

Mutational analysis of the [4Fe–4S]-cluster converting iron regulatory factor from its RNA-binding form to cytoplasmic aconitase

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The control of cellular iron homeostasis involves the coordinate post-transcriptional regulation of ferritin mRNA translation and transferrin receptor mRNA stability. These regulatory events are mediated by a soluble cytoplasmic protein, iron regulatory factor (IRF), which binds specifically to mRNA hairpin structures, termed iron-responsive elements (IREs), in the respective mRNAs. IRF is modulated by variations of cellular iron levels and exists as either an apo-protein or a [4Fe–4S]-cluster protein. The two conformations show distinct, mutually exclusive functions. High-affinity IRE binding is observed with the apo-form induced by iron deprivation, but is lost under high iron conditions when IRF is converted to the [4Fe–4S]-cluster form which shows cytoplasmic aconitase activity. Moreover, IRE binding is inactivated by the sulfhydryl-oxidizing agent diamide and fully activated *in vitro* by 2% 2-mercaptoethanol, whereas alkylation of IRF inhibits IRE binding. In the present study, we analyzed each of the above features using site-directed mutants of recombinant human IRF. The results support the bifunctional nature of IRF. We conclude that cysteines 437, 503 and 506 anchor the [4Fe–4S]-cluster, and are essential to the aconitase activity. Mutagenesis changing any of the cysteines to serine leads to constitutive RNA binding in 0.02% 2-mercaptoethanol. Cysteine 437 is particularly critical to the RNA–protein interaction. The spontaneous or diamide-induced formation of disulfide bonds between cysteines 437 and 503 or 437 and 506, in apo-IRF, as well as its alkylation by *N*-ethylmaleimide, inhibit binding to the IRE. Unlike wild-type human IRF, the cysteine mutants are not regulated in their IRE-binding activity by cellular iron levels in transiently transfected mouse L cells. Thus, our data provide direct evidence for the role of the [4Fe–4S]-cluster insertion in modulating the RNA–protein interaction of this unique post-transcriptional regulatory system and explain the *in vitro* effects on IRE–IRF binding by oxidation/reduction of cysteines. **Key words:** aconitase/Fe–S cluster/iron-responsive element/post-transcriptional regulation/RNA–protein interactions

Introduction

The coordinate control of cellular iron uptake, storage and utilization is among the best documented examples of post-transcriptional regulation of gene expression. It involves

high-affinity RNA–protein interactions in the cytoplasm (Kühn and Hentze, 1992; Leibold and Guo, 1992; Klausner *et al.*, 1993). A soluble 98 kDa protein, iron regulatory factor (IRF), also called iron-responsive element-binding protein (IRE-BP), binds to specific RNA hairpin structures known as iron-responsive elements (IREs) (Leibold *et al.*, 1988; Rouault *et al.*, 1988; Koeller *et al.*, 1989; Müllner *et al.*, 1989). The IRE consists of a stable stem ~10 nucleotides long, which is interrupted by an unpaired C positioned five nucleotides 5' of the loop. The loop consists invariably of six nucleotides with the conserved sequence CAGUGN. IREs are present in several mRNA species coding for key proteins in iron metabolism. Ferritin H- and L-chain (Aziz and Munro, 1987; Hentze *et al.*, 1987), erythroid 5-aminolevulinic acid synthase (May *et al.*, 1990; Cox *et al.*, 1991; Dandekar *et al.*, 1991) and mitochondrial aconitase mRNAs (Dandekar *et al.*, 1991; Zheng *et al.*, 1992b) all carry an IRE in their 5' untranslated region (UTR) and are inhibited in translation by the binding of IRF (Walden *et al.*, 1989; Goossen *et al.*, 1990; Bhasker *et al.*, 1993; Melefors *et al.*, 1993). In contrast, transferrin receptor mRNA, which contains five highly related IREs in its 3' UTR (Casey *et al.*, 1988), is stabilized by the IRE–IRF interaction, and therefore receptor synthesis is enhanced (Müllner and Kühn, 1988; Müllner *et al.*, 1989). High-affinity IRE binding by IRF is induced only under low-iron conditions and is inactivated by high cellular iron levels (Haile *et al.*, 1989; Müllner *et al.*, 1989). The factor is therefore considered to sense a free cellular iron pool and to exert its effects on RNA accordingly. Thus, cells growing in low-iron medium show diminished iron storage in ferritin and an induction of transferrin receptor synthesis, thereby enhancing iron uptake via receptor-mediated endocytosis of serum transferrin. These coordinate regulatory mechanisms tend to compensate cellular iron deprivation. At high iron, the regulation is accordingly inversed. It can therefore be considered as a feedback in which iron controls its own cellular homeostasis (Müllner *et al.*, 1989).

Evidence based on the sequence homology of IRF with mitochondrial and bacterial aconitases (Zheng *et al.*, 1990; Hentze and Argos, 1991; Rouault *et al.*, 1991; Prodromou *et al.*, 1992) and computer modeling of IRF based on the crystal structure of mitochondrial aconitase (Robbins and Stout, 1989; Rouault *et al.*, 1991) has led to the discovery that IRF can adopt the form of a cytoplasmic aconitase at the expense of its RNA-binding properties (Kaptain *et al.*, 1991). First documented by *in vitro* conversion of apo-IRF to a [4Fe–4S]-cluster-containing enzyme with aconitase activity (Constable *et al.*, 1992; Haile *et al.*, 1992a,b; Emery-Goodman *et al.*, 1993), it has subsequently been shown that isolated cytoplasmic aconitase is identical to the holo-form of IRF (Kennedy *et al.*, 1992). Therefore, the inactivation of RNA binding by iron *in vivo* most likely correlates with the insertion or completion of a [4Fe–4S]-cluster. It was postulated that this insertion induces a con-

formational change such that the IRE-binding site on IRF becomes inaccessible to the RNA (Emery-Goodman *et al.*, 1993; Klausner *et al.*, 1993). This model accommodates well the dual function of IRF, but so far has not revealed the site of the RNA–protein interaction. Moreover, the model has not given a satisfactory explanation of previous data indicating that the IRE-binding site is inactivated by the sulfhydryl-oxidizing agent azodicarboxylic acid bis[*dimethylamide*] (diamide) and fully activated by reduction with 2% 2-mercaptoethanol (Hentze *et al.*, 1989; Neupert *et al.*, 1990). In particular, the identification of redox-sensitive sites in IRF and their relative importance versus the [4Fe–4S]-cluster with respect to modulating RNA-binding properties have remained unsolved issues.

