Oxidation-Reduction and the Molecular Mechanism of a Regulatory RNA-Protein Interaction

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Iron-responsive elements (IREs) are RNA motifs that have been identified within the 5′ untranslated region of ferritin messenger RNA and the 3′ untranslated region of transferrin receptor mRNA. A single IRE mediates iron-dependent control of ferritin translation, whereas multiple IREs are found in the region of the transferrin receptor mRNA responsible for iron-dependent control of mRNA stability. A cytosolic protein binds in vitro to the IREs of both mRNAs. The IRE-binding protein (IRE-BP) is shown to require free sulfhydryl groups for its specific interaction with the IRE. Treatment of lysates with reducing agents increases the binding activity, whereas agents that block sulfhydryls inhibit binding. Iron starvation, leading to decreased ferritin translation, results in increased binding activity, which is explained by an increase in the fraction of the IRE-BP that is in a fully reduced state.

THE FORMATION OF SPECIFIC NUcleic acid–protein complexes has been shown to play a key role in DNA transcription, pre-mRNA processing, and translation (*1–3*). Structural principles underlying DNA-protein interactions are emerging from studies of specific DNA-binding proteins that are involved in transcriptional regulation (*1, 3*). In contrast, less is known about protein interactions with RNA, although amino acid consensus sequences within some RNA-BPs have been recognized (*4*). The expression of cytoplasmic RNA can be controlled at the level of mRNA stability and translation, and numerous examples of each type of regulation have been observed (*5*). A family of regulatory RNA sequences, IREs, was recently identified (*6, 7*). IREs are responsible for the iron-dependent control of ferritin mRNA translation (*7, 8*) and of the stability of transferrin receptor (TfR) mRNA (*9*). The IREs are phylogenetically conserved stem-loop structures with a characteristic six-membered loop (CAGUGN) (*9*). We used a gel retardation assay to show (i) that cytoplasmic extracts from human cells contain a protein that binds specifically to the ferritin (*10*) and TfR (*11*) IREs and (ii) that the specific activity of the IRE-BP is higher in lysates prepared from iron-starved [treated with desferrioxamine (Df)] than those from untreated cells (*10*). We now describe the molecular nature of this alteration in IRE-BP activity.

Studies on the interaction between the coat protein of R17 coliphage and the phage replicase mRNA (*12*) and between the *Escherichia coli* Ala-tRNA-synthetase and its target tRNA (*13*) have implicated the critical role of free sulfhydryl groups. In both cases the interaction depends on a nucleophilic attack by a sulfhydryl on the C6 of a uracil within the RNA to form a transient covalent bond, referred to as a Michael adduct (*14*). In view of these findings, we examined the potential role of sulfhydryl groups in the interaction between the IRE and its binding protein.

N-ethylmaleimide (NEM) alkylates and thus blocks free sulfhydryl groups; it irreversibly inactivates the ability of the IRE-BP to form a stable complex with an IRE. Thus when cytosolic extracts are treated with 1 m*M* NEM before the addition of a labeled IRE, the formation of an RNA-protein complex (as assessed by gel mobility shift) is completely abolished within minutes (Fig. 1A). Half-maximal inactivation occurs at 75 μ*M* NEM. Once the RNA-protein complex is formed, however, it is resistant to subsequent addition of NEM (Fig. 1A, lane 3). Next, we examined reagents that can oxidize free sulfhydryls and reagents that can reduce disulfide bonds for their effects on IRE-BP activity. The diazene carbonyl derivative diamide can chemically catalyze the oxidation of free sulfhydryl groups (*15*) as can copper orthophenanthroline (*16*), although by different mechanisms. Incubation of crude cellular extracts with either one of these reagents abolished IRE-BP activity. In contrast to inactivation with NEM, the effect of diamide or copper orthophenanthroline was fully reversible with the reducing agents 2-mercaptoethanol (2-ME) or dithiothreitol (DTT). That the diamide-induced inactivation is stable over 24 hours and persists after removal of diamide by dialysis indicates that it is acting by producing a protein disulfide bond. Interestingly, the addition of 2-ME or DTT alone to untreated lysates resulted in a dose-dependent increase in IRE-binding activity. To assess whether the reversible oxidation-reduction phenomenon was an intrinsic property of the IRE-BP or required additional components of the crude cytosol, we examined partially purified IRE-BP. The IRE-binding activity was enriched by affinity chromatography on a solid support to which an IRE-containing RNA was coupled (*17, 18*). The partially purified protein was tested for its response to reversible sulfhydryl perturbations. The partially purified IRE-BP is fully responsive to treatment with 2-ME and diamide; the reversible nature of these responses is fully preserved (Fig. 1B). The oxidized and reduced forms of the IRE-BP elute in the same peak fraction on an ACA 34 sizing column (*19*). The responsiveness to sulfhydryl perturbations is unaffected by prolonged dialysis of lysates. Thus, neither small (dialyzable) nor large molecules (that would change the elution profile from the tested sizing column) participate in

