The bifunctional iron-responsive element binding protein/cytosolic aconitase: The role of active-site residues in ligand binding and regulation

(mutagenesis/RNA binding)

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ABSTRACT   The iron-responsive element binding protein/cytosolic aconitase functions as either an RNA binding protein that regulates the uptake, sequestration, and utilization of iron or an enzyme that interconverts citrate and isocitrate. These mutually exclusive functions are regulated by changes in cellular iron levels. By site-directed mutagenesis we show that (i) ligation of a [4Fe-4S] cluster is necessary to inactivate RNA binding and activate enzyme function in vivo, (ii) three of four arginine residues of the aconitase active site participate in RNA binding, and (iii) aconitase activity is not required for iron-mediated regulation of RNA binding.

The iron-responsive element binding protein (IRE-BP) is an example of a single gene product serving two broadly different and seemingly unrelated functions. This protein is an RNA binding protein that regulates the expression of genes involved in the uptake, sequestration, and utilization of iron. IRE-BP binds with high affinity to RNA stem–loops known as iron-responsive elements (IREs) present in the 5' untranslated region of the mRNAs of ferritin and the erythroid form of aminolevulinic acid synthase and in the 3' untranslated region of the mRNA of the transferrin receptor (reviewed in refs. 1–3). Binding of IRE-BP prevents translation of ferritin and aminolevulinic acid synthase (2, 3) and protects the mRNA of transferrin receptor from degradation. Other genes of iron metabolism may also contain IREs and may be regulated through interaction with IRE-BP. This protein performs an important metabolic function in response to low intracellular iron levels by interacting with cognate mRNAs to increase net iron uptake (transferrin receptor) and decrease sequestration (ferritin) and utilization (aminolevulinic acid synthase) of iron.

IRE-BP is identical in primary amino acid sequence to cytosolic aconitase, an enzyme that interconverts citrate and isocitrate, and the protein functions either as an RNA binding protein or as a functional enzyme depending on cytosolic iron levels. The RNA binding form of the protein predominates in iron-deprived cells, and IRE-BP isolated from these cells has little aconitase activity. Conversely, when the protein is isolated from iron-replete cells, it is a fully active aconitase with low IRE binding activity (4). Because this protein switches between the two functions of aconitase and IRE-BP, it has been proposed that the protein be referred to as the iron regulatory protein (IRP). We shall refer to the original IRE-BP as IRP1 to distinguish it from a second homologous IRE-BP, which is not an aconitase (ref. 5; J. Chin, F. Samaniego, and T.A.R., unpublished observations).

Several lines of evidence have suggested that the switch from the RNA binding form (IRE-BP) to the enzymatically active form (aconitase) results from ligation of an iron-sulfur

cluster (4, 6–8). In vitro manipulations that assemble or disassemble a [4Fe-4S] cluster result in the interconversion between the two mutually exclusive states, and complete disassembly of the [4Fe-4S] cluster appears to be required for acquisition of RNA binding activity (4, 6). Here we address genetically the prediction that ligation of the Fe-S cluster is required for in vivo iron regulation of RNA binding and aconitase activity by using site-directed mutagenesis of the three predicted cluster-ligating cysteine residues.

While experimental results obtained from these mutations strongly support the essential role of the cluster in the switching mechanism, the mechanism by which ligation of the cluster inactivates RNA binding remains unclear. A possible answer is suggested by recent evidence indicating that the active site for IRE binding may overlap the enzymatic active site of IRP1 (9). A model in which the active-site cleft binds either enzyme substrate or RNA predicts that conserved residues within the cleft may perform dual ligation functions. Within the active site, the tricarboxylic acid substrates are bound by four arginine residues. Arginine has been clearly implicated as essential for RNA recognition in other proteins, such as human immunodeficiency virus Tat (10, 11). We mutated each of the four active-site arginine residues to assess whether any or all might play a role in RNA recognition.

The results of these studies confirm that the dual function of this protein is reflected in the dual function of the active-site region. What remains a puzzle is why IRP1 has enzyme activity and, indeed, why cytosolic aconitase is maintained in eukaryotic cells. We considered two possible explanations: (i) the aconitase catalytic mechanism is required for the unusual process of cluster disassembly proposed to be required to turn on the RNA binding activity of IRP1 (4), and (ii) it is advantageous for the cell to coordinately regulate both cytosolic citrate/isocitrate metabolism and the fate of IRE-containing RNA transcripts in response to alterations in iron levels. To investigate this issue, we expressed an IRP1 containing a single amino acid substitution predicted to eliminate enzyme activity without affecting cluster ligation or substrate binding, and we then evaluated the iron-regulated RNA binding activity of this protein.

