Regulation of the 75-kDa Subunit of Mitochondrial Complex I by Iron*

Eva Lin‡, Joseph H. Graziano‡§, and Greg A. Freyer§¶

From the ‡Department of Pharmacology and the §Division of Environmental Health Sciences, ¶Anatomy and Cell Biology, Columbia University, New York, New York 10032

Iron homeostasis is tightly regulated, as cells work to conserve this essential but potentially toxic metal. The translation of many iron proteins is controlled by the binding of two cytoplasmic proteins, iron regulatory protein 1 and 2 (IRP1 and IRP2) to stem loop structures, known as iron-responsive elements (IREs), found in the untranslated regions of their mRNAs. In short, when iron is depleted, IRP1 or IRP2 bind IREs; this decreases the synthesis of proteins involved in iron storage and mitochondrial metabolism (e.g. ferritin and mitochondrial aconitase) and increases the synthesis of those involved in iron uptake (e.g. transferrin receptor). It is likely that more iron-containing proteins have IREs and that other IRPs may exist. One obvious place to search is in Complex I of the mitochondrial respiratory chain, which contains at least 6 iron-sulfur (Fe-S) subunits. Interestingly, in idiopathic Parkinson’s disease, iron homeostasis is altered, and Complex I activity is diminished. These findings led us to investigate whether iron status affects the Fe-S subunits of Complex I. We found that the protein levels of the 75-kDa subunit of Complex I were modulated by levels of iron in the cell, whereas mRNA levels were minimally changed. Isolation of a clone of the 75-kDa Fe-S subunit with a more complete 5’-untranslated region sequence revealed a novel IRE-like stem loop sequence. RNA-protein gel shift assays demonstrated that a specific cytoplasmic protein bound the novel IRE and that the binding of the protein was affected by iron status. Western blot analysis and supershift assays showed that this cytosolic protein is neither IRP1 nor IRP2. In addition, ferritin IRE was able to shift assays showed that this putative IRP. These results suggest that the 75-kDa Fe-S subunit of mitochondrial Complex I may be regulated by a novel IRE-IRP system.

The mitochondrial respiratory chain is composed of five large complexes. Complexes I–IV transport electrons from NADH or succinate to oxygen and pump protons out of the mitochondria to form an electrochemical gradient that is then used by Complex V to synthesize ATP from ADP. Complex I is the first and largest protein of this chain and consists of at least 43 subunits (1). Within these subunits are three key iron sulfur (Fe-S) proteins. These 75-, 51-, and 24-kDa Fe-S subunits together form the structural and functional unit of the complex (2). Of interest, Complex I activity has been shown to be modified in a number of neurodegenerative diseases, including idiopathic Parkinson’s disease (IPD) (3).

Parkinson’s disease is associated with the death of dopaminergic neurons in the substantia nigra pars compacta. The notion that mitochondrial dysfunction might be an etiologic factor in IPD was driven by the observation that the active metabolite 1-methyl-4-phenylpyridinium of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is taken up into nigral dopaminergic neurons by the dopamine transporter and specifically inhibits Complex I (4, 5). MPTP rapidly induces parkinsonism, a syndrome strikingly similar to IPD (6–8). Moreover, in the substantia nigra compacta of IPD patients, a selective 30–40% decrease of Complex I activity has been reported (9). In addition, brain iron homeostasis has also been found to be markedly abnormal in IPD and MPTP-induced parkinsonism. Although it is not clear how, a number of studies have demonstrated that, in comparison to controls, iron levels are increased 35–77% in the substantia nigra of IPD patients (10, 11).

Cellular levels of iron, a potentially toxic nutrient, are regulated post-transcriptionally by mRNA-protein interactions (12, 13). Iron regulatory proteins, IRP1 and IRP2, control the synthesis of a number of proteins involved in iron metabolism by binding a stem loop structure in the untranslated region (UTR) of their mRNAs, known as iron-responsive element (IRE) (14–18). For example, when cellular iron is low, IRPs bind to the IRE in the 5’-UTR of ferritin and block its translation. In contrast, transferrin receptor, which brings iron into the cell, has IREs located in its 3’-UTR. Here, IRP binding stabilizes the mRNA, increasing its half-life, which increases protein production (13). The mRNAs of other iron-containing proteins involved in mitochondrial metabolism have also been found to contain IREs in their mRNA and consequently fall under IRP regulation. These include erythroid aminolevulinic acid synthase (19), mitochondrial aconitase (20), and succinate dehydrogenase (SDH) (21). Thus, there are a growing number of iron-containing proteins that have IREs, and there may be additional IRPs as well.