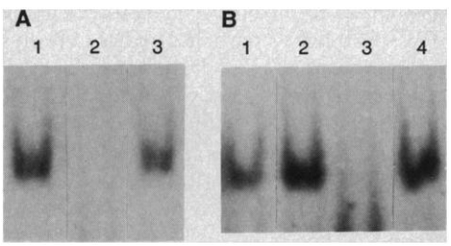

Fig. 1. Requirement for free sulfhydryl groups for IRE-BP activity. Cellular extracts from human K562 cells were prepared by lysis in 1% Triton X-100, 25 m*M* tris-HCl, *p*H 7.4, 40 m*M* KCl, 0.1 m*M* phenylmethylsulfonyl fluoride, and leupeptin (10 μg/ml) followed by centrifugation at 100,000*g*. Protein concentrations of the supernatants were determined by the BCA method (Bio-Rad, Richmond, California). Gel retardation assays were performed as described (*10, 21*), except that an IRE transcript was generated by transcription from a DNA oligonucleotide with T7 RNA polymerase (*18*). This transcript encoded the human ferritin H-chain IRE from nucleotide −181 to −147 (*6, 7*) and was labeled with [^{32}P]CTP to a specific activity of 6×10^6 cpm/μg of RNA. The preparation was diluted to 1.6 μg of RNA per milliliter, heated to 95°C for 5 min, and 1 μl was added with 1 unit of ribonuclease inhibitor (Inhibit-ACE, 5 Prime 3 Prime, Paoli, Pennsylvania) to 10 μg of protein extract in a 15-μl reaction. After a 30-min incubation at 25°C, heparin was added to a final concentration of 3 mg/ml for 10 min. Glycerol (2 μl) was added and the sample was separated by electrophoresis through a 4% polyacrylamide gel (*10*). (**A**) (Lane 1) The complex formed between the IRE and the IRE-BP from 10 μg of cellular extract and saturating amounts of IRE transcript. Preliminary incubation of cellular extracts with 1.0 m*M* NEM prevents complex formation (lane 2), whereas the same concentration of NEM does not affect a complex that has already formed (lane 3). (**B**) The IRE-BP was affinity-purified as described (*17*). Equal portions were analyzed by a gel retardation assay after a preliminary incubation with 2.5% 2-ME (lane 2), 100 m*M* diamide (lane 3), 100 m*M* diamide followed by 2.5% 2-ME (lane 4), or no incubation before the addition of the IRE probe (lane 1). The RNA-protein complex is shown.

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the oxidized form of the IRE-BP. Rather, it is likely that the oxidized form of the IRE-BP contains an intramolecular disulfide. These findings suggest that: (i) There is a functionally critical sulfhydryl available to be oxidized. (ii) Oxidative inactivation is readily reversible. (iii) Cellular extracts, as isolated, contain an oxidized fraction of the IRE-BP that can be activated in vitro by reduction. (iv) The responsiveness to sulfhydryl perturbation is fully retained after partial affinity purification of the IRE-BP and, therefore, the components required for this responsiveness appear to be contained within (or closely associated with) the IRE-BP.

