Novel Roles for Iron Regulatory Proteins in the Adaptive Response to Iron Deficiency1,2,3

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ABSTRACT Iron regulatory proteins (IRP) modulate the use of mRNA-encoding proteins that are involved in the transport, storage and use of iron. Several new potential mRNA targets for IRP were recently identified: divalent metal transporter-1 (DMT-1) and ferroportin, which are critical regulators of iron absorption in the gut and of iron cycling between various tissues of the body. Although this may extend the reach of IRP to other processes that are important for maintaining body iron homeostasis, the extent to which IRP modulate other physiological processes that are altered in response to changes in iron availability is not clear. However, in the past several years, targets for IRP and IRP-like proteins were identified in eukaryotes and prokaryotes in the tricarboxylic acid (TCA) cycle and electron-transport chain. In mammals, this includes the mRNA that encodes the TCA-cycle enzyme mitochondrial aconitase (m-acon). Recent work established that m-acon expression is translationally regulated by iron in a manner that is strongly correlated with IRP RNA-binding activity. Interestingly, these studies also demonstrate that IRP regulate their mRNA targets in a hierarchical manner. The changes in m-acon synthesis and abundance in liver during iron deficiency fail to affect TCA-cycle capacity but are associated with a significant upregulation of mitochondrial export of radiolabeled citrate. We conclude that IRP are required for the regulation of physiological pathways that include but are not limited to iron metabolism, and as such, IRP are critical factors in the adaptive response to iron deficiency. J. Nutr. 133: 1510S–1516S, 2003.

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

Iron deficiency is a global health problem of immense proportions. Over the past four or five decades, much interest and effort has focused on understanding the metabolic consequences of iron deficiency (1,2). Organisms mount an array of responses to iron deficiency to maintain various essential functions as well as possible until the opportunity for iron repletion arises. There are tissue-specific responses to iron deficiency that likely reflect the hierarchical role of organs in supporting critical functions for the organism. The adaptive response to iron deficiency includes but is not limited to upregulation of iron-transporter activity in the small intestine, rapid decline of iron stores in liver and other storage depots, depletion of the functional iron pool in mitochondria (hemoglobin, cytochromes, and iron-sulfur proteins, etc.) and increase in transferrin receptor (TfR) expression in multiple tissues such as in immature red cells. Classical thought holds that organs such as the erythron and brain are the sites that are spared as much as possible when mammals are exposed to long-term iron deficiency. However, work from several laboratories (3–5) indicates that in the liver, except for the rapid loss of iron stores, many iron-containing proteins are spared even though in skeletal muscle the same proteins become rapidly depleted.

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5 Abbreviations used: c-acon, cytosolic aconitase; DMT-1, divalent metal transporter-1; eALAS, erythroid 5-aminolevulinate synthase; eIF4F, eukaryotic translation initiation factor-4F; ETC, electron transport chain; IRP, iron regulatory protein; m-acon, mitochondrial aconitase; IRE, iron responsive element(s); TCA, tricarboxylic acid; TIR, transferrin receptor; UTR, untranslated region.

6 A second TIR, TIR-2, was recently discovered that is ~45% identical to TIR-1, lacks iron-responsive elements in the 3′ untranslated region (UTR) of its mRNA and is not downregulated by iron overload, at least in liver. All other TIR references in this review are to TIR-1.
and blood hemoglobin levels fall. Although there are undoubtedly multiple reasons for this response, it is likely that the selective responses of various tissues are controlled to meet the needs of the organism. Prime candidates to help orchestrate an integrated response to variations in iron availability are the iron regulatory proteins (IRP), which are considered to be central regulators of iron metabolism. Recent evidence indicates that the action of IRP is not limited to regulating the expression of proteins of iron metabolism but is extended to include proteins that function in specific aspects of intermediary metabolism. In this manner, we contend that IRP are critical players in the adaptive response to iron deficiency.

