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Modulation of Iron Regulatory Protein-1 by Various Metals

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Iron regulatory protein-1 (IRP-1) is known as a cytosolic aconitase and a central regulator of iron (Fe) homeostasis. IRP-1 regulates the expression of Fe metabolism-related proteins by interacting with the Fe-responsive element (IRE) in the untranslated regions of mRNAs of these proteins. However, it is less known whether IRP-1 modulates various non-Fe metals. In the present study, we showed that treatment of homogenously purified IRP-1 with non-Fe metals decreased the affinity to IRE in RNA band shift assays and increased aconitase activity. Non-Fe metals also inhibited ^{55}Fe incorporation into the fourth labile position of the Fe–S cluster of IRP-1. In PLC hepatoma cells, metal loading inactivated binding activity and activated enzyme activity. It also suppressed transferrin receptor mRNA expression in the cells. These results suggest that various non-Fe metals modulate IRP-1 by conversion of the 3Fe–4S apo-form to a [1 non-Fe metal + 3Fe]–4Fe holo-form.

Key Words: **iron regulatory protein-1; aconitase; iron-responsive element; iron; non-iron metals; essential metals; transition metals; heavy metals; transferrin receptor.**

Iron regulatory proteins (IRPs) are cytosol mRNA binding proteins that control the stability or the translation rate of mRNAs of several proteins (transferrin receptor (Tf-R), ferritin and 5-aminolevulinic acid synthetase, etc.) in response to availability of cellular iron (1–3) after the uptake (4). The regulatory mechanism involves the interaction between the iron-responsive element (IRE) in 3′ or 5′ untranslated regions of the transcripts and cytosolic IRPs (IRP-1 and -2). IRP-1 is an iron–sulfur (Fe–S) protein with aconitase activity containing a cubane 4Fe–4S cluster. When Fe is plentiful, IRP-1 prevails in a 4Fe–4S form and is an active cytoplasmic aconitase. When Fe is scarce, it readily loses one Fe to become a 3Fe–4S cluster and in this state has little enzymatic activity (5, 6). In contrast, IRP-2 possesses neither the Fe–S cluster nor aconitase activity (7). The binding activity of IRP-1 to IRE is modulated by intracellular Fe levels. It is an interesting question whether non-Fe metals—including essential, transition, heavy and toxic metals—are regulated by IRP-1 in the cells. Constable *et al.* reported that non-Fe metals fail to mimic the Fe effect; they could not observe that Zn, Mn, and Cd bind to IRP-1 to decrease the IRP-1/IRE binding activity as Fe does (8). This report appeared to contradict the finding of an IRE in the 3′ untranslated region of the divalent cation/metal ion transporter (DCT1) cDNA. The translation product takes up Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Pb^{2+} (9). DCT1 involves the excessive absorption of hereditary hemochromatosis (HH) (10). HH patients also accumulate other metals, such as Co^{2+} and Mn^{2+} (11). These findings proved that mammalian cells take up iron by both Tf-dependent and Tf-independent iron (Fe) uptake systems (4). As the regulation of DCT1 mRNA stability is considered to be analogous to the regulation of Tf-R mRNA, it is possible that IRP-1 post-transcriptionally regulates the expressions of DCT1 and Tf-R mRNAs. From these reports, we may hypothesize that IRP-1 senses both cellular Fe and non-Fe metal levels to modulate them. In the present experiment, we obtained results in support of this hypothesis.

Evidence has accumulated that Fe and aluminum (Al) may play toxic roles in the pathogenesis of Parkinson's and Alzheimer's diseases (PD and AD) (12–18). Accumulation of Fe and Al might be responsible for nigral dopamine cell death in PD and the vulnerability of cerebral cortex in AD because unbound Fe causes membrane lipid peroxidation in neurons and Al can accelerate the peroxidation by Fe. However, how these

