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The sequence selectivity of KSRP explains its flexibility in the recognition of the RNA targets

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ABSTRACT

K-homology (KH) splicing regulator protein (KSRP) is a multi-domain RNA-binding protein that regulates different steps of mRNA metabolism, from mRNA splicing to mRNA decay, interacting with a broad range of RNA sequences. To understand how KSRP recognizes its different RNA targets it is necessary to define the general rules of KSRP–RNA interaction. We describe here a complete scaffold-independent analysis of the RNA-binding potential of the four KH domains of KSRP. The analysis shows that KH3 binds to the RNA with a significantly higher affinity than the other domains and recognizes specifically a G-rich target. It also demonstrates that the other KH domains of KSRP display different sequence preferences explaining the broad range of targets recognized by the protein. Further, KSRP shows a strong negative selectivity for sequences containing several adjacent Cytosines limiting the target choice of KSRP within single-stranded RNA regions. The in-depth analysis of the RNA-binding potential of the KH domains of KSRP provides us with an understanding of the role of low sequence specificity domains in RNA recognition by multi-domain RNA-binding proteins.

INTRODUCTION

K-homology (KH) splicing regulator protein/fuse binding protein 2 (KSRP/FBP2) is a multi-functional RNA-binding protein that is involved in different steps of mRNA metabolism. The protein was originally identified as an important factor in the alternative splicing of N1 neuronal RNA (1) and subsequently shown to be important in the decay of a subset of mRNAs containing AU-rich stretches within their 3′-untranslated regions (UTRs) [the so-called adenosine–uridine-rich elements, or AREs (2)]. A later study has uncovered an interaction between the rat orthologue of KSRP (Marta1) and the dendritic targeting element (DTE) in MAP2 mRNA 3′-UTR that is necessary to localize the mRNA at the dendritic synapsis (3). RNA binding is central to the different functions of KSRP and is mediated by four KH domains that occupy the middle region of the protein (Figure 1) (4).

The role of KSRP in ARE-mediated mRNA decay (AMD) has been studied in depth. KSRP targets a subset of mRNAs that contain AREs within their 3′-UTR, recruiting the exosome and other de-adenylation factors. Recruitment of these complexes results in polyA shortening followed by 3′–5′ exonucleolytic digestion of the mRNAs (2,4). The comparison of the AREs targeted by KSRP reveals that despite being AU-rich, these elements have very different sequences (5). Further, although the role of the protein in pre-mRNA splicing and mRNA localization is still largely to be explored, it seems clear that, in those contexts, protein–RNA binding does not involve ARE-like sequences (1,3). No unifying element has been identified so far amongst the RNA targets of KSRP.

Functional data suggest that KSRP binds to RNA using multiple domains (4). Indeed the KH-fold can recognize a core 4 nt sequence within single-stranded nucleic acid regions (6) and the long, single-stranded RNA (ssRNA) can potentially fit several of these domains. This hypothesis is also consistent with *in vitro* dissociation constants of KH3, KH4 and KH3–KH4 in complex with the RNA targets. The isolated KH domains bind to short AU-rich sequences with K_d values in the high micromolar range but when two (or more) domains are joined a sub-micromolar affinity is reached (7) (I. Díaz-Moreno *et al*., submitted for publication).

The broad range of sequences recognized by KSRP indicates that if we are to understand the rules directing KSRP–RNA recognition, we cannot limit ourselves to analyse the details of the interaction with a single RNA partner but we need to dissect the full capability of the protein to discriminate between different RNA sequences. Although KSRP has been shown to directly interact with the AREs (4), we have only little information on the binding specificity and affinity of the isolated KH domains of the protein for the RNA. The only equilibrium data available show that the relative positioning of A's and U's

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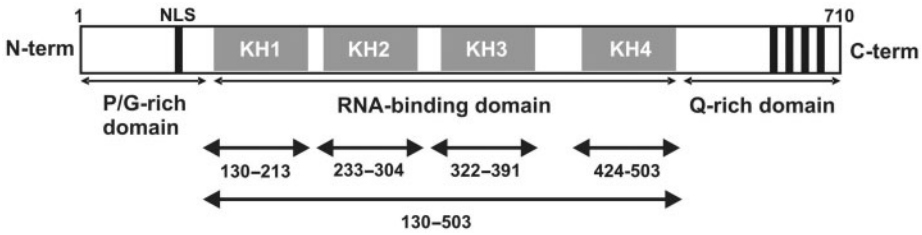

Figure 1. Domain organization of KSRP.

within the TNF-α ARE does not change significantly the binding affinity of KH3 and KH4 (7). No equivalent data are available on the binding of the domains of KSRP to non-AU-rich sequences.

