The Intracellular Location of Iron Regulatory Proteins Is Altered as a Function of Iron Status in Cell Cultures and Rat Brain

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ABSTRACT Iron regulatory proteins (IRPs) are proteins involved in the regulation of intracellular iron homeostasis that bind to specific mRNA structures termed iron responsive elements (IREs). Because the target mRNAs for the IRPs are both cytosolic and membrane associated, we hypothesize that movement of IRPs between the cytosolic and the membrane associated subcellular fractions occurs in response to intracellular iron changes. We tested this hypothesis in a cell culture model, using mouse fibroblast cells (NIH 3T3) and macrophage cells (J774), and in a rat model of early iron deficiency and excess. This presented the first opportunity to examine IRP binding activity in rat brain during states of dietary iron deficiency and excess. Binding activity for IRPs was demonstrated in both membrane and cytosolic fractions in the cell lines and the rat brain homogenates. Although IRP binding activity is predominantly located in the cytosol (90%), there was increased IRP/IRE binding activity in both cytosolic and membrane fractions when the cells were treated with deferoxamine, and decreased binding activity after treatment with iron. In the rat study, brain cortex, hippocampus and striatum homogenates had more IRP binding activity in iron-deficient rats and less in iron-supplemented rats in a region- and time-specific manner. The intracellular distribution of IRPs also changed between the cytosolic and membrane fractions of the brain homogenates in conjunction with changes in iron. These in vivo studies are consistent with the cell culture analyses showing intracellular redistribution of IRPs as a function of iron status. The results of these experiments extend our understanding of cytoplasmic mRNA binding protein activity and raise questions regarding the mechanism by which mRNA binding proteins can locate their target mRNAs within cells. The elucidation of this mechanism will have a significant impact on our understanding of eukaryotic gene regulation. J. Nutr. 131: 2831–2836, 2001.

KEY WORDS: • iron regulation • post-transcriptional regulation • rats • iron regulatory proteins • iron deficiency • cell culture

Iron poses a challenge to the cell because of the adverse effects of both iron deficiency (ID)³ and excess. Thus, maintenance of intracellular iron homeostasis is essential for cell survival. Cellular iron homeostasis is achieved through the controlled synthesis of several proteins involved in the movement, storage and utilization of iron. The mRNAs of some of these proteins contain specific sequences termed iron responsive elements (IREs) that interact with cytoplasmic iron regulatory proteins (IRPs)-1 and -2, which are the key regulators of intracellular iron homeostasis. The control of IRP/IRE interaction has been the subject of a number of investigations (see recent reviews by Cairo and Pietrangelo (1) and Eisenstein (2)]. In the cell, IRPs are thought to exist in three states: 1) bound to an IRE, 2) free and available to bind to an IRE and 3) free but unavailable to bind IRE (i.e., cytoplasmic aconitase activity pool). Variations in the size of the intracellular labile iron pool produce a change in the IRP/IRE binding activity and, as a consequence, a change in the levels of the proteins involved in iron homeostasis. Of the target mRNAs for the binding of IRPs, transferrin receptor mRNA is membrane associated (3), while ferritin mRNA is found in both the cytosol and the endoplasmic reticulum (4,5). Thus, we hypothesize that the IRPs are found in more than one intracellular compartment and that the post-transcriptional regulation of IRP/IRE interaction involves intracellular translocation of IRPs in relation to their target mRNAs between the cytosolic and the membrane-associated subcellular fractions.

In the brain, the regulation of iron metabolism provides challenges not matched by any other organ. The brain has an uneven distribution of iron, with some regions having a high concentration (substantia nigra, ventral pallidus and globus pallidus) (6). In the brain, there is a high requirement for iron during development, as evidenced in animal studies (7–9). If this requirement is not met, as in early ID, it could have devastating long-lasting effects (10–12). Furthermore, iron excess in the brain has been associated with neurodegenerative diseases such as Alzheimer’s (13–16) and Parkinson’s (16–18).

The metabolism of iron in the brain involves the same
proteins as elsewhere in the body but, because of the tightly regulated environment behind a vascular barrier, it is unclear that the molecular regulation is similar. IRPs have been demonstrated in human brain (19) and the IRP/IRE interaction may be altered in Alzheimer’s disease (20). Recently a mouse model in which IRP2 was rendered null has been reported. These mice may have altered brain iron status and motor function but this requires more rigorous evaluation (21). We previously established a developmentally sensitive rat model of dietary ID and excess, which allowed us to report on the changes in brain iron, transferrin, transferrin receptor and ferritin levels (22). In this manuscript, we report the results of the evaluation of the IRP/IRE binding activities in cortex, hippocampus and striatum homogenates. In the context of this analysis, an additional question was addressed regarding the intracellular distribution of the IRPs. This question was posed because of the compartmentalization of ferritin and transferrin receptor synthesis.