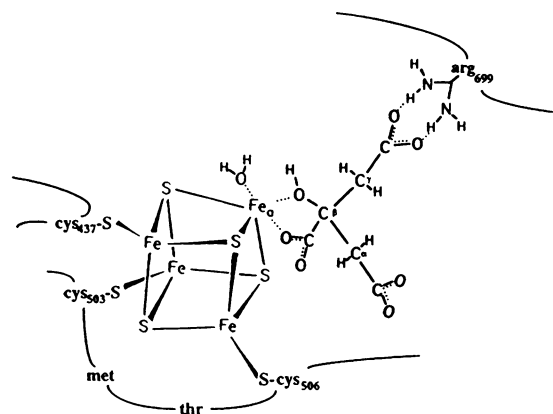
In the present study, we investigated how the insertion of the [4Fe–4S]-cluster and redox reagents modify the structure of the regulatory protein, such that its interaction with RNA is modulated. In order to address this question, we mutated specific amino acids and analyzed their importance to the acotinase and IRE-binding activities of IRF, as well as the response to 2-mercaptoethanol and diamide. The cysteines which coordinate the iron–sulfur cluster and an arginine involved in the substrate–enzyme interaction were replaced by site-directed mutagenesis. Mutants were analyzed for their ability to respond to cellular iron levels in transiently transfected L cells. Alternatively, mutant proteins were synthesized in a bacterial expression system or by coupled transcription–translation prior to *in vitro* assays with diamide or 2-mercaptoethanol. The results confirm the direct involvement of the iron–sulfur cluster in the switching of IRF between its RNA-binding and enzymatic forms. In addition, our data explain the effects of reduction or oxidation on this RNA–protein interaction. Finally, we are able to locate part of the RNA-binding site of IRF.

Results

Mutagenesis of [4Fe–4S]-cluster coordinating cysteines prevents the formation of cytoplasmic aconitase

The questions concerning the regulation of IRF by a [4Fe–4S]-cluster, redox reagents and alkylation of free thiols prompted us to mutate the three specific cysteines 437, 503 and 506 in IRF. They are thought to anchor the [4Fe–4S]-cluster based on comparison with mitochondrial aconitase (Robbins and Stout, 1989; Rouault *et al.*, 1991). Site-directed mutations were introduced into the full-length human IRF cDNA (Hirling *et al.*, 1992) by changing the cysteines to serines either singly or pair-wise (Figure 1). Furthermore, to analyze the effect of substrate–enzyme interactions on RNA binding, arginine 699, which is homologous to the citrate binding arginine 580 in mitochondrial aconitase (Zheng *et al.*, 1992a), was modified to lysine. Each of the mutant cDNAs was reconstructed in the bacterial expression vector pGEX-2T (Smith and Johnson, 1988) to enable isolation of the recombinant human IRF by the NH₂-terminal glutathione-*S*-transferase tag.

To test aconitase activity, wild-type and mutant IRF fusion proteins were purified over a glutathione–Sepharose column. At least two independent clones for each mutant were analyzed by measuring spectrophotometrically the disappearance of *cis*-aconitate at 240 nm (Table I). The IRF-wt showed a specific aconitase activity which was close to the values reported for mitochondrial aconitase (Zheng



| name | mutation |
|--------------|----------------------------------|
| IRF-wt | wildtype |
| IRF-S437 | cys 437→ser 437 |
| IRF-S503 | cys 503→ser 503 |
| IRF-S506 | cys 506→ser 506 |
| IRF-S503/506 | cys 503→ser 503, cys 506→ser 506 |
| IRF-K699 | arg 699→lys 699 |

Fig. 1. Schematic representation of the iron–sulfur cluster in IRF. The model is based on the crystal structure and substrate–enzyme interaction of mitochondrial aconitase (Beinert and Kennedy, 1989; Robbins and Stout, 1989; Zheng *et al.*, 1992a). The iron–sulfur cluster coordinating cysteines 437, 503 and 506, as well as arginine 699 involved in substrate binding, are conserved in IRF and therefore are likely to play a role in its cytoplasmic aconitase activity (Rouault *et al.*, 1991; Zheng *et al.*, 1992a). They were mutated by site-directed mutagenesis as indicated in the list of mutants.

Table I. Aconitase activity of wild-type and mutant IRF

| Mutant | Aconitase activity ^a |
|---------------|---------------------------------|
| IRF-wt | 0.36 ± 0.02 (4) |
| IRF-S437 | <0.003 (4) |
| IRF-S503 | <0.003 (4) |
| IRF-S506 | <0.003 (4) |
| IRF-S503/S506 | <0.003 (4) |
| IRF-K699 | 0.18 ± 0.014 (5) |

Recombinant human IRF with a glutathione-*S*-transferase tag was prepared from both wild-type and mutant cDNA constructs expressed in bacteria. The protein was isolated on a glutathione–Sepharose column and analyzed for aconitase activity. For each mutant, two independent isolates were measured at least twice.

^aThe specific activity is expressed as nmol *cis*-aconitate converted/s·μg IRF at 37°C. The detection limit of the assay was at 3 pmol *cis*-aconitate converted/s·μg IRF.

et al., 1992a). This suggests that most of the recombinant IRF-wt contained the [4Fe–4S]-cluster. In contrast, none of the preparations with similar amounts of protein carrying a cysteine mutation had detectable activity. Moreover, no aconitase activity could be measured after an attempt to reconstitute the iron–sulfur cluster *in vitro* by exposing recombinant mutant protein under anaerobic conditions to FeSO₄, Na₂S and dithiothreitol (Emery-Goodman *et al.*, 1993). Apparently, mutagenesis of single cysteine residues prevented insertion of the cluster into IRF.

In contrast to the cysteine mutants, the IRF-K699 mutant showed aconitase activity, although at a significantly reduced

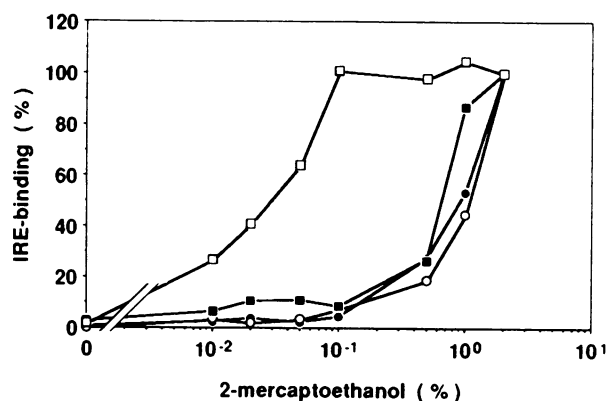


Fig. 2. *In vitro* activation of IRE binding by 2-mercaptoethanol measured after pre-incubation of wild-type IRF with various chemical agents. Purified recombinant human IRF-wt (100 ng) expressed in bacteria was incubated for 30 min at 23°C with either 10 mM $K_3Fe(CN)_6$ /0.1 mM EDTA, or 2% 2-mercaptoethanol, or 1.5 mM diamide/0.1 mM EDTA. IRF was then separated from low molecular weight substances by a Bio-Gel P-6DG spin column. Aliquots (1 ng) of the pre-treated samples were incubated with various concentrations of 2-mercaptoethanol and analyzed by the RNA gel-retardation assay using radiolabeled ferritin H-chain IRE as a probe. Autoradiograms were quantitated by densitometry and values (means of three experiments) expressed as the percentage of activation obtained with 2% 2-mercaptoethanol. ●, IRF untreated; ○, IRF pre-incubated with 2-mercaptoethanol; ■, with 1.5 mM diamide/EDTA; □, with $K_3Fe(CN)_6$ /EDTA.

rate (Table I). The K_m for *cis*-aconitate was 2.5-fold higher than that of wild-type and the V_{max} for its conversion to isocitrate two-fold reduced. Thus, the effect of this mutation was less pronounced than that of the one at arginine 580 of mitochondrial aconitase, where the K_m is affected 30-fold (Zheng *et al.*, 1992a). Conditions that reconstitute [Fe-S]-clusters increased the aconitase activity of IRF-wt and IRF-K699 by 10%.

