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Differences in the RNA binding sites of iron regulatory proteins and potential target diversity

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ABSTRACT **Posttranscriptional regulation of genes of mammalian iron metabolism is mediated by the interaction of iron regulatory proteins (IRPs) with RNA stem–loop sequence elements known as iron-responsive elements (IREs). There are two identified IRPs, IRP1 and IRP2, each of which binds consensus IREs present in eukaryotic transcripts with equal affinity. Site-directed mutagenesis of IRP1 and IRP2 reveals that, although the binding affinities for consensus IREs are indistinguishable, the contributions of arginine residues in the active-site cleft to the binding affinity are different in the two RNA binding sites. Furthermore, although each IRP binds the consensus IRE with high affinity, each IRP also binds a unique alternative ligand, which was identified in an *in vitro* systematic evolution of ligands by exponential enrichment procedure. Differences in the two binding sites may be important in the function of the IRE–IRP regulatory system.**

Regulation of iron metabolism in mammalian cells is achieved primarily through posttranscriptional gene regulation. RNA stem–loops known as iron-responsive elements (IREs) are found in transcripts of genes of iron metabolism, including ferritin, the transferrin receptor (TfR), and erythroid 5-aminolevulinate synthase, the enzyme that carries out the rate-limiting step in heme biosynthesis (reviewed in refs. 1–3). Two distinct proteins known as iron regulatory proteins (IRPs), IRP1 and IRP2, function as IRE-binding proteins when cells are depleted of iron. Binding to transcripts results in either attenuation of translation when the IRE is located in close proximity to the 5′ cap of mRNAs (4) or protection from endonucleolytic cleavage when IRP1 is bound to IREs in the 3′ untranslated region (UTR) of the TfR (5).

Phylogenetic comparisons, mutational analyses, and comparisons among functional IREs in different transcripts have permitted definition of the features of the IRE that are necessary for high-affinity binding by IRPs. The consensus structure for an IRE consists of a base-paired stem interrupted by an unpaired cytosine 5 bp removed from a six-membered loop. The sequence of the loop is almost always CAGUGN, where N can be any base but G (reviewed in ref. 1).

Both IRP1 and IRP2 bind consensus IREs with high affinity (6–9), and both proteins are equally efficacious as translational repressors *in vitro* (10). A single IRP, IRP1, can influence expression of both ferritin and the TfR *in vivo* (11). Since each protein is expressed in all cell types examined to date, and differential effects of the two IRPs on gene regulation have not been described, it is unclear why there are two IRPs in cells. Although the RNA binding activity of each of the two proteins decreases when cells are iron-replete, the mode of regulation differs, and this feature may be of importance in specialized circumstances. IRP2 is rapidly degraded in cells that are iron-replete (8, 9, 12), whereas IRP1 is stable and its function is determined by the presence or absence of an iron–sulfur cluster (reviewed in ref. 1).

The versatility of the IRE–IRP regulatory system would be greatly enhanced if each protein had, in addition to high binding affinity for consensus IREs, unique target specificities *in vivo* that could be combined with specific activation of RNA binding of one of the two proteins. In this study, we have characterized the RNA binding specificities for IRP1 and IRP2 by systematic evolution of ligands by exponential enrichment (SELEX), using previously established methodology (13) to determine whether there are additional potential target specificities for IRPs that could be physiologically relevant. In addition, we have compared the RNA binding site of IRP2 with that of IRP1 by using mutagenesis and found unexpected differences between the two proteins.

MATERIALS AND METHODS

Generation of IRP2 Mutants Using a Two-Step PCR Protocol. All mutants were constructed using a two-step PCR method. Briefly, oligonucleotides containing the desired sequence (underlined) were used to generate by PCR fragments containing the desired mutation that extended either 5′ or 3′ in the IRP2 gene. These fragments were annealed to each other and PCR was used to generate a fragment containing mutations in both strands, which was then cloned into the expression vector pCDSRα, which contains a *myc* epitope-tagged IRP2 using the restriction enzymes *Nsp* V and *Sse* 8387I for K611R and R616Q, *Sst* I for R855Q and N853S, and *Sse* 8387I and *Kpn* I for R774Q (14). The oligonucleotides used were as follows: K611Q, 5′-TTATCTGGAAACCAAAATTTTGAAGGTCGTCT-3′; R616Q, 5′-GAAGGTCAGCTTTGTGATTGTGTTCGTGCC-3′; R774Q, 5′-TATTTGACAAACCAGGGCCTTACCCCTCGT-3′; R855Q, 5′-CAGGAAACTCCCAAGACTGGGCTGCCAAAGGACCGTAT-3′. N853S, 5′-AATATGGTTCAGGATCCTCCAGAGACTGGGCTGCCAA-3′, and K611R, 5′-TTATCTGGAAACAGAAATTTTGAAGGTCGTC-3′. The PCR-generated mutations were confirmed by sequencing.

