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REGULATION OF IRON METABOLISM: Translational Effects Mediated by Iron, Heme, and Cytokines

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CONTENTS

INTRODUCTION 240
RNA STRUCTURES INVOLVED IN IRON RESPONSE 243
PROTEINS THAT BIND SPECIFICALLY TO IREs 244
EFFECTS OF REGULATORY MOLECULES OTHER THAN IRON ON FERRITIN AND TfR PROTEIN SYNTHESIS 246
Cytokines 246
Nitric Oxide 249
Phosphorylation 249
FUTURE QUESTIONS 250
IRE Stability: Correlation with IRP Binding and Translational Efficiency 250
Role of Ferritin mRNA Sequences Distinct from the IRE 250
Role of Sulfhydryl Groups in IRP Activity 251
Heme and Iron as Inducers of IRP Dissociation from IREs 252
Role of Citrate and Isocitrate in the Cytoplasm 254

ABSTRACT

Recent advances in the knowledge of iron metabolism underscore its complex relationship to overall cell metabolism. One of the key components of the iron uptake and storage pathway is ferritin, a protein that sequesters iron in a nontoxic form. Ferritin synthesis is translationally regulated by iron. Molecules such as nitric oxide and cytokines also affect transcriptional and/or posttranscriptional ferritin synthesis. Conversely, iron-containing molecules affect expression of mitochondrial aconitase, erythroid aminolevulinic acid synthase,

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and nitric oxide synthase. This observation indicates a complex linkage between iron metabolism and a variety of other important cell activities. The finding that the cytoplasmic iron-responsive protein (IRP) has two forms also raises intriguing questions about the relationship between the cytoplasmic aconitase and translational regulation of mRNAs such as ferritin. At least one of the IRPs can be phosphorylated. These recent discoveries open exciting new avenues for research that should lead to a better understanding of cellular iron metabolism.

INTRODUTION

Regulation of iron metabolism is central to overall cell function. Control is accomplished in part by adjusting the levels and activities of specific proteins. The best-studied example of such a protein is ferritin, a multimeric protein that is evolutionarily conserved from prokaryotes to eukaryotes (49). It is thought that ferritin provides a storage depot for iron that can later be used in a variety of metabolic pathways (29, 30, 32). The ubiquity of ferritin likely results from the reactivity of iron in an aerobic environment wherein toxic oxygen free radicals are produced via Fenton-type reactions (reviewed in 55, 84, 110). Thus, ferritin apparently acts both as an iron-storage repository and as a cytosolic defense against generation of potentially toxic free radicals (3, 4, 14, 31).

For cells in the steady state, a rapid influx of iron would necessitate an equally rapid response in the synthesis of ferritin, since apoferritin is normally not present in excess. Higher eukaryotes have evolved a strategy wherein the response to iron excess is achieved primarily by posttranscriptional mechanisms (reviewed in 56, 77, 85, 95, 100, 126, 128); however, transcriptional induction plays a role under some conditions (10, 139). In contrast, prokaryotes, yeast, and plants respond to iron influx chiefly via transcriptional mechanisms (127). This dissimilarity is perhaps related to different mechanisms of iron transport.

In this review, we focus on the posttranscriptional mechanisms employed by nonerythroid cells in animal systems. Erythroid cells destined to synthesize large amounts of hemoglobin respond to iron levels somewhat differently (108, 118). The specialized nature of these cells is beyond the scope of this review, and we direct the reader to other literature on this subject (20, 108, 109, 118). Another emerging topic to which we unfortunately cannot devote more space is transferrin-independent uptake of iron by cells. This process has a number of implications, primarily for the specialized requirements of intestinal epithelial cells. We encourage the reader to examine recent work in this area (23, 24, 71, 72).

Originally conceived by Munro and colleagues (141), a model to account for the rapid, transcription-independent induction of ferritin synthesis in response to iron has evolved. In this model, iron-poor cells contain ferritin mRNA that is translationally inactive owing to the binding of a *trans*-acting factor to

a *cis*-acting repression site within the mRNA. Treatment of these cells with iron salts, transferrin, or heme induces the translation of these previously inert mRNAs by releasing or altering the *trans*-acting factor. The *cis*-acting sequence found within the mRNAs for both the heavy (H) and light (L) forms of ferritin was subsequently identified (2, 65). This sequence is a conserved stem-loop structure referred to as the iron-responsive element (IRE) (reviewed in 77, 85, 95, 100, 128; see Figure 1). An IRE located in the 5′ untranslated region (5′ UTR) of an mRNA serves as a binding site for the *trans*-acting factor which, when bound to the IRE, represses translation of that mRNA (reviewed in 77, 85, 95, 100, 128). This factor is a 98-kDa protein that has been found in organisms as diverse as chickens, frogs, fish, flies, humans, and mice (114). We refer to it as iron regulatory protein (IRP), first referred to as such by Hentze (62), although the term was coined jointly by Lukas Kühn and Richard Klausner. IRP is also known variously as iron-responsive element binding protein (IRE-BP) (51), iron regulatory factor (IRF) (98), ferritin repressor protein (FRP) (15, 132), and P90 (57). Some investigators have proposed that binding of IRP to the IRE prevents access of eIF-4F (the cap binding protein) to the 5′ cap structure, resulting in a blockage of initiation (13, 45, 125). IRP bound to a 5′ UTR IRE prevents association of the 43S ribosomal