Detecting the presence of a [4Fe-4S]-cluster in IRF

The above results are compatible with the idea that the aconitase activity of IRF depends on the proper formation of a [4Fe-4S]-cluster attached to cysteines 437, 503 and 506. However, it cannot be concluded that inhibition of aconitase activity is in every case due to a missing [4Fe-4S]-cluster. In order to define an independent criterion for the presence or absence of the cluster, we analyzed recombinant IRF-wt with or without its [4Fe-4S]-cluster. For this purpose, IRF-wt with significant aconitase activity (Table I), and hence largely loaded with its [4Fe-4S]-cluster, was pre-incubated either with $K_3Fe(CN)_6$ which is known to destroy the iron-sulfur cluster (Kennedy and Beinert, 1988; Haile *et al.*, 1992b), or with 2-mercaptoethanol which activates IRE binding *in vitro*, or with the thiol-oxidizing agent diamide which inhibits IRE binding. The protein was then separated from the reagents by gel filtration and analyzed for aconitase activity. We found that the aconitase activity was entirely lost after incubation with 10 mM $K_3Fe(CN)_6$, whereas only 3% of the activity was lost after 2% 2-mercaptoethanol treatment and 20% after incubation with 1.5 mM diamide. Similar data have been reported for mitochondrial aconitase where 0.1 mM $Fe(CN)_6^{3-}$ in the presence of EDTA converts the [4Fe-4S]-cluster into a [3Fe-4S]-cluster without aconitase activity, while high concentrations are

known to remove the cluster (Kennedy and Beinert, 1988).

To test whether the loss of the [4Fe-4S]-cluster activates IRE binding, we analyzed the pre-treated IRF-wt samples described above by RNA gel-retardation assays. Aliquots were incubated with various concentrations of 2-mercaptoethanol and then tested with radiolabeled human ferritin H-chain IRE probe (Figure 2). RNA-protein complexes were resolved on non-denaturing polyacrylamide gels. Quantitation of autoradiograms showed that 2-mercaptoethanol- or diamide-pre-treated IRF was inactive after gel filtration, but could be totally reactivated by 2% 2-mercaptoethanol. In contrast, IRF-wt pre-incubated with 10 mM $K_3Fe(CN)_6$ was half-maximally reactivated by 0.02% 2-mercaptoethanol, a concentration that is 50- to 100-fold lower than that required to reactivate untreated IRF-wt (Figure 2). These results confirm the observation that $Fe(CN)_6^{3-}$ alters the [4Fe-4S]-cluster permanently (Haile *et al.*, 1992b), and indicate that the effects of 2-mercaptoethanol or diamide are reversible.

Our data indicate that conditions which convert IRF into the apo-form by removing the [4Fe-4S]-cluster, such as treatment with high $Fe(CN)_6^{3-}$ concentrations, produce a conformational change which facilitates the *in vitro* activation of IRE binding by 2-mercaptoethanol. In contrast, in the presence of the cluster a high 2-mercaptoethanol concentration is required for activation. This difference can therefore serve to distinguish between the presence and absence of the cluster. All IRF mutants were subsequently analyzed with respect to their response to 2-mercaptoethanol.

Reduction of apo-IRF is required for IRE binding

The cysteine mutants which are apparently unable to insert a [4Fe-4S]-cluster should bind IREs constitutively. In order to test this hypothesis, we analyzed recombinant wild-type and mutant IRFs for their binding to a ferritin IRE *in vitro* (Figure 3). In the absence of 2-mercaptoethanol, only the recombinant mutant IRF-S437 showed strong RNA binding, suggesting that in this case the prediction was correct. However, for IRF-S503 and IRF-S506, the results were not anticipated, as both mutants were inactive. Therefore, each mutant was re-analyzed for its sensitivity to 2-mercaptoethanol-induced IRE binding (Figure 3). IRF-wt became half-maximally activated at ~1% 2-mercaptoethanol, as described previously (Hentze *et al.*, 1989; Rothenberger *et al.*, 1990). An almost identical result was obtained for the mutant IRF-K699. In contrast, mutants IRF-S503 and IRF-S506 required only a small amount of 2-mercaptoethanol in order to gain activity. Their half-maximal response occurred at ~0.02% 2-mercaptoethanol. This feature, previously observed for cyanolized wild-type IRF (Figure 2; Haile *et al.*, 1992b), is reminiscent of apo-IRF. It indicates that mutants IRF-S503 and IRF-S506 are also apo-proteins like mutant IRF-S437. The differences between mutants in their response to 2-mercaptoethanol can therefore not be attributed to the presence or absence of a [4Fe-4S]-cluster. Instead, it seemed rather that cysteines in IRF-S503 or IRF-S506 might be disulfide linked to a nearby cysteine (Figure 1). Computer predictions based on the structure of mitochondrial aconitase (Robbins and Stout, 1989) indicated cysteine 437 as the only likely partner for the remaining cysteines 506 and 503, respectively. We therefore decided to make the double mutant IRF-S503/506 (Figure 1). This mutant, similar to IRF-S437, was >60% active in the

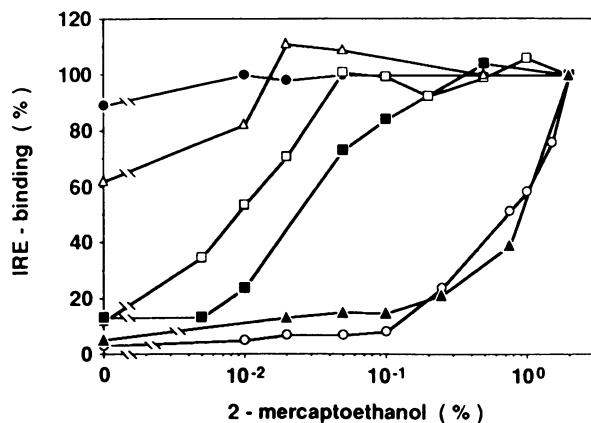


Fig. 3. *In vitro* induction of the IRE-binding activity by 2-mercaptoethanol. The IRE-binding activities of wild-type or mutant recombinant IRF were measured in crude extracts from bacteria (1 μ g protein) by the gel-retardation assay using human ferritin H-chain IRE (Müllner *et al.*, 1989), either in the absence or 2-mercaptoethanol or after 10 min incubation with increasing concentrations of 2-mercaptoethanol. Autoradiograms of IRE-IRF complexes were scanned densitometrically and plotted as a percent of the value obtained with 2% 2-mercaptoethanol (mean of three experiments). \circ , IRF-wt; \bullet , IRF-S437; \blacksquare , IRF-S503; \square , IRF-S506; \triangle , IRF-S503/506; \blacktriangle , IRF-K699.

absence of 2-mercaptoethanol, and was activated to 100% *in vitro* by very low concentrations of reductant (Figure 3). This result is best explained by the absence in IRF-S503/506 of a partner with which cysteine 437 might form a disulfide bridge.

