS1	LRLNIV	RGQVFVEY	RMF	y/y	4.2
PUF60	AAF05605	MVLRNM	IVKIFVEF	RWF	y/y	4.6
SPF45	AAH39322	VLLRNM	AVRIFLEF	RYF	y/y	4.5
DRT111	AAA73382	LLLRNM	AVRIFVQF	RYF	y/y	4.4
TAT-SF1	AAB18823	VIIKNM	DGVASVSF	RWF	y/y	4.6
UAP2	CAB38682	VVLKHI	DGVVTVRF	RYF	y/y	4.5
CUS2	CAA96203	VIFANV	KGEATVVF	RYF	y/y	4.2
MAN1	AAF73293	LKIRNM	EGCVYVKC	SWF	y/y	5.8
HCC1	Q14498	FQLSNM	QGNVYVKC	RWF	y/y	5.7
PAD1	AAD22102	VVLHNM	AGDIYLKF	RYF	y/y	4.8
MUD2	CAA81911	LLLNC	AGNIYIKF	TQF	y/y	4.2
UHM candidates distantly related to U2AF						
PRP24	AAA76605	VFVSNL	STVALVEF	KYF	n/n	5.8
NGR1	CAA85176	ADLLSL	RCFGFVRV	KWF	n/n	9.9
SAP49	AAH04273	IFIGNL	KGYAFINF	QYL	y/n	4.4
MATRIN3	P43243	IHLNL	KSQAFIEM	LWF	n/n	9.5
SRP38	AAN65380	LFVRNV	RGFAYVQF	KWI	y/y	7.0
PES4	P39684	IFIKNL	YLWAFVTY	FYF	y/n	9.7
TIA1	P31483	VFVGDL	KGYGFSVF	QWL	y/n	9.2
Canonical RRM with known RNA complex structures						
U1A-RRM1	JQ1528	IYINNL	RGQAFVIF	FPF	n/n	10.0
SXL-RRM1	AAO39587	LIVNYL	YGYAFVDF	ITV	y/n	9.7
SXL-RRM2	AAO39587	LYVTNL	RGVAFVRY	VIP	n/n	9.2
PAB-RRM1	P11940	LYVGDL	LGYAYVNF	DVI	n/n	7.0
PAB-RRM2	P11940	IFIKNL	KGYGFSVF	MLL	n/n	8.4
HUD-RRM1	AAA58396	LIVNYL	LGYGFSVNY	LRL	y/n	9.3
HUD-RRM2	AAA58396	LYVSGL	RGVGFIRF	QKP	y/n	9.0
Nucleolin-RRM1	A27441	LFIGNL	RKFGYVDF	LKV	y/n	5.2
Nucleolin-RRM2	A27441	LLAKNL	KGIAYIEF	AIE	y/n	4.4
RRM consensus ^b		ΨΘΨXXL	⊕GΘΩΘΨXΘ	XXX	n/n	>9
UHM consensus ^b		ΨΨΨXXΨ	XGXΨΘΨXΘ	⊕ [WX] F	y/y	<7

Canonical RRM were selected based on the availability of known RNA complex structures.

^aResidues corresponding to U2AF³⁵-Glu84/Glu88 and U2AF⁶⁵-Asp401/Glu405.

^bΨ represents aliphatic amino acids (I/L/V), Θ aromatic amino acids (F/Y), ⊕ basic amino acids (R/K), Ω small amino acids (G/A), and X any amino acid. Shown in bold are characteristic features of UHM domains, including divergence in the second and sixth positions of the RNP2-like motif, divergence in the first and third positions of the RNP1-like motif, matches to the RXF motif, and low isoelectric point (pI).

the UHM candidates play important roles during RNA splicing. Interestingly, a few of the candidates (PUF60, TAT-SF1, and HCC1) play a dual role in regulating transcription. Because RNA splicing factors can influence the efficiency of transcription, and conversely, transcription often influences the efficiency and products of alternative RNA splicing (Auboeuf et al. 2002; Rosonina and Blencowe 2002), these UHM candidates may couple RNA splicing with transcription to coordinate gene expression. Other candidate UHM proteins, namely, the membrane protein MAN1 and the regulatory kinase KIS, are important for signal transduction, but have not yet been shown to affect RNA processing. It remains to be determined whether KIS and MAN1 couple pre-mRNA splicing with other cellular pathways via

their established roles in signal transduction. Demonstrating the significance of their functions, misregulated UHM-containing proteins are associated with several human diseases, including human immunodeficiency virus type 1 (HIV-1; Zhou and Sharp 1996), and certain cancers (Liu et al. 2001; Bieche et al. 2003; Sampath et al. 2003).