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metals accumulate in the brains of patients with the neurodegenerative disease. We previously reported that Al suppresses Tf-R mRNA expression in rat cortical cells, suggesting that this non-Fe metal decreases IRE-binding activities of IRP-1 to affect the iron metabolism by binding to iron regulator, IRP-1 (19). Therefore, we postulated that IRP-1 is regulated by various metals having chemical similarities to Fe. In the present study, we examined whether non-Fe metals directly inactivate the IRE-binding activity and activate the enzyme activity of IRP-1 purified from beef liver cytosol *in vitro*, as well as the RNA-binding and enzyme activities of cytosolic IRP-1 in PLC hepatoma cells after metal treatment. Our findings suggest that IRP-1 plays vital roles as one of the sensor proteins for various cellular metals. The present study may contribute to understand molecular mechanisms of the abnormalities of iron homeostasis in HH and of the metal neurotoxicity in brains of PD and AD as well.

MATERIALS AND METHODS

Preparation of IRP-1. IRP-1 was homogeneously purified from beef liver cytosol by the established method (20) with modifications. Fresh beef liver was homogenized in 2 mM Hepes (pH 7.2) containing 2 mM citrate using a glass homogenizer with a Teflon-pestle. The homogenate was spun at 1300*g* for 60 min and at 77,000*g* for 30 min. The supernatant was used as a starting extract. The soluble fraction was brought to 35% saturation with solid ammonium sulfate and allowed to stand for 2 h. After centrifugation (27,000*g* for 25 min), the supernatant was brought to 75% saturation with ammonium sulfate and allowed to stand overnight. The protein fraction was applied to a Sephacryl S-200 column (2.5 × 90 cm) equilibrated with the above-described buffer. The fractions containing enzyme activity were combined and applied to a column (2.5 × 30 cm) of Sp Sepharose HP in 2 mM Hepes (pH 6.5) containing 2 mM citrate. IRP-1 was eluted by a linear gradient of 4–10 mM citrate in 20 mM Hepes buffer (pH 6.5). After concentration, the active fractions were further purified by gel filtration on a column (2.0 × 90 cm) of Sephadex G-100 superfine (Amersham–Pharmacia Biotech, Buckinghamshire, UK) equilibrated with 2 mM Hepes (pH 7.2) containing 2 mM citrate. IRP-1 thus purified was electrophoretically homogenous. When the Sephadex G-100 fraction was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), a single band showing an apparent molecular weight of 98,000 daltons was observed as shown in Fig. 1. A summary of a typical run of the purification method developed is given in Table 1.

Cell culture. Human PLC hepatoma cells were purchased from the American Type Culture Collection.

RNA band shift assay. The RNA band shift assay was carried out as described previously (21–23). After incubation with 150 μM Fe-, Mn-, Ni-, Cu-, Cd-, and Hg–nitrilotriacetates (NTA) at 37°C for 24 h, cell lysates from PLC cells were used as samples for the assay.

Competition on non-Fe metals with ^{55}Fe incorporation into the Fe–S cluster of IRP-1 in vitro. After the fourth labile Fe in the 4Fe–4S cluster of IRP-1 was removed under reducing conditions (24), ^{55}Fe was inserted into the vacant position. IRP-1 protein purified from beef liver was incubated with 150 μM ^{55}Fe–NTA in the absence or the presence of non-Fe metals at the concentration 1, 2, 5, and 10 times the concentration of ^{55}Fe–NTA for 30 min at room temperature, The labeled IRP-1 proteins and free ^{55}Fe–NTA were separated on a 5% native acrylamide gel. The dried gels were then exposed to imaging plate to quantify with an image analyzer.

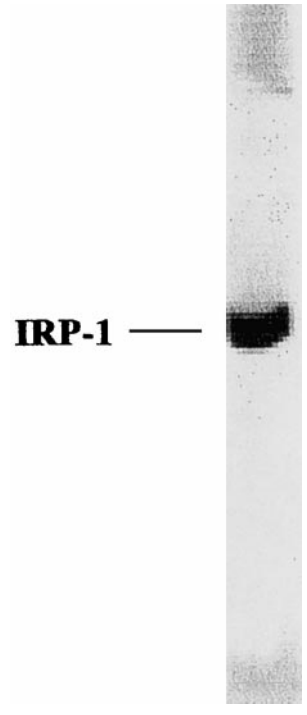


FIG. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified beef liver IRP-1. The protein (30 μg) was denatured with 2% SDS at 95°C for 10 min and resolved by electrophoresis in an SDS/7.5% polyacrylamide gel. The gel was stained with Coomassie brilliant blue. The molecular weight was determined by comparing its mobility with those of myosin heavy chain, β-galactosidase, phosphorylase *b*, bovine serum albumin, and carbonic anhydrase.

