Expression and Biochemical Characterization of Iron Regulatory Proteins 1 and 2 in *Saccharomyces cerevisiae*†

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ABSTRACT: Iron-regulatory proteins (IRPs) 1 and 2 are cytosolic RNA-binding proteins that bind to specific stem−loop structures, termed iron-responsive elements (IREs) that are located in the untranslated regions of specific mRNAs encoding proteins involved in iron metabolism. The binding of IRPs to IREs regulates either translation or stabilization of mRNA. Although IRP1 and IRP2 are similar proteins in that they are ubiquitously expressed and are negatively regulated by iron, they are regulated by iron by different mechanisms. IRP1, the well-characterized IRP in cells, is a dual-function protein exhibiting either aconitase activity when cellular iron is abundant or RNA-binding activity when cellular iron is scarce. In contrast, IRP2 lacks detectable aconitase activity and functions exclusively as an RNA-binding protein. To study and compare the biochemical characteristics of IRP1 and IRP2, we expressed wild-type and mutant rat IRP1 and IRP2 in the yeast *Saccharomyces cerevisiae*. IRP1 and IRP2 expressed in yeast bind the IRE RNA with high affinity, resulting in the inhibition of translation of an IRE-reporter mRNA. Mutant IRP2s lacking a 73 amino acid domain unique to IRP2 and a mutant IRP1 containing an insertion of this domain bound RNA, but lacked detectable aconitase activity, suggesting that the presence of this domain prevents aconitase activity. Like IRP1, the RNA-binding activity of IRP2 was sensitive to inactivation by *N*-ethylmaleimide (NEM) or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), indicating IRP2 contains a cysteine(s) that is (are) necessary for RNA binding. However, unlike IRP1, where reconstitution of the 4Fe-4S cluster resulted in a loss in RNA-binding activity, the RNA-binding activity of IRP2 was unaffected using the same iron treatment. These data suggested that IRP2 does not contain a 4Fe-4S cluster similar to the cluster in IRP1, indicating that they sense iron by different mechanisms.

Regulation of cellular iron homeostasis is controlled by cytosolic RNA-binding proteins known as iron-regulatory proteins (IRP1 and IRP2).[1] IRPs bind to stem−loop structures termed iron-responsive elements (IREs), that are located in the 5′ or 3′ untranslated regions (UTRs) of specific mRNAs that encode proteins involved in iron metabolism [for reviews, see Klausner et al. (1993), Leibold and Guo (1992), and Mascotti et al. (1995)]. IREs are located in the 5′ UTR of mRNAs encoding the iron storage protein ferritin (Aziz & Munro, 1987; Hentze et al., 1987; Leibold & Munro, 1987, 1988; Rouault et al., 1988), the Krebs cycle enzyme mitochondrial (mt) aconitase (Zheng et al., 1992b), and the heme biosynthetic enzyme erythroid aminolevulinate synthase (Cox et al., 1991; Dandekar et al., 1991). The binding of IRPs to the 5′ IREs in ferritin and erythroid aminolevulinate synthase mRNAs (Bhasker et al., 1993; Goossen et al., 1990; Gray & Hentze, 1994; Guo et al., 1994; Melefors et al., 1993; Walden et al., 1989) represses translation of the mRNA. Five IREs are located in the 3′ UTR of the transferrin receptor mRNA where IRP binding stabilizes the mRNA (Casey et al., 1989; Mullner & Kuhn, 1988; Mullner et al., 1989). IRPs are regulated by changes in cellular iron levels. When cells are iron-deplete, IRPs bind with high affinity to IREs, leading to either translational repression or stabilization of mRNA. Whereas, when cells are iron-replete, IRPs bind with low affinity to IREs, leading to the derepression of translation or the destabilization of mRNA. The net result of IRP regulation of ferritin translation and TfR mRNA stabilization by the IRPs provides a mechanism to modulate uptake, utilization, and storage of iron by cells, balancing the cells requirement for iron with the toxicity of iron.

IRP1 has been purified and cloned from a variety of mammalian tissues and cells (Rouault et al., 1990; Walden et al., 1989; Yu et al., 1992). IRP1 has a molecular mass of 98 000 Da and shares about 30% amino acid identity with porcine mitochondrial (Rouault et al., 1991; Zheng et al., 1990) and bacterial (Prodromou et al., 1992) aconitases. Aconitase is a mitochondrial enzyme that contains a 4Fe-4S cluster that catalyzes the conversion of citrate to isocitrate via the intermediate *cis*-aconitate in the Krebs cycle. IRP1 contains a 4Fe-4S cluster and exhibits aconitase activity (Constable et al., 1992; Emery-Goodman et al., 1993; Haile et al., 1992a,b; Kaptain et al., 1991) and is thought to be

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[1] Abbreviations: IRP1 and IRP2, iron-regulatory proteins 1 and 2; IRE, iron-responsive element; UTR, untranslated region(s); NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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identical to cytosolic aconitase (Kennedy et al., 1992). IRP1 can therefore be considered a dual-function protein exhibiting aconitase activity when cellular iron is abundant and RNA-binding activity when iron is scarce. In addition to being regulated by iron, the RNA-binding activity of IRP1 is increased in cells stimulated with cytokines to produce nitric oxide (Drapier et al., 1993; Pantopoulos & Hentze, 1995b; Weiss et al., 1993) and in cells subjected to oxidative stress (Pantopoulos & Hentze, 1995a).

IRP2 has been purified from rat liver (Guo et al., 1994) and cloned from human and rat cDNA libraries (Guo et al., 1995a; Rouault et al., 1990). IRP2 has a molecular mass of about 104 000 Da (Guo et al., 1994; Henderson et al., 1993) and shares 61% overall amino acid identity with IRP1 (Guo et al., 1995a; Rouault et al., 1992). Although IRP2 contains the three conserved cysteine ligands for the 4Fe-4S cluster of IRP1 and mt-aconitase, IRP2 lacks two of the identified aconitase active-site residues (Guo et al., 1995a; Samaniego et al., 1994) and lacks aconitase activity (Guo et al., 1994). Like IRP1, IRP2 binds IREs with high affinity (Guo et al., 1994; Henderson et al., 1993; Samaniego et al., 1994) and functions as a translational repressor of IRE-containing RNAs *in vitro* (Guo et al., 1994; Kim et al., 1995). IRP1 and IRP2 differ in three aspects. First, IRP1 and IRP2 are regulated by iron by different mechanisms. Whereas IRP1 is regulated by iron by the conversion between high- and low-affinity RNA-binding forms, IRP2 is regulated by iron by proteolysis (Guo et al., 1994, 1995b; Henderson & Kuhn, 1995; Samaniego et al., 1994). Iron-mediated degradation of IRP2 requires the presence of a 73 amino acid domain present only in IRP2 (Iwai et al., 1995). Second, IRP2 shows a preference for binding specific IRE sequences, suggesting that they may regulate different IRE mRNAs *in vivo* (Butt et al., 1996; Henderson & Kuhn, 1995; Henderson et al., 1996). Third, the RNA-binding activity of IRP2, unlike IRP1, is not modulated by nitric oxide in hepatoma cells (Phillips, 1996).

To investigate the biochemical characteristics of IRP2 and to compare these properties with those of IRP1, we overexpressed rat liver IRP1 and IRP2 in the yeast *Saccharomyces cerevisiae*. We report on the characterization of RNA-binding and aconitase activities of wild-type and mutant IRP1 and IRP2 and the IRP-dependent inhibition of translation of IRE-containing mRNAs in a yeast-reconstituted IRP·IRE system. We analyzed the effects of sulfhydryl-modifying and oxidizing compounds and iron reconstitution on the RNA-binding activity of purified IRP1 and IRP2. These results are discussed in relationship to the different mechanisms by which IRP1 and IRP2 sense iron.

MATERIALS AND METHODS

Yeast Strains, Plasmid Constructions, and Transformations. The *S. cerevisiae* strain W303-1A (MATα *his3*-11 leu2-3, 112 ura3-1 trp1-1 ade2-1 can1-100) was used for all constructions. Yeasts were grown in synthetic complete medium (0.67% yeast nitrogen base, ammonium sulfate, amino acids, and either 2% dextrose or 2% galactose).