Specificity of protein-protein interaction by UHM domains

As stated above, the structures of the U2AF³⁵-UHM and U2AF⁶⁵-UHM complexed with their ligands revealed remarkably similar modes of protein interaction. Notably, both the U2AF⁶⁵ and SF1 ligands bind their respective

Figure 4. Identification of candidate UHM-containing proteins. (A) Structure-based alignment of candidate UHM sequences, U2AF³⁵, U2AF⁶⁵, and representative canonical RRM domains whose structures in complex with RNA are known. Structures were aligned using the program TOPP from the CCP4 suite (Collaborative Computational Project, no. 4, 1994). Secondary structure elements are indicated above the sequences. The positions of key residues that distinguish UHM from RRM domains are highlighted yellow. Consensus UHM residues including acidic residues and the Arg-X-Phe loop are red. Residues in the RNP-like motifs that match the canonical RRM consensus are blue. (B) Phylogenetic tree of candidate UHM domains.

UHM domains via a similar arrangement of basic and Trp residues. A search for the ligand consensus pattern [RK]-X-[RK]-W, shared by both the SF1 and U2AF⁶⁵ ligands, found >1000 matches within the SWISS-PROT database, indicating that predicting protein ligands of candidate UHMs is impractical without further experimental information to identify their functional binding partners. Given that all 12 compelling UHM candidates possess the signature sequences predicted to recognize ligands containing the [RK]-X-[RK]-W motif, it remains an open question whether each UHM domain specifi-

cally recognizes a single target, or could promiscuously interact with the intended ligand of a different UHM in the absence of temporal or spatial regulation. Toward answering this question, the U2AF⁶⁵-UHM has been found not to interact with the N terminus of the U2AF⁶⁵-ligand in two-hybrid assays (Tronchere et al. 1997) and pull-down experiments (C. Kielkopf, unpubl.), suggesting that individual UHMs may, indeed, specifically recognize a unique target. By analogy with other modular peptide binding domains (Pawson and Nash 2003), UHM sequences flanking the Trp-binding pocket

Table 2. Representative homologs of UHM candidates

Human protein	Accession number	Homolog ^a	Source organism	Sequence identity ^b (%)
U2AF³⁵	Q01081	Q9D883	<i>M. musculus</i>	100
		AAM34646	<i>D. rerio</i>	97
		AAB17271	<i>D. melanogaster</i>	82
		AAA08115	<i>A. gambiae</i>	82
		AAM97101	<i>A. thaliana</i>	54
		CAB55137	<i>C. elegans</i>	66
U2AF⁶⁵	P26368	P26369	<i>M. musculus</i>	99
		AAH65869	<i>D. rerio</i>	85
		A48249	<i>D. melanogaster</i>	72
		AAA08228	<i>A. gambiae</i>	61
		E96634	<i>A. thaliana</i>	42
		AAC26982	<i>C. elegans</i>	59
KIS	Q8TAS1	AAH58732	<i>M. musculus</i>	99
PUF60	AAF05605	AAH10601	<i>M. musculus</i>	95
		CAD61099	<i>D. rerio</i>	76
		AAA09944	<i>A. gambiae</i>	53
		AAF47501	<i>D. melanogaster</i>	49
		AAF60676	<i>C. elegans</i>	48
SPF45	AAH39322	AAC64085	<i>M. musculus</i>	100
		AAH45473	<i>D. rerio</i>	78
		AAA15153	<i>A. gambiae</i>	39
		EAA46008	<i>D. melanogaster</i>	36
		CAA97799	<i>C. elegans</i>	35
Tat-SF1	AAB18823	AAH37711	<i>M. musculus</i>	100
		AAH55565	<i>D. rerio</i>	55
		AAF51719	<i>D. melanogaster</i>	45
		EAA10753	<i>A. gambiae</i>	44
		AAK29956	<i>C. elegans</i>	37
HCC1	Q14498	Q8VH51	<i>M. musculus</i>	99
		AAH44487	<i>D. rerio</i>	79
		AAF52478	<i>D. melanogaster</i>	61
		EAA12873	<i>A. gambiae</i>	53
		AAM97977	<i>C. elegans</i>	47
		AAM20703	<i>A. thaliana</i>	42
MAN1	AAF73293	Q9WU40	<i>M. musculus</i>	70

^aCandidates identified from *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Anopheles gambiae*, *Drosophila melanogaster*, *Arabidopsis thaliana*, and *Caenorhabditis elegans*, based on similar domain structure and >35% sequence identity in matching regions identified by the PSI-BLAST program (Altschul et al. 1997).