Transfections, Gel-Retardation Assays, Overexpression, and Competition Assays. COS cells were transiently transfected with pCDSRα expression plasmids encoding IRP2 by electroporation (7). After transfection (48 h), lysates were prepared in 25 mM Tris·HCl (pH 8.0), 40 mM KCl, 1% Triton X-100, and 1 mM dithiothreitol with the protease inhibitors *p*-nitrophenyl *p*′-guanidinobenzoate and leupeptin (Sigma),

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Abbreviations: SELEX, systematic evolution of ligands by exponential enrichment; UTR, untranslated region; TfR, transferrin receptor; IRE, iron-responsive element; IRP, iron regulatory protein.

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and gel-retardation assays were performed as described (6, 10). Reaction mixtures for the gel-retardation assay contained lysate (1–5 μg of protein), 2 μg of tRNA, 20 units of RNasin (Promega), and ^{32}P-labeled IRE probe (2 ng) in a reaction volume of 20 μl.

Overexpression and purification of IRP1 and IRP2 were performed as described (7, 15). To assess the binding affinities of the several different ligands that were identified by the SELEX procedure (described below), competition assays were performed by adding *in vitro*-transcribed RNA ligands (unlabeled) of the indicated sequences to radiolabeled consensus IRE and purified IRPs in a filter binding assay as described (10).

Preparation of RNA Libraries for SELEX. The DNA oligonucleotides (human ferritin H-chain IRE) used as a template were constructed so that an equal amount of each nucleotide was incorporated at the randomized positions, indicated by N (5′-GGATCCAAGTATACAATTCCNTCC-AANNNNNNTTGAANCAGGAATTGTATGAGCTCTC-ACTCT**CCCTATAGTGAGTCGTATTA**-3′). This 83-nt template contained the antisense sequence of the T7 promoter primer, (boldfaced) to which a 20-mer was annealed to create a promoter that results in transcription of RNA complementary to the remaining sequence. The ^{32}P-labeled RNA was purified on an 8% denaturing polyacrylamide gel (6, 16).

Selection of Randomized RNA That Is Bound by IRPs. The ^{32}P-labeled RNA was subjected to three rounds of *in vitro* selection and amplification. ^{32}P-labeled RNA (1 μg) was incubated with IRP1 or IRP2 (100 ng) in reaction buffer containing 25 mM Tris·HCl (pH 8.0), 40 mM KCl, 5% glycerol, 1 mM dithiothreitol, and 5 μg of tRNA in 20 μl for 15 min at 25°C, and the components of the reaction mixture were electrophoretically separated as described (17). After electrophoresis in 1× TBE buffer, IRP–IRE complexes were detected by exposure to x-ray film, eluted in 0.5 M ammonium acetate 0.1% SDS 10 mM magnesium acetate, extracted with phenol/chloroform, and precipitated with ethanol. cDNA was prepared from selected RNAs using 100 units of reverse transcriptase (Perkin–Elmer) for 1 h at 42°C, and the cDNA was amplified by 30 cycles of a PCR protocol in a 50-μl reaction mixture. The products were purified using a PCR cleaning kit (Qiagen) and subsequently transcribed *in vitro* for use in the next round of selection (18, 19).

Cloning and Characterization of SELEX Products. After three rounds of selection, the cDNAs were amplified by PCR, cloned into pCR-SK(+) (Stratagene), and sequenced with Sequenase (Amersham). Binding activity of the individual variants was compared in a binding assay in which IRP1 or IRP2 was present in excess (Fig. 1).