We previously reported that perturbations in the regulatory signal (iron status) of human erythroleukemia K562 cells give rise to a quantitative alteration in the IRE-binding activity in cytoplasmic extracts prepared from these cells (*10*). At saturating levels of IRE-containing probe, the amount of complex formed between the IRE and the IRE-BP is five to ten times higher in lysates from iron-starved than from untreated cells. Thus the concentration of reactive IRE-BP is higher in these lysates. These results suggest an intriguing hypothesis: that the Df-induced alteration in IRE-binding activity may possibly be induced by a change in the ratio between the reduced and oxidized form of the IRE-BP. If this were the case, the total amount (reduced plus oxidized) of IRE-BP would be constant, and lysates from iron-starved cells with a higher detectable IRE-binding activity should contain a smaller fraction of oxidized (that is, can be activated by 2-ME) IRE-BP than lysates from untreated cells.

A representative example of the effect of 2-ME on complex formation between the IRE and the IRE-BP in lysates prepared from iron-starved or untreated K562 cells are shown in Fig. 2, A and B. The lysates from untreated cells responded by a sharp increase in binding activity that reached a maximum at 2 to 3% 2-ME. Higher concentrations of 2-ME resulted in a decrease in binding activity, possibly a consequence of denaturation. In contrast, analysis of the lysates from iron-starved cells revealed that the addition of 2-ME led to only a slight increase in binding activity that reached a peak at 2 to 3% 2-ME and then decreased at higher concentrations. The rise is much less than that observed with lysates from untreated cells and, in the presence of reducing agents, the maximal obtainable binding activity from control lysates approaches that of the lysate from iron-starved cells. Similar results have been obtained with human rhabdomyosarcoma (RD4) cells. This demonstrates that the total amount of IRE-BP is not significantly changed in response to the iron status of the cell. Instead, the iron status of the cell determines the IRE-binding activity by altering the ratio between the active (reduced) and inactive (oxidized) form of the IRE-BP. We propose that the regulatory signal (iron status) is transduced by the cell to the regulatory IRE-BP by changing the redox state of a sulfhydryl group, or groups, that is critical for the activity of the IRE-BP.

We then examined the relation between the ability of Df to reduce and activate the IRE-BP population and its effect on ferritin mRNA translation within the cell. Ferritin biosynthetic rates decrease within 2 hours after the addition of Df and reach their lowest levels between 15 and 25 hours of treatment. Since the IRE-BP has been envisioned to function as a translational repressor (*8, 20*), we would predict that IRE-BP activity should rise concomitantly with the fall in the ferritin biosynthetic rate; this is indeed the result (Fig. 3A). Thus the kinetics and quantitation of the Df-induced changes in ferritin biosynthetic rates precisely mirror the changes in IRE-BP activity. We propose that the IRE-binding activity is regulated post-translationally by reversible oxidation or reduction of sulfhydryl groups critical to the protein-RNA interaction (Fig. 2). Accordingly, the oxidized (2-ME inducible) fraction of the IRE-BP should decrease as the measured binding activity increases in response to Df treatment. This prediction was tested (Fig. 3B). There is a decrease in the 2-ME–inducible IRE-BP activity that closely follows the increase in binding activity that occurs after the addition of Df to cells. We have been unable to observe Df-induced activation of IRE-BP activity when cell lysates are treated with the iron-chelator. Surprisingly, we have not detected stable, enhanced oxidation of the IRE-BP population in lysates derived from cells treated with an iron source, hemin, although the biosynthesis of ferritin and TfR responds to hemin administration in these cells. A complex response of IRE-binding activity has been reported with iron treatment of rodent cells (*21*). However, addition of hemin antagonizes the Df effect, as is predicted by previous studies (*22*), demonstrating that the Df effect is due to iron chelation. There may be many reasons for our inability to observe the predicted enhanced oxidation of the IRE-BP after hemin administration, including the possible instability of the fully oxidized state during the preparation of cellular extracts. Although Df acts by perturbing the cellular iron status, an iron chelator may

