# Iron Regulatory Proteins, Iron Responsive Elements and Iron Homeostasis[1,2]

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**ABSTRACT The discovery of iron regulatory proteins (IRPs) has provided a molecular framework from which to more fully understand the coordinate regulation of vertebrate iron metabolism. IRPs bind to iron responsive elements (IREs) in specific mRNAs and regulate their utilization. The targets of IRP action now appear to extend beyond proteins that function in the storage (ferritin) or cellular uptake (transferrin receptor) of iron to include those involved in other aspects of iron metabolism as well as in the tricarboxylic acid cycle. To date, it appears that IRPs modulate the utilization of six mammalian mRNAs. Current studies are aimed at defining the mechanisms responsible for the hierarchical regulation of these mRNAs by IRPs. In addition, much interest continues to focus on the signaling pathways through which IRP function is regulated. Multiple factors modulate the RNA binding activity of IRP1 and/or IRP2 including iron, nitric oxide, phosphorylation by protein kinase C, oxidative stress and hypoxia/reoxygenation. Because IRPs are key modulators of the uptake and metabolic fate of iron in cells, they are focal points for the modulation of cellular iron homeostasis in response to a variety of agents and circumstances. J. Nutr. 128: 2295–2298, 1998.**

KEY WORDS: • iron • iron regulatory protein • iron responsive element • ferritin • transferrin receptor

The ability to obtain and safely use iron for various biochemical processes is a central requirement for nearly all forms of life. Iron is a component of proteins required for crucial cellular processes including respiration and cell division. However, the biological use of iron is limited by its low solubility as the uncomplexed metal and its propensity to participate in formation of potentially lethal oxidizing agents. Given the important biological roles of iron it is not surprising that variations in body iron status influence human and animal health. Iron regulatory proteins (IRPs)[4] and the proteins whose synthesis they regulate form a homeostatic network that allows mammals to make use of the essential properties of iron while reducing its potentially toxic effects.

***Homeostatic Regulation of Mammalian Iron Metabolism.*** The major processes responsible for modulating mammalian iron homeostasis are intestinal absorption, interorgan transport and uptake, and cellular utilization. Mammals have a number of proteins that facilitate the safe and efficient transport, uptake, use and storage of iron. Alterations in the abundance and/or activity of these proteins provides a key mechanism for modulating interorgan and cellular iron utilization. In this review we focus on the control of cellular iron homeostasis by IRPs. The reader is referred to more comprehensive reviews of IRP function for the details covering work before 1996 (Eisenstein et al. 1997, Hentze and Kuhn 1996, Rouault and Klausner, 1996).

Interorgan transport and uptake of nonheme iron is largely performed by the transferrin (Tf)/ transferrin receptor (TfR) system. Tf is a serum glycoprotein that binds up to two iron atoms. TfR is a disulfide-linked homodimer present in the plasma membrane that binds one Tf molecule per TfR monomer. The Tf-TfR complex is internalized by receptor mediated endocytosis ultimately entering the endosomal compartment of the cytoplasm. Endosomal acidification, to a pH of ~5.5, is required for release of iron from Tf. Transfer of iron across the endosomal membrane into the cytosol is poorly understood, but recent observations suggest that two proteins, natural resistance associated macrophage protein 2 (nRAMP2), also know as divalent cation transporter 1 (DCT1), (Fleming et al. 1997, Gunshin et al. 1997) and stimulator of iron transport (Gutierrez et al. 1997), may have important roles in this process. The immediate fate of iron leaving the endosome is an even less well understood aspect of iron metabolism. It seems likely that, as is the case for copper, proteins will be identified that traffic iron to specific cytoplasmic locations.

Many circumstances including alterations in iron availability, the level of specific hormones, growth factors and cytokines, as well as the state of cell proliferation or differentiation, influence the uptake and metabolic fate of iron. The size of the intracellular chelatable iron pool influences ferritin and TfR gene expression largely at the post-transcriptional level through the action of two iron-regulated RNA binding proteins, the IRPs. Elucidation of the central role of IRPs, and the iron responsive elements (IREs) to which they bind, in regulating ferritin and TfR synthesis has been instrumental in elucidating how iron-dependent and iron-independent signaling pathways influence cellular iron homeostasis.

