ANTIOXIDANTS & REDOX SIGNALING
Volume 13, Number 10, 2010

DOI: 10.1089/ars.2009.2983

COMPREHENSIVE INVITED REVIEW

Iron Regulatory Proteins: From Molecular Mechanisms to Drug Development

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Abstract

Eukaryotic cells require iron for survival but, as an excess of poorly liganded iron can lead to the catalytic production of toxic radicals that can damage cell structures, regulatory mechanisms have been developed to maintain appropriate cell and body iron levels. The interactions of iron responsive elements (IREs) with iron regulatory proteins (IRPs) coordinately regulate the expression of the genes involved in iron uptake, use, storage, and export at the post-transcriptional level, and represent the main regulatory network controlling cell iron homeostasis. IRP1 and IRP2 are similar (but not identical) proteins with partially overlapping and complementary functions, and control cell iron metabolism by binding to IREs (i.e., conserved RNA stem-loops located in the untranslated regions of a dozen mRNAs directly or indirectly related to iron metabolism). The discovery of the presence of IREs in a number of other mRNAs has extended our knowledge of the influence of the IRE/IRP regulatory network to new metabolic pathways, and it has been recently learned that an increasing number of agents and physiopathological conditions impinge on the IRE/IRP system. This review focuses on recent findings concerning the IRP-mediated regulation of iron homeostasis, its alterations in disease, and new research directions to be explored in the near future. *Antioxid. Redox Signal.* 13, 1593–1616.

Reviewing Editors: *Greg Anderson, Richard K. Bruick, Z. Ioav Cabantchik, Raymond Regan, Kevin L. Schalinske, and Xi-Ming Yuan*

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I. Introduction

Iron readily exchanges electrons and is therefore widely used in nature for a number of enzymatic functions requiring the transfer of electrons via oxidation–reduction reactions. This makes it an essential nutrient that is indispensable for various redox and nonredox functions, including oxygen transport, cell respiration, and DNA synthesis (11, 228). In addition to the oxygen transporting proteins (hemoglobin and myoglobin) that account for most of the iron in the body, there is an impressive list of iron-containing enzymes that encompasses proteins of vital physiological significance (see Table 1 for representative examples of iron-dependent enzymes grouped by physiological functions or pathways). For example, iron is a component of heme in cytochromes but also an essential co-factor for nonheme enzymes such as ribonucleotide reductase, the enzyme limiting DNA synthesis. The large number of members of the Fe(II) and 2-oxoglutarate-dependent dioxygenase family of enzymes suggests the existence of still unknown mechanisms by which iron controls cell functions. In metazoans, the enzymes of this superfamily are involved in a number of processes including oxygen sensing, DNA repair, fatty acid metabolism, and post-translational modifications (26, 87). Moreover, a recent study predicting the existence of novel enzymes involved in nucleic acid modifications has extended the role of iron into epigenetic processes that are important for gene regulation (87).

It is therefore clear that iron deficiency can cause cell growth arrest and death (4, 225, 230), whereas excess iron generates highly reactive and toxic "free" iron in the so-called labile iron pool (LIP) (10). Poorly liganded ferrous iron reacts with reactive oxygen species (ROS, superoxide and hydrogen peroxides) or lipid peroxides, which are inevitably and continuously generated during the normal activity of every cell living under aerobic conditions, and produces hydroxyl or lipid radicals. In turn, these radicals damage biological molecules such as lipids, proteins, and nucleic acids, thus leading to cell and tissue damage and, ultimately, death (59, 73, 191) (Fig 1).

II. Overview of Iron Homeostasis

A. Systemic iron metabolism

The dual role of iron has led to the evolution of elegant control mechanisms that maintain appropriate iron levels by means of a complex network of transporters, storage molecules, and regulators that coordinately govern iron absorption, iron recycling, and the mobilization of stored iron (Fig. 2). Any disruption in these processes causes a variety of disorders associated with iron deficiency (e.g., anemia) or overload (e.g., siderosis) (4, 20, 40, 119, 155).

1. Iron absorption. Two different forms of dietary iron (inorganic and heme iron) are absorbed in the duodenum to compensate for daily iron loss (Fig. 3) (3). After being reduced on the apical membrane of intestinal enterocytes (DcytB is a candidate reductase), dietary nonheme iron (mainly Fe^{3+}) (123) crosses the apical membrane of absorptive epithelial cells, transported by divalent metal ion transporter 1 (DMT1) (118). The iron that is not used or stored by intestinal enterocytes is exported to the blood by the basolateral iron exporter ferroportin (FPN), and incorporated into the major iron transport protein transferrin (Tf). The export of iron by FPN depends on the conversion of Fe^{2+} to Fe^{3+} by means of multicopper oxidases (ceruloplasmin in the circulation and hephaestin on the basolateral membrane of enterocytes) in order to incorporate iron into Tf (220). Heme uptake and release in

Table 1. Physiological Functions or Pathways Based on Iron-Dependent Enzymes

Functions/Pathways	*Proteins involved*
Oxygen handling	Hemoglobin
	Myoglobin
Heme synthesis	Ferrochelatase
Energy production	Proteins of the mitochondrial respiratory chain (heme-containing cytochromes and Fe–S proteins)
	Enzymes of the TCA cycle (the Fe–S protein aconitase)
Neurotransmission	Monoamine oxidase
	Tyrosine hydroxylase
Detoxification, redox control	Cytochrome P450
	Xanthine dehydrogenase/ oxidase
	Catalase
Nucleic acids replication, repair, and expression	Pirin
	DNA primase
	DNA helicase
	ABCE1/2
	Fe(II) 2-oxoglutarate-dependent dioxygenases
Inflammatory response	Enzymes of arachidonic acid metabolism
	Nitric oxide synthase
	Indoleamine 2,3-dioxygenase
Cell growth	Ribonucleotide reductase
Extracellular matrix, bone deposition/remodeling	Collagen prolyl hydroxylase and lysyl hydroxylase
	Purple acid phosphatase
	Tartrate resistant phosphatase (Fe III)
Oxygen sensing	Prolyl hydroxylase
	Asparagine hydroxylase (FIH)
Second messenger signaling	Soluble guanylate cyclase

The table summarizes examples of iron-dependent enzymes involved in essential functions or pathways.

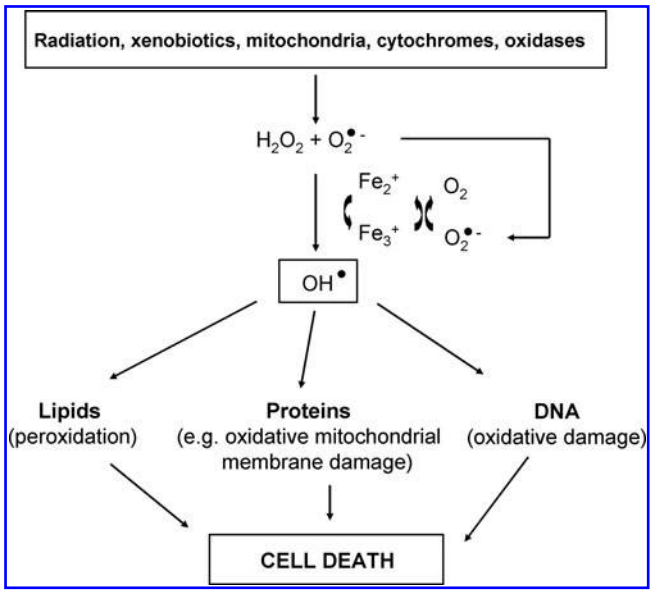

FIG. 1. Effects of reactive oxygen species on cell damage and death. Various stimuli can lead to the formation of moderately reactive peroxide and superoxide ($O_2^{\bullet -}$ and H_2O_2) which, however, can react with iron and catalyze the formation of the very reactive and toxic hydroxyl radical ($OH^\bullet$). This can damage important cell macromolecules such as lipids, proteins, and nucleic acids, and eventually cause cell death.

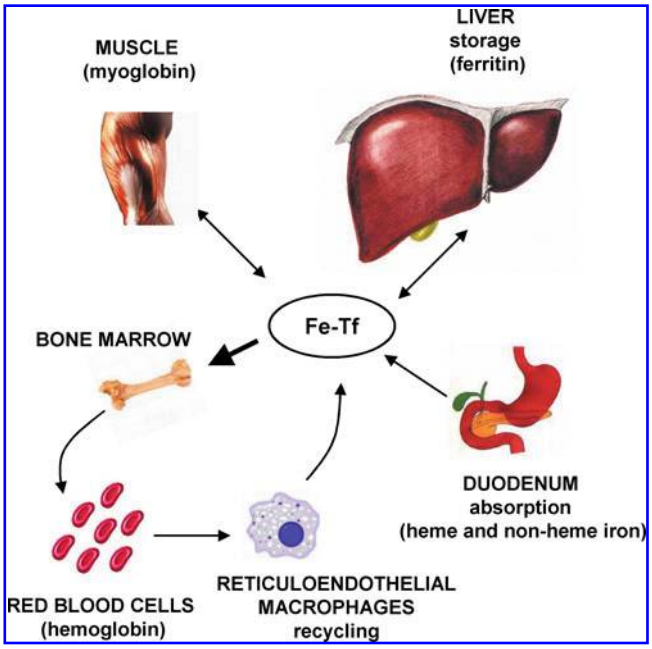

FIG. 2. Systemic iron homeostasis. Dietary iron absorbed by the duodenum is exported to the blood where it is incorporated into transferrin (Tf). The Tf-bound iron is then delivered to all tissues and cells, but primarily to the erythroid precursors in bone marrow that incorporate the metal into heme. Old or damaged erythrocytes are phagocytosed by macrophages, which degrade hemoglobin and recycle iron back into the plasma where it binds Tf. In parenchymal tissues such as the liver, excess iron is deposited in ferritin, whereas, in muscle, the iron is mainly incorporated into myoglobin. Iron may also be mobilized from storage sites in order to meet increased iron demand. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

the intestine is less clearly defined because, although various transporters have been identified (104, 157, 158, 187), their role is still unclear.

2. Iron distribution and recycling within the body. Tf-bound iron is the major source of iron for most tissues. As approximately 70% of body iron is found in hemoglobin, most of the circulating iron is consumed for heme biosynthesis by immature bone marrow erythrocytes, which internalize Tf through the transferrin receptor (TfR1) (Fig. 2) (81). The liver stores 10%–20% of body iron in the form of ferritin, which can be easily mobilized when needed. About 10% of body iron is heme-bound to myoglobin in striated muscle; the rest is distributed in other tissues. The main iron supply for hemoglobin synthesis comes from the iron recycled from senescent erythrocytes by macrophages in the liver and spleen (Figs. 2 and 4). Hemoglobin-derived heme is transported in a still undefined manner to the cytoplasm, where it is catabolized by cytosolic heme oxygenase-1 to release the iron that is subsequently exported into the circulation by FPN. Heme can also be exported directly into the circulation via the feline leukemia virus subgroup C receptor (FLVCR) on macrophage plasma membranes (91) (Fig. 4), which also plays a critical role in exporting excess heme from immature erythrocytes and hepatocytes (91). Under conditions of increased erythropoiesis (when there is an increase in iron demand by erythropoietic bone marrow), iron may be mobilized from hepatic storage sites (Fig 2).

Skeletal muscle may also be a source of iron after the stimulation of red blood cell production, as observed in subjects exposed to high altitudes (166). However, muscle iron mobilization has not been found in subjects treated with erythropoietin (167) despite the similar hematological responses. These findings indicate that enhanced erythropoietic activity is not the only trigger of muscle iron release and suggest that the hypoxic environment may play a role.