Determination of K_d for Binding of the IREs to Wild-Type and Mutated IRP1s and IRP2s. Lysate (2 μg) from COS cells transiently transfected with different IRP1 and IRP2 constructs in which expression was confirmed by Western blot analysis was incubated in triplicate with various concentrations of radiolabeled IRE (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M) in band shift buffer [40 mM KCl, 25 mM Tris (pH 8.3), 1 mM dithiothreitol, 2% 2-mercaptoethanol, and bovine serum albumin at 0.5 mg/ml]. For radioligand concentrations $\geq 10^{-9}$ M, the specific activity of the probe used was 19.6×10^4 dpm/pmol of IRE and for concentrations of radioligand $\leq 10^{-9}$ M, the specific activity of the probe used was 15.9×10^6 dpm/pmol IRE, as described (17). Data were analyzed using the LIGAND program in which statistical analysis and goodness-of-fit tests are performed automatically and graphic fit can be visually evaluated (20). The sequence of the consensus IRE used in Scatchard analysis was AGAGGAUCCUGCUUCAACAGU-GCUUGGACGGAUCCCAU, and the sequence of the alternative ligand used was AGAGGAUCCUGCUUCAAGGG-AGUUUGGACGG-AUCCCAU.

A

	IRP1	IRP2
C-----CAGUG(A/C/U)-----C	100	100
C-----CAGAGA-----C	42	45
C-----CAGGG(A/C/U)-----C	83±4	81±2
C-----UAGUA(C/U)-----C	38±4	14±3

B

C-----CAGUG(A/C/U)-----C	100	100
C-----CCGUGC-----C	7	22
C-----CAGAGA-----C	42	45
C-----CAGGG(A/C/U)-----C	83±4	81±2
C-----GAGAG(G/U)-----C	11±3	21±4
C-----GAGUCA-----C	22	31
C-----CCGAGA-----C	0	32
C-----GGGAG(A/C/G/U)-----C	12±8	73±7

FIG. 1. Relative binding efficiencies of ligands generated by SELEX with IRP1 (*A*) and IRP2 (*B*). The sequences of IRE ligands resulting from the SELEX procedure, as described in *Materials and Methods*, are represented. As diagrammed in Fig. 2, RNA ligands were generated for SELEX by allowing random incorporation of all four ribonucleotides at eight positions within the stem–loop: the six loop positions, the 5′ bulge C, and the position 6 nucleotides 3′ to the loop. The sixth nucleotide position of the loop was a site of frequent variation, and variants are shown in parentheses. RNA binding activity was quantitated by performing densitometry of gel-retardation complexes generated by mixing radiolabeled ligand (each sequence listed) with lysates from COS cells that were transiently transfected with expression plasmids for either IRP1 or IRP2. Values for binding to the consensus IRE were assigned a value of 100%, and the values of the alternative ligands were calculated relative to that. Corrections for the specific activity of each radiolabeled ligand were made where appropriate. When there was variation at the sixth nucleotide of the loop, the values presented represent an average of single point values obtained for the different variants. Values not associated with error bars were measured a single time to assess which variants would merit full affinity assessments by Scatchard analysis. Sequences depicted were independently isolated a minimum of three times from the ligand pool. A total of 43 or 63 separate clones were analyzed for IRP1 and IRP2, respectively. Twenty of the clones selected by IRP2 contained the sequence GGGAG(X).

Search of GenBank Data Bases for Sequence Matches to RNA Ligands Identified by SELEX. Identification of alternative RNA ligands for IRP1 and IRP2 prompted us to search data bases for matching sequences. The GenBank data base (19) was scanned using a customized exhaustive search program (created by S.A.) for sequences listed in Fig. 1 and their reverse complements. The algorithm used required seven Watson–Crick base pairs to either side of the loop, excluding the bulge C. Sequences that matched the consensus IRE or any of the alternative ligands were considered candidates for further evaluation if their position within the transcript was determined to be within the 5′- or 3′-UTR.

RESULTS

IRP1 and IRP2 Bind Consensus IREs with Equal Affinity. Values for the K_d of binding of the consensus IRE to IRP1 ranged from 5 to 50 pM in this study (data not shown), which is comparable to those previously reported (6, 7). The K_d for binding of the consensus IRE to IRP2 was measured as 19 ± 15 pM, a value that is comparable to those measured previously (8).

