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IRON REGULATORY PROTEINS AND THE MOLECULAR CONTROL OF MAMMALIAN IRON METABOLISM

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■ **Abstract** Mammalian iron homeostasis is maintained through the concerted action of sensory and regulatory networks that modulate the expression of proteins of iron metabolism at the transcriptional and/or post-transcriptional levels. Regulation of gene transcription provides critical developmental, cell cycle, and cell-type-specific controls on iron metabolism. Post-transcriptional control through the action of iron regulatory protein 1 (IRP1) and IRP2 coordinate the use of messenger RNA-encoding proteins that are involved in the uptake, storage, and use of iron in all cells of the body. IRPs may also provide a link between iron availability and cellular citrate use. Multiple factors, including iron, nitric oxide, oxidative stress, phosphorylation, and hypoxia/reoxygenation, influence IRP function. Recent evidence indicates that there is diversity in the function of the IRP system with respect to the response of specific IRPs to the same effector, as well as the selectivity with which IRPs modulate the use of specific messenger RNA.

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INTRODUCTION

The nutritional requirement for iron arises because it is an essential component of proteins that perform redox and nonredox roles in many cellular functions. However, there are two significant problems regarding the use of iron in biological systems—its low solubility and potential toxicity from iron's ability to promote formation of reactive oxygen intermediates. A variety of mechanisms have evolved that permit organisms to acquire and make use of iron for essential functions while simultaneously reducing the incidence of inappropriate effects of this micronutrient on cell viability. Mammals use a combination of transcriptional and post-transcriptional mechanisms to control the abundance of proteins that modulate the transport, cellular uptake, and metabolic fate of iron. Basal and inducible differences in the rates of transcription of genes encoding the proteins of iron metabolism likely play a key role in permitting organs to meet their specialized requirements for iron and to fulfill their function in body iron homeostasis. A significant component of the means by which cellular iron metabolism is controlled in mammals occurs through directed changes in the use of specific messenger RNA (mRNA). Iron regulatory proteins (IRPs) are critical components of a sensory and regulatory system that coordinates the use of mRNA-encoding proteins that are required to maintain cellular and organismal iron homeostasis in a variety of circumstances. Hence, mammals use a combination of transcriptional and post-transcriptional mechanisms to control the abundance of proteins that modulate the transport, cellular uptake, and metabolic fate of iron. This review focuses on the molecular control of cellular iron homeostasis, and the reader is referred to other reviews for more extensive analysis of the molecular or physiological aspects of whole-body iron metabolism (14, 25, 70, 133).

IRON—AN ESSENTIAL BUT POTENTIALLY TOXIC NUTRIENT

The chemical versatility of iron has made it one of the most commonly used metals in biological systems. Iron, in the form of iron sulfide, may have been one of the critical catalysts for formation of complex organic compounds in the primordial environment that was present on Earth several billion years ago (109). Alterations in the prebiotic environment followed by the formation and evolution of living organisms resulted in a proliferation of the uses of iron in cellular processes and the development of cellular mechanisms to promote its acquisition and appropriate use (49, 108, 126, 226). In vertebrates, multiple physiological processes including oxygen transport, respiration, DNA synthesis, formation of some neurotransmitters and hormones, xenobiotic metabolism, and certain aspects of host defense use iron-containing proteins.

The concept that iron could influence human health was first apparent ~3000 BC, in China, where it was believed that iron could prevent symptoms similar to anemia (22). The essential role of iron in human and animal health became apparent in the mid-eighteenth and nineteenth centuries with the identification of iron as a body constituent and the realization of the relationship between adequate iron intake and the prevention of certain diseases (22, 159). Today, the nutritional importance of iron is apparent, given the worldwide prevalence of physiological disorders arising from iron deficiency and the demonstration of the central role of iron-containing proteins in multiple cellular processes (25, 51, 131). However, while iron is clearly an essential component of a healthy diet, when present at levels that exceed the capacity of organisms to safely use it, iron can be toxic because of its ability to promote oxidation of lipids, proteins, and other cellular components. High levels of iron within the body have been associated with increased incidence of certain cancers and dysfunction of organs, such as heart, pancreas, or liver, and high iron levels may be associated with development of some neurodegenerative disorders (4, 10, 95, 222). These potentially deleterious effects of iron as well as the beneficial biochemical roles of iron-containing proteins have likely been a significant driving force for the evolution of systems to transport, store, and use iron in a safe and available manner. The specific binding of iron by proteins provides a means to increase its solubility, reduce the potential toxicity, and exert control over the metabolism of this essential nutrient.

OVERVIEW OF THE PROTEINS OF IRON METABOLISM

Several physiological mechanisms promote the maintenance of iron homeostasis, and these mechanisms have similar purposes in many organisms. (*a*) Some systems scavenge iron from the environment, and a number of transport systems have evolved to promote the transfer of iron into cells. (*b*) Specific mechanisms are used to increase the solubility of iron and, in multicellular eukaryotes, to facilitate

the intercellular transport of iron. (*c*) Eukaryotes can store iron in a safe and available manner. (*d*) Mechanisms must exist that promote cellular export of iron. (*e*) Organisms have developed sensory and regulatory systems to coordinate the uptake, metabolic utilization, storage and, in some cases, export of iron.

Iron Transport and Uptake

Both genetic and biochemical evidence has demonstrated the presence of multiple pathways for iron uptake by eukaryotic cells (12, 56; see article by Wessling-Resnick in this volume). In mammals, changes in iron absorption are the major control point for altering the iron content of the body and of individual cells. Iron absorption occurs primarily in the duodenum and is inversely related to body iron stores but directly related to the rate of erythropoiesis. Significant recent progress has been made in identifying new or potential participants in the intestinal absorption of nonheme iron, including proteins implicated in the lumenal uptake [divalent metal transporter 1 (DMT1)], serosal release (ferroportin 1, which is also referred to as IREG1; hephaestin), and overall regulation (HFE) of body iron absorption (60, 71, 73, 89, 133, 149, 218). Additional systems may also modulate intestinal absorption of iron (217).

Cellular iron uptake can occur through multiple mechanisms. Except in exceptional circumstances, transferrin (Tf) is the primary means for interorgan transport of nonheme iron. Tf, a serum glycoprotein of M_r ~80,000, binds a maximum of two iron atoms. Normally about one third of the Tf-binding sites in serum are iron saturated, with the apo form present to prevent accumulation of toxic non-protein–bound iron. The high-affinity pathway of uptake of Tf by receptor-mediated endocytosis of transferrin receptors (TfRs) is a key pathway of iron uptake for reticulocytes, hepatocytes, and other cell types. Inactivation of the gene encoding this TfR greatly impairs development of the erythroid and the nervous systems (140). Recent evidence indicates that there are two mammalian TfRs (119; unless noted otherwise, all references to TfR refer to the first TfR discovered). The first TfR characterized has an $M_r \sim 95{,}000$ and forms a homodimer that is capable of binding two Tf molecules. At the pH of the extracellular fluid, diferric Tf preferentially binds to cell surface TfRs. The Tf-TfR complex is internalized by receptor-mediated endocytosis; the endosomal compartment that is so formed is acidified, and iron is released from Tf and pumped into the cytoplasm apparently through the action of DMT1 (74). The apoTf-TfR complex is returned to the cell surface, where, at extracellular pH, apo-Tf dissociates from TfR and can then acquire iron elsewhere in the body.

Tf-dependent delivery of iron to cells is influenced by changes in the percent saturation of Tf with iron, alterations in the total serum concentration of Tf, and modulation of the cell surface display of TfRs. Tf gene expression is influenced by multiple factors, and the abundance of serum transferrin is inversely related to iron status (110, 155, 232). In iron deficiency, Tf gene transcription is increased roughly two- to threefold in liver, the major site of Tf synthesis (111, 150). Reduced

translation of Tf mRNA may occur when iron levels are high (46). Because the K_D ($\sim$10–100 nM) for the interaction of diferric Tf (Fe_2Tf) with TfRs is well below the blood concentration of Fe_2Tf ($\sim$10 μM), TfRs on the cell surface should always be saturated with Fe_2Tf (3). Therefore, it is not clear how relatively modest changes in Tf abundance and/or percent saturation with iron would influence iron delivery to cells through the action of TfRs. In these situations, uptake of Tf iron through pathways not involving TfRs may exhibit increased importance (215).