The +IRELacZ and −IRELacZ strains were constructed as follows. A *Pst*I site located in the 5′ UTR of the *URA3* gene on plasmid pRS306 (Sikorski & Hieter, 1989) was digested with *Pst*I, and the ends were blunted with T4 DNA polymerase. Two complementary 33 base oligonucleotides were synthesized corresponding to the consensus sequence for the IRE (GTATCTTGCTTCAACAGTGTTTGGACG-GAACAG), ligated into the blunt *Pst*I site, and sequenced. These plasmids were designated py+IREURA3 and py−IREURA3. The IREs inserted into the *Pst*I site were located 21 nt downstream from the major start of transcription of the *URA*3 gene. A functional *HIS3* gene was inserted into the polylinker of these plasmids to be used for integration. The *LacZ* gene from pMC1871 (Pharmacia) was cloned into py+IREURA3 and py−IREURA3 plasmids described above by digesting the DNA with *Eco*RV and *Nsi*I and inserting a *Sma*I−*Pst*I fragment of *LacZ* in-frame to the *URA3* gene. These plasmids, py+IRELacZ and py−IRELacZ, were integrated into W303-1A by digestion with *Hin*dIII which is located in the *HIS3* gene.

An aconitase deletion strain was constructed by polymerase chain amplification reaction (PCR) of the *ACO1* gene using primers corresponding to the 5′ and 3′ ends of the published *ACO1* sequence (Gangloff et al., 1990). The PCR fragment was cloned into Bluescript KSII(+) (Stratagene). A 0.6 kb *Kpn*I//*Xba*I fragment of the *ACO1* coding region was replaced with a 1.8 kb *Kpn*I/*Xba*I fragment of *HIS3* to create the *aco1* deletion allele. The *aco1* strain, JDP10, was constructed by transformation of W303-1A with the deletion allele and selecting for histidine prototrophy. Yeast transformations were performed using the lithium acetate method (Ito et al., 1983).

The IRP1 yeast expression plasmid pJVIRP1 was constructed by cloning a 2.7 kb *Bam*HI fragment containing the coding region of rat IRP1 (Yu et al., 1992) into plasmid pJV1 (a gift of Dr. J. Johnston, University of Utah) downstream of the GAL1/10 promoter. As a control, an IRP1 cDNA containing a premature stop codon leading to the production of an unstable protein was also cloned into pJV1 to create pJVtIRP1. pJV1 contains the *LEU2* selectable marker and a copy of the 2μ sequence.

A full-length IRP2 cDNA was constructed by amplification of three plasmids containing various portions of the rat liver IRP2 coding sequence (Guo et al., 1995a). pSL800 contained the first 800 bp of the amino terminus, pBG contained 1000 bp of internal sequence, and pUZBP2-1 contained the last 1017 bp of the carboxyl terminus. PCR was carried out using Pfu Polymerase (Stratagene) according to the manufacturer. After amplification of each fragment, a DNA fragment containing internal and 3′ coding region sequences was amplified by PCR using primers corresponding to the 5′ and 3′ ends of the internal and 3′ region sequences, respectively. A full-length IRP2 cDNA was amplified by PCR by mixing the 5′ coding region fragment and the amplified internal and 3′ coding region fragments with primers corresponding to the 5′ and 3′ ends of the amino and carboxyl region sequences, respectively. The IRP2 PCR product was purified from an agarose gel, cloned into Bluescript SKII(+), and sequenced. This plasmid was called pBG5. An IRP2 yeast expression plasmid was constructed by inserting a *Bam*HI fragment of pBG5 that contains the entire coding region of IRP2 into the yeast expression vector pJV1 to create pJVIRP2.

IRP2 mutant plasmids were constructed as follows. (1) IRP2DM (IRP2 double-mutant) was constructed by mutating Lys611 to Arg by changing the codon from AAA to AGA and Asn853 to Ser by changing the codon from AAT to AGT. Changes were introduced *in vitro* using the unique

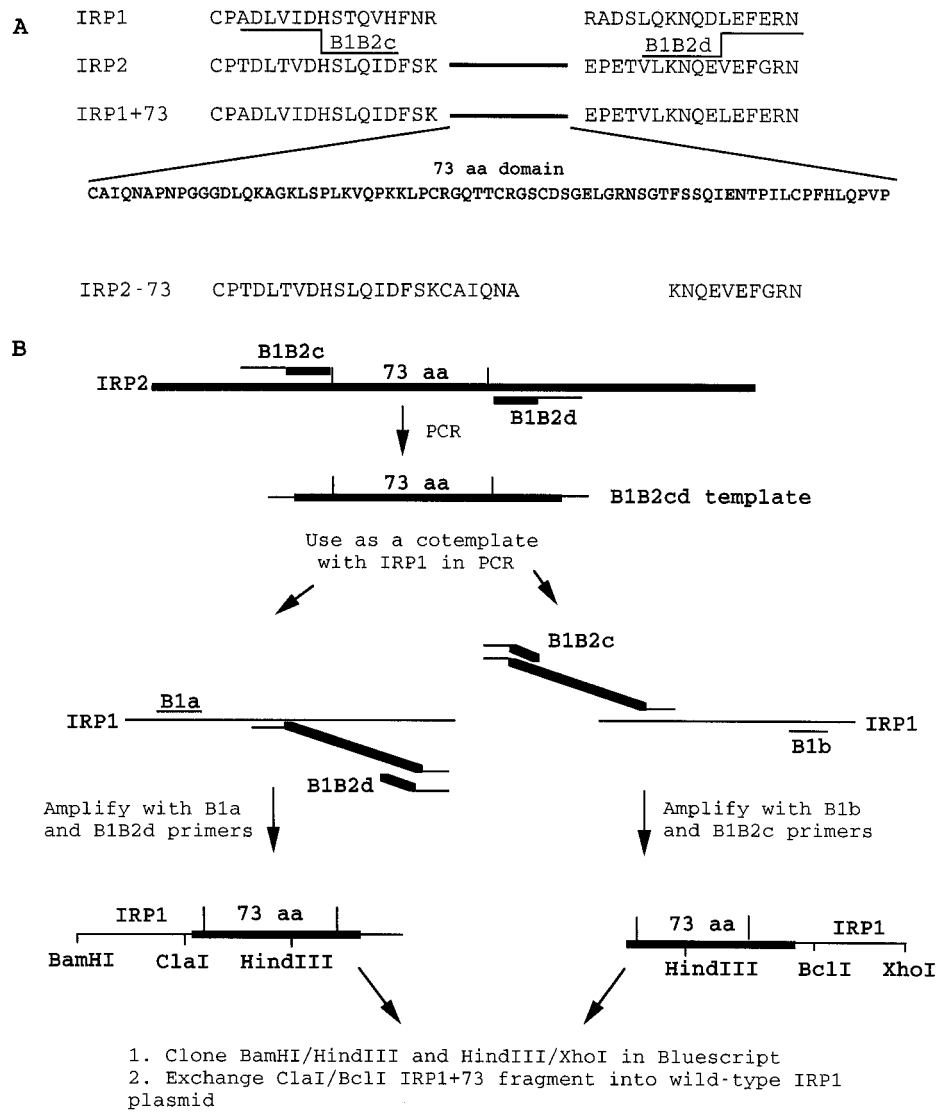

FIGURE 1: Construction of IRP1+73 and IRP2−73 mutant genes. (A) Amino acid sequences of rat IRP1 and IRP2 surrounding the 73 amino acid domain in IRP2. The sequence of the 73 amino acid domain is shown, and its location in IRP2 is indicated by the black bar. The primers used in the crossover PCR to construct IRP1+73 are B1B2c and B1B2d (see Materials and Methods). The position of the crossover from IRP1 to IRP2 and then back again into IRP1 to create IRP1+73 is indicated by the black lines. The location of the 73 amino acid domain in IRP1 (IRP1+73) and the deleted portion of the 73 amino acid domain in IRP2 (IRP2−73) are also indicated. (B) Crossover amplification and cloning strategy for IRP1+73 (see Materials and Methods for details). IRP1 sequences are indicated by the thin black lines, and IRP2 sequences are indicated by the thick black lines. The primers used in the crossover PCR are B1B2c, B1B2d, B1a, and B1b. The 73 amino acid domain is located within the vertical bars in IRP2 and is designated 73 aa.

