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Regulation of Interaction of the Iron-Responsive Element Binding Protein with Iron-Responsive RNA Elements

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The 5′ untranslated region of the ferritin heavy-chain mRNA contains a stem-loop structure called an iron-responsive element (IRE), that is solely responsible for the iron-mediated control of ferritin translation. A 90-kilodalton protein, called the IRE binding protein (IRE-BP), binds to the IRE and acts as a translational repressor. IREs also explain the iron-dependent control of the degradation of the mRNA encoding the transferrin receptor. Scatchard analysis reveals that the IRE-BP exists in two states, each of which is able to specifically interact with the IRE. The higher-affinity state has a K_d of 10 to 30 pM, and the lower affinity state has a K_d of 2 to 5 nM. The reversible oxidation or reduction of a sulfhydryl is critical to this switching, and the reduced form is of the higher affinity while the oxidized form is of lower affinity. The in vivo rate of ferritin synthesis is correlated with the abundance of the high-affinity form of the IRE-BP. In lysates of cells treated with iron chelators, which decrease ferritin biosynthesis, a four- to fivefold increase in the binding activity is seen and this increase is entirely caused by an increase in high-affinity binding sites. In desferrioxamine-treated cells, the high-affinity form makes up about 50% of the total IRE-BP, whereas in hemin-treated cells, the high-affinity form makes up less than 1%. The total amount of IRE-BP in the cytosol of cells is the same regardless of the prior iron treatment of the cell. Furthermore, a mutated IRE is not able to interact with the IRE-BP in a high-affinity form but only at a single lower affinity K_d of 0.7 nM. Its interaction with the IRE-BP is insensitive to the sulfhydryl status of the protein.

Virtually all cells must acquire iron from the environment in order to accomplish a wide range of metabolic processes. The necessity for this nutrient coupled with the severe toxicity associated with excess cellular iron demand a metabolic system that is highly regulated. In higher eucaryotic cells, two well-characterized proteins responsible for the uptake and detoxication of iron are the transferrin receptor and ferritin, respectively. The expression of both of these proteins is highly regulated by iron. Interestingly, the information for this regulation is carried out by the mRNA encoding each of the proteins. The RNA element that provides the target for the regulation by iron of the fate of these two mRNA species was first identified in the 5′ untranslated region (UTR) of ferritin (1, 5, 6). In this mRNA, approximately 30 bases are necessary and sufficient for the ability of the ferritin message to be translationally controlled by changes in intracellular iron. This regulatory motif has been named the iron-responsive element (IRE). Although we have not fully defined the details of the RNA that are required for IRE function, present data suggest that both the structure and sequence of this element are important to its function (2, 3, 13). All known functional IREs form a moderately stable stem-loop structure. The stem is broken by an unpaired cytidine found 6 bases 5′ of a six-membered loop whose sequence is CAGUGX. A cytosolic protein that binds to the IRE has been identified by gel shift assays (9, 13). A point mutation in the sequence of an IRE that results in a loss of binding to the IRE binding protein (IRE-BP) also results in the loss of the ability to function in iron-dependent translational control when placed in the 5′ UTR of a reporter gene (13). Recently, the rabbit IRE-BP has been shown to function as a translational repressor in a wheat germ in vitro translation system (14).

The transferrin receptor (TfR) limits the amount of iron taken up by the cell and is also regulated by iron. The regulation of this gene is phenotypically distinct from the regulation of ferritin. Whereas ferritin synthetic rates drop in response to iron deprivation, the synthesis of the TfR goes up. Furthermore, whereas ferritin is regulated at the level of translational initiation, the major regulation of the TfR occurs at the level of mRNA stability. Thus, changes in the rate of synthesis of the receptor are mirrored in the cytoplasmic levels of TfR mRNA. The region of the TfR mRNA responsible for iron responsiveness is localized to its 3′ UTR (2, 7a). Despite these differences, we have recognized that the regulatory region of the TfR mRNA contains five stem-loop structures that resemble our description of IREs (2). These sequences within the 3′ UTR are highly conserved in human and chicken genes (4). We have demonstrated that several of these stem-loop structures confer ferritin-like translational control when placed in isolation in the 5′ UTRs of reporter genes (2). Furthermore, each of these sequences binds to the same cytosolic binding protein that recognizes the ferritin IREs (7a).