Because of the constraints of the animal model, namely the developmentally related response to dietary iron changes, and the number of different cells in a given brain region, a cell culture model was developed to more directly examine this intracellular distribution. The main objective of this set of experiments was to characterize the intracellular distribution of IRPs and to correlate this distribution with that of their target mRNAs. We hypothesize that movement of IRPs between the cytosolic and the membrane-associated subcellular fractions occurs in response to intracellular iron changes. We tested this hypothesis in the animal model and the cell culture system.

**MATERIALS AND METHODS**

**Cell culture model.** The following sets of experiments were performed to evaluate the effect of iron levels on the intracellular distribution of IRPs. Mouse fibroblast cells (NIH 3T3) and mouse macrophage cells (J774) were purchased from American Type Culture Collection (Rockville, MD). These cells were chosen to expand the observations from the brain homogenates to non-neural tissues. The cells were grown in a 5% CO2 atmosphere at 37°C in Dulbecco’s modified Eagle’s medium (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (Biocell, Rancho Dominguez, CA) 4 mM L-glutamine (Sigma, St. Louis, MO), 10% ultrapure penicillin and 1 μg/mL streptomycin (Gibco). The cells were plated at 70% confluence, and the experiments were performed 24 h later. To determine the effects of changes in intracellular iron levels, the cells were exposed for 16 h to either ferric ammonium citrate (FAC; 100 mg/mL, Sigma) or the iron chelator deferoxamine (DFO; 100 μmol/mL, Sigma). The results were consistent for both cell types.

**Separation of cytosolic and membrane fraction.** The cells were washed with Hank’s solution (Sigma), trypsinized and collected. To separate membrane bound organelles from cytosol, the cells were washed and homogenized in lysis buffer (10 mmol/L HEPES, pH 7.4, 40 mmol/L KCl, 5% glycerol, 3 mmol/L MgCl2, 0.1 mmol/L EDTA, 10 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride and 0.1 g/L leupeptin, all reagents from Sigma). Homogenization was carried out on ice in a Dounce homogenizer. The homogenates were kept at −70°C for further analyses. The intracellular distribution of IRP1 was subjected to densitometric analysis using the Collage Image Analysis System (Fotodyne, Hartland, WI).

**Northern blot analysis of the distribution of L-ferritin mRNA in the sucrose gradient fractions.** Total RNA was isolated in each of the fractions by the method described by Chomczynski and Sacchi (23). Equal amounts of total RNA were separated on a 1% agarose gel and transferred overnight to a nylon hybridization membrane (Amersham, Piscataway, NJ) by capillary elution. After RNA was immobilized by UV crosslinking, the membrane was placed between two 586 filter papers (Schleicher & Schuell, Keene, NH) saturated in hybridization solution containing 5× saline sodium citrate buffer, 118 mM NaCl, 17 mmol/L SDS and 2× Denhardt’s solution (all reagents from Sigma). For RNA measurement, an aliquot of the hybridization solution was collected by a 22-gauge needle and the RNA was extracted with phenol and precipitated with ethanol. Ferritin probes were derived from human ferritin cDNA clones (provided by J. Drysdale, Tufts University, Boston, MA), labeled with 32P-CTP using polynucleotide kinase and subjected to Southern blots with a cDNA probe specific for human ferritin. For autoradiography, the membranes were exposed to X-ray film at −70°C.

**Animal model.** The animals, dietary treatment and specimen collection are the same as in Piñero et al. (22) and the details for each are presented in that manuscript. In brief, the experimental design allowed us to obtain rats that had early ID/excess, between postrnatal day (PND) 10 and 21, late ID/excess, between PND 21 and 35, or long-term ID/excess, between PND 10 and 35. At PND 21 or PND 35 the rats were killed by exsanguination while being perfused, and the brains removed and dissected into several regions. These regions were suspended in 0.32 mol/L sucrose solution, homogenized and stored at −70°C for further analyses. The intracellular distribution of IRP1 was evaluated in three different brain regions (cortex, hippocampus and striatum) of early ID/excess and long-term ID/excess rats. These regions and times were chosen because they showed the most dramatic responses in iron, transferrin, transferrin receptor (TfR) and ferritin concentrations to changes in dietary iron status (22). Homogenates were separated into cytosolic and membrane fractions using the method described above. IRP binding activity was determined using autoradiography. Each sample used to separate the cytosolic and membrane fractions was the result of pooling samples from three different animals from each group. The