site elimination method (Pharmacia Biotech). The mutagenic oligonucleotides used were K611R (GGAAACAGAAATTTC-GAAGGT-3′), N853S (GGTTCAGGAAGTTCAAGAGAC), and the selective oligonucleotide PL-X-K (CTCGATGGG-GGGCCCGTTACC). (2) IRP2−73 was constructed by removal of a portion of the 73 amino acid domain (Guo et al., 1995a; Rouault et al., 1992) as follows. A 62 nt primer, JDP/unique, (GACTTCAGTAAATGTGCAATACAGAAT-GCAAAAAATCAAGAAGTAGAATTTGGCAGAAATC-G), was synthesized that matched the rat IRP2 sequence from positions 400 to 429 and then again from 648 to 680. The primers JDP/unique and BG24 (TCAACAAACTTTC-CAGCC), corresponding to nucleotides 1047−1064 in the rat IRP2 sequence, were used to amplify a 399 nt fragment of IRP2 using full-length IRP2 cDNA (pBG5) as a template. The PCR product was gel-isolated and used as a primer for a second round of PCR with the T7 primer at the 5′ end of IRP2 pBG5. The resulting 900 nt product was then cut with *Not*I and *Avr*II, cloned back into pBG5, and sequenced. (3) IRP2DM-73 was created by inserting the same *Not*I−*Avr*II fragment used to construct IRP2-73 into IRP2DM. IRP2DM, IRP2−73, and IRP2DM-73 were then subcloned into pJV1 for expression in yeast.

An IRP1 plasmid containing an insertion of the IRP2 73 amino acid domain (IRP1+73) at a homologous position was constructed by using a crossover PCR strategy (Figure 1). Two primers (B1B2c and B1B2d) were synthesized that contain 18 nt of rat IRP1 sequence at their 5′ ends and 18 nt of rat IRP2 sequence at their 3′ ends. B1B2c (5′-GCTGAC-CTCGTAATCGATCATTCTCTACAAATTGAC-3′) encodes amino acids ADLVID$_{IRP1}$HSLQID$_{IRP2}$, and B1B2d (5′-ATTCCTTTCAAATTCCAGTTCTTGATTTTTTAACAC-3′) encodes ADLVID$_{IRP1}$HSLQID$_{IRP2}$. Primers B1B2c and B1B2d flank the 5′ and 3′ ends of the 73 amino acid domain, respectively.

These primers were used to amplify the 73 amino acid domain from the IRP2 cDNA (pBG5), resulting in a fragment consisting of the 73 amino acid domain with 18 nt of IRP1

sequence at each end. This fragment (B1B2cd) was used as a cotemplate with an IRP1 cDNA along with IRP1 primer B1a (5′-GCGGATCCTGTGACGAGTTTTTGGTG-3′) which is located at nucleotides 148−171 in the rat IRP1 sequence and B1B2d. The only way a productive amplification could occur is if the B1B2cd template annealed through the 18 nt at its end to the IRP1 sequence and was extended along the IRP1 sequence producing a crossover template that could be amplified with B1a and B1B2d. The product of this reaction is the IRP2 73 amino acid domain with 227 nt of 5′ IRP1 sequence. Likewise, a similar amplification reaction was performed using the rat IRP1 primer B1b (5′-GACCTCGAGCAATCATGGTGGTGTGAG-3′) which corresponds to sequences located at nucleotides 617−634 in the rat IRP1 cDNA. The products from these amplification reactions were cloned together using a unique *Hin*dIII site located in the 73 amino acid domain, and the crossover product was cloned into Bluescript KSII(+) using *Bam*HI and *Xho*I sites engineered at the ends of the B1a and B1b primers, respectively. The crossover product was excised from Bluescript using unique *Cla*I and *Bcl*II sites in the IRP1 sequence that flank the crossover region. This fragment was then cloned into the corresponding region of rat wild-type IRP1 cDNA. IRP1+73 was excised from Bluescript and cloned into pJV1 for expression in yeast.

LacZ Assays. Yeast were grown in liquid culture to an OD_{600} of 0.6, and total cell extracts were prepared using glass beads. Protein concentrations were determined using the Lowry assay (Lowry et al., 1951). LacZ assays were carried out by adding 20 μL of each protein extract to 980 μL of Z buffer (100 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol) and 200 μL of *o*-nitrophenyl β-D-galactoside at 4 mg/mL. Reactions were incubated at 30 °C and stopped by the addition of 0.5 mL of 1 M sodium carbonate. Specific activity was calculated as OD_{420} x 1000 × (1/time) × (1/mg of protein).

Purification of Recombinant IRP1 and IRP2 from Yeast. The rat liver IRP1 (Yu et al., 1992) and the IRP2 coding regions (Guo et al., 1995a) were separately cloned in-frame to the HIS tag in pET16b (Novagen). The fusion genes were subcloned into the yeast expression plasmid pJV1 and transformed into W303-1A. To purify IRP1 and IRP2, 2−4 L of cells was grown to late log phase in medium containing galactose to induce expression of IRP1 and IRP2. Cells were harvested and washed in sonication buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl) at 4 °C and resuspended in 3 mL/g wet weight of sonication buffer containing 1 mM phenylmethanesulfonylfluoride (PMSF) and 1 mM diisopropyl fluorophosphate (DFP). Cells were disrupted by passage though a French press, and the effluent was spun at 3000*g* for 10 min at 4 °C. The supernatant was clarified by an additional centrifugation spin at 100 000*g* for 30 min at 4 °C. The supernatant was added to 4 mL of nickel nitriloacetic acid (Ni^{2+}-NTA) (Qiagen) and incubated overnight at 4 °C to absorb IRP2. The slurry was poured in a column and washed with 100 mL of sonication buffer followed by 80 mL of wash buffer (50 mM sodium phosphate, pH 6.5, 300 mM NaCl, 70 mM imidazole, and 10% glycerol). IRP1 and IRP2 were eluted by washing with 10 mL of wash buffer containing 200 mM imidazole, 1 mM PMSF, and 1 mM DFP at room temperature. The protein was dialyzed in dialysis buffer (25 mM HEPES, pH 7.5, 1 mM DTT, and 10% glycerol). Approximately 1 and 2 mg of purified IRP1 and IRP2, respectively, was obtained from 1 L of yeast culture.

Immunoblotting and RNA Band Shift Assays. Protein samples from yeast expressing IRP1 and IRP2 were fractionated on an 8% SDS−polyacrylamide gel, and the proteins were transferred to a nitrocellulose membrane. IRP1 and IRP2 were detected by incubation of the membranes with chicken polyclonal antibodies directed against the coding region of rat IRP1 or rabbit polyclonal antibodies directed against the 73 amino acid insertion of rat IRP2 (Guo et al., 1994). After the primary antibody incubation, the membranes were incubated with horseradish peroxidase-conjugated goat anti-chicken or goat anti-rabbit antibodies for 1 h, and protein was detected using the Enhanced Chemiluminescent Western blotting system (Amersham).

RNA band shift gels were performed as described previously (Leibold & Munro, 1988). Equal amounts of protein were incubated with a ^{32}P-labeled ferritin IRE RNA (Leibold & Munro, 1988) in a 20 μl reaction for 20 min at 24 °C and treated sequentially for 10 min each with 2 units of RNase T1 and 20 μg of heparin. RNA−protein complexes were separated on 5% nondenaturing polyacrylamide gels, and the gels were dried down and subjected to autoradiography.