***In Vitro* SELEX Defines an IRP1-Specific Ligand and an IRP2-Specific Ligand.** To attempt to identify potential alternative ligands that might be physiologically relevant, a SELEX procedure was performed for IRP1 and IRP2. Based on the fact that IRP1 and IRP2 share extensive sequence homology and bind consensus IREs with equal and high affinity, we expected that the IRE binding sites would be very similar. However, in a SELEX procedure previously completed for IRP1, an alternative ligand was described that bound with high affinity only to IRP1 (13). In the SELEX procedure reported

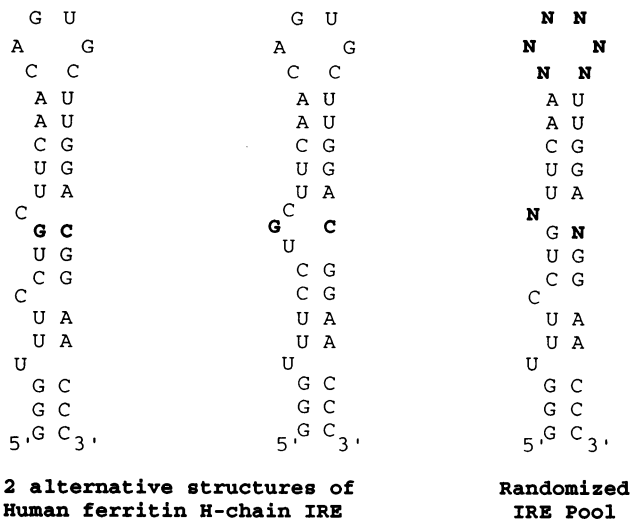

FIG. 2. Ferritin H-chain IRE drawn showing a G·C base pair in bold-face type below the bulge C of the consensus IRE. An alternative structure that would be theoretically possible is drawn to the right. The randomized sequence is depicted to the far right, and each nucleotide that was permitted to vary is represented as an N. In addition to permitting variation in the sequence corresponding to the bulge C and the six-membered loop of the IRE, variation was also permitted at the nucleotide that apposes a G residue 5′ of the bulge C (in bold-faced type), to assess whether base pairing was favored at this position.

here, variations from the consensus IRE were permitted in the residues of the loop and bulge through synthesis of a degenerate oligonucleotide template. Purified IRP1 or IRP2 was used to partition a starting pool of 65,536 sequences as outlined in Fig. 2. In view of the work showing that the sequence of the nucleotides of the upper stem is not important as long as base pairing is maintained (21, 22), variation was not introduced at most positions in the stem.

Selection was performed by adding radiolabeled probe to purified IRP1 or IRP2, followed by purification and amplification of gel-shift complexes. Sequences of cloned ligands were determined after three rounds of selection. Many sequences were repeatedly identified, while others were found only once. If a sequence was identified in more than three independent clones from the entire pool of cloned sequences, radiolabeled RNA of the same sequence was synthesized, and the relative binding efficiency was determined (Fig. 1). All sequences that were selected contained a cytosine residue at the position of the 5′ bulge for ligands bound by either IRP1 or IRP2. In addition, at the 3′ mid-stem position that was permitted to vary, only sequences containing a cytosine residue were selected, implying that the guanine nucleotide 5′ of the "bulge C" of the stem is base paired in IREs.

As was expected on the basis of known affinity measurements, consensus IREs were selected for both IRP1 and IRP2 (top three lines of *A* and *B* of Fig. 1). An alternative ligand was selected for IRP1 in which the loop sequence was UAGUAC, thus repeating the results of a previous SELEX procedure (13). Single nucleotide deviations from the consensus sequence were common at the fourth nucleotide position of the loop, with substitutions of A or G for U in ligands selected by both IRP1 and IRP2. Every sequence selected by either IRP1 or IRP2 contained a guanine at position 3 of the loop, consistent with the possibility that the third position of the loop may be an important binding determinant for both IRP1 and IRP2.