Northern blot analysis. After the cultured cells were exposed to 150 μM metal–NTA at 37°C for 24 h, cytoplasmic RNAs were isolated from the cultured cells according to previous reports (25) and samples of 5 μg were analyzed on Northern blots with human Tf-Rc (26) and β-actin cDNA probes.

Aconitase activity. Aconitase activity was measured with the coupled aconitase/isocitrate dehydrogenase assay (6, 27). The reaction components were 100 mM Tris–HCl (pH 8.0), 1 mM citrate, 1 mM $MgCl_2$, 1 mM NADP, and isocitrate dehydrogenase (100 μg/ml). Production of NADPH was measured by change in absorbance at 340 nm.

RESULTS AND DISCUSSION

Noniron Metals Decrease IRE-Binding Activity and Increase Aconitase Activity of IRP-1 in the Cytosol Fraction from Hepatoma PLC Cells

It has previously been believed that non-Fe metals fail to mimic the iron effect (8); and indeed, in the present study, IRP-1 binds non-Fe metals *in vitro*. We also previously reported that aluminum (Al) suppresses transferrin receptor (Tf-R) mRNA expression in rat neuronal and glial cells and showed that Al decreases the IRE-binding activity of IRP-1 in the cytosol fraction (19, 28). To confirm this finding, we here investigated whether non-Fe metals affect both the binding activity to iron-responsive element (IRE) using

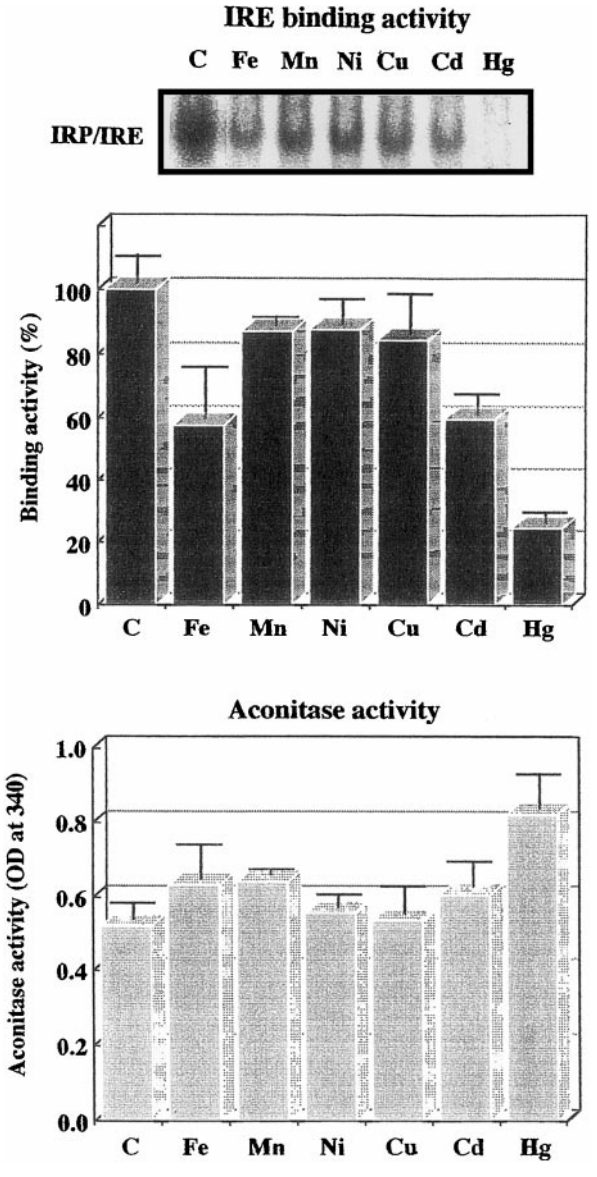


FIG. 2. Effect of various metals to EDTA ratio on the inactivation of IRE binding activity. Purified aconitase (10 μg) was activated in 80 mM Hepes (pH 7.5) containing 5 mM dithiothreitol, 100 μM EDTA, and various concentrations of metals. The samples were incubated for 30 min at room temperature before determining the binding activity. Binding activities were quantified with an image analyzer. Results are means ± SD of three to five experiments.

