Loops and Bulge/Loops in Iron-responsive Element Isoforms Influence Iron Regulatory Protein Binding

FINE-TUNING OF mRNA REGULATION?*

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A family of noncoding mRNA sequences, iron-responsive elements (IREs), coordinately regulate several mRNAs through binding a family of mRNA-specific proteins, iron regulatory proteins (IRPs). IREs are hairpins with a constant terminal loop and base-paired stems interrupted by an internal loop/bulge (in ferritin mRNA) or a C-bulge (in m-aconitase, erythroid aminolevulinate synthase, and transferrin receptor mRNAs). IRP2 binding requires the conserved C-G base pair in the terminal loop, whereas IRP1 binding occurs with the C-G or engineered U-A. Here we show the contribution of the IRE internal loop/bulge to IRP2 binding by comparing natural and engineered IRE variants. Conversion of the internal loop/bulge in the ferritin-IRE to a C-bulge, by deletion of a single base U6, decreased IRP2 binding 20-fold, with only a small effect on IRP1 binding. Similarly, a C-bulge in the natural IREs (m-aconitase, erythroid ALS (eALAS), and the transferrin receptor (TfR)) decreased IRP2 binding 10-fold, compared with the ferritin-IRE. Natural IRP1 and IRP2 in a cell extract produced results similar to those observed with recombinant IRPs. The results coincide with structural differences observed by NMR spectroscopy (11, 12) and Cu(phen)2 probing (6). The differential sensitivity of IRP1 and IRP2 binding to natural variations in IREs at the junction of the two helices (internal loop/bulge or C-bulge) suggests that the presence of two IRPs broadens the regulatory range of IREs and emphasizes the importance of the internal loop/bulge region in RNA-protein interactions.

EXPERIMENTAL PROCEDURES

RNA Preparation—RNA, transcribed using T7 RNA polymerase and a chemically synthesized DNA template (9, 29), was purified on 12% polyacrylamide/urea gels; concentrated by ethanol precipitation, resuspended in water and stored at −80 °C until use. 5′-32P labeling of RNA was carried out as described previously (6, 30), with purification through NENSORB™ columns (DuPont).

Band-shift and Supershift Assays—5′-32P-Labeled RNAs were heated to 85 °C for 5 min in 100 mM KCl, 40 mM Hepes, pH 7.2 and annealed to 25 °C before each use. In competition experiments, unlabelled RNAs were heated and annealed as described for labeled RNA before adding to the binding reaction. If RNA was only heated to 65 °C, the percentage that bound IRP1 was greatly decreased (50–60%).

Binding of recombinant IRPs was accomplished by incubation of RNA (0.9 fmol, ~1.5 × 108 cpm) and protein at 10 °C for 30 min in 20 mM Hepes, pH 7.2, 4 mM MgCl2, 5% glycerol, 2% 2-mercaptoethanol; protein:RNA was 15:1. Almost all (80–90%) of the ferritin-IRE was bound by IRP1, but only 30–45% of the RNA was bound by IRP2.

The iron-responsive element (IRE), present in the 5′- or 3′-noncoding regions of animal mRNAs encoding proteins of iron and oxidative metabolism, regulates synthesis of the encoded proteins posttranscriptionally. Iron regulatory proteins (IRPs) bind to the IREs to inhibit ribosome binding or protect mRNA from ribonuclease cleavage (1–5). The predicted secondary structures of the IRE family are hairpins with a six-nucleotide terminal loop (CAGUGN*, N* = A, C, or U), interrupted by an internal loop/bulge (UGC/C) (ferritin-IRE) or a C-bulge (TIR, eALAS, and m-aconitase IREs), that is generally supported by enzymatic cleavage and chemical probing (6–8); NMR spectroscopy shows a G-C base pair in the hairpin loop and in the internal loop/bulge (9–12).

Two IRE-binding proteins, IRP1 and IRP2, have a high sequence identity except for a 73-amino acid insertion unique to IRP2, and each of them has 30% sequence identity to m-aconitase; IRP1 can have aconitase activity (13–17). IRP1 and IRP2 binding to IREs in iron-depleted cells is abrogated when iron is in excess, with IRP1 forming an [4Fe-4S] cluster (16–19), and IRP2 being degraded (14, 20–22). IRP phosphorylation (23, 24), indicates that IRP functions may be integrated with more general metabolic signals.