Inhibition of RNA binding induced by diamide involves cysteine 437

Binding of IRF to IREs is inhibited by the sulfhydryl-oxidizing agent diamide (Hentze *et al.*, 1989; Neupert *et al.*, 1990) which catalyzes disulfide bridging between thiol groups (Kosower and Kosower, 1987). Based on the above results, it seemed likely that this inhibition involves cysteines 437, 503 or 506. In order to test how thiol oxidation affects the cysteine mutants as compared to IRF-wt, we transcribed the different cDNAs *in vitro* and translated the mRNAs in wheat germ extracts. We have previously shown that this system synthesizes reduced apo-IRF, which is fully active in IRE binding and can be inhibited by treatment with diamide (Hirling *et al.*, 1992). Translation products were then incubated with the radiolabeled IRE probe and analyzed in gel-retardation assays. As shown in Figure 4, freshly translated IRF-wt showed properties of a reduced apo-IRF and bound the IRE to the same extent in the absence and presence of 2-mercaptoethanol (Hirling *et al.*, 1992). However, the RNA-binding activity was readily inhibited by incubation with 1.5 mM diamide, and only recovered after incubation with 2-mercaptoethanol. As IRF-wt, all three cysteine mutants were translated as fully active IRE-binding proteins. Pre-incubation of the mutants IRF-S503 and IRF-S506 with 1.5 mM diamide abolished IRE binding to the same extent as in IRF-wt, and this inactivation was reversed by 2% 2-mercaptoethanol. In contrast, IRF-S437 did not respond to oxidation with diamide. This suggests that disulfide bridging of cysteine 437 is involved in the inhibition of IRE-binding. Indeed, in contrast to the single-site mutants at cysteines 503 or 506, the double mutation at both residues (mutant IRF-S503/506) prevented inactivation by diamide.

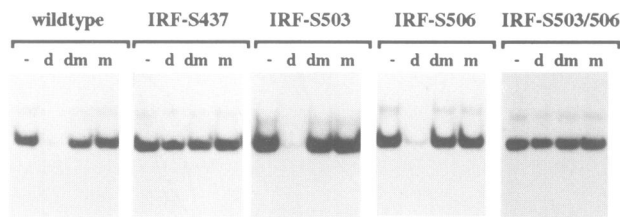


Fig. 4. Response of wild-type and mutant IRF to oxidation by diamide. IRF RNA was transcribed *in vitro* with T7 polymerase and then translated in the wheat germ system. Synthesized IRF was analyzed by gel-retardation assay with a radiolabeled ferritin H-chain IRE probe (Müllner *et al.*, 1989). These assays were performed either without treatment of the samples (-), after pre-incubation for 10 min on ice with 1.5 mM diamide (d), after diamide pre-incubation followed by reduction with 2% 2-mercaptoethanol (dm), or in the presence of 2% 2-mercaptoethanol without diamide pretreatment (m). Note that *in vitro* translated IRF binds IRE to the same extent in the absence or presence of 2-mercaptoethanol, suggesting that IRF is initially made in its reduced apo-protein conformation. One experiment out of three, all of which gave similar results, is shown.

Therefore, oxidation of IRF by diamide requires cysteine 437 and either one of cysteines 503 or 506. This is a strong indication that diamide facilitates disulfide bonds of cysteine 437 to either 503 or 506 and thereby inhibits the interaction with the IRE.

An identical inhibition with diamide could also be observed with recombinant wild-type apo-IRF prepared from bacterial extracts after exposure to 10 mM $K_3Fe(CN)_6$, 0.1 mM EDTA, followed by 0.1% 2-mercaptoethanol (data not shown).

Alkylation of cysteine 437 by *N*-ethylmaleimide inhibits the IRE-IRF interaction

In order to establish whether cysteine 437 plays a role in the RNA-protein interaction, we decided to test the effect of alkylation by *N*-ethylmaleimide. This alkylating agent has previously been found to abolish the IRE-IRF interaction (Hentze *et al.*, 1989). IRF-wt and the three cysteine mutants made as glutathione-*S*-transferase-tagged recombinant proteins were incubated with various concentrations of *N*-ethylmaleimide. Figure 5A shows that all the mutants except IRF-S437 are readily inhibited by the alkylating agent at <100 μ M. It must be concluded that alkylation at cysteine 437 is responsible for the loss of IRF-binding activity by alkylation. Replacement of this amino acid by serine prevents the effect fully, at least at *N*-ethylmaleimide concentrations sufficient to inhibit IRF-wt and the other mutants. These results strongly suggest that the close environment of cysteine 437 constitutes one of the targets for the RNA-protein interaction.

Since serine at position 437 permits a strong constitutive RNA-protein interaction (Figure 3), it is not the cysteine *per se* which is recognized by the RNA. Thus, *N*-ethylmaleimide might affect RNA binding through steric hindrance or protein backbone distortion rather than the modification of a free sulfhydryl. In order to elucidate this point further, IRF-wt was alkylated with 5 mM iodoacetamide (Figure 5B). Interestingly, this alkylating agent was unable to block the RNA-protein interaction. To prove that IRF had been alkylated in the assay, we also incubated the protein sequentially with iodoacetamide and *N*-ethylmaleimide. Under these conditions *N*-ethylmaleimide did not show the usual inhibitory effect (compare lanes 2 and 4 of

