Modification of a Free Fe-S Cluster Cysteine Residue in the Active Iron-responsive Element-binding Protein Prevents RNA-binding Binding* 

(Received for publication, May 10, 1993, and in revised form, June 22, 1993)

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The iron-responsive element-binding protein (IRE-BP) binds to specific RNA stem-loop structures called iron-responsive elements (IREs), which mediate the post-transcriptional regulation of a variety of genes involved in iron metabolism. The IRE-BP is cytosolic aconitase, and a [4Fe-4S] cubane cluster is required for aconitase activity but is associated with loss of IRE binding affinity. Chemical modification of the IRE-BP can abrogate RNA binding and the 3 cysteines predicted to coordinate the Fe-S cluster in the IRE-BP could be targets for modification. We report the expression of recombinant IRE-BP in which the three putative cluster cysteines (Cys-437, Cys-503, and Cys-506) have been mutated to serine residues. Replacement of any or all of these cysteine residues results in a complete loss of aconitase activity. While all of the mutants bind RNA, substitution of Cys-437 specifically renders the IRE-BP resistant to inactivation by low concentrations of N-ethylmaleimide or diamide. These results identify Cys-437 as the target of in vitro regulation of RNA binding in the IRE-BP and suggest that, in the RNA-binding form of the protein, Cys-437 is free and therefore available for modifications that inhibit RNA binding.

Post-transcriptional regulation of expression of proteins of iron metabolism is modulated by the interaction of the iron-responsive element-binding protein (IRE-BP) with specific stem-loop structures called iron-responsive elements (IREs) located in the 5′-untranslated region of ferritin mRNA and the 3′-untranslated region of transferrin receptor mRNA. The binding of the IRE-BP to ferritin mRNA results in inhibition of initiation of translation and a decrease in ferritin protein, while binding of IRE-BP to transferrin receptor mRNA confers stability on the message and increases receptor levels. IRE-BP binding affinity is regulated by cellular iron levels. When iron is abundant, binding affinity is low; when iron is scarce, binding affinity is high (reviewed by Klausner et al., 1993; Klausner and Rouault, 1993, and references therein). This switch occurs in the absence of protein synthesis or any change in the level of the IRE-BP protein and thus represents a post-translational modification of the protein (Tang et al., 1992).

The IRE-BP is identical to cytosolic aconitase (Kennedy et al., 1992), and recent experiments have allowed us to formulate a model for iron-dependent regulation of the IRE-BP. When the IRE-BP is isolated from cells replete with iron, it fails to bind to RNA and has full aconitase activity, demonstrating that it contains a [4Fe-4S] cluster. In contrast, the active RNA-binding protein isolated from iron-deprived cells has no aconitase activity (Haile et al., 1992a, 1992b). We have recently attempted to determine the exact status of the cluster in the RNA-binding form of the protein. These studies have pointed to the apoprotein as the form that most closely resembles the protein derived from cells deprived of iron (Haile et al., 1992b; Emery-Goodman et al., 1993). Thus, we postulate that reversible assembly and disassembly of the cluster is the mechanism for in vivo iron regulation of RNA binding.

The RNA binding activity of the IRE-BP can be shut off by two types of in vitro manipulations. Iron loading protocols that assemble a full cluster and thus reconstitute aconitase activity abrogate RNA binding (Haile et al., 1992a; Constable et al., 1992). Alternatively, chemical modification of 1 or more cysteine residues will inactivate RNA binding (Hentze et al., 1989). The effects of these modifications can be irreversible, using the alkylating agent NEM, or reversible, using the sulfhydryl modulating agent diamide. Both these modifications and the assembly of Fe-S clusters have in common the ligation of cysteines. In mitochondrial aconitase the Fe-S cluster is coordinated by 3 cysteine residues located in the active site cleft (Lauble et al., 1992; Robbins and Stout, 1989b). These residues are conserved in the IRE-BP and are predicted to serve a similar function (Rouault et al., 1992). In this study, we report the expression of recombinant mutant IRE-BPs in which cluster cysteiny1 residues have been replaced with serines. This work supports the assignment of the predicted cluster ligating cysteines and, furthermore, provides the identification of one of these cysteine residues as the primary target of in vitro regulation of RNA binding.