**RNA band shift assay.** A synthetic RNA transcript containing the IRE was generated from the oligonucleotide template 5’-ttatgcgggtgatctcccttccttcgggaaggctggctgcggaagttgtcacgaacctgcctagg-3’ with T7 polymerase in the presence of CTP, GTP (NEN Life Sciences, Boston, MA). Binding reactions were carried out as described previously (19). Briefly, equal amounts of protein for the cytoplasmic and membrane fractions were incubated with the synthetic radiolabeled IRE probe. To measure total IRP binding activity, a subset of samples were treated with 2% β-mercaptoethanol (BME, Sigma) for 5 min before the addition of the probe. The RNA-protein complexes were separated on a 6% native polyacrylamide gel and visualized by autoradiography. The autoradiograms were scanned and the bands were subjected to densitometric analysis using the Collage Image Analysis System (Fotodyne, Hartland, WI).

**Analysis of polysomal distributions.** To investigate the relationship between IRPs and ferritin mRNA in the ribosomal and nonribosomal fractions, analysis of ribosomal distribution was performed in both cell types. After treatment, the cells were lysed by the addition of a solution containing 10 mmol/L Tris-HCl, pH 7.4, 40 mmol/L NaCl and 1.5 mmol/L MgCl2 and then homogenized in a Dounce homogenizer. The lysates were centrifuged at 10,000 × g for 10 min and the resulting postmitochondrial supernatants were collected. An aliquot of these supernatants (500 μL containing 1 mg total protein) was loaded on top of 11 mL of a 0.22- to 0.66-mol/L linear sucrose gradient and centrifuged at 145,000 × g for 2 h. Twenty fractions were collected with an ISCO fractionator (Lincoln, NE), which resulted in a gradient from the top to the bottom. The first fraction represents the top of the gradient and the last fraction represents the bottom of the gradient. Ribosomal distribution was monitored at 254 nm during this collection. The relative amount of ribosomal mRNA was determined in each fraction using 32P-labeled cDNA to the 28S ribosomal mRNA. Free and total IRP binding activities were measured in the same fractions by RNA band shift assay as described above.
mRNA (L-ferritin) as a function of iron status. The postmitochondrial distribution of IRP (measured by binding activity) and a target mRNA was obtained to demonstrate the movement of the IRPs between membrane and cytosolic fractions in response to treatment. The uneven distribution of total binding activity in untreated control and DFO-treated samples, except after BME supplementation was a decrease in the cytosolic fraction of the untreated group was IRP binding activity observed in the ribosomal-containing fractions. These data indicate a shift in the IRPs in the cytosolic fractions as a function of iron availability.

RESULTS

Effect of iron on IRP binding activity. The results of the gel shift assay (Fig. 1) showed that IRP binding activity was found in both the cytosolic and membrane fractions of the cells. Exposing the cells to FAC decreased the IRP binding activity in each fraction, while DFO treatment increased the binding in both fractions. There was an increase in binding activity of both IRP1 and IRP2 in the membrane fraction after DFO treatment compared with the untreated membrane fraction. The total IRP binding activity measured after treatment with BME remained unchanged after treatment in the cytosolic fraction. In the membrane fraction, by contrast, total IRP binding activity was decreased after iron treatment and increased after DFO treatment with respect to the untreated cells. IRP2 was not detected in the membrane fraction of the untreated control and DFO-treated samples, except after BME treatment. The uneven distribution of total binding activity in both membrane and cytosolic fractions in response to treatment.

Distribution of IRPs and ferritin mRNA in sucrose gradient. A sucrose gradient was obtained to demonstrate the distribution of IRP (measured by binding activity) and a target mRNA (L-ferritin) as a function of iron status. The postmitochondrial fraction of the cytosol was separated on a sucrose gradient and the non–ribosome- and ribosome-containing fractions were identified using 28S ribosomal RNA as a probe. Neither the percentage of total protein in each fraction nor the ribosomal distribution were altered by treatment (data not shown). The IRP binding activity in the different fractions was measured by gel shift assay, and the results are shown in Figure 2. Panels A and B are the same samples but the films were exposed for 12 and 48 h, respectively. The exposure times were modified in an attempt to detect IRP2 and also to detect the presence of even minimal amounts of IRP in the various fractions. IRP binding activity (for both IRP1 and IRP2) was most prominent in the ribosome free fractions in the untreated samples. Deferoxamine treatment was associated with more IRP-binding activity in both the non–ribosome- and ribosome-containing fractions than in the control samples. FAC-treated samples had less IRP binding activity than control or DFO-treated groups in all of the fractions. Only in the DFO-treated group was IRP binding activity observed in the ribosome-containing fractions. These data indicate a shift in the IRPs in both the iron-treated and DFO-treated samples (Fig. 2C).