A number of guanine-rich alternative ligands were selectively bound by IRP2. These sequences are noteworthy because, unlike the consensus IRE and the IRP1 specific alternative ligand UAGUAC (13), they do not offer the possibility

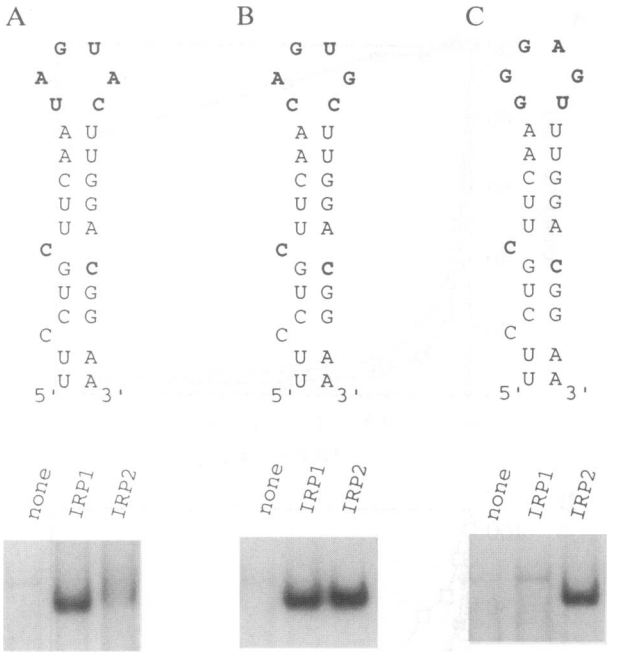

FIG. 3. The alternative ligand GGGAGU is bound exclusively by IRP2, and the alternative ligand UAGUAC is bound preferentially by IRP1. Radiolabeled ligands for the UAGUAC ligand (*A*), the consensus IRE (*B*), and the GGGAGU ligand (*C*) (30 ng) were mixed with purified IRP1 or IRP2 (100 ng) and resolved on a native gel (8%) as described in *Materials and Methods*. Gel-retardation complexes of the UAGUAC alternative ligand (*A*), the consensus IRE (*B*), and the GGGAGU ligand (*C*) are shown.

of Watson–Crick base pairing between the nucleotides in the first and fifth positions of the loop.

As shown in Fig. 3, the alternative ligand UAGUAC is bound preferentially by IRP1 (Fig. 3*A*), and the GGGAGU ligand is bound only by IRP2 (Fig. 3*C*), whereas the consensus IRE is bound equally well by both IRPs (Fig. 3*B*). The K_d for binding of the GGGAGU ligand to IRP2 was unexpectedly high (326 ± 170 pM vs. 19 ± 15 pM for the consensus IRE), a result that was qualitatively further affirmed in competition studies.

Competition Studies Indicate That the RNA Binding Site of the Alternative Ligands Overlaps with the Binding Site for the Consensus IRE. Competition studies were performed to determine whether the alternative ligands occupied the binding site of the consensus IRE and to further confirm direct affinity measurements. These analyses are based on an equivalent competition principle for comparison of nonidentical ligands and interaction with a common receptor. In Fig. 4*A*, competitors for the binding of IRP1 for consensus IRE included unlabeled consensus IRE, UAGUAC, and the IRP2 ligand GGGAGU. The UAGUAC ligand was equally efficacious as the consensus IRE for binding to IRP1, whereas the GGGAGU ligand did not compete for the IRE binding site of IRP1. Competition for the IRP2 binding site with consensus IRE and alternative ligands was performed (Fig. 4*B*), and the concentrations required for 50% inhibition of IRE binding (IC_{50}) for the consensus IRE, the GGGAGU ligand, and the UAGUAC ligand were 32 ng/20 μl, 140 ng/20 μl, and 335 ng/20 μl, respectively. The apparent affinity of the GGGAGU ligand for IRP2 was greater when assessed by competition curves vs. Scatchard analyses, possibly because competition analyses are often more accurate than direct binding curves in assessing the relative affinities of lower affinity ligands (23).

Arginine Residues Are Important in Binding of Consensus IREs to IRP2. Initial information regarding the RNA binding site of IRP1 came from RNA–protein cross-linking data (15).