In Vitro Iron Reconstitution and Aconitase Assays. Reconstitution of the 4Fe-4S cluster in IRP1 and IRP2 was carried out by the addition of 10 mM DTT, 125 μM ferrous ammonium sulfate, and 125 μM sodium sulfide to 100 μg of total yeast extract or 1−10 μg of purified IRP1 or IRP2 in 100 μL of 20 mM HEPES, pH 8.0, 5% glycerol, and 20 mM KCl or a combination of these reagents as indicated. All reactions were carried out in an anaerobic chamber for 1 h at room temperature. Aconitase activities were determined by following the disappearance of *cis*-aconitate measured at 240 nm (Kennedy et al., 1983). The reaction was started by the addition of 100 μL of 2 mM *cis*-aconitate to 100 μg of total yeast extract in 900 μL of 100 mM Tris, pH 8.0. For analysis of RNA-binding activity, the reconstituted proteins were diluted 1:1 with RNA band shift buffer without prior purification. These experiments have been carried out several times, and one representative experiment is shown.

In Vitro Oxidation and Alkylation of Sulfhydryl Groups in IRP1 and IRP2. Alkylation of free sulfhydryl groups in IRP2 was carried out by treating 10 μg of purified protein with 0.5 or 5 mM *N*-ethylmaleimide (NEM) for 30 min at room temperature. Ten millimolar DTT was added for 30 min as indicated. Oxidation of sulfhydryl groups in purified IRP1 and IRP2 was carried out by treating purified protein with 1.0 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for 30 min at room temperature. Half of each DTNB-oxidized sample was treated with 10 mM DTT for an additional 30 min. DTNB-treated IRP1 and IRP2 and NEM-treated IRP2 were analyzed for RNA-binding activity by RNA band shift gels described above. These experiments have been carried out several times, and one representative experiment is shown.

RESULTS

Overexpression of IRP1 and IRP2 in Yeast. To compare the biochemical properties of IRP1 and IRP2, we overexpressed rat IRP1 and IRP2 in *S. cerevisiae*. Yeast were chosen for expression of IRP2 since the expression of IRP1

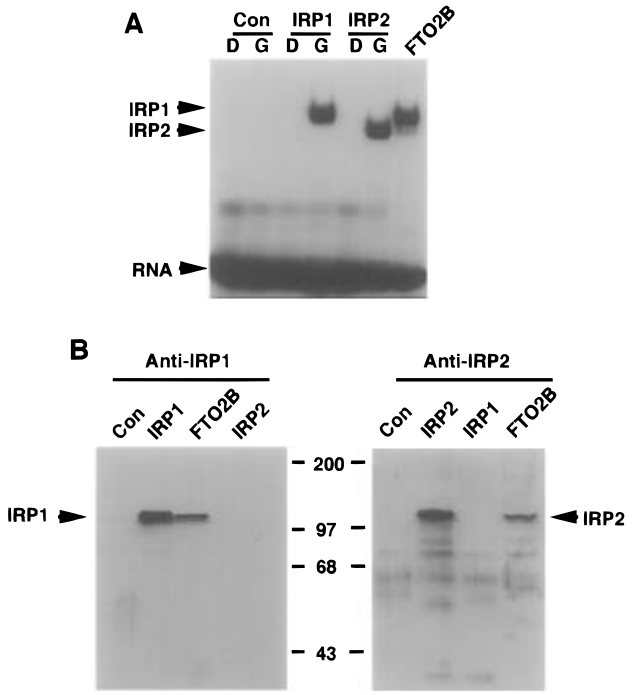

FIGURE 2: Overexpression of recombinant rat IRP1 and IRP2 in yeast. Yeast were transformed with plasmids containing IRP1 (pJVIRP1), IRP2 (pJVIRP2), or a control plasmid (pJVtIRP1) under the control of the GAL1/10 promoter. The control plasmid contains an IRP1 sequence with a stop codon within the coding region, leading to production of an unstable protein (Con). Cells were grown in either dextrose (D) or galactose (G) to induce expression of IRP1 and IRP2. (A) RNA band shift gels of extracts from yeast expressing IRP1 or IRP2 (10 μg) or rat FTO2B hepatoma cells (40 μg). Aliquots of protein were incubated with a ^{32}P-labeled IRE, and the RNA−protein complexes were separated by a 5% nondenaturing polyacrylamide gel. The IRP1·IRE and IRP2·IRE complexes and free RNA are indicated. (B) Protein (40 μg) from samples in (A) was separated by 8% SDS−polyacrylamide gels. The protein was transferred to nitrocellulose, and the membranes were probed with either a chicken anti-IRP1 antisera or a rabbit anti-IRP2 antisera. Positions of IRP1 and IRP2 bands are indicated.

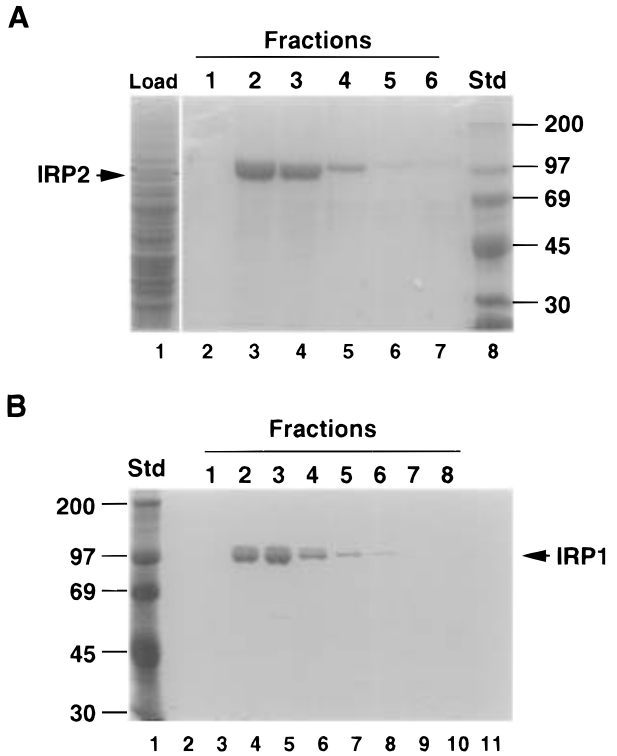

FIGURE 3: Purification of recombinant rat IRP1 and IRP2 from yeast. IRP1 and IRP2 containing a His_{10}-tag were purified by Ni^{2+}-NTA agarose. IRP1 and IRP2 were eluted from Ni^{2+}-NTA−agarose using 200 mM imidazole, and aliquots from the fractions were analyzed by 8% SDS−polyacrylamide gels. Protein was visualized by Coomassie-blue staining of the gel. (A) IRP2 purification by Ni^{2+}-NTA agarose. Total yeast load, (lane 1); imidazole-eluted fractions containing IRP2 (lanes 2−7). (B) IRP1 purification by Ni^{2+}-NTA-agarose. Imidazole-eluted fractions containing IRP1 (lanes 2 −11). Positions of molecular weight standards (Std) and of IRP1 and IRP2 are indicated.

or IRP2 in *E. coli* resulted in the production of insoluble proteins that lacked RNA-binding activity (data not shown). Yeast were transformed with plasmids containing rat IRP1 (pJVIRP1) or IRP2 (pJVIRP2) cloned under the control of a galactose promoter. A plasmid containing an inactive truncated form of IRP1 (pJVtIRP1) was also transformed into yeast and used as a control. The yeast were grown in galactose to induce protein expression, and extracts were prepared and analyzed for the presence of IRP1 or IRP2 by immunoblot analysis using anti-IRP1 or anti-IRP2 antibodies and RNA-binding activity by RNA band shift gels (Figure 2). Recombinant IRP1 and IRP2 expressed in yeast bound the IRE RNA (Figure 2A) and migrated with similar mobilities to rat FTO2B hepatoma cell IRP1 and IRP2 (Figure 2C,D).