MATERIALS AND METHODS

Construction of Mutant IRP1s. Mutant IRP1s were constructed using the two-step PCR method (12) in the mammalian expression vector pCDL-SRα (13) with a carboxyl-terminal Myc epitope (14). Mutant and wild-type constructs were also subcloned into an episomal expression vector

Abbreviations: IRE, iron-responsive element; IRP, iron regulatory protein; 2-ME, 2-mercaptoethanol; IRE-BP, iron-responsive element binding protein.

containing the glucocorticoid-inducible promoter GRE-5 (15).

Cell Culture. Calcium phosphate transient transfections were performed in murine fibroblasts (B6) using wild-type and mutant IRP1 constructs in pCDL-SRα (16). For *in vivo* iron regulation studies, calcium phosphate transfections of HeLa cells (American Type Culture Collection) were performed using wild-type and mutant constructs in the GRE-5/episomal vector. Transfected cells were selected in hygromycin B (250 μg/ml; Calbiochem), and pooled populations of resistant cells were studied. Stable cell lines were induced with 20 nM dexamethasone (Sigma) for 48 hr and then treated with either 20 μM hemin (Sigma) or 100 μM desferrioxamine (CIBA–Geigy) for 12 hr.

Gel Retardation Assays and Scatchard Analysis. Cells were lysed and IRE binding activity was assessed by the gel retardation assay (17) performed with the following modifications: 0.5 μg of lysate from transient transfections was incubated with 2 ng of radiolabeled IRE in the presence of 1% 2-mercaptoethanol (2-ME) and 250 ng yeast tRNA and then subjected to electrophoresis on 8% polyacrylamide gels. Samples for Scatchard analysis were incubated for 30 min with 20 ng or 500 ng of lysate and radiolabeled IRE concentrations of 1 pM to 1 nM at a specific activity of 1.60×10^{19} dpm/mol and concentrations of 1 nM to 0.1 μM at a specific activity of 1.96×10^{17} dpm/mol. After electrophoresis, gels were fixed, dried, and quantitated by using a PhosphorImager (Molecular Dynamics). Under these conditions, the recombinant IRP1–IRE complex is easily resolved from the endogenous murine complex, which is present in 100-fold lower amounts. Experiments were performed in duplicate or triplicate and data were analyzed using the program LIGAND (18). Because of the technical limitations of the gel retardation assay, K_d for the lower affinity mutant IRP1s was estimated from the lower specific activity data points. Gel retardation assays using lysates from stable HeLa cell lines were performed with the "supershift" modifications: 10 μg of lysate was incubated with 1 μg of protein A-Sepharose-purified anti-Myc antibody from murine ascites at 4°C for 2 hr or 30°C for 1 hr prior to the addition of 2 ng of radiolabeled IRE. These samples were assayed in the absence or presence of 2% 2-ME and subjected to electrophoresis on 6% gels.

In Vitro Iron Loading and Aconitase Assay. Aconitase assays were performed with or without *in vitro* iron loading. To iron load, 0.5–1.0 mg of lysate was incubated with ferrous ammonium sulfate (0.5 mM), sodium sulfide (0.5 mM), sodium citrate (0.5 mM), and dithiothreitol (10 mM) in 25 mM Tris (pH 8.0) for 1 hr at 4°C. Iron-loaded lysates (30 μg) were desalted over a G-50 column and analyzed for IRE binding by the supershift gel retardation assay. For aconitase assays, lysates (0.5–1.0 mg) were immunoprecipitated on 10 μl of protein A-Sepharose to which monoclonal anti-Myc antibody had been chemically crosslinked. Immunoprecipitates were washed in 25 mM Tris (pH 8.0), 40 mM KCl, and 1 mM sodium citrate. The coupled aconitase/isocitrate dehydrogenase assay (19, 20) was performed on the precipitated IRP1 in a 300-μl reaction volume with constant agitation, and the production of NADPH in the supernatant was monitored by the absorbance at 340 nm. Untransfected HeLa cells were used as a negative control. Specific activities were determined by SDS/PAGE (21) and silver staining of immunoprecipitated IRP1s using purified IRP1 from insect cells as a standard (22). Units are millimoles of isocitrate formed per minute with citrate as substrate.