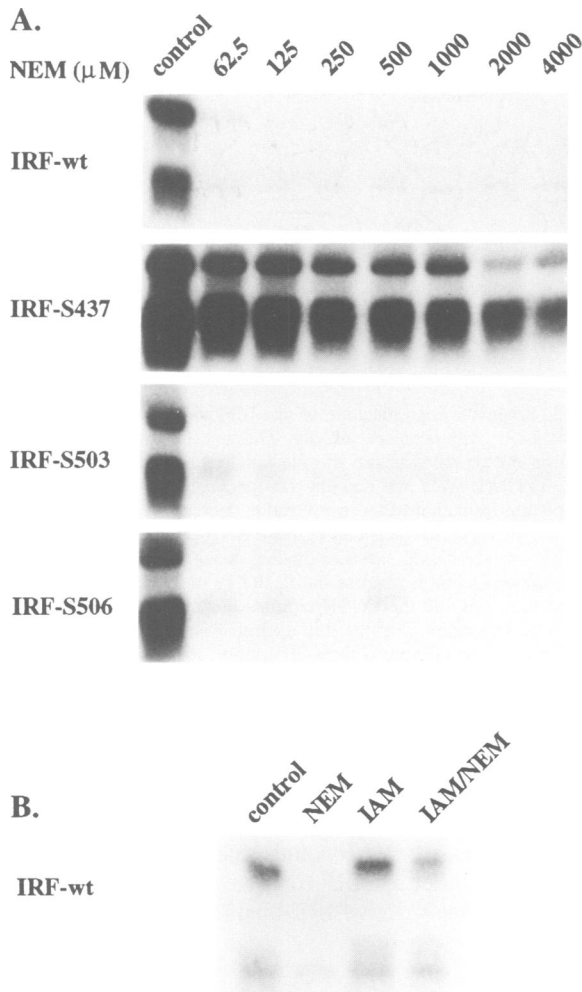


Fig. 5. Inhibition of IRE binding by alkylation. (A) Recombinant IRF-wt and mutants expressed in bacteria (1 or 2 μg protein) were incubated with various concentrations of *N*-ethylmaleimide prior to the gel-retardation assay using the ferritin H-chain IRE. Two RNA-protein complexes were observed in each case. The lower one corresponds to glutathione-*S*-transferase-tagged IRF complexed to an IRE. The upper complex was always proportional in quantity. It contains the same components and may represent a dimer. (B) Recombinant IRF-wt was incubated for 30 min at 23°C either with 1 mM *N*-ethylmaleimide (NEM), or 5 mM iodoacetamide (IAM) alone or followed by *N*-ethylmaleimide (IAM/NEM). The gel-retardation assay was performed as above. One of three experiments with the same results is shown.

Figure 5B), indicating that the cysteines had indeed been occupied by acetamide. The results favor the hypothesis of steric hindrance by *N*-ethylmaleimide.

The substrate *cis*-aconitate inhibits *in vitro* activation of IRF by 2-mercaptoethanol

When analyzed for its response to 2-mercaptoethanol, IRF-K699 showed a response curve identical to that of IRF-wt (Figure 3), indicating that this mutant contains a fully assembled [4Fe-4S]-cluster. The reduced aconitase activity in IRF-K699 is therefore most likely due to a poorer interaction with the substrate rather than to absence of the cluster. It was of interest to analyze this mutant further in order to confirm a finding recently reported by Haile *et al.* (1992b). These authors suggested that aconitase substrate can inhibit the activation of IRF by 2-mercaptoethanol, presumably by

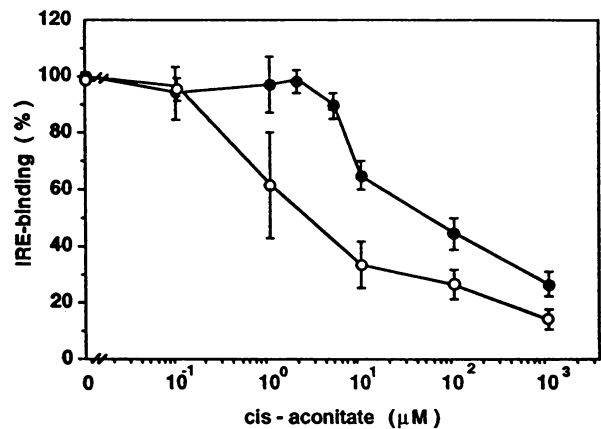


Fig. 6. Conversion by 2-mercaptoethanol of the [4Fe-4S]-cluster containing cytoplasmic aconitase to an IRE-binding form depends on the substrate concentration. Wild-type IRF (○) or the IRF-K699 mutant (●) were expressed in bacteria, and total extracts (1 μg) incubated with various concentrations of *cis*-aconitase immediately before adding the probe in 2% 2-mercaptoethanol. IRE binding was analyzed by the gel retardation assay and quantitated by densitometric scanning of autoradiograms. Results are expressed in percent IRE binding as compared to the fully activated IRF (in 2% 2-mercaptoethanol) without *cis*-aconitase. Error bars indicate the standard deviation obtained in three experiments.

protecting the [4Fe-4S]-cluster. In order to analyze this point, we examined the activation of IRF-wt and IRF-K699 by 2% mercaptoethanol in the presence of different concentrations of *cis*-aconitase (Figure 6). With IRF-wt, substrate concentrations of $\sim 1 \mu\text{M}$ prevented *in vitro* activation by 2% 2-mercaptoethanol to at least 50%. In contrast, with mutant IRF-K699, an ~ 30 -fold higher concentration of substrate was required to observe the same inhibition. This indicates that the mutant has indeed a lower affinity for *cis*-aconitase than wild-type IRF. The results are in line with the idea that bound substrate prevents activation by 2-mercaptoethanol.

Cysteine mutants of human IRF are not regulated by cellular iron levels in transfected L cells

In order to test in living cells whether [4Fe-4S]-cluster insertion was required for regulation of the IRE-binding activity by iron levels, we expressed wild-type and mutant hIRF in transfected mouse Ltk⁻ cells. Stable transfection with plasmid carrying the hIRF cDNA behind an SV-40 late promoter showed good expression of the human wild-type protein in L cells (Figure 7A). Upon modulation of iron levels, IRE binding of hIRF was apparently induced as for the endogenous mouse IRF. A difficulty arose, however, in the interpretation of these data, since mouse extracts contain a second IRE-binding protein (mIRF_B; Henderson *et al.*, 1993) which forms an RNA-protein complex with H-chain ferritin IRE that co-migrates with the complex of human IRF (Figure 7A; Rothenberger *et al.*, 1990). Recently, we have found a solution to this problem by selecting an IRE variant that binds well to IRF, but does not recognize mIRF_B. This IRF-specific probe was designated IRE mut.1 and has an IRE structure identical to the ferritin IRE, but a loop sequence 5'-GAGAGU-3' instead of 5'-CAGUGC-3'. As seen by comparison of the left and right panel in Figure 7A, the IRE mut.1 probe measures faithfully endogenous mouse and transfected human IRE-binding activities, but fails to reveal the faster

