Structural requirements of iron-responsive elements for binding of the protein involved in both transferrin receptor and ferritin mRNA post-transcriptional regulation

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ABSTRACT

The synthesis of both transferrin receptor (TfR) and ferritin is regulated post-transcriptionally by iron. This is mediated by iron responsive elements (IREs) in the 5'- and 3'-untranslated regions, respectively, of TfR and ferritin mRNAs. Although these IREs have different sequences, they both form a characteristic stem-loop. We used competition assays and partial peptide mapping of UV-crosslinked ferritin and TfR IRE-protein complexes to show that the cytosolic protein binding to the ferritin 5'-IRE, the iron-responsive element binding protein (IRE-BP), also binds to TfR 3'-IREs. To identify the structural requirements necessary for RNA-protein binding, ferritin IRE RNAs were synthesized which contained altered secondary structures and base substitutions. Affinities of these RNAs for IRE-BP were assayed in RNA-protein binding gels. Substitutions disrupting base-pairing of the stem prevented IRE-BP binding. Substitutions which restored base-pairing also restored IRE-BP binding. We conclude that the IRE-BP binds to both ferritin and TfR IREs and recognizes a particular IRE conformation.

INTRODUCTION

Regulation of gene expression is primarily controlled at the level of transcription. Transcriptional regulation occurs by the interaction of trans-acting factors with specific DNA sequences causing either the activation or inactivation of genes. The expression of a growing number of genes is known to be post-transcriptionally controlled either through translational activation and repression of mRNAs (1) or through the regulation of mRNA stability (2,3). Little is known, however, about the trans-acting factors controlling these regulatory events. Two proteins which are coordinately regulated by iron via post-transcriptional mechanisms are ferritin, an intracellular iron storage protein (4), and the transferrin receptor (TfR), a membrane receptor protein mediating uptake of iron into the cell (5,6). Ferritin synthesis is induced by iron and occurs by the rapid mobilization of stored ferritin heavy subunit (H) and light subunit (L) mRNAs from the cytosolic ribonucleoprotein fraction to polysomes for translation (7–9). This effect is mediated by highly conserved sequences, termed the iron responsive elements or IREs, in the 5'-untranslated region of all ferritin H- and L-subunit mRNAs (10,11). In contrast, TfR synthesis is decreased when intracellular iron levels are low and increased when iron levels are high. This regulation occurs via the selective destabilization of IREs by iron (12–15). The 3'-untranslated region of the TfR mRNA contains five palindromic IREs which form stem and loop structures similar to the ferritin 5'-IRE (13–15). The sequences responsible for the iron-dependent destabilization of TfR mRNAs have been localized to three of the five IREs (16).

We previously identified two cytosolic proteins in rat liver which bind to the IRE in the 5'-untranslated region of ferritin mRNAs. These proteins, termed iron-responsive element binding proteins, or IRE-BP B1 and B2, were identified using a RNA-protein gel electrophoresis assay to resolve RNA-protein complexes from free RNA (17). Other IRE-BPs, corresponding to our IRE-BP B1, have been identified in human K562 erythroleukemia cells (18,19), mouse L cells (15), and rabbit liver (20). The rabbit liver IRE-BP specifically represses ferritin synthesis in an in vitro translation system (20–22). The five IREs in the 3'-untranslated region of the TfR mRNA bind a cytosolic protein in human K562 erythroleukemia cells (23) and mouse L cells (15). The ferritin IRE competes for binding of this protein, suggesting that the same or a similar IRE-BP binds to both elements. The function of the rat IRE-BP B2 protein complex is unknown.

We show here by competition studies and partial proteolytic peptide mapping that the rat liver IRE-BP B1 binds both the ferritin and TfR IREs. To determine the structural requirements necessary for RNA-protein binding, we synthesized ferritin IRE RNAs containing base substitutions which disrupt the base-pairing of the stem. We find that disruption of the base-paired stem of the IRE prevents protein binding. Substitutions which restore base-pairing also restore protein binding. Our results indicate that a specific conformation of the IRE is required for protein binding.