RNA-band shift assay and its aconitase activity of IRP-1 in the cytosol fraction obtained from hepatoma PLC cells after metal loading. As shown in Fig. 2, when the cells were incubated with 150 μM metal–NTA at 37°C for 24 h, non-Fe metals decreased IRP binding and increased aconitase activities in the cytosol fraction of PLC cells. For the RNA-binding activity, Mn, Ni, and Cu slightly inactivated IRP-1 in the fraction. However, Cd inactivated it to the same degree as Fe did and Hg inhibited IRP-1 to approximately twice the level done by Fe. For the enzyme activity, the effect of Mn and Cd were similar to those of Fe. Ni and Cu had less effect on the activity. However, Hg significantly increased the enzyme activity. These results suggest that IRP-1 in the cytosol fraction may directly bind non-Fe metals after non-Fe metals taken up by the cells.

Non-Fe Metals Directly Bind to Purified IRP-1 in Vitro

To examine whether non-Fe metals directly bind to IRP-1 *in vitro,* we homogeneously purified IRP-1 (Table 1 and Fig. 1). As a preliminary experiment, we checked the relation of the enzyme, IRE-binding activities and the Fe/EDTA ratio under reducing conditions (24). For this experiment, the concentrations of IRP-1, dithiothreitol (DTT) and EDTA were kept constant, and only the Fe^{2+} concentrations were varied. When the fourth labile iron in the 4Fe–4S cluster is removed with EDTA, a 3Fe–4S cluster is formed. As Fe is titrated to apo IRP-1, a 4Fe–4S cluster is formed. As a result of Fe titration, IRE/IRP-1 binding activity decreased and aconitase activity increased (Figs. 3a and 4). Next, we examined the effect of each non-Fe metal on the binding and enzyme activities of IRP-1 under the same conditions (Figs. 3b–3f and 4). All non-Fe metals used here decreased the binding activity in a dose-dependent manner. The effects of Mn and Ni on IRP-1 binding activity were similar to those of Fe. Cu and Cd inactivated binding activity to a slightly greater degree than Fe. Hg as known a toxic metal completely inactivated IRP-1 function as a RNA-binding protein suggesting that not only essential and transition metals but heavy and toxic metals bind to IRP-1. To confirm

TABLE 1

Purification of IRP from Beef Liver

Purification step	Total activity (units)[a]	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Soluble fraction	3265	65,300	0.05	100	1.0
$(NH_4)_2SO_4$ precipitate	1292	11,754	0.11	40	2.2
Sephacryl S-200	620	258	2.45	19	49.0
Sp Sepharose Hp	490	99	6.43	15	128.6
Sephadex G-100	417	13	32.1	13	650

[a] Units are μmol NADPH/min/mg protein at 25°C after enzyme activation.

this, we measured their aconitase activities (Fig. 4). Hg, Cd, and Cu activated IRP-1 to approximately twice the level activated by Fe. However, Mn and Ni induced less enzyme activity than Fe. Thus, results from the enzyme activities were comparable to those of binding activities. These results showed that non-Fe metals directly bind to purified IRP-1.

Non-Fe Metals Suppress the Expression of Tf Receptor mRNA in PLC Cells

Iron metabolism in higher eukaryotes is regulated at the posttranscriptional level by RNA–protein interactions (1). When the cellular Fe concentration is high, IRP-1 binds with low affinity as an apoprotein to the IRE of Tf-R mRNA and destabilizes it by targeted RNase attack (23). When the cellular Fe concentration increases, the mRNA level decreases. To confirm the suppression of Tf-R mRNA expression by non-Fe in PLC cells, we performed Northern blot analysis (Fig. 5). As shown in Fig. 5, all non-Fe metals decreased the amount of Tf-Rc mRNA in metal-loaded cells. The relative amount of Tf-Rc mRNA in Cd- and Hg-loaded cells was less than those in Fe-, Mn-, or Ni-loaded cells. The results from Northern blot analysis support our hypothesis.