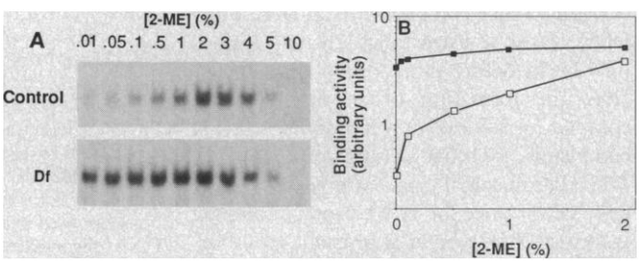

Fig. 2. Effect of in vivo iron perturbations on the redox state of the IRE-BP. Detergent lysates were prepared as described (Fig. 1), and portions (10 μg) were treated with increasing concentrations of 2-ME and analyzed with a saturating amount of labeled IRE transcript. (**A**) A comparison of the amount of complexes that were formed from control lysates and from lysates from cells that were treated for 20 hours with 100 μ*M* Df. (**B**) The quantitation by laser densitometry of the RNA-protein complex from the autoradiographs of (A). ■, Df; and □, control.

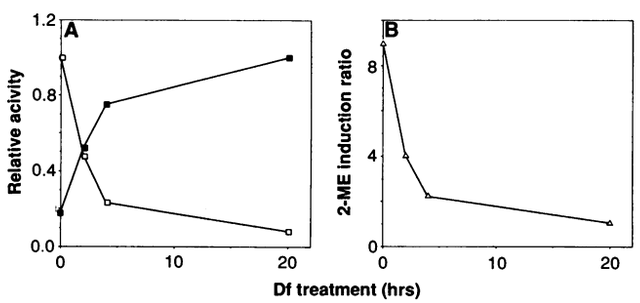

Fig. 3. Correlation between ferritin biosynthesis and IRE-BP activity in human RD4 cells. The RD4 cells were treated with 100 μ*M* Df for 2, 4, or 20 hours, or remained untreated. Subsequently, cellular extracts were prepared for determination of IRE-BP activity (Fig. 1) or cells were metabolically labeled with ^{35}S (Trans-label; ICN Biochems) for 30 min, and ferritin was immunoprecipitated and analyzed by gel electrophoresis (*6*). A separate experiment with RD4 yielded results consistent with the ones shown here. (**A**) Ferritin biosynthetic rates and IRE-BP activity were determined from autoradiographs by laser densitometry (*10*) and plotted as relative fractions of their maximal levels. ■, IRE-binding activity; and □, ferritin biosynthesis. (**B**) Equal portions of cellular extracts were treated with 2.5% 2-ME or remained untreated. Subsequently, samples were analyzed for IRE-BP activity by the gel retardation assay. RNA-protein complexes were quantitated by laser densitometry and the 2-ME induction ratio (ratio of binding activity with 2-ME to binding activity without 2-ME) was calculated for each cellular extract.

have a more stabilizing effect on the IRE-BP than direct iron starvation or administration. Finally, treatment of RD4 cells with puromycin at doses that completely inhibit protein synthesis has no effect on the induction of IRE-binding activity in response to Df (*19*).