***Iron Responsive Elements Modulate mRNA Translation or Stability.*** Ferritin and TfR synthesis are linked to cellular iron status through the regulated interaction of one of two IRPs with a 28 nucleotide (nt) structural motif, the IRE, present in their mRNAs. IREs are RNA stem-loop structures composed of a conserved loop sequence, CAGUGX where X is usually U or C but can be an A. Five basepairs (bp) 5′ of the loop is a bulge nt region that may vary in size depending on the IRE examined (Bettany et al. 1992, Theil 1994). This bulged nt region contains an unpaired C residue that is a critical determinant of IRE function. Ferritin IREs appear to have a different structure in this region compared to the IRE(s) in other mRNAs (Bettany et al. 1992, Gdaniec et al. 1998). Diversity in IRE sequence and structure, as well as the presence of mRNA specific sequences that influence IRE/IRP function, may provide a mechanistic basis for selective regulation of IRE containing mRNA.

One or more IRE(s) are present in the 5′ or 3′ untranslated region (UTR) of specific mRNAs where they influence mRNA translation

[1] Supported by National Institutes of Health Grant DK42719 (RSE), U.S. Department of Agriculture 97–35200–4232 (RSE) and 96–35200–3273 (KPB).
[2] Manuscript received 22 September 1998. Revision accepted 28 September 1998.
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[4] Abbreviations used: bp, basepair; c-acon, cytosolic aconitase; DCT1, divalent cation transporter 1; eALAS, erythroid 5-aminolevulinate synthase; IRE, iron responsive element; IRP, iron regulatory protein; m-acon, mitochondrial aconitase; nRAMP2, natural resistance associated macrophage protein 2; nt, nucleotide; PKC, protein kinase C; RTD, rapid turnover determinant; TCA, tricarboxylic acid; Tf, transferrin; TfR, transferrin receptor; UTR, untranslated region.

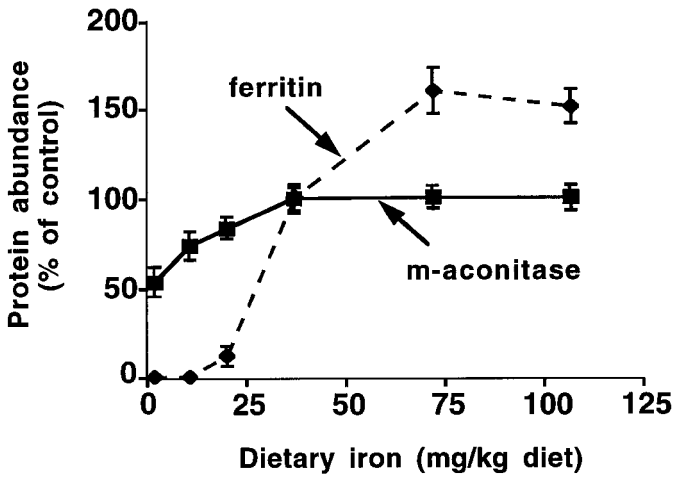


**FIGURE 1** Putative differences in iron responsive element (IRE) structure may promote differential regulation of the targets of IRP action. Iron deficiency differentially affects the abundance of ferritin and m-acon protein in rat liver (adapted from Chen et al. 1997).

or stability, respectively **(Table 1)**. Ferritin mRNAs contains a single IRE in their 5′ UTR which is necessary and sufficient for iron-regulation of ferritin mRNA translation. In contrast to ferritin, TfR mRNA contains five IREs in its 3′ UTR where they regulate its stability. Two regions within the 3′ UTR of TfR mRNA are required for iron regulation. This includes three of the five IREs as well as a second region, the rapid turnover determinant (RTD). Thus, IREs participate in the regulation of mRNA translation or stability depending on their location within the RNA target as well as on their functional interaction with other sequences within an mRNA, such as the RTD.