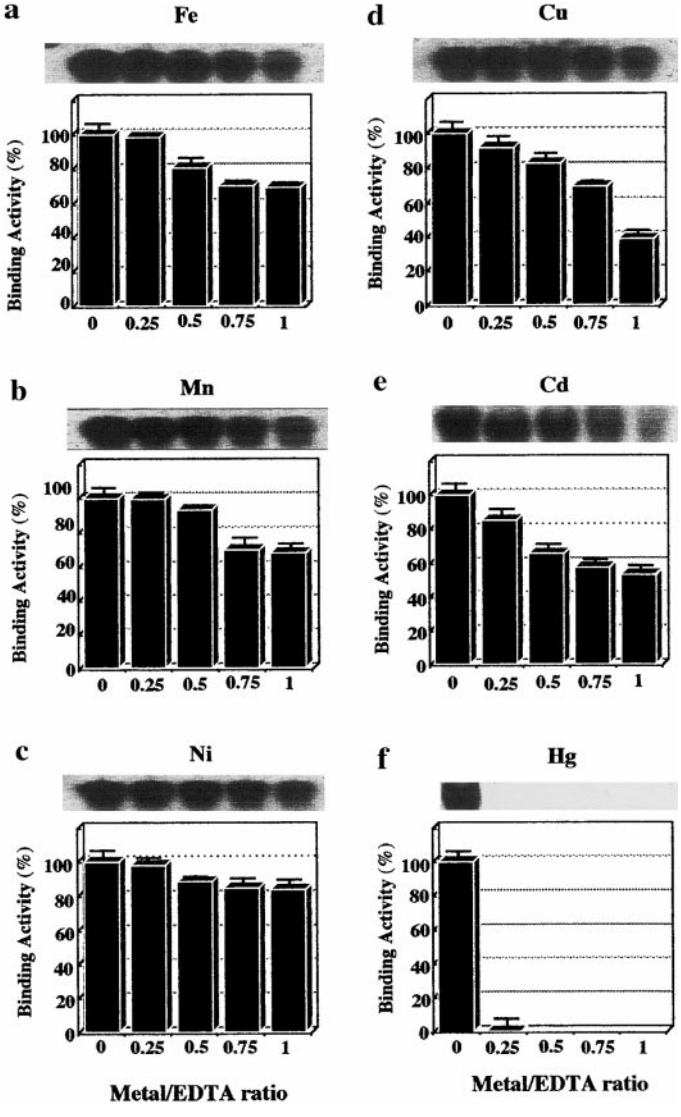


FIG. 3. Effect of various metals to EDTA ratio on the inactivation of IRP binding activity to IRE. RNA band shift assays for the binding activity were performed as described under Materials and Methods. Results are means ± SD of three to five experiments.

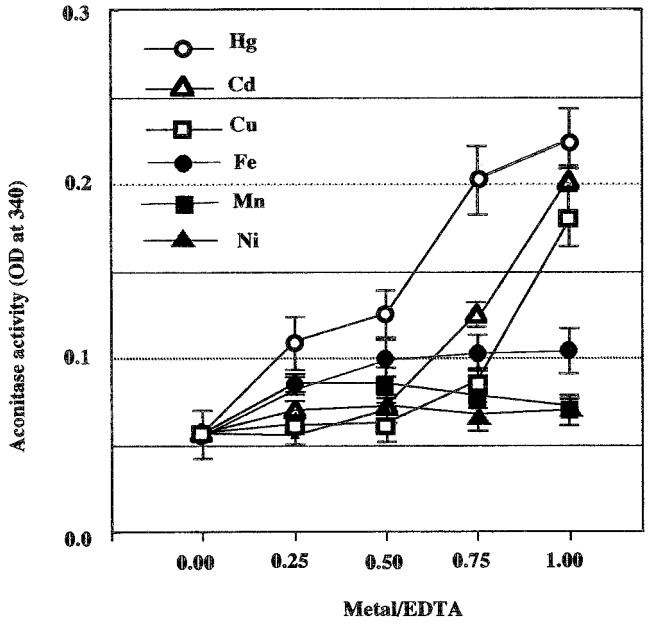


FIG. 4. Effect of various metals to EDTA ratio on the activation of aconitase activity. The enzyme activation of IRP-1 was performed as described under Materials and Methods. Results are means ± SD of three to five experiments.