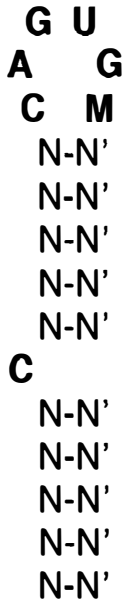

Figure 1 The consensus secondary structure of the functional IRE found in ferritin mRNA among others. In the sequence shown, M represents any nucleotide except guanosine, and N-N′ indicates two base-paired nucleotides within a stem structure. The structure of this RNA element has been determined from phylogenetic as well as mutagenic studies (12, 59, 73, 133).

preinitiation complex with ferritin and erythroid aminolevulinic acid synthase RNAs (47). When iron levels are increased in the cell, IRP dissociates from the IRE, which enables initiation of translation of ferritin mRNA (reviewed in 77, 85, 95, 100, 128). A second IRP, originally discovered in rodents, is referred to here as IRP-2 (50, 60, 87, 140). The significance and characteristics of this variant of IRP will be discussed in more detail in the following sections.

Ferritin is not the only gene that contains an IRE. Other messages that contain functional IREs in their 5′ UTRs encode erythroid δ-aminolevulinic acid synthase (δ-ALAS) (13, 28) and mitochondrial aconitase (mAcon) (33). Although the transferrin (Tf) mRNA contains an IRE-like sequence in its 5′ UTR, the effect of this sequence on translation is minimal (less than a 50% rate change), and its response to iron is opposite to that of ferritin (27). IRE-like sequences have also been reported in the 5′ UTRs of the *Drosophila melanogaster* toll transcript (33) and the *Azobacter vinelandii* bacterioferritin (85); however, neither of these IREs confers an iron response in vitro or in vivo. An IRE-like sequence has also been reported within the open reading frame (ORF) of the human β-amyloid protein (124). The putative IRE within the message for this Alzheimer's disease–related polypeptide is unrelated to pathogenicity and is not considered functional (143).

Although the transferrin receptor (TfR) message contains several functional IREs, these are located in the 3′ untranslated region (3′ UTR) of the mRNA (18, 86, 97). IRP also binds to these IREs in the absence of iron and dissociates in the presence of added iron. While bound to the IREs in TfR mRNA, IRP confers protection from degradation by nucleases (19, 56, 79, 98). Dissociation of IRP by iron uptake results in rapid degradation of TfR mRNA, which is correlated in time with an activation of ferritin translation. Thus, a coordinately regulated cycle for iron uptake and storage is created (77, 82).

Several investigators have addressed the question of how the IRP senses iron availability. From the observations that reductants such as 2-mercaptoethanol (2-ME) can activate binding of IRP to the IRE in vitro and that sulfhydryl oxidants such as diamide inactivate RNA-binding affinity, these investigators postulated that a "sulfhydryl switch" governed activation of IRP (66). This first hypothesis concerning the mechanism of IRP activation was expanded when the gene for IRP was cloned and sequenced from different sources (67, 103, 107, 116, 140). Sequence comparison of the human IRP to the human mitochondrial aconitase (mAcon) showed a high degree of homology (~30%) (63, 115). IRP has since been determined to be the apo version of the cytosolic aconitase (cAcon) (11, 25, 52, 54, 75, 76). The mAcon is a Krebs cycle iron-sulfur enzyme that catalyzes the conversion of citrate to isocitrate via a *cis*-aconitate intermediate. This exciting finding has led to the conclusion that iron is directly involved in the iron-sensing mechanism of IRP. This model is based on the ability of apo-mAcon to form an iron-sulfur cluster

(three or four iron atoms in conjunction with four sulfur atoms) and the fact that IRP (apo-cAcon) can also form an iron-sulfur cluster (25, 52, 54, 63, 76, 115). An IRP with a fully loaded iron-sulfur cluster cannot bind to IREs, presumably because the RNA-binding domain of IRP is occluded. In such a state, the molecule also exhibits aconitase activity.

In addition to the possible role of a sulfhydryl switch and the influence of the presence of the iron sulfur cluster, a hypothesis not related to aconitase homology has been put forth to explain the inactivation of IRP. This mechanism postulates that heme can bind to IRP and induce dissociation of IRP from IREs (43, 44, 88–90). The early observation that maximal induction of ferritin synthesis in response to iron salts is not as great as maximal induction by heme led to the development of this mechanism (8, 38, 69). A key observation was that heme specifically inactivates IRP in vitro (88–91). IRP was subsequently shown to bind to heme in vitro and in vivo (90, 93a). It then apparently forms several high molecular weight species (HMS) (43, 44) that are subsequently degraded proteolytically in an ill-defined pathway. Additionally, Eisenstein et al (38) have shown that heme can induce ferritin synthesis by serving as a source of iron that can be released by the action of heme oxygenase.