^bSequence identity between the human candidate and the homolog from the indicated source organism.

may ensure specific and directional interactions (N-to-C orientation) with the ligand.

In support of this analogy, structure-based modeling suggests that variation of the central X residue in the UHM Arg-X-Phe loop may provide one mechanism for UHM recognition of diverse C-terminal ligand sequences. Several of the UHM candidates (hURP, PUF60, Tat-SF1, and HCC1) share a Trp residue within the Arg-X-Phe loop that is essential in the U2AF³⁵-UHM for specific recognition of C-terminal U2AF⁶⁵-ligand-Pro

residues (Kielkopf et al. 2001). Other UHM sequences vary from similar Arg-Trp-Phe motifs (SPF45, DRT111, UAP2, CUS2, and PAD1) to divergent Lys (U2AF⁶⁵) and Met (KIS) residues. Besides recognizing the ligand C terminus via the Arg-X-Phe loop, distinct U2AF⁶⁵-UHM or U2AF³⁵-UHM residues make specific contacts with N-terminal ligand residues. In particular, the bulky U2AF⁶⁵-ligand Tyr 91 stacks against unique U2AF³⁵ aromatic residues (Tyr 52 and Phe 81), and forms a specific hydrogen-bond with His 77 of the U2AF³⁵-UHM (Kielkopf et al. 2001). Similar or identical residues in the URP-UHM (Phe 206, Phe 239, and Gln 235) suggest that a bulky, hydrophobic residue preceding the consensus ligand-Trp would be recognized in an analogous manner, consistent with an interaction between hURP and U2AF⁶⁵ in pull-down and yeast two-hybrid assays (Tronchere et al. 1997). The smaller size of the corresponding U2AF⁶⁵-UHM residues (Ile 398 and Val 384) would leave the hydrophobic side chain of a ligand-Tyr in an unfavorable, solvent-exposed environment (Selenko et al. 2003). Considering the variety of cellular roles played by UHM candidates and the consequent requirement to recognize diverse protein ligands, it will be important to determine whether variation in the positions corresponding to U2AF³⁵ Tyr 52, Phe 81, and His 77, and the central position of the Arg-X-Phe loop enables recognition of distinct ligand sequences by UHM domains.

In addition to recognizing short peptide ligands, UHM domains can self-associate to form protein homodimers. For example, the PUF60-UHM domain interacts with itself in two-hybrid assays (Poleev et al. 2000) and forms SDS-resistant homodimers during electrophoresis (Page-McCaw et al. 1999). The U2AF³⁵-UHM has been shown to form weak homodimers by gel filtration, analytical ultracentrifugation, dynamic light scattering (Kielkopf et al. 2001), and two-hybrid assays (Wentz-Hunter and Potashkin 1996), whereas homodimers of the U2AF⁶⁵-UHM have not been observed (Tronchere et al. 1997). Homo- or heterotypic oligomerizations also have been observed for classical protein-protein interaction domains, with several different effects on ligand recognition. For example, the nNOS-PDZ/syntrophin heterodimer prohibits peptide recognition (Hillier et al. 1999), whereas GRIP or Shank PDZ homodimers leave the peptide-binding pockets free (Im et al. 2003a,b) and the Eps8-SH3 homodimer alters the ligand specificity (Kishan et al. 1997). Although a U2AF³⁵-UHM homodimer can be modeled with the solvent exposed Arg-Trp-Phe loop binding to the Trp-binding site on a second UHM domain, alternative interfaces are possible that would allow the oligomer to simultaneously recognize peptide ligands, as observed for established protein-protein interaction domains.

Do UHM domains recognize RNA?

Modeling of the U2AF-UHM/ligand structures with RNA has revealed that peptide binding to the helical surface of the RRM-like fold is not predicted to physi-