Recent work from this laboratory has attempted to define the relationship between the binding of this protein and the regulated fate of the target mRNA molecule (7). The amount of binding activity increases when cells are deprived of iron by treatment with an iron chelator, compared with the activity in iron-replete cells. Furthermore, inactive IRE-BPs found in the cytosol of iron-replete cells can be activated in vitro by reducing agents. In contrast, iron-starved cells possess little, if any, IRE-BPs that can be activated with reducing agents. The mechanism by which iron status deter-

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mines the binding activity in the cytosol of a cell can be explained by the reversible interconversion between oxidized and reduced forms of the IRE-BP. Therefore, free sulfhydryl groups are required for the RNA-protein interaction, and this can be blocked either irreversibly with alkylating agents or reversibly with oxidizing agents.

A better understanding of the interaction of the IRE and the IRE-BP is critical to elucidating the mechanism by which this protein regulates ferritin mRNA translation and TfR mRNA stability. In this report, we demonstrate, by using both crude cytoplasmic extracts and affinity-purified IRE-BP (13a) that the IRE-BP has two distinct binding affinities for the IRE. A high-affinity binding (K_d, 10 to 30 pM) is observed only for biologically active IREs, while a lower affinity is observed both for functional and for at least one nonfunctional mutant IRE. Neither class of IRE-specific binding sites is observed for nonspecific stem-loop RNAs. When the iron status of the cell is perturbed, one observes a change specifically in the number of high-affinity binding sites. This physiologic change can be mimicked in vitro by sulfhydryl active agents (7).

MATERIALS AND METHODS

Cell growth and cytoplasmic lysate preparation. A human rhabdomyosarcoma cell line (RD4) was grown, and detergent lysates were prepared as previously described (7). In some experiments, a human erythroleukemia cell line (K562) was used (7, 13). Final protein concentrations were 2 to 10 mg/ml in a buffer consisting of 40 mM KCl, 25 mM Tris hydrochloride (pH 7.4), 1% Triton X-100, 10 μg of leupeptin per ml, and 25 μM *p*-nitrophenyl-*p'*-guanidinobenzoate (lysis buffer). Protein determination was by the BCA method (Bio-Rad Laboratories, Richmond, Calif.).

Treatment of cells with hemin or desferrioxamine was accomplished by overnight treatment of RD4 cells with media containing 10 μM hemin or 100 μM desferrioxamine. Control cells were given fresh media without additions.

RNA preparation. Partially single-stranded DNA molecules containing a double-stranded region of the 17-base T7 promoter served as templates for the synthesis of the required RNA sequences (10). The T7 polymerase was a kind gift from David Draper and Sarah Morse, Johns Hopkins University, Baltimore, Md. All DNA oligonucleotides were made on an Applied Biosystems 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). Labeling was with [α-^{32}P]CTP (ICN Pharmaceuticals Inc., Irvine, Calif.) to specific activities of 3,000 to 140,000 cpm/ng of RNA. Full-length RNA transcripts were separated from shorter fragments and free nucleotides by electrophoresis on a 20% acrylamide (acrylamide:bisacrylamide of 20:1)-8 M urea gel. The full-length band was cut out after visualization by UV shadowing and eluted overnight in a buffer consisting of 0.5 M ammonium acetate, 20 mM magnesium acetate, 0.5 mM EDTA, and 0.1% sodium dodecyl sulfate. The eluate was extracted with phenol-chloroform and ethanol precipitated to recover the RNA.

The sequence of the DNA molecule used to synthesize the IRE was 5'-GGGTTCCGTCCAAACACTGTTGAAGCAG GAAACCCTATAGTGAGTCG-TATTA-3'. The sequence of the DNA oligonucleotide used to synthesize the ΔC-IRE differed by the deletion of the G at position 25.

The sequence of the DNA template used to synthesize the so-called random stem loop was 5'-ATAGCTAGCTAGC TATGTCATAGCTAGCTAGCTATTATAGTGAGTCGT ATTA3'. The underlined sequences were double stranded and represent the T7 RNA polymerase promoter.

β-Globin 5' UTR mRNA was prepared from a plasmid, using SP6 polymerase (Promega Biotec, Madison, Wis.) as described in a prior publication (13).

RNA gel retardation assay and quantitation. Gel retardation assay reaction mixtures were prepared by mixing 1 to 20 μg of lysate in 13 μl of lysis buffer with 1 μl of radiolabeled RNA probe and 1 μl of Inhibitase (5 Prime→3 Prime, Westchester, Pa.) and incubated at 25°C for 30 min. In some experiments, heparin was then added to a final concentration of 3 mg/ml and the incubation continued for an additional 10 min. Where indicated, lysates were pretreated for 5 to 10 min with 2% 2-mercaptoethanol (2-ME) prior to addition of RNA probe. Two microliters of 50% glycerol was added to the reaction mixture, and the entire mixture was loaded onto a 4% native polyacrylamide gel and electrophoresed as described previously (7, 9, 13).