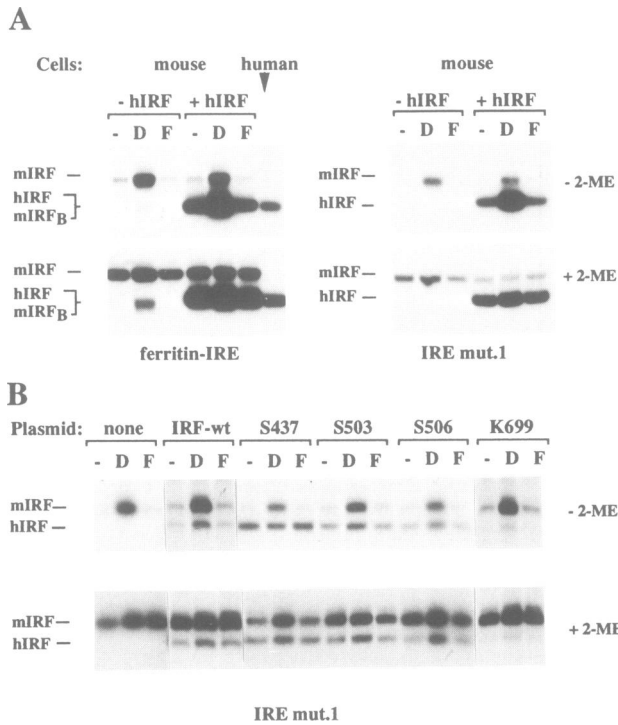


Fig. 7. IRE-binding activity of wild-type and mutant human IRF in transfected mouse L cells. Gel-retardation assays were performed with cytoplasmic extracts from stably (panel A) or transiently transfected Ltk⁻ cells (panel B). Extracts were made from cells grown in either normal culture medium (-), iron-deprived medium with 50 μM desferrioxamine (D), or medium containing 20 μg/ml ferric ammonium citrate (F). **Panel A** depicts gel-retardation complexes obtained with extracts from human cells, non-transfected mouse Ltk⁻ cells (-hIRF) or a stably transfected L cell population expressing human wild-type IRF (+hIRF). On the left panel, RNA-protein complexes were formed with a ³²P-labeled human H-chain ferritin IRE, whereas on the right panel, the same extracts were analyzed with an IRF-specific mutant IRE (IRE mut.1) that does not form complexes with the fast-migrating IRE-binding protein of mouse cells (mIRF_B). In **panel B**, plasmids encoding human wild-type or mutant IRFs were transiently transfected into Ltk⁻ cells. Extracts were again analyzed with the IRE mut.1 probe which detects both the endogenous mouse IRF (mIRF) and the additional human IRF (hIRF) expressed from transfected plasmid DNA. Several stably transfected cell populations and at least two transient transfection assays gave similar results.

migrating mouse IRF_B band visible in a gel-retardation assay with non-transfected L cells.

The results show that IRE binding of human IRF in transfected mouse cells is regulated by iron levels to the same extent as that of endogenous IRF (Figure 7A). Although this experiment indicated a way to analyze IRF mutants by the stable transfection procedure, we failed in several attempts to obtain such transfectants for the cysteine mutants. The reason for this remains unexplained. We therefore transfected plasmid DNA transiently rather than stably, and analyzed human IRE-binding activity 48–60 h after transfection by taking advantage of the IRE mut.1 (Figure 7B). IRE-binding activity was quantified for each sample relative to endogenous mIRF after 2-mercaptoethanol activation and was clearly regulated for human wild-type IRF as a function of iron availability. A similar result was obtained for the IRF-K699 mutant which was predicted to contain an iron-sulfur cluster. In contrast, human IRF with mutations at any of the three cysteine residues showed a phenotype that was not regulated. Interestingly, the cysteine mutants were fully

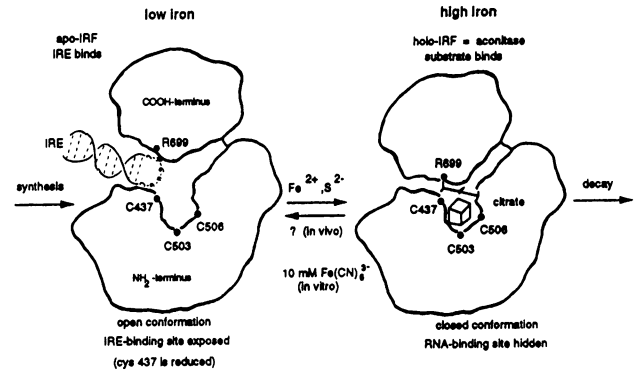


Fig. 8. Schematic representation of apo-IRF with RNA-binding activity and holo-IRF with aconitase activity. The model drawn for IRF is based on the crystal structure of mitochondrial aconitase (Robbins and Stout, 1989). It takes into account that the availability of cellular iron controls the formation of the iron-sulfur cluster. We propose that insertion of the cluster converts an open RNA-binding conformation of apo-IRF into the closed substrate-binding aconitase form. The conformational change requires the flexibility of the hinge region that connects the NH₂- to the COOH-terminal domain. Mutations preventing the insertion of the iron-sulfur cluster inhibit the maturation of the enzymatic form. Therefore, IRF remains in the open apo-protein conformation, and the IRE gains access to the cleft where it interacts with the NH₂-terminal domain near cysteine 437.

active in RNA binding immediately after lysis (compare band shifts in the absence and presence of 2-mercaptoethanol), indicating that cysteine residues were fully reduced in the cellular cytoplasm. We conclude that the inability to insert a [4Fe-4S]-cluster into IRF correlates with the accumulation of a constitutively active IRE-binding form of IRF.

Discussion

In the present study, we confirm the bifunctional nature of IRF which adopts the conformation of an IRE-binding apo-protein or a [4Fe-4S]-cluster containing holo-protein with aconitase activity (Figure 8). Our data support the model according to which the [4Fe-4S]-cluster is responsible for the iron-dependent regulation of the mRNA-IRF interactions. In addition, the results from site-directed mutagenesis lead to new conclusions concerning the structure-function relationships of this post-transcriptional regulatory factor. In particular, we are able to identify the amino acids which coordinate the [4Fe-4S]-cluster, to define the contribution of reduction/oxidation to IRF activities and to locate part of the RNA-binding site.

The amino acid sequence of IRF shows significant similarity to mitochondrial aconitase, which contains a [4Fe-4S]-cluster involved in the enzymatic conversion of citrate to isocitrate (Beinert and Kennedy, 1989; Robbins and Stout, 1989; Hentze and Argos, 1991; Rouault *et al.*, 1991). This homology includes amino acids of the active center with three cysteines holding the cluster. Based on these comparisons, it can be predicted that IRF is an aconitase with a similar three-dimensional structure. Here we show that sites known to play a role in mitochondrial aconitase activity are structurally and functionally homologous in cytoplasmic aconitase/IRF. Mutagenesis of the cysteines predicted to hold the [4Fe-4S]-cluster gives rise to molecules that have properties of apo-IRF. They lack aconitase activity and are easily converted to IRE-binding proteins by low concentrations of 2-mercaptoethanol. This

confirms the prediction that cysteines 437, 503 and 506 represent the sites of cluster attachment. Moreover, we conclude that arginine 699 in IRF is important to cytoplasmic aconitase activity, since it interacts with the substrate, as does the homologous site (arginine 580) in mitochondrial aconitase (Zheng *et al.*, 1992a). This is documented by the differential inhibition by substrate of the *in vitro* activation by 2-mercaptoethanol (Figure 6). Unlike the cysteine mutants, IRF-K699 can bind the [4Fe-4S]-cluster since high 2-mercaptoethanol concentrations are required to activate its IRE binding.