The co-occurrence of altered iron metabolism along with the decrease of Complex I activity in IPD led us to explore the possible regulation of the Fe-S subunits of Complex I via the IRE-IRP model. By using COS-7 cells, we first examined whether protein synthesis and mRNA levels of the 75-kDa Fe-S subunit were affected by iron. Because the subunit protein; IRE, iron-responsive element; UTR, untranslated region; IPD, idiopathic Parkinson’s disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine; SDH, succinate dehydrogenase; PCR, polymerase chain reaction.

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† To whom correspondence should be addressed: Columbia University, 722 W. 168th St., P. 1. Annex, Rm. 114, New York, NY 10032. Tel.: 212-543-4125; Fax: 212-781-4993; E-mail: gaf1@columbia.edu.

¶ The abbreviations used are: Fe-S, iron sulfur; IRP, iron regulatory
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Iron-dependent Regulation Studies—The effect of iron on the 75-kDa subunit IRE-binding protein was examined through gel shift assays using protein extracts prepared from HeLa cells in which iron content had been manipulated. HeLa cell cultures from the National Cell Culture Center were either treated with 100 μM desferrioxamine, 20 μM hemin, or left untreated overnight. Cytoplasmic extracts and gel shift assays were carried out as described above.

Identification of the Cytoplasmic Binding Protein—Partial purification of 75-BP from total extract was undertaken. HeLa S100 extracts (a gift from Dr. Bruce Stillman) were applied to a Mono Q-Sepharose (Amersham Pharmacia Biotech) column equilibrated in Digman’s buffer D (without glyceral), (22, and bound proteins were eluted with a stepwise GdnHCl salt gradient (0.15 to 0.6M). The presence of 75-BP in fractions was determined by gel shift assays, and the presence of IRP1 and IRP2 was determined by comparable assays using ferritin IRE. The fractions containing 75-BP were pooled and applied to a Sephacryl 200 sizing column (Sigma), and the fractions containing the binding protein were again revealed via gel shift assays.

Western blot analysis was performed on the sizing column fractions with IRP1 and IRP2 antibody (gifts from both Dr. Elizabeth Lebold and Dr. Tracey Rouault). Also, supershift assays were conducted with IRP1 and IRP2 antibody as follows. IRP1 or IRP2 antibody was added to the partially purified protein column fractions prior to addition of radiolaabeled IRE probe. The supershift complexes (antibody-protein-RNA) were detected by autoradiography. The IRP1 antibody capable of producing a supershift was generously provided by Dr. Richard Eisenstein, and both IRP1 and IRP2 antibodies were gifts from Dr. Leibold and Rouault.

RESULTS

Iron—To investigate the effect of iron on protein levels, cytoplasmic and mitochondrial extracts were prepared from COS-7 cells either untreated or treated with the iron chelator desferrioxamine (100 μM) or the iron loader hemin (20 μM). Treatment was for 6, 24, 48, and 72 h. These extracts were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with the specified antibodies (Fig. 1). In the mitochondrial extracts levels of the 75-kDa subunit protein decreased to undetectable levels within 6 h after treatment and remained undetectable or increased with hemin treatment (Fig. 1, lanes 2–5 and 6–9, respectively). As a control, the levels of another Complex I subunit (the non Fe-S 39-kDa subunit) were simultaneously measured. No change in its protein levels was detected nor were there any detectable changes in the level of tubulin with either desferrioxamine or hemin treatment. Changes in tubulin levels were therefore referred to as nifold. The National Center for Biotechnology Information’s (NCBI) computer Basic Local Alignment Search Tool (BLAST) and the Berkeley Drosophila Genome Project’s Neural Network Promoter Prediction tool programs were also used to locate potential cap sites and additional 5′-UTR sequences.