3. Regulation. Iron homeostasis is controlled at both systemic and cell level. Hepcidin is the key regulator of systemic iron homeostasis (142), whereas the main regulators of intracellular iron metabolism are the iron regulatory proteins IRP1 and IRP2 (138). However, the recent construction of mouse lines with disrupted IRP1 and/or IRP2 throughout the body or in specific tissues has shown that IRPs are also key elements in systemic homeostasis (see below) (54).

An important breakthrough was made with the recent discovery of hepcidin, a peptide that is primarily produced and secreted by hepatocytes (Fig. 5) (142). Hepcidin is the central regulator of systemic iron homeostasis and exerts its function by controlling the presence of FPN (the principal or only cell iron exporter) on the cell plasma membrane. Hepcidin binding induces FPN internalization and degradation (41, 143), which inhibits the release of iron (mainly from enterocytes, macrophages, hepatocytes, and placental cells) into plasma, thus leading to the retention of cell iron and decreasing the levels of circulating iron. Hepatic hepcidin

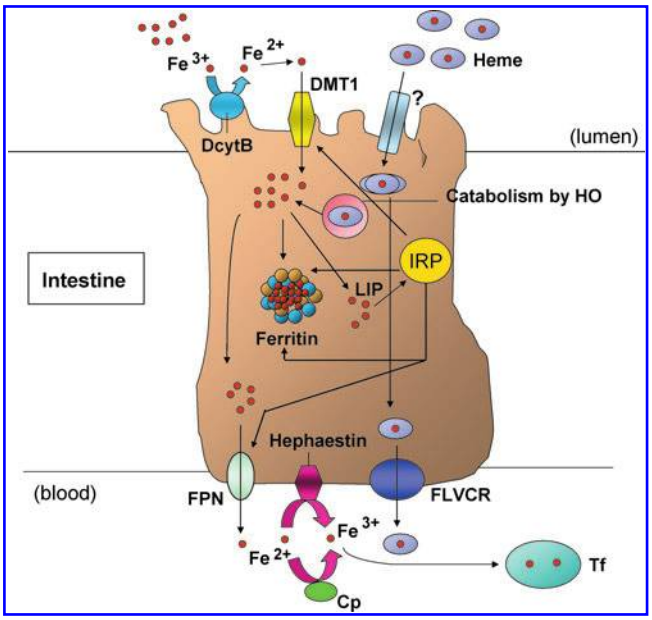

FIG. 3. Intestinal iron absorption. Iron absorption in the duodenal mucosa of the gut requires transport across the apical and basolateral membranes of duodenal enterocytes. The dietary nonheme iron in the duodenal lumen is reduced by a ferrireductase (possibly Dcytb) and thus made available for DMT1, which transports ferrous iron across the apical brush border membrane. The transporter(s) involved in heme iron absorption on the apical side have not been clearly identified or characterized. The amount of iron not retained by the cell inside the iron storage protein ferritin is transferred to the bloodstream. The basolateral release of nonheme iron (which is also derived from heme catabolized by heme oxygenase, HO) is mediated by ferroportin (FPN, which transports the metal across the membrane) and hephaestin, which re-oxidizes iron as a necessary step for binding to the plasma carrier protein Tf. This step may be favored by the presence of the soluble oxidase ceruloplasmin (Cp), and it is also possible that a portion of heme is released intact through the action of the heme exporter FLVCR. The main proteins involved in iron absorption are controlled by iron regulatory proteins (IRPs), whose activity is regulated by the levels of the metal in the labile iron pool (LIP). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

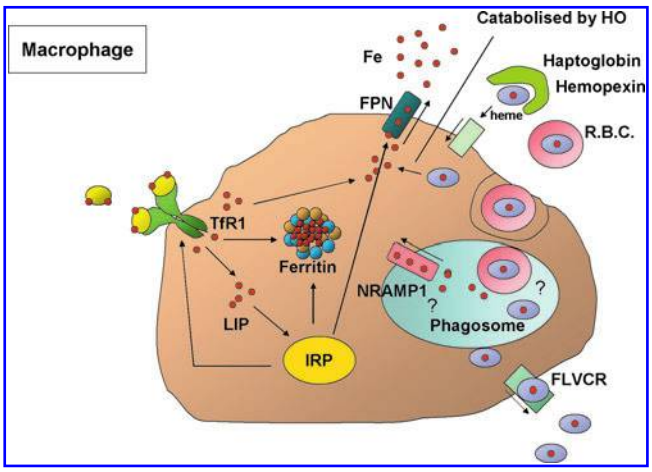

FIG. 4. Macrophage iron metabolism. A simplified view of macrophage iron homeostasis showing the most important molecules involved in iron traffic, most of which are controlled by IRPs. Macrophages acquire iron through a variety of distinct and specialized systems: the major source is heme iron (derived from the phagocytosis and degradation of red blood cells or from hemopexin and haptoglobin scavenging), but the TfR1-mediated uptake of nonheme iron is also important. The iron that is not required by cells or stored in ferritin is mainly exported by FPN but also by FLVCR (as heme iron). Iron traffic in the phagosome has not been completely clarified, although Nramp1 is used to pump iron across the phagosome membrane in the case of bacterial infection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

expression is downregulated in response to iron deficiency (142) anemia or hypoxia (152) (which increases iron release and availability), and upregulated in response to iron overload, inflammation (142), or endoplasmic reticulum stress (149, 210). Inadequate hepcidin expression in relation to body iron stores underlies the dysregulated duodenal iron absorption that characterizes most genetic iron overload disorders (155). The regulated expression of FPN, which is controlled at post-translational level by liver-derived circulating hepcidin and at translational level by the IRE/IRP system, may be a key element connecting systemic and intracellular iron regulation.

B. Cell iron metabolism

Like systemic iron homeostasis, appropriate levels of iron in the cellular LIP (10) are maintained by the coordinated

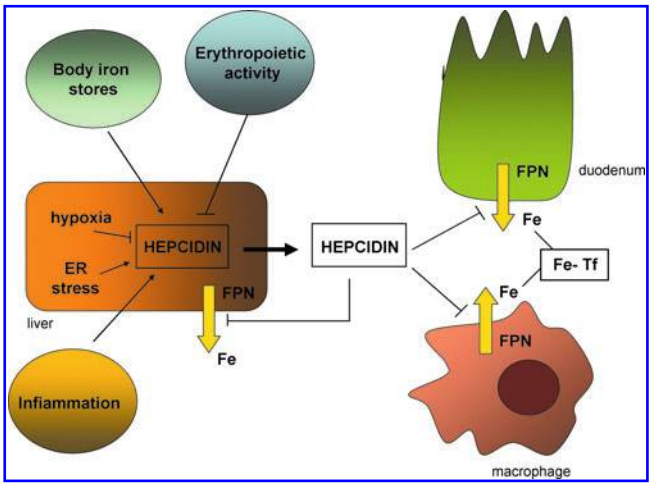

FIG. 5. Hepcidin-mediated regulation of iron homeostasis. Hepcidin, the iron "hormone" that controls systemic iron homeostasis, modulates Tf iron saturation by inhibiting FPN-mediated iron efflux from the duodenum, macrophages, and other body cells. Hepcidin synthesis in the liver is positively regulated by body iron stores, endoplasmic reticulum stress, and inflammatory signals, and negatively regulated by erythropoietic activity and hypoxia. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

expression of the proteins involved in iron uptake, export, storage, and use. This system is controlled at multiple steps, but the post-transcriptional control mediated by the IRE/IRP system is central and essential (138) because cell iron metabolism is coordinately controlled by the binding of IRP1 and/or IRP2 to cis-regulatory mRNA motifs called iron responsive elements (IREs) in various target mRNAs.

IRE/IRP interactions regulate the expression of the mRNAs encoding proteins for iron acquisition (TfR1, DMT1), storage (H and L ferritin), use (erythroid 5 aminolevulinic acid synthase (eALAS), mitochondrial aconitase, *Drosophila* succinate dehydrogenase (SDH), and hypoxia-inducible factor (HIF-2α)), and export (FPN) (78, 108, 138, 172, 204, 214, 216) (Fig. 6). The excess capacity of the IRP system (218) ensures that all of these (and probably other still unknown) mRNAs are appropriately regulated, at least under normal conditions. Although other signals and conditions can modulate IRP activity (see Section IV), iron is the major regulator of the IRE/IRP network: the combined IRE-binding activity of both IRPs is high in iron-deficient cells and low in iron-replete cells (Fig. 7). The molecular details of iron-mediated IRP regulation are discussed below.

Although the role of the IRE/IRP regulatory system in cell iron homeostasis has been extensively investigated in cultured cells, its *in vivo* functions have only recently been addressed. Mouse embryos lacking both IRPs die early during embryonic life (60, 194), thus indicating that the IRE/IRP regulatory system is essential for development. On the other hand, mice lacking only IRP1 or IRP2 are viable, which indicates that the two IRPs can compensate for each other. This has been confirmed by similar results showing a compensatory increase in IRP1 in cells in which IRP2 was knocked down (and *vice versa*) (218).

A number of comprehensive reviews of the IRP-mediated control of iron metabolism have been recently published (10, 14, 16, 78, 108, 138, 172, 204, 214) and readers can also refer to other excellent reviews for more specific aspects of the IRE/IRP regulatory network (14, 154, 212).

In this article, after re-examining the major features of the system, we will focus on the more recently discovered aspects of the role of IRPs in iron homeostasis and other cell functions.

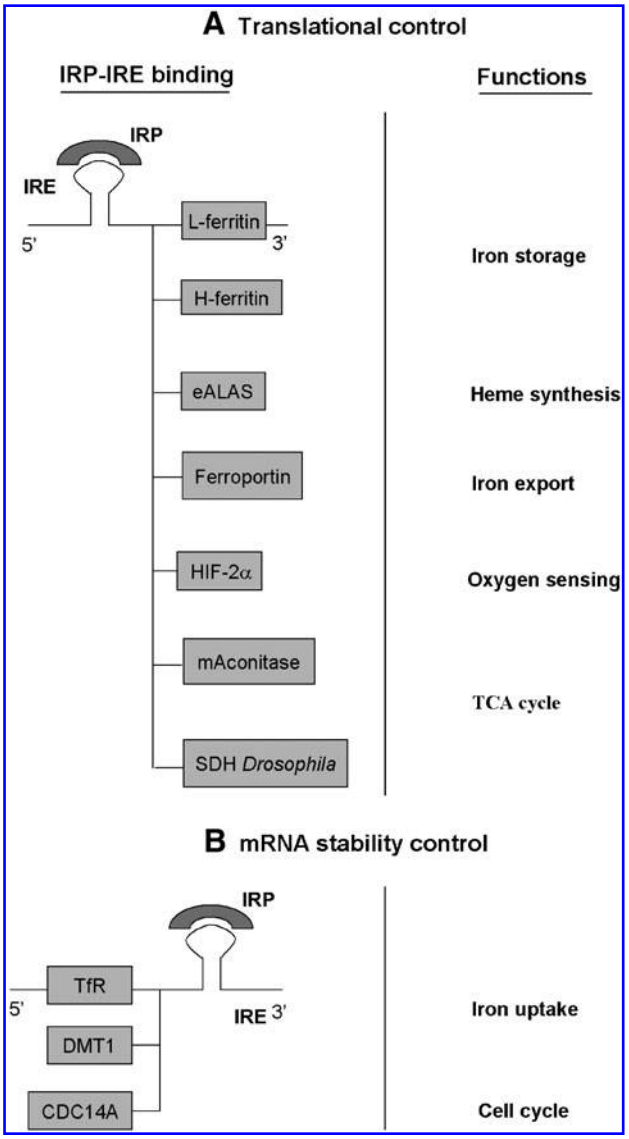

FIG. 6. Regulation of cell iron homeostasis by IRPs. IRP1 and IRP2 recognize and bind to the IREs located in the 5′ (**A**) or 3′ (**B**) untranslated regions of a variety of mRNAs involved in cell iron metabolism and other functions.