To purify IRP1 and IRP2 from yeast, the IRP1 and IRP2 expression vectors, pJVIRP1 and pJVIRP2, were modified by cloning in a sequence encoding 10 histidine residues (His_{10}) at their N-termini. These constructs were expressed in yeast and affinity purified by nickel nitriloacetic acid−agarose (Ni^{2+}-NTA) (see Materials and Methods). IRP1-His_{10} and IRP2-His_{10} were eluted from a Ni^{2+}-NTA column using an imidazole buffer, and each fraction was analyzed by Coomassie staining of SDS−polyacrylamide gels (Figure 3). Using this approach, IRP1 and IRP2 were purified to homogeneity in a one-step purification resulting in approximately 1 and 2 mg of IRP1 and IRP2, respectively, from 1 L of yeast culture.

IRP1- and IRP2-Dependent Inhibition of Translation of an IRE−β-Galactosidase mRNA in a Yeast IRE·IRP Reconstituted System. IRP1 expressed in yeast is capable of repressing translation of a reporter gene containing an IRE in its 5′ UTR (Oliveira et al., 1993). To determine whether the IRP2 expressed in yeast is also able to repress translation of IRE-containing mRNAs *in vivo*, the ability of IRP2 to repress translation of an IRE-*LacZ* mRNA expressed in yeast was examined. An IRE sequence was inserted in the 5′ UTR of the reporter gene *LacZ* (+IRELacZ). As a negative control, the IRE was also cloned in the inverse orientation in the 5′ UTR of *LacZ* (−IRELacZ) since this sequence does not form an IRP binding site. The +IRELacZ and −IRE-LacZ reporter constructs were integrated into the yeast genome (see Materials and Methods). These yeast strains were then transformed with the IRP1 and IRP2 expression vectors, pJVIRP1 and pJVIRP2, and as a control, the inactive truncated IRP1 plasmid, pJVtIRP1. The yeast were then assayed for their ability to affect the translation of the −IRELacZ or +IRELacZ mRNAs. Three independent colonies from each transformation were assayed, in duplicate, in either dextrose- or galactose-containing medium to induce IRP1 or IRP2 expression. Units of β-galactosidase activity were calculated for each independent isolate, and the data are presented as the average and the standard deviation for

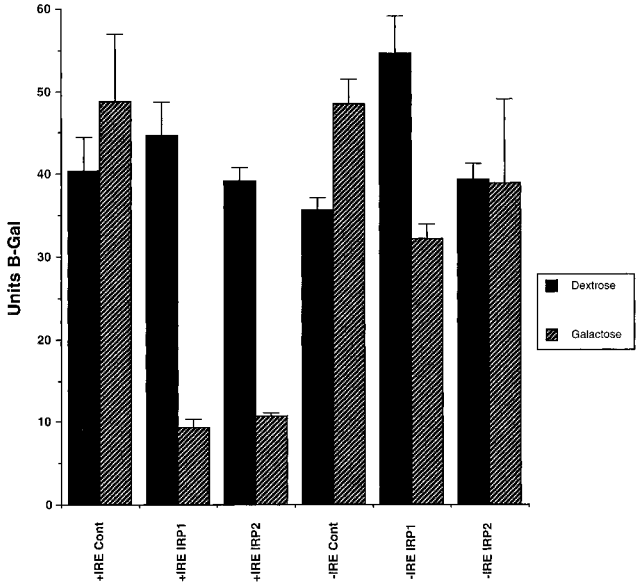

FIGURE 4: IRP1- and IRP2-dependent inhibition of β-galactosidase translation *in vivo*. Yeast containing an integrated copy of the LacZ gene containing an IRE (+IRE) or an inverted IRE (−IRE) were transformed with plasmids expressing either recombinant rat IRP1 (pJVIRP1) or IRP2 (pJVtIRP2) or control truncated IRP1 (pJVtIRP1) under the control of the GAL1/10 promoter. Three independent isolates for each transformant were assayed in duplicate, and the bars represent the average and the standard deviation for each set. Units of β-galactosidase activity are calculated as change in OD_{420nm} per minute per milligram of protein.

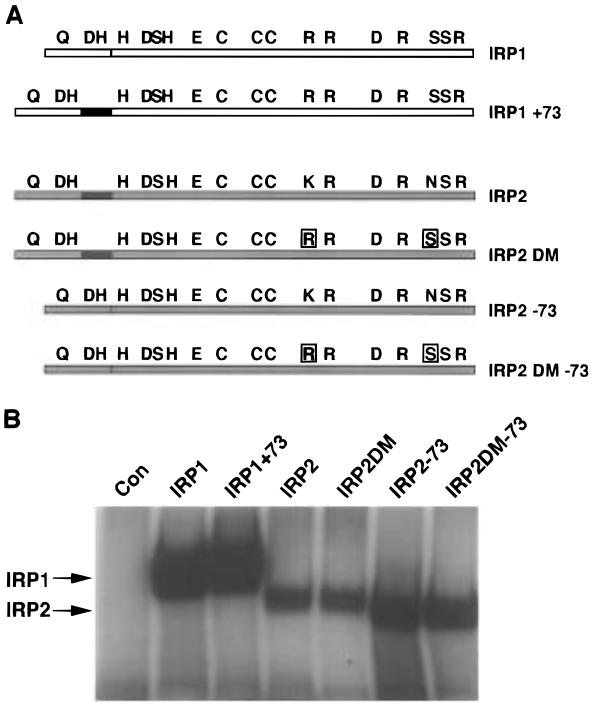

FIGURE 5: RNA-binding activities of mutant IRP1 and IRP2. (A) The structures of wild-type and mutant IRP1 and IRP2 are schematically drawn. Single-letter amino acids above each mutant sequence indicate aconitase active-site residues, and the boxes around amino acids R611 and S853 in IRP2 indicate changes from the wild-type sequence to aconitase active-site residues. IRP1 sequences are indicated by the white boxes, and IRP2 sequences are indicated by the gray boxes. The 73 amino acid insertion in IRP2 is indicated by the black box. Wild-type IRP1; IRP1+73, containing an insertion of the IRP2 73 amino acid domain; wild-type IRP2; IRP2DM, IRP2 double-mutant containing aconitase active-site substitutions K>R and N>S; IRP2−73, IRP2 lacking the 73 amino acid domain; IRP2DM-73, IRP2 lacking the 73 amino acid domain and containing active-site substitutions K>R and N>S. (B) Extracts (10 μg) isolated from yeast expressing wild-type or mutant IRP1 and IRP2 proteins were incubated with a ^{32}P-labeled IRE, and RNA−protein complexes were separated by 5% nondenaturing gels. The positions of IRP1·IRE and IRP2·IRE complexes are indicated.

each set. Figure 4 shows that the induction of IRP1 or IRP2 expression with galactose results in translational repression of +IRE-LacZ mRNA about 3-fold and 4-fold, respectively. As predicted, IRP1 and IRP2 expression had no effect on the translation of the −IRE-LacZ mRNA, indicating that repression is specific for the sense IRE-containing mRNA. Since IRP1 and IRP2 levels could not be accurately quantitated in these studies, the data do not address whether IRP1 and IRP2 are equally effective in repressing translation of IRE-mRNAs in yeast. These studies indicated that IRP2, like IRP1, bound to an IRE inserted in a reporter mRNA and was capable of repressing translation of IRE-containing RNAs in yeast. These data indicated that yeast contains the necessary factors for expressing IRP2 in a form capable of translational repression.