The binding assay consisted of the gel shift assay with the following modifications. Five micrograms of cytoplasmic lysate in 15 μl of lysis buffer, 5% glycerol, and 0.1 U of Inhibitase per μl was incubated with 5 μl of RNA probe in lysis buffer for final RNA concentrations ranging from 25 pM to 10 nM at 25°C for 10 min. Aliquots of 18 μl of the reaction mix were loaded onto native polyacrylamide gels as described previously (7, 9, 13) and electrophoresed for 40 to 50 min.

2-ME treatment was done by preincubating lysates with 2 or 2.5% 2-ME for 5 to 10 min prior to addition of RNA. Diamide (Calbiochem-Behring, La Jolla, Calif.) was made as a 2 M solution in 25 mM Tris hydrochloride, pH 7.4, and lysates were pretreated with a 100 mM concentration for 10 min prior to addition of RNA.

IRE-BP was purified by affinity chromatography as described in a previous publication (13a). The purified IRE-BP was eluted from IRE-agarose beads in a mixture of 2 M KCl, 25 mM Tris hydrochloride (pH 7.6), 0.5 mM dithiothreitol, and 1.5 M $MgCl_2$ precipitated with 70% ammonium sulfate. The precipitate was dissolved in a mixture of 40 mM KCl and 25 mM Tris hydrochloride (pH 7.6) buffer. Approximately 0.1 ng of purified IRE-BP in 15 μl of buffer containing 40 mM KCl, 25 mM Tris hydrochloride (pH 7.4), 5% glycerol, and 0.1 U of Inhibitase per μl was incubated with serial dilutions of 5 μl of IRE probe in the same buffer, and the binding assay was done as described above.

After electrophoresis, gels were fixed and dried. Quantitation of bound and free RNA was done with autoradiography on preflashed Kodak X-AR film and densitometry on an LKB Ultrascan XL (LKB Instruments, Inc., Rockville, Md.) or using a Betascope 603 blot analyzer (Betagen, Waltham, Mass.). Results with either method were similar. With each experiment, internal standards of known amounts of RNA probe were run on a similar gel and quantitated alongside the experimental gels. This allowed quantitation of the amounts of probe in the experimental gels and ensured that quantitation was performed only in regions of exposures where both the film and densitometer were within their linear ranges. The background was estimated by scanning the area between shifted and free probe by densitometer or the blot scanner and was subtracted from the bound probe to arrive at the specific bound probe concentration. Data was analyzed by using the LIGAND program package and fitted to one or two binding site models (11). Errors reported for the dissociation constants represent the standard deviations of the number of experiments noted.

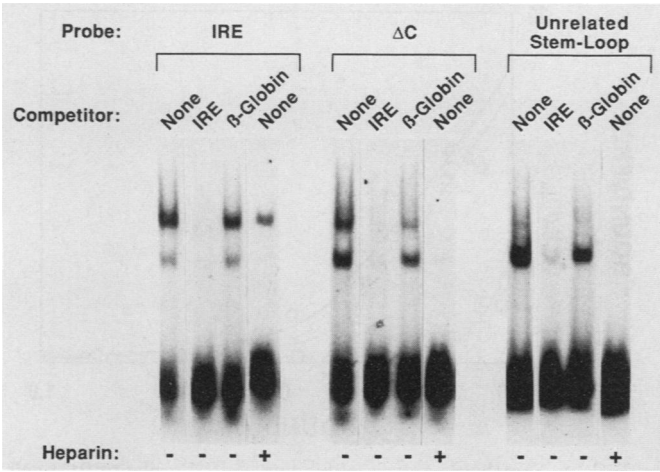

FIG. 1. Gel retardation assay of IRE probe, ΔC-IRE probe (ΔC), and unrelated stem-loop RNA probes with RD4 cell lysates. RD4 lysate (20 μg) was incubated with 0.5 ng (specific activity, 5,300 cpm/ng) of RNA probe in the presence (+) or absence (−) of 3 mg of heparin per ml as described in Materials and Methods. Competitions were with 500 ng of unlabeled IRE probe or unlabeled β-globin mRNA 5′ UTR.