Table 3. Function of human UHM candidates and their potential disease relevance

UHM candidate	Full Name	Domain Organization	Function	Relevance to human disease	References
URP	U2AF-Related Protein		Involved in pre-mRNA splicing.	Altered methylation of U2AF1-RS1 is associated with developmental defects.	(Kitagawa et al. 1995; Tronchere et al. 1997; Dean et al. 1998).
KIS	Kinase Interacting with Stathmin		Phosphorylates a variety of proteins <i>in vitro</i> including the cyclin-dependent kinase inhibitor p27 ^{Kip1} .	KIS mRNA levels are misregulated in neurological tumors associated with neurofibromatosis type I (NF1).	(Maucuer et al. 1995; Maucuer et al. 2000; Boehm et al. 2002; Bieche et al. 2003).
PUF60	Poly-Uridine Factor 60KD		Involved in pre-mRNA splicing; an alternatively spliced form, FBP-Interacting-Protein (FIR), blocks activator-dependent <i>c-myc</i> transcription.	The <i>Drosophila</i> homologue regulates splicing of certain ovarian genes during development; cells from xeroderma pigmentosa (XP)B and XPD patients are defective in FIR repression.	(Page-McCaw et al. 1999; Liu et al. 2000; Poleev et al. 2000; Liu et al. 2001; Van Buskirk and Schubach 2002; Zhou et al. 2002).
SPF45	Splicing Factor 45KD		Involved in pre-mRNA splicing; putative homologues from <i>Arabidopsis thaliana</i> (DRT111) and <i>Toxoplasma gondii</i> suggest a second function in DNA repair.	SPF45 is over-expressed in a variety of human cancers.	(Pang et al. 1993; Neubauer et al. 1998; Dendouga et al. 2002; Lallena et al. 2002; Sampath et al. 2003).
TAT-SF1	Tat Stimulatory Factor 1		Stimulates transcription elongation; human and putative yeast homologues (CUS2, UAP2) associate with splicing factors.	Involved in HIV-1 pathogenesis by stimulating Tat-dependent transcription.	(Zhou and Sharp 1996; McKinney et al. 1997; Li and Green 1998; Yan et al. 1998; Fong and Zhou 2001).
MAN1	MAN antigen 1		<i>Xenopus laevis</i> homologues regulate bone morphogenetic protein (BMP) signaling.	Disruption of BMP/TGF- β signaling pathways mediated by MAN1 are associated with vascular diseases and cancers.	(Lin et al. 2000; Osada et al. 2003; Raju et al. 2003; Waite and Eng 2003).
HCC1	Hepatocellular Carcinoma 1		Involved in pre-mRNA splicing; mouse homologue is a transcriptional coactivator.	Originally identified as a nuclear autoantigen from a patient with hepatocellular carcinoma.	(Imai et al. 1993; Kulda et al. 1998; Jung et al. 2002; Auboeuf et al. 2004)

Domains are annotated according to CDD (Marchler-Bauer et al. 2002). (RS) Arg-Ser rich; (Zn) zinc binding; (S/T kinase) Ser/Thr phosphokinase; (G-patch) Gly rich; (acidic) Glu/Asp rich; (TM) transmembrane region; (LEM) LAP2-Emerin-Man1 homology.

cally interfere with putative RNA interactions on the opposite β -sheet face (Kielkopf et al. 2001; Selenko et al. 2003). Although the U2AF³⁵-UHM/U2AF⁶⁵-ligand complex binds RNA weakly ($K_d > 6 \mu\text{M}$), accessory protein factors and adjacent domains in the full-length U2AF³⁵ sequence (e.g., flanking zinc fingers and an RS domain) are required to assist the weak interaction (Rudner et al. 1998a; J. Valcarcel, pers. comm.). Likewise, the U2AF⁶⁵-UHM domain is not required for Py-tract recognition (Banerjee et al. 2003), and does not appear to interact with RNA (Selenko et al. 2003). These results indicate that UHM domains are not likely to be involved in RNA interactions.

Instead, the UHM family has evolved sequence characteristics that have no benefit for RNA binding, while optimizing the interaction with peptide ligands. In most

canonical RRM-RNA structures, conserved aromatic Phe/Tyr residues at the third RNP1 position or second RNP2 position stack with RNA bases or sugars, and a basic Arg/Lys residue at the first position of the RNP1 motif frequently forms a salt bridge with the phosphate backbone (Fig. 5A; Oubridge et al. 1994; Price et al. 1998; Deo et al. 1999; Handa et al. 1999; Allain et al. 2000; Wang and Tanaka Hall 2001). In contrast, the corresponding U2AF³⁵-UHM (Fig. 5B) and U2AF⁶⁵-UHM (Fig. 5C) residues are replaced with aliphatic substitutions that are not predicted to interact favorably with RNA. Moreover, UHMs display unexpectedly low isoelectric points for optimal binding of basic peptides.