Induction of IRP by heme or iron salts in vivo was recently shown to be essentially irreversible under standard growth conditions in the absence of iron chelators or nitric oxide (NO) (43, 44). This observation is consistent with the fact that heme induces proteolytic degradation of IRP. However, it raises interesting questions about the fate of the fully iron-loaded cAcon. If the latter is considered a stable end product, one wonders whether it continues to serve some important function. These and other questions are discussed below.

RNA STRUCTURES INVOLVED IN IRON RESPONSE

The minimal consensus structure of the IRE is shown in Figure 1. The loop portion of the IRE contains the consensus sequence CAGUGM, where M may be C, U, or A (12, 73, 133). Because the variant loop sequence UAGUAM functions just as well as the consensus sequence in vitro, it has been proposed that a base-pair interaction occurs between positions 1 and 5 (C–G, in the consensus sequence) (59). Altering the intervening AGU reduced affinity for IRP (59). Adjacent to the loop is a canonical 5–base pair (bp) stem. In addition, a conserved unpaired cytosine residue is located on the 5′ side of the IRE immediately preceding the 5-bp stem. Figure 1 depicts a simplified model of the IRE; however, sequences other than the IRE are also required for optimal ferritin translational induction (13, 26, 34–36, 128). For example, endogenous ferritin synthesis undergoes a 20- to 30-fold induction upon iron stimulation (26, 45, 46). However, in constructs where a heterologous ORF is placed downstream from an IRE, only a 4- to 5-fold induction by iron has been noted

(26, 34). Data presented elsewhere are in agreement with these observations (45, 46). The base-paired regions flanking the IRE account for an approximately 2-fold effect in translational induction of ferritin in vitro (36, 128). This effect is paralleled by an increase in affinity of IRP for the IRE with intact flanking regions (5).

PROTEINS THAT BIND SPECIFICALLY TO IREs

IRP has been purified to various extents from several natural sources, including human placenta (101), rabbit liver (132), and beef liver (76). Small amounts of pure IRP have been produced by in vitro transcription and translation (67, 68). IRP has been overproduced in transfected mouse fibroblasts (52, 75), in baculovirus-infected insect cells (6, 41), and in vaccinia virus–infected HeLa cells (48). In addition, IRP has been produced in bacteria (48, 68). These various methods of production allow the study of individual (e.g. apo or iron-loaded) IRP states.

Treatment of either intact organisms (134) or cultured cells with iron salts or with heme induces ferritin synthesis while decreasing production of TfR (4, 5, 8, 43, 44, 69, 99). These phenomena are temporally coordinated with a switch from IRP to cAcon (48, 68, 77-78, 105). To reverse these changes, new IRP must ordinarily be synthesized. These and other observations indicate that the iron-loaded form of IRP (cytoplasmic aconitase) does not bind RNA in vivo, thus permitting ferritin synthesis and destabilization of the TfR mRNA.

This in vivo inactivation of IRE binding capacity can be mimicked in vitro by treating IRP with iron salts under reducing, anaerobic conditions in the presence of S^{-2} (25, 54, 76). RNA binding by IRP can also be inactivated by treatment with oxidizing agents such as ferricyanide (52, 68) or diamide (66, 68, 105, 121). IRP, once oxidized, can be reactivated for IRE binding by the addition of very low concentrations of 2-ME (0.02% ≈ 2.8 mM) (52, 68). This level falls within the normal range of the cellular redox state (1).

In vitro, high concentrations of reductants such as 2-ME or 2-mercaptoethanolamine induce the iron-loaded form of IRP to bind to the IRE. This effect can be largely prevented by the addition of aconitase substrates (e.g. citrate) (52, 54). The addition of dithiothreitol (DTT) is curious in that it has anomalous effects: At neutral pH, even 50 mM DTT cannot induce the cAcon to bind IRE (99). However, 100 mM DTT restores some IRE-binding activity. In contrast, other authors have found that at pH 8.9, 100 mM DTT approximates closely the effects of 2-ME (54). Interestingly, Zheng et al (142) have reported that the apo, 3-Fe, and 4-Fe forms of IRP all bind the IRE within the message for mitochondrial aconitase with similar affinity. These studies were conducted near neutral pH; however, the reactions contained 50 mM DTT, which may account for this result.

Variants of IRP have been created by using site-directed mutagenesis (68, 105, 106). By observing the effects of thiol-modifying agents on these variants of IRP, investigators determined that three cysteines were required for coordination of the iron-sulfur cluster: Cys 437, Cys 503, and Cys 506. Additionally, Cys 437 must be reduced in the apo form for IRE binding to occur. The switching of IRP to cAcon reduces the affinity of the protein for RNA. However, substitution of the aconitase active-site serine (S778) with alanine shows that it is the formation of the iron-sulfur cluster, not aconitase activity per se, that inactivates RNA binding (106).

Recent experiments using UV crosslinking of radiolabeled IREs have identified RNA-binding active sites on IRP (7, 122). The regions of IRP that are crosslinked to the IRE include the site where the aconitase iron-sulfur cluster normally forms. This finding is consistent with the existence of two mutually exclusive states of IRP, namely RNA binding and aconitase activity (11). Additional site-directed mutagenesis studies have implicated three arginines, R536, R541, and R780, as critical to high-affinity RNA-binding (106). Only R536 can be replaced by lysine, suggesting that it is necessary for RNA binding solely by virtue of its positive charge. The other two arginines presumably also have hydrogen-bonding capability.