TfRs have been found on many but not all cell types in the body (84). The importance of TfRs in iron uptake is supported by the observations that the number of receptors displayed on the cell surface is proportional to iron uptake and that iron deficiency induces TfR gene expression (reviewed in 3). Surface display of TfR is affected by its total cellular concentration, as well as its distribution and rate of recycling between the cell surface and cell interior. The efficiency of TfR function can be affected by other proteins, including SFT (stimulator of iron transport) and HFE. SFT stimulates iron uptake by the Tf and non-Tf pathways, whereas HFE appears to reduce Tf-dependent iron uptake in some cell systems (93, 137, 169, 189).

Factors including and independent of iron have an influence on TfR expression transcriptionally and post-transcriptionally. In response to an increased need for iron, as occurs during iron deficiency, the onset of cell proliferation, or as a consequence of differentiation of some hematopoietic cells, the level of TfRs is increased (reviewed in 3, 134). Variations in iron status of cells in culture alters the total cellular abundance of TfR protein through changes in its rate of synthesis (30, 147). The predominant affect of iron in influencing TfR synthesis occurs through alterations in TfR mRNA stability, although some modulation of TfR gene transcription also occurs (66, 105, 126).

Cellular Iron Use

Generally speaking, there are three possible fates for iron once it is delivered to the cytoplasm: (*a*) It is used metabolically for the synthesis of iron-containing proteins; (*b*) it is stored; or (*c*) it is exported from the cell. The extent to which iron delivered to the cytoplasm is then directed along metabolic pathways rather than being incorporated into the iron storage protein ferritin likely depends on the amount of iron taken up, as well as the iron status and metabolic needs of the cell. Iron-containing proteins are present in numerous locations within the cell, including the cytosol and a number of organelles. One critical location of intracellular iron use is the mitochondrion. This organelle is the site of iron incorporation into protoporphyrin IX (PPIX) to form heme, as well as the location where metal centers for heme and/or nonheme (e.g. iron-sulfur proteins) are assembled and inserted into proteins that are required for mitochondrial function. In liver and especially erythroid cells, a large portion of iron delivered to the cytoplasm is ultimately used for heme formation. Heme formation is modulated by coordinating the synthesis of PPIX with the availability of iron and/or the intracellular level of heme (57, 176). The

rate-limiting enzyme for heme formation in most if not all cells is 5-aminolevulinate synthase (ALAS). Control of ALAS synthesis is a key factor in modulating heme formation by erythroid and nonerythroid cells. Understanding the mechanisms of intracellular transport of iron and its incorporation into heme and nonheme iron proteins represents a critical area for future research in iron metabolism.

Although the sites of and machinery used for formation of iron centers in iron-containing proteins have not been fully elucidated, important advances are being made. The components required for formation of iron centers in mitochondrial and cytosolic iron-sulfur proteins are beginning to be defined (125, 135, 197, 203, 231). It is interesting that recent work in yeasts and humans in relation to the disease Friedrich's ataxia has indicated that mitochondria have a dynamic non-protein-bound, nonheme iron pool that functionally interacts with the cytosolic iron pool (127, 180, 190). This and other work has identified components of mitochondrial iron uptake and efflux pathways (7, 50, 136). However, much remains to be understood concerning the transport of iron to the intracellular sites where these proteins are assembled. It seems likely that protein chaperones will transport iron to specific intracellular sites, as is the case for copper (171).

In addition to controlling the flow of iron towards metabolic use, an important component of the means for establishing iron homeostasis is through alterations in iron storage capacity. Modulation of iron storage can occur through changes in synthesis and, in some cases, degradation (9, 157, 178, 184, 207) of ferritin. In most vertebrates, ferritin is a multisubunit shell composed of 24 subunits of two types, H and L. Ferritin provides a means for protecting cells from the toxic effects of iron and for storing the metal in a safe and available manner. Ferritin shells with different subunit composition are called isoferritins, and both heteropolymers and homopolymers exist (reviewed in 99). Each ferritin shell is capable of storing several thousand iron atoms. The two ferritin subunits have different roles in iron metabolism. H-ferritin is a ferroxidase, and this activity promotes the more rapid incorporation of iron into ferritin shells that are rich in this subunit. Isoferritins rich in L-ferritin take up iron more slowly but appear to hold onto it longer, perhaps explaining why iron storage tissues like liver and spleen are L-rich with respect to their ferritin shells. Production of ferritin protein is directly coupled to iron status, primarily through changes in translation of a pre-existing pool of H- and L-ferritin mRNAs, thereby allowing cells to respond rapidly to iron excess or deficiency (68, 105, 126). Translational regulation of H- and L-ferritin synthesis by iron occurs in a coordinated manner, because of the similar mechanism for regulating translation of these two mRNAs (see below). When ferritin synthesis is activated, the actual rate of production of the two subunits depends on the abundance of their mRNA, which varies in a tissue- and cell-type-specific manner.

Iron Export

Although much work has focused on the mechanisms by which iron uptake can be regulated, comparatively less attention has been given to the pathways by which

the mineral is released from cells. In liver, Kupffer cells appear to release iron that is processed from senescent erythrocytes, in the form of ferritin, so that it can be taken up by hepatocytes (202). Hepatocytes in culture release iron spontaneously and in response to chelators (11). However, it is not entirely clear how iron in hepatocyte ferritin is transferred to extracellular apo-transferrin (230). In addition, much remains to be understood concerning how iron is transported through duodenal mucosal cells, bound to Tf, and released to the circulation. Unlike other cell types, apo-Tf can be internalized at the basolateral surface of intestinal epithelial cells to pick up iron for export (8). This event may well be coordinated with the action of the iron exporter ferroportin 1/IREG1 (60, 149). In addition, recent studies on the physiological relationship between iron and copper metabolism have provided important details that have expanded our understanding of these phenomena. Copper deficiency leads to an accumulation of iron in several tissues (reviewed in 65). Depending on the tissue, the multicopper oxidases ceruloplasmin and hepaestin may have critical roles in iron export.

CONTROL OF IRON METABOLISM AT MULTIPLE LEVELS OF GENE REGULATION

Animal cells differ somewhat from plants and lower eukaryotes in the means through which they control iron metabolism. Plants and lower eukaryotes maintain iron homeostasis primarily through transcriptional means. For instance, in *Saccharomyces cerevisiae*, the iron-regulated transcription factor AFT1 controls production of multiple gene products that are needed to assemble the high-affinity iron transport system (64). In a similar way, in plants the iron storage protein ferritin is transcriptionally regulated by iron, which contrasts with its means of regulation in animal cells (179). Animal cells appear to use post-transcriptional control of iron metabolism in nearly all cell types in the body. Regulation of gene transcription appears to have a more important role in cell-type-specific modulation of iron homeostasis. This includes the tissue-specific regulation of the expression of H- and L-ferritin, an erythroid-specific isoform of 5-aminolevulinate synthase, or in controlling the relative expression of IRP1 and IRP2 between tissues. Hence, mammalian iron homeostasis is maintained through integrated use of sensory and regulatory systems that operate at multiple levels of gene regulation.

Transcriptional Control of Mammalian Iron Metabolism

Alteration of ferritin gene transcription provides an important means through which the relative abundance of the ferritin subunits can be modified to meet the unique iron storage and/or detoxification needs of specific tissues. The ratio of the abundance of the H- and L-subunits varies between tissues, and this variation likely arises owing to tissue-specific differences in rates of transcription of the ferritin genes (21, 216). Ferritin gene transcription can be modulated by iron-dependent

and iron-independent factors. Iron excess can have a discordant effect on transcription of the ferritin genes as shown by the selective increase in L-ferritin gene transcription in liver in response to iron excess (225). However, in other systems, H- and L-ferritin transcription is altered to a similar extent by iron (34). Transcription of the ferritin genes is also modulated by a number of iron-independent signaling pathways. Several hormones, including tumor necrosis factor-α, thyrotropin, thyroid hormone, and insulin, preferentially affect H-ferritin gene transcription (42, 115, 145, 221, 229). The *E1A* oncogene and c-*MYC* protooncogene repress H-ferritin expression, and these changes appear to be part of a controlled program for altering cellular iron metabolism in relation to cell proliferation (216, 227). It is interesting that the reduction in H-ferritin transcription appears to be necessary for c-*MYC* to increase cell proliferation and transformation. Transcriptional regulation of ferritins is also important during differentiation of some hematopoietic cells (16, 45, 209).