***Iron Regulatory Proteins–Central Regulators of Iron Homeostasis.*** The mechanism by which IREs regulate mRNA utilization occurs due to interaction with IRP1 or IRP2. IRPs are sequence- and structure-specific RNA binding proteins found in the cytosol of all mammalian tissues examined to date. When iron supply is low, IRPs are high affinity ($K_d$ ~20–100 pmol/L) RNA binding proteins. When bound to the IRE in H- or L-ferritin mRNA, IRP1 or IRP2 blocks the initiation of protein synthesis **(Fig. 2).** The current model for the regulation of TfR mRNA stability suggests that IRP binding hinders interaction of a ribonuclease with the RTD (Fig. 2). When iron is in excess, the high affinity RNA binding function of IRPs is inactivated. Consequently, synthesis of ferritin is initiated while the level of TfR mRNA declines. By providing a common locus for modulation of ferritin and TfR synthesis, IRPs are central regulators of cellular iron homeostasis.

***Additional IRE-Containing Messenger RNA.*** IREs, or IRE-like motifs, have been identified in many other mRNA, and it appears that IRPs also modulate the utilization of these mRNA (Table 1). IREs have been identified in the 5′ UTR of mRNAs encoding the erythroid isoform of 5-aminolevulinate synthase (eALAS), mitochondrial aconitase (m-acon) and the iron-protein subunit of succinate dehydrogenase in *Drosophila*, but not in humans. The mRNA encoding DCT1, the rat homolog of nRAMP2, contains an IRE-like sequence in its 3′ UTR. These observations suggests that IRPs influence multiple aspects of iron metabolism and cell function.

eALAS is the rate limiting enzyme in heme formation in red cells. The presence of an IRE in 5-aminolevulinate synthase mRNA suggests that IRPs link the formation of protoporphyrin IX with the availability of iron in order to coordinate these two key aspects of heme formation [referenced in (Hentze and Kuhn 1996)]. Because erythroid heme formation represents the major daily use of body iron, it would appear that IRPs are important modulators of body iron flux.

nRAMP2 appears to function both in intestinal absorption and endosomal release of iron (Fleming et al. 1997 and 1998, Gunshin et al. 1997). nRAMP2 mRNA has a single IRE-like element in its 3′ UTR, although it is of interest to note that multiple splice variants of this mRNA exist, including one which lacks this element (Lee et al. 1998). nRAMP2 mRNA is increased in iron deficiency, suggesting that IRP may modulate nRAMP2 mRNA in a manner analogous to TfR mRNA (Gunshin et al. 1997). The exact mechanism through which iron regulates nRAMP2 expression remains to be determined. Such studies will need to consider the physiological impact of the expression of an nRAMP2 mRNA that lacks an IRE.

m-Acon converts citrate to isocitrate in the tricarboxylic acid (TCA) cycle. There is strong evidence that IRPs modulate translation of m-acon mRNA (Chen et al. 1997, Gray et al. 1996, Kim et al. 1996, Schalinske et al. 1998). However, the purpose of IRP-mediated changes in m-acon abundance remains unclear. The evidence suggests that IRPs may modulate m-acon synthesis not to regulate ATP production, but perhaps to alter the use of citrate in other cellular processes possibly including its use as an iron chelator or as a means to alter fuel utilization during iron deficiency (Chen et al. 1998).

***Functional Diversity in Iron Responsive Elements.*** Considering the varying functions of the cellular targets of IRP action, it is likely that there is selectivity in the timing and/or degree of regulation of the numerous IRE-containing mRNA. For instance, given the difference in the functions of iron storage protein and TCA cycle enzymes, it is likely that ferritin and m-acon expression would not vary in an identical manner in response to alterations in cellular iron status. A central issue concerning the extent to which various IRE-containing mRNA are regulated is what factors influence the formation of a complex between a given target mRNA and an IRP. Considered together, these points raise the question: Are all IRE functionally equivalent?