Here we use scaffold-independent analysis (SIA) (8) to dissect the capability of the four KH domains of KSRP to recognize RNA. Our experiments clarify that the four domains of KSRP have different sequence specificities, although they share a negative selectivity for polyC sequences. Based on these results, we probe the affinity range of the different domains using NMR-monitored equilibrium binding assays and we define the role of KH3 as a high affinity RNA-binding domain. In KSRP, the sequence preference of the single domains is conserved in the multi-domain constructs (M. F. Garcia-Mayoral *et al*., unpublished data and I. Diaz-Moreno *et al*., unpublished data). The SIA data on a single domain are used to discuss a general model for KSRP–RNA recognition that has implications for other similar proteins.

MATERIALS AND METHODS

Cloning, expression and purification of the protein constructs and preparation of the RNA oligonucleotides

KSRP KH1 (130–218), KH2 (233–305), KH3 (323–418) and KH4 (423–525) proteins were obtained as described (7). Briefly, ^{15}N-labeled proteins were expressed in *Escherichia coli* BL21 (DE3) (Invitrogen Corp, Carlsbad, USA) as His–GST fusion proteins and initially purified using nickel affinity chromatography according to the manufacturer's instructions. The bulky His–GST fusion tags were then cleaved with TEV protease and removed using a second nickel affinity step. The constructs were then further purified and buffer exchanged by gel filtration (Superdex 75 16/60 column, Pharmacia, Pharmacia Corp, Peapack, USA). Protein purity (always >95%) was assessed using SDS–PAGE and Coomassie staining. Protein quantification was achieved by a combination of spectrophotometry using predicted extinction coefficients and ninhydrin analysis of protein hydrolysates.

All RNA oligonucleotides were chemically synthesized (Dharmacon).

NMR spectroscopy

All NMR spectra were recorded on a Varian Inova (Varian Inc, Palo Alto, USA) spectrometer operating at 800 MHz ^{1}H frequency. The spectra were processed with the NMRPipe package (9) and analysed with Sparky (10). Solutions of 25–50 μM ^{15}N-labeled samples of KH1, KH2, KH3 and KH4 in 10 mM Tris–HCl buffer, 50 mM NaCl, 1 mM TCEP, pH 7.4, were titrated with the relevant RNA oligonucleotides. ^{15}N-HSQC spectra were recorded at each point of the titration at 27°C. Amide chemical shift changes as a function of RNA/protein ratio were fitted to obtain the K_d values for the complexes using in-house software as described in (11).

SIA

SIA experiments were carried out and analysed as previously described (8). In brief, the 16 RNA pools (1 μmol synthesis) that allowed the analysis of the sequence preference in 4 nt positions were purchased as 5′ biotinylated-HPLC-purified oligos from Curevac, Germany and reconstituted in RNAse-free 10 mM Tris–HCl pH 7.4.

Titrations of 25 μM KH1, KH2, KH3 and KH4 samples with the different RNA pools (i.e. uANNN, uCNNN, uGNNN, etc.) were carried out at 27°C in 10 mM Tris–HCl buffer, 50 mM NaCl, 1 mM TCEP, pH 7.4. ^{15}N–^{1}H HSQC spectra were recorded at each step of the titration (RNA:protein ratios of 0:1, 1:1 and 3:1) and averaged chemical shift differences were measured as described above for a subset (8–12) of the peaks affected by binding. Full assignments of backbone resonances of free and bound KH domains were available from previous work (7) (I. Díaz-Moreno *et al*., submitted for publication, I. Díaz-Moreno *et al*., unpublished data). Analysis of the perturbed resonances confirmed that the selected residues are scattered across the protein–RNA interface.