Synthesis of Tf and TfR is restricted to fewer tissues than are the ferritins, suggesting a key role for transcription in dictating their expression in specific tissues (29, 117, 172, 192, 232). The transcription of both genes is induced in iron deficiency (143, 150, 151, 181, 209). However, regulation of mRNA stability is the primary determinant of TfR expression in iron deficiency in many cell types. In contrast, transcription appears to be a more critical determinant of iron regulation of Tf expression in iron deficiency, although translational regulation may be important in reducing Tf synthesis when iron is in excess (2, 46, 47, 150). Tf and TfR gene transcription are enhanced during hypoxia to increase iron delivery to the erythron, thus to promote erythropoiesis and increase oxygen-carrying capacity (142, 186, 206). Transcription has a greater role in modulating TfR expression during erythropoiesis than in many nonerythroid cells. This may allow for increases in TfR expression without maximal induction of IRP activity and thereby permit simultaneous expression of the erythroid isoform of ALAS [eALAS (32, 41)]. TfR gene transcription is also influenced by the growth state of cells, such as when lymphocytes are stimulated to proliferate (162, 170, 199). Elements of the TfR gene promoter required for modulating its expression during growth stimulation are being defined (15, 162). Clearly, regulation of the transcription of the H- and L-ferritins, Tf, and TfR genes makes important contributions to the maintenance of cell and organismal iron homeostasis.

Iron Regulatory Proteins and the Coordination of Iron Homeostasis

Although in many situations changes in rates of transcription help establish the final level of mRNA-encoding proteins that control the uptake or metabolic fate of iron, IRPs perform a critical role in maintaining body iron homeostasis by coordinating the use of many of these mRNAs. IRPs are considered central regulators of mammalian iron metabolism because they regulate the synthesis of proteins that is

TABLE 1 Known and potential targets of iron regulatory protein action

mRNA	Location of iron regulatory element	Function of encoded protein
Known targets		
Transferrin receptor	3′ UTR	Iron uptake
H- and L-ferritin[a]	5′ UTR	Iron storage
eALAS	5′ UTR	Heme formation
Mitochondrial aconitase	5′ UTR	Tricarboxylic acid cycle
Succinate dehydrogenase iron protein subunit[b]	5′ UTR	Tricarboxylic acid cycle
Potential targets		
DMT1[c]	3′ UTR	Iron transporter
Ferroportin 1/IREG[d]	5′ UTR	Iron transporter

[a]In *Drosophila* spp., splice variants of ferritin mRNAs exist that lack the iron regulatory element (IRE) (141).

[b]Only in *Drosophila* spp., not in humans (128).

[c]Divalent metal transporter 1 (DMTl) transports a number of divalent metals including iron. It is required for lumenal uptake of iron in the duodenum and endosomal release of iron in erythroid and perhaps other cell types. The IRE-like sequence in DMTl mRNA may be functional in some but not all circumstances (74, 76, 89, 96, 219).

[d]Ferroportin 1/IREGl contains a 5′ IRE but iron modulates its mRNA level. It is an iron exporter present at the basolateral side of intestinal mucosal cells (60, 149).

required for the uptake, storage, and use of iron by cells (Table 1). First, it is well accepted that IRPs are critical determinants of the post-transcriptional regulation of TfR expression. Second, IRPs have a major role in determining the iron storage capacity of cells by regulating translation of both H- and L-ferritin mRNA. Third, translation of the mRNA for the eALAS also appears to be regulated by IRPs. In this role, IRPs may coordinate PPIX formation with the availability of iron. Therefore, IRPs may be important modulators of iron cycling in the body. In addition, the mRNA-encoding DMT1 and ferroportin 1/IREG1 contain iron-responsive element (IRE)-like sequences, suggesting that IRP might possibly affect the use of these mRNAs (60, 89, 149). DMT1 expression is iron regulated in some but not all situations (76, 96, 219), whereas other studies indicate that ferroportin 1/IREG1 mRNA abundance responds to alterations in iron status (149). Should the IREs in DMT1 and ferroportin1/IREG1 mRNA prove to be functional, as is the case for TfR mRNA, than IRPs would likely be major modulators of the transmembrane transport of Tf and non-Tf iron. Whereas IRP action is a critical factor regulating mRNA use, alterations in gene transcription between tissues or in response to specific stimuli have a critical effect on the ultimate level of production of the proteins of iron metabolism. In some circumstances alterations in ferritin and TfR gene transcription can alter or override the post-transcriptional regulation of these genes by IRPs (16, 37, 170, 187, 209).

CYTOPLASMIC CONTROL OF FERRITIN AND TRANSFERRIN RECEPTOR SYNTHESIS

In the mid-1960s, several investigators discovered that iron induction of ferritin synthesis did not require transcription. The elucidation of the mechanism by which iron regulates ferritin synthesis led to the discovery of IREs and iron-regulatory proteins (IRPs) and the role that this unique system has in the modulation of cellular iron utilization. A number of other reviews have covered the distant and more recent history of this area (68, 105, 126). This review first provides a summary of the regulation of ferritin and TfR expression by IRPs and primarily focuses on developments in this field in the last 5 years.

Regulation of Ferritin and Transferrin Receptor Synthesis by the Iron-Regulatory Element/Iron-Regulatory Protein System

Iron regulates the synthesis of ferritin and TfRs largely through the regulated interaction of cytosolic RNA-binding proteins, IRPs, with the 28-nucleotide IRE in their mRNA (Figure 1). IRPs recognize IRE in a sequence- and structure-specific manner. The IRE is a stem-loop structure composed of a 6-nucleotide CAGUGX loop at the end of an RNA helix. Recent evidence suggests that internal base pairing between the first and fifth nucleotides of the loop results in the formation of a smaller three-nucleotide loop with a bulge nucleotide on its 3′ side (1, 33, 85, 101, 102). The RNA helix or stem contains a bulged-nucleotide region that is five base pairs 5′ of the first nucleotide of the loop, which is a critical determinant in the recognition of IREs by IRPs. H- and L-ferritin mRNAs have a single IRE in their 5′-untranslated region (UTR), very close to the 5′ cap structure, whereas TfR mRNAs contain five IREs in their 3′ UTRs. Subtle differences in IRE structure appear to be important in determining the hierarchy of regulation of various IRE-containing mRNAs (see below). Additional sequences flanking the IREs may also influence ferritin production in response to iron and other factors (59, 185).

When IRPs are active for RNA binding, as is the case under iron-limiting conditions, IRP1 or IRP2 binds to the IRE in ferritin mRNA and blocks its translation. When IRPs are bound to a 5′ IRE, the cap-binding complex (eIF4F) binds to the mRNA but cannot make functional contact with the 43S preinitiation complex (156). Hence, in the repressed state, ferritin mRNAs appear to have entered the initiation pathway but are blocked at an early step. The presence of the cap-binding complex on the repressed mRNA may provide a mechanism that promotes efficient competition for translation after IRP inactivation (156). In a similar manner, evidence suggesting that IREs may be stimulators of translation, in the absence of IRP binding, also may explain the efficiency with which ferritin mRNAs compete with other cellular mRNAs for translation when the iron level is high (58). The efficiency with which the IRPs and IREs modulate mRNA translation is reflected in the 50- to 100-fold range of iron regulation of ferritin expression (44, 201).