The majority of L-ferritin mRNA (58%) was found in the non–ribosomal-containing fractions in the untreated samples (Fig. 3 A and B). After iron treatment, 60% of the L-ferritin mRNA was found within the ribosomal-containing fraction, while iron chelation with DFO resulted in the majority (70%) of the L-ferritin mRNA appearing in the non–ribosome-containing fractions (Fig. 3C). The intracellular redistribution of L-ferritin mRNA demonstrates that our treatment induced changes in a manner consistent with synthesis of ferritin in response to iron status.

IRP binding in different brain regions of rats. There was an increase in the IRP binding activity in brain regions of iron-deficient rats compared with controls in both the cytosolic and membrane fractions in rats of all ages and brain regions examined except for the cytosolic fraction of striatum at PND35 (Fig. 4). The only change produced by dietary iron supplementation was a decrease in the cytosolic fraction of the cortex at PND 35 and in the membrane fraction of the hippocampus at PND 21.

When the IRP binding activity was expressed as a percentage of the BME-induced IRP binding activity, i.e., active IRP, as percentage of total available IRP, between 85 and 90% of the IRP in the cytosolic fractions was available for all the brain regions of rats.
regions and dietary treatments. For the membrane fractions, only 60–70% of the total was available across the different dietary treatments. Iron supplementation resulted in a 20–30% increase in the total BME-induced IRP binding activity compared with the controls in all brain regions and in both age groups. There were no differences in total available IRP between the controls and the iron-deficient groups.

**DISCUSSION**

The main objective of this set of experiments was to characterize the intracellular distribution of IRPs as a function of iron status and compare this distribution with that of a target mRNA. We used both an in vivo and a cell culture approach, and the results show that IRP binding activity is found in two subcellular locations, a cytosolic fraction and a membrane-associated fraction. Furthermore, our results demonstrate that the amount of binding activity in each subcellular location can be altered by cellular iron status. An additional question raised in this study is the relationship between the IRP and their target IREs. We showed that distribution of I-ferritin mRNA was determined in each fraction using total and free IRP binding activity was measured by RNA band shift assay and the data were analyzed densitometrically. The relative amount of ribosomal mRNA was determined in each fraction using 32P-labeled cDNA to the 28S ribosomal mRNA (see Fig. 3A). These results demonstrate that the majority of the IRP binding activity is found in the non-ribosome-containing fraction in the untreated samples. Treatment with the iron chelator DFO was associated with movement of the IRP into the ribosomal pool. Panel B is identical to panel A but was subjected to a longer exposure time (48 vs. 12 h) in order to detect the presence of any radioactivity in the ribosome-containing fractions. The shorter exposure time (panel A) was necessary to differentiate IRP1 from IRP2. In panel C, BME was used to induce total IRP binding activity. The results in panel C should be compared with those in panel A. Total IRP binding activity was increased in the nonribosomal fractions (1–3) in both the iron-exposed (+ Iron) and the iron-chelated (+ DFO) groups. There was a greater increase in the iron-treated group than in the DFO-treated group.

![Figure 2](https://academic.oup.com/jn/article/131/11/2831/4686747)

**FIGURE 2** Changes in IRP distribution in sucrose gradient after treatment with ferrum ammonium citrate (FAC, 100 mg/L for 16 h) or deferoxamine (DFO, 100 μmol/L for 16 h) in J774 macrophages. For this analysis, postmitochondrial lysates were centrifuged through a 7.5–22.5% sucrose gradient and 20 fractions were collected. Only the first 10 fractions are shown in the figure since they contained >95% of the mRNA. Free and total IRP binding activity was measured by RNA band shift assay and the data were analyzed densitometrically. The relative amount of ribosomal mRNA was determined in each fraction using 32P-labeled cDNA to the 28S ribosomal mRNA (see Fig. 3A). These results demonstrate that the majority of the IRP binding activity is found in the non-ribosome-containing fraction in the untreated samples. Treatment with the iron chelator DFO was associated with movement of the IRP into the ribosomal pool. Panel B is identical to panel A but was subjected to a longer exposure time (48 vs. 12 h) in order to detect the presence of any radioactivity in the ribosome-containing fractions. The shorter exposure time (panel A) was necessary to differentiate IRP1 from IRP2. In panel C, BME was used to induce total IRP binding activity. The results in panel C should be compared with those in panel A. Total IRP binding activity was increased in the nonribosomal fractions (1–3) in both the iron-exposed (+ Iron) and the iron-chelated (+ DFO) groups. There was a greater increase in the iron-treated group than in the DFO-treated group.