We therefore propose that chelation of iron by Df results in the activation of the IRE-BP by leading to the reduction of an intramolecular disulfide in the IRE-BP. At least one of the now free cysteinyl residues is required for a high affinity interaction between the protein and the IRE which, in turn, is responsible for the repression of ferritin mRNA translation. In essence, alteration in cellular iron status operates a "sulfhydryl switch" by reversible oxidation or reduction of critical sulfhydryl group or groups in the IRE-BP. This hypothesis raises the question of whether such a switch could be physiologically relevant in the reducing environment of the cytosol. The major redox buffer in the cytosol is the glutathione system. The vast excess of reduced over oxidized glutathione is largely responsible for the reducing potential of the cytosol. A study on the reversible oxidation-reduction of 3-hydroxy-3-methylglutaryl coenzyme A reductase demonstrates that oxidized sulfhydryls can exist and even predominate within the cytosolic glutathione redox buffer system (*23*). Two factors can determine the redox state of a protein sulfhydryl within the cytosol. One is the ratio of reduced to oxidized glutathione, which can change significantly under physiologic conditions (*23*). The second is the oxidation equlibrium constant (K_{ox}) for a particular sulfhydryl group within a protein. Equilibrium constants for protein sulfhydryls can vary over many orders of magnitude, reflecting the effects of the local environment around the cysteinyl moiety on its K_{ox} (*24*). These local effects may reflect the stabilization or destabilization of the thiolate anion. Conformational changes that alter this local environment can therefore affect the K_{ox} of a particular cysteine sulfhydryl group. In this way allosteric effectors can perturb the K_{ox} of sulfhydryls on specific proteins and thereby alter the redox state of the protein, even in the presence of a constant cytosolic redox buffer. Our data on the IRE-BP provide an example of the utility of oxidation-reduction as a reversible covalent modification in the regulation of cellular protein function.

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17. Partial purification of the IRE-binding protein was achieved by RNA affinity chromatography. An IRE-containing RNA was transcribed in vitro by RNA polymerase T7 (*18*) with two synthetic oligodeoxynucleotides that when hybridized constituted an RNA polymerase T7 promoter and a template for the ferritin IRE (Fig. 1) plus the 3′ nucleotides $UCUCUCUCU_{24}$. The reaction included 3 m*M* biotin-11–uridine triphosphate (UTP) (Bethesda Research Laboratories) and 1 m*M* UTP, in addition to [α-^{32}P]guanosine triphosphate. The RNA was purified by hybridization chromatography on poly(A) Sepharose (Pharmacia). RNA was added to a lysate of K562 cells and incubated for 30 min at room temperature before addition of 5 mg of sodium heparin (Hynson, Westcott, and Dunning) per milliliter. This sample was then added to streptavidin agarose (Bethesda Research Laboratories) and the mixture was incubated for an additional 15 min before the resin was washed five times with 20 volumes of 40 m*M* KCl, 25 m*M* tris-Cl, *p*H 8, and 1% Triton X-100. Elution of IRE-BP was accomplished with 1*M* KCl, 5 mg of sodium heparin per milliliter, 25 m*M* tris-Cl, *p*H 8, and 1% Triton X-100. Based on recovery of IRE-binding activity and the recovery of labeled K562 protein from a parallel experiment with a [^{35}S]methionine lysate, we estimate that the IRE-binding protein was purified approximately 50-fold by this procedure.
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Isolation of a cDNA Clone Derived from a Blood-Borne Non-A, Non-B Viral Hepatitis Genome

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A random-primed complementary DNA library was constructed from plasma containing the uncharacterized non-A, non-B hepatitis (NANBH) agent and screened with serum from a patient diagnosed with NANBH. A complementary DNA clone was isolated that was shown to encode an antigen associated specifically with NANBH infections. This clone is not derived from host DNA but from an RNA molecule present in NANBH infections that consists of at least 10,000 nucleotides and that is positive-stranded with respect to the encoded NANBH antigen. These data indicate that this clone is derived from the genome of the NANBH agent and are consistent with the agent being similar to the togaviridae or flaviviridae. This molecular approach should be of great value in the isolation and characterization of other unidentified infectious agents.

WITH THE DEVELOPMENT OF SPECIFIC diagnostics for the hepatitis A virus (HAV) and the hepatitis B virus (HBV) in the 1970s, it became clear that most cases of hepatitis arising from blood transfusion were not caused by infections with these or other known viral agents (*1–4*). Despite over a decade of research, the agent or agents responsible for this so-called non-A, non-B hepatitis (NANBH) remains unidentified (*5, 6*), although there is evidence that one blood-borne NANBH agent may be a small, enveloped virus that is readily transmissible to chimpanzees (*7, 8*). A major impediment to progress in studies of this virus has been that despite intensive work, conventional immunological methods have consistently failed to identify specific viral antibodies and antigens (*5, 6*). Although this failure could be interpreted in terms of a lack of viral antibody, we consid-

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