III. The IRE/IRP Regulatory System

IRP1 and IRP2 are cytoplasmic proteins belonging to the aconitase superfamily that regulate intracellular iron metabolism by binding with high affinity and specificity to conserved IREs in the untranslated regions (UTR) of mRNA (10, 14, 16, 78, 108, 138, 172, 204, 214). Plants use iron-dependent transcription as their primary means of regulating iron metabolism, whereas IRPs are conserved proteins found in mammals (in which they have evolved into two different forms) and other species: for example, IRP1 homologues have been found in ticks (72), *Drosophila*, zebrafish, and *Caenorhabditis* (137, 171). Both IRPs are expressed in all tissues, but IRP1 is particularly abundant in kidney and brown fat, and IRP2 expression is higher in intestine, brain, and the cells of the reticuloendothelial system (14).

Despite the predominantly cytosolic localization of IRPs, microscopic and biochemical approaches have identified a minor fraction of IRP1 (but not IRP2) that is associated with the Golgi and endoplasmic reticulum membranes in a phosphorylation- (151) and hypoxia-dependent manner (25), but the role of membrane-bound IRP1 is still undefined.

When iron is scarce, active IRPs bind to IREs. This stabilizes the mRNAs that have IREs in their 3′ UTR (such as TfR1 and DMT1) and decreases the translation of the mRNAs for ferritin and FPN, which have IREs in their 5′ UTR; however, the extent of the regulation varies between different transcripts (67, 179). This eventually increases iron uptake and both its intracellular and whole-body availability. Conversely, high iron levels decrease IRE-binding activity, which leads to the efficient translation of ferritin and FPN mRNAs, decreases the stability of TfR1 and DMT1 mRNAs, and ultimately decreases intracellular iron levels (Figs. 6 and 7). Other mRNAs that are apparently not immediately related to cell iron homeostasis, such as myotonic dystrophy kinase-related Cdc42 binding kinase (MRCKα) (29), cell division cycle 14A (CDC14A) (176), mitochondrial aconitase (179), SDH (in *Drosophila*) (68, 99),

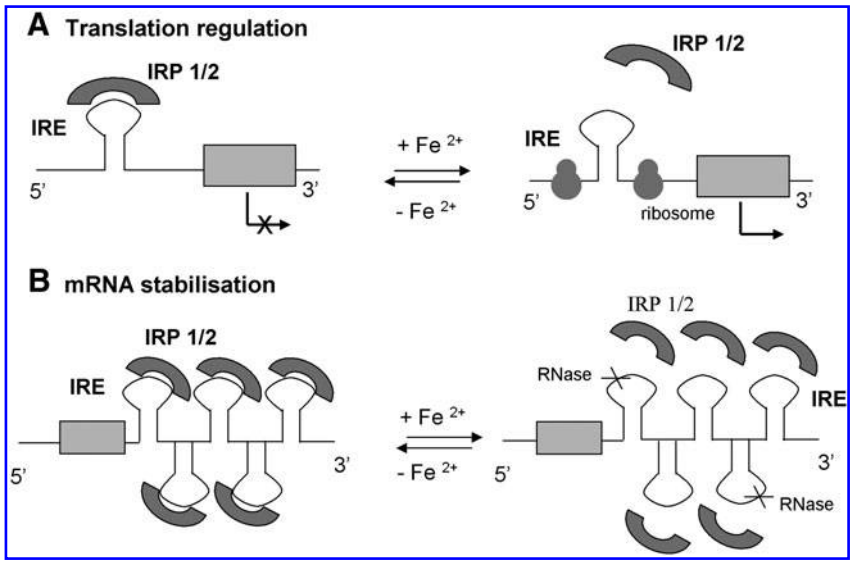

FIG. 7. Iron-dependent regulation of IRP binding activity. Under conditions of iron deficiency, IRP1 and IRP2 bind to IREs, and thus repress mRNA translation (**A**) or prevent mRNA degradation (**B**). Increased iron levels lead to the loss of IRP affinity for IREs, and increase the translation of 5′ IRE-containing mRNAs and the degradation of 3′ IRE-containing mRNAs.

HIF2α (177, 231), and eALAS (35, 39) are regulated in a similar manner (Fig. 6). New approaches based on genome-wide transcriptome and proteome analyses of the IRE/IRP regulatory network are currently being used to expand the present list of about a dozen mRNAs with a functional IRE (Hentze MW *et al*., 2009, Abstract, page 69, International BioIron Society Meeting, Porto, Portugal). It has recently been demonstrated that IRP1 can bind *in vitro* to RNA stem-loops that are significantly different from those of the consensus IRE (67), thus suggesting that other IRE-bearing mRNAs can exist.

Recent studies have shown that some genes have elegant means of evading IRP-dependent regulation in particular settings. In order to sustain the extreme demand for iron required to ensure proper hemoglobinization, erythroid precursor cells must reconcile a high degree of TfR1 mRNA stability (accompanied by the repression of ferritin and FPN mRNA translation) with the efficient synthesis of the eALAS that catalyzes the first step of heme biosynthesis. Consequently, the "standard" post-transcriptional regulation of iron metabolism via the IRE/IRP system is modified to permit the efficient use of iron in heme synthesis during erythropoiesis (181). The recent finding of an alternative FPN mRNA lacking the 5′ IRE as a result of alternative splicing has provided a molecular explanation of the by-passing of IRP-dependent repression (27, 229). Moreover, the discovery that duodenal epithelial cells highly express the same transcript has shown that duodenal cells can continue to export iron into the circulation even when they are iron depleted.

A. Structure and general regulation of IRPs

1. IRP1. IRP1 is a functional aconitase that interconverts citrate to isocitrate through a *cis*-aconitate intermediate in the cytosol and has 30% amino acid sequence identity with mitochondrial aconitase, which catalyzes the same reaction in the mitochondrial matrix (7).

Studies of the crystal structure of IRP1 have shown that mitochondrial and cytosolic aconitases are very similar (49). The active forms of both isoforms contain a [4Fe–4S] cubane cluster with three iron atoms bound by cysteines and the remaining labile iron available for the enzymatic reaction.

In iron-replete cells, the cluster is assembled and IRP1 displays aconitase activity; in iron-depleted cells, the cluster cannot form and apo-IRP1 acts as an RNA-binding protein (Fig. 8). When iron availability changes, IRP1 protein levels remain nearly constant, and the same occurs under other conditions known to alter IRP1 binding activity, although a STAT5-dependent (195) decrease in IRP1 protein levels has been reported in nitric oxide-treated cells (148). IRE binding is also controlled by the oxidation–reduction state of the cysteine residues involved in cluster coordination (79, 80), which is why it was initially proposed that a reversible switch between a cluster-containing holoprotein and a cluster-deficient apoprotein allows aconitase/IRP1 to sense iron levels constantly and adapt them to cell requirements without any changes in protein levels. However, it has since been shown that, although with slower kinetics than IRP2, apo-IRP1 is also subject to iron-dependent degradation (28, 217), possibly upon FBXL5 recognition (173, 209), and crystallographic studies have failed to predict the direct insertion of the cluster in the

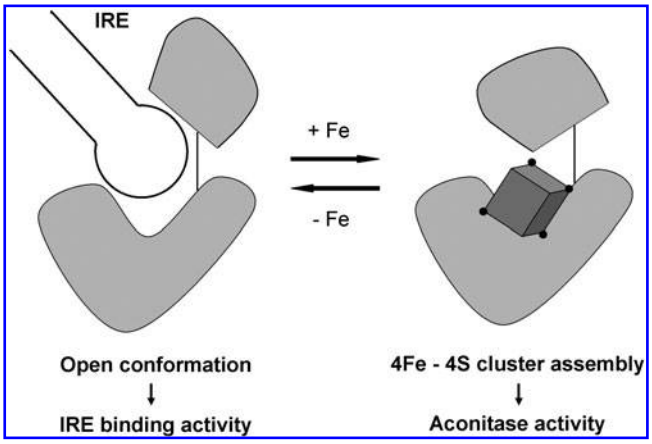

FIG. 8. The structure of IRP1. Low iron levels prevent iron–sulfur cluster assembly, thus allowing IRE entry and facilitating IRP1 binding. Conversely, high iron levels favor the formation of the iron–sulfur cluster [4Fe–4S], which impedes IRE binding and permits aconitase activity.

apo-IRP1 bound to mRNA because of the existence of overlapping sites (213). Moreover, although the insertion of a [4Fe–4S] cluster in IRP1 is favored by iron availability, it is not clear how high iron concentrations lead to the dissociation of IRE–RNA/IRP complexes, and the results of recent *in vitro* experiments suggest the possibility that iron directly weakens the binding between IRP1 and IRE (94).

The idea that a single IRP1 molecule can reversibly assume both forms may therefore need to be revised; IRP1 exists predominantly in the aconitase form (92, 159, 178), and the formation of IRP1 or aconitase, respectively, depends on the scarcity or availability of iron. In response to increased iron levels, the cluster-free form is probably more prone to degradation than cluster assembly, whereas the loss of all four iron atoms that converts cytoplasmic aconitase to the RNA-binding form can probably only occur in response to a direct oxidative or nitrative attack on the cluster (see below).

It has also been shown that protein kinase C-dependent phosphorylation controls both the RNA binding and aconitase activities of IRP1, and influences the mechanisms of IRP1 regulation in response to iron. Phosphorylation can therefore be considered an indirect means of cluster destabilization insofar as phosphorylated IRP1 seems to be more sensitive to decreased iron availability, a mechanism that allows cluster removal in the absence of strong perturbants such as ROS or reactive nitrogen species (RNS) (42).

Recent significant advances in defining the processes and molecular participants underlying the assembly of IRP1 [Fe–S] clusters indicate that iron availability is not the only requirement. Detailed analyses of the pathways of [Fe–S] cluster assembly in mammalian cells have shown that both mitochondrial and cytosolic components play a key role (discussed in (111, 172)) (Fig. 9), and that their impairment induces IRP1 binding activity and may also alter iron homeostasis (38). For example, by shifting the IRP1/aconitase balance in favor of the apoform, a mutation in mitochondrial glutaredoxin (a protein involved in [Fe–S] cluster biogenesis (221)) is sufficient to induce anemia in zebrafish by repressing eALAS translation and hence heme formation.