A second IRP (IRP-2) has been identified in RNA band-shift experiments using rodent cell lysates (60, 87, 98, 114). IRP-2 is distinct from IRP in that antibodies raised against a 75–amino acid peptide within IRP-2 do not recognize IRP (50). The complex of rodent IRP-2 with the IRE migrates more rapidly to the anode during electrophoresis than the complex of IRP with the IRE (16, 60). However, the rodent IRP-2-IRE complex migrates in a manner identical to the human IRP-IRE complex (60). Whether IRP is expressed at detectable levels in humans is unknown; however, two human cDNA clones with similar sequences exist for IRP (40, 116). Recently, the second human IRP was shown to be homologous to rat IRP-2 (50).

The function of IRP-2 appears to be similar to that of IRP. IRP-2 binds to the same wild-type IREs as does IRP, but its specificity varies slightly. Guo et al (50) found that IRP-2 binds to the rat L (light chain)-ferritin IRE with an affinity equivalent to that of IRP. They also noted that the affinities of IRP and IRP-2 were equal in binding to two different TfR IREs. However, using a variety of IREs mutated in the loop sequences, Henderson et al (59) found a hierarchy of affinities of IRP-2. For instance, IRP binds to an IRE where C1/G5 have been replaced by A1/U5 (59). In contrast, IRP-2 does not bind to this IRE variant in vitro, indicating a greater specificity of IRP-2 for the native sequence. Moreover, IRP-2 binds in vitro more strongly to the ferritin H (heavy chain)-IRE than to any other (59). It would be helpful to test the binding of IRP and IRP-2 with all known native IREs to determine the biological significance of the observed specificities.

The iron response of IRP-2 also differs strikingly from that of IRP (16, 16a, 50, 60, 116a). Recent evidence indicates that IRP-2 cannot acquire aconitase activity (50). Furthermore, addition of 2% 2-ME does not increase the affinity of IRP-2 for IREs in vitro (50). Iron administration downregulates IRP-2 function; however, this process apparently occurs by way of a specific decrease in the protein level. A similar decrease in IRP levels has been noted under certain conditions (43, 44). This effect was thought to be triggered by heme. The effect of heme administration on IRP-2 has not been tested, but such studies might help determine how the two distinct versions of IRP are regulated by different means.

Treatment of purified IRP with heme in vitro also reduces its RNA-binding affinity (88, 90, 99, 121). This effect of heme can be highly specific for IRP, provided that reducing agents and at least 0.1 mg/ml carrier protein are present (91). Of the variety of metalloporphyrins tested, only heme and Co^{+3} protoporphorin IX had significant activity (53, 88). The inactivation of RNA-binding ability is thought to result from the spontaneous association of heme with IRP. Heme binds to IRP both in vitro (88–90) and in vivo (43, 93a). A highly specific complex that is stable to boiling in sodium dodecyl sulfate (SDS) forms within 15 min of mixing the pure components in vitro (90, 91). Digestion of this complex with V8 protease yields a 15-kDa peptide that is tightly bound to heme, which is also stable in SDS (90). These results suggest that heme may link covalently to IRP. Heme also catalyzes the cross-linking of IRP molecules to each other in vitro. However, the physiological significance of this observation is questionable: Whether these multimeric forms of the protein are related to HMS observed in vivo is not clear, although their mobilities during electrophoresis are similar.

Treatment of growing cells with heme also induces the irreversible degradation of IRP (43, 44; see also Figure 2). In contrast, under conditions in which cells are confluent (i.e. stationary phase), little or no IRP degradation is observed (44, 123). This growth-state dependence of heme effects may reflect tissue and cell specificity in vivo (37, 44, 99). The molecular nature of the different effects of heme on IRP in growing vs stationary cells might result from differences in IRP phosphorylation state (40).

EFFECTS OF REGULATORY MOLECULES OTHER THAN IRON ON FERRITIN AND TfR PROTEIN SYNTHESIS

Cytokines

Cytokines have been implicated in modulating synthesis of proteins that are iron regulated (42, 96, 111–113, 117, 129, 130). For instance, induction of ferritin synthesis by cell differentiation or by cytokines results in the selective