MATERIALS AND METHODS

Fractionation of IRE-BP B1 and B2 from Rat Liver

Rat liver IRE-BP B1 and B2 were isolated from the livers of 25 male Sprague Dawley rats. S100 (100,000×g supernatant)
liver extract was prepared as described by Digman et al. (24). The S100 extract (10 g) was loaded onto a DEAE-Sephacel CL-6B column (5 cm × 25 cm) equilibrated with 25 mM HEPES pH 7.2/10% glycerol/1 mM dithiothreitol (HGD) + 25 mM KCl. The column was washed with HGD + 25 mM KCl and developed with a linear KCl gradient (25 mM−500 mM). Fractions were assayed for RNA-binding activity. IRE-BP B1 elutes in flow-through fractions, whereas B2 elutes at approximately 300 mM KCl. Fractions containing B1 and B2 activity were separately pooled and protein was determined by the BioRad protein assay using bovine serum albumin as a standard.

RNA Preparation

Ten RNA transcripts were synthesized representing IRE sequences from the rat L-ferritin 5'-untranslated region (25) and the human TfR 3'-untranslated region (14). The sequences and structures of wild type ferritin IRE RNA, Ftr-IRE (29 bases), and transferrin receptor IRE RNAs, TIR-B IRE and TIR-C IRE (29 bases), are shown in Figure 1. Variant ferritin IRE RNAs (Table 1) were: 29-IRE Δ 7, ferritin IRE containing a C > A base substitution at position 7 in the bulge nucleotide in the stem (29 bases); 29-IRE Δ 10, ferritin IRE containing three base substitutions, UUG > AAC, at positions 19, 20, and 21 in the stem (29 bases); 29-IRE Δ 19, ferritin IRE containing three base substitutions, UUG > AAC, at positions 19, 20, and 21 in the stem (29 bases); 29-IRE Δ 10 + 19, ferritin IRE containing combined base substitutions of 29-IRE Δ 10 and 29-IRE Δ 19 (29 bases); 18-IRE, ferritin IRE RNA with a stem shortened by four base pairs, containing nucleotides 7–24 of the IRE (18 bases).

Labeled IRE RNA transcripts were synthesized by in vitro transcription of synthetic DNA deoxyoligonucleotide templates and T7 RNA polymerase according to the procedure of Milligan et al. (26). The 32P-labeled RNAs were prepared by incubating 1 mM deoxyoligonucleotide, 1.5 mM each of unlabeled ATP, CTP and UTP, 0.1 mM unlabeled GTP, and 250 Ci [α-32P]GTP (Amersham, > 410 Ci/mmol) in a 40 μl reaction mix. RNAs used for competition studies were synthesized using 1 μM DNA templates and 3 mM unlabeled nucleotides, including tracer amounts (10 μCi) of [α-35S]UTP (Amersham, > 650 Ci/mmol) to permit quantitation of RNA yield. Labeled RNAs were purified in a 7% SDS-polyacrylamide gel. 32P-labeled RNAs were visualized by autoradiography, while 35S-labeled RNAs were detected by UV-shadowing. Specific activities were 2.0–7.0 × 107 cpm/μg and 1.3 × 108 cpm/μg for 32P-labeled RNAs and 35S-labeled RNAs, respectively.

A 32P-labeled 118-LRNA (118 bases) was synthesized from Sma I-digested pGL-66, a rat L-subunit pseudogene (25), containing the first 65 bases of the 5′-untranslated region (including the IRE), 33 bases of the 5′-flanking sequence, and 20 bases from pGEM2 (Promega Biotec). For use in competition experiments, 32P-labeled ferritin-30 IRE RNA was synthesized from Stu I-digested pET7E, a plasmid containing 30 bases of the rat ferritin L-IRE sequence (provided by Rick Eisenstein and Hamish Munro). This RNA is similar to the ferritin 29-IRE RNA in Figure 1 except that it contains an extra U nucleotide at its 5′ end. Transcription reactions were performed in the presence of 250 μCi [α-32P]GTP (Amersham, > 410 Ci/mmol), 0.1 mM GTP, 1.0 mM each ATP, CTP, and UTP, and SP6 RNA polymerase for pGL-66 and T7 RNA polymerase for pET7E. 118-LRNA was purified in a 5% urea-polyacrylamide gel and pET7E was purified in an 18% urea-polyacrylamide gel.