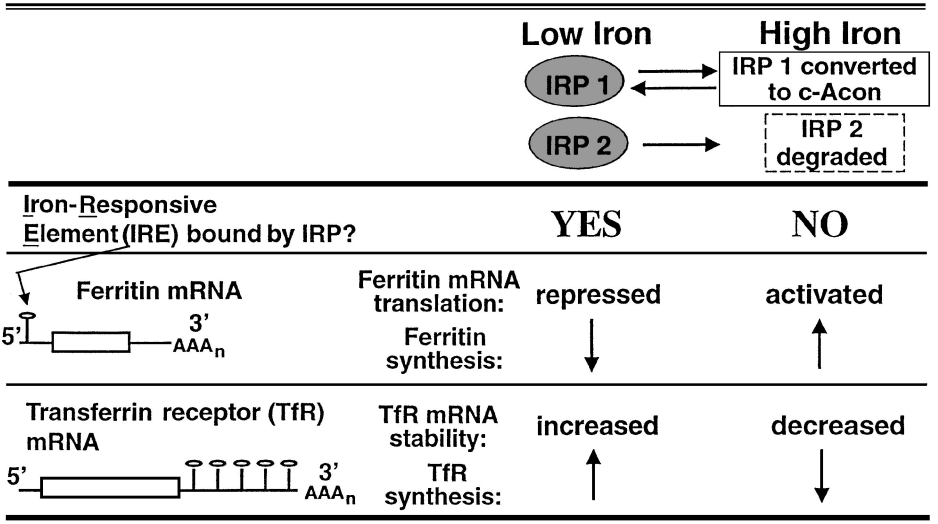

Figure 1 Iron regulatory proteins (IRPs) control the translation or stability of ferritin and transferrin receptor (TfR) mRNA, respectively. Ferritin mRNA contains a single iron-responsive element (IRE) in its 5′ untranslated region. IRP binding blocks the binding of the 43S preinitiation complex to the mRNA. TfR mRNA contains five IREs in its 3′ untranslated region. IRP binding to the TfR IRE stabilizes the mRNA presumably by blocking access of a nuclease system to the rapid-turnover determinant in the 3′ untranslated region. When iron levels are high, IRP1 is converted to cytosolic aconitase (c-Acon), which fails to bind RNA with high affinity. In contrast, IRP2 is degraded when iron levels rise.

In contrast to ferritin mRNAs, IRPs indirectly modulate TfR mRNA translation by influencing its rate of degradation (Figure 1). Two regions within the 3′ UTR of TfR mRNA are required for iron regulation of its stability. These include the IRE, of which only three appear to be required, and a region referred to as the rapid turnover determinant (reviewed in 105, 126). When IRPs are bound to TfR mRNA, they retard its degradation, and the message has a half life of ∼6 h. When IRPs are not bound to the mRNA, it has a much shorter half life (<1 h). Unlike most eukaryotic mRNAs examined to date, the degradation of TfR mRNA does not appear to be initiated by deadenylation (24). Instead, TfR mRNA degradation appears to be initiated by endonucleolytic cleavage at a site near one of the IREs and within the rapid turnover determinant (24). TfR mRNA degradation requires other *trans*-acting factors, including a putative RNA polymerase III transcript as well as a short-lived protein that is required for IRP inactivation (177, 198, 199). Much remains to be understood about the control of TfR mRNA stability, including the role of other post-transcriptional steps (199) and the identification of a nuclease system that degrades the mRNA when iron levels are high. Finally, the newly discovered protein TfR2 is encoded by an mRNA

that lacks IREs, indicating that in some circumstances regulation of the number of Tf-binding sites in the cell would not be controlled by IRP (119). In contrast, expression of TfR2 appears to be refractory to alterations in iron status, at least in liver (75).

Additional Targets for Iron-Regulatory Protein Action

Further appreciation for the role of IRPs as central regulators of iron metabolism has come from the discovery that additional mRNAs possess functional or putative IRE. In the context of discussing these additional real or potential mRNA targets of IRPs, it is important to delineate what defines an IRE. They must be defined not only by structural comparison to accepted IREs such as those in ferritin and TfR mRNA but also by results demonstrating the functionality of the element in question. The prime structural determinants of known functional IREs are the CAGUGX loop sequence and the presence of an unpaired or "bulged" cytosine residue that is five basepairs 5′ of the first nucleotide of the loop. Recent evidence with RNA selection procedures indicates that alterations in the loop sequence can be tolerated in vitro, but no natural IREs containing these modified sequences have yet been identified (33, 100, 102). The second critical area in defining a new IRE concerns function. For translational regulation, the following issues are important. Is the natural mRNA translationally regulated by iron in intact cells? Can IRPs affect translation of the mRNA in vitro? Can the putative IREs confer iron dependence on the translation of a heterologous mRNA? For IREs suspected of modulating mRNA stability, is there evidence that the stability of the natural mRNA is iron regulated? Does the natural mRNA contain an instability determinant such as the rapid turnover determinant of TfR mRNA? Whether one suspects regulation of stability or translation, does mutation of the putative IRE in its native sequence context lead to loss of iron regulation? Can a *cis* effect of the putative IREs on transcription of the natural gene be excluded? Does the putative IRE bind IRPs with high affinity? Is the element conserved evolutionarily? Both structural and especially functional evidence are required to identify new targets of IRP action.

Dierks was the first to identify an IRE-like element in the 5′ UTR of the mRNA encoding eALAS (57). In 1991, two other groups reported the functional analysis of this IRE (48, 53; reviewed in 105). These observations generated significant excitement because erythroid heme formation represents the major daily use of iron in the body. More recently it has been shown that the IRE in eALAS mRNA is evolutionarily conserved among vertebrates (63). The eALAS IRE binds IRP specifically but with ∼10-fold–lower affinity than the ferritin IRE. When linked to a heterologous mRNA, the eALAS IRE confers iron regulation on the translation of the reporter mRNA in transfected cells (53). Translation of eALAS mRNA is iron regulated in erythroleukemia cells, and the eALAS synthesis rate is stimulated by iron (152). Purified IRP1 represses translation of eALAS mRNA in vitro and is much less efficient at repressing translation

of an eALAS mRNA with a mutated IRE (23). These observations predict that eALAS synthesis is regulated by iron to coordinate PPIX synthesis with iron availability. This contrasts with the regulation of the housekeeping form of ALAS, which appears to be primarily feedback regulated by intracellular heme level (176).

The mRNAs encoding two enzymes in the tricarboxylic acid cycle appear to be targets of IRP action. In *Drosophila* species, but not humans, the mRNA for the succinate dehydrogenase iron-protein subunit has an IRE in its 5′ UTR (129). In several mammals, the mRNA encoding the mitochondrial isoform of aconitase (m-acon) also contains an IRE in its 5′ UTR (53, 88, 124, 233). m-Acon is the tricarboxylic acid cycle enzyme that converts citrate to isocitrate. Like the majority of mitochondrial proteins, m-acon is encoded by a nuclear gene, its mRNA is translated in the cytoplasm, and the primary translation product is targeted to the mitochondria. IRP binds with high affinity to the m-acon IRE, resulting in translational regulation of the mRNA in cell-free systems (88, 124). Iron deficiency results in a decrease in the abundance and activity of m-acon in rat liver in a time- and dose-dependent manner (43, 44, 124). Furthermore, m-acon synthesis is translationally regulated by iron in HL 60 cells (195). Whereas the evidence strongly suggests that m-acon synthesis is modulated by IRP, the metabolic purpose remains unclear. The theories advanced include proposals that IRP modulates ATP production, reduces mitochondrial generation of free radicals, or modulates the use of citrate in iron metabolism or other aspects of cell function (43, 88, 124).

Although the evidence strongly suggests that eALAS and m-acon mRNA are targets of IRP action, the case is far from clear for other mRNA. One poignant example is DMT1. As noted previously, DMT1 has a critical role in transport of iron across the brush border membrane of the small intestine, as well as across the endosomal membrane of erythroid cells (39, 83, 204). The putative IRE in DMT1 is in the 3′ UTR and contains an unusual bulge nucleotide structure that is not present in other IREs (89). It is of interest that two DMT1 RNA transcripts are produced, with only one containing an IRE (138). The level of the IRE-containing transcript is increased sevenfold in duodenal cells from iron-deficient mice, whereas the DMT1 mRNA lacking an IRE is not affected (76). In a similar manner, the level of DMT1 mRNA was decreased in Caco2-cells treated with iron-transferrin (96). However, in mouse fibroblasts, DMT1 mRNA is not regulated by iron even though IRP activity and TfR mRNA levels changed markedly (219). Thus, it is not possible to conclude at this time that the DMT1 IRE is functional. Further caution in concluding that DMT1 mRNA is IRP regulated is in order, given the case of glycolate oxidase (GOX) (129). GOX complementary DNA was cloned after its mRNA was purified, using an IRP1 affinity column. GOX mRNA contains an IRE-like sequence, but, somewhat like DMT1, it contains alterations in the stem structure. However, this stem-loop region in GOX mRNA appears not to be an IRE because it fails to confer iron regulation on the translation of a heterologous mRNA. Hence, at this time it

is not possible to conclude that DMT1 or ferroportin 1/IREG1 mRNA is regulated by IRP because the functionality of their IREs has not been directly demonstrated.