Maintenance of iron homeostasis by IRP and targets of IRP action

IRP are cytosolic RNA-binding proteins that bind to and regulate the translation or stability of mRNA that contains iron-responsive elements. Iron-responsive elements (IRE) are small stem-loop structures that were originally proposed to contain a loop sequence of CAGUGX and a bulged C nucleotide located five basepairs 5' of the first nucleotide of the loop (Fig. 1A). In response to low-iron conditions, IRP bind the IRE regions of specific mRNA with high affinity (K_D = 20–100 pm). The mRNA that encodes the H and L subunits of the iron-storage protein ferritin contains a single IRE near the 5' end. When bound to the ferritin iron-responsive element, IRP fail to interfere with one of the first steps in the initiation of translation of most mRNA, which is the binding of the translation initiation factor complex [eukaryotic initiation factor-4F (eIF4F)] with the 5' end of ferritin mRNA (Fig. 2). Instead, it appears that IRP block the ability of eIF4F to recruit the 40S subunit with its associated factors (the 43S preinitiation complex) to the mRNA (6). Interestingly, the presence of the eIF4F complex on ferritin mRNA even when IRP is bound suggests that upon derepression of ferritin mRNA (dissociation of IRP), the mRNA is set to recruit the 43S preinitiation complex, which perhaps gives it a competitive advantage over other mRNA.

In contrast to ferritin mRNA, TfR mRNA contains five IRE in its 3' UTR. Binding of IRP to iron-responsive elements in TfR mRNA protects the mRNA from degradation, which leads to increased mRNA stability and enhanced synthesis of TfR protein (7,8). In addition to the IRE, TfR mRNA contains a "rapid-turnover determinant" in the 3' UTR that contains a site for endonucleolytic cleavage of the mRNA (9). When IRP are inactive (as occurs in iron-replete cells), the rapid-turnover-determinant region of TfR mRNA is accessible, and the mRNA is degraded starting with the endonucleolytic cleavage event in the rapid-turnover determinant (10). When cells are iron deficient, and IRP bind the iron-responsive elements in the TfR mRNA, the rapid-turnover determinant is not accessible for cleavage, and the RNA accumulates. Hence, IRP regulation of mRNA stability requires two elements: the turnover determinant and the IRE. Together, these elements confer iron regulation of mRNA stability.
One of the original proposals (11) for a canonical structure of the IRE proposed a 6-nucleotide CAGUGX loop and the bulged C nucleotide located 5 bp 5′ of the loop (Fig. 1A). These and other reports also suggested that all IRE were equivalent with respect to the ability to bind IRP and therefore implied that all mRNA with 5′ IRE would be identically regulated. However, it has become apparent (12) that differences with respect to the sequence and structure of the stem region both within and flanking the highly conserved 28-nucleotide IRE region have an impact on IRE function (Fig. 1B-D). Interestingly, the sequences that compose the stem region of IRE are well conserved (~90% identical) within an iron-responsive-element “species” (e.g., ferritin IRE), but tend to be much less well conserved (~35–90%) between IRE species (e.g., ferritin vs. mitochondrial aconitase [m-acon; (13)]. It has also been demonstrated that IRP-1 and IRP-2 differ with respect to which natural IRE they prefer to bind, and this is influenced by the structure of the stem region of each IRE (14). Furthermore, selection of artificial iron-responsive elements for a large randomized pool of RNA molecules revealed that RNA could be isolated that specifically bound to IRP-1 or IRP-2 but not both (15,16). These findings provide support for the concept that various IRE are not equipotent in influencing mRNA fate, and that IRP-1 and IRP-2 may selectively regulate the use of overlapping but not identical sets of mRNA.

New and emerging targets of IRP action

Since the elucidation of the role of the IRE in modulating the synthesis of ferritin and transferrin receptors, IRE or IRE-like elements have been discovered in the mRNA that encodes several other proteins that have critical roles in iron metabolism. Among these is the erythroid isoform of 5-aminolevulinate synthase (eALAS) (17,18). Synthesis of eALAS is translationally regulated by iron in murine erythroleukemia cells and also when iron is added to an in vitro protein-synthesis system programmed by eALAS mRNA. The presence of an IRE only in the erythroid isoform of eALAS suggests that IRP have a critical role in the cycling of iron between the erythron, plasma and reticuloendothelial system, which represents the major use of iron on a daily basis. However, the extent to which IRP modulate erythroid heme formation in vivo has not been elucidated.