Competition Experiments

RNA-protein binding was carried out according to the procedure described by Leibold and Munro (17). Competition experiments were carried out with DEAE-fractionated B1 extracts (2.5 μg) and 32P-labeled ferritin IRE RNA (0.007 pmoles, 3.5 × 104 cpm). These conditions were chosen based on experiments using a fixed amount of IRE RNA with increasing IRE-BP B1 concentration to determine the linearity of 32P-labeled ferritin IRE binding with the IRE-BP B1. As competitors, 0, 0.1, 1, 10, 100 and 1000 nM (0–2000 fold molar excess) variant ferritin IRE RNAs trace labeled with 35S[UTP] were used. 32P-labeled and 35S-labeled IRE RNAs and IRE-BP B1 were incubated in a reaction volume of 15 μl for 30 min at 25°. RNase T1 and heparin additions were omitted in competition experiments. The protein-RNA complexes were analyzed in a 5% native-polyacrylamide gel (acrylamide/methylene bisacrylamide ratio, 14:1), dried, and subjected to autoradiography using preflashed film. Peak areas were calculated using a LKB laser densitometer and plotted. Binding affinity of the variant ferritin IRE for IRE-BP was measured in terms of the concentration of variant ferritin IRE RNA (nM) at which specific binding of 32P-labeled wild type ferritin IRE was inhibited by 50%. Each RNA competition experiment was repeated a total of two to five times and average areas were calculated.

UV-Crosslinking and Partial Proteolytic Peptide Mapping of UV-Crosslinked Complexes

To prepare 32P-labeled RNA-protein complexes for UV-crosslinking and partial proteolytic peptide mapping, DEAE-fractionated IRE-BP B1 (50 μg) was incubated for 30 min with 1 × 105 cpm of either 32P-labeled ferritin IRE RNA or 32P-labeled TIR-C IRE RNA. In some samples, RNase T1 (1 U) and heparin (5 mg/ml) were added before crosslinking. The complexes were crosslinked by UV-irradiation as described previously and analyzed in a 7% SDS-polyacrylamide gel (17). The gel was dried and subjected to autoradiography. Partial peptide mapping of UV-crosslinked protein-RNA complexes was carried out according to the procedure of Cleveland et al. (27). The crosslinked 32P-labeled RNA-protein complexes were excised from unfixed dried 7% SDS-polyacrylamide gels and soaked in 125 mM Tris-HCl (pH 6.8) /0.1% SDS/1 mM EDTA for 2 h with occasional shaking. After removing the filter paper, the gel slices were placed into the wells of 15% SDS-polyacrylamide gels filled with running buffer (25 mM Tris-HCl/200 mM glycine/1.0% SDS). 20 μl of 125 mM Tris-HCl, pH 6.8/0.1% SDS/1 mM EDTA/20% glycerol/0.1 mg/ml bromophenol blue was added to each well. Each gel slice was overlaid with 40 ng of Staphylococcus aureus V8 protease (Miles) in 20 μl of 125 mM Tris-HCl (pH 6.8) /0.1% SDS/1 mM EDTA/10% glycerol. The gels were run at 100 mV/gel for approximately 1 h, stained with Coomassie Blue to visualize the protein standards (BioRad), dried, and subjected to autoradiography.

RESULTS

Rat Liver IRE-BP B1 binds both Ferritin and TfR IREs

To determine if the same or a similar IRE-BP binds both ferritin and TfR IREs, we carried out partial proteolytic peptide mapping of IRE-BP B1 crosslinked by UV-irradiation with ferritin and TfR IRE RNAs. IRE-BP B1 was separated from a lower affinity IRE-BP complex, IRE-BP B2, by chromatography on DEAE-
Figure 1. Computer-predicted secondary structures of wild type ferritin IRE RNAs and TfR IRE RNAs. Frt-IRE, rat ferritin L-IRE RNA, 29 bases; TfR-B IRE RNA, transferrin receptor IRE RNA, 29 bases; TfR-C IRE RNA, transferrin receptor TfR-C IRE RNA, 29 bases.