The chemical shift changes observed upon addition of the 16 pools for each of the residues selected were displayed in four histograms (Figure 2b). To rank the differences between pools in a simple format that allows an immediate understanding of the binding preference, the four shifts displayed in each histogram for each peak were normalized to the highest value of the four. Then the normalized value obtained for one peak in one titration was averaged over all the peaks for the same titration that are reported in each histogram. Comparison of this final score for the 4 nt in the same position reports on the binding preference(s) of the protein(s) (Table 1).

RESULTS AND DISCUSSION

SIA is a recently established method that ranks the nucleotide preference of an RNA-binding domain for each position of the single-stranded target sequence (8). The method is designed for domains that bind to RNA with low-to-intermediate affinity and provides not just

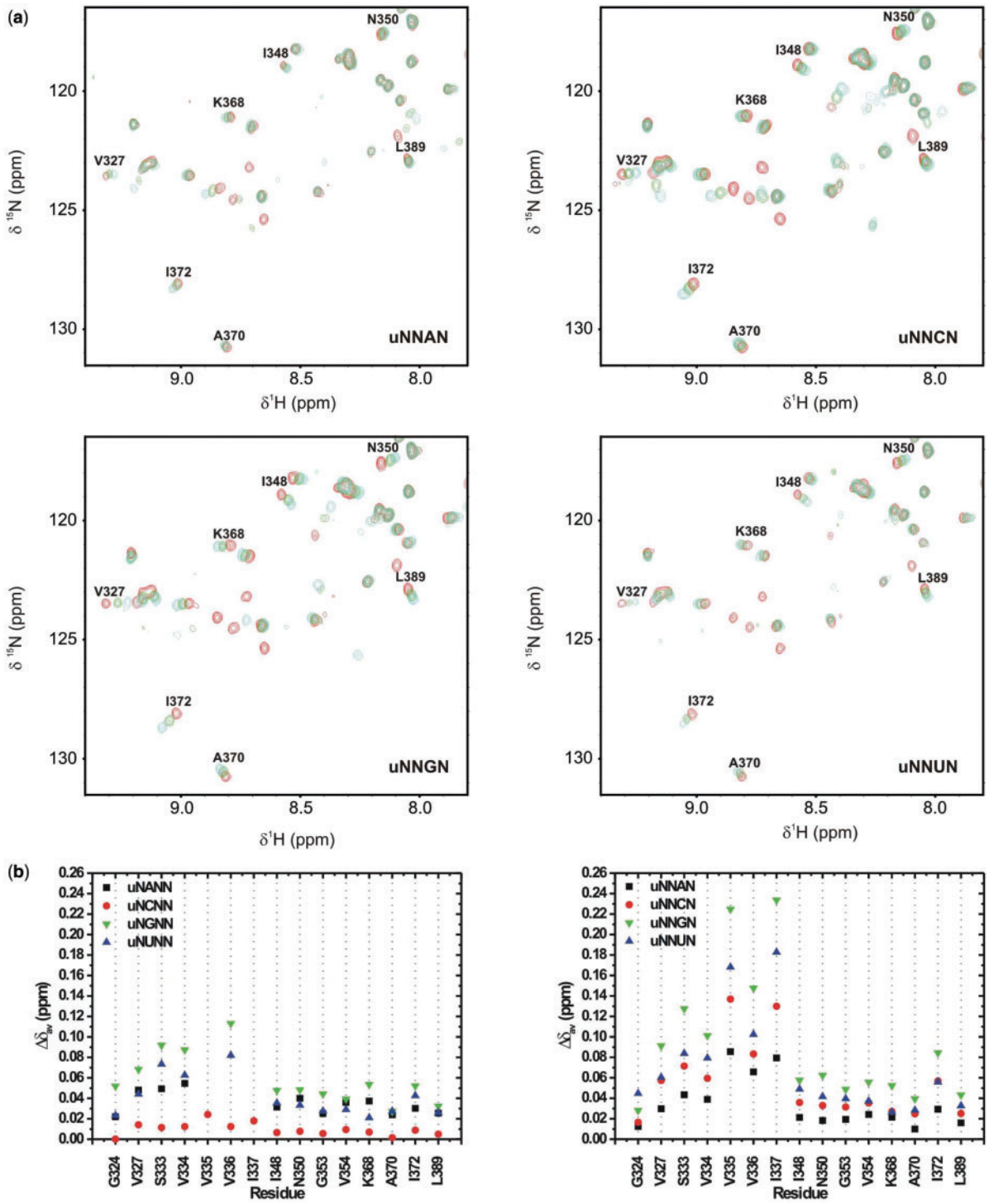

Figure 2. SIA assays for KSRP KH3. (**a**) Overlays of a downfield region from ^{15}N-HSQC spectra recorded during four titrations of the KH3 domain of KSRP with the uNNAN, uNNCN uNNGN, uNNUN pools. Each panel displays the superimposition of three spectra corresponding to the free protein (red), 1:1 (green) and 1:3 (cyan) protein–RNA ratios. Within the ensemble of resonances that shift, we observe peaks in both fast and intermediate exchange. This is true for all of the titrations/pools, although for the highest scoring pools we observe a larger number of resonances in intermediate exchange (7–8 versus 2–3). In order to analyse the data comparatively, we choose only peaks that are in fast exchange in all four titrations. As all the peaks shift because of the same protein–RNA binding event, the exclusion of some peaks does not bias the results (8). (**b**) Chemical shift changes of 15 selected residues in the eight protein–RNA titrations with the non-randomized pools in the second (left) and third (right) positions. The weighted chemical shift changes ($\Delta\delta_{av}$) for the 1:3 protein:RNA ratio are plotted versus the residue numbers and normalized to the highest value for those residues. For each residue the four values reported correspond to the uNANN, uNCNN, uNGNN, uNUNN (left) and uNNAN, uNNCN, uNNGN, uNNUN titrations (right). KH3 prefers binding to a G both in position 2 and in position 3 of the target sequence.