RNA Gel Shift Assays—A gel shift assay was conducted to measure the interaction between binding proteins and IREs. Approximately 40 μg of protein from total cytoplasmic extract was analyzed for binding by incubation with 0.2 ng (10^5 cpm) of radiolabeled 75-kDa subunit and ferritin transcripts. These RNAs were transcribed in vitro according to published methods (23, 24) with T7 RNA polymerase in the presence of [α-32P]UTP using oligonucleotides containing single-stranded IRE stem loop sequences and a double-stranded T7 promoter (75-IRE, ferritin-IRE, T7-1). A nonspecific stem loop run-off 32P-labeled transcript (pBS-Cla, Table I) was also generated from a Clal-cleaved pBluescript SK(+) vector (Stratagene) with T7 RNA polymerase. To form RNA-protein complexes, cytoplasmic extracts were incubated for 20 min at 25 °C with the labeled RNA. Unprotected probe was degraded by incubation of 1 unit of RNase T1 for 10 min followed by addition of heparin (5 mg/ml) for 10 min to prevent nonspecific protein binding to the RNA probe. RNA-protein complexes were then analyzed on 4% non-denaturing polyacrylamide gels. Competition binding assays were conducted by addition of varying molar excesses of specified unlabeled competitor RNA to the cytoplasmic extract 2 min prior to the addition of labeled transcript. Shifted complexes were visualized by autoradiography.

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Iron levels of the Fe-S cluster-containing subunit changes in response to cellular iron status. As for the positive controls, predictably, ferritin ferritin mRNA is minimally affected by iron manipulation. Northern blot analysis of 75-kDa subunit and ferritin mRNA is shown. COS-7 cells were either untreated (lanes 1–3) or treated for 24, 48, or 72 h with desferrioxamine (lanes 4–6) or hemin (lanes 7–9). Approximately 10 μg of total RNA was loaded into each lane of the 1.2% agarose, 2.2 M formaldehyde gel. The blot was probed with 32P-labeled random primed 75-kDa subunit and ferritin probes. Upper arrow denotes 75-kDa subunit mRNA and lower arrow denotes ferritin mRNA.

**Fig. 2.** 75-kDa subunit mRNA is minimally affected by iron manipulation. Western blot analysis of 75-kDa subunit and ferritin mRNA is shown. COS-7 cells were either untreated (lanes 1–3) or treated for 24, 48, or 72 h with desferrioxamine (lanes 4–6) or hemin (lanes 7–9). Approximately 10 μg of total RNA was loaded into each lane of the 1.2% agarose, 2.2 M formaldehyde gel. The blot was probed with 32P-labeled random primed 75-kDa subunit and ferritin probes. Upper arrow denotes 75-kDa subunit mRNA and lower arrow denotes ferritin mRNA.

Suggesting that, in response to iron status, a post-transcriptional mechanism controls the change in protein levels of the 75-kDa subunit as it does for ferritin, i.e. IRP1 or IRP2 binding to the IRE in its mRNA.

**Complex I 75-kDa Subunit Contains a Novel IRE-like Stem Loop Sequence—**Complex I 75-kDa subunit synthesis was shown by Western blot analysis to be regulated by iron, and 75-kDa subunit mRNA levels were minimally changed by iron status as seen by Northern blot analysis. Since these same results were found for ferritin protein synthesis and mRNA levels, we searched for an IRE sequence in the 5′-UTR. 5′-Rapid amplification of cDNA ends was performed on a Marathon ready human fetal liver cDNA library (CLONTECH). The PCR products of the 5′-UTR of 75-kDa subunit were cloned into a T/A cloning vector, and clones that screened positive with a 32P-labeled probe (75 screen, Table I) were sequenced. The sequence of the longest cDNA provided 95 additional bases in the 5′-UTR, as compared with GenBank™ sequence, accession number X61100 (Fig. 3a).