The significance of two IRPs, apparently equivalent in terms of RNA binding and posttranscriptional regulation, is a puzzle, since exclusivity of IRP1 or IRP2 binding for one or another natural IRE sequence has not yet been observed (25–28). IRP binding specificity for the internal loop/bulge and C-bulge of IREs examined in this study, showed that conversion of the ferritin-IRE internal loop/bulge to a C-bulge, by deletion of a single base U6, decreased IRP2 binding 20-fold, with only a small effect on IRP1 binding. Similarly, a C-bulge in the natural IREs (m-aconitase, erythroid ALS (eALAS), and the transferrin receptor (TfR)), decreased IRP2 binding 10-fold, compared with the ferritin-IRE. Natural IRP1 and IRP2 in a cell extract produced results similar to those observed with recombinant IRPs. The results coincide with structural differences observed by NMR spectroscopy (11, 12) and Cu(phen)2 probing (6).

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¶ The abbreviations used are: IRE(s), iron-responsive element(s); IRP, iron regulatory protein(s); m-aconitase; mitochondrial aconitase; TfR, transferrin receptor; eALAS, erythroid aminolevulinate synthase.

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bound by IRP2, suggesting that inactive IRP2 was present in preparations of IRP2; 2% mercaptoethanol does not decrease binding by IRP1 or IRP2 (14). RNA-protein complexes were separated from RNA in 4% non-denaturing acrylamide gels (acylamide: bis = 19:1) in Tris borate-EDTA buffer (90 mM Tris borate, 2 mM EDTA, pH 8.0, 8 volts/cm for 1 h at 10 °C). Binding of IRPs in rabbit reticulocyte lysates (30–50 µg/20 µl reaction mixture), prepared as before (31), used the same binding buffer, but with tRNA (50 µg/ml). The IRP2:IRE complex was identified with anti-IRP2 serum, generated against the 73-amino acid insertion in IRP2 (20); 2 µl of the serum were added after 20 min of incubation, followed by 10 min incubation, addition of heparin (7.5 mg/ml) (20, 32, 33) and electrophoresis in a 5% native acrylamide gel (acrylamide:bis 19:1), 12 volts/cm at 4 °C. Order of antiserum addition had no significant effect on the results.

Protein Purification—Recombinant IRP1 was isolated from the cytosol of Saccharomyces cerevisiae BJ5465 (34) containing the rabbit liver IRP1 sequence (35) in plasmid pYES-His (Invitrogen, Inc.), grown in minimal medium without uracil, and with 3% glycerol, 2% galactose (36). The IRP1 was purified as His-tagged protein with a nickel-chelate column (Amersham Pharmacia Biotech), followed by heparin-agarose chromatography in 20 mM Tris-Cl, pH 7.4, 50 mM KCl, 1 mM EDTA, 2 mM sodium citrate, 10% glycerol, 7 mM mercaptoethanol, and stored at −80 °C. Recombinant IRP2-His, which appears to be less stable than IRP1 (22, 37), was prepared on nickel-chelate columns as described by Phillips et al. (33).

**RESULTS**

Previous studies that compared IRP1 and IRP2 binding had shown that differential IRP binding occurred only with mutations in the hairpin loop (25–28), but not in natural IREs (20, 38–40). The hairpin loop is the most conserved part of the IREs; evolutionary divergence occurs in the stem and internal loop regions (1–5). Recent studies of IREs by NMR and other approaches, which showed significant structural differences in the internal loop/bulge and C-bulge IREs (11, 12, 41), stimulated reexamination of whether IRP1 and IRP2 differentially bind to the internal loop/bulge and C-bulge IREs. To enhance RNA conformational homogeneity, we synthesized RNA of comparable size (28–30 nucleotides), purified the RNA using denaturing gel electrophoresis, heated the purified RNA to 85 °C, and annealed before each use (see “Experimental Procedures”).

The influence of the internal loop/bulge characteristic of the ferritin IRE was investigated with recombinant IRPs, by examining the effect of the deletion of U6, which converted the internal loop/bulge into the C-bulge (Fig. 1, a and c). IRP2 recognizes the ferritin-IRE ΔU6 much more poorly than the ferritin-IRE (Fig. 2A and Table I) in contrast to IRP1. Mutated ferritin-IRE HL1, HL2, and C8A (Fig. 1, f–h and Fig. 2A) were controls, to show that results under the conditions used were comparable to those previously observed (25, 26, 28, 42).