More recently, the mRNA that encodes the iron-transport proteins ferroportin and DMT-1 was shown to contain an IRE or IRE-like element (19–24). DMT-1 is a proton-coupled iron transporter that is responsible for iron uptake at the apical surface of the duodenum as well as, apparently, iron transport out of the endosome (25). Ferroportin is the basolateral iron transporter of the duodenal mucosal cell that also has a critical role in iron export from the reticuloendothelial system. In the case of DMT-1, several alternatively spliced mRNA species are produced, some of which contain an IRE-like element in their 3′ UTR (65). In this case, the iron-responsive element contains a bulged U residue on the 3′ side of the CAGUGX loop that is not present in other iron-responsive elements (21). Dietary iron intake, genetic iron overload and changes in iron levels in the internal media can result in significant changes in DMT-1 expression. It is of interest to note that the IRE-containing mRNA tends to be the RNA species that responds most extensively to changes in iron availability in vivo or in cell culture (20,21,26–28). Although it is interesting that the DMT-1 IRE-like element preferentially binds to IRP-1 (21), it is far from clear whether this RNA element is required for the iron-dependent changes in DMT-1 mRNA expression. Furthermore, no direct changes in stability have been demonstrated for either DMT-1 mRNA species, and consequently, the role of IRP in the regulation of DMT-1 expression remains unclear.

Ferroportin was discovered as a gene whose expression was linked to alterations in intestinal iron transport activity, anemia in mutant zebrafish or a gene that encoded an mRNA that contained a 5′ IRE that bound to IRP (22–24). Recent works that elucidate the molecular causes of a less-common form of hemochromatosis (29) and reticuloendothelial iron overload (30) further demonstrate that ferroportin is a critical protein for the maintenance of mammalian iron homeostasis. Ferroportin mRNA contains an apparent IRE in its 5′ UTR that can bind IRP in vitro (22–24). However, iron-dependent changes in ferroportin expression do not always occur in a direction that is consistent with the presence of a 5′ IRE (22). In liver, iron regulation of ferroportin expression mimics ferritin, which is as expected, because ferroportin mRNA (like ferritin mRNA) contains a 5′ IRE. However, in duodenum, ferroportin expression is regulated in a manner opposite of ferritin (22). It appears that the form of ferroportin mRNA expressed in intestine has a longer 5′ UTR such that the iron-responsive element is further from the 5′ end of the mRNA (22). This may allow the IRE in this longer form of ferroportin mRNA to be ignored by the translation machinery, as is the case with synthetic constructs, in which the IRE is moved progressively farther from the 5′ end of the mRNA (22,31). Although the IRE in ferroportin mRNA may not be operative in intestine, recent evidence reveals a clear and dramatic change in ferroportin mRNA translation in liver as assessed by polysome profile analysis (S.L. Clarke, K.L. Ross and R.S. Eisenstein, unpublished observations). In iron-deficient liver, ferroportin mRNA translation is as effectively repressed as ferritin mRNA, and conversely, iron injection stimulates the translation of both mRNA.

Potential role of IRP or IRP-like proteins in controlling energy metabolism in diverse organisms

Recent evidence provocatively suggests that IRP may control the synthesis of proteins that are not direct regulators of iron metabolism. Evidence in support of this arises from studies of the aconitase family of proteins in prokaryotes and eukaryotes. As a result of these studies, it appears that IRP and their bacterial homologs may also influence the synthesis of specific enzymes in the tricarboxylic acid (TCA) cycle and/or the electron transport chain (ETC) (3,4,32–39). The enzymes that are targets of this regulation vary among organisms and include mammalian m-acon, Escherichia coli aconitase A and B, Drosophila melanogaster succinate dehydrogenase and Bacillus subtilis cytochrome oxidase, all of which contain an IRE or IRE-like element in the mRNA. Although the consequences of regulating their expression would seem to be obvious, the exact reasons that the expression of these specific enzymes is modulated by iron status (as opposed to other iron-containing enzymes in the same pathways) are not entirely clear. Furthermore, that there is little direct evidence linking iron-related changes in the abundance of these proteins with alterations in cellular metabolism makes it difficult to draw firm conclusions in this regard. Because in mammals m-acon is the only TCA cycle enzyme whose mRNA contains a canonical IRE, we focused on elucidating the physiologic consequences of this regulatory scenario. Our ultimate goal is to understand the physiologic basis as to why the aconitase family of proteins (e.g., IRP-1 and bacterial aconitases) controls the expression of enzymes in the TCA cycle and ETC in prokaryotes and eukaryotes.
Mitochondrial aconitase as a target of IRP action