The present data resolve a long-standing issue about the involvement of reduction and oxidation in the activation or inactivation of IRF. Our experiments with the cysteine mutants demonstrate clearly that in the apo-protein conformation these cysteines can undergo either spontaneous or diamide-induced disulfide linkages. In particular, the bridging of cysteine 437 to either 503 or 506 has an inhibitory effect on the IRE-IRF interaction. Another candidate, cysteine 300, proposed as a possible intramolecular partner for the fourth iron in the [4Fe-4S]-cluster (Leibold and Guo, 1992), was also mutated, but had no influence on the RNA binding or the aconitase activity (P.Kaldy and L.C.Kühn, unpublished results). The results obtained with the double mutant IRF-S503/S506 revealed that cysteine in apo-IRF must be in its reduced state in order to allow for the binding to RNA. It implies that spontaneous or induced disulfide bridging alters the apo-protein conformation, and that a small amount of 2-mercaptoethanol is sufficient in this case to reactivate IRE binding. This agrees with data for cyanolized IRF-wt (Haile *et al.*, 1992b; and Figure 2). The concentration of reductant required to keep apo-IRF in a reduced state with RNA-binding competence is in the range of naturally occurring glutathione levels. The present data explain well the effect of 1.5 mM diamide on total cellular extracts: a fraction of the IRF molecules is in the reduced apo-protein conformation when cells are broken, and these molecules undergo intramolecular disulfide bridging upon addition of diamide (Hentze *et al.*, 1989; Neupert *et al.*, 1990). The formation of intramolecular disulfide bonds can also account for slow inactivation/oxidation observed during the purification of IRF (Neupert *et al.*, 1990). It cannot be ruled out that diamide oxidizes IRF elsewhere, but this would not have an effect on IRE binding. We also conclude that diamide has no measurable effects on holo-IRF.

The explanation concerning the *in vitro* activation of holo-IRF by high 2-mercaptoethanol concentrations is less evident. Although activation of the IRE binding by 2% 2-mercaptoethanol is clear cut, this induces no permanent destruction of the [4Fe-4S]-cluster. The treated protein is recovered as an active aconitase when 2-mercaptoethanol is washed away by gel filtration (Haile *et al.*, 1992b; and data presented here). Thus, 2-mercaptoethanol has only a transient effect on RNA binding, perhaps by modulating one of the cysteine-Fe interactions. Either the chemical reaction induced by 2-mercaptoethanol or the access of RNA to its binding site is prevented by substrate, as demonstrated with mutant IRF-K699 (Figure 6). Reasoning in terms of the results obtained for apo-IRF, it seems possible that a transient reduction of cysteine 437 might also be needed to induce high-affinity RNA-binding properties of holo-IRF. However, since substrate is generally present *in vivo* and reductant concentrations are far below those required *in vitro*, it is unlikely that reduction of holo-IRF is a mechanism that plays

a role in the cell. The fact that iron deprivation is the main activator for the post-transcriptional regulation of ferritin and transferrin receptor points rather to the apo-IRF as the naturally active RNA-binding form.

The *in vitro* results indicate that reduced apo-IRF, but not disulfide-linked apo-IRF, binds to the IRE. This is highly relevant to the definition of the RNA-binding site. It appears that cysteine 437 must be accessible and in an appropriate, reduced state for IRE binding to be observed. It is interesting to notice, however, that serine can replace cysteine at position 437 without any noticeable loss of RNA binding. It is therefore almost certain that the RNA does not undergo a covalent linkage with IRF, as originally suggested by Hentze *et al.* (1989). It seems nevertheless that cysteine 437 and/or its surroundings are critical to the RNA-protein interaction. This hypothesis is further supported by the data showing that alkylation by the bulky *N*-ethylmaleimide, but not the smaller acetamide group, is inhibitory to the IRE-IRF interaction. Among the mutants tested, only the one replacing cysteine 437 prevents the effect of *N*-ethylmaleimide, suggesting that alkylation of this site hinders sterically the RNA-protein interaction or distorts the amino acid backbone such that the IRE can no longer bind at neighboring sites. Together with the data on the inhibitory effect of disulfide bridging of cysteine 437, the evidence argues strongly in favor of a direct contact between the RNA and the NH₂-terminal globular domain of IRF at the inner surface of its cleft (Figure 8). In holo-IRF, this surface would not be exposed: the [4Fe-4S]-cluster constitutes the active site such that substrate can bridge the NH₂- and COOH-terminal domains and close the cleft, leaving no access for the IRE hairpin. In support of this view, we have previously shown that the IRE can cross-link to the NH₂-terminal domain of IRF (Hirling *et al.*, 1992). However, the COOH-terminal domain was also necessary for IRE binding (Hirling *et al.*, 1992), and further studies are needed to define precisely the contact points between the RNA and the regulatory protein.

According to our view, two conditions must be fulfilled in order to obtain an IRE-IRF interaction: (i) the absence of the iron-sulfur cluster which determines the access of the IRE to cysteine 437 or its surroundings; (ii) the free/reduced state of this cysteine (Figure 8). This model is strongly supported by the data from transient transfection of mutant constructs into mouse L cells. The *in vivo* situation correlates perfectly with the prediction from *in vitro* measurements with recombinant IRF, according to which the loss of the [4Fe-4S]-cluster or a failure in its insertion results in constitutive IRE binding. The findings also agree with previous results from our laboratory and another group showing that variation of iron in the culture medium determines the ratio between the apo- and holo-IRF (Haile *et al.*, 1992b; Emery-Goodman *et al.*, 1993). Thus, 'sensing' of iron by IRF as the explanation of post-transcriptional feedback regulation is clearly reflected in the formation of the [4Fe-4S]-cluster. The question arises whether *in vivo* this rate of cluster formation is entirely controlled by the availability of Fe²⁺ or whether it can be influenced by other factors. Moreover, it is unclear whether iron-sulfur cluster insertion into IRF is a reversible process. At the present time, several findings indicate that Fe²⁺ levels are directly limiting to iron-sulfur cluster formation. On the one hand, it is established that high iron levels induce a rapid and rather permanent inactivation of IRE binding in L cells (Müllner

et al., 1989, 1992). On the other hand, low cellular iron conditions induce the accumulation of IRE-binding apo-IRF by a slow process (taking 15 h) which is inhibited by cycloheximide in murine L cells (Müllner *et al.*, 1989). This suggests that most likely *de novo* synthesis of IRF is required in these cells to increase the IRE-binding pool. However, protein synthesis inhibition has remained without effect on the appearance of IRE-binding activity in other cell lines (Tang *et al.*, 1992). Therefore we cannot formally exclude that holo-IRF might be converted into apo-IRF (Figure 8). The continuous presence of substrate and the difficulty in accessing the cluster in the aconitase are considerations which argue against a holo- to apo-IRF conversion. Recent evidence from this group and others indicates, however, that nitric oxide can induce IRE-binding activity in macrophages and other cells (Drapier *et al.*, 1993; Weiss *et al.*, 1993) and the possibility that small mediators might be capable of disrupting the cluster remains open. Another possible mechanism to control IRE binding *in vivo* might take advantage of the oxidation–reduction state (Hentze *et al.*, 1989) of the sensitive cysteine 437 in the apo-IRF. In this case, the natural reduction potential of glutathione would need to be overcome. We have previously shown that addition of diamide to iron chelator-treated L cells decreases transiently the active IRE-binding form (Müllner *et al.*, 1992). In view of our present results, this would correspond to a disulfide linkage in apo-IRF. Noticeably, the oxidation was rapidly reduced when diamide was removed from L cells (Müllner *et al.*, 1992). The results of the present study indicate that the intracellular reduction potential of glutathione is sufficient to maintain the IRF-S503 and IRF-S506 mutants in a constitutive IRE-binding conformation (Figure 7B). Thus, it can be expected that newly made apo-IRF is in the reduced and therefore competent RNA-binding conformation.