In addition to poor conservation of RNP-like motifs and overall negative charge, RNA binding by the U2AF⁶⁵-UHM structure is further inhibited by a C-ter-

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Figure 5. RNA recognition by an RRM domain compared with the U2AF-UHM domains. The UHM or RRM domains are shown in blue, protein ligands are yellow, and RNA ligands are purple. (A) The U1A-RRM recognizing an RNA oligonucleotide. The RNP1-Arg recognizes the RNA phosphates, a nucleotide base stacks on RNP2-Tyr, and RNP2-Leu is folded in the hydrophobic core. (B) The U2AF³⁵-UHM/U2AF⁶⁵-ligand complex (PDB code 1JMT) rotated 180° about the Y-axis with respect to Figure 3 to show the putative RNA interaction surface. (C) Similar view of the U2AF⁶⁵-UHM/SF1-ligand structure (PDB code 1O0P). The RNP2-Ile packs with the ligand-Trp. The small aliphatic residues in the RNP-like motifs are predicted to be unable to form favorable RNA interactions. Schematic representation of RNP residues that differ between UHMs compared with canonical RRM is shown to the right.

minimal α -helix that forms a tight hydrophobic interface with the putative RNA-binding surface of the RRM-like fold (Selenko et al. 2003). In contrast, the C-terminal extensions of canonical RRM more often strengthen rather than inhibit RNA binding. For example, the C-terminal helical extension of the N-terminal U1A-RRM not only contributes to RNA binding (Oubridge et al. 1994; Zeng and Hall 1997) but also mediates dimer formation for recognition of tandem RNA elements (Klein Gunnewick et al. 2000; Varani et al. 2000). Based on the

U2AF⁶⁵-UHM structure, Phe 433 in the RNP1-like motif and Tyr 463 within the preceding turn interact with Tyr 469, Phe 474, and Trp 475 in the C-terminal α -helix (Selenko et al. 2003). Although counterparts of Phe 474 and Trp 475 are absent among the UHM candidates, aromatic residues at positions corresponding to Phe 433, Tyr 463, and Tyr 469 are observed for the UHM domains of KIS, PUF60, SPF45, and HCC1. This raises the possibility that some of the UHM candidates may have a hydrophobic C-terminal extension that may either inter-

ferre with RNA binding as for the U2AF⁶⁵-UHM, or contribute to homodimer formation in a manner similar to U1A.

Conclusions

The canonical RRM domain was a relatively late evolutionary addition to the array of RNA-binding folds that emerged in response to the needs of complex pre-mRNA processing pathways (Anantharaman et al. 2002). As processes that were originally based on the RNA world became progressively more regulated and reliant on protein interactions, the RRM fold further developed specialized sequence characteristics for protein recognition to form the UHM subfamily. These UHM signature sequences included divergent residues in the RNP-like motifs, an Arg-X-Phe loop sequence, and key acidic residues that collectively recognized the Trp residue and positive charge of the protein ligand. Convincing UHM candidates have been discovered in association with a variety of fundamental cellular processes, ranging from pre-mRNA splicing to transcription, DNA repair, and signal transduction. The large number of proteins that share the signature protein-protein interaction residues of UHM domains supports the proposal that the U2AF-UHMs represent a novel family of modular protein interaction domains.

Because protein interaction domains are attractive modules for communication among a network of pathways, the UHM domain may be an evolutionary extension of RRM domains that couples pre-mRNA processing with other nuclear processes. Protein recognition by so-called RNA-binding domains is an emerging theme in molecular recognition. An early example of RRM-protein interactions was observed in the structure of U2B'/U2A', in which the α -helical surface of the U2B'-RRM interacts with the U2A' leucine-rich repeat motif (Price et al. 1998). Several recent structures of the β -sheet surfaces of heterodimeric RRM domains interacting with α -helical protein ligands have revealed a second mode of RRM-protein recognition distinct from that of UHM/protein complexes (Fribourg et al. 2003; Lau et al. 2003; Shi and Xu 2003; Kadlec et al. 2004). In addition to distinguishing protein recognition domains within the RRM family, a growing list of fold families such as the Sterile α -Motif (SAM; Kim and Bowie 2003), LEM (Cai et al. 2001; Laguri et al. 2001), Pumilio/HEAT-repeat domains (Wang et al. 2002), and zinc fingers (Morgan et al. 1997) have been found to bind either nucleic acids or protein ligands through slight variations of a common scaffold. Furthermore, RS domains have been shown to contact the pre-mRNA during splicing (Valcarcel et al. 1996; Shen et al. 2004), and have also been reported to mediate protein-protein interactions (Wu and Maniatis 1993). Because a major goal of the "postgenomic" era is the ability to predict protein functions even in the absence of corroborating experimental results (Thornton et al. 2000), it will be essential to compile a lexicon of signature sequences, such as those that distinguish UHMs from canonical

RRMs, for other fold families whose members play diverse functional roles.

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