In 1991 two novel observations were made that established critical links between mammalian iron metabolism and the aconitase family of proteins. First, it was suggested and later borne out that IRP-1 is the cytoplasmic isoform of aconitase (c-acon) and that modification of its Fe–S cluster regulated its RNA-binding function (40,41). In fact, we now know that IRP-1 is a bifunctional protein with activities as an iron-responsive-element binding protein when it lacks an Fe–S cluster and it is c-acon when it contains a [4Fe–4S] Fe–S cluster. Second, a computer search for new IRE revealed the unexpected finding that m-acon mRNA appears to contain an IRE in its 5’ UTR (42). To date, this remains the only example of an mRNA in mammals that contains a canonical IRE and appears to be a target of IRP action yet does not code for a protein with a clear function in iron metabolism.

The observation that m-acon mRNA contains an IRE led to the development of several hypotheses, some of which suggest a novel role for m-acon regulation in aspects of iron metabolism. Included among these was the suggestion that IRP-dependent regulation of m-acon expression represents a means to modulate the generation of oxidative stress through directed changes in TCA-cycle flux. This hypothesis was attractive given that in E. coli, it appeared that the cellular aconitase could act as a throttle to control TCA-cycle flux. The basis for this response is that the Fe–S cluster of aconitases is accessible to low–molecular weight oxidants such as superoxide, which induces disruption of the cluster and loss of enzymatic activity (43). Therefore, by analogy with the situation of E. coli, it was suggested that increased oxidant stress would promote removal of the Fe–S cluster in c-acon and thereby lead to activation of IRP-1 RNA-binding activity and repression of m-acon synthesis (33). A second proposal suggested that the IRE in m-acon mRNA was merely one example of many mRNA that encode Fe–S proteins that contain IRE, and that this represents a general mechanism to coordinate Fe–S protein synthesis with iron availability (34). The third proposal suggested that IRP modulates m-acon synthesis as part of an adaptive response to coordinate iron and energy metabolism through iron-dependent changes in citrate metabolism (3,4). Citrate is a key metabolite in a number of metabolic pathways, an allosteric regulator of carbohydrate and lipid metabolism and a physiologically relevant iron chelator. Hence, one could envision a potential role for IRP-dependent regulation of m-acon as a means to control the use of citrate in specific aspects of intermediary or iron metabolism. Whichever of these or other hypotheses proves to be correct, it is important to demonstrate that m-acon mRNA translation is regulated by IRP and then determine whether this is associated with alterations in citrate metabolism.

Before the discovery of IRP, at least two reports in the literature demonstrated that total cellular aconitase and/or m-acon activity was decreased during iron deficiency (44,45). Since the demonstration that IRP exist and that m-acon mRNA contains an IRE, several lines of evidence have emerged that lead to the conclusion that m-acon synthesis is regulated by IRP. First, m-acon mRNA translation can be specifically repressed when IRP are added to a cell-free protein-synthesis system (33,34). Second, dietary iron deficiency in rats leads to a selective dose- and time-dependent decrease in m-acon abundance in liver that is strongly correlated with the increase in IRP RNA-binding activity (3,4). Third, iron stimulates the association of m-acon mRNA with polysomes in HL60 cells and thereby shows that m-acon synthesis is translationally regulated (38). This is associated with changes in the synthesis rate of m-acon. Finally, we recently found that dietary iron deficiency leads to significant repression of m-acon mRNA translation in rat liver (K.L. Ross, S.L. Clarke and R.S. Eisenstein, unpublished observations). In conclusion, changes in cellular iron status lead to predictable alterations in m-acon mRNA synthesis, which occur through a translational mechanism. These changes are strongly correlated to the modulation of IRP RNA-binding activity.

Another important aspect of these studies is the finding that although changes in iron status influence m-acon synthesis translationally, it is also clear that m-acon expression is less robustly regulated compared to ferritin expression (Fig. 3) (3,4,34,38). Given their differing physiologic roles, the differential regulation of expression of these proteins is not surprising; yet it also provides a clear demonstration that there is a hierarchical regulation of the targets of IRP action. Apparent differences in iron-responsive-element structure and the sequences that flank the iron-responsive elements likely contribute to this differential regulatory effect (Fig. 2B–D) (12,38). Taken together, it is apparent that m-acon activity, abundance and mRNA-translation rate are coordinately regulated in rat liver in response to changes in iron availability apparently through the action of IRP. The regulation of m-acon expression in rat liver provides a well-defined systems where it is clear that the expression of proteins encoded by IRE-containing mRNA was being selectively regulated, that was used further to investigate the basis for IRP-dependent regulation of m-acon expression.