Competition of Non-Fe Metals with ^{55}Fe Incorporated into the Fe–S Cluster of IRP-1

To reconfirm this hypothesis, we further performed a competition experiment by non-Fe metals with ^{55}Fe incorporation into the Fe–S cluster of IRP-1. The fourth labile Fe of IRP-1 was labeled by ^{55}Fe in the absence of non-Fe metals as designated in control (metal concentration at 0 mM) of Figs. 6a–6f after the labile Fe was removed by EDTA under reducing condition. Incorporation of ^{55}Fe to the Fe–S cluster was inhibited by non-Fe metals in a dose-dependent manner (Figs. 6b–6f). Ni, Cu, Cd, and Hg weakly inhibited IRP-1 labeling with ^{55}Fe, compared with Fe. Unexpectedly, among of non-Fe metals, Mn has markedly inhibited IRP-1 labeling as similar as Fe. It is noteworthy that Mn strongly did IRP-1 labeling.

So far, studies on the interaction between RP-1 and non-Fe metals other than Mn have not been reported yet. Therefore, in the present study, we have system-

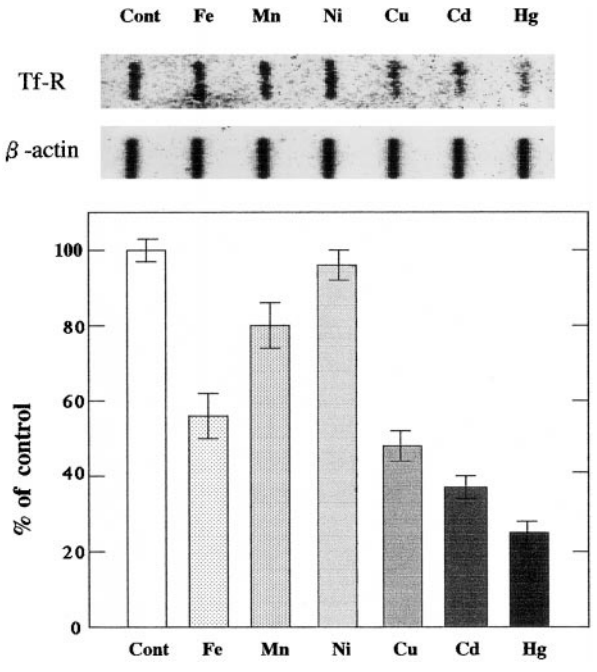


FIG. 5. Northern blot analysis of Tf-Rc mRNA in metal-loaded PLC cells. PLC cells were exposed to metal–NTA at 150 μM for 24 h at 37°C. The lower data were quantified and normalized to the amount of β-actin mRNA. Results are expressed as means ± SD of three experiments.

atically investigated the modulation of non-Fe metals by IRP-1 *in vitro* and *in vivo* experiments.

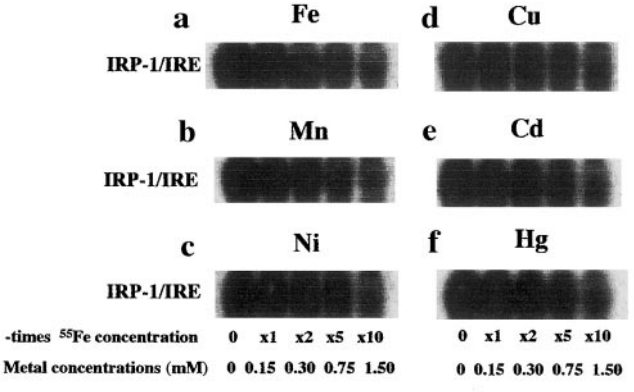
Figures 2 to 5 show that Hg and Cd bind IRP-1 with high affinity, compared with Fe. These metals may cause the disruption of Fe metabolism by inhibiting posttranscriptional regulation of iron-related proteins, such as ferritin and Tf-R. The effects of these toxic metals on inactivation of IRP-1/IRE binding and activation of aconitase may explain a part of the cell toxicity.