Biochemical Characterization of Recombinant IRP1 and IRP2 Mutants. Although IRP2 shares 61% identity with IRP1 and contains the three cysteines that coordinate the 4Fe-4S cluster in both IRP1 and aconitase, IRP2 contains substitutions at two residues essential for aconitase activity (Zheng et al., 1992a). Lys611 is substituted for the active-site residue Arg, and Asn853 is substituted for the active-site residue Ser (Guo et al., 1994, 1995a; Rouault et al., 1992). It is therefore not surprising that IRP2 lacks detectable aconitase activity. In addition to these active-site substitutions, IRP2 contains a 73 amino acid insertion that may block aconitase activity. This region is essential for iron-mediated proteolysis of IRP2 and when inserted into a similar position in IRP1 resulted in iron-mediated degradation of IRP1 (Iwai et al., 1995). To determine whether the presence of the 73 amino acid domain prevented IRP2 from being an aconitase, mutant IRP2 plasmids were constructed that either lacked a portion of the 73 amino domain (IRP2−73) or contained restored active-site amino acids Lys611>Arg and Asn853>Ser (IRP2DM) (Figures 1 and 5A). A third IRP2 mutant was also constructed that contained the restored active-site residues and a deletion of the 73 amino acid domain IRP2DM-73 (Figures 1 and 5A). These plasmids were transformed individually into the aconitase deletion strain (JDP10), and RNA-binding and aconitase activities of mutant IRPs were determined. The use of the aconitase deletion strain for expression of these constructs was chosen since aconitase and RNA-binding activities can be measured in cell lysates that lack endogenous mt-aconitase activity without purification of IRP1 or IRP2. All extracts were incubated anaerobically with ferrous ammonium sulfate/DTT/sodium sulfide, which are reagents shown to be effective in reconstituting the 4Fe-4S cluster in mt-aconitase and IRP1 (see Materials and Methods). IRP1 showed slight aconitase activity in yeast extracts that were not subjected to the reconstitution procedure (Figure 6), indicating that a small number of IRP molecules contain an 4Fe-4S cluster. After iron reconstitution, IRP1 aconitase activity increased significantly (Figure 6). IRP2 mutants lacking part of the 73 amino acid domain (IRP2−73) or containing restored active-site residues (IRP2DM) bound RNA, but lacked detectable aconitase activity (Figures 5B and 6). Surprisingly, aconitase

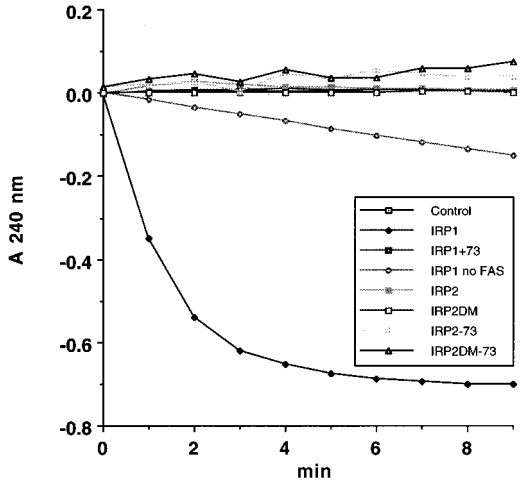

FIGURE 6: Aconitase activity of mutants IRP1 and IRP2. Wild-type or mutant IRPs were expressed in an aconitase deletion strain, and aconitase activity was measured in each yeast extract. Samples were first reconstituted with iron by the addition of 125 μM ferrous ammonium sulfate (FAS), 125 μM sodium sulfide, and 10 mM DTT. Aconitase activity was also measured in extracts from yeast expressing IRP1 without prior iron reconstitution (IRP1 no FAS). The samples were then assayed for aconitase activity by the addition of 2 mM *cis*-aconitate, and the change in absorbance at 240 nm was monitored over time. The samples were assayed in duplicate, and the data shown are the average from a representative experiment. A description of the mutant IRP1 and IRP2 genes is described in Figure 5A; control, IRP1 plasmid (pJVtIRP1) containing a premature stop codon.

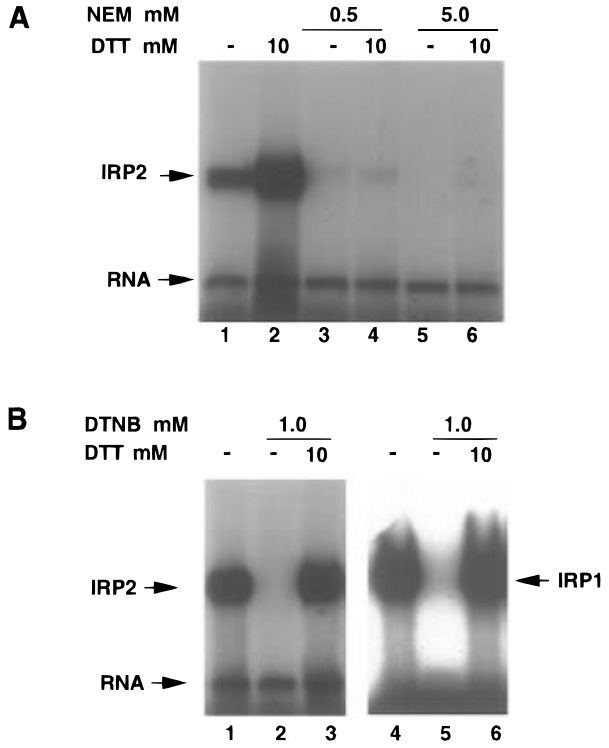

FIGURE 7: Inactivation of RNA-binding activities of IRP1 and IRP2 by sulfhydryl-modifying compounds and oxidants. (A) Purified IRP2 (10 μg) was treated with 0.5 or 5.0 mM NEM for 30 min. In some samples, 10 mM DTT was added to the reaction mixtures after NEM treatment. (B) Purified IRP2 (10 μg) (lanes 1−3) and IRP1 (10 μg) (4−6) were treated with 1.0 mM DTNB for 30 min followed by treatment with 10 mM DTNB for 30 min. After treatments, an aliquot of the NEM-modified protein and the DTNB-oxidized protein were incubated with a ^{32}P-labeled IRE, and the RNA−protein complexes were separated by 5% nondenaturing polyacrylamide gels. The positions of IRP1·IRE and IRP2·IRE complexes and free RNA are indicated.

activity could not be restored in IRP2DM-73, although it bound RNA (Figures 5B and 6). The amount of RNA bound by IRP1 and IRP2 mutants varied, but correlated well with the amount of protein synthesized (data not shown). These data showed that deletion of a portion of the 73 amino acid domain and restoration of the active-site residues in IRP2 were not sufficient to restore aconitase activity, suggesting that IRP2 may adopt a structure incompatible with aconitase activity.

To determine if the presence of the 73 amino acid domain blocks aconitase activity in IRP1, this domain was inserted at a similar position in IRP1 (IRP1+73) (Figure 5A). IRP1+73 bound RNA indicating, that this region does affect the tertiary structure of the protein in a way which would block RNA binding (Figure 5B). IRP1+73, however, did not exhibit aconitase activity (Figure 6). These data suggested that the lack of aconitase activity in IRP1+73 and in IRP2 is due in part to the presence of the 73 amino acid domain. Although the loss of two aconitase active-site substitutions in IRP2 would severely inhibit aconitase activity, these substitutions are not sufficient to account for the lack of aconitase activity.

Recombinant IRP1 and IRP2 Are Sensitive to Sulfhydryl-Modifying Compounds and Oxidants in Vitro. During purification and storage of IRP2, we observed that the RNA-binding activity of IRP2 steadily decreased over time. The loss of IRP2 RNA-binding activity occurred whether IRP2 was purified from yeast or from mammalian cells or tissues (data not shown). By treating IRP2 with 10 mM DTT, RNA-binding activity could be readily restored (Figure 7A, lanes 1 and 2). In contrast, the RNA-binding activity of IRP1 expressed in yeast was stable during purification and storage.