In the rat studies, we have showed that IRP binding activity is present in the rat brain and is altered in brain subsequent to dietary ID and excess. These data agree with the reports by Chen et al. (24,25) that the activity of liver IRP responds to changes in dietary iron levels in rats. However, in the brain, the change in binding activity is region specific and developmentally sensitive. Also, in our study, excess dietary iron had a limited effect on IRP binding activity in the brain, but IRP binding activity was dramatically decreased in liver after excess dietary iron in the studies of Chen et al. (24,25). The changes in IRP binding activity in the brain regions analyzed
are consistent with the changes in TfR, ferritin, transferrin and iron status found in the same brain regions (22). For example, during ID, there is an increase in TfR associated with the increased IRP binding activity for each of the different brain regions. The lack of an effect of dietary iron on brain IRP binding activity probably reflects the action of the blood-brain barrier to limit iron acquisition by the brain. The changes in IRP binding activity in this study indicate that regional and age-related changes in iron metabolism found in the previous report are regulated at the molecular level. The data further suggest that the molecular regulatory mechanism is age and brain region sensitive.

Because of the number of different cell types in the brain and the heterogeneous distribution of iron in the brain, the layers of complexity in this organ are too many to understand the mechanism underlying redistribution. Since we began this study, the cellular and regional distribution of IRPs in the mouse brain have been reported and confirm our notion that the IRP expression in brain is heterogeneous (26). Thus, the cell culture models not only complement the brain analyses but are essential to identifying the intracellular relationship between IRPs and their target mRNAs.

In summary, we have shown that the IRPs exist in different subcellular locations and the location is associated with cellular iron status. These results suggest that intracellular movement of IRPs may be critical to post-transcriptional regulation.

**FIGURE 3** Distribution of L-ferritin mRNA in a sucrose gradient. The same subcellular fractions used in Fig. 2 were probed for L-ferritin mRNA to demonstrate that the paradigm of iron treatment and iron chelation could induce movement of L-ferritin mRNA. This approach also allows direct comparison of the relationship between ferritin mRNA, an IRP target, and the IRPs. In panel A, the relative amount of ribosomal mRNA in each sucrose fraction is shown using 32P-labeled cDNA to the 28S ribosomal mRNA. The results of a representative Northern blot are shown as both a gel (panel B) and in graphic form (C). In the graph, the fractions are collapsed to indicate non–ribosome-containing (1–3) and ribosome-containing (4–10) fractions. In the untreated samples, the majority of ferritin mRNA is in the non–ribosome-containing fractions. After iron exposure (+Iron), the majority of ferritin mRNA was found within the translatable pool. In iron chelation, the ferritin mRNA was found predominantly in the nontranslatable pool.

**FIGURE 4** IRE/IRP binding activity in rat brain homogenates. The data in this graph were generated by first obtaining the density of the bands on the autoradiographs for IRE/IRP binding activity in each brain region. This density was then compared with the total IRP activity in the same homogenate after treatment with BME. This analysis resulted in a percentage of total IRP binding for each sample. To evaluate the changes with respect to the control rats, the results for each region, treatment and age were expressed as a percentage of the respective controls (CN). The results for cortex, hippocampus and striatum are shown in panels A, B and C, respectively. The results show that iron deficiency (ID) increased IRP binding activity in both the membrane and cytosolic fractions from each brain region at each age studied except for postnatal day 35 (35ID) in the cytosolic fraction of the striatum. Iron supplementation (SU) decreased IRP binding activity in only the cortex (cytosolic fraction, 35SU) and the membrane fraction from the hippocampus (21SU). Values are means ± SEM, n = 6. *P < 0.05 and **P < 0.01 vs. controls.
of ferritin and transferrin receptor mRNAs. We also dem-
strate that IRPs exist in rat brain and are responsive to dietary
ton iron changes in a manner that is consistent with changes in
TfR and ferritin. Thus, it appears that IRPs are involved in
regulation of brain iron status, but the region and age sen-
tivity suggest that the IRPs may themselves be subjected to
currently unidentified regulatory mechanisms. Furthermore,
because IRPs regulate iron acquisition in the brain, elucidation
of the regulation of these proteins may provide insight into the
underlying defects in regulation that permits excess iron to
accumulate in Alzheimer’s and Parkinson’s diseases.

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