TO DEGRADE OR NOT TO DEGRADE: THAT IS THE QUESTION!

IREs influence mRNA translation or stability because of their sequence-specific and iron-dependent interaction with IRPs. Under the appropriate conditions, IRPs bind with high affinity ($K_D \sim 0.02$–0.1 nM) to the IRE stem-loop structure. Factors that affect the heme and nonheme "chelatable" iron pools influence IRP function (67, 146, 213). However, there is a fundamental difference in the mechanism of iron regulation of IRP1 and IRP2.

The Aconitase Model of Iron Regulatory Protein1 Function

IRP1 is a bifunctional protein with mutually exclusive functions as an IRE RNA-binding protein or as the cytoplasmic isoform of aconitase (c-acon). Aconitases are iron-sulfur [Fe-S] proteins and a [4Fe-4S] cluster is required for their enzymatic activity (19). The crystal structure of m-acon indicates that its Fe-S cluster sits in a solvent-accessible cleft between one of two regions of the protein. One region contains domains 1 to 3, and a second region containing domain 4. The solvent accessibility of the Fe-S cluster is important in the regulation of aconitase function by low-molecular-weight perturbants such as nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet -}$). m-Acon is known to have a labile iron atom, Fe_a, which is susceptible to attack converting the protein to the [3Fe-4S] form (19). IRP1 is predicted to have a similar cleft, and the extent to which the cleft is closed or open is believed to be affected by the formation or complete loss of the [4Fe-4S] cluster (126; Figure 2). It was initially proposed that an iron-dependent switch between the [3Fe- 4S] and [4Fe-4S] forms regulated the RNA-binding activity of IRP1. However, it is clear that the presence or complete absence of the Fe-S cluster regulates the RNA-binding function. The putative cleft in IRP1/c-acon is believed to contain functionally overlapping regions required for RNA binding or aconitase activity (13, 107, 118, 175, 205). The reduced apoprotein binds RNA with high affinity (K_D 20–100 pM), whereas the c-acon form with its [4Fe-4S] cluster exhibits low affinity for RNA ($K_D \sim 2$–4 nM) but has aconitase activity. The [3Fe-4S] form does not bind RNA with high affinity (94). Taken together, these studies have expanded the appreciation of the biological roles of Fe-S clusters and focused attention on the aconitase family of proteins (78). The recent demonstration that the aconitases from *Bacillus subtilus* and apparently from *Escherichia coli* are RNA-binding proteins and that they may modulate energy and/or iron metabolism in this organism suggests that roles for aconitases in metabolism and gene regulation have been present for some time (5, 52, 208).

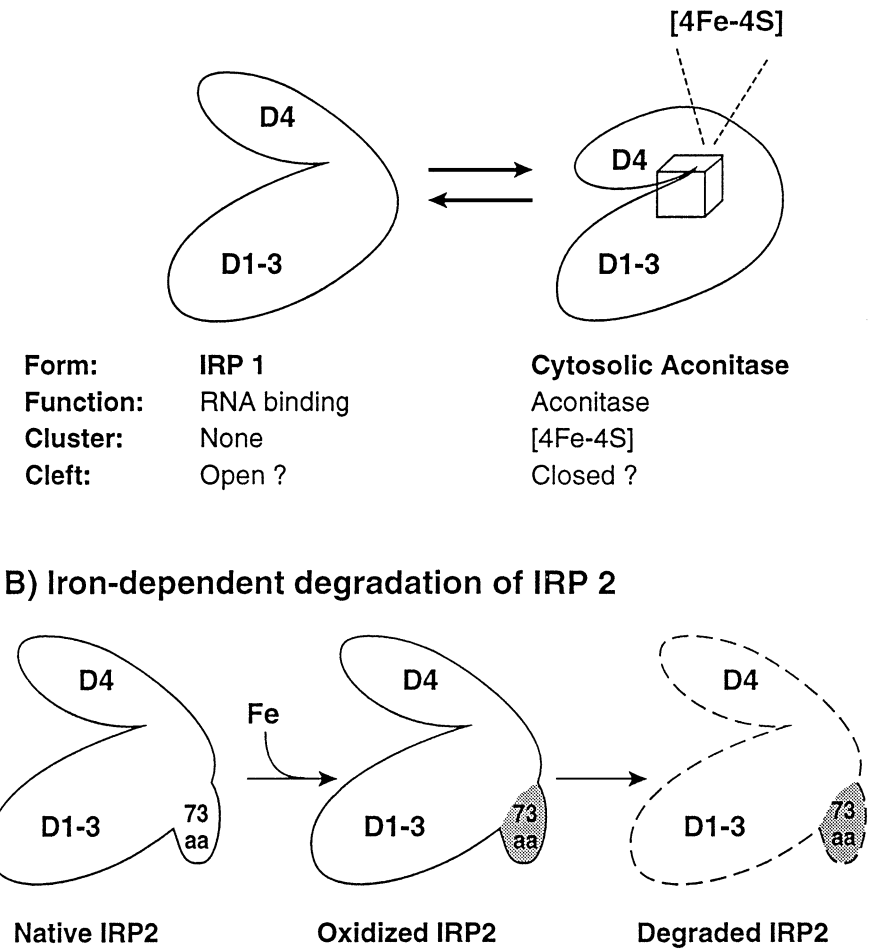

Figure 2 Iron regulates the RNA binding activity of iron regulatory proteins 1 (IRP1) and 2 (IRP2) by different mechanisms. *A.* IRP1 is believed to have a structure similar to that of mitochondrial aconitase. The crystal structure of mitochondrial aconitase indicates that it contains four domains with a cleft forming between domains 1–3 (D1-3) and domain 4 (D4). IRP1 does not contain an Fe-S cluster but binds iron regulatory element RNA with high affinity. When iron levels increase, a [4Fe-4S] Fe-S cluster is assembled in IRP1, converting it to cytosolic aconitase. Cytosolic aconitase converts citrate to isocitrate. The total cellular level of IRP1/cystolic aconitase does not change when iron levels change; the percentage of the protein in the IRP1 or cystolic aconitase state does change. *B.* IRP2 is believed to have a similar structure to IRP1 except for the presence of a 73-amino-acid insert. Iron is believed to bind to Cys residues in the 73-amino-acid region of IRP2 and induce oxidation of this and/or other regions of the protein (*shaded area*). Oxidation is followed by proteasomal degradation of IRP2.

Iron Regulates Iron Regulatory Protein 2 Through a Protein Degradation Mechanism

IRP1 is a long-lived protein whose degradation rate and cellular abundance do not appear to be altered by cellular iron status (92, 163, 191). In contrast to IRP1, the abundance of IRP2 is decreased, and its rate of degradation is greatly increased in iron-treated cells (92, 191). IRP2 is 61% identical to IRP1 in amino acid sequence (90, 191). A notable exception is the presence of a unique 73-amino-acid sequence in IRP2. This motif is an iron-dependent degradation signal for IRP2, and it can confer iron-regulated degradation when transferred to a heterologous protein (114). The 73-amino-acid sequence contains three Cys residues that appear to be required for iron regulation of IRP2 degradation (114). Iron appears to induce oxidation of IRP2 perhaps after binding to the 73-amino-acid region, and this is followed by ubiquitination and proteasomal degradation of the binding protein (91, 112, 113; Figure 2). It is interesting that IRP2 expression can also be controlled through transcriptional means (132).