Figure 2. Competition assays using ferritin and TfR IRE RNAs with DEAE-fractionated B1 extract. 32P-labeled 118 ferritin-LRNA, Frt-IRE, TfR-C IRE and 18-IRE, a mutant ferritin IRE RNA (Table 1), were incubated with either a S100 rat liver extract or DEAE-fractionated B1 extract. The protein-RNA complexes were treated with RNase T1 and heparin, crosslinked by UV-irradiation, and analyzed in a 7% SDS-polyacrylamide gel. Lanes 1–3, ferritin 118-LRNA alone, treated with UV or RNase T1; lanes 4 and 5, 118 LRNA with S100 extract and DEAE-B1 extract; lanes 6 and 7, Frt-IRE RNA with S100 extract and DEAE-B1 extract; lanes 8 and 9, TfR-C IRE RNA with S100 extract and DEAE-B1 extract; lanes 10 and 11, 18-IRE RNA with S100 extract and DEAE-B1 extracts. Prestained molecular weight standards are shown in kilodaltons.

Figure 3. UV-crosslinking of ferritin-IRE and TfR-IRE RNAs with rat liver S100 extract and DEAE-fractionated B1 extract. 32P-labeled 118 ferritin-LRNA, Frt-IRE, TfR-C IRE and 18-IRE, a mutant ferritin IRE RNA (Table 1), were synthesized. The affinities of these RNAs for IRE-BP B1 were determined by competition assays in which the binding of 32P-labeled wild type ferritin IRE RNA to IRE-BP B1 was measured in the presence of 0–2000 fold molar excess of ferritin IRE, TfR-B IRE, and TfR-C IRE RNAs (Fig. 2). Yeast tRNA was used as a control competitor RNA. A 20-fold molar excess of competitor ferritin IRE, TfR-B, and TfR-C IRE RNAs markedly diminished binding of 32P-labeled ferritin IRE RNA to IRE-BP B1. In contrast, yeast tRNA did not compete even at the highest RNA concentration.

Partial proteolytic peptide mapping studies were carried out using 32P-labeled ferritin 118-LRNA (containing the first 70 bases in the ferritin 5’-untranslated region, including the IRE), ferritin IRE RNA and TfR-B IRE RNA crosslinked by UV-irradiation with either rat liver S100 extract or IRE-BP B1 extract. The complexes were analyzed in a 7% SDS-polyacrylamide gel (Fig. 3). An 100-kDa protein-RNA complex was formed for both rat liver S100 extract and DEAE-fractionated B1 extract with ferritin 118-LRNA, ferritin IRE, and TfR-C IRE RNAs. The ferritin IRE and TfR-C IRE crosslinked complexes were excised from the 7% SDS-polyacrylamide gel and the gel slices placed
complexes were identical. The peptide pattern generated by V8 protease digestion of the ferritin 118-LRNA complex was identical to the ferritin IRE complex indicating that the binding sites in the larger ferritin 118-LRNA and smaller ferritin IRE RNA were the same (data not shown). Also, the peptide pattern generated by V8 protease digestion of the ferritin and TIR IREs after incubation with rat liver S100 extract was identical to that of DEAE-fractionated B1 extracts (data not shown). These results indicate that the IRE-BP B1 recognizes and bind both ferritin and TIR IREs.