2. IRP2. Although IRP2 is highly homologous to IRP1, it is unable to assemble a [4Fe–4S] cluster and therefore lacks aconitase activity. Furthermore, unlike those of IRP1, IRP2 protein levels oscillate widely as the protein accumulates in

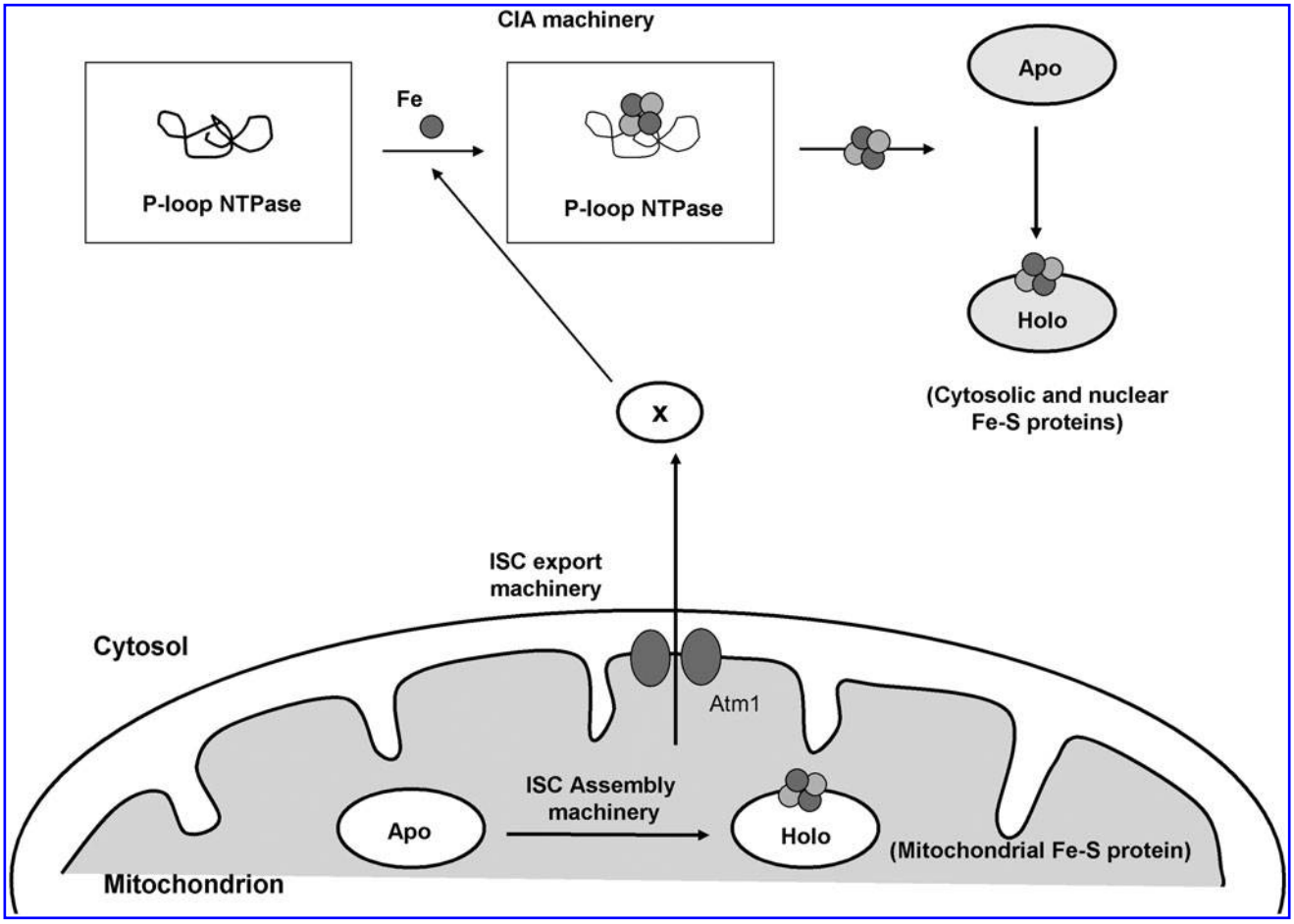

FIG. 9. A simplified view of the role of mitochondria and the CIA machinery in mammalian iron–sulfur cluster formation. Cytosolic and nuclear Fe–S–protein biogenesis requires the mitochondrial iron–sulfur cluster (ISC) assembly and export machinery, and the cytosolic iron–sulfur cluster assembly (CIA) machinery. The Atm1 ABC transporter on the mitochondrial inner membrane exports an unknown compound (X) to the cytosol for use in Fe–S–protein assembly. In the cytosol, the components of the CIA machinery catalyse two-step Fe–S–protein maturation by transiently assembling labile Fe–S clusters on a scaffold (the P-loop NTPase complex) before they are transferred to apoproteins.

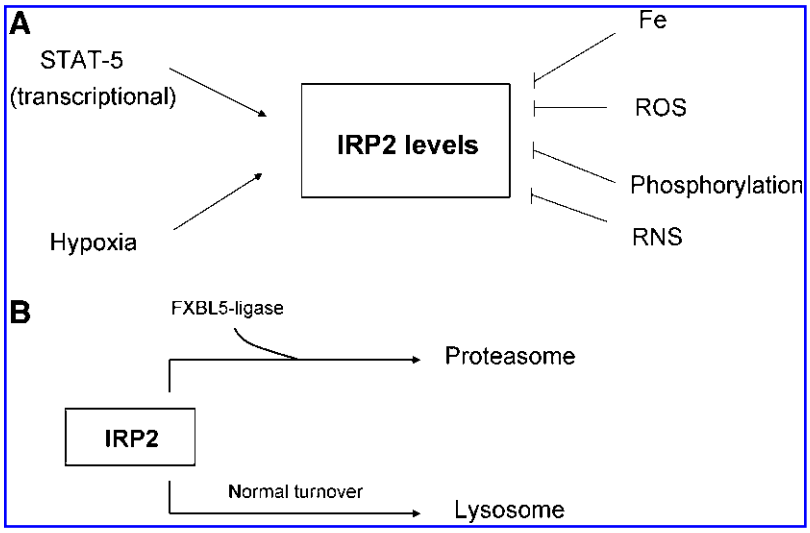

FIG. 10. Regulation of IRP2. (**A**) Factors controlling IRP2 levels. Increased iron levels, phosphorylation, and exposure to conditions favoring the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) promote the degradation of IRP2, whereas hypoxia-induced IRP2 stabilization and increased STAT-5-dependent IRP2 gene transcription upregulate IRP2 expression. (**B**) Pathways of IRP2 degradation. The lysosomal pathway seems to be involved in the normal turnover of IRP2, whereas FBXL5 ligase-mediated mechanisms target IRP2 to proteasomal degradation in response to the stimuli shown in (**A**).

iron-deficient cells and is rapidly targeted for degradation in iron-replete cells (15, 138, 172, 214) (Fig. 10).

The mechanism by which IRP2 undergoes iron-dependent degradation is still incompletely understood. In comparison with IRP1, IRP2 contains an extra exon coding for a cysteine-rich domain that has been referred to as the iron-dependent degradation domain, and which may be responsible for characteristic iron-dependent degradation because of its ability to facilitate iron-dependent oxidation, ubiquitination, and proteasomal degradation (70, 71, 86, 174). However, the first step of the iron-dependent degradation of IRP2 has still not been fully characterized. It was initially proposed that the starting iron-sensing mechanism may be the binding of heme to the degradation domain, but most recent work indicates that this domain is a protease-sensitive region, which does not support its function as a heme sensor (50).

Iron could function indirectly by favoring oxidative stress, but the protein degradation triggered by iron-dependent oxidative damage is probably not the only mechanism underlying the effect of oxidative stress on the RNA binding activity of IRP2. It has recently been demonstrated that two cysteines lying in the RNA-binding cleft of IRP2 must be unmodified or reduced to interact with the IRE, and so their redox status seems to be a key factor regulating IRP2 binding activity (and iron homeostasis) under conditions of oxidative stress (233).

Redox control, which seems to be the main player regulating IRP2, may also function through 2-oxoglutarate-dependent dioxygenases, which require oxygen, ferrous iron, and ascorbate, and may be involved in targeting IRP2 to degradation (Hausmann A. *et al*., 2009, Abstract, page 128, International BioIron Society Meeting, Porto, Portugal).

Furthermore, other factors (such as phosphorylation status) may also be involved (Fig. 10) as the iron-independent reversible phosphorylation of IRP2 Ser-157 occurs during a specific phase of the cell cycle and leads to IRP2 degradation, thus modulating cell iron homeostasis (215).

The pathway of IRP2 destruction also remains controversial: ubiquitination-dependent degradation in the proteasome seems to be the prevalent form of IRP2 breakdown, but the ubiquitin ligases involved have not been definitely identified and characterised. It has been shown that the originally proposed heme-oxidized IRP2 ubiquitin ligase-1 HOIL-1 (84, 224) is not required for iron-dependent degradation (75, 216, 232). However, very recently, two groups have independently reported the identification of a protein (FBXL5) that participates in another ubiquitin ligase complex that promotes the iron-dependent polyubiquitination and degradation of IRP2 (173, 209) (Fig. 10). The iron-dependent degradation domain is not required for the interaction, as is demonstrated by the fact that apo-IRP1 is also bound by FBXL5, although IRP1 binding activity is not significantly affected by FBXL5 knockdown. As the stability of FBXL5 itself is also controlled by iron and/or oxygen levels through an iron-binding hemerythrin-like domain located in the N terminus, FBXL5 accumulates when iron is plentiful and is degraded upon iron depletion, thus functioning as a key sensor in the regulation of iron homeostasis. The complexity of the process has been made even clearer by the demonstration that the lysosomal pathway may also play a role, especially in normal turnover (Fig. 10), whereas proteasomal degradation becomes predominant under conditions of excess iron (51).

Finally, the control of protein stability is not the only mechanism involved in IRP2 expression as recent studies have shown that the IRP2 gene is a direct transcriptional target of Stat5 in erythroid cells (93), a finding that is in line with the previous demonstration that the IRP2 gene is a transcriptional target of c-myc (223) (Fig. 10).

B. Iron responsive elements

IREs are highly conserved binding sites for IRPs with a stem-loop structure; it seems that five residues in the exanucleotide loop and base pairs in the stem are necessary for a fully functional IRE. The sequence and structural features of IREs (of which 150 homologues belonging to a dozen different types have been identified (154)) have recently been reviewed (212), and will therefore not be discussed here.

Early experiments showed that different IREs maintained the same post-transcriptional regulation for various transcripts and that the two IRPs bind consensus IREs with similar affinity *in vitro* (reviewed in (14, 78)). However, there is evidence indicating that the regulation is more potent in the case

of some mRNAs than others. For example, the translation of ferritin mRNA is more sensitive than that of mitochondrial aconitase to IRP-mediated regulation in HL-60 cells (179) and the intestine (60). Moreover, Theil *et al*. found that IRP2 preferentially binds to ferritin mRNA (90), and data obtained using a SELEX procedure to identify novel IRE sequences (reviewed in (14, 78)), together with the analysis of the binding activity of the IRE variants detected in patients with hyperferritinemia (see below), have shown that each IRP recognizes an exclusive IRE subset. Using a more sensitive assay than the RNA-bandshift assay traditionally used to detect IRP binding activity, it has been shown that the small variations in the IREs of H and L ferritin mRNAs are responsible for the preferential response of the L subunit to iron, a difference that is more pronounced under hypoxic conditions (175). Moreover, by determining the affinity of IRP1 for a number of 5′ IRE variants, Goforth *et al*. have recently precisely characterized the structural determinants within IREs that account for their wide range of binding affinities (67). Wallander *et al*. (214) have extensively discussed this question and proposed that only combinatorial control involving slight differences in the structural requirements of IRP1 and IRP2 for RNA recognition, and a small diversity in IRE sequence (and structure), can explain the different regulation of specific transcripts (*in vivo*).

C. Role of the IRE–IRP regulatory network in iron uptake

All cells require iron to maintain normal function. The main iron uptake pathway is mediated by TfR1, which internalizes iron-laden Tf. TfR1 expression is controlled by a variety of cell conditions, including iron and oxygen status (14, 16, 78, 108), and regulated at transcriptional level by the hypoxia-inducible factor HIF1 in response to low oxygen levels and iron starvation (8, 115, 197) or inflammatory mediators (199). It is also a direct transcriptional target of Stat5 in erythroid cells (93), and its promoter region contains potential binding sites for many transcription factors, such as c-myc (146). However, TfR1 expression is mainly controlled post-transcriptionally by means of the interaction between IRPs and five IREs, which convey regulation of expression by cellular iron concentration. These IREs are located in the 3′ UTR, and allow IRP binding to stabilize the mRNA and thus upregulate TfR1 expression under iron-deficient conditions (Figs. 6 and 7).