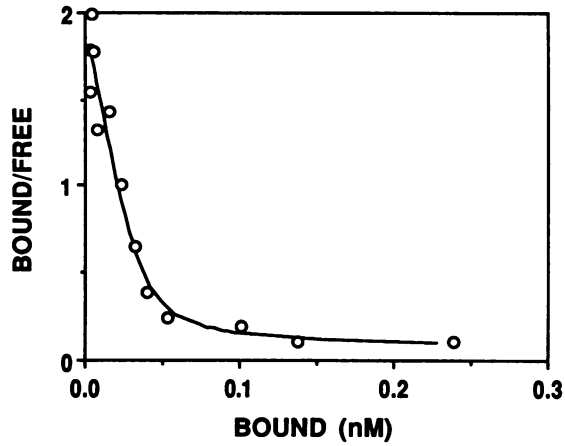

FIG. 2. Scatchard analysis of binding of the IRE probe to RD4 cell cytoplasmic lysates. Cytoplasmic lysate (5 μg) was incubated with IRE probe (specific activity, 72,000 cpm/ng) of concentrations ranging from 45 nM to 25 pM, and the gel retardation and binding quantitation assay were performed as described in Materials and Methods. For the high-affinity binding site, K_d 1 = 12.5 ± 5.5 pM (n = 3), and for the low-affinity site, K_d 2 = 3.7 ± 4.0 nM (n = 3).

RESULTS

Band shifts of IRE and ΔC-IRE with cell lysates. The IRE specifically binds to the IRE-BP, and this can be demonstrated by a gel shift assay. Figure 1 shows a representative assay. In addition to uncomplexed RNA, two bands are seen in most experiments when the assay is performed in the absence of heparin. When heparin was included, only the upper band remained. The lower band showed no specificity for IRE stem-loop structures, and we conclude that it represents a relatively low affinity nonspecific RNA-protein interaction. The upper band can be specifically eliminated by competition with excess unlabeled IRE but not with RNA corresponding to the 5′ UTR of the β-globin mRNA or with an unrelated stem-loop RNA. Next, the binding of the IRE-BP to the ΔC-IRE was examined. The ΔC-IRE is a mutated IRE with the unpaired cytosine deleted in the stem. When the ΔC-IRE was used as a probe in this assay, two bands were again seen, with the upper one corresponding in migration to the specific IRE/IRE-BP complex. This band was eliminated in the presence of excess unlabeled RNA that contains either a functional IRE or a ΔC-IRE but not in the presence of nonrelated RNA sequences. In contrast to our previous experiments (13), no heparin was included here. When the band shift assays were done in the presence of 3 mg of heparin per ml, two effects were seen: the intensity of the shifted band observed with the native IRE-containing probe was diminished, and the shifted band observed with the ΔC-IRE-containing probe was abolished. These observations suggested that the heparin may preferentially inhibit low affinity (albeit specific) binding and that the affinity of the interaction with the ΔC-IRE is too low to be preserved in the presence of heparin.

Scatchard analysis of binding of IRE to IRE-BP. In order to specifically address this possibility and to obtain more quantitative information on the interaction between the IRE-BP and its cognate RNA, we established conditions to apply Scatchard-type analysis to this interaction. This was accomplished by using the gel shift assay, which allowed the separation and quantitation of both the protein-bound and free RNA probe. The binding data were analyzed by using computer-based modeling. To assure that, under the conditions used, the binding reactions had reached equilibrium, we examined the amount of complex formed as a function of the time of incubation of the reactants. For all concentrations of reactants used in these studies, the binding had reached its steady state well within the time of incubation chosen (data not shown).

Previously, all the reported IRE gel retardation assays had been performed in the presence of heparin in order to decrease nonspecific binding. With the shorter probes used in this study (35 bases), nonspecific binding was not a great problem and we chose to eliminate the heparin from the assay. A representative Scatchard analysis of the IRE/IRE-BP interaction in the absence of heparin is shown in Fig. 2. The curve is concave upward, consistent with either more than one binding site or negative cooperativity. In order to distinguish between these two possibilities, we examined the dissociation of the preformed complex. In particular, we tested whether the rate of dissociation upon dilution differed if excess unlabeled IRE-containing RNA was present. The lack of effect of excess ligand mitigated against interpreting the Scatchard plots as representing negative cooperativity (data not shown). Computer-assisted analysis suggested that the data could be well modeled by a two-site model. Therefore, we will refer to two binding sites. The high-affinity binding displayed a K_d of 10 to 30 pM, while the low-affinity interaction had a K_d of approximately 2 to 7 nM. There are approximately 50,000 to 100,000 total (high- and low-affinity) binding sites per cell in these cytosolic preparations of RD4 cells. In the absence of heparin, about 10 to 20% of the total number of binding sites were high affinity. In the presence of heparin, the low-affinity binding is preferentially eliminated, although many of the high-affinity sites are lost as well and 50 to 100% of the total number of sites are high affinity (data not shown). The effect of heparin on the apparent K_d is dose dependent (data not shown), and we suspect that heparin is acting as a mixed antagonist for binding. In the presence of heparin we observed high-affinity binding sites, while in the absence of heparin we observed predominantly low-affinity, albeit specific, sites.