Figure 3a demonstrates that apo-IRP-1 was converted to holo-IRP-1 by Fe titration. When a labile Fe in the 4Fe–4S cluster was removed by EDTA in this experiment, the 3Fe–4S cluster could be prepared. When we substituted a labile Fe (24) of IRP-1 with each non-Fe metal, we observed inactivation of IRP-1 binding and activation of aconitase activity (Figs. 3 and 4). These results may represent the conversion of the 3Fe–4S to a [1 non-Fe metal + 3Fe]–4Fe cluster, which corresponds to the 4Fe–4S cluster of holo-IRP-1. The results of competition experiments (Fig. 6) also support this hypothesis.

Mitochondrial and cytosol aconitases contain not only a 4Fe–4S cluster with one particularly labile Fe atom (24, 29) but IRE stem-loop structure (1, 27). Zheng *et al.* recently reported that Mn treatment significantly inhibits aconitase activity in mitochondrial fraction from rat brain though they did not show the data of RNA-band shift assay (30). At the concentration of 0.6 to 2.5 mM, the inhibitory effects of Mn on the aconitase are 24 to 81% of the control values. The inhibitory effect is reversible, Mn concentration-dependent, and due to the competitions of Mn with Fe and/or citrate binding. Their results suggest that the alteration of aconitase by Mn may lead to the disruption of mitochondrial energy production and cellular Fe metabolism. The difference between cytosol and mitochondrial aconitases in effect of Mn is under investigation.

As shown in Figs. 3–6, our *in vitro* experiments demonstrate that Mn has a high affinity to the fourth labile position of Fe–S cluster of IRP-1 as similar as Fe (Figs. 3–6). Our present study may underlie Mn-induced neurotoxicity in Parkinson's disease (PD) (12, 31–33) as the symptoms of Mn-induced neurotoxicity resemble those of PD (31, 34, 35).

Taken together, these results suggest that non-Fe metals modulate IRP-1 by binding to the fourth posi-



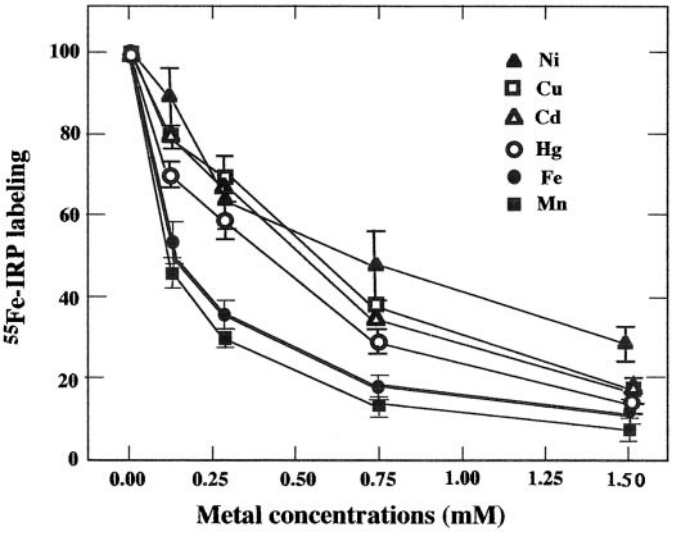


FIG. 6. Effect of non-Fe metals on ^{55}Fe incorporation into the Fe–S cluster of IRP-1 *in vitro* The competition experiment was performed as described under Materials and Methods. The amount of [1 ^{55}Fe + 3Fe]–4S equal to 4Fe–4S holo form of IRP-1 were quantified with a image analyzer (lower data). Results are expressed as means ± SD for three determinations.

tion of labile Fe in the Fe–S cluster to regulate the expressions of iron-related proteins and their transcripts in mammalian cells.

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