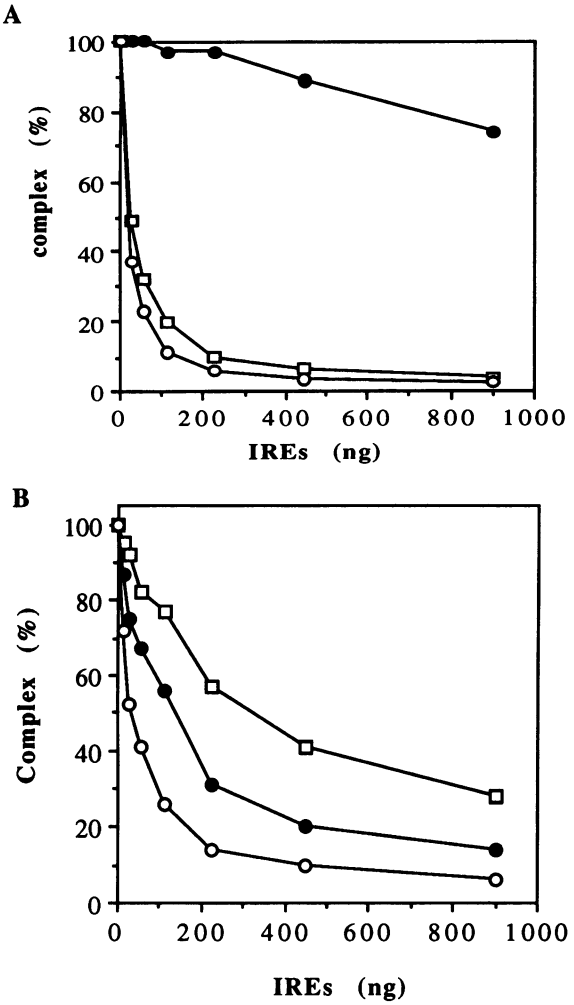

FIG. 4. Competition assays for binding of ligands to IRP1 (*A*) or IRP2 (*B*). Increasing amounts of unlabeled ligands (30–900 ng) were added in the indicated amounts to a filter-binding assay in which radiolabeled consensus IRE (30 ng) was incubated with purified IRP1 (*A*) or IRP2 (*B*) (100 ng) as indicated in *Materials and Methods*. Unlabeled ligands included the consensus IRE (○), the GGGAGU ligand (●) and the UAGUAC ligand (□). Representative examples of experiments that were performed three times are shown.

Based on the expectation that the tertiary structure of IRP1 would be similar to the known crystal structure of mitochondrial aconitase, these data implicated residues of the presumed active-site cleft in RNA binding. Subsequently it was shown that IRP1 active-site residues R536, R541, and R780 contributed to binding of the consensus IRE as summarized in Table 1 (14). Residue R780 was indispensible for IRE binding, whereas mutations at positions R536 and R541 measurably decreased the binding affinity.

In view of the observation that IRP2 binds consensus IREs with similarly high affinity as IRP1 and is highly related at the primary amino acid sequence level, IRP2 residues that align with those evaluated in IRP1 were mutagenized (24). The residues analogous to residues R536, R541, R699, and R780 of IRP1 are K611, R616, R774, and R855 of IRP2, respectively (24). In marked contrast to IRP1, substitution of glutamine individually for IRP2 residue K611, R616, R774, or R855 did not change the affinity for consensus IREs as judged by Scatchard analyses (K_d values were 40, 13, 10, and 29 pM, respectively; Table 1). However, a decrease in the binding affinity of >100-fold was observed for the double mutation R616Q/R855Q ($K_d \approx 2000$ pM). These two residues align with those that maximally affect IRE binding by IRP1 as single mutations. The combination of K611Q and R855Q also increased the K_d (K_d = 243 pM), although less substantially than the R616Q/R855Q pair.

IRP2 contains a 73-aa insertion relative to IRP1, which is predicted to form a surface loop. Deletion of this sequence from IRP2 (IRP2–73) did not measurably alter the affinity toward IRE binding (12). However, the R855Q mutation in the IRP2–73 context resulted in the loss of detectable binding to consensus IRE (data not shown). These results indicate that the 73-aa domain in IRP2 may contribute to the IRE binding site of IRP2.

The measured K_d values for binding of IRP2 by the GGGAGU ligand were as follows: single mutations R855Q, R616Q, and R774Q were 264, 369, and 710 pM, respectively; double mutations R611Q/R855Q and R616Q/R855Q were 785 and 191, pM respectively, and IRP2–73 was 1097 pM (see Table 1 for a qualitative summary). Arginine residues are clearly not indispensable in binding of the lower affinity GGGAGU ligand by IRP2.