Table 1. SIA scores for the four KH domains of KSRP

	uXNNN	uNXNN	uNNXN	uNNNX
KH1				
A	0.66	0.35	0.29	0.53
C	0.61	0.36	0.32	0.51
G	0.73	0.98	1	1
U	1	0.79	0.62	0.66
+	U	G	G	G
−	A/C	A/C	A/C	A/C
KH2				
A	0.57	0.76	0.86	0.93
C	0.45	0.33	0.38	0.63
G	0.76	0.64	0.94	0.98
U	0.97	0.98	0.79	0.69
+	U	U	A/G	A/G
−	C	C	C	C/U
KH3				
A	0.83	0.72	0.37	0.80
C	0.85	0.56	0.58	0.71
G	0.95	0.99	0.98	0.95
U	0.81	0.71	0.71	0.91
+	N	G	G	G/U
−	N	C	A	A/C
KH4				
A	0.98	0.67	0.21	0.79
C	0.74	0.64	0.28	0.39
G	0.89	0.96	0.95	0.88
U	0.68	0.65	0.57	0.91
+	A/G	G	G	G/U
−	C/U	A/C/U	A/C	C

Depending on the size of the ensembles used for the scoring, small variations in the SIA score (<0.1) may occur. The results of each analysis have been summarized in the '+' and '−' lines. '+' is assigned to nucleotides with SIA score within 0.1 from the top one. '−' is assigned to the ones with SIA score within 0.1 from the bottom one. When overlap exists between '+' and '−' the position is assigned an 'N'.

a preferred binding sequence, but a full analysis of the domain sequence specificity. The information obtained allows us to approach the problem of RNA target choice from orthogonal perspectives; that is allows us to identify not only the sequence(s) that a domain selects for but also the ones that the same domain selects against. As the mRNA is by and large coated by proteins, negative selection against specific 'naked' targets could represent a powerful regulatory tool. The SIA method has been validated on the well-characterized interaction between the KH3 domain of Nova-1 protein and its RNA target (8). We now apply this method to each of the four domains of the KSRP protein.

SIA is based on a comparative analysis of the NMR-detected interactions between an array of short semi-randomized RNA sequences and the target protein (Figure 2). The SIA score reflects the preference for a nucleotide in a specific position of the binding site on a 0–1 scale (8); comparison of this score for the four nucleotides provides an evaluation of the binding preference(s) of the protein. For example, to understand the nucleotide preference of KSRP KH3 for the second position within the target RNA, we compare the SIA scores for the four quasi-randomized uNANN, uNGNN, uNCNN and uNUNN pools (Table 1). The highest scorer is uNGNN (0.99), while the scores of the other three pools are significantly lower. This indicates that the preferred nucleotide in the second position of the KH3-binding site is a G.