To determine the secondary structure of the extended 5′-UTR of the 75-kDa subunit protein, two computer modeling programs (GGG foldRNA and mfold program) were employed. The 5′-UTR was predicted to fold into an IRE-like stem loop/ hairpin structure with a calculated initial free energy of −54.5 kcal/mol (GGG foldRNA) and an energy of −56.0 kcal/mol for the hairpin loop (mfold) (Fig. 3b). Unlike the consensus IRE, this putative 75-kDa subunit IRE contains a 5-member loop (ACAGAG), rather than a 6-member loop, and an A bulge, instead of a C bulge, in the stem. The location of this novel IRE is 90 bases proximal to the translational start site, ATG, placing it within the range of 0–150 bases for the known functional IREs. Since an even longer 5′-UTR could contain other IREs, we sought to determine more 5′ end sequences of the 75-kDa subunit by using the genomic DNA sequence added to the human genome data base during the course of our studies (GenBank™ accession number AC007383). Four possible cap sites 5′ of the 75-kDa subunit were identified using the computer programs, BLAST and Neural Network Promoter Prediction. Three of the predicted cap sites maintained the stem loop structure, and those cap sites were determined to be 14, 285, and 384 bases proximal to the IRE stem. Based on the size of the 75-kDa subunit mRNA calculated from the Northern blot analysis (Fig. 2), the cap site is likely to be 384 bases from the start of the IRE.

A Specific Cytoplasmic Protein Binds the Novel 75-kDa Subunit IRE—Gel shift analysis was used to determine if the putative 75-kDa subunit IRE could bind a cytosolic protein, by using various amounts of S100 extract incubated with 32P-
The IRE sequence is 9-rapid amplification of cDNA ends products. Novel a* -UTR IRE subunit 5 subunit cDNA and the predicted secondary structure of 75-kDa 9-UTR up to the atg start codon (GenBank TM accession number X61100).

Right loop, upper stem, bulge, and lower stem.

To examine the binding specificity of 75-BP for 75-kDa IRE, a 32P-labeled nonspecific stem loop structure was utilized in gel shift experiments as a positive control to compete for the protein that binds the 75-kDa IRE or ferritin IRE (lanes 6–10), the ferritin IRE could compete for binding of the protein was found for cells treated with desferrioxamine or hemin or left untreated. Cytoplasmic S100 ferritin IRE (Fig. 7, lanes 1–5), increased binding of the 75-BP was again followed by gel shift analysis with labeled ferritin IRE, the ferritin IRE–ferritin IRE complex migrated to a position lower (Fig. 4, lanes 1–5), whereas the 75-kDa IRE did not compete for binding to IRP1 or IRP2 (lanes 15–17).

Interaction of 75-BP with the Novel 75-kDa Subunit IRE Is Affected by Iron—The binding of IRP1 and IRP2 to IREs are both affected by cellular iron levels. To investigate the effect of iron on the 75-BP, HeLa cells were treated overnight with desferrioxamine or hemin or left untreated. Cytoplasmic S100 extracts were then prepared and used in gel shift assays with 32P-labeled 75-kDa IRE (75-IRE, Table I). In comparison to controls (Fig. 6a, lanes 1–5), increased binding of the 75-BP was detected in the desferrioxamine-treated extracts (lanes 6–10), whereas decreased binding of the protein was found for cells treated with hemin (lanes 11–15). This difference is seen more clearly in competitive binding experiments using unlabeled 75-kDa IRE (lanes 7–10, 12–15). Ferritin IRE-binding proteins, IRP1 and IRP2, behaved as reported previously (16–18). To summarize, not only is the 75-kDa subunit synthesis regulated by iron but 75-BP binding is also regulated by iron.

Separation and Identification of the 75-BP—Ion exchange columns were used as an initial purification method to separate 75-BP from cytoplasmic extracts. The elution profile revealed that fraction 18 appeared to be most enriched for the 75-BP (Fig. 7, a and b, solid arrow). In contrast, IRP1 and IRP2 eluted from the column earlier, as demonstrated by the gel shifts with ferritin IRE (Fig. 7b, dashed arrow, lanes FT, W2, W4, 2, 4, 6, 10, and 14). Interestingly, in the absence of IRP1 and IRP2, we found strong binding of ferritin IRE to 75-BP (Fig. 7b, solid arrow, lanes 18, 22, 26, 30, and 34).