Materials and methods

Construction of IRF mutants

Point mutations in the human IRF cDNA were constructed by site-directed mutagenesis of plasmid pGEM-hIRF (Hirling *et al.*, 1992) deleted of its 3'-terminal *KpnI* fragment. According to the method of Kunkel *et al.* (1987), the plasmid was transformed into the *Escherichia coli* strain CJ236, and single-stranded DNA prepared by using the helper phage VCS13 (Stratagene, Heidelberg, Germany). Oligonucleotides (19mers) carrying specific mismatches were hybridized to the single-stranded DNA in order to introduce the following amino acid changes: Cys437 to Ser437 (TGC to AGC), Cys503 to Ser503 (TGC to AGC), Cys506 to Ser506 (TGC to AGC), Arg699 to Lys699 (AGA to AAA). The second strand was synthesized by 1 U T4 DNA polymerase (Boehringer, Mannheim, Germany) in the presence of 0.5 mM each of dATP, dCTP, dGTP and dTTP, 3 mM MgCl₂, 2 mM dithiothreitol, 1 mM ATP, 3 U T4 DNA ligase (Boehringer), 50 mM NaCl and 20 mM Tris–HCl (pH 7.6). This double-stranded DNA was retransformed into the *E. coli* strain BZ234. Single-stranded DNA was prepared from several independent colonies and the presence of desired point mutations confirmed by sequencing (Sanger *et al.*, 1977). The 3'-terminal *KpnI* fragment was then reinserted into the double-stranded DNA of mutated cDNA clones.

In vitro transcription/translation

Different plasmids containing the cDNAs for wild-type and mutant IRF forms were linearized with *HindIII* at position 3040 (Hirling *et al.*, 1992) and transcribed *in vitro* by T7 RNA polymerase (Promega, Madison, WI) as previously described (Hirling *et al.*, 1992). This RNA was translated in the wheat germ translation system (Promega) using a 25 μ l reaction mixture (Hirling *et al.*, 1992). *In vitro* translated IRF showed properties of a reduced apo-protein, presumably due to the presence of 10 mM dithiothreitol in the commercial translation mixture and the lack of iron–sulfur cluster insertion. Aliquots of the reaction were tested for the presence of IRE-binding activity by the gel retardation assay.

Expression of IRF in *E. coli*

The full-length cDNA coding for human IRF (Hirling *et al.*, 1992) was cloned into the bacterial expression vector pGEX-2T (Pharmacia, Uppsala, Sweden) in frame behind the coding region of a 26 kDa fragment of glutathione-S-transferase under the control of the IPTG-inducible tac promoter. This plasmid was transformed into the bacterial strain HB101. Induction with IPTG and purification of the expressed fusion protein by a glutathione-Sepharose CL4B column (Pharmacia) was carried out as previously described (Smith and Johnson, 1988). Bacteria were lysed either by sonication in phosphate-buffered saline (PBS)/1% Triton X-100 (large-scale preparation) or by digestion with 1 mg/ml lysozyme in 5% sucrose, 30 mM Tris–HCl (pH 8) before lysis in 0.3% Nonidet P-40 (purification from small cultures). The yield of the expression system was ~100 μ g recombinant IRF/l bacterial culture. The purification was performed under anaerobic conditions in an argon-saturated atmosphere.

Expression of human IRF in mouse L cells

Murine Ltk⁻ cells, grown in α -MEM with 10% fetal calf serum, were stably transfected with the wild-type human IRF cDNA (Hirling *et al.*, 1992) by the calcium phosphate method of Graham and van der Eb (1973) using a previously established protocol (Owen and Kühn, 1987). Precipitates contained 3 μ g hIRF-wt cDNA subcloned behind the SV-40 late promoter of pSVL (Pharmacia), 100 ng of a herpes simplex thymidine kinase-encoding plasmid and 12 μ g carrier mouse DNA. For transient expression assays, Ltk⁻ cells were exposed for 16 h to calcium phosphate precipitates of plasmid pSVL DNA (without carrier DNA) carrying either human wild-type (2.5 μ g) or mutant (15 μ g) IRF cDNA constructs. Transfected cells were split 24 h later, left 6 h to re-attach and then incubated for 16 h with normal medium or 50 μ M desferrioxamine-containing medium. One of two desferrioxamine-treated cultures was washed and further incubated for 4 h with 20 μ g/ml ferric ammonium citrate. Cytoplasmic extracts were prepared from ~10⁶ cells by lysis in 100 μ l buffer A [10 mM HEPES (pH 7.5), 40 mM KCl, 5% glycerol, 3 mM MgCl₂] with 0.3% Nonidet P-40, followed by 5 min centrifugation at 15 000 g. Protein content was measured by the Bio-Rad assay and 2 μ g of each extract analyzed by RNA gel retardation assay. Relative IRE-binding activity was quantitated by cutting slices of dried gels and counting RNA–protein complexes after addition of scintillation fluid.

RNA gel-retardation assay

In order to measure the IRE-binding activity, different forms of IRF, crude bacterial extracts, purified recombinant protein or *in vitro* translated IRF were incubated in buffer A with a molar excess of [α -³²P]CTP-labeled RNA probe corresponding to the IRE from the 5' untranslated region of human ferritin H-chain mRNA [transcript of plasmid pSPT-fer in Müllner *et al.*, 1989]. In experiments with purified IRF, the samples were in buffer A containing bovine serum albumin (BSA) (20 μ g/ml). Where indicated, radiolabeled IRE probe was added together with 2-mercaptoethanol. The incubations and separation of IRE–IRF complexes from unbound probe were carried out by non-denaturing gel electrophoresis as previously described (Rothenberger *et al.*, 1990). In some experiments, IRF was pre-incubated with either 1.5 mM azodicarboxylic acid bis[*dimethylamide*] (Sigma, St Louis, MO), known as diamide, or different concentrations of *N*-ethylmaleimide, or 5 mM iodoacetamide, or 10 mM K₃Fe(CN)₆ with 0.1 mM EDTA, or 2% 2-mercaptoethanol, or various concentrations of *cis*-aconitate as mentioned in the results. Where indicated, these reagents were removed by gel filtration over Bio-Gel P-6DG (Bio-Rad) spin columns prior to the gel-retardation assay.

Aconitase activity

Aconitase activity of wild-type and mutant forms of recombinant IRF was determined by measuring the disappearance of *cis*-aconitate at 240 nm (Drapier and Hibbs, 1986). IRF (160–760 ng) was incubated with 0.2 mM *cis*-aconitate in 50 mM Tris–HCl (pH 7.2), 100 mM NaCl, 0.02% BSA at 37°C in the cuvette of a Uvikon 940 spectrophotometer (Kontron, Zürich, Switzerland).

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