RESULTS AND DISCUSSION

We designed three series of single amino acid substitutions that were predicted to (*i*) destroy the protein's ability to fully ligate the [4Fe-4S] cluster, (*ii*) assess whether enzymatic

active-site residues participate in RNA recognition, or (*iii*) eliminate the catalytic activity of the enzyme, independent of cluster ligation. These mutations were based upon the amino acid alignment of human IRP1 with active-site residues identified in mitochondrial aconitase, a highly related protein that has been crystallized and extensively characterized (23–26). The three cysteine residues predicted to ligate the Fe-S cluster in human IRP1 (Cys-437, -503, and -506) and a fourth cysteine not predicted to ligate the cluster (Cys-300) were individually changed to a serine residue to form mutant IRPs C437S, C503S, C506S, and C300S (26, 27). The mutually exclusive functions of this protein led us to look for enzymatic active-site residues that participate in RNA recognition. Arginine residues have been previously identified as important in several RNA binding interactions because of their charge, their ability to participate in the formation of a "base triple" (as in the interaction of Tat with TAR RNA) (10, 11), and their ability to mimic guanosine binding properties (28). In mitochondrial aconitase, four active-site arginine residues are involved in substrate recognition. The corresponding arginine residues in IRP1 (Arg-536, -541, -699, and -780) were replaced with lysine or glutamine, and the RNA binding capacity of the mutant proteins was assessed. Finally, in mitochondrial aconitase a single serine mutation virtually eliminates enzymatic activity (26); Ser-778 in the human cytosolic aconitase sequence, which corresponds to the critical catalytic serine in mitochondrial aconitase, was changed to alanine in mutant S778A.

Because overexpression of IRP1 in mammalian cells is associated with loss of iron regulation and interference with the formation of stable cell lines that express the recombinant protein, the mutant and wild-type IRP1 constructs were cloned into an episomal expression vector under the control of a glucocorticoid inducible promoter, GRE-5 (15). Stably transfected HeLa cell lines were produced that had virtually no expression of recombinant protein prior to induction with dexamethasone. After induction, recombinant wild-type IRP1 demonstrated *in vivo* iron-regulated RNA binding comparable to that of endogenous protein (see Figs. 1 and 3).