RNA-binding activity of mammalian IRP1 can be inactivated by treatment with sulfhydryl-modifying compounds and oxidants (Henderson & Kuhn, 1995; Hentze et al., 1989; Hirling et al., 1994; Kim et al., 1995; Philpott et al., 1993). Cys437 was shown to be a target for the sulfhydryl-modifying compound *N*-ethylmaleimide (NEM) (Hirling et al., 1994; Philpott et al., 1993). Recent studies have indicated that the RNA-binding activity of recombinant IRP2 synthesized in a transcription/translation extract is insensitive to NEM (Kim et al., 1995), whereas another study indicated that the RNA-binding activity of IRP2 in cell lysates was sensitive to NEM (Henderson & Kuhn, 1995). One difference between these studies is the source of protein used for NEM modification. We therefore wanted to test whether the RNA-binding activity of yeast recombinant IRP1 and IRP2 was affected by alkylation and oxidation. Purified IRP2 was incubated with NEM, and the effect of alkylation on the RNA-binding activity of IRP2 was determined by RNA band shift gels (Figure 7A). RNA-binding activity of IRP2 was inactivated by treatment with either 0.5 or 5.0 mM NEM. Treatment of the NEM-modified IRP2 with 10 mM DTT did not restore activity. Oxidation of IRP1 or IRP2 with 1.0 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) completely inactivated their RNA-binding activities (Figure 7B, lanes 2 and 5). The inactivation of IRP1 and IRP2 RNA-binding activity by DTNB could be readily reversed by treatment of the protein with 10 mM DTT, indicating that the loss of RNA binding was not due to disruption of the

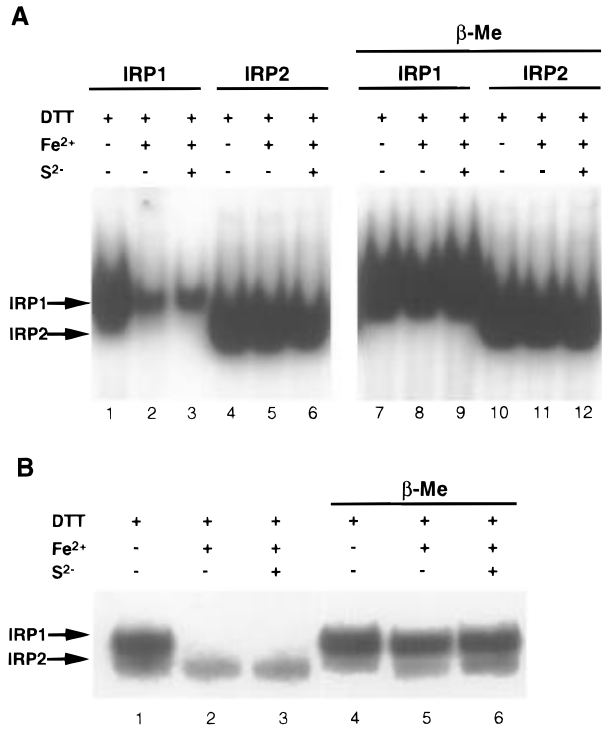

FIGURE 8: Treatment of recombinant rat IRP1 and IRP2 with iron and reducing agents *in vitro*. (A) Purified IRP1 (10 μg) (lanes 1−3 and 7−9) and IRP2 (lanes 4−6 and 10−12) were treated with 125 μM ferrous ammonium sulfate (FAS) and 10 mM DTT in the presence or absence of 125 μM sodium sulfide. After iron treatment, an aliquot of protein from each sample was incubated with a ^{32}P-labeled IRE, and the RNA−protein complexes were separated by 5% nondenaturing polyacrylamide gels. Some samples were treated with 2% β-ME for 10 min prior to the addition of ^{32}P-labeled IRE (lanes 7−12). (B) An FTO2B extract (100 μg) containing IRP1 and IRP2 was treated with iron as described in (A). An aliquot of protein (10 μg) from each sample was used for RNA band shift gels as described in (A). Some samples were treated with 2% β-ME prior to the addition of ^{32}P-labeled IRE (lanes 4−6).

protein structure (Figure 7B, lanes 3 and 6). Collectively, these data suggested that oxidation of either one or more cysteines in IRP2 inhibited RNA binding.

RNA-Binding Activities of Recombinant IRP1 and IRP2 after Treatment with Ferrous Ammonium Sulfate and Reducing Agents. The 4Fe-4S cluster of IRP1 can be reconstituted anaerobically using ferrous ammonium sulfate (FAS) and reducing agents. Reconstitution of the 4Fe-4S cluster results in an increase in aconitase activity and a decrease in RNA-binding activity (Constable et al., 1992; Emery-Goodman et al., 1993; Haile et al., 1992a, 1992b). Likewise, we wanted to determine if standard procedures used for the reconstitution of the 4Fe-4S cluster in IRP1 and mt-aconitase (Kennedy & Beinert, 1988; Kennedy et al., 1983) would result in a decrease in the RNA-binding activity of IRP2. IRP1 and IRP2 were purified from yeast using Ni^{2+}-NTA and incubated with DTT and FAS in the presence or absence of sodium sulfide in an anaerobic chamber (see Materials and Methods). The ability of IRP1 and IRP2 to bind RNA was determined by RNA band shift gels (Figure 8A). As expected, the RNA-binding activity of IRP1 decreased after *in vitro* reconstitution with either FAS/DTT or FAS/DTT/sulfide (Figure 8A, lanes 2 and 3). RNA-binding activity of IRP1 could be restored with β-ME as previously shown (Constable et al., 1992; Emery-Goodman et al., 1993; Haile et al., 1992a,b) (Figure 8A, lanes 8 and 9). When IRP2 was incubated with FAS/DTT or FAS/DTT/sulfide under the same conditions used for IRP1, no loss in RNA-binding activity was observed (Figure 8A, lanes 5 and 6) nor did treatment with β-ME affect RNA-binding activity (Figure 8A, lanes 11 and 12). To determine if iron can be detected in IRP2, IRP2 was incubated anerobically with FAS/DTT and analyzed by atomic absorption spectroscopy. These studies did not detect iron bound to IRP2 (data not shown); however, further spectroscopic studies are required to determine if IRP2 binds iron. These data suggested that if IRP2 binds iron it does not result in decreased RNA-binding activity.

To eliminate the possibility that the inability to reconstitute IRP2 with iron was due to an altered conformation of yeast recombinant IRP2, we incubated a cytosolic extract prepared from rat FTO2B hepatoma cells with FAS/DTT or FAS/DTT/sulfide and assayed IRP1 and IRP2 RNA-binding activity. As expected, the RNA-binding activity of IRP1 decreased after iron reconstitution (Figure 8B, lanes 2 and 3), but was restored with treatment with β-ME (Figure 8B, lanes 5 and 6). In contrast, the RNA-binding activity of IRP2 was unaffected by treatment with FAS/DTT or FAS/DTT/sulfide (Figure 8B, lanes 2 and 3). These data are consistent with our results obtained with yeast recombinant IRP2, indicating that if IRP2 binds iron it does not form a 4Fe-4S cluster like the cluster in IRP1 or mt-aconitase. One possibility is that IRP2 may contain a novel cluster whose formation does not interfere with RNA-binding activity.

DISCUSSION

IRP1 and IRP2 are key regulators of iron homeostasis in higher eukaryotes. Iron regulation is achieved by the binding of IRP1 or IRP2 to IREs located in the 5′ or 3′ UTRs of mRNAs encoding proteins involved in iron metabolism. The binding of IRPs to IREs results in translational repression or stabilization of mRNA, providing a mechanism to coordinately regulate expression of genes by changes in iron levels. The following question arises: Why are two IRPs required to regulate iron homeostasis? Overall, IRP1 and IRP2 share 61% amino acid identity with each other, and each shares about 35% identity with mt-aconitase (Rouault et al., 1992; Samaniego et al., 1994; Guo et al., 1995a). In contrast to IRP1 where regulation is achieved by changes in RNA-binding activity, IRP2 is regulated by iron-mediated proteolysis (Guo et al., 1994, 1995b; Samaniego et al., 1994; Henderson & Kuhn, 1995). It is not clear why IRP1 has evolved two distinct biological activities which are dependent on cellular iron levels. One possibility is that IRP1 has evolved as a bifunctional protein regulating IRE-mRNAs when iron is scarce and citrate metabolism when iron is abundant (Philpott et al., 1994). In contrast, IRP2 lost aconitase activity, perhaps due to the acquisition of the 73 amino acid degradation domain, and consequently functions solely as an RNA-binding protein. Because of the loss of aconitase activity, IRP2 was not under selective pressure to maintain aconitase active-site residues. One clue to the different roles of IRP1 and IRP2 comes from studies showing that they have preferences for specific *in vitro* synthesized IREs, suggesting that they may regulate different IRE-mRNAs *in vivo* (Henderson & Kuhn, 1995; Butt et al., 1996; Henderson et al., 1996).