Effects of iron and redox perturbations on binding of the

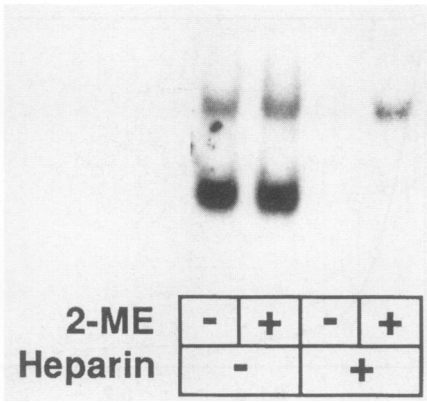

FIG. 3. Gel retardation assay of K562 cell lysates with IRE probe demonstrating elements of 2-ME enhancement of binding in the presence (+) or absence (−) of heparin. Cytoplasmic lysate (0.83 μg) was incubated with 2 ng of RNA (3500 cpm/ng) probe under conditions described in Materials and Methods.

IRE to IRE-BP. As described in the Introduction, binding activity measured in the cytosol derived from cells in culture could be increased either by treating the lysates in vitro with reducing agents or by pretreating the cells with the iron chelator desferrioxamine (7). In comparing the gel shifts in the presence or absence of heparin, we noted that at saturating IRE probe concentrations, the effects of either sulfhydryl reduction or desferrioxamine were not apparent when IRE-BP activity was assessed in the absence of heparin (Fig. 3). At saturating probe concentration, all total binding sites should be occupied. Therefore, in the absence of heparin, the same total amount of binding activity is present, irrespective of treatment of cells or lysate. Again, this finding suggested that the effect of 2-ME or desferrioxamine might be limited to the high-affinity, and thus relatively heparin-insensitive, binding sites.

Scatchard analyses of the binding activity presence in control lysates, control lysates treated with 2-ME, and lysates from cells treated with desferrioxamine were performed. While the total binding activity observed in the absence of heparin did not respond to these treatments (Fig. 3), the number of high-affinity binding sites specifically rose with respect to untreated lysates from control cells (Fig. 4). After these treatments, the number of high-affinity sites rose about four- to fivefold without a change in the apparent K_d of this site. The maximal 2-ME induction was seen at 1 to 4% 2-ME, and this varied within this range among preparations. We have previously been unable to identify a decrease in IRE-BP activity in lysates derived from cells treated with an iron source, compared with activity in control lysates. We reassessed this with the greater resolution and sensitivity of the Scatchard analysis. Cells were treated overnight with 100 μM hemin as an iron source before cytosolic lysates were prepared. These lysates were analyzed for IRE-BP activity and compared with lysates derived from untreated or desferrioxamine-treated cells (Fig. 5). Iron pretreatment resulted in a marked drop in the number of high-affinity binding sites compared with the number in either untreated or chelator-treated cells. Computer analysis of the binding curves with a two-site model demonstrated no significant differences in the number of total binding sites in lysates derived from treated or untreated cells. However, the fraction of total sites (high plus low affinity) demonstrating the high-affinity binding decreased from about 25 to 50% for desferrioxamine-treated cells to less than 1% for hemin-