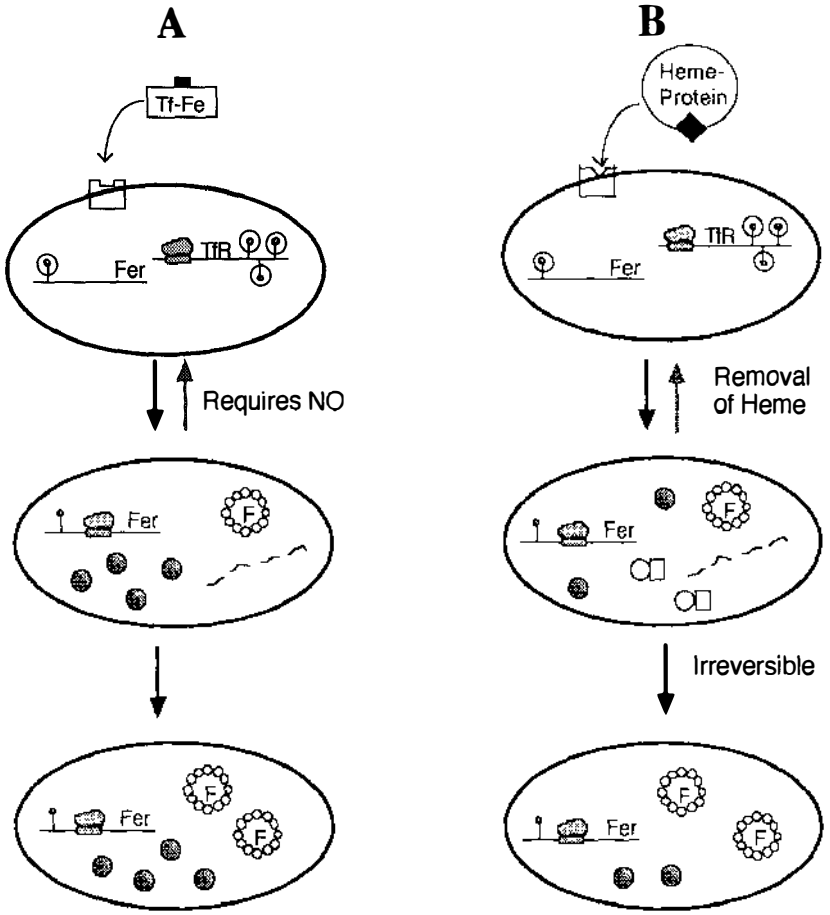

Figure 2 Two proposed pathways for how iron and heme affect ferritin and TfR translation, as well as their differential effect on IRP in nonerythroid cells, are depicted. (*a*) Initially, iron enters an iron-poor cell via the TfR receptor. Open circles indicate IRP, and the ferritin and transferrin receptor mRNAs are shown. Iron uptake promotes formation of iron-sulfur clusters in IRP (i.e. to create cAcon, depicted as shaded circles), causing release of the proteins from the IREs to which they had been bound. The release of proteins from IREs enables translation of the ferritin mRNA (ferritin is shown as a ring of small subunits encircling the letter F) and degradation of the TfR mRNA (degraded TfR mRNA is shown as fragments). For re-repression of the ferritin mRNA to occur under ordinary conditions, new IRP must be synthesized (42). However, some cells respond to nitric oxide, which can induce IRP rebinding to IREs, presumably by disrupting iron-sulfur clusters of cAcon (37, 120, 137). (*b*) The pathway by which heme enters an iron-poor cell involves carrier proteins (indicated by "heme-protein") such as hemopexin or haptoglobin (bound to hemoglobin). Some of the heme is degraded by heme oxygenase, which releases iron. This intracellular iron can act in a manner similar to that depicted in (*A*). However, undegraded heme can also bind to IRP directly (93a). The interaction of heme with IRP induces the formation of a poorly defined material termed high molecular weight species (HMS) shown as a covalent complex of IRP molecules with each other or with other unknown proteins (depicted as open squares) (43, 93a). Whether IRP within this HMS contains an iron-sulfur cluster is not known. Although IRP within HMS may still be able to bind IREs, it is a short-lived species and is degraded within a few hours. If heme influx is brief, undegraded HMS can revert to form functional IRP. Longer treatment with heme (>1 h) causes the irreversible loss of IRP.

stimulation of H- vs L-ferritin. This process is accomplished by both transcriptional (96, 135) and translational mechanisms (111–113). In these studies, the effect of iron, which is translational, appears to be independent of the cytokine effects.

The individual effects of cytokines vary. The acute response polypeptide, interleukin-1α (IL-1α), has been shown to induce the preferential synthesis of the ferritin H-chain in both myoblasts and myotubes, resulting in an increased H:L subunit ratio (135). These authors also found that another acute response protein, tumor necrosis factor-α (TNF-α), has the same effect on ferritin synthesis, which is additive with the effect of IL-1α. The action of IL-1α is not decreased by the addition of anti–TNF-α antibodies, which precludes the possibility of IL-1α induction of TNF-α. Torti and coworkers also found that TNF-α preferentially induces ferritin H-chain synthesis in muscle cells (129) and myoblasts (96). In myoblasts, the action of TNF-α was determined to be independent of iron and can be arrested by actinomycin D, indicating an effect on transcription of the ferritin H-chain.

Recent studies of the effects of cytokines on monocytes, which are major sites of cytokine synthesis as well as response, are generally in agreement with earlier studies on other cells (42). Different cytokines elicit selective effects on these cells. In these studies, interferon-γ (IFN-γ) and TNF-α induced ferritin H-chain mRNA levels, whereas interleukin-1β (IL-1β) had no effect on these levels. None of these cytokines had much effect on the levels of mRNA for L-ferritin. Thus, the H:L mRNA ratio was increased by TNF-α and IFN-γ, but not by IL-1β. Although TNF-α and IL-1β also decreased TfR levels, IFN-γ had no effect on expressed TfR (42). IL-1β, IFN-γ, and TNF-α all decreased the overall rate of iron uptake substantially, but TNF-α induced a greater fraction of the iron that was taken up to become incorporated into ferritin than did the other two cytokines (42). The reduction of TfR expression by TNF-α and IL-1β correlates with decreased iron uptake; however, IFN-γ reduced iron uptake despite its lack of effect on TfR levels. The reason for this effect is at present unknown.