RNA Structural Requirements Necessary for IRE-BP B1 Binding

Despite sequence differences in the stem structure, both ferritin and TIR IREs bind to IRE-BP B1. This suggests that a particular secondary structure is required for IRE-BP B1 binding. To address this question, four variant ferritin IRE RNAs containing altered secondary structures were synthesized (Table 1). Ferritin 29-IRE A 10 RNA contains three base substitutions, CAA > GUU, at positions 10–12 in the stem. Ferritin 29-IRE Δ 19 RNA contains three base substitutions, UUG > AAC, at the complementary positions 19–21 in the stem. These RNAs can no longer base pair in this region. The double variant 29-IRE Δ 10+19 contains both of the substitutions of 29-IRE Δ 10 and Δ 19 and restores base pairing in the stem. Ferritin 18-IRE RNA contains a stem containing the bulge cytosine, but is shortened by four base pairs. The binding of 32P-labeled wild type ferritin IRE RNA to IRE-BP B1 was measured in the presence of 0–2000 molar excess of these variant IRE RNAs (Fig. 5). The RNA-protein complexes were analyzed in a 5% native-polyacrylamide gel, subjected to autoradiography, and the autoradiograms scanned by densitometry. The binding affinities of these variant IREs for the IRE-BP B1 were measured by calculating the concentration of variant IRE at which 32P-labeled wild type ferritin IRE was inhibited by 50% (Table 1). Ferritin 29-IRE Δ 10 or ferritin 29-IRE Δ 19 did not compete with 32P-labeled wild type ferritin-IRE RNA even at the highest RNA concentration (2000-fold molar excess) (Fig. 5). When base-pairing of the stem was restored, as in 29-IRE Δ 10 + 19 RNA, IRE-BP B1 binding also was restored, but with a binding affinity approximately 10-fold less (30 nM) than that of wild type ferritin IRE RNA (3 nM). These data indicate that in addition to a base-paired stem, specific nucleotides in the IRE RNA may be required for the optimal binding of IRE-BP B1. When the IRE stem was

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**Table 1. Variant ferritin IRE RNAs**

<table>
<thead>
<tr>
<th>IRE Mutant</th>
<th>Base Substitution</th>
<th>Relative Affinity*</th>
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<tr>
<td>29-IRE, wild type</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>29-IRE Δ 10 -12</td>
<td>CAA &gt; GUU</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>29-IRE Δ 19 -21</td>
<td>UUG &gt; AAC</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>29-IRE Δ 10 +19</td>
<td>GUU &gt; AAC</td>
<td>30</td>
</tr>
<tr>
<td>29-IRE Δ 7</td>
<td>C &gt; A</td>
<td>10</td>
</tr>
<tr>
<td>29-IRE Δ 15</td>
<td>G &gt; A</td>
<td>20</td>
</tr>
<tr>
<td>18-IRE</td>
<td>-</td>
<td>200</td>
</tr>
</tbody>
</table>

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*The relative binding affinities of the variant ferritin IRE RNAs to the IRE-BP were measured in terms of the concentration of variant IRE RNA (nM) at which maximum specific binding of 32P-labeled wild type ferritin-IRE RNA was inhibited by 50%. Each variant IRE RNA was measured 3–5 times. Results were quantitated by RNA-protein gel electrophoresis assay and densitometry.

**a** Variant ferritin IRE RNA containing nucleotides 7–24 of the IRE, but missing the lower four base pairs in the stem.
shortened by four base pairs, as in ferritin 18-IRE. IRE-BP B1 binding decreased by 66-fold (200 nM).

Base substitutions in the stem and loop were studied by synthesizing variant ferritin IRE RNAs which contained either a G > A substitution at position 15 in the loop, as in 29-IRE Δ 15, or a C > A substitution at position 7 in the stem bulge nucleotide, as in 29-IRE Δ 7 (Table 1). Ferritin 29-IRE Δ 7 and 29-IRE Δ 15 bound IRE-BP B1 with approximately 3-fold (10 nM) and 6-fold (20 nM) less affinity than wild type ferritin IRE RNA (Fig. 5 and Table 1). These results show that these nucleotides can be substituted by other nucleotides without a significant decrease in IRE-BP binding.