Fe-S Cluster Ligand Residue Mutations. Mutation of the putative cluster-ligating cysteine residues (Cys-437, -503, and -506) and the noncluster ligating cysteine residue Cys-300 to serine did not affect high-affinity RNA binding (27). Using stable cell lines, we experimentally assessed whether treatment with iron would result in the normal loss of RNA binding capacity *in vivo* if the predicted cluster-ligating cysteine residues were mutated. Endogenous IRP1 has little RNA binding activity when assayed from cells treated with hemin (an iron source), but full RNA binding activity can be restored when assayed in the presence of 2% 2-ME (19, 29). Desferrioxamine-treated cells are iron starved; IRP1 from these cells has high RNA binding affinity, and little additional binding can be recruited in the presence of 2-ME. Cell lines expressing recombinant IRP1s were treated with hemin or desferrioxamine, and the regulation of RNA binding was assessed by the supershift gel retardation assay (Fig. 1A). Wild-type and C300S IRP1s from hemin-treated cells show a loss of binding activity that can be restored with 2-ME (lanes 1–4) while C437S, C503S, and C506S have high binding activity regardless of the iron status of the cell (lanes 5–8). The aconitase activity of each of these mutants was measured after *in vivo* iron loading (Fig. 1B). The aconitase specific activity of recombinant wild-type IRP1 and C300S was calculated to be 11 units/mg, whereas the activity of mutant IRPs C437S, C503S, and C506S was not detectable above background. Thus, iron-regulated RNA binding requires all three cluster-ligating cysteine residues; mutation of any one results in an IRP1 that is always in the RNA binding form. This finding supports the prediction that Cys-437, -503, and -506 ligate the [4Fe-4S] cluster and that incorporation of a
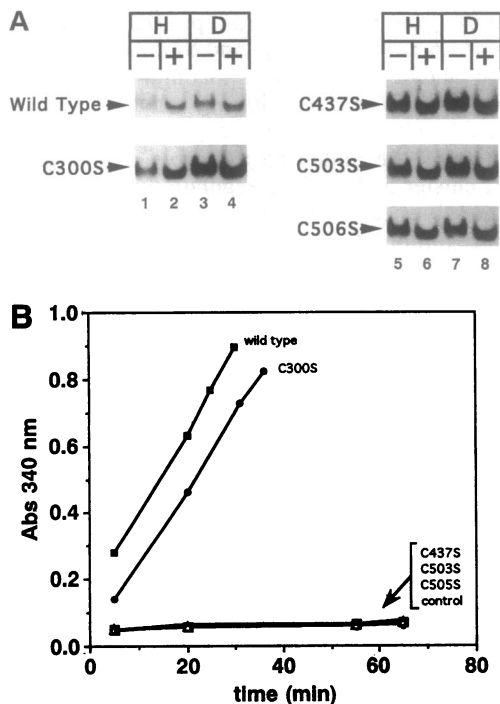
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FIG. 1. *In vivo* iron regulation of RNA binding and aconitase activity in cysteine mutants of IRP1. Stably transfected cell lines were induced with dexamethasone for 48 hr and then treated with either hemin (H) or desferrioxamine (D) for 12 hr. Cells were then lysed. (*A*) IRE binding activity determined by the gel retardation assay (17) performed with the supershift modification. Samples were assayed in the absence (−) or presence (+) of 2% 2-ME and subjected to electrophoresis on 6% polyacrylamide gels. (*B*) The aconitase assay was performed by immunoprecipitating 1.0 mg of lysate from hemin-treated cells with 10 μl of anti-Myc antibody chemically crosslinked to protein A-Sepharose. The coupled aconitase/isocitrate dehydrogenase assay (19, 20) was performed on the precipitated IRP1 in 300 μl, and the production of NADPH in the supernatant was monitored by the absorbance at 340 nm. Untransfected HeLa cells were used as a negative control. The change in absorbance at 340 nm per min indicates the relative aconitase activity. Each sample contained 100–200 ng of recombinant IRE-BP as determined by SDS/PAGE and silver staining (data not shown).

cluster is required *in vivo* to turn off RNA binding and to restore aconitase activity as the cell accumulates iron.

Active-Site Arginine Mutations Affect RNA Binding. Recent studies (9, 27) have indicated that the IRE binding site may include residues located in the solvent-filled cleft between domains 1–3 and 4 in the structure predicted by the crystal structure of mitochondrial aconitase. We considered that active-site arginine residues might contribute to RNA binding because arginine residues have been shown previously to play an important role in RNA binding (10, 11, 28). Accordingly, Arg-536, -541, -699, and -780 were individually mutated to both lysine and glutamine, expressed transiently in murine fibroblasts, and assayed for RNA binding activity by gel retardation (Fig. 2*A*) and Scatchard analysis (Table 1). Quantitative immunoprecipitation (Fig. 2*B*) demonstrated that each of the mutant IRP1s was expressed in approximately equal amounts in each transfection. Substitution of Arg-699 with either lysine (R699K; Fig. 2*A*, lane 7) or glutamine (R699Q; Fig. 2*A*, lane 8) did not diminish RNA binding affinity, which suggests that this residue does not participate in IRE binding. R536K IRP1 had an RNA binding affinity equal to that of wild type while R536Q had a dramatically reduced affinity (Fig. 2*A*, lanes 3 and 4), indicating that side chain charge rather than structure is important in the residue's interaction with the IRE. In contrast, either change at
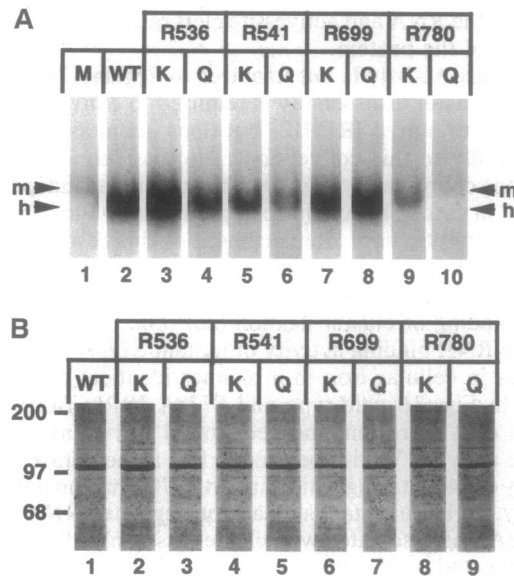