DMT1 is a glycoprotein that consists of twelve transmembrane domains with ion channel transporter characteristics. It is expressed in the apical domain of duodenal enterocytes (118) (Fig. 3), where it is required for intestinal iron absorption, and is also involved in transporting iron from endocytic vesicles to the cytoplasm after the TfR1-mediated internalization of Fe-Tf. The DMT1 gene encodes four transcripts that are the result of alternative splicing at the 3′ or 5′ end. One splicing form, which contains an IRE structure in its 3′ UTR, is upregulated by iron deficiency (118, 214) (Figs. 6 and 7); however, the regulation of DMT1 is complex (discussed in (214)) and, despite many studies, there is still no clear and direct evidence that it is mediated by the IRE itself. The ablation of both IRPs in intestinal epithelial cells reduces the levels of IRE-containing DMT1 mRNA and DMT1-dependent intestinal iron uptake (60), but transcriptional regulation may be important, as is also suggested by recent studies showing that HIF2α–dependent DMT1 transcription plays a role in intestinal iron uptake (121, 186).

D. Role of the IRE–IRP regulatory network in iron storage

Ferritin, the major iron storage protein, is found in the cytoplasm, mitochondria, and nucleus of cells (6). In vertebrates, cytoplasmic ferritin is expressed in almost all tissues. Ferritin molecules consist of heavy (H) and light (L) chain subunits. The 24-mer ferritin shell has variable proportions of H or L chains, which give rise to isoferritins that are either more acidic (H-rich) or more basic (L-rich) (5). The ratio of H and L subunits can vary: tissues such as liver and spleen are predominantly L-rich, whereas heart and kidney are predominantly H-rich (76, 205). Ferritin is also present in serum and other biological fluids, although its secretion mechanisms are still unclear. Serum ferritin is increased in cases of iron overload and inflammation, but its function is obscure, although it may play a role in regulating blood vessel formation (30) and immunity/autoimmunity (161).

The best characterized system regulating ferritin expression is the post-transcriptional, iron-dependent machinery based on the interaction between the IRPs and IREs localized in the 5′ untranslated region of H- and L-ferritin mRNA. The rate of synthesis and abundance of ferritin can change over a 50-fold range in response to variations in iron availability, and so the transcripts of the H and L ferritin chains are the prototypes of IRP-mediated translational control, as first suggested more than 30 years ago (226). IRP1 and IRP2 bind to the stem-loop structure of ferritin IRE and inhibit mRNA translation, and the system similarly regulates both the H and L chains, whose IRE sequences are almost identical (discussed in (14)). This occurs under normoxic conditions, but it has been found that the L subunit responds to iron more strongly under hypoxic conditions (175) (Figs. 6 and 7).

Mitochondrial ferritin, which arises from a retrotranscribed pseudogene and "transforms" the untranslated IRE into a translated domain coding for the mitochondrial targeting peptide, lacks an IRE and does not respond to iron (109).

E. Role of the IRE–IRP regulatory network in iron release

The transmembrane protein FPN is the only known iron exporter (45). It is ubiquitously expressed but more abundant on the basolateral membrane of polarized enterocytes in the duodenum (220) (Fig. 3) and reticuloendothelial cells (219), which means it is important for iron absorption (iron transport from enterocytes into the plasma) and reuse (the transport of about 20 mg of iron out of macrophages coming from the destruction of senescent or damaged erythrocytes) (222) (Figs. 3 and 4).

FPN is post-translationally regulated by hepcidin, which plays a central role in controlling systemic iron levels. Upon hepcidin binding, FPN is internalized and degraded (41, 143), thus inhibiting iron efflux from enterocytes, macrophages, and other cells (Fig. 5). However, it must be remembered that FPN mRNA bears a functional IRE motif in its 5′UTR that allows it to respond to iron manipulations in hepatic, intestinal, and monocytic cell lines (116). Moreover, the IRP-dependent translational control of FPN expression has been

found in response to nitric oxide (˙NO) (113) and erythrophagocytosis (43). Data showing that the simultaneous ablation of IRP1 and IRP2 in mouse markedly increases intestinal FPN expression despite the increase in hepatic hepcidin expression indicate that IRPs are as critical as hepcidin for physiological FPN expression in the intestine (60). A further indication of the importance of IRP/IRE interactions in the expression of FPN is the demonstration that a microdeletion in the promoter region of the *Fpn1* gene that eliminates the IRE leads to increased FPN protein levels in polycythemic mice (135). Moreover, a point mutation close to the FPN IRE (and possibly altering its structure) has been found in a hemochromatosis patient (112).

F. Role of the IRE–IRP regulatory network in other pathways

Recent findings obtained using a variety of approaches to identify novel IRE-containing genes indicate that the influence of IRPs extends over a number of regulatory pathways that are not directly related to iron homeostasis (Fig. 6).

MRCKα, a kinase that acts downstream of the small GTPases known to regulate the cytoskeleton, has an IRE in the 3′ UTR that may mediate a response to iron that is similar to (but less than) that of TfR1 (29), and suggests that iron plays a role in maintaining the cytoskeleton. Furthermore, a functional IRE has been found in a differentially spliced mRNA isoform coding for CDC14A, a phosphatase involved in the regulation of critical cell cycle proteins that may be a tumor suppressor (176). Although it is not clear how IRPs regulate CDC14A expression, this finding opens up interesting links between iron metabolism and cell cycle regulation.

As iron is essential for cell proliferation, the role of IRPs in cancer growth is highly interesting. It has been reported that IRP1 overexpression inhibits the growth of tumour xenografts in nude mice (24) and, as ferritin and FPN expression were unchanged and TfR1 levels were increased (an effect that should favor cell growth), other still unidentified mRNAs coding for proteins relevant for cell multiplication are probably targeted by IRP1. However, the relationship between IRPs and cell growth still requires further investigation because recent findings by the same group have shown that the overexpression of IRP2 increases the growth of transplanted lung cancer cells in mice (Maffettone C. *et al.*, 2009, Abstract, page 139, International BioIron Society Meeting, Porto, Portugal).

It has recently been demonstrated that a sequence in the 3′ region of the mRNA coding for α-hemoglobin stabilizing protein (AHSP), which functions as a chaperone for free α-globin during hemoglobin synthesis, is similar to IRE. Despite its deviation from the consensus sequence and poor binding in bandshift assays, the results of other assays led the authors to suggest that AHSP should be added to the list of genes regulated by IRPs. These findings extend the range of functional IRE structures to noncanonical sequences, and propose a new post-transcriptional mechanism linking hemoglobin synthesis to iron availability (46).

Another recent study found the presence of a functional IRE in the mRNA for hypoxia-inducible factor HIF2α, a transcription factor activated by a lack of oxygen or iron (177). HIF forms heterodimers consisting of the oxygen-regulated HIF1α or HIF2α subunit and a constitutively expressed HIF1β subunit (184). Under normoxic conditions, IRP1 binds to the HIF-2α IRE to repress basal translation, whereas hypoxia impairs the IRP1/IRE interaction and allows the efficient translation of the message (231). The fact that an IRE is present in HIF2α mRNA and that the system is activated by low iron levels suggests a negative feedback control of the HIF-mediated response under conditions of limited iron availability. This finding may shed further light on the link between iron and oxygen homeostasis. Hypoxia derepresses HIF2α mRNA translation by impairing IRP1 binding activity to its 5′ IRE and prevents HIF2α degradation by inhibiting prolyl hydroxylase (PHD) activity: the combination of these two mechanisms greatly increases HIF2α protein levels. Low iron levels hinder PHD activity, but also increase IRP1 activity, thus reducing HIF2α translation. It has been suggested that the IRE in HIF2α mRNA indirectly

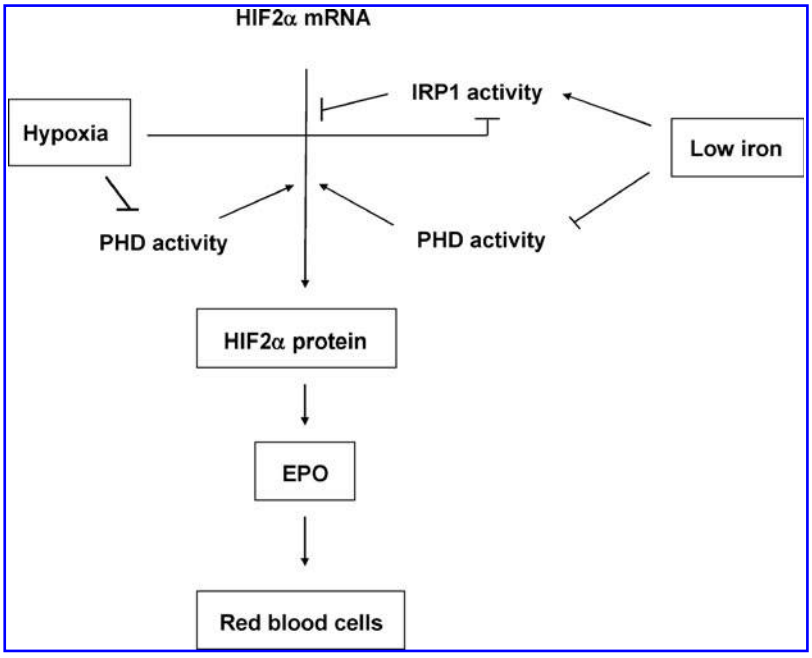

FIG. 11. IRP-mediated regulation of HIF2α. In order to increase HIF2α protein levels (and consequently the expression of target genes such as erythropoietin, [EPO]), hypoxia derepresses HIF2α mRNA translation by impairing IRP1 binding to its 5′ IRE, and prevents HIF2α degradation by inhibiting prolyl hydroxylase (PHD) activity. Low iron levels hinder PHD activity, but increase IRP1 activity, thus reducing HIF2α translation. It has been suggested that this mechanism modulates EPO levels, thus adjusting erythropoiesis to iron availability.

modulates the levels of erythropoietin (a major target of HIF2α) and may thus allow the rate of red blood cell production to be adjusted to iron availability (Fig. 11).

As brain iron homeostasis is disrupted in a number of neurodegenerative disorders (227), the reported presence of an IRE-like motif in the 5′ UTR of the mRNAs for α-synuclein (58) (a presynaptic protein that accumulates in several brain disorders including Parkinson's disease) and Alzheimer's amyloid precursor protein (169) is particularly interesting, and may have implications for iron homeostasis in Alzheimer's disease (168). However, the sequences are not canonical IREs, and IRE-like motifs previously found in other mRNAs have proved to be nonfunctional (100, 164).