The effect of another cytokine, interleukin-2 (IL-2), on TfR expression in a mouse T-cell line was recently described (113). This cytokine activates transcription of the TfR gene by approximately threefold and induces stabilization of the TfR mRNA. This process occurs via activation of IRP-binding activity and by an undefined mechanism that inhibits the degradation of the RNA.

In addition to their transcriptional effects (described above), cytokines also affect translation of ferritin (111, 113). In human hepatoma cells, IL-1β increases the synthesis of both H- and L-ferritin (113). The mRNA levels of L-ferritin are not increased by IL-1α or IL-1β (42, 96, 129, 135). The induction of L-ferritin synthesis is accompanied by a shift of L-ferritin mRNA from monosomes to polysomes, indicating an enhancement of its translatability

(113). In that study, Rogers et al found that the rate of iron uptake was unchanged by IL-1β. However, Fahmy & Young (42) observed that iron incorporation into ferritin was slightly increased upon IL-1β stimulation. These results together suggest that intracellular iron is rerouted during this treatment.

A recent report showed that a 20-nucleotide sequence within the ferritin H-chain 5′ UTR, distinct from the IRE, is responsible for an approximately twofold enhancement of translation following treatment by IL-1β (112). This sequence has been termed the acute box and appears to function at the level of translational initiation. When the iron chelator desferal is added in conjunction with IL-1β, no induction of L-ferritin translation is observed (113). Thus, the translational induction by IL-1β must occur after release of IRP from the IRE.

Nitric Oxide

The actions of cytokines should be analyzed in concert with the effects of nitric oxide (NO), an extremely potent second messenger of many cytokine actions (61, 92, 138). Furthermore, NO affects iron-containing proteins (19a, 57a, 61, 120, 138), including IRP (37, 72a, 136). The mechanism of NO action on ferroproteins can occur either directly or via metabolites of NO and oxygen (19a, 57a, 61). The disruption of an iron-sulfur cluster in cAcon by NO would be expected to induce RNA-binding ability of IRP, as has been observed experimentally (37, 72a, 136). Indeed, it has been suggested that the primary function of cAcon may be to sense NO and transduce this signal to alter iron metabolism (120). Thus, an elegant mechanism for connecting iron metabolism to inflammation response may be mediated by NO. Recent evidence shows an elaborate feedback mechanism relating NO synthase production to intracellular iron availability in murine macrophages (137).

Phosphorylation

IRP can undergo phosphorylation mediated by protein kinase C (PKC) (40). This phosphorylation correlates with increased IRE-binding affinity of IRP in vitro. Whether IRP or the cAcon version of the protein is preferentially phosphorylated is not known. It would be of interest to determine whether phosphorylation of the protein occurs in vivo and whether it affects the assembly or disassembly of the iron-sulfur cluster.

Because mitosis is linked to PKC activity, the phosphorylation of IRP may be related to cell proliferation and to cytokine stimulation of cells. Upon partial hepatectomy of rat livers, levels of ferritin decline and levels of TfR increase (16). This outcome could be related to phosphorylation of IRP, since hepatectomy induces a state of rapid cell division when enhanced PKC activity would

be expected. The phosphorylated serines of human IRP are not present in human IRP-2 (40), but the significance of this observation is not yet clear.

As noted above, the effects of heme on IRP stability are strongly dependent on cell growth rate, suggesting that phosphorylation of IRP may be required for heme interaction.

FUTURE QUESTIONS

As indicated above, the effects of cytokines, NO, and phosphorylation on the functions of IRP are obviously of great interest and underscore the linkage between iron metabolism and overall cell activities. Exploration of the effects of these agents should be a fruitful endeavor. However, other questions remain regarding aspects of the IRE and IRP that are less easy to interpret.

IRE Stability: Correlation with IRP Binding and Translational Efficiency

In addition to its role as a binding site for IRP, the IRE may act as a translational enhancer element for ferritin in vitro (35, 128). A recent report by Coulson & Cleveland (26) indicates that in the presence of iron, polysome formation in vivo on IRE-bearing mRNAs is more efficient than on cognate mRNAs from which the IRE has been deleted. Thus, the IRE can confer both positive and negative regulation of translation efficiency, depending on the binding status of IRP. Exactly how the IRE might facilitate translational initiation is unknown; however, it has been postulated that an IRE adjacent to a 5′ cap structure could enhance binding of translation initiation factors, perhaps simply by enhancing cap accessibility (125, 128). The significance of the correlation of translational efficiency with the presence of an IRE is interesting and merits further exploration to determine the underlying mechanism. A number of mRNAs that have interesting structures in the 5′ UTR have been identified, raising the possibility that such structures may be of general regulatory significance (125). Preliminary evidence makes direct binding of global translation factors to the IRE seem unlikely. For example, in vitro RNA-bandshift experiments designed to detect binding of IRP to a minimal IRE (~30 nt) using lysates from iron-depleted cells always show specific binding of IRP to the IRE. In contrast, when lysates from heme- or iron-treated cells are used, no specific complex formation is found.