FIG. 2. Active-site arginine mutations affect RNA binding. Murine fibroblasts were transiently transfected with wild-type human IRP1 (WT) or mutants of Arg-536, -541, -699, and -780 in the expression vector pCDL-SRα. (*A*) The gel retardation assay was performed using 0.5 μg of lysate and 2 ng of radiolabeled IRE in the presence of 250 ng of yeast tRNA. Arrowheads indicate the specific IRE–IRP1 complex of endogenous murine origin (m) or transfected recombinant human (h). Untransfected murine cell lysates (M) were used as a control. (*B*) SDS/PAGE and silver staining were performed on anti-Myc immunoprecipitates from 50 μg of lysate containing recombinant wild-type or mutant IRP1. Molecular size standard migrations (in kDa) are indicated. K, lysine; Q, glutamine.

position 780 or 541 resulted in a complete loss of high affinity binding, and R780Q binding was almost undetectable (Fig. 2*A*, lanes 5, 6, 9, and 10). This result is consistent with the concept that the unique structure of these arginine residues mediates the RNA–protein interaction. Previous work demonstrated that the IRE was UV crosslinked to a region of the protein adjacent to an active-site residue in domain 1 (9). Arg-780 is located in the fourth domain, and the requirement of this arginine for RNA binding suggests that the transition from enzyme to RNA binding protein must involve opening of the cleft via a linker peptide to accommodate the IRE stem–loop structure. Therefore, the fourth domain must assume a new orientation in order to participate in RNA binding (1, 30). The UV crosslinking data coupled with the arginine mutagenesis indicates that direct contacts form

Table 1. IRE binding affinities of recombinant arginine mutant IRP1s

Arginine residue	K_d, pM	
	Lysine	Glutamine
536	17	>100*
541	>100*	>1,000*
699	7	6
780	>100*	>10,000†

Affinities were determined by Scatchard analysis using the program LIGAND. Gel retardation assays were performed using 20 ng of lysate from transiently transfected murine fibroblasts and 1 pM to 0.1 μM radiolabeled IRE in 20 μl. Quantification of bound and free IRE was determined by using a PhosphorImager. In this assay, wild-type IRP1 had a K_d of 14 pM. All experiments were performed in duplicate and the standard error (percentage coefficient of variations) was ≤30%.
*Because of the lower affinities, 500 ng of lysate was used and the K_d was estimated after quantification.
†The R780Q–IRE complex was inconsistently detectable in the gel retardation assay.

between the RNA and active-site cleft residues in domains 1, 3, and 4 of the protein.

Aconitase Activity Is Not Required to Regulate IRE Binding in Vivo. Cluster disassembly is required to convert cytosolic aconitase to active IRE-BP, but does aconitase contribute catalytically to this conversion? Alternatively, are the two functions coordinately regulated but otherwise independent? Ser-778 in cytosolic aconitase is predicted to function as the critical catalytic base. Replacement of this residue in mitochondrial aconitase results in a dramatic reduction in enzymatic activity, although the mutation does not affect substrate binding or cluster ligation (26). Fig. 3A demonstrates that the RNA binding activity of mutant S778A is altered by changes in cellular iron in a manner identical to wild-type IRP1. In Fig. 3B wild-type and S778A IRP1s from hemin-treated cells were immunoprecipitated and assayed for aconitase activity. Wild-type protein had an aconitase specific activity of 14 units/mg while mutant S778A had no detectable activity. To better establish that iron-regulated RNA binding in S778A was associated with cluster assembly/disassembly, we subjected wild-type and mutant IRP1 to an *in vitro* iron-loading procedure, which will assemble a [4Fe-4S] cluster. The RNA binding activity of IRP1s from desferrioxamine-treated cells is shown in Fig. 4A before and after *in vitro* iron loading. Again wild-type and S778A IRP1s are identical in their diminished RNA binding activities after iron loading. The ability of a cluster-building protocol to restore aconitase activity in desferrioxamine lysates is demonstrated in Fig.