MATERIALS AND METHODS

Recombinant Mutant IRE-BPs—All mutants were constructed by the polymerase chain reaction method (Higuchi et al., 1988), cloned directly into pCDLsRA containing IRE-BP/Myc cDNA (Kaplan et al., 1991) and confirmed by sequencing of the polymerase chain reaction-generated portion. Murine fibroblast cells (3T3) were transiently transfected with mutant constructs by the calcium-phosphate method (Casey et al., 1988). Cys64 lysates were prepared in 1% Triton, 25 mM Tris (pH 8.0), 40 mM KC1 as described (Haile et al., 1989).

Acidic Assay and Iron Loading Procedure—Lysates (500 μg) were incubated in diethylene triamine (100 mM) and ferrous ammonium sulfate (1 mM) for 30 min on ice. After the addition of EDTA (1 mM), the lysates were diluted 30-fold in buffer (25 mM Tris (pH 8.0), 40 mM KC1, 1 mM sodium citrate) and incubated with 5 μl of anti-Myc monoclonal antibody from ascites (Kaplan et al., 1991; Pelham, 1986) for 30 min on ice. This was followed by 2 μl of rabbit anti-mouse antibody (Organon Technika) with an additional 30-min incubation. Protein A-Sepharose (50 μl) was added and the samples incubated at 4 °C for 30 min. The samples were washed twice with 1 ml of buffer and resuspended in aconitase assay solution (Haile et al., 1992a; Rose and O’Connell, 1967), and the
absorbance at 340 nm was followed.

\textit{N}-Ethylmaleimide, Iodoacetamide, and Diamide Treatment—Cytosolic lysates from hemin- or desferrioxamine-treated murine fibroblast (B6) cells were prepared as previously described (Haile et al., 1989). For iodoacetamide (IAM) evaluation, desferrioxamine lysates were incubated in 10 mM IAM for 30 min on ice, followed by the addition of NEM to 1.0 mM for 30 min, and then by dilution and desalting over a G-50 column. Protein concentrations were then determined on column eluates. Lysates were treated with NEM alone at concentrations of 0.1, 1.0, and 10 mM in a total volume of 10 µl for 30 min on ice. Samples were diluted with 40 µl of 25 mM Tris (pH 8.0), 40 mM KCl before the gel retardation assay. For diamide treatment, lysates were incubated in 1.0 and 10 mM diamide for 30 min on ice, then diluted 5-fold before use in the gel retardation assay.

\textbf{Gel Retardation Assay—}Gel electrophoresis of IRE-IRE-BP complexes was performed as described (Haile et al., 1989) using 0.5–1 ng of radiolabeled IRE probe, 1–5 µg of lysate, and an 8% acrylamide gel. The final concentration of radiolabeled IRE was 200 nM. The specific IRE-IRE-BP complex is indicated by the arrow.