DISCUSSION

Since iron is toxic to cells, its cytosolic concentration is tightly regulated both by its uptake via the TIR and by its storage in ferritin. Iron induces ferritin synthesis by the translational mobilization of inactive ferritin mRNA to actively translating polysomes (7–9). Ferritin synthesis is repressed by the binding of the IRE-BP to the IRE in 5'-untranslated region in ferritin mRNAs (20–22). Iron also regulates TIR synthesis. In contrast to ferritin, iron reduces TIR synthesis by the destabilization of TIR mRNA (12–14). Three of the five IRES in the 3'-untranslated region of the TIR mRNA, TIR-B, TIR-C, and TIR-D, are essential for this regulation (16). Recent studies have shown that the deletion of single cytosine residues in the loop of the three TIR IRES abolishes interaction with the IRE-BP in vitro (16). Introduction into cells of a TIR IRE construct containing a single base deletion in the loop of the IRE resulted in decreased levels of TIR mRNA (16), suggesting that the IRE-BP protects the TIR mRNA from degradation. IRE-BP binding activity is increased when intracellular iron levels are low and decreased when intracellular iron levels are high (15,18,28). The interaction of IRE-BP with the IRE requires the presence of free sulfhydryl groups in the IRE-BP (28,29). IRE-BPs can exist in two forms, an oxidized form with lower affinity for the IRE, and a reduced form with higher affinity for the IRE (29). These data suggest that the iron status of the cell determines whether IRE-BP are in a high affinity or a low affinity form.

Although the nucleotide sequences of the stems of the ferritin and TIR IRES differ, they both bind IRE-BP with similar affinities. A cytosolic protein in human K562 cells (23) and mouse L cells (15) was recently shown by cross-competition studies to bind both ferritin and TIR IRES. Our peptide mapping studies confirm that IRE-BP B1, which corresponds to the IRE-BP studied by other investigators (15,19,20), binds both ferritin and TIR IRES. The assembled data permits model in which the IRE-BPs in response to iron levels within the cell, coordinately affects both ferritin and TIR mRNAs. When intracellular iron levels are low, IRE-BPs bind both the ferritin 5'-IRE and the TIR 3'-IRES. The IRE-BP suppresses ferritin translation, whereas the IRE-BP stabilizes TIR mRNAs, resulting in an increase in TIR synthesis. When intracellular iron levels are high, IRE-BP is released from the ferritin and TIR IRES. Ferritin mRNAs are free to bind the 40S ribosome and initiation factors and to commence translation. TIR mRNAs are destabilized, perhaps by forming a different RNA conformation more susceptible to RNase attack.

Our data indicate that the binding of IRE-BP B1 to the ferritin and TIR IRES requires the presence of a base-paired stem. Variant ferritin IRE RNA containing a disrupted stem do not bind the IRE-BP B1, whereas restoration of base-pairing of the IRE stem restores binding. In addition, a minimum stem length is necessary for binding, suggesting that a particular stable RNA conformation is required. RNA secondary structure has been shown to be important in protein recognition and translational repression in prokaryotes. For example, the coat protein of the R17 phage represses translation of the R17 replicase gene by binding to a RNA stem-loop structure (30). Translational repression of the ribosomal proteins in Escherichia coli is due binding of the S4 proteins to a pseudoknot structure in the mRNA (31).

The IRE loop contains six nucleotides with a consensus of CAG U/A GN. The first three bases (CAG) and the fifth nucleotide (G) are invariant in both ferritin H- and L- IRESs and TIR IRESs. A variant ferritin IRE (29-IRE Δ 15) containing a G > A
substitution in the loop bound the IRE-BP with 6-fold less affinity than wild type ferritin IRE RNA. Other data have shown that deletion of the first nucleotide (C) in the loop of ferritin IRE (18) or TfR IRE (16) results in an IRE RNA which no longer binds protein and is not regulated by iron. The bulge nucleotide in the stem (C) is invariant among ferritin and TfR IRE RNAs. Substitution of the bulge nucleotide C > A (29-IRE A 7) does not diminish IRE-BP binding significantly. While the loop nucleotide and the bulge nucleotide in 29-IRE A 15 and the 29-IRE A 7, respectively, can be substituted by other nucleotides without a significant decrease in IRE-BP binding, the minor decreases observed in relative binding affinities of these variants by comparison with that of wild type ferritin IRE suggest that certain nucleotides may be important for optimal protein binding. These data imply that certain nucleotide substitutions may be tolerated in the IRE RNA, as long as the combined stem and loop conformation remains unaltered. Further studies are needed to determine critical nucleotides important for IRE-BP binding. These studies will provide a basis for the correlation of structure of the IRE with biological function.

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