Functional effects of the repression of m-acon expression

To understand why IRP modulate the expression of m-acon, we focused on first determining whether there were perturbations in m-acon–dependent pathways involved in cellular citrate metabolism in iron-deficient liver. It was also of interest to determine whether potential changes in citrate metabolism during iron deficiency could be traced to downregulation of m-acon expression in iron-deficient liver. First, we examined the effects of iron deficiency on TCA-cycle capacity in liver. There was no effect of consumption of a very low–iron diet
Our results suggest that IRP-dependent downregulation of m-acon expression in iron deficiency occurs to enhance citrate efflux from the mitochondria without alteration of TCA-cycle flux. These results are not surprising given the observation that m-acon does not have a significant control strength over TCA-cycle flux in most tissues, which is in contrast to citrate synthase, isocitrate and α-ketoglutarate dehydrogenases (46). However, that Liu et al. (49) have observed enhanced citrate export from prostate cells when m-acon is downregulated due to hormonal influences indicates that m-acon activity can influence extramitochondrial citrate metabolism in multiple tissues. Our novel findings expand the circumstances where directed changes in citrate efflux from the mitochondria provide a mechanism for metabolic cooperation between the mitochondria and cytosol.

In recent years, much interest has focused on new ways in which the mitochondria and cytosol integrate information about cellular or organismal status and act to modulate specific metabolic pathways to maintain homeostasis of specific nutrients (50–52). Of special relevance to this review are the findings that have broadened our view of the role of mitochondria in influencing cellular iron metabolism (53). We propose that IRP-dependent regulation of m-acon expression and aspects of cellular citrate metabolism represent a new and novel example with respect to how cells and organisms orchestrate an adaptive response to a stress (e.g., iron deficiency) to promote survival until the stress is relieved.

Why enhance citrate delivery to the cytosol in iron-deficient liver? Formally, a number of possible fates for cytosolic citrate exist. These include use of citrate for lipogenesis or gluconeogenesis but not both simultaneously, because these pathways are usually divergently regulated (e.g., fatty acid oxidation fuels gluconeogenesis). As noted above, our published and more recent results argue against a use of citrate for lipogenesis. In terms of gluconeogenesis, it is clear that iron-deficient skeletal muscle becomes more reliant on glucose as a fuel source and there is enhanced whole-body glucose oxidation in iron deficiency (54). In this regard, it is of interest that citrate can be a significant source of carbon for gluconeogenesis, which perhaps indicates that m-acon regulation may augment glucose formation by iron-deficient liver (55). However, once again our current results that citrate lyase is downregulated in iron deficiency argue against the use of mitochondrially derived citrate for gluconeogenesis. These findings point toward other uses of citrate in cellular metabolism in iron-deficient animals.

The relationship between the aconitase family of proteins and the regulation of the expression of enzymes in the TCA cycle and ETC in a wide diversity of organisms leads us to speculate that the process or processes being regulated by the aconitases may be similar across these species.

What common physiologic processes are altered when this wide array of organisms is faced with long-term iron deficiency? During iron deficiency, it is suggested that there could be detrimental consequences of a selective downregulation of the formation of essential iron-containing proteins in the TCA cycle and ETC while the expression of other non-iron-containing proteins is not altered (56). In fact, after extended periods of iron deficiency, mitochondria from muscle and liver exhibit a greater degree of uncoupling, which perhaps leads to greater generation of oxygen radicals from incomplete reduction of oxygen by the ETC (57–59). In light of this, it is relevant to note that recent evidence indicates that there is increased oxidant stress in iron-deficient liver and muscle (60,61) and that in E. coli, expression of one of the superoxide dismutases (SOD A) appears to be posttranscriptionally regulated by aconitases A and B in this organism (62). Taken
together, it is tempting to speculate that IRP-dependent modulation of m-acan may regulate citrate efflux to the cytoplasm to generate reducing equivalents for antioxidant defense. Current efforts are focused on tracing the route of cytosolic citrate utilization in iron-deficient liver and determining the consequences of dysregulation of m-acan during iron deficiency.

LITERATURE CITED


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