\textbf{RESULTS AND DISCUSSION}

\textit{Inhibition of RNA Binding by NEM and Diamide—}The cellular regulation of IRE-BP activity is observed by studying the protein derived from lysates of cells that have been treated with an iron source (hemin) or an iron chelator (desferrioxamine). The IRE-BP from the iron-treated cells has full aconitase activity and no RNA binding activity (Haile et al., 1992a). The latter activity can be fully recovered by assaying the interaction with a target RNA in the presence of 2% 2-ME. In contrast, the protein derived from the iron-deprived cells has no aconitase activity and has full RNA binding activity. The addition of reducing agents has no effect on the RNA binding activity of such “active” IRE-BP. We will refer to the “active” IRE-BP as that form which binds IRE-containing RNAs with high affinity. \textit{In vitro} regulation of RNA binding can be achieved through chemical modification of cysteine residues of the IRE-BP. Treatment of the active IRE-BP from iron-deprived cells (Fig. 1a) with 1.0 mM diamide resulted in the complete loss of RNA binding activity as assessed by a gel retardation assay (lane 5). The lost activity could be recovered by treatment with 2% 2-ME (lane 6). RNA binding activity could also be lost by treatment with 0.1 or 1.0 mM NEM. In this case, however, no recovery was seen with the subsequent addition of reducing agent (lanes 3 and 4). The RNA binding activity of IRE-BP from iron-replete cells was, in contrast, relatively resistant to NEM inactivation (Fig. 1b). A slight reduction in binding was seen after treatment with 0.1 mM NEM (lanes 3 and 4), and partial resistance to inactivation was seen at a 10-fold higher concentration of NEM (lanes 5 and 6). In other words, the form of the protein that contains the [4Fe-4S] cluster is resistant to NEM inactivation of RNA binding. This could either reflect the direct protection of one or more target cysteines by cluster ligation or represent a more indirect conformational effect of the cluster that prevents alkylation of the critical cysteine(s). In order to test these possibilities, we turned to site-directed mutagenesis of the cysteines predicted to ligate the cluster in the IRE-BP.

\textit{Mutagenesis of the Cluster Cysteines—}A cunbe [4Fe-4S] cluster must be present in mitochondrial aconitase and the IRE-BP in order to measure aconitase activity (Beinert, 1990; Beinert and Kennedy, 1989; Kennedy et al., 1992), and, based upon alignment of the IRE-BP sequence to porcine mitochondrial aconitase (Rouault et al., 1991; Hentze and Argos, 1991), 3 cysteine residues (Cys-437, Cys-503, and Cys-506) are predicted to coordinate this cluster. The presence of the predicted [4Fe-4S] cluster in the IRE-BP has been confirmed by spectroscopy (Kennedy et al., 1992). Each of these cysteines was individually mutated to serine (C437S, C503S, and C506S) and expressed transiently in murine fibroblasts. Additionally, a double mutant (C503S/C506S) and a triple mutant (C437S/C503S/C506S) were constructed and expressed. Following an iron-loading procedure, which has been shown to activate both mitochondrial aconitase and the IRE-BP (Haile et al., 1992a; Kennedy et al., 1993), none of these mutants developed aconitase activity, whereas wild type IRE-BP demonstrated good aconitase activity. Mutation of a nearby cysteine residue not predicted to ligate the cluster, Cys-300, resulted in no loss of aconitase activity (data not shown). These data support the model that Cys-437, Cys-503, and Cys-506 coordinate the Fe-S cluster in the IRE-BP and that loss of a single ligating cysteine residue results in an inability to incorporate a [4Fe-4S] cluster and therefore in a loss of aconitase activity. All of the cysteine mutants bound RNA with a specificity and affinity comparable to active IRE-BP isolated from the cytosol of human cells.

We next asked whether any of the cysteine mutations rendered the RNA-binding form of the protein resistant to either NEM or diamide. The effect of NEM on wild type IRE-BP and cysteine mutants C437S, C503S, C506S, C503S/C506S, and C437S/C503S/C506S is shown in Fig. 2. Treatment with 0.1

**Figure 1.** Gel retardation assay of NEM and diamide treated lysates from desferrioxamine- and hemin-treated cells. Murine fibroblast (B6) cells were treated with 100 µM desferrioxamine (a) or hemin (b) for 16 h and cytosolic lysates prepared as described. Lysates were treated with 0.1 or 1.0 mM NEM for 30 min or with 1.0 mM diamide for 30 min, then subjected to gel electrophoresis after addition of radiolabeled IRE. 2-ME was added to 2% where indicated prior to the addition of IRE. The specific IRE-IRE-BP complex is indicated by the arrow.