Mutagenesis of Enzymatic Active-Site Residues of IRP2 Does Not Produce a Functional Aconitase. Of the 23 residues that make up the enzymatic active site of porcine mitochondrial aconitase, all are conserved in IRP1. In IRP2, two of these residues are not conserved.§ To attempt to develop a sensitive assay for potential assembly of an iron–sulfur cluster in IRP2, the residues that differed from the aconitase active-site residues were mutated as follows: N855 was mutated to serine to restore the catalytic base (27, 28), and K611 was mutated to arginine, as is found in porcine mitochondrial aconitase (25). These restorations of active-site residues to match those of mitochondrial aconitase, along with deletion of the 73-aa domain, did not result in acquisition of aconitase activity in the modified IRP2 when expressed in stable cell lines or when subjected to *in vitro* loading procedures (data not shown).

DISCUSSION

In this paper, we have attempted to define differences between the two IRPs that might be of physiologic relevance. We have evaluated the RNA binding sites using a SELEX procedure and site-directed mutagenesis. Because of the observation that IRP1 and IRP2 bind consensus IREs with equal and high affinity, we expected that residues that were important in binding of the consensus IRE of IRP1 would be equally indispensable to IRE binding by IRP2. To our surprise, none of the mutations of individual arginine residues important to binding of IRP1 affected RNA binding of IRP2. However, while the effect of single mutations was not discernible in IRP2, results of double mutations indicated that several of these arginine residues are important in IRE binding of IRP2. These results, together with the failure to convert IRP2 into an active aconitase, suggest that there are other uncharacterized differences between the enzymatic active-site region of IRP1 and the analogous region of IRP2.

Since the time of the initial description of the IRE as a functional motif in ferritin (29, 30), functional IREs have been recognized in a number of other transcripts important in iron metabolism (31–34). Here we report that, in addition to the consensus sequence, an alternative ligand is bound preferentially by each IRP. Evaluations of these alternative ligands reveal that the UAGUAC ligand binds with an affinity comparable to that of the consensus IRE, and though no sequence matches were found in the data base, it is likely that the UAGUAC ligand could mediate translational regulation of

§Further sequencing has revealed that a previously reported third active-site residue differing from that of aconitase, P88, (24), encodes a Q, as has been reported for mitochondrial aconitase and rat IRP1 and (25, 26).

Table 1. Comparison of mutations of active-site arginine residues on binding affinities of IRP1 and IRP2 for consensus and alternative IREs

IRP1		IRP2		
IRP1 mutation	Effect on IRE binding	Equivalent IRP2 mutation	Effect on IRE binding	Effect on GGGAGU binding
R780Q	1000-fold decrease*	R855	None	None
R541Q	100-fold decrease*	R616Q	None	None
R536Q	10-fold decrease*	K611Q	None	None
R699Q	None	R774Q	None	2-fold decrease
R780Q/R541Q	ND	R855Q/R616Q	100-fold decrease	None
R780Q/R536Q	ND	R855Q/K611Q	10-fold decrease	2-fold decrease
		IRP2–73	None	3-fold decrease

Values for binding of IRE ligands and the IRP2 alternative ligand GGGAGU indicated are relative to values of wild-type IRP1 and IRP2. Previously described mutants of IRP1 (14), indicated with an asterisk, are reported here for comparison only. ND, Not determined. All values for IRP2 were determined by Scatchard analysis as described in *Materials and Methods* using lysates from COS cells transiently transfected with an expression plasmid containing the indicated mutation. K_d values are indicated in the text.

transcripts. It is not clear at this time whether the K_d of the IRP2-specific ligand tested, which is at least 5-fold higher than that for the consensus IRE, would be sufficient to mediate translational repression of transcripts in cells. For regulation by IRPs, a range of K_d values of between 5 and 50 pM is sufficient for regulation, whereas a K_d of 5 nM is not sufficient (6). It is unlikely that the GGGAGU ligand mediates translational regulation of transcripts *in vivo*, as it does not mediate translational regulation *in vitro* when placed in the 5′-UTR of several reporter constructs (H.-Y.K, unpublished results).

Although data base searches for the alternative ligands have not yielded any obvious candidates for transcripts that are regulated specifically by IRP1 or IRP2, the possibility remains that endogenous transcripts may be specifically bound by either IRP1 or IRP2. If this were the case, then differences in the sensitivities of the two proteins to environmental stress and differences in the mode of regulation (35, 36) could result in independent regulation of some transcripts, thus expanding the range and versatility of this posttranscriptional regulatory system.

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