Analysis of the SIA scores in Table 1 indicates that the four KH domains of KSRP have different sequence preferences and that different positions within the target sequence are responsible for specificity. In KH3 and KH4, the most specific positions within the RNA target are the two central ones, but this does not apply to KH1 and KH2. The SIA scores for KH1, show that the domain selects negatively for A and C in all positions. This may explain why KH1 binds to AU-rich sequences with an affinity significantly lower than that of the other domains. KH3 and KH4 that do bind to AU-rich sequences with measurable affinity (7), show a positive selectivity for G-rich sequences, while KH2 is the only domain that shows a preference for an AU-rich RNA. There is also a clear negative selection for C in most positions for all the domains. This is different from what we observed for the Nova-1 KH3–RNA interaction: in this domain C is the favoured nucleotide in two of the four positions, consistently with SELEX and *in vivo* data (8). Further, the KH domains of proteins from the PCBP family (including αCP1–4 and hnRNPs K/J) are known to specifically target single-stranded C-rich sequences in the 3′-UTR of several mRNAs (12) with nanomolar affinity. And even the chicken orthologue of KSRP, ZBP2, has been reported to transiently interact with the C-rich Zipcode in β-actin mRNA (13). Therefore, the negative selectivity for C observed for the KH domains of KSRP does not reflect a structural constraint imposed by the KH-fold but a very specific feature of the KSRP protein.

To validate the rank ordering of our SIA data and to relate them to actual affinities (8) we measured the dissociation constants of the complexes formed between the different domains and a representative set of the preferred sequences indicated by SIA (Table 1). We also measured the affinity of the domains for an RNA oligo of sequence uCCCC that according to SIA is selected against by all four KSRP domains. The results of these binding assays (Figure 3) confirm the SIA data and provide a clear picture of the RNA-binding capability of the four domains. We have previously reported that KH3 binds to AU-rich RNA sequences with a K_d of ∼100 μM, ∼3-times stronger than KH2 and KH4, while KH1 binds to the same sequences with very low affinity (7) (I. Díaz-Moreno *et al.*, submitted for publication). We now show that KH3 binds to an SIA-defined sequence (uGGGU) ∼100-fold stronger than to canonical UAUUAU or UAUUUA elements (Figure 3a). In contrast, KH2 shows a binding affinity for the SIA sequence (uUUAG) which is similar (within the error) to the binding affinity for the AU-rich elements described above. KH4 binding to the preferred SIA sequence (uAGGG) is only marginally stronger (∼2-fold) than binding to the AU-rich elements. Finally, KH1 binds to the AU-rich elements very weakly but discriminates effectively in favour of the SIA targets (uUGGG) (Figure 3a). This is consistent with the SIA data described above, which show that this domain does not favour A in any of the positions of the target. Further, it shows that KH1 binds to the RNA target with low

(a)

	uUGGG	uUUAG	uGGGU	uAGGG	uCCCC	UAUUAU
KH1	318 ± 46	---	---	---	nb	>1000
KH2	---	540 ± 85	---	---	nb	396 ±54
KH3	---	---	1.5 ± 1	---	nb	140 ± 20
KH4	---	---	---	170 ± 30	nb	350 ± 30

(b)