Some of the first evidence suggesting that all 5′ IRE are not equivalent came from structural studies of the ferritin IRE. The canonical IRE structure contains a conserved CAGUGX sequence and a conserved single nucleotide C bulge separated from the terminal loop by 5 bp. The evidence suggests that the ferritin IRE does have a structure in the bulge nt region that is different from that proposed for other IRE (Bettany et al. 1992, Gdaniec et al. 1998, Theil 1994). The relevance of these observations lies in the fact that the size and/or structure of such bulge nt regions affects the tertiary structure of RNA and can influence their interaction with proteins

**TABLE 1**

*Known and emerging mRNA targets of IRP action*

| mRNA | IRE Location | Function of encoded protein |
|---|---|---|
| H- and L-ferritin | 5′UTR | Iron storage |
| eALAS | 5′UTR | Erythroid heme formation |
| m-Aconitase | 5′UTR | Tricarboxylic acid cycle |
| SDH iron protein[1] | 5′UTR | Tricarboxylic acid cycle |
| Transferrin receptor | 3′UTR | Cellular uptake of Tf iron |
| nRAMP2/DCT1 | 3′UTR | Intestinal absorption/endosomal releases of iron |

[1] An IRE is present in the Drosophila mRNA but not in the human mRNA.

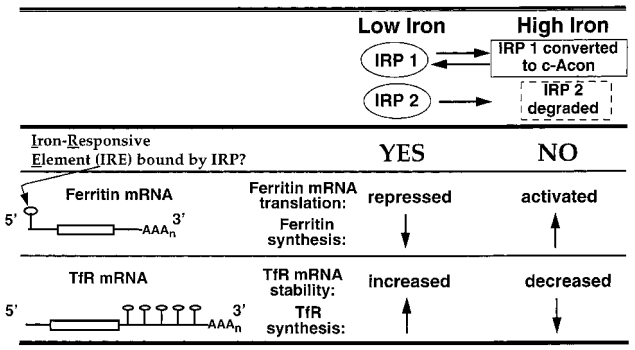


**FIGURE 2** Post-transcriptional regulation of ferritin and transferrin receptor (TfR) by iron regulatory proteins (IRPs).

(Bettany et al. 1992, Gdaniec et al. 1998). Interestingly, the size of this bulge nt within the IRE greatly influences its interaction with IRP2, but not IRP1(Ke et al. 1998).

Much of the evidence suggesting that 5′ IRE are not functionally equivalent comes from studies comparing the regulation of m-acon and ferritin synthesis by iron. It has been known for many years that alterations in iron status can lead to changes in the rate of ferritin synthesis of as much as 100-fold. We have demonstrated a similar response of ferritin protein abundance in liver in relation to changes in dietary iron intake (**Fig. 1**) (Chen et al. 1997). However in contrast to ferritin, m-acon abundance and its synthesis rate are much less responsive to changes in iron status (Fig 1) (Schalinske et al. 1998). The differential regulation of ferritin and m-acon synthesis by iron appears to be largely due to much less efficient repression of m-acon synthesis in response to low iron levels, and this may relate to differences in the structure of their IRE (Kim et al. 1996, Schalinske et al. 1998). These studies illustrate that the synthesis and abundance of the targets of IRP action are not equally affected by iron. Whereas the exact role of IRE structure in the differential regulation of ferritin and m-acon synthesis remains to be fully defined, these studies indicate that there is significant versatility in the IRE/IRP system.

***Factors Regulating IRP RNA Binding Activity.*** Since their discovery in 1988, it has become apparent that the RNA binding activity of IRPs is regulated by numerous factors. IRP1 and IRP2 respond to the same as well as to different effectors. In addition to iron, the activity of IRP 1 and/or IRP2 is affected by nitric oxide (Bouton et al. 1998, Kennedy et al. 1997), phosphorylation by protein kinase C (PKC), oxidative stress (Pantopoulos et al. 1996), hypoxia/reoxygenation (Hanson and Leibold 1998) and to changes in cell proliferation or differentiation. In most if not all cases, iron-independent pathways for modulating IRP function impinge on their regulation by iron.