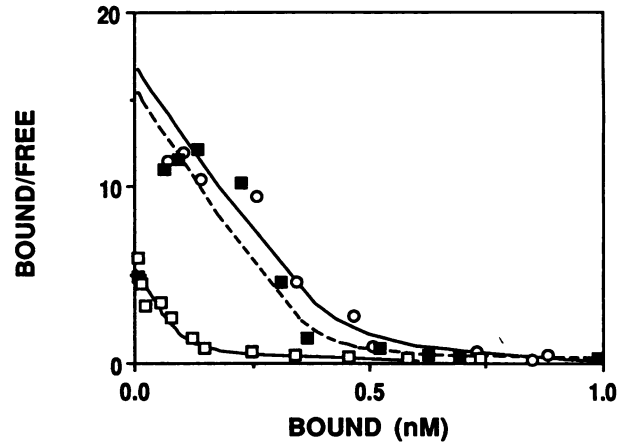

FIG. 4. Scatchard analysis of binding of IRE probe to RD4 cell cytoplasmic lysates; effects of 2-ME and desferrioxamine. Lysates of cells were treated overnight with 100 μM desferrioxamine (○), control lysate (□), or control lysate treated with 2.0% 2-ME (■). Lysate (5 μg) was incubated with IRE probe (specific activity, 72,000 cpm/ng) at concentrations ranging from 5 nM to 25 pM. The gel retardation binding assay and quantitation assay were performed as described in Materials and Methods. For desferrioxamine-treated cell lysates, K_d 1 = 20.1 ± 10.3 pM (n = 3) and K_d 2 = 2.5 ± 1.6 nM (n = 3). For control cell lysates treated with 2-ME, K_d 1 = 18.3 ± 8.0 pM (n = 3) and K_d 2 = 3.5 ± 3.2 nM (n = 3).

treated cells. The high affinity K_d was between 15 and 30 pM, and the low-affinity K_d was between 2 and 7 nM. The high-affinity sites can be reactivated from the hemin-treated lysate by the addition of 2-ME and can be inactivated by the addition of diamide to the lysate derived from desferrioxamine-treated cells (data not shown).

Scatchard analysis of the binding of IRE-BP to ΔC-IRE. We noted that at very high concentrations of ΔC-IRE RNA, the

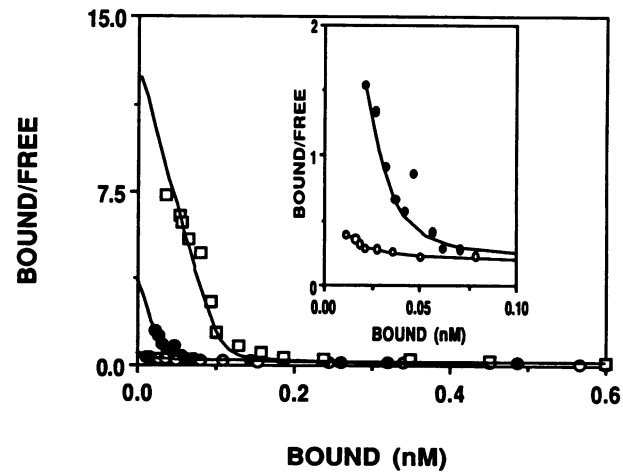

FIG. 5. Scatchard analysis of binding of the IRE probe to RD4 cytoplasmic lysates; effects of hemin and desferrioxamine. Lysates of cells grown overnight in the presence of 100 μM hemin (○), control (●), or desferrioxamine (□). Lysate (5 μg) was incubated with IRE probe (specific activity, 144,000 cpm/ng) at concentrations ranging from 7.5 nM to 50 pM, and gel retardation and binding quantitation assays were performed as described in Materials and Methods. For hemin-treated cell lysates, K_d 1 = 40 pM (n = 1) and K_d 2 = 7.2 nM (n = 1). Inset shows the high-affinity region for hemin (○) and control (●) lysates.

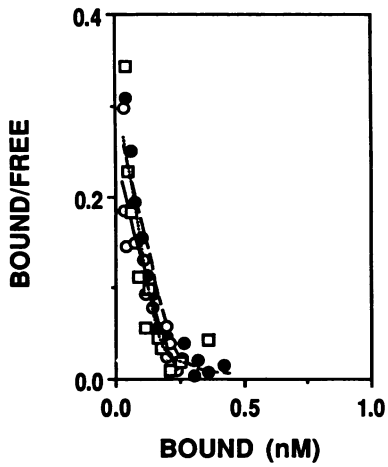

FIG. 6. Scatchard analysis of binding of ΔC-IRE probe to RD4 cell cytoplasmic lysates. Cells were treated overnight with 100 μM desferrioxamine (●), control lysate (□), or control lysate treated with 2.0% 2-ME (○). Lysate (5 μg) was incubated with ΔC-IRE probe (specific activity, 3,200 cpm/ng) at concentrations ranging from 50 nM to 10 pM. The gel retardation binding assay and quantitation were performed as described in Materials and Methods. For the control cell lysate interaction with the ΔC-IRE probe, K_d = 0.65 ± 0.21 nM (n = 2); for control lysates treated with 2-ME, K_d = 0.90 nM (n = 1); and for desferrioxamine-treated lysates, K_d = 0.79 nM (n = 1).