Natural IREs all have the same C-G base pair and the pentameric sequence, CAGUG in the terminal hexaloop, but vary in structure at the interhelix junction (internal loop/bulge or C-bulge). Conversion of the ferritin internal loop/bulge to a C-bulge by deletion (ΔU6) differentially altered IRP recognition (Fig. 2A and Table I). Thus, IRP1 and IRP2 should also have different interactions with the natural C-bulge IREs (m-aconitase, TfR, and eALAS IREs) compared with the natural internal loop/bulge IRE (ferritin-IRE). The results proved our prediction. All natural C-bulge IREs bind IRP2 much more poorly than IRP1 (10–12%) when compared with ferritin-IRE binding (Fig. 2A and Table I) and are similar to the ferritin-IRE ΔU6.

The observation that the internal loop/bulge enhanced IRP2 binding was confirmed by competition experiments with unlabeled RNAs. A 10-fold molar excess of unlabeled ferritin-IRE (internal loop/bulge IRE) prevented binding of the labeled ferritin-IRE to IRP2, whereas >50-fold molar excess of unlabeled TfR, m-aconitase, or eALAS IREs (C-bulge IREs) were required (Fig. 2B and Table I).

To ensure that the differential binding of recombinant IRP2 to IREs with an internal loop/bulge or a C-bulge (Fig. 2 and Table I) was a property of IRPs, and independent of possible differences between natural and recombinant proteins, IRE-protein binding was examined with IRPs in rabbit reticulocyte lysates comparing of the ferritin-IRE to the TfR-IRE. IRPs in such cell extracts can regulate translation of IRE-containing mRNAs (43–46). The ferritin-IRE formed two RNA-protein complexes in the red cell extracts, in relatively equal amounts (Fig. 3A, lane 3), whereas the TfR-IRE formed only one RNA-protein complex (Fig. 3A, lane 4). The complex in the lower band formed with the ferritin IRE was identified as an IRP2:RNA complex with IRP2 antibody (compare lanes 5, 6, and 3, 4 in Fig. 3A). The binding of TfR-IRE to IRP1 is 0.52 ± 0.05 of that of the ferritin-IRE (Fig. 3A, lanes 5 and 6, upper bands). (Similar results were obtained with fresh preparations of purified natural IRP1, but with recombinant IRP1, ferritin-IRE and TfR-IRE binding was the same, either because of the His tag used or the absence of posttranslational modifications such as phosphorylation or both.) The binding of TfR-IRE to
endogenous IRP2 in red cell extracts was 0.10 ± 0.03 that of the ferritin-IRE (Fig. 3A), which was comparable with binding to recombinant IRP2 (Fig. 2A).

**DISCUSSION**

IRP isoforms IRP1 and IRP2 showed quantitative differences in binding to IREs from different mRNAs (Figs. 2 and 3 and Table I). The ferritin IRE is recognized best by both IRP1 and IRP2, compared with the m-aconitase, TIR, and eALAS IREs. Accordingly, a larger fraction of the ferritin IRE is likely to be complexed with IRPs than other IREs, which explains the observation that IRE-dependent regulation in vivo and in vitro has the greatest range for the ferritin IRE (38, 40, 47–49).

Among IRE isoforms, variations in the IRE/IRP interaction were greatest for IRP2, suggesting that IRP2 will make the major contribution to differential IRE-dependent regulation in vivo. IRP1/IRP2 ratios vary considerably in different cell types, exemplified by liver, kidney, and intestine: IRP1 > IRP2 (50), in RRL. IRP1–IRP2 (Fig. 3A) and in a pro-B-lymphocyte cell line, which appears to have only IRP2 (51).

IRP2 is sensitive to engineered changes in the IRE hairpin loop and internal loop/bulge. Because the hairpin loop structure is conserved in all natural IREs, its contribution to IRP2 binding will be constant. However, the variation in structure of IREs, with C-bulge or the internal loop/bulge, will differentially influence IRP2 binding to natural IREs (Figs. 2 and 3 and Table I). The ferritin-IRE ΔU6 with the C-bulge was an even poorer competitor for IRP2 binding than natural IRE isoforms with a C-bulge (Fig. 2B), suggesting context effects even within the group of IREs with a C-bulge. NMR studies suggest more conformational flexibility at the internal loop/bulge than at the C-bulge in IREs (11, 12). The IRE consensus sequence designed for NMR studies (11), contained a C-bulge, ΔU6, and 3 G-C base pairs next to the bulge in the lower stem.
creating an analogue of the AU6 ferritin-IRE, which likely behaves similarly in IRP2 binding. Note that in addition to the C-bulge or internal loop/bulge, effects of IRF flanking regions have been observed on the predicted structure (m-aconitase IRE) (52) and on both solution structure and translation regulation (ferritin-IRE) (53).

RNA conformational flexibility which is matched to differences in the binding proteins, as recently emphasized for BIV-IRE and IRP Isoforms (ferritin-IRE) (53).

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