Iron Regulates Cytoplasmic Levels of a Novel Iron-responsive Element-binding Protein without Aconitase Activity*

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Iron-responsive element-binding proteins (IRE-BPs) are cytosolic proteins that bind to a conserved RNA stem-loop, termed the iron-responsive element (IRE), that is located in the 5' or 3'-untranslated regions of mRNAs involved in iron metabolism. Binding of the IRE-BP to 5'-IREs represses translation, whereas binding to 3'-IREs stabilizes the mRNA. The previously identified IRE-BP (BP1) contains a 4Fe-4S cluster and has sequence homology to mitochondrial aconitase. The 4Fe-4S cluster is important for iron-dependent regulation: BP1 containing iron has low affinity for the IRE and contains aconitase activity, whereas BP1 lacking iron has high affinity for the IRE, but lacks aconitase activity. A second IRE-BP (BP2) has been identified in rat tissues and cells and exhibits many of the hallmarks of an IRE-BP, including binding to the IRE and functioning as a translational repressor of IRE-containing RNAs. BP1 and BP2 RNA binding activities are decreased in extracts from cells treated with iron, indicating that BP1 and BP2 are negatively regulated by iron. Although BP1 and BP2 share similar characteristics, they differ in two significant ways. Unlike BP1 levels, which do not change when RNA binding activity decreases in response to iron, BP2 decreases to undetectable levels in extracts from cells treated with iron; and unlike BP1, BP2 does not have aconitase activity. These data indicate that BP1 and BP2 are distinct proteins that have similar specificity for IRE binding and that function similarly in translation, but are regulated by iron via different mechanisms.

Iron is an essential element for many organisms and is required for their growth and survival. The importance of iron is implicit in the role it plays in many biological processes, including oxygen and electron transport, nitrogen fixation, and DNA synthesis. Free iron, however, is toxic to cells. This toxicity is due to the ability of free iron to form reactive hydroxyls that can cause peroxidation of lipid membranes and other cellular components. Cells regulate the levels of free iron by controlling the uptake of iron via the transferrin receptor (TfR)† and by sequestering iron into ferritin. Ferritin synthesis increases in iron-repleted cells and occurs by a translational mechanism whereby pre-existing ferritin mRNA becomes translationally active (1, 2). The translational regulation of ferritin mRNA depends on the presence of a specific sequence called an iron-responsive element (IRE) in its 5'-untranslated region (3, 4). In contrast to ferritin synthesis, TfR synthesis decreases in iron-repleted cells and is due to destabilization of the TfR mRNA (5). The sequences responsible for the destabilization of TfR mRNA are located in the 3'-region that contains five IREs (6, 7). The IRE forms a stable stem-loop structure containing ~30 nucleotides and binds a cytosolic protein termed the iron-responsive element-binding protein (IRE-BP) (8–11). Binding of the IRE-BP to 5'-IREs results in the repression of ferritin mRNA translation (12), whereas binding of the IRE-BP to 3'-IREs prevents degradation of the TfR mRNA (13).

Intracellular iron mediates the post-transcriptional regulation of IRE-containing mRNAs. The IRE-BP binds the IRE with high affinity in iron-depleted cells and with low affinity in iron-repleted cells. Thus, the regulation of TfR and ferritin synthesis by the IRE-BP allows cells to maintain iron homeostasis by coordinately regulating the amount of iron taken up by the cell via the TfR and the amount of iron sequestered in the cell as ferritin. The net result of ferritin and TfR regulation by the IRE-BP is a decrease in intracellular free iron levels, thereby preventing iron toxicity. IREs have also been identified in the mRNAs of the Krebs cycle enzyme mitochondrial aconitase (14) and the heme biosynthetic enzyme 5-aminolevulinate synthase (15, 16).

IRE-BPs have been cloned from rats (17), humans (18), rabbits (19), and mice (20) and share at least 95% amino acid homology among these different species. The IRE-BP also shares ~30% identity with the 4Fe-4S enzyme mitochondrial aconitase (21). The 18 active-site residues in mitochondrial aconitase, including the 3 cysteines that serve as ligands for the 4Fe-4S cluster, are conserved in the IRE-BP. Spectroscopic studies on bovine IRE-BP demonstrated that the IRE-BP contains a 4Fe-4S cluster similar to that of mitochondrial aconitase (22), and biochemical studies demonstrated that the IRE-BP has aconitase activity (23, 24). The function of aconitase activity in the IRE-BP is unknown, but one hypothesis proposed is that it may be involved in the destabilization of the 4Fe-4S cluster during the enzymatic conversion of substrate (10). IRE-BP activity can be modulated in vitro by oxidants or reductants that either perturb the 4Fe-4S cluster or alter the conformation of the protein, suggesting that the assembly and disassembly of the Fe-S cluster may be a mechanism of regulating RNA binding activity in vivo (24, 25). One model of IRE-BP activation based on the above data proposes that the IRE-BP is post-translationally regulated by iron through interconversion between iron-loaded and apo-protein forms. In iron-depleted cells, the IRE-BP lacks aconitase activity and iron, but binds the IRE with high affinity. In contrast, in iron-repleted cells, the IRE-BP possesses aconitase activity and contains iron, but binds the IRE with low affinity. Thus, the IRE-BP may act as an iron sensor protein and may function through the

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The abbreviations used are: TfR, transferrin receptor; IRE, iron-responsive element; IRE-BP, IRE-binding protein; DTP, dithiothreitol.
assembly and disassembly of its 4Fe-4S cluster. The interconversion of the IRE-BP between iron-loaded and apoprotein forms results in the modulation of IRE-BP activity and, consequently, in regulating rates of translation and turnover of IRE-containing mRNAs.

Recent experiments have suggested that there may be more than one IRE-BP in the cell. We have previously identified a second IRE-BP-IRE complex in the extracts of rat tissues and cultured cells (8) and have termed this protein IRE-BP B2 or BP2, while designating the previously characterized IRE-BP as BP1. A complex similar to the rat BP2 IRE complex has been identified in mouse tissues by RNA band-shift analysis and UV cross-linking analysis (26). Whether the protein in this complex is similar to rat BP2 has not been established. Finally, Rouault et al. (18) have isolated an IRE-BP-like cDNA from a human T cell library that has a predicted amino acid sequence that has 57% identity to human BP1. The function of these IRE-BP-like proteins in the iron-dependent regulation of IRE-containing mRNAs has not been determined.

In this report, we