***The Aconitase Model of IRP1 Function.*** Iron status modulates the affinity with which IRP1 binds to the IRE, without changing the total cellular level of the binding protein. This suggested that IRP1 RNA binding activity could be regulated in the absence of the synthesis of new protein, i.e. posttranslationally. A crucial clue concerning the mechanisms of iron action was provided by the observation that IRP1 was found to be similar in amino acid sequence to m-acon, the [4Fe-4S] TCA cycle enzyme that converts citrate to isocitrate. Ultimately, IRP1 was shown to be a bifunctional protein, sequence specific RNA binding protein or the poorly characterized cytosolic isoform of m-acon, cytosolic aconitase (c-acon). Of particular interest was the fact that m-acon was known to contain a solvent accessible and labile Fe-S cluster, which in the presence of oxygen or other agents was reversibly converted to the [3Fe-4S] form. It was first proposed that regulated interconversion between the 3Fe and 4Fe forms of a putative Fe-S cluster in IRP1 represented the mechanism for iron-dependent modulation of its RNA binding activity. Subsequently IRP1, in its iron-loaded form, was shown to contain a [4Fe-4S] iron-sulfur cluster and to exhibit substantial aconitase activity. The Fe-S cluster is now known to be an important determinant of the capacity of IRP1 to bind the IRE. However, generation of high affinity RNA binding activity requires complete loss of the Fe-S cluster; both the [4Fe-4S] and [3Fe-4S] forms bind RNA poorly ($k_D \sim 3$ nmol/L). Hence, formation or loss of the Fe-S cluster is a means by which changes in cellular iron status could be transmitted into alterations in RNA binding capacity of IRP1.

The crystal structure of m-acon is known and it appears that IRP1, in its iron-loaded form (c-acon), has a tertiary structure similar to m-acon. On this basis IRP1 was predicted to contain a cleft, a region of the protein required for formation and ligation of the Fe-S cluster or in the binding of RNA. The presence or absence of the cluster alters IRP1 structure and is believed to affect the extent to which the cleft is open and accessible for interaction with RNA [(Schalinske et al. 1997) and references therein]. Several amino acids in the putative cleft are required for both the RNA binding and aconitase functions. Taken together, overlapping use and/or altered accessiblity of amino acids within the putative cleft of IRP1 act as key determinants of its mutually exclusive functions, enzyme or RNA binding protein.

Whereas it is well accepted that conversion of IRP1 to c-acon occurs, and can be iron-regulated in cells, it is not clear how the Fe-S cluster is removed from c-acon in order to generate RNA binding activity. It is of interest that changes in dietary iron intake do not lead to as extreme a change in abundance of the two forms of the binding protein as seen in cell culture. In rat liver, dietary iron deficiency elicited a robust three- to four-fold increase in IRP1 RNA binding activity with no change in c-acon activity (Chen et al. 1997). These results suggest that only a small pool of c-acon need respond to perturbations in iron availability in order to influence IRP1 RNA binding activity. However, considered from another perspective, how and why is it that most of c-acon is resistant to iron deficiency? These results suggest that either the Fe-S cluster in IRP1 is not easily removed, at least in response to iron deficiency, or there exist distinct forms of the binding protein that differ with respect to the ease with which the Fe-S cluster can be removed. These studies illustrate some of the unresolved issues concerning the function of IRP1. Given the important role of the Fe-S cluster in affecting IRP1 function, much interest will continue to focus on the mechanisms of its formation and removal.

***Factors Influencing the Interconversion of IRP1 and Cytosolic Aconitase.*** In order for the Fe-S cluster of IRP1 to serve as a biosensor, its assembly and/or disassembly must be regulated. In this regard, it is important to realize that Fe-S clusters do not spontaneously disassemble. In other words, iron deficiency per se is not sufficient to promote loss of the cluster. Consequently, several issues such as stability of the Fe-S cluster as well as whether cluster removal is, or is not, protein-mediated need be considered. One factor in the loss of the Fe-S cluster in aconitases may be that one of the iron atoms of the [4Fe-4S] cluster, denoted $Fe_a$, is not ligated directly to the protein.