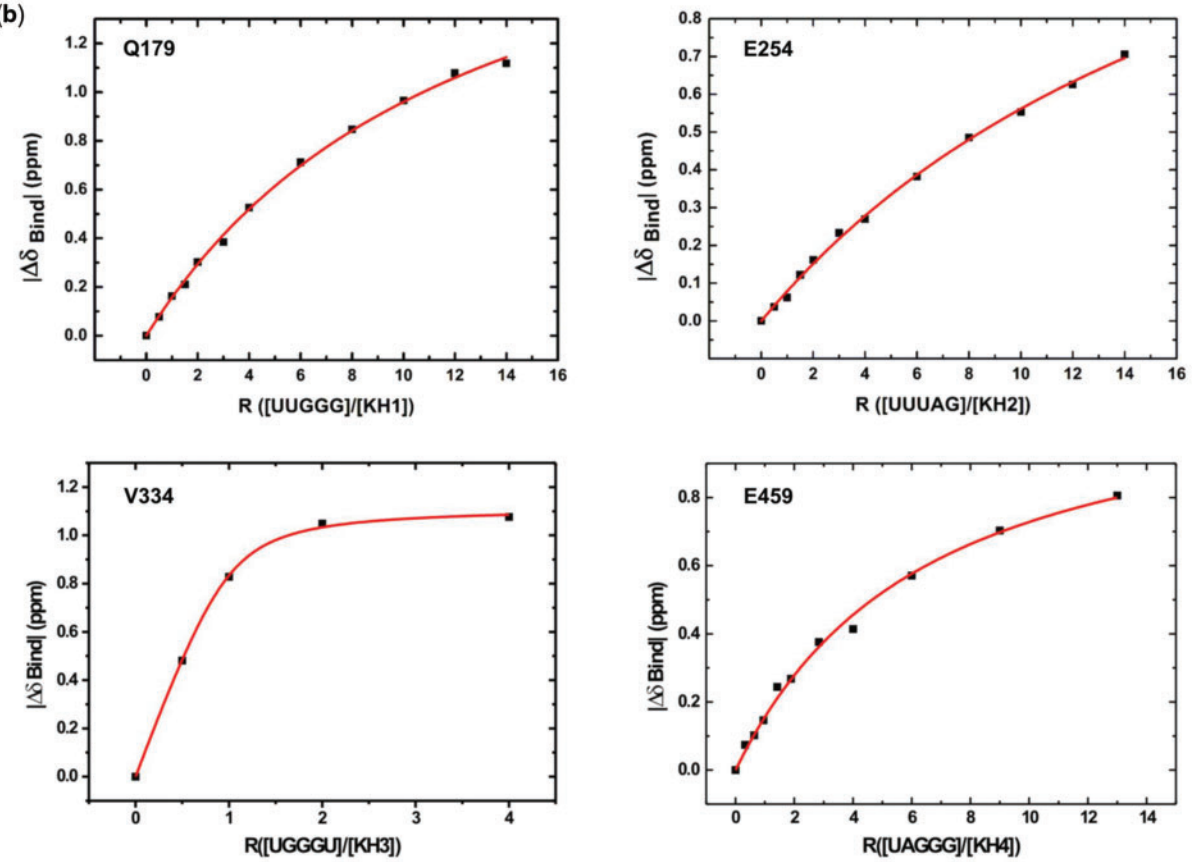

Figure 3. KSRP KH domains bind to their target sequences with very different affinities—(**a**) K_d values (μM) of the complexes between KSRP KH domains and selected RNA oligos (nb: no significant binding observed). These oligos include one of the 'best' sequences, an AU-rich sequence and one of the worse sequences for each domain, as defined by the SIA scores of Table 1. Below, the sequence preferences of the domains are summarized in a Weblogo (http://berkeley.edu/logo.cgi) graphic format (a larger letter and top position indicates a higher score). (**b**) Binding isotherms for selected residues of KH1 (K_d, 318 ± 46; top left), KH2 (K_d, 540 ± 85; top right), KH3 (K_d, 1.5 ± 1; bottom left) and KH4 (K_d, 170 ± 30; bottom right) exemplify the different binding affinities of the KH domains of KSRP. The absolute values of the amide ^{15}N chemical shift changes are represented as a function of the increasing RNA:protein ratios.

affinity and yet discriminates effectively in favour of the wanted sequence, a combination that may be useful in exploring the binding space within a large RNA target. Our binding data also confirm that the four individual domains select strongly against C-rich sequences as no measurable shift is observed during NMR titrations (Figure 4a). To evaluate the effect of this negative selectivity in the context of the four-domain protein, we tested KSRP–RNA interactions using a construct comprising all four domains (KH1234) and two RNAs: a polyC 25-mer