4B. Before iron loading, wild-type IRP1 had a specific activity of 1.6 ± 0.4 units/mg, and S778A had no detectable aconitase activity. After iron loading, wild-type activity increased to 15.6 ± 2.1 units/mg while S778A remained undetectable above background. These data prove that S778A retains the ability to assemble and disassemble a [4Fe-4S] cluster and achieve regulation of RNA binding *in vivo* in the absence of catalytic activity.

The recognition of specific cis-acting regulatory elements by cytosolic RNA binding proteins plays a central role in determining the fate of specific transcripts. With the IRP1 system we observe a remarkable compaction of function whereby the active site of a highly conserved cytosolic enzyme can serve either to recognize specific tricarboxylic acid substrates or the target RNA structure that allows multiple transcripts to be regulated in response to changes in iron and/or oxidants (31–33). The central feature of this regulatory network has been proposed to be the labile Fe-S
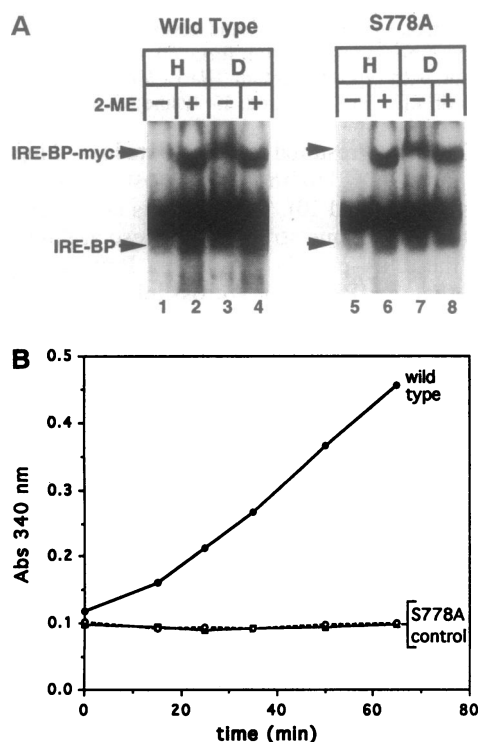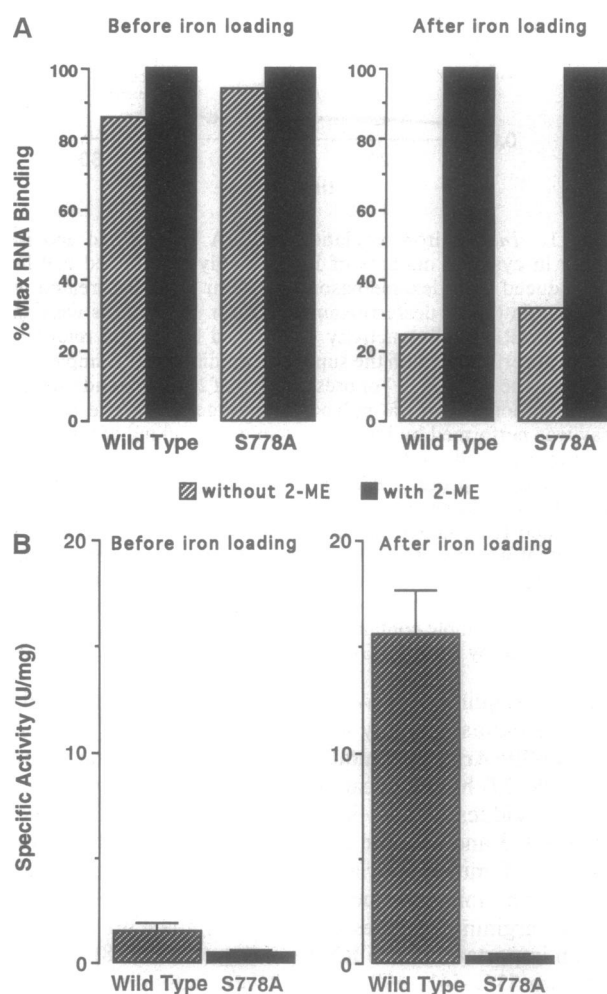

FIG. 3. *In vivo* iron regulation of RNA binding in an IRP1 mutant deficient in aconitase activity. Hemin (H) and desferrioxamine (D) lysates from stable cell lines expressing recombinant wild-type and mutant S778A IRP1 were prepared as described. (*A*) The supershift gel retardation assay in the absence (−) or presence (+) of 2-ME was performed as described in Fig. 1. The arrows indicate the supershifted recombinant IRP1–IRE (IRE-BP-myc) or the endogenous IRP1–IRE (IRE-BP) complex. (*B*) The aconitase activity of recombinant wild-type and S778A IRP1 from hemin-treated cells was determined. Lysates (500 μg) were immunoprecipitated, and aconitase activity was determined as described in Fig. 1. Representative data from a single experiment are presented. This experiment was repeated three times, and aconitase activity was not detectable above background for S778A.