Several lines of evidence indicate that agents that influence the removal of $Fe_a$ could represent one pathway for initiating cluster disassembly [reviewed in (Eisenstein et al. 1997)]. First in the presence of oxygen, the Fe-S cluster of aconitases is labile; $Fe_a$ is lost forming the [3Fe-4S] cluster. Second, NO and $O_2^{\cdot -}$-induce cluster disassembly in aconitases more rapidly than oxygen and

this can involve formation of the [3Fe-4S] cluster (Flint et al. 1993, Gardner 1997, Kennedy et al. 1997). Third, in cell culture NO induces RNA binding activity by IRP1. It will be of interest to determine whether cluster perturbants such as NO and $O_2^{\cdot -}$ play a role in the generation of IRP1 RNA binding activity in general, and specifically in response to iron deficiency.

***Iron Regulates IRP2 Function through a Protein Degradation Mechanism.*** IRP2 is 57% identical in amino acid sequence to IRP1 with the noted exception of the presence of a 73 amino acid insertion. The steady state level of IRP2 protein is inversely related to cellular iron level, and the 73 amino acid insert serves as an iron-dependent degradation motif. It appears that iron mediates metal-catalyzed oxidation of IRP2 prior to its degradation (Iwai et al. 1998).

***Phosphorylation-Dependent Modulation of IRP Function.*** Because they are central regulators of iron metabolism, IRPs can serve as focal points for a variety of extracellular and intracellular agents acting to alter the uptake and metabolic fate of iron. In recent years, evidence has accumulated suggesting that IRP function is modulated by PKC-dependent phosphorylation. Both IRPs are phosphoproteins, and the PKC activator phorbol 12-myristate 13-acetate rapidly stimulates IRP phosphorylation and increases IRE binding activity in HL60 cells. Purified rat liver IRP1 is an efficient substrate for PKC and Ser 138 and Ser 711 are the apparent sites of PKC action. Recent results provide strong evidence that phosphorylation of Ser138 reduces the stability of the Fe-S cluster of IRP1 in response to cluster perturbants and suggests that phospho-IRP1 preferentially exists in the RNA binding form (Brown et al. in press). Hence, phosphorylation may affect the interconversion of IRP1 and c-acon and, in this manner, alter the set-point for iron regulation of the binding protein.

IRP2 is more highly phosphorylated in cells than is IRP1, suggesting the phosphorylation is necessary for the basal function of IRP2 (Schalinske and Eisenstein 1996). Phosphorylation appears to regulate IRP2 function through a redox mechanism with the reduced form of the protein binding RNA. Several putative PKC sites exist in the 73 amino acid degradation motif, and phosphorylation may affect the redox state of critical Cys therein.

These results have novel implications for the regulation of iron metabolism in physiological and pathological situations. Regulation of IRP function through changes in their phosphorylation state provides a molecular basis by which agents other than iron can selectively modulate iron metabolism in cells and tissues in the presence or absence of changes in iron availability induced by diet or other physiological situations. This may include changes in the uptake and metabolic fate of iron that occurs during cell proliferation, erythroid differentiation or the inflammatory response where factors other than iron act as important determinants of cellular or whole body iron metabolism.

***Summary and Perspectives.*** In the little more than 10 years since they were discovered it has become apparent that IRPs are key regulators of many aspects of iron homeostasis in higher eucaryotes. However, there are still many unresolved issues and exciting avenues to explore concerning the structure and function of IRPs. How is the assembly/disassembly of the Fe-S cluster in IRP1 regulated? What factors influence the hierarchal regulation of IRE-containing mRNA by IRPs? Does the ratio of IRP1 and IRP2 in cells influence the extent to which specific mRNA are regulated? What extracellular factors influence IRP phosphorylation state? Why do IRPs apparently modulate m-acon synthesis? Do other IRE-containing mRNA and/or IRPs exist? Does HFE, the hemochromatosis gene product, communicate with IRPs in order to modulate cellular iron metabolism? The future is sure to provide novel observations that shed further light on the biological roles of these central regulators of iron homeostasis.

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