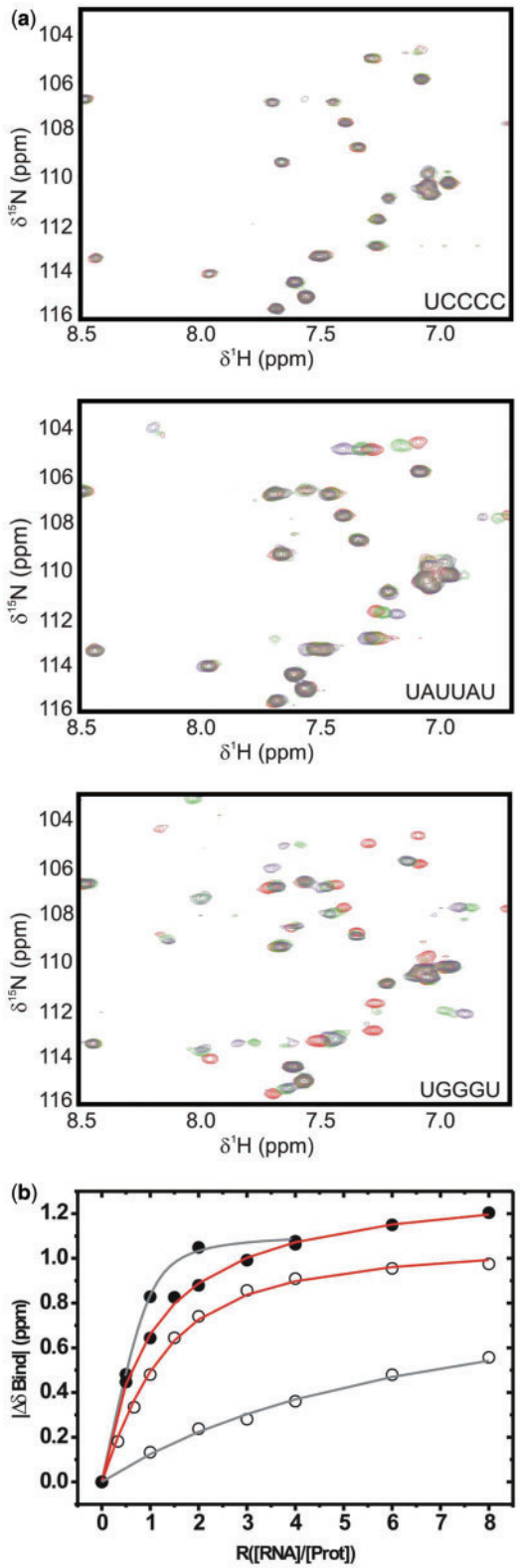

Figure 4. KH3 binding to target and non-target sequences. (**a**) Overlays of a downfield region from ^{15}N-HSQC spectra recorded during the titrations of the KH3 domain of KSRP with, respectively, the UCCCC (top), UAUUAU (middle) and UGGGU (bottom) oligos. Each panel displays the superimposition of three spectra corresponding to the free protein (red) and to 1:1 (green), and 1:4 (purple) protein–RNA ratios. (**b**) Binding isotherms of KH3 to its different RNA targets exemplifies the increase of affinity as we build the consensus sequence. The curves report on the titrations of KH3 with UAUUAU (grey fit, hollow circles), UAGUAU (red fit, hollow circles), UAGGUA (red fit, filled circles) and UGGGU (grey fit, filled circles).

and the TNF-α ARE 25-mer. CD and EMSA show that, while binding to the ARE sequence is very tight [K_d in the low nanomolar range (7)] the interaction with the polyC is weak ($K_d >> \mu M$) (data not shown), confirming that the KH domains of KSRP also select against multiple adjacent Cs in the four-domain protein.

It is worth mentioning that the oligo-nucleotides used in our binding assays are both 6-mer (TNF-α-derived) and 5-mers (SIA-derived). In principle, it is possible that the sixth nucleotide is involved in additional important contacts with the protein. However, we do not see additional chemical shift changes for a 6-mer with respect to a 5-mer indicating that a similar range of contacts is likely to take place. In fact, the largest number of shifts is observed when titrating the uGGGU and uAGGG 5-mers into, respectively, KH3 and KH4 and during titrations with the AU-rich 6-mers. Further, the SIA-derived specific 5-mers bind the KH domains with higher affinity than the TNF-α-derived 6-mers, so any possible difference due to the sixth nucleotide would increase the gap between specific and non-specific sequence reported here. The negative specificity observed for the C-rich pentamers instead has been validated using RNA 25-mers, as described above.