FIG. 4. (*A*) *In vitro* iron regulation of RNA binding of wild-type IRP1 and mutant S778A with and without iron loading. Desferrioxamine lysates were prepared as described in Fig. 1 and underwent iron loading. To iron load, lysates (30 μg) were incubated with ferrous ammonium sulfate (0.5 mM), sodium sulfide (0.5 mM), sodium citrate (0.5 mM), and dithiothreitol (10 mM) in 25 mM Tris (pH 8.0) for 1 hr at 4°C. Iron-loaded lysates were desalted over a G-50 column and analyzed for RNA binding by the supershift gel retardation assay. RNA binding was quantitated by using a PhosphorImager and expressed as the percent of maximal RNA binding in the presence of 2% 2-ME. Hatched bars indicate binding without 2-ME, and solid bars indicate binding with 2-ME. (*B*) *In vitro* iron regulation of aconitase activity of wild-type IRP1 and mutant S778A. Desferrioxamine lysates (500 μg) were assayed for aconitase activity without and with iron loading as described in Fig. 1.

cluster that provides the posttranslational switch that underlies the environmental responsiveness of this system. Here we have provided evidence using site-directed mutagenesis that the ability to ligate a [4Fe-4S] cluster via three cysteine residues is required for both *in vivo* iron regulation of RNA binding and acquisition of aconitase activity in IRP1. The mapping of three substrate binding arginines in the enzymatic active site as important in RNA binding supports the model that cluster disassembly mediates the activation of RNA binding by allowing the local conversion of the enzyme active site to an RNA binding site and that the essential conformational change is the opening of the cleft via the linker peptide that connects domains 3 and 4 of the protein.

Although aconitase activity is present in mammalian cells in the mitochondria, the conservation of all active-site residues in mammalian cytosolic aconitase suggests that the enzymatic activity has been specifically retained in the cytosol. The mutagenesis reported here shows that enzymatic activity is not required for cluster assembly/disassembly or regulated RNA binding; thus, we must conclude that cytosolic aconitase function and, therefore, citrate metabolism itself are iron regulated. Why does the cell actively regulate cytosolic aconitase in response to environmental stresses such as iron availability and oxidants (31–33)? Perhaps the answers lie in the role of citrate in intracellular iron delivery (34) and/or the pathways of citrate metabolism—for example, in production of acetyl CoA via citrate lyase (35). It seems most likely that the direct posttranslational regulation of cytosolic aconitase activity is part of a regulatory metabolic network that also requires the regulation of expression of IRE-containing transcripts.

The regulation of protein activity is generally thought of in terms of a simple on/off switch. IRE-BP/cytosolic aconitase is a bifunctional protein where the switch (provided by the labile Fe-S cluster) turns off one activity while simultaneously turning on the other. We suggest the term "ambifunctional" to refer to the particular type of either/or bifunctionality that has been demonstrated for this protein. The recent report that glyceraldehyde-3-phosphate dehydrogenase functions as a specific tRNA binding protein in which the ability to bind tRNA is blocked by the binding of NAD, an essential enzymatic cofactor, may indicate the existence of another ambifunctional enzyme/RNA binding protein (36). Similarly, *Neurospora* mitochondrial tyrosyl tRNA synthase can bind either tRNA or intronic RNA sequences, thus allowing the protein to function as either a synthase or a splicing protein (37). Ambifunctional proteins may be widespread, but their identification will require development of assays for novel functions of proteins in which the known function is turned off. Why did the cell evolve such a complex posttranslational iron regulatory system in the cytosol when most genes are transcriptionally regulated in the nucleus? Conceivably, the intrinsic iron-sensing capability, based on a labile iron-sulfur cluster, of the preexisting cytosolic aconitase provided the ideal foundation on which to build an iron-dependent gene regulatory system, thus leading to the evolution of the ambifunctional cytosolic aconitase/IRE-BP.

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