To study the structure and function of IRP2, we overexpressed IRP2 in yeast and investigated the biochemical properties of wild-type and mutant proteins. Like IRP1 (Oliveira et al., 1993), IRP2 synthesized in yeast bound an IRE-reporter mRNA and inhibited its translation. These data indicated that yeast contain the necessary machinery for translational inhibition of an IRE-containing mRNA by IRP2 and that IRP2 does not require other components for translational repression. The mechanism by which IRP2 inhibited translation of IRE-containing mRNAs is therefore similar to IRP1.

Although IRPs expressed in yeast were functional in binding IREs and repressing translation, they were not appropriately iron-regulated. When yeast were grown in high iron-containing medium, the RNA-binding activity of IRP1 was not decreased nor was IRP2 degraded (J.D.P. and E.A.L., unpublished observations). The reason for the lack of iron regulation of recombinant IRP1 and IRP2 in yeast is not known. Yeast recombinant IRP1 exhibited detectable aconitase activity without prior to iron reconstitution. However, after iron reconstitution, IRP1 exhibits increased aconitase activity, indicating that recombinant IRP1 was functional. These data suggested that the inability of yeast to efficiently assemble an 4Fe-4S in IRP1 may be due to the lack of proteins required for 4Fe-4S cluster formation or that iron may not be bioavailable for cluster formation. Although recent studies have shed light about the mechanism of iron uptake in yeast (de-Silva et al., 1996), little is known about how yeast traffic and store cellular iron. Whether the lack of iron regulation of recombinant IRP2 in yeast was due to the lack of iron binding, the lack of specific proteins required for iron-mediated degradation, or sequestration of iron into an unavailable form is not known.

Our data indicated that when IRP2 was incubated with iron *in vitro* under identical conditions used for IRP1, no loss in RNA-binding activity was observed. This was not a property of yeast recombinant IRP2, since RNA-binding activity of IRP2 in hepatoma extracts was also not affected by iron reconstitution. One conclusion from these studies was that IRP2 does not contain a 4Fe-4S cluster similar to the cluster in IRP1. However, IRP2 contains a region of 73 amino acids that contains 4 cysteines and 1 histidine (Guo et al., 1995a; Rouault et al., 1992). Site-specific mutation of cysteines within this region prevented iron-mediated degradation of IRP2, indicating the importance of these residues in iron regulation (Iwai et al., 1995). The cysteine(s) and the histidine residue could serve as metal ligands and participate in the formation of a novel iron cluster. In this scenario, binding of iron to IRP2 would not affect RNA-binding activity, but increase the susceptibility of IRP2 for degradation. Our studies (Guo et al., 1995b) and those of others (Iwai et al., 1995) showed that IRP2 RNA-binding activity was not decreased in cells treated with iron in the presence of proteasome inhibitors, supporting our idea that if iron binds IRP2, RNA-binding activity is not affected. From these data, two models can be put forth to account for IRP2 regulation by iron. One model proposes that iron binds to cysteines and/or the histidine in the 73 amino acid domain, altering the conformation of IRP2 and rendering it susceptible to degradation. Most proteins targeted for degradation by the proteasome are ubiquitinated (Ciechanover, 1994; Rechsteiner et al., 1993); however, it is not known whether IRP2 is ubiquitinated. It is possible that iron binding unmasks a sequence in IRP2 that binds a specific protein that is required for targeting of IRP2 to the proteasome. This idea is consistent with data demonstrating that protein synthesis is required for IRP2 iron-mediated degradation (Guo et al., 1995b; Henderson & Kuhn, 1995). Another role for iron in IRP2 degradation is that iron binding may result in metal-catalyzed oxidation of the cysteines or the histidine in the 73 amino acid domain marking IRP2 for degradation. The accumulation of oxidized proteins has been shown to occur in cells during aging and in some pathological states and is thought to be due to an increase in metal-catalyzed oxidation of proteins (Stadtman & Oliver, 1991). Our data reported here indicated that IRP2 RNA-binding activity was highly susceptible to oxidative inactivation *in vitro*. Whether IRP2 is regulated by an oxidative mechanism *in vivo* remains to be determined.

Alternatively, a second model to explain IRP2 degradation by iron proposes that IRP2 does not bind iron and that the role of iron is to induce the synthesis of a protein or activate a latent protein required in the IRP2 degradation pathway. Although our preliminary studies using atomic absorption spectroscopy have not detected iron in IRP2 treated with reagents known to reconstitute the 4Fe-4S cluster in mt-aconitase and IRP1 (J.D.P. and E.A.L., unpublished observations), further spectroscopic studies of IRP2 are required to conclusively determine if IRP2 binds iron, and if it does what type of cluster is present.

A functional difference between IRP1 and IRP2 was that IRP2 lacked detectable aconitase activity (Guo et al., 1994). This is not surprising given the fact that IRP2 contains a Lys at residue 611 which is substituted for the active-site residue Arg, and an Asn at residue 853 which is substituted for the active-site residue Ser. In addition, IRP2 contains an insertion of 73 amino acids which is not present in IRP1. Substitution of Lys611 and Asn853 with Arg and Ser, respectively, in IRP2DM did not restore aconitase activity. In fact, aconitase activity could not be restored in the double mutant, IRP2DM-73, which contained a deletion of the 73 amino acid domain in addition to the active-site substitutions. Furthermore, IRP1 containing an insertion of the 73 amino acid domain lacked aconitase activity, suggesting that the presence of this domain might preclude substrate binding or 4Fe-4S cluster formation. Collectively, these data suggested that the lack of detectable aconitase activity in IRP2 may be due not only to substitutions at the two aconitase active-site residues and the presence of the 73 amino acid domain, but also to other alterations in either the sequence or structure of the protein.

RNA-binding activity of IRP1 can be inactivated using the sulfhydryl-modifying reagents NEM, phenylmaleimide, or iodoacetamide or the oxidant diamide either in crude lysates (Hentze et al., 1989) or with purified recombinant IRP1 (Henderson & Kuhn, 1995; Hirling et al., 1994; Kim et al., 1995; Philpott et al., 1993). The target of NEM alkylation is a cysteine at residue 437 in the active-site cleft. The mechanism of inactivation is due to the inhibition of IRE binding by steric hindrance (Hirling et al., 1994; Philpott et al., 1993). Conflicting studies have been reported as to whether IRP2 binding of RNA is inactivated by NEM. One study showed that the RNA-binding activity of a recombinant IRP2 synthesized *in vitro* was not inactivated by NEM (Kim

et al., 1995), whereas another study reported that in mouse cell lysates, the RNA-binding activity of IRP2 is inactivated by NEM or with diamide (Henderson & Kuhn, 1995). Our studies showed that the RNA-binding activity of yeast recombinant IRP2 can be inactivated with NEM or DTNB, indicating that both recombinant and endogenous IRP2 were similarly affected by these treatments.

One difference between IRP1 and IRP2 is that the RNA-binding activity of purified IRP2 isolated from either mammalian or yeast sources steadily decreased over time. RNA-binding activity of IRP2 can be restored by treatment with DTT. IRP2 contains 18 cysteines, 6 of which are conserved in IRP1 and 11 of which are unique to IRP2. Cysteine 437, which has been shown to be the target of NEM in IRP1 (Hirling et al., 1992; Philpott et al., 1993), is conserved in IRP2; however, it is unknown whether this cysteine is also the target for NEM inactivation or oxidation in IRP2. Given the sensitivity of IRP2 to sulfhydryl group oxidation, this suggested that cysteine(s) unique to IRP2 may be the target of oxidation. Redox regulation has been observed to regulate the binding of activator proteins to the *psb*A mRNA *in vivo*. In *Chlamydomonas reinhardtii*, the translation of the *psbA* mRNA is controlled by binding of activator proteins to a stem−loop in its 5′ UTR. The binding of these proteins to *psb* mRNA *in vitro* and translational regulation *in vivo* are dependent on the redox state of these proteins (Danon & Mayfield, 1994). Further studies are required to understand the role of redox regulation in modulating the RNA-binding activity of IRP1 and IRP2.

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