We have previously shown that KH3 affinity for AU-rich sequences is higher than that of KH2 or KH4 for the same sequences. We show here that the domain is able to recognize a G-rich sequence with micromolar K_ds. This affinity is equivalent to that of the isolated Nova-1 KH3 for a short RNA 5-mer containing its target sequence (8) and indicates that KSRP KH3 discriminates between specific and non-specific RNA sequences with more than 100-fold efficiency. In summary, while KH1, KH2 and KH4 bind to the favoured ssRNA sequences with K_ds $>100\,\mu M$, the K_d of KH3 in complex with the specific target is 1–2 μM. This suggests that, in targets containing G-rich sequences, KH3 defines the frame of recognition of the whole KSRP. This general model is validated by the recent discovery that KH3 binding docks KSRP to the GGG-containing apical loop of Let7a miRNA precursor and stimulates pri (and pre)-miRNA processing (M. Trabucchi *et al.*, submitted for publication).

However, a three-G stretch is rare within the ARE targets of KSRP: the AU-rich sequences bound by the protein must be in a single-stranded conformation and insertion of three consecutive Gs is likely to favour base pairing with other 3′-UTR sequences. We therefore wondered if the recognition of G-rich sequences by KSRP was relevant mostly to the interaction with its non-ARE targets. SIA data report on nucleotide preference in one position of the target and are largely uncoupled from the sequence context. Therefore, we can expect that the insertion of isolated Gs (or GG elements) within AU-rich elements would stabilize a specific complex favouring KH3 binding. Single Gs do not promote the formation

of stable secondary structure elements in an AU-rich context and are indeed found in many of the ARE targets of KSRP. We examined the binding affinity of KH3 for single and double G motifs embedded within ARE sequences. The KH3-UAGUAU and UAGGUU complexes have K_ds of 26 ± 5 and $6 \pm 2\,\mu M$, respectively (Figure 4b). Insertion of an isolated G within the TNF-α ARE-derived AU-rich sequence leads to a 5-fold increase in KH3 affinity; insertion of a second G leads to a further 4-fold increase. This clearly shows that KH3 would recognize single and double Gs in an AU-rich context, and that this recognition is likely to be a general feature of the RNA targets of KSRP.

KSRP recognizes its RNA targets with the combined action of multiple domains (4,7) and the discriminatory role of KH1, KH2 and KH4 in RNA binding is subtle but not necessarily less important than the one of KH3. A combination of two or three of the KH domains of KSRP, including KH3, is necessary to reach a K_d in the nanomolar range, but the KH domains of KSRP show a strong negative selectivity towards a range of sequences. Among these sequences are the ones containing multiple Cs. PolyC mRNA regions within the 3′-UTR are expected to be single-stranded, as are AU rich ones, and are targeted by multi-KH regulatory proteins such as hnRNP K and other Poly-C-binding proteins. A negative selectivity for Cs restricts binding of KSRP to a subset of the possible ssRNA targets and contributes to the specificity of recognition. It is important to point out that KSRP can bind sequences with interspersed Cs and it is only when several sequential Cs are present that negative selectivity applies. It is worth mentioning that the *in vitro* selected RNA targets of HuR, an RRM-containing ARE-binding protein that acts antagonistically to KSRP often contain isolated Gs but very rarely Cs (14).

The role played by non-sequence-specific RNA-binding domains is a long-standing issue in RNA recognition by multi-domain proteins. It has been proposed that some multi-domain RNA-binding proteins like hnRNP K use a combination of sequence-specific and non-sequence-specific domains to select their RNA targets (15,16). Our data show that KSRP also uses a combination of high and low specificity domains to recognize the target and that, if a G-rich sequence is present KH3 would assume a leading role in defining the binding frame on the target RNA. However, the functional binding of the RNA needs further interactions and it is interesting to consider the effect of a negative selectivity by the other domains in discriminating between target and non-target RNAs. As a more in-depth analysis of sequence preference in ssRNA-binding proteins is undertaken, negative selectivity may be recognized as an effective answer to the problem of preventing unwanted protein–RNA interactions.

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