Consequences of Dysregulation of the Iron Regulatory Element-Iron Regulatory Protein System

Mutations in the IRPs or in the IRE sequences with which they interact may lead to a failure to properly maintain some aspects of cellular iron metabolism. Cys437 of IRP1 is a residue that is critical for the formation of the Fe-S cluster in c-acon and for RNA binding by IRP1 (reviewed in 105, 126). When this or one of the other Cys that ligate the Fe-S cluster to the protein is mutated to Ser, the Fe-S cluster cannot form, and IRP1 is constitutively present as an RNA-binding protein. Expression of the Cys437Ser mutant of IRP1 leads to loss of regulation of ferritin and TfR expression (55). Even in the presence of excess iron, these cells maintain high levels of TfR mRNA and low levels of ferritin protein. Studies of the effect of expression of a TfR mRNA lacking the IRE region in its 3′ UTR have also demonstrated the importance of the IRP-dependent modulation of TfR expression in the maintenance of cellular iron homeostasis. Cells expressing this mutant TfR mRNA become hypersensitive to oxidative stress (158). Finally, recent evidence indicates that overexpression of the ferritin IRE itself can perturb cellular iron levels (79).

A remarkable natural example of dysregulation of the IRE/IRP system is seen in the hyperferritinemia-cataract syndrome (17, 87). Numerous reports have identified novel mutations within or in the region flanking the L-ferritin IRE (summarized in 6, 139). Mutation of the L-ferritin IRE is associated with high levels of the serum and tissue forms of L-ferritin in the absence of iron overload. There appears to be a significant relationship between the clinical severity of the disease and the degree to which the IRE/IRP interaction is disrupted (6, 40). Overexpression of L-ferritin in the lens frequently leads to development of cataracts in the first decade of life, perhaps owing to derangement of the solubility of other lens proteins or to redox effects of iron (139). No significant effects on body iron metabolism are

apparent, and it has been suggested that this relates to the different roles of the ferritin subunits in iron metabolism (139).

DIVERSITY IN THE FUNCTION OF THE IRON REGULATORY ELEMENT–IRON REGULATORY PROTEIN SYSTEM

Several potential metabolic advantages of using two IRE-binding proteins to help maintain iron homeostasis can be envisioned. First, variety in the means by which individual IRPs are regulated expands the circumstances in which these proteins may help maintain iron homeostasis. Second, specificity in the targets that each IRP acts on may permit selective and/or hierarchical regulation of specific mRNA. Third, the presence of multiple IRPs permits redundancy in their function and might allow for continued maintenance of iron homeostasis in the absence of the expression of a given IRP (194). Here I first examine the signaling pathways that lead to selective and/or divergent regulation of IRP1 and IRP2 and then examine the evidence that IRPs appear to discriminate in the IRE-containing mRNA on which they act.

Selective Effects of Nitric Oxide on Iron Regulatory Protein 1 and Iron Regulatory Protein 2

It has been apparent for many years that many Fe-S proteins are sensitive to agents that disrupt their Fe-S cluster, such as free radicals, nitric oxide, and superoxide ($NO^{\bullet}$, $O_2^{\bullet -}$) and other perturbants like oxygen or hydrogen peroxide (O_2, H_2O_2; 18, 77, 82). It has been suggested that the solvent accessibility of the Fe-S cluster of aconitases and some other Fe-S proteins permits them to be modulators of cellular energy metabolism in response to oxidative stress, as well as be a target of cytotoxic or signaling agents (61, 62, 77, 82, 223). The role of the Fe-S cluster in the switch between IRP1 and c-acon suggested a new function for perturbants that disrupt the Fe-S cluster as signaling agents that could influence iron metabolism. Oxygen and/or $O_2^{\bullet -}$ were known to induce conversion of the [4Fe-4S] form of aconitases to the [3Fe-4S] form, and this process can be reversible in vivo (77, 80, 122). However, generation of IRP1 requires complete loss of the cluster, and until recently the extent to which these and other agents could directly or indirectly induce formation of the apo form of c-acon (IRP1) in vivo was not clear.

The effects of $NO^{\bullet}$ on IRP with consequent effects on iron metabolism appear to be important in macrophage activation (62, 223) and hepatic inflammation (36, 174), and they may influence erythropoiesis (161). Several investigators have focused on the extent to which $NO^{\bullet}$ influences the conversion of c-acon to IRP1, because of the known ability of $NO^{\bullet}$ to bind transition metals and the fact that m-acon was an established target of $NO^{\bullet}$ released by activated macrophages (26, 61, 103).

Activation of macrophage cell lines with interferon-γ and lipopolysaccharide results in generation of NO$^\bullet$, an increase in IRP1 activity, and a decrease in c-acon activity (62, 223). It is interesting that iron status can influence expression of the inducible nitric oxide synthase in the J774 macrophage cell line in a manner that suggests the presence of a regulatory loop between iron metabolism and NO$^\bullet$ production (224). NO$^\bullet$ gas and chemical generators of NO$^\bullet$ can convert recombinant c-acon to IRP1 (62, 160), and it has been established that NO$^\bullet$ can directly cause disassembly of the Fe-S cluster of c-acon (81, 121). NO$^\bullet$ and its derivatives, such as peroxynitrite, can convert c-acon to IRP1 as long as a proper reductant is present (26, 27, 160). The ability of NO$^\bullet$ to influence IRE-binding activity depends on the proportion of IRP1 that is in the aconitase vs RNA binding forms (220). However, the effects of NO$^\bullet$ on IRP2 appear to be cell type specific because NO$^\bullet$ can activate or have no effect on this binding protein, perhaps owing to various effects of NO$^\bullet$ on iron release from cells (157, 168, 174). Other redox-related species of NO$^\bullet$ may modulate IRP functions through alternative mechanisms (183). The activation of IRP1 induced by interferon-γ and lipopolysaccharide or by chemical sources of NO$^\bullet$ was associated with a decrease in ferritin synthesis and an increase in TfR mRNA levels (164, 168, 174). However, many physiological agents that stimulate NO$^\bullet$ production ultimately influence the expression of ferritin and/or TfRs by both IRP-dependent and IRP-independent pathways (36, 174, 182). For instance, TfR mRNA increased in response to chemical generators of NO$^\bullet$ but not to interferon-γ plus lipopolysaccharide, even though IRP1 activity was increased in both situations (28, 174, 182). How interferon-γ and lipopolysaccharide reduced TfR mRNA expression independently of IRP1 activation remains to be fully determined, but this may be related to a selective down-regulation of IRP2 in response to these effectors (28, 182). Perhaps TfR mRNA stability is primarily modulated by IRP2 in some cells. These studies also highlight the fact that IRP1 and IRP2 do not always respond identically to the same stimulus.

Oxidative Stress Preferentially Influences Iron Regulatory Protein 1

The presence of the Fe-S cluster in IRP1 provides a mechanism whereby IRP may directly respond to alterations in the level of oxygen or oxygen metabolites. Furthermore, because the mitochondrial electron transport chain is a major source of oxygen radicals in vivo, IRP may be part of a regulatory loop to modulate the level of oxidative stress through their potential ability to influence the synthesis of the tricarboxylic acid cycle enzyme m-acon (88). Despite the apparent predictability of the response of IRP1 to oxidative stress (i.e. loss of the Fe-S cluster), the responses of both IRPs to oxidants are varied. This likely relates to a role of antioxidant defense systems as well as to changes in cellular iron levels overlapping with the oxidative stress effect (38). The evidence suggests that IRP function can be directly or indirectly influenced by oxidants in vitro and in vivo (104, 188).

In 1995 it was reported that treatment of cultured cells with H_2O_2 (50–400 μM) rapidly enhanced IRP1 RNA-binding activity (144, 165) and repressed ferritin

biosynthesis while increasing TfR mRNA levels (165). From these and other findings, it has been concluded that a signaling pathway(s) is activated by H_2O_2 that leads to removal of the Fe-S cluster of IRP1 (166–168). The physiological implications of these results are not completely apparent, because there are multiple possible effects of H_2O_2 and other oxidants on cell function. Activation of IRP1 per se would be expected to lead to enhanced iron uptake and reduced iron storage, which by itself would not seem to be beneficial. Hence, it has been suggested that the H_2O_2 effect is reflective of the action of macrophages in killing target cells or the response to intracellular oxidative stress as may occur owing to mitochondrial dysfunction (167). Oxygen radicals can also stimulate cell growth or induce apoptotic cell death (72, 116). The effect of H_2O_2 on ferritin and TfR expression is also consistent with the iron requirements of proliferating cells. It is relevant to note that H_2O_2 stimulates the proliferation of a number of cell types, perhaps by activating one or more growth factor receptors (54, 106, 200, 212, 228). Hence, the implications of the activation of IRP1 function by H_2O_2 remain to be determined.