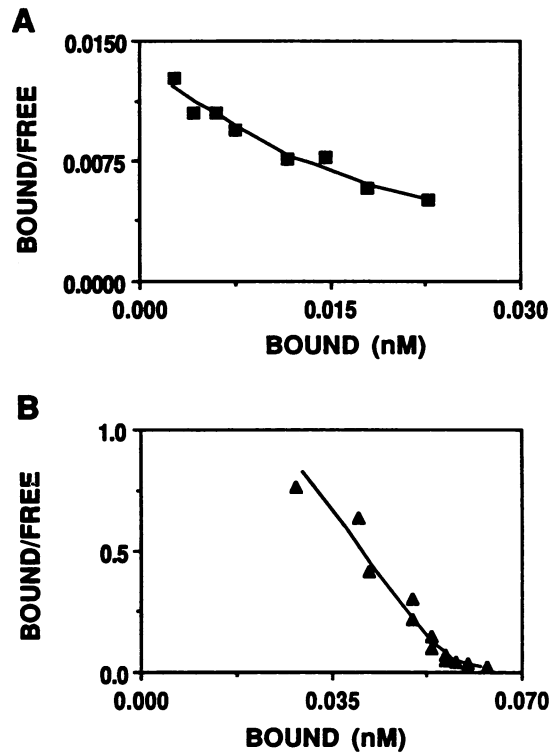

FIG. 7. Scatchard analysis of IRE probe binding to the purified IRE-BP in the absence of reducing agents (■) and in the presence of 1% 2-ME (▲). (A) In the absence of reducing agents, K_d = 1.4 nM (n = 1). (B) In the presence of 1% 2-ME, K_d = 26 ± 10 pM (n = 3). Gel retardation binding assay conditions and quantitation were as described in Materials and Methods.

complex formed with a functional IRE could be inhibited. In the absence of heparin, we observed a specific complex, with mobility identical to that seen with a functional IRE, with this mutated IRE. We examined the ΔC-IRE complex by Scatchard analysis. In contrast to a wild-type IRE, the ΔC-IRE probe gives only a single-site binding curve whose affinity is approximately 0.7 nM (Fig. 6). No high (pM)-affinity interaction is seen. Furthermore, there is no response to the addition of 2-ME even in lysates that displayed a marked response when a functional IRE was used as a probe. Likewise, we could not distinguish the binding activity present in lysates from control and desferrioxamine-treated cells with a ΔC-IRE probe.

Scatchard analysis of binding of the purified IRE-BP to the IRE. The IRE-BP has been purified to homogeneity by affinity chromatography with IRE bound to a solid matrix (13a). The purified IRE-BP can be inactivated by diamide and reactivated by the addition of 2-ME (data not shown). Furthermore, Scatchard analysis of the binding of IRE to IRE-BP showed a single site of 1 to 2 nM affinity in the absence of reducing agents and a single site of 30 pM in the presence of 2-ME (Fig. 7).

DISCUSSION

In this report, we have examined the quantitative aspects of the interaction of iron-responsive RNA regulatory elements with their specific cytosolic binding protein. Previous work has suggested that the binding of this protein is responsible for the repression of translation initiation (3, 14). Whether it is simply the presence of this binding protein on the 5′ UTR of a message that is sufficient for repression, perhaps by sterically interfering with the early events of initiation, is not clear. Observations have allowed us to correlate the binding of this protein with the ability to mediate iron-responsive translational control. We have observed a correlation between increased binding activity and decreased translation in cells treated with the iron chelator desferrioxamine. We have recently provided evidence that the effect of iron deprivation is to activate a pool of inactive IRE-BP within the cytosol via a posttranslational mechanism and that this activation involves the reduction of at least one critical intramolecular disulfide bond (7).