Role of Ferritin mRNA Sequences Distinct from the IRE

Early studies elegantly demonstrated that the 28-nt IRE was both necessary and sufficient to confer iron inducibility to mRNAs (64). However, more recently, several groups have shown that the full range of iron inducibility is not always observed for foreign mRNAs that have been added to the IRE alone

(26, 34, 36, 57, 112, 128). For instance, an IRE placed within the 5′ UTR of the human growth hormone (hGH) transfected into HeLa cells confers only a 5- to 8-fold iron induction of hGH production, whereas endogenous ferritin is induced 20- to 30-fold (46). Similarly, an IRE placed within the 5′ UTR of a cytomegalovirus (CMV)-promoted chloramphenicol acetyltransferase (CAT) gene transfected into Chinese hamster ovary (CHO) cells confers only a 3-fold iron induction compared with a 27-fold induction for the endogenous ferritin (26). A slightly better (8- to 16-fold) iron inducibility was obtained in HepG2 cells when a construct containing the ferritin H-chain IRE was placed at the 5′ end of a message encoding CAT (112); however, this level is still lower than that expected for endogenous ferritin.

A recent report showed that a 20-nucleotide sequence within the ferritin H-chain 5′ UTR, which is downstream from the IRE, is responsible for an approximately twofold enhancement of translation following treatment with IL-1β (112). This 20-nucleotide sequence is similar to sequences found in many mRNAs that encode acute-phase response proteins and has therefore been termed the acute box (111, 112). The acute box appears to function at the level of translational initiation.

Another interesting observation has been made using bicistronic messages in mouse C127 cells, in which a ferritin ORF is 5′ proximal and a CAT ORF is situated downstream (34). Using this system, investigators have demonstrated that inclusion of ferritin ORF sequences in addition to an IRE results in increased iron inducibility. This increase is accompanied by mRNA stabilization and strong translational suppression of the downstream CAT ORF (34). The basis for this effect of the ferritin ORF is unknown; however, sequences within the ferritin ORF may anchor the binding of mRNA proteins other than IRP. These proteins could interact with IRP to stabilize its binding, thereby increasing the range of iron inducibility. They may also mask the mRNA from nucleases as well as inhibit the translation of downstream ORFs. Thus elements within the 5′ UTR, as well as the ORF of the ferritin mRNA, appear to be important for achieving the full range of translational induction.

Role of Sulfhydryl Groups in IRP Activity

The earliest hypothesis as to how IRP senses iron abundance invoked a sulfhydryl switch (66). This hypothesis has since been largely superseded by the concept of iron-sulfur cluster formation and of heme cross-linking and heme-dependent degradation mechanisms. It is possible, of course, that the observed dependence on a reduced sulfhydryl is only seen in vitro. However, there are now several well-known examples of redox-regulated transcription factors, including the well-studied NF-κB (58). Activation of NF-κB can be observed in T cells during oxidative stress and/or cytokine stimulation. The activation

of NF-κB results from its reduction by thioredoxin (Trx) (9, 70). Trx levels increase in T cells upon treatment with H_2O_2 or TNF-α (58), indicating a correlation between the effects of oxidative stress and TNF-α administration (17 and references therein; 93).

Other examples of redox regulation of RNA-binding proteins are the binding of thymidylate synthase (TS) to its own mRNA (21), and an unnamed protein that stabilizes the catalase mRNA during hyperoxic shock (22). Whether Trx or a Trx-like protein is involved in either of these cases is unknown.

Although considerably more reduced glutathione than oxidized glutathione is present in normal cells (1), local sequence effects surrounding cysteines can render them unusually redox sensitive (94). For IRP, Cys-437 is the residue responsible for its sensitivity to reductants in vitro (68, 105). The primary sequence surrounding this cysteine contains no charged residues; however, according to the X-ray structure of mAcon, there are four arginine and three histidine residues in the vicinity of this cysteine (83). Cysteines placed within the basic environment conferred by those residues are likely to be more reactive toward oxidants than would be the case for cysteinyl residues in an acidic environment (119). Thus, the sulfhydryl switch might be relevant to the activation or inactivation of the cAcon in certain cases, provided that an enzyme such as Trx were capable of catalyzing this event.

Heme and Iron as Inducers of IRP Dissociation from IREs

Why do there appear to be two distinct pathways for the inactivation of IRP (Figure 2)? What advantage is to be gained from the heme pathway? Heme is not normally found free in serum but rather is complexed primarily to hemopexin and albumin (3, 4, 118). In addition, under oxidative conditions, reticulocytes can be ruptured to release hemoglobin, which is readily bound by haptoglobin (118). The hemoglobin-haptoglobin complex is then taken up by the liver (118). Once inside the cell, heme induces synthesis of heme oxygenase, which rapidly accrues to high levels (3, 4, 38). The presence of heme oxygenase desensitizes the cells to the oxidative damage that occurs during chronic heme challenge (3). This desensitization is correlated with an increase of apoferritin production and a decrease of TfR synthesis. Therefore, heme entry appears to upregulate iron storage and antioxidant capacity (14).