Oxidative stress can also inactivate IRPs, apparently by oxidizing critical Cys in IRP1. Generation of $O_2^{\bullet -}$ and H_2O_2 in liver cell extracts with xanthine (X) and xanthine oxidase (XO) results in a major reduction in IRP1 RNA-binding activity (35). Because there appears to be no inactivation of lysate aconitase activity by X and XO and the RNA-binding activity can be recovered by 2-mercaptoethanol, it appears that IRP1 activity can be modulated by reversible oxidation. Subsequently, other investigators showed that oxidized IRP1 binds the IRE poorly (27, 193). These studies suggest that, in the presence of sufficient levels of $O_2^{\bullet -}$ and H_2O_2, IRP1 can be temporarily inactivated. The potential for a reduction in iron uptake and an increase in iron storage capacity would presumably help alleviate the oxidant stress. However, the $O_2^{\bullet -}$ generator menadione can, at low levels, activate IRP1, but when it is present at high levels, it irreversibly inactivates the binding protein (86). Whether this level of oxidative stress can be obtained in physiological systems remains to be determined. A somewhat similar situation is seen with the compound doxorubicinol, a metabolite of the anticancer drug doxorubicin, which irreversibly inactivates the c-acon and IRP1 activities when added to human myocardial extracts (153). It has been suggested that this may be a contributor to the cardiotoxicity of anthracycline drugs. Thus, oxidative stress appears to influence IRP function through direct effects on the Fe-S cluster of c-acon and oxidation of critical cysteines in IRP, as well as through indirect influences such as activation of specific signaling pathways. Further research is needed to fully understand the physiological implications of modulation of IRP function through these means.

Hypoxia and the Modulation of Iron Regulatory Protein Function

The evidence suggests that the damage that occurs to tissues in response to ischemia/reperfusion (i.e. stroke) is due, in part, to iron-catalyzed production of radicals and other toxic oxidants that damage cellular macromolecules (148). Cell culture studies have demonstrated that IRPs respond to changes in oxygen tension.

In rat hepatoma cells, hypoxia causes a rapid post-translational reduction in IRP1 RNA-binding activity without altering IRP2 (98). It is interesting that IRP1, but not IRP2, is refractory to iron regulation during reoxygenation of hepatoma cells (98). It appears that low oxygen tension favors conversion of IRP1 to c-acon, perhaps by decreasing oxidant-induced disassembly of the Fe-S cluster. It is not known whether the failure of iron to reduce IRP1 RNA-binding activity during reoxygenation relates to the known increase in production of oxygen radicals that occurs (148). However, these results contrast with those obtained in rat liver, perhaps because of the short duration of the ischemia (206). Hypoxia results in an increase in total IRE-binding activity in human hepatoma and embryonic kidney cells, but there are differing views of the response of IRP1 (97, 214). There is a significant contribution of IRP2 activation in this situation. In contrast to IRP1, exposure to hypoxic conditions leads to an increase in the level of IRP2 protein (97). It has been suggested that the divergent response of the two IRPs may be indicative of an adaptive response of the cells wherein the use of mRNAs that are preferentially modulated by one IRP could be selectively altered (97).

Novel Effects of Phosphorylation on Iron Regulatory Proteins

Investigations of the role of protein kinase C (PKC) or other kinases in influencing IRP functions were initiated as a result of observations that suggested a relationship between PKC and iron metabolism (reviewed in 68). Treatment of HL60 cells with phorbol 12-myristate-13-acetate stimulates IRE-binding activity twofold within 30 min and results in an increase in TfR mRNA abundance (69, 196). Phosphorylation of and RNA binding by both IRPs were activated (196). Based on the results of a number of biochemical analyses, two potential PKC sites, Ser138 and Ser711, were identified in IRP1 (69). Both sites are within or near regions of the protein known or thought to be involved in the RNA binding or aconitase functions of IRP1. Sequence alignment suggested that these sites were near the entrance to the putative cleft in IRP1. Hence, it is of interest that the apo- or RNA-binding form of IRP1 was a fivefold better substrate for PKC phosphorylation than was the c-acon form (193). These results suggest that IRP1 is the preferred form that is phosphorylated by PKC in vivo, but they do not preclude c-acon as a kinase substrate. However, these results also suggest that phosphorylation by PKC might affect the interconversion of IRP1 and c-acon. Phosphomimetic mutants of IRP1 at position 138 (Ser138Asp or Ser138Glu) displayed a cluster-instability phenotype when converted to c-acon both in vitro and in vivo (31). On exposure to oxygen, the Fe-S cluster in the phosphomimetic mutants of IRP1 disassembled 5- to 20-fold faster than the cluster in the wild-type protein (31). Hence, PKC-dependent phosphorylation may provide a means to alter the set- point at which iron regulates RNA binding by IRP1, by altering the sensitivity of the Fe-S cluster in c-acon to low-molecular-weight perturbants.

These observations have led to the development of a model proposing that phosphorylation of IRP1 permits the protein to be converted to the c-acon form

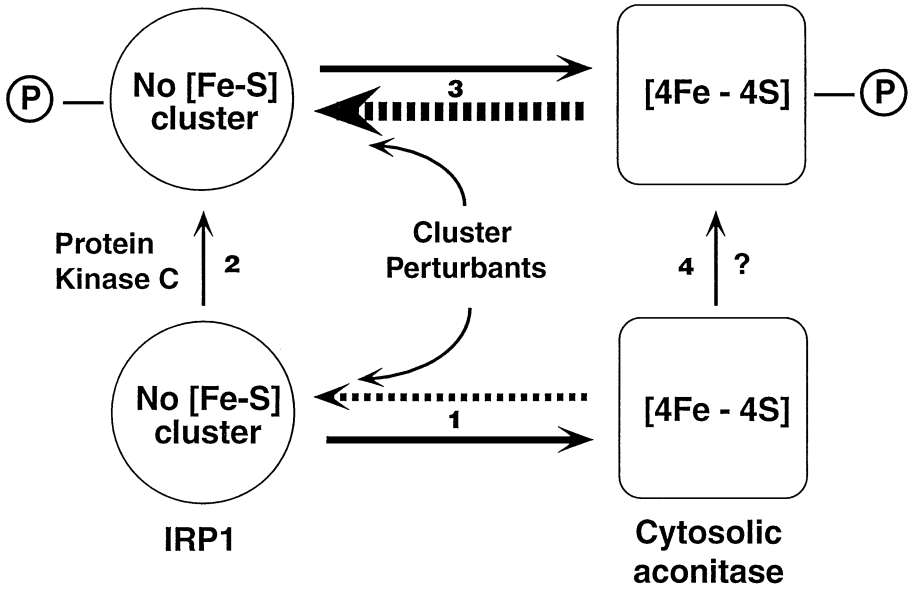

Figure 3 Phosphorylation may alter the sensitivity of cystolic aconitase to cluster perturbants. Changes in cellular iron status lead to formation or loss of the Fe-S cluster (step 1), but phosphorylation of IRP1 (step 2) or possibly cystolic aconitase (step 4) may influence the interconversion of the RNA-binding and aconitase forms of the protein (step 3). IRP1 is the preferred substrate for phosphorylation, compared with cystolic aconitase (step 2). Based on structure function analyses, phosphorylated IRP1 is believed to form an Fe-S cluster that gives rise to phosphorylated cystolic aconitase. Phosphorylated cystolic aconitase appears to display much greater sensitivity to cluster perturbants such as nitric oxide, oxygen, superoxide, or hydrogen peroxide. Certain signaling pathways may lead to phosphorylation of cystolic aconitase, resulting in loss of the Fe-S cluster owing to the action of cluster perturbants (step 4).