In order to assess the binding characteristics of the IRE-BP, we altered the assay used previously for IRE/IRE-BP interactions by synthesizing a short (35-nucleotide) probe and including neither heparin nor RNase T1. These modifications allowed us to observe two binding affinities. Both of these were specific by the criteria that they were inhibited by RNA containing an IRE but not by random RNA such as the 5′ region of β-globin or by a so-called unrelated stem loop. The latter probe consisted of a 35-nucleotide sequence (described in Materials and Methods) predicted to assume a stem-loop structure lacking both the unpaired C in the stem and the six-membered loop sequence characteristic of naturally occurring IREs. In lysates from control cells, the percentage of total binding represented by the high-affinity sites varied between 5 and 20%. The presence of these two binding affinities allowed us to redefine our previous observations that correlated binding with IRE function. First of all, the ΔC-IRE does show specific binding and results in an IRE/IRE-BP complex whose migration in these nondenaturing gels is identical to that given by a functional IRE. However, this probe is incapable of engaging the IRE-BP in a high-affinity interaction. Second, we have previously demonstrated that treatment of cells with desferrioxamine or of lysates with reducing agents activates an inactive pool of

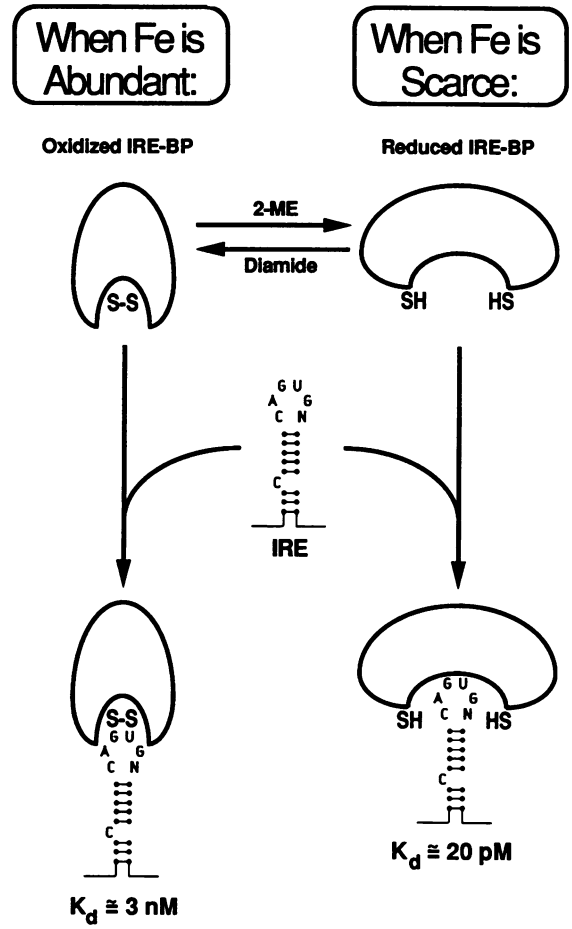

FIG. 8. Schematic diagram of a proposed model for IRE/IRE-BP interaction. We do not imply knowledge of actual contact points of IRE to IRE-BP.

cytosolic IRE-BP from one that fails to bind to one that is capable of binding to IREs (7). The modified assay used in this study allowed us to define this activation step.

The model we favor for the interaction between the IRE and the IRE-BP, based upon the data presented in this report, is that this protein contains a single binding site for its cognate RNA (Fig. 8). This site can exist in two configurations depending upon the status of at least one sulfhydryl pair. When this pair is oxidized, the site is available for low-affinity (nM) interaction with a suitable RNA. When those groups are reduced, the site can interact with an appropriate RNA with a 100-fold–higher affinity. Thus, the sulfhydryl switch, which we have recently proposed, serves to change the IRE/IRE-BP interaction from a low (nM) to a high (pM) affinity. The ΔC-IRE allows us to distinguish these two binding configurations on the basis of the RNA rather than the protein. The ΔC-IRE stem-loop is capable of being specifically recognized by the IRE-BP as an IRE. However, it is not capable of high-affinity interaction within the binding site even when the protein is in its reduced state. The interaction between the free sulfhydryl and the IRE can only be conjectured about at this time. One intriguing possibility is that it can participate in a Michael addition with the RNA (8, 12). The Michael addition involves a nucleophilic addition of a free thiol group to the 6 carbons of a pyrimidine to form a covalent bond. The formation of such a bond, while only speculation for the IRE-BP, would provide a satisfying explanation for the role of a free sulfhydryl in the very high-affinity RNA-protein interaction. The ability of the ΔC-IRE to interact with the protein allows us to formulate the question as to what constitutes an IRE with some greater precision. Thus, we now need to define what the minimal and critical structure or sequence is that allows specific binding as well as what is required to engage in a high-affinity interaction.

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