G. Roles and importance of IRP1 and IRP2

Most IRP1 in normal tissues with unrestricted iron availability is in the aconitase form (159, 172) which, together with evidence that IRP1 knockout mice do not show abnormal iron metabolism (60, 105, 194), suggests that its main role is related less to iron homeostasis than to its enzymatic function; however, there is still no experimental evidence to support this hypothesis. Moreover, the absence of an overt phenotype in IRP1 knockout mice under standard conditions (60, 105, 194) or in response to chemotherapy (33) or inflammation (211) is in line with earlier indications that IRP2 is more sensitive than IRP1 to the modulation of iron levels (160) and, when solely (178) or abundantly expressed (165), may be the only regulator of cell iron homeostasis. This has been confirmed by experiments in cell lines in which IRP1, IRP2, or both were knocked down (218). Body iron balance therefore seems to be mainly controlled by IRP2, probably because it is the predominant IRE-binding protein at physiological oxygen tension (128). The results obtained in animals and tissue cultures have been confirmed by extensive studies of human tissues and blood cells (159). It is interesting to note that the high iron demand accompanying the increased mitochondrial biogenesis required for osteoclast differentiation leads to low iron levels that are sensed by IRP2 (but apparently not IRP1) (83). Iron deficiency consequently upregulates TfR1 and thus stimulates the iron uptake necessary for the formation of mitochondria-rich mature osteoclasts and the activity of iron-containing enzyme tartrate-resistant acid phosphatase, which is essential for efficient osteoclast function (83).

However, recent findings challenge the idea that IRP2 dominates iron metabolism and indicate that the function of IRP1 is not limited to compensating for the lack of IRP2 when it is genetically ablated (32, 60, 105): it has been demonstrated that IRP1 is essential for the regulation of eALAS and heme synthesis in zebrafish (221) and in controlling HIF2α translation (231).

The roles of the two IRPs in cancer growth are still not completely understood, as is shown by the previously mentioned studies indicating that IRP1 overexpression efficiently suppresses tumor growth (24), whereas the overexpression of IRP2 stimulates it (Maffettone C. *et al*., 2009, Abstract, page 139, International BioIron Society Meeting, Porto, Portugal).

These different results can be reconciled by considering that the expression of IRP1 and IRP2 varies in different cell types (14), and that their distinct affinities for different IREs may establish a regulatory hierarchy, as suggested by Goforth *et al*. (67). For example, under conditions of IRP1 overactivation, even mRNAs with lower-affinity IREs may be strongly repressed (221), whereas the preferential expression of highly repressed mRNAs (e.g., ferritin) is expected when one of the two IRPs is inactivated.

IV. Regulation of IRP Function by Stimuli Other than Iron

Iron was the first regulator of the IRE/IRP regulatory network to be identified and characterized, and remains the strongest known effector; but various other agents and pathobiological conditions may affect IRP activity.

A. Hypoxia

The two IRPs are also regulated by oxygen tension (reviewed and discussed in (14, 16, 172)), which differentially controls their binding activity. Although some studies have found that hypoxia increases IRP1 binding to IREs (25, 206), the results of most suggest that it actually decreases the binding to IRE elements, and leads to IRP1 acquiring aconitase activity, but increases IRP2 activity (74, 128, 180, 197). Moreover, hypoxia greatly reduces IRP1 binding to HIF-2α, but slightly increases the binding of IRP2 (231). However, this divergent modulation can go in opposite directions even in cells belonging to the same organ, as in the case of neurons and glial cells (82).

Why IRP1 and IRP2 respond to hypoxia in an opposite manner remains to be established but, as IRP2 is the predominant IRE binding protein at physiological oxygen tension (3%–5%) (128), which may explain its importance in regulating iron metabolism, it has been suggested that its upregulation is the most important response to hypoxia (172). However, the finding of Zimmer *et al*. that hypoxia derepresses HIF-2α translation by impairing the ability of IRP1 to bind mRNA (231) suggests that IRP1 acts as a direct or indirect sensor of hypoxia. While not excluding the possibility that endogenous IRP2 binds to HIF2α IRE *in vivo*, these authors therefore challenge the prominent role of IRP2 and suggest that the main regulatory role of hypoxia in HIF translation is due to IRP1, at least in the context of renal cells in which IRP1 is particularly abundant (14). The recent finding that IRP2 degradation is controlled by FBXL5 in an iron- and oxygen-dependent manner (173, 209) suggests one possible explanation for the differential regulation of the two IRPs by hypoxia. At low oxygen concentrations, which favor the assembly of the 4Fe–4S cluster of IRP1 and stabilise IRP2, the latter regulates iron homeostasis but, at high oxygen concentrations, FBXL5 is stabilized, interacts with IRP2, and induces its degradation, whereas the apoform of IRP1 can bind and regulate the mRNAs encoding proteins of iron metabolism. These mechanisms may therefore allow cells to regulate iron metabolism effectively over a broad range of oxygen availability.

The effects of low oxygen levels on the binding activity of IRPs are not the only connection between oxygen and iron homeostasis. A lack of oxygen and/or iron abrogates the HIF-1/2α hydroxylation that would lead to proteasomal degradation, thus increasing protein stability and transcriptional activity (184). This supports the existence of a physiologically important overlap between oxygen and iron sensing in the regulation of HIF-1. The findings of recent studies indicate that changes in cell iron status under physiological and pathophysiological conditions can have a significant impact on the

activity of the HIF-1 system (136, 152). The idea of an interaction between oxygen and iron homeostasis is further reinforced by evidence of IRP-mediated HIF-2α expression (231) (see above), and the fact that the genes coding for many of the proteins involved in iron metabolism are among the target genes of hypoxia (136, 184) (Table 2). The importance of the HIF-2α-mediated regulation of DMT1 and DcytB for the maintenance of body iron homeostasis has been recently shown by two *in vivo* studies showing the negative effect of intestine-specific HIF inactivation on iron absorption (121, 186).

HIF1-dependent transcriptional upregulation may combine with IRP-dependent post-transcriptional control to expand the extent of the response of iron metabolism genes to a scarcity of oxygen or iron. This has been demonstrated in the case of TfR1 (Fig. 12), which, in response to iron chelation, is induced less in HIF-1-deficient hepatoma cells (which only rely on IRP-mediated regulation) than in their wild-type counterparts (8), and is strongly upregulated in renal carcinoma cells that have lost the VHL gene and hence overexpress HIF-1 (2). However, the physiological meaning of TfR1 upregulation in response to hypoxic conditions is still unclear. It is possible that it has evolved to ensure that red cell precursors receive sufficient iron for erythropoiesis under conditions that threaten oxygen availability, and has been conserved despite its possibly dangerous consequences for nonerythropoietic cells (i.e., the increase in iron uptake could trigger ROS formation during reoxygenation or post-ischemic reperfusion, both of which may follow hypoxia *in vivo*) (198).

Table 2. Iron-Related Genes Regulated by Hypoxia

HIF-dependent	*HIF-2 dependent*	*HIF-independent*
Transferrin (170)	DMT1 (186)	GDF15 (103)
Transferrin Receptor 1 (115, 197)	DcytB (186)	
Ceruloplasmin (140)	Frataxin (147)	
Ferrochelatase (114)	Ferroportin (?) (121)	
Furin (125, 192)		
Erythropoietin (185)		
Heme oxygenase (106)		
Hepcidin (144, 153)		
Ferroportin (?) (124, 153)		

For the genes listed in the central lane, a HIF2-mediated specific regulation has been demonstrated.

B. Oxidative stress

Given the role of iron in redox reactions (see above), the regulation of IRPs under conditions of oxidative stress (i.e., when the amount of ROS exceeds cell antioxidant capacity) has been intensively investigated. Early studies showed that exposure to exogenous H_2O_2 upregulates IRP1 in cell lines (reviewed in Refs. 14, 16, 78), a transient event leading to the later induction of ferritin (207). However, this seems to be due to the phosphorylation-dependent signaling pathways involved in the pleiotropic effects of H_2O_2, rather than to a direct effect of oxidative stress on IRP1 activity (19), and the oxidative removal of the fourth iron atom from the cluster is not sufficient to produce the iron binding apoform of IRP1. Nevertheless, studies of various cell lines and *in vivo* studies have shown that a number of conditions or agents known to increase cell H_2O_2 and $O_2^{\cdot-}$ levels induce the reversible inactivation of IRP1 (12, 18, 63, 193, 200), and this makes it possible to conclude that IRP1 downregulation (aimed at decreasing TfR1 while increasing ferritin levels, and hence diminishing the LIP) is a common response designed to prevent the enhanced formation of ROS. In line with this, increased ferritin synthesis has been documented in a number of cell types exposed to oxidative stimuli (14, 205), whereas the overexpression of either the ferritin H (36, 52, 150) or ferritin L subunit (110, 150) reduces oxidative damage.

Probably more relevant for the antioxidant response involving iron modulation is the attack of ROS produced under conditions oxidative stress to IRP2. The results of a number of *in vitro* and *in vivo* experiments suggest that IRP2 is highly sensitive to ROS-induced downregulation, which may be triggered by oxidative modifications of sensitive residues, and can lead to ubiquitin-dependent proteasomal digestion (85) (Fig. 10). IRP2 is therefore rapidly inactivated in rat liver exposed to glutathione depletion or ischemia/reperfusion (18, 200), as well as in cells exposed to menadione (63). On the other hand, IRP2 is not downregulated in cells exposed to exogenous H_2O_2 (16, 78), thus confirming that extracellular H_2O_2 acts through mechanisms that are independent of oxidative stress.

In conclusion, the downmodulation of both IRPs by intracellularly produced ROS provides cells with a regulatory loop that reduces intracellular iron availability and prevents the amplification of oxidative damage.

C. Reactive nitrogen species

Both IRPs may also be direct or indirect targets of RNS. $^{\cdot}NO$ promptly reacts with iron sulfur clusters and attacks the [4Fe–4S] cluster of cytoplasmic aconitase/IRP1. It also modifies the -SH residues exposed on the cluster-free IRP2, thus targeting it for proteasome-mediated degradation (Fig. 10). Most studies investigating the effects of endogenously generated or exogenous $^{\cdot}NO$ on iron metabolism have used macrophages because the NO-mediated modulation of their IRPs is involved in important pathophysiological conditions such as inflammation. A number of findings indicate that $^{\cdot}NO$ production enhances IRP1 activity, but the precise mechanism(s) of this activation remain unclear. Some investigators have suggested that $^{\cdot}NO$ attacks the [Fe–S] cluster of cytoplasmic aconitase, thus inducing its disassembly and switching it to IRP1 (reviewed in Refs. 16, 48, 78), and others that $^{\cdot}NO$ operates by inducing cell iron release (16, 48, 78), although these two events may not be mutually exclusive. Experiments showing that the modulation of FPN in *S. typhimurium*-infected macrophages reduces iron availability, and thus increases IRP binding activity, support the idea that increased IRP activity under pathological conditions *in vivo* may be due to a reduced cytoplasmic LIP (141). Other RNS, such as the nitrosonium ion (NO^+) that nitrosylates thiol groups of proteins, may have different effects because it has been reported that treating K562 cells with an NO^+ donor decreases the RNA binding capacity of both IRPs (reviewed in (98)). It seems that IRP1 is susceptible to activation under certain conditions, but IRP2 is almost invariably inactivated by RNS, which cause redox modifications of -SH residues followed by proteasome-mediated degradation. (14, 16, 48, 78, 97) (Fig. 10).