but that the phosphorylated form of c-acon is much more sensitive to cluster perturbants such as oxygen, oxygen radicals, and $NO^{\bullet}$ (Figure 3). Phosphorylation may partition c-acon between one form (unstable Fe-S cluster) used for iron-regulated post-transcriptional gene regulation and another form (stable Fe-S cluster) used for metabolic purposes. Furthermore, it is possible that phosphorylation of c-acon might provide a means to promote removal of the Fe-S cluster. In some systems, H_2O_2 can activate isoforms of PKC (72, 130). Perhaps a combination of H_2O_2-induced phosphorylation of c-acon followed by oxidant-induced loss of the cluster puts c-acon on a pathway converting it to IRP1. In addition, these results suggest that constitutively ($O_2^{\bullet -}$) or inducibly ($NO^{\bullet}$) produced cluster perturbants may have a key role in the normal regulation of IRP1 function. They also suggest that iron may regulate IRP1 by a default process. Cluster perturbants that are constitutively produced may continually favor accumulation of the phosphorylated protein in the IRP1 form. If iron levels are low, the IRP1 form predominates; only when

iron levels are sufficiently elevated is the protein converted to c-acon. Support for this model comes in part from studies of the effect of iron deficiency on rat liver IRP (43, 44). In rat liver, most (∼95%) of this protein appears to be in the c-acon form, and very little is present as IRP1. Severe iron deficiency completely represses liver ferritin expression. IRP1 RNA-binding activity is elevated severalfold, but c-acon levels do not decline as would be expected. These results suggest that only a very small pool of c-acon responds to iron deficiency, further suggesting that multiple forms of the protein with inherent differences in stability of the Fe-S cluster may exist.

The activity of IRP2 is also modulated by PKC-dependent phosphorylation (196). Phorbol 12-myristate-13-acetate treatment of HL60 cells stimulates IRP2 phosphorylation and RNA-binding activity. A number of investigators have shown that IRP2 can be redox regulated, with the oxidized form being inactive for RNA binding (27, 100, 173, 196). Phosphorylation stimulates IRP2 RNA-binding activity by increasing the amount of the protein present in a stably reduced and active form (196). The 73-amino-acid region responsible for iron regulation of IRP2 protein degradation contains five Cys residues, a number of which are predicted to be redox active given their proximity to charged amino acids. The presence of putative PKC and other phosphorylation sites close to these Cys residues suggests that phosphorylation may influence the redox potential of these Cys residues and possibly alter the RNA-binding activity of IRP2 (196).

Selectivity in the Modulation of the Targets of Iron Regulatory Protein Action

Given the various functions of the proteins encoded by IRE-containing mRNA, it seems likely that their use would not be regulated in an identical manner by IRP. Two situations stand out. First, both eALAS and the ferritin mRNAs have a 5′ IRE. However, eALAS is involved in iron utilization because it is the rate-limiting enzyme in heme formation, and ferritin is for iron storage. Thus, it seems unlikely that the synthesis of these proteins would exhibit differences in the degree or timing of their regulation by iron. Second, m-acon mRNA also has an IRE in the 5′UTR. Given its role in the tricarboxylic acid cycle, one of the central pathways of cellular energy metabolism, it would be surprising if the translation of its mRNA was as extensively repressed as is the case for ferritin mRNA in response to either low or normal iron levels. In fact, evidence accumulated over the past several years indicates that there is selectivity in the control of the use of various IRE-containing mRNAs. The mechanistic basis for this selectivity appears to lie in subtle variations in IRE sequence or structure between different mRNA species, differences in what each IRP prefers to "see" in an RNA, and perhaps additional, as yet unidentified regulatory sequences in the mRNA of interest that influence IRE function (20, 44, 85, 118, 124, 211).

The evidence indicates that the ferritin IREs differ from the m-acon, eALASs and TfR IREs in the size of the bulge nucleotide region 5 base pairs 5′ of the first nucleotide of the loop (20, 85, 120, 195, 211). The size of a bulged nucleotide

region, sometimes referred to as an internal loop, has significant effects on the secondary and tertiary structure of an RNA helix. These regions affect the extent to which an RNA helix "kinks," as well as the extent to which the major groove of the RNA helix is open and accessible for interaction with a protein or other compounds (154). Evidence from a variety of approaches indicates that the stem of the ferritin IRE has an altered structure compared with the m-acon, eALAS, and TfR IREs (20, 85, 210). Hence, variations in RNA structure may affect the degree of regulation of an IRE-containing mRNA. Additional evidence suggests that IRP1 and IRP2 exhibit selectivity in the RNAs they prefer to bind (33, 101, 102, 120). Changes in the bulge nucleotide region in synthetic IREs selectively influence the binding of IRP1 and IRP2 (120). In this regard, there are a number of examples of differential regulation of targets of IRP action. Most notable is the case of m-acon and ferritin. In response to changes in dietary iron intake, ferritin protein levels undergo a much broader range of regulation than does m-acon in rat liver (66). A similar phenomenon is seen in the effects of hemin on ferritin and m-acon synthesis in tissue culture cells and in a cell-free system (124, 195). Other examples include the observation that IRPs appear to bind less tightly to the eALAS IREs compared with the ferritin IREs (48) and the fact that changes in IRP2, but not IRP1, appear to dictate the changes in TfR mRNA expression which are seen in certain situations (36, 182). It seems likely that differences in IRE structure and the abundance of the mRNA targets of IRP action, as well as the absolute and relative levels of IRP1 and IRP2 and possibly their phosphorylation state (120), will influence the use of specific IRE-containing mRNA.

Unresolved Aspects of Iron Regulatory Protein Function Although it is well established that IRP activity is a critical determinant of the expression of a number of proteins of iron metabolism, many issues are yet to be fully understood. Because the evidence indicates that there can be selective regulation of IRP1 and IRP2, then it is of interest whether there are specific mRNA targets for one or the other IRP (120). These findings also raise the question: Is there "crosstalk" between IRP1 and IRP2 to modulate iron metabolism? Concerning the regulation of IRP function, a number of issues must be addressed. What delivers iron (and sulfide for IRP1) to IRP to promote their inactivation? Recent developments indicate that humans have a homolog of *nif*S to provide sulfide for Fe-S cluster formation (135). However, the state of the apoenzyme (IRP1) in the cytoplasm is not fully defined (68). Does RNA-bound IRP1 contain sulfide? The presence of sulfide in a form of IRP1 that can bind RNA with high affinity would presumably permit a more rapid inactivation of RNA-binding activity than if sulfide were not present. Similar issues arise concerning how the cluster is removed from c-acon to generate RNA-binding activity. Some evidence suggests that iron oxidatively removed from clusters is cytotoxic; it raises the question of whether accessory proteins are required for safe removal of the components of the Fe-S cluster (123). Concerning phosphoregulation of IRP, a number of issues must be addressed. Is phosphorylation required for cluster removal from IRP1 or for RNA binding and/or iron regulation of IRP2? Do phosphorylated IRPs preferentially associate with RNA?

Answers to these and other questions are sure to provide further insight into the post-transcriptional regulation of cellular iron metabolism.

PERSPECTIVE

Knowledge of the mechanisms that are responsible for the maintenance of iron homeostasis has increased considerably since the observations nearly 50 years ago that iron influences ferritin accumulation through changes in its synthesis rate. Advances at the cellular and organismal levels demonstrate the importance of transcriptional and post-transcriptional controls in establishing a regulatory network that permits the safe and efficient use of iron in mammals. IRPs have a critical role in orchestrating the use of numerous mRNAs that encode proteins required for the maintenance of iron homeostasis. Tissue-specific and developmentally regulated differences in transcription are a key modulator of the level of mRNA transcripts on which IRPs can act, in addition to the levels of IRPs themselves. Furthermore, the presence or absence of accessory proteins such as HFE or SFT can influence the function of proteins (e.g. TfR) whose abundance is modulated by IRP activity. Control of iron metabolism at multiple levels of gene regulation provides for specificity and variety in the overall use of iron by various cell types in the body. Future studies are likely to further define the means by which iron and other factors regulate the expression of proteins that influence iron metabolism at the transcriptional and post-transcriptional levels. However, elucidation of the mechanisms responsible for intracellular transport of iron, the proteins required for iron export from cells, and the means by which accessory proteins such as SFT or HFE affect transmembrane transport of iron will be critical in furthering our understanding as to how iron metabolism is controlled. Research in these and other areas is sure to provide further insight into the molecular control of mammalian iron homeostasis.

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