**Figure 2.** Treatment of recombinant mutant IRE-BP with NEM. Lysates from cells transiently transfected with cysteine mutants were treated with 0.1, 0.1, or 1.0 mM NEM and subjected to the gel retardation assay as described under "Materials and Methods." 2-ME was added to each lane at 1% prior to the addition of labeled IRE. C503,6S, C503S/C506S, 3CS, C437S/C503S/C506S.
Cysteine 437 Is Not Required for RNA Binding—The retention of full RNA binding activity in the C437S mutant rules out any absolute requirement of the thiol group for interaction with RNA. On the other hand, because three distinct modifications (NEM alkylation, diamide modification, and cluster assembly) of this specific cysteine block the interaction with RNA, Cys-437 is likely very close to the RNA binding site. To address this point in greater detail, we asked whether a smaller modifying group might fail to block RNA binding. We therefore treated the endogenous protein with iodoacetamide (IAM). This alkylating agent adds a much smaller group (M, 57) than does NEM (M, 126). Indeed, IAM treatment has no effect on RNA binding activity (Fig. 4, lanes 5 and 6). Pretreatment with IAM, however, protects the IRE-BP from NEM inactivation (lanes 7 and 8). This can be explained if the alkylation of Cys-437 by IAM fails to interfere with RNA binding, perhaps because IAM adds a less bulky group than does NEM.

Although the IRE-BP contains 9 cysteinyl residues, only the 3 which are predicted to ligate the Fe-S cluster are conserved between the IRE-BP and mitochondrial aconitases (Rouault et al., 1992). Because the IRE-BP is known to contain at least one reactive cysteine residue whose modification regulates RNA binding in vitro, and because in vivo and in vitro alterations within the active site cleft result in changes in RNA binding affinity, these cysteine residues were felt to be good candidates for the targets of in vitro modifications that change RNA binding affinity. Our data demonstrate that Cys-437 is the primary target for inactivation of RNA binding by NEM or diamide and suggest that the protein is protected from NEM inactivation when a cluster is present. We can further suggest an explanation for the ability of high concentrations of reducing agent to activate RNA binding, even in the presence of a cluster (Haile et al., 1992a). If the iron-sulfur bond at residue 437 is the site of the reversible reduction, then reduction may free Cys-437 from the cluster and alleviate the steric block to RNA binding. The cluster would presumably remain bound to the protein via the two remaining covalent bonds with cysteines 503 and 506, and subsequent removal of the high levels of reducing agent would allow the cluster to re-establish the ligation with cysteine 437.

We have previously proposed a model in which the cluster inhibits RNA binding because of interference with the RNA binding site at or near the active site cleft (Klausner et al., 1993, Klausner and Rouault, 1993). While more direct structural studies will be required to define precisely the RNA-protein contact residues, the cysteine mutagenesis studies reported here are fully consistent with this model. The simplest interpretation of the effects of NEM and diamide modification is that the RNA binding site includes a region near cysteine 437, but we cannot rule out the possibility that an indirect

**Fig. 3.** Treatment of recombinant mutant IRE-BP with diamide. Lysates of cysteine mutants were treated with 0 or 10 mM diamide for 30 min then analyzed by the gel retardation assay in the absence or presence of 1% 2-ME. Abbreviations are defined in Fig. 2 legend.

**Fig. 4.** Protection by IAM of endogenous IRE-BP after treatment with NEM. Desferrioxamine lysates were treated with nothing (C), 1.0 mM NEM (NEM), 10 mM IAM (IAM), or 10 mM IAM followed by 1.0 mM IAM (IAM, IAM) for 30 min (each agent). Lysates were then desalted over a G-50 column and assayed in the absence or presence of 2% 2-ME as indicated.
conformational change caused by NEM and diamide modification results in loss of RNA binding. This indirect effect of NEM has been described in mitochondrial aconitase (Kennedy et al., 1988). The x-ray structure of mitochondrial aconitase in its active enzymatic form cannot accommodate the IRE stem-loop within the active site cleft (Robbins and Stout, 1989a, 1989b). Opening of the cleft, an alteration that would require motion around the flexible linker between protein domains 1 through 3 and 4, would allow binding of the IRE. Whether the fully ligated cluster prevents RNA binding by direct steric interference via a local conformational change or by maintaining the cleft in a “closed” state cannot be distinguished in these studies, and the different possibilities are not mutually exclusive.

REFERENCES