As noted above, high levels of intracellular heme can regulate iron uptake and storage by triggering the irreversible degradation of IRP (43, 44). Yu et al (140) found that IRP in rat liver is not degraded after ferric ammonium citrate treatment, which strongly suggests that the heme-dependent degradation of IRP is not the major pathway involved in liver tissue. However, the fact that IRP-2 iron-dependent degradation occurs in cells in which IRP is not

degraded but converted to cAcon suggests that two different signaling pathways may coexist in the same cells.

One advantage of a heme-induced mechanism is that it would shorten the overall processing time of excessive amounts of intracellular heme by ~50%. The processing time is reduced because the induction of ferritin synthesis by iron is slow, requiring 4–6 h to attain a maximum rate. Thus, if heme can also act as an inducer, then the induction of ferritin and heme oxygenase synthesis can occur simultaneously rather than in tandem. Allowing these two processes to take place simultaneously should therefore decrease the duration of cell exposure to high levels of intracellular iron and heme by several hours. The advantages gained by such shortened response times may not be trivial, especially when the presence of oxygen would enhance the production of free radicals through the Fenton reaction (110). Indeed, in view of the high toxicity of iron and heme, an excess of these substances may well be expected to elicit a stress response. The fact that heme oxygenase is one of the most common stress proteins is consistent with this view. Another advantage of a heme-induction mechanism is that cellular uptake of iron via the transferrin pathway could be rapidly shut down soon after the influx of heme and before elaboration of the heme oxygenase pathway.

Of course, the implication that iron and iron compounds may be considered essential metabolites as well as stressors does not necessarily mean that more than one inductive mechanism exists. Nevertheless, this duality of roles for iron makes the evolution of multiple levels of control plausible.

An interesting comparison can be made between iron metabolism and metabolism of cadmium. The primary mechanism of storage and/or detoxification of these cadmium ions is through chelation by metallothionein. However, a secondary detoxification mechanism was recently identified (74). This mechanism involves one or more protein-degradative steps, as inferred by the utilization of a special ubiquitin-conjugating enzyme in the detoxification process. Thus, the two pathways of cadmium metabolism share striking features with the two iron-dependent pathways shown in Figure 2; however, whereas IRP is clearly a regulatory protein, metallothionein is not. Nevertheless, because the target(s) of the cadmium-induced degradation is not known, one or more regulatory proteins may be preferentially degraded in the presence of cadmium. This notion that a stress response may be mediated by the preferential degradation of a regulatory protein that normally functions to keep the stress-response pathway in the "off" mode is inherently satisfying. An especially attractive aspect of this hypothesis is the ease with which specific stress-response mechanisms could evolve, especially under conditions of repeated challenge by high levels of the stressing agent.

These considerations may explain why both heme and iron can act as primary inducers in vivo. When iron is present at a low level, its toxicity is

minimal, and so it could easily be processed by a slow mechanism suitable for handling metabolites. In contrast, when heme, which is much more active than iron in Fenton chemistry, is present at a high concentration, the rapid mechanism that is more closely identified with stress responses would be expected to predominate (3, 4). In accordance with this rationale, iron is taken up and stored by liver tissue without concomitant degradation of IRP (140). In contrast, evidence of IRP degradation was inferred in brain tissue exposed to lysed erythrocytes that generate free heme (80, 81). The proposal by Eisenstein & Munro (39) that macrophages and other reticuloendothelial cells might also employ the heme-dependent degradative pathway is consistent with this hypothesis.

Role of Citrate and Isocitrate in Cytoplasm

For a number of years, the question of how iron is transported within cells has remained unanswered. Iron has a high affinity for nucleotides, phosphate, and carboxylic acids, such as citrate and isocitrate; however, radioactive iron tracer experiments have led to the conclusion that iron is nearly always bound to some protein and, conversely, that very little is associated with low molecular weight chelators (LMC) (102, 104). An exception to this finding is apparent during postischemic reperfusion of tissue, when transient increases of the LMC have been observed (131). Similar increases of the LMC occur in liver when rats are treated with the prooxidant phorone (16a).

The iron-dependent creation of active cAcon has led to intriguing conjectures regarding the role of citrate or isocitrate in iron transit. The formation of cAcon is generally irreversible in vivo in the absence of NO or iron chelators (44), and cAcon is stable in nongrowing cells. Thus, the longevity of cAcon suggests that it has some specific function. Because cAcon comprises only a small fraction of all intracellular iron, it is unlikely to be an iron-storage depot. However, cAcon catalyzes the conversion of citrate to isocitrate. Therefore, its ability to make isocitrate may constitute an important role. However, why this reaction is necessary in the cytoplasm is unclear. A potential role of citrate and/or isocitrate can be surmised. For example, these substrates could serve as "porters" for iron, either in the free form or when bound to cAcon. Alternatively, cAcon could interact with the mitochondria to transfer its product, isocitrate, into the mitochondria and thereby enhance Krebs cycle activity.

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