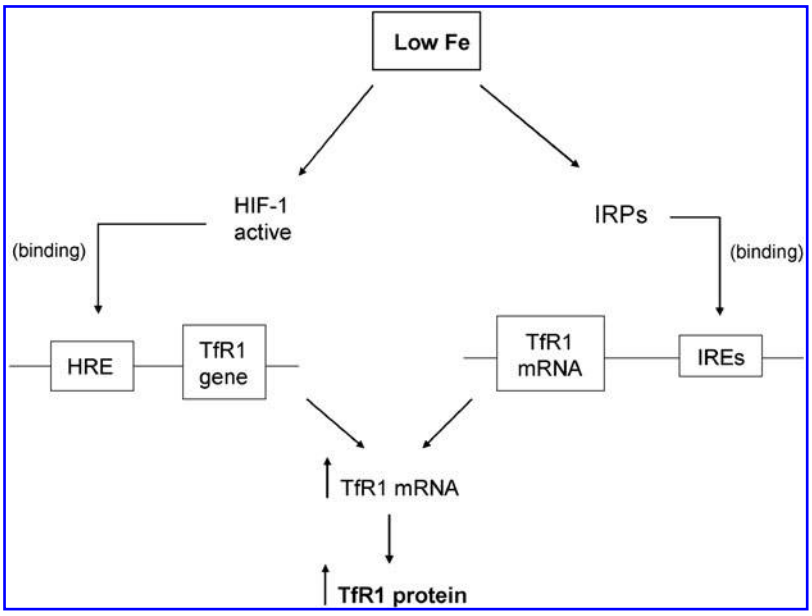

FIG. 12. Different molecular pathways contribute to enhancing TfR1 expression when iron levels are low. When iron is scarce, HIF-1 degradation is prevented: this leads to increased HIF-1 binding to hypoxia responsive elements (HRE) in the TfR1 promoter and therefore high levels of TfR1 transcription. At the same time, low iron levels increase IRP binding to the IRE sequences in the 3′ end, thus stabilizing TfR1 mRNA.

As macrophages (key cells in inflammatory processes) preferentially express IRP2, the RNS-induced inactivation of IRP2 may be more pathophysiologically relevant than the concomitant activation of IRP1 binding activity. IRP2 downregulation may account for the increased ferritin synthesis and reduced TfR1 mRNA expression found in macrophages producing $^{\cdot}NO$ or $ONOO^{-}$ after stimulation with cytokines or LPS (97, 165), and the described downregulation of IRP1 protein levels may contribute to maintaining such responses (148). The total IRP activity (IRP1 + IRP2) of human monocytes/macrophages tends to increase shortly after treatment with cytokines or $^{\cdot}NO$ donors, but then markedly declines while ferritin content increases (163). These effects are similar to those observed when IRP2 undergoes downregulation in mouse macrophage lines (165). The effects of RNS on IRPs may therefore concur with hepcidin-dependent FPN downmodulation to explain the iron sequestration that characterizes macrophages under inflammatory conditions, as confirmed by the lack of downmodulated IRP binding activity in activated human M2 macrophages, which have a different immunological function and do not withhold iron (162).

D. Xenobiotics

The cardiotoxicity induced by doxorubicin (DOX), an anticancer anthracycline that also causes a severe form of chronic cardiomyopathy, provides an excellent model for showing that IRPs may also be a target of xenobiotics (129, 131). Independent studies have demonstrated that the alcohol metabolite of DOX (DOXol), which delocalizes iron from the [4Fe- 4S] cluster of cytoplasmic aconitase, and the $O_2^{\cdot-}$ and H_2O_2 derived from the redox activation of DOX cooperate to convert IRP1 into a "null" protein that lacks both aconitase and RNA binding activities (9, 133, 134). On the other hand, another study (101) has shown that the formation of the "null" protein does not depend on the action of DOXol but is due to anthracycline-iron complexes attacking aconitase/IRP1. IRP2 (which lacks the cluster) is not attacked by DOXol but is targeted to degradation by the action of the ROS produced in this setting (34, 134) (Fig. 10).

The actions of DOXol and ROS on IRPs have pathological effects. DOX treatment increases ferritin synthesis in H9c2 cardiomyocytes (34) and mouse hearts (33), and more markedly induces the antioxidant ferritin H subunit (6, 76, 205). The downmodulation of IRP2 in response to ROS formation may therefore act protectively to facilitate ferritin expression and sequester free iron before it converts $O_2^{\cdot-}$ and H_2O_2 into more potent oxidants. IRP1 ablation does not alter DOX-induced changes in iron metabolism or DOX-induced oxidative damage of the myocardium, thus indicating that the high level of cardiac IRP1 activity in DOX-treated animals does not counteract the potential cardioprotective effect of IRP2 downmodulation (33). However, by making cardiac cells unable to sense iron levels, the loss of the aconitase and RNA binding activities of IRP1 may play an important role in inducing the chronic cardiomyopathy that coincides with the gradual conversion of DOX to DOXol.

The ability of DOX to modulate the RNA binding activity of IRP1 and IRP2, and hence the expression of IRE target genes, may be involved in its antitumoral and cardiotoxic properties, and has led to some important clinical findings. Combining DOX with other antineoplastic drugs (e.g., taxanes) enhances chronic cardiotoxicity because the conversion of DOX to DOXol is accelerated (132). Conversely, anthracyclines that form fewer alcohol metabolites have been found to be less cardiotoxic in preclinical settings, and are now being used in clinical trials aimed at comparing their efficacy and safety with those of DOX (130, 132).

E. Cell growth

Iron is an essential component of many of the proteins involved in cell growth and replication, and neoplastic cells require more iron than normal cells because they generally proliferate more rapidly, as indicated by their higher expression of TfR1 and greater uptake of Tf-bound iron. This is why iron chelators have been found to have promising antineoplastic activity in cell cultures and clinical trials (reviewed in (225)). Furthermore, it is interesting to note that recruiting pathways that reduce the availability of intracellular iron is

one of the mechanisms used by p53 to induce cell cycle arrest (230).

Iron homeostasis and cell growth are therefore interrelated, as has been shown by recent findings relating iron depletion-mediated growth suppression at the G1/S transition to a mechanism regulating cyclin D1 expression (145), and the fact that CDC14A mRNA has an IRE (see above). Interestingly, IRP2 is phosphorylated (at Ser 157) regardless of iron by Cdk1/cyclin B1 during the G2/M phase of the cell cycle, and then dephosphorylated by CDC14A after mitosis. This modification reduces its RNA binding activity in the G2/M phase of the cell cycle, thus increasing ferritin synthesis and impairing TfR1 mRNA stability (215). By modulating iron availability, this reversible phosphorylation of IRP2 seems to facilitate cell cycle progression.

It has been previously shown that IRP activity is high not only in mitogen-stimulated cells (183, 201, 202), but also in a classical model of non-neoplastic cell growth such as liver regeneration (13). Cell proliferation seems to induce IRP2 particularly, possibly because of the combined effect of high iron consumption in growing cells and IRP2's preferential sensitivity to iron deprivation (160). However, the demonstration that IRP2 transcription is specifically stimulated by an oncogene (223) suggests that the induction of IRP2 activity (with consequent ferritin repression and TfR1 upregulation) may be specifically aimed at ensuring sufficient iron for the metabolic requirement of proliferating cells.

F. Others

Hormones are among the iron-independent factors regulating IRP binding activity, as is shown by the fact that thyroid hormone modulates the interaction between IRPs and the ferritin IRE mRNA (107). Moreover, in line with the earlier demonstration that IRP1 is upregulated in differentiating preadipocytes (55), it has recently been found that estrogens regulate the RNA binding activity of IRP1 in adipose tissue, and consequently the expression of ferritin and TfR1 (122). It has also been reported that rat livers are characterized by the cGMP-mediated attenuation of IRP binding activity induced by atrial natriuretic peptide (95).

Given the role of iron in viral infection (47), a number of studies have investigated virus–iron interactions at molecular level. Virus infections may affect the RNA binding activity of IRPs, as has been shown in the case of BHV-1 infection in MDBK cells (120). However, this may often be due to other factors, such as increased ROS production in the case of the IRP1 downmodulation induced by hepatitis B virus X protein (69). Similarly, TfR1 downregulation and increased FPN levels reduced the LIP and concomitantly induce IRP2 in a cell culture model of hepatitis C virus replication (56).

Studies of the effects of vitamins on iron metabolism have led to contrasting results concerning the role of IRPs. It has been demonstrated that retinoic acid (vitamin A) supplementation inhibits IRP binding activity and thus modulates hepatic iron homeostasis in rats (182), whereas the cobalamin (vitamin B12)-dependent regulation of ferritin expression in rat spinal cord is not accompanied by any changes in IRP binding activity (17).

V. Role of IRPs in Physiology and Disease

A. Effects of IRP1 and IRP2 deficiency

Gene targeting experiments have provided valuable information concerning the physiological and pathological role of IRPs (particularly IRP2), the deficiency of which dysregulates a number of organs and functions. In two different and independent mouse models, $Irp2^{-/-}$ mice have shown mild microcytosis and impaired hematopoiesis (32, 61). The relatively mild phenotype might be due to the compensatory effect of IRP1, as indicated by the more severe phenotypical alterations observed in $Irp2^{-/-}Irp1^{+/-}$ mice (194). The anemia in $Irp2^{-/-}$ animals is characterized by high serum ferritin levels, normal transferrin saturation, and an accumulation of erythrocytic protoporphyrin IX that is possibly due to ferrochelatase deficiency (38). These mice also have abnormal body iron distribution, with increased hepatic and intestinal loading (32, 61), and either a mild neurological phenotype (62) or late-onset neurodegeneration with severe locomotor abnormalities accompanied by neuronal iron accumulation (64, 105). These findings have been recently confirmed in a novel IRP2 knockout mouse line characterized by locomotor dysfunction and iron accumulation in the brain regions coordinating muscle function (Zumbrennen K. *et al.*, 2009, Abstract, page 69, International BioIron Society Meeting, Porto, Portugal). It has also been reported that IRP2 deletion affects brain copper levels in young mice (139), which may have significant implications for neurodegenerative diseases characterized by altered copper levels, such as Alzheimer's disease. Interestingly, IRP2-deficient mice do not seem to accumulate iron in the pancreas, but have hyperglycemia and impaired pancreatic beta cell function (Zumbrennen K. *et al.*, 2009, Abstract, page 81 International BioIron Society Meeting, Porto, Portugal).

The embryonic lethalness of the deletion of both IRPs (32, 61) has limited the study of the IRE/IRP regulatory network *in vivo*, but the use of Cre/Lox technology has recently allowed the generation of viable animals lacking IRP expression in single tissues. The specific ablation of IRP2 in cells that play a substantial part in iron metabolism has recently made it possible to define the role of IRP2 in regulating tissue-specific and systemic iron homeostasis (54, 60). Knocking out IRP2 in enterocytes, hepatocytes or macrophages does not affect erythropoiesis. Selective ablation in enterocytes or hepatocytes respectively leads to duodenal and liver iron accumulation, but does not alter intestinal iron transport or systemic iron homeostasis. By contrast, the loss of IRP2 binding activity in macrophages does not recapitulate the relatively iron-deficient phenotype observed in the spleen of mice with total and constitutive IRP2 deficiency, thus suggesting that these abnormalities depend on IRP2 function in other cell types.

B. IRP-related clinical situations

High serum ferritin levels are frequently encountered in clinical practice and, once iron overload or inflammation has been ruled out, it is worth seeking a genetic cause involving the disruption of the IRE/IRP regulatory network. Hyperferritinemia with autosomal dominant congenital cataract, a disorder characterized by early-onset, bilateral nuclear cataracts and high serum ferritin levels, is the most widely studied human disease involving the IRE/IRP regulatory system (reviewed in (1, 189)). Patients affected by this disorder

have normal or low serum iron and transferrin saturation, which excludes iron overload as the underlying cause of the hyperferritinemia, but molecular studies have identified multiple point mutations in the IRE of L-ferritin mRNA affecting the highly conserved CAGUGU motif in the IRE loop recognized by IRPs (reviewed in (1, 189)). An inability to block ferritin translation in this disorder has been demonstrated by experiments using cultured lymphoblastoid cells from affected patients: the mutation abolishes the binding of IRPs and leads to constitutively high levels of L-ferritin synthesis that correlate with serum ferritin levels (23). The affected subjects show no hematologic or biochemical abnormalities, and the phenotype of this mutation is only characterized by cataracts associated with the accumulation of L-type ferritin in the lens, although no direct relationship between the mutation and this deposition has been formally demonstrated. Hyperferritinemia caused by a mutation in the IRE has recently been found in a patient without a family history despite reduced L-ferritin mRNA levels, thus underlining the preeminent role of translational control in ferritin expression (22).

The point mutation in the IRE of the human H ferritin gene found in members of a Japanese family affected by iron overload (89) was not identified in another study of a large number of subjects with abnormal serum ferritin values and an abnormal iron status (37).

Another example of the clinical implications of alterations in the IRE/IRP network has been provided by the demonstration that the expression of FPN may also be altered by the loss of its 5′ IRE, as in the case of polycythemic mice (135), or by mutations in the 5′UTR that have been detected in a patient with iron overload due to FPN disease (112), and which may depend on altered IRE-IRP recognition.

A large study combining microarray screening and single nucleotide polymorphism (SNP) genotyping has recently found an association between SNPs in the IREB2 gene encoding IRP2 and susceptibility to chronic obstructive pulmonary disease (44). It has also been reported that polymorphisms in the promoter region of the IRP2 gene are statistically associated with Alzheimer's disease (31). The relevance of dysregulated iron metabolism in the neurological disorders mentioned above makes this finding potentially interesting, but it needs to be confirmed in an independent and larger population, and the functional significance of the haplotype determined. In addition, the high level of IRP1 activity observed in patients with Parkinson's disease may account for the low levels of ferritin in the neurones of the substantia nigra despite the accumulation of iron (53). Another recent study showing IRP1 and IRP2 alterations in the brains of scrapie-infected mice (an animal model of transmissible spongiform encelopathies or prion disease) (96) underlines the role of IRPs in neurodegenerative disorders

VI. Pharmacological Modulation of Iron-Mediated Oxidative Damage

Attempts to translate the "oxidative stress hypothesis of disease" into clinical facts have been quite disappointing. It is certainly true that a given patient may present with reduced circulating levels of one or more antioxidants, but there is no conclusive evidence to support the idea that antioxidant supplementation would benefit that patient. There is robust empirical evidence that diseases such as diabetes or anti-tumor chemotherapy-induced toxicity deplete cell antioxidant defense systems and/or increase the steady-state levels of free radical formation. Nevertheless, interventional trials have almost invariably failed to document any measurable benefit of antioxidants in diminishing the metabolic, neurological, or cardiovascular complications of diabetes or anti-cancer therapies.

Such a disappointing discrepancy between what we see in the laboratory and the lack of therapeutic benefit in clinical medicine has sometimes been referred to as the "antioxidant paradox" (188). It is not too ungenerous to conclude that similar considerations may well apply to many other human diseases in which the overproduction or defective elimination of free radicals is thought to play a causative role. The involvement of iron in experimental models of disease poses similar problems of interpretation.

It is not possible here to cover the whole spectrum of diseases supposedly mediated by oxidative stress and hence curable by means of iron chelators (or molecules modulating IRPs regardless of iron chelation) and/or antioxidants, and so concentrating on the well-defined cardiotoxicity induced by anthracycline may be a more pragmatic approach. Anthracyclines are DNA-intercalating and topoisomerase II inhibitors used to treat breast cancer, lymphomas, and many other human tumors. However, their clinical use is limited by life-threatening dose-related cardiotoxicity, which has been attributed to oxidative stress because of the limited antioxidant defenses of cardiomyocytes in comparison with other cell types (127). Anthracyclines can generate ROS and impair iron homeostasis by releasing iron from ferritin or by inducing an inappropriate degradation of IRP2 or inactivation of IRP1 (126). Moreover, recent evidence suggests that they can also interact with the IRE regions of ferritin mRNAs that are critical to interactions with IRPs (21). It therefore seems that they are more than likely to promote the conversion of $O_2^{\cdot-}$ and H_2O_2 to more damaging hydroxyl radicals or equivalent iron–peroxo complexes (Fig 1) and, on the basis of these premises, iron chelators and antioxidants should both prevent anthracycline-induced cardiotoxicity. However, this is not the case. Cardiotoxicity is prevented by dexrazoxane, an FDA-approved *bis*-ketopiperazine that diffuses into cells, hydrolyses to an EDTA-like diacid-diamide, and thus meets the structural requirements needed to chelate iron before it catalyzes the conversion of $O_2^{\cdot-}$ and H_2O_2 to more damaging oxidants (77).

By contrast, some antioxidants offer protection in animal models but not in patients (131). Carvedilol, a β-blocker with antioxidant properties, was initially found to be protective in lymphoma patients treated with DOX (88), but the study design was questioned (57) and no effort was made to characterize the similar protection offered by β-blockers lacking antioxidant properties (66).

In exploring the discrepancy between the lack of protection provided by antioxidants and the protection offered by dexrazoxane, it is necessary to consider problems related to the appropriateness of antioxidant supplementation and the action of iron chelators. One major barrier to interpreting the presence or absence of an effect of antioxidants is the variability of the doses, timing, and duration of supplementation in published studies. The recommended dietary allowances of vitamins, for example, are intended to provide guidelines for preventing nutrient deficiencies and promoting health in

healthy individuals. However, the dietary supplements that maintain steady-state levels of a given antioxidant in the general population may not have similar effects in individuals exposed to stressors or suffering from chronic illnesses or metabolic diseases (102). Diseased patients probably require higher or more aggressive antioxidant doses and schedules than those recommended by nutritional guidelines (102). On the other hand, antioxidant levels that are high enough to intercept the free radicals generated by anthracyclines or other stressors would convert to secondary radicals which, at least in principle, retain oxidizing potential against cell constituents (208). Finally, the least elegant but most pragmatic conclusion might be that free radicals are not involved in inducing tissue damage—a possibility supported by the observation that N-acetylcysteine decreases oxidative stress but not the cell death induced by DOX in isolated cardiomyocytes (190).

But how can the limited or negative evidence of antioxidant protection be reconciled with the unquestionable protection provided by dexrazoxane? Once again, there are a number of possibilities. First of all, dexrazoxane may protect by means of mechanisms other than iron chelation, such as by interfering with topoisomerase II beta-mediated DNA double-strand breaks (117). Second, if it protects by means of canonical iron chelation, this may introduce benefits that go beyond eliminating the formation of hydroxyl radicals or their equivalents. Looking at this second possibility, it is worth noting that other lipophilic iron chelators have either failed to afford protection (as was the case of deferiprone) (156), or have offered protection at low-intermediate but not higher doses (as was the case of pyridoxal 2-chlorobenzoyl hydrazone and its structurally related cogeners) (196). There is therefore something unique to dexrazoxane that is not shared by other iron chelators and casts doubts as to whether cardiac protection depends on the ligand binding efficacy of one compound or another in diverting iron from oxidative stress.

There is a steady accumulation of preclinical studies of the potential benefits of modulating IRPs, and it seems that both their inhibition and activation may have protective effects. The knockdown of both IRPs enhances the resistance of HeLa cells to H_2O_2 by increasing ferritin synthesis and repressing TfR1 expression (218), whereas IRP1 activation by the investigational antioxidant Tempol has been found to prevent the neurodegeneration associated with functional cell iron depletion in IRP2 KO mice (65). On pharmacological grounds, high-throughput screening has identified low-molecular-weight compounds that activate IRP1 regardless of iron chelation and generate effects linked to the repression of HIF2α mRNA translation (231). Finally, there is promising evidence that compounds such as Fe-bleomycin, cobalt hexammine, $Cu(phen)_2$ (1,10-phenanthroline-Cu), and Ru tpy(bpy) may quite selectively bind at or near the internal loop/bulge of the ferritin IRE, or at the hairpin loop (203); as some ferritin mRNAs remain untranslated even when cytoplasmic iron levels are high, it can be predicted that the abrogation of ferritin IRE regulation by such complexes could further promote the ferritin synthesis that protects cells from iron toxicity (203).

Only the future will show whether all of these compounds and strategies are suitable for study in animal models of disease and eventually patients.

VII. Conclusions and Future Directions

Over the last few years, a stream of new findings has increased our understanding of the complexity of the regulatory network based on IRE/IRP interactions. These include the identification and characterization of a number of upstream regulating factors other than iron, and new target mRNAs in addition to the prototypical ferritin and TfR1 (Fig 6). As the precise control of iron levels is essential for a variety of biological processes at both cell and systemic levels, it is becoming clear that this complexity (two dissimilarly regulated IRPs with small but significant differences in target recognition, and a set of IREs with distinct sequence and structural features) is necessary to ensure sufficient sensitivity and specificity to enable the body to adjust and react to many physiological and physiopathological stimuli.

There has been significant progress in identifying the molecules (most regulated by IRPs) controlling iron homeostasis and their mode of action. Nevertheless, much remains to be learned about their roles and mechanisms of action, as well as those of other molecules that have yet to be discovered. One major prediction arising from the above is that future investigation will need to range from basic structural, biochemical, and cell biological research to physiology and molecular medicine.

New technological approaches based on the high-throughput screening of (synthetic) molecules, "omic" technologies, and modern genetics will presumably increase the number of known IRP-regulated mRNAs. The identification of new molecular targets has shown that the impact of the IRE/IRP regulatory network extends to multiple pathways and pathophysiological conditions. Moreover, we can expect to obtain important information from the generation of mouse lines with the cell-specific, constitutive or inducible ablation (or overexpression) of one or both IRPs. These will provide invaluable animal model systems for unravelling the functions of IRP1 and IRP2 and improving our understanding of the pathophysiological consequences of IRP defects.

In particular, it seems likely that the main areas in which we will learn from future discoveries are brain iron homeostasis (and its derangement in neurodegenerative disorders), mitochondrial iron traffic (and its implications for hematopoiesis), and the connection between oxygen homeostasis and iron metabolism (together with its role in angiogenesis, cancer growth, and host defense).

The contributions from such (only apparently) disparate fields will help us to understand in more detail the role of the IRE/IRP system in the complex and interacting network of iron biology, although the widespread influence of the IRP system may hamper the development of therapeutic interventions aimed at modifying specific IRE/IRP interactions.

Acknowledgments

This study was supported by grants from the Italian Ministry of Health to S.R., and from University Campus Bio-Medico, Rome (Special Project CardioOncology), to G.M.

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Date of first submission to ARS Central, November 5, 2009; date of final revised submission, February 23, 2010; date of acceptance, March 9, 2010.

Abbreviations Used

AHSP = alpha hemoglobin stabilizing protein
CDC14A = cell division cycle 14A
CIA = cytosolic iron–sulfur cluster assembly
Cp = ceruloplasmin
DMT1 = divalent metal-ion transporter 1
DOX = doxorubicin
DOXol = doxorubicinol
eALAS = erythroid 5 aminolevulinic acid synthase
EPO = erythropoietin
FLVCR = feline leukemia virus subgroup C receptor
FPN = ferroportin
HIF = hypoxia inducible factor
HO = heme oxygenase
HRE = hypoxia responsive elements
IRE = iron responsive element
IRP = iron regulatory proteins
ISC = iron sulfur cluster
LIP = labile iron pool
MRCKα = myotonic dystrophy kinase-related Cdc42 binding kinase alpha
NO = nitric oxide
PHD = prolyl hydroxylase
RNS = reactive nitrogen species
ROS = reactive oxygen species
SDH = succinate dehydrogenase
Tf = transferrin
TfR1 = transferrin receptor
UTR = untranslated region

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