Labeled 75-kDa subunit IRE (75-IRE, Table I). Indeed, a cytoplasmic protein clearly bound the putative 75-kDa subunit IRE (Fig. 4, lane 1). Ferritin IRE (ferritin-IRE, Table I) was also used in these gel shift experiments as a positive control to demonstrate binding to IRP1 and IRP2 (lane 6), previously shown to co-migrate with each other (32). The 75-BP-75-kDa IRE complex migrated to a position lower (Fig. 4, solid arrow) than that of the IRP1-IRP2-ferritin IRE complex(es) (dashed arrow), suggesting a different protein that we refer to as 75-BP. Interestingly, when cold competitor ferritin IRE was added to the binding assay with labeled ferritin IRE, the ferritin IRE–protein complex shifted to the lower migrating form, apparently binding to the 75-BP once IRP1 and IRP2 were saturated with ferritin IRE (Fig. 4, lanes 7 and 8). This suggested that ferritin IRE is able to bind to 75-BP. Furthermore, although the 75-kDa IRE could not compete for IRP1 or IRP2 binding (lanes 9 and 10), the ferritin IRE could compete for binding of 75-BP (lanes 4 and 5).

Treatment with proteinase K during the incubation of cytoplasmic extract and labeled IRE in the gel shift assay completely eliminated the shifted complexes in both COS-7 and HeLa cells (data not shown).

To examine the binding specificity of 75-BP for 75-kDa IRE, a 32P-labeled nonspecific stem loop structure was utilized in the gel shift assay. No shifted band was seen when this non-specific loop was incubated with cytoplasmic extracts (Fig. 5, lanes 21–30). In addition, the nonspecific stem loop did not compete for the protein that binds the 75-kDa IRE or ferritin IRE (lanes 8–10 and lanes 18–20). Thus, 75-BP appears to have specificity for iron-responsive elements.

Fig. 8. Gel shift assay with the labeled IRE and the gel shift assay with the labeled ferritin IRE (lanes 5–7), whereas the 75-kDa IRE did not compete for binding to IRP1 or IRP2 (lanes 15–17).
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To test further that 75-BP was not either IRP1 or IRP2, Western blot analyses with IRP1 and IRP2 antibodies were carried out. Fractions from the Sephacryl sizing column with positive gel shifts for 75-BP were used in a Western blot and probed with IRP1 and IRP2 antibodies (provided by Drs. Lembold and Rouault). Total extract and wash fractions revealed the presence of IRP1 and IRP2 (Fig. 9, a and b, arrows, lanes C, and W2), whereas the fractions with the 75-BP did not (Fig. 9, a and b, lanes 24 and 26).

The supershift assay with IRP1 and IRP2 antibodies demonstrated that 75-BP is yet a third IRE-binding protein. Total extract, Sephacryl sizing column fraction 24 (which contains 75-BP, see Fig. 8), and fraction 30 (which likely contains IRP1 or IRP2, see Fig. 8) were used in the supershift assay. With the 32P-labeled 75-kDa IRE (Fig. 10, lanes 1–9), no supershift was observed with the addition of IRP1 antibody (lanes 2, 5, and 8) or IRP2 antibody (lanes 3, 6, and 9) in either total extract, fraction 24, or fraction 30, respectively. It remains unclear what caused the apparent "subshift" in lanes 2 and 3 when IRP1 and IRP2 antibody were added to total extracts and incubated with 75-kDa subunit IRE, however, this result was reproduced with addition of tubulin antibody, and we concluded it is a nonspecific effect.

In contrast, with 32P-labeled ferritin IRE (Fig. 10, lanes 10–18), supershifted bands were seen in total extract as expected with either IRP1 or IRP2 (lanes 12 and 13, respectively). However, no supershifted bands were found with fraction 24, where 75-BP was present (lanes 15 and 16). Curiously, despite the absence of supershifts, the original complex of ferritin IRE-75-BP as seen in lane 14 appeared more faint in lanes 15 and 16, as if the formation of the complex was somehow hindered with the addition of IRP1 or IRP2 antibody. In addition, the supershifted band seen with IRP1 antibody and fraction 30 (lane 18) confirms that fraction 30 contains IRP1 and not IRP2.

DISCUSSION

Two major biochemical abnormalities are observed in IPD. Elevated iron levels have been found in substantia nigra of deceased IPD patients, and mitochondrial Complex I function is reduced. Because several mitochondrial Complex I subunits contain iron, we considered the possibility that these two observations were related. We undertook the current study to determine whether the iron sulfur proteins in Complex I were regulated by the iron status of the cell and, if so, by what mechanism.

The regulation of several iron-containing proteins has been shown to be regulated at the level of translation by the IRE-IRP system. In addition to ferritin and transferrin receptor, proteins involved in mitochondrial metabolism (mitochondrial acetyl-CoA synthase and SDH), and heme synthesis (erythroid aminolevulinic acid synthase) also fall under the control of the IRE-IRP system. In addition to ferritin and transferrin receptor, proteins involved in mitochondrial metabolism (mitochondrial acetyl-CoA synthase and SDH), and heme synthesis (erythroid aminolevulinic acid synthase) also fall under the control of the IRE-IRP interaction. All of these proteins share the common feature of having a 4Fe-4S cluster. Therefore, we focused on the regulation of the 4Fe-4S 75-kDa subunit of mitochondria Complex I. To our surprise, we found strong evidence that a novel IRE and a novel IRP are involved in the regulation of the 75-kDa subunit.

In our experiments, the regulation of the expression of Complex I 75-kDa subunit was consistent with that which would be predicted for an iron-utilizing protein. The 75-kDa subunit of Complex I was regulated by levels of iron in the cell. When iron...
was depleted by desferrioxamine treatment, the protein levels of the 75-kDa subunit decreased; when iron was added, protein levels increased (Fig. 1). Together these results clearly show this protein to be regulated by cellular iron levels. The levels of positive and negative control proteins responded predictably in this assay, demonstrating that the effect was specific for iron status. The fact that another Complex I protein, 39-kDa subunit, which does not contain an Fe-S cluster, is not reduced with reduced cellular iron levels, clearly demonstrates that this regulation is specific for the Fe-S-containing protein and not just a nonspecific decrease in general protein syntheses or Complex I levels. As stated earlier, this is analogous to SDH where only the Fe-S cluster-containing subunit, which has an IRE, is regulated by cellular iron status (25), but the levels of the other two non-Fe-S proteins in the complex are unaffected (25). This was demonstrated in rats fed an iron-poor diet where the SDH-iron subunit was decreased, and the levels of the other two proteins of the SDH complex were unchanged.

Studies carried out over 15 years ago (25, 33, 34) demonstrated that in iron deficiency, Complex I and SDH exhibit decreased enzymatic activity. For Complex I, the concentration of the 75-kDa subunit in iron-deficient membranes was lower than in control membranes. Furthermore, the decrease in activity in Complex I was found not to be due to the absence of Fe-S clusters but to less membrane-bound protein (25). Our study shows that this decreased activity is likely the result of reduced protein synthesis.

We did see that the 75-kDa subunit mRNA levels were altered with modified iron status as were ferritin mRNA levels (Fig. 2). The slight change in mRNA levels cannot account for the significant decrease seen in protein levels with desferrioxamine treatment as illustrated by the Western blot (Fig. 1). This revealed that regulation of the 75-kDa subunit synthesis by iron was mainly post-transcriptional, similar to other proteins regulated under the IRE-IRP system. The 75-kDa subunit contains a novel IRE-like stem loop sequence in its 5'-UTR.

This structure differs from the consensus sequence in that it contains a 5-member loop (1CAGAG5) and an A bulge in the stem instead of the 6-member loop and the C bulge (Fig. 3). In recent work, the 6-member loop (1CAGUGN6, where N = any nucleotide except G) in the consensus IRE has been shown by nuclear magnetic resonance studies to form an interloop base pair between the C1 and G5 (35). Formation of this structure would create a 3-member loop, containing nucleotides at positions 2–4 (2AGU4), accessible for interaction with IRP1, IRP2, or other binding proteins (36). By extension in the 75-kDa...
subunit, if the C¹ and G⁵ nucleotides form a CG pair, it would leave a 3-member loop of 2AGA⁴ to bind to IRPs. Transferrin receptor contains five IRE stem loop structures in the 3'-UTR of its mRNA (A-E), one of which (A) has a loop sequence of 1CAGAGU⁶ (Fig. 11) (14). With interloop pairing of this transferrin receptor IRE-A loop, the novel 75-kDa subunit IRE loop would have the exact sequence and structure (1CAGAG⁵ (37, 38). Furthermore, like the recently cloned mouse glycolate oxidase (Fig. 11) (39), a change from the C bulge to the A bulge in the 75-kDa subunit IRE may merely affect the strength of the translational regulation of the 75-kDa subunit but not the binding of IRPs (40).

In addition to the actual IRE sequence, conservation of the IRE position has also been shown to be important in translational control (41). The distance of our novel IRE from the translational start site is 90 bases, which falls between the effective range of 0 nucleotides for mitochondrial aconitase mRNA and 150 nucleotides for ferritin mRNA. Placement of the IRE greater than 67 nucleotides downstream of the cap structure permits the association of the 43 S preinitiation complex and diminishes the translational regulation of IRPs binding to IRE (42). By using gel shift analysis, we found a cytoplasmic protein (75-BP) which bound to the putative 75-kDa subunit IRE. Our studies showed that the shifted product formed with the 75-kDa IRE migrated to a different position on gels than that formed by IRP1/IRP2 binding to ferritin IRE (Fig. 4). By using antibodies we further demonstrated that this protein was neither IRP1 nor IRP2. Finally this protein appears to bind more specifically to the 75-kDa subunit IRE than to the ferritin IRE as the binding to ferritin IRE occurs only when IRP1 and IRP2 are missing or depleted. Therefore, we hypothesized that 75-BP is a novel iron regulatory protein.

In further support of the validity of 75-BP as a novel iron regulatory protein, we examined the effect of iron status on the binding of 75-BP to the 75-kDa subunit IRE. If the 75-kDa subunit IRE may merely affect the strength of the translational regulation of the 75-kDa subunit but not the binding of IRPs (40).

Although it may be premature to describe 75-BP as IRP3, our studies have shown that it seems to have much in common with the profiles of IRP1 and IRP2. Further studies will be needed to establish unequivocally that this 75-BP is a bona fide IRP. Enhanced oxidative damage, decreased Complex I activity, and increased iron levels each have been implicated in the cascade of events that lead to nigral cell death in IPD (43). The establishment of the sequence of these biochemical changes should provide a better understanding of the cause(s) of IPD. The reciprocal relationship between respiratory chain dysfunction and altered iron metabolism as cause and effect of overall oxidative stress in IPD has been studied at length (43–49). Our observation that the 75-kDa subunit of Complex I is regulated by iron via a novel IRE and novel IRP adds another level of complexity to the model of iron homeostasis. Other lines of investigation also point to the interplay between Complex I, iron metabolism, and IPD. MPTP, the first environmental toxicant to be shown to cause parkinsonism in patients and animals, inhibits Complex I activity. Moreover, elevated iron levels exacerbate the effects of MPTP in cells, and conversely, MPTP increases nigral uptake of iron (50). The observation that the pesticide rotenone, a classic Complex I inhibitor, was also able to produce clinical, pathological, and biochemical characteristics of parkinsonism in rats further supports the suggestion that Complex I indeed plays a key role in the molecular pathology of the disease.

Finally, a recent paper (51) described the phenotypic characteristics of a mouse strain in which a targeted disruption of the IRP2 gene (IREB2) was generated. These mice showed misregulation of iron metabolism in the brain and intestinal
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mucosa. Excessive iron accumulation in the cytosol of brain neurons preceded neurodegeneration, demonstrated by ataxia, bradykinesia, and tremor. These results dramatically strengthen the argument that misregulation of iron may indeed be at the basis of IPD, a model that we have long favored. Obviously, we are now anxious to determine if a connection exists between defects in the novel IRP described in this paper and IPD. We hope that the diverse lines of investigation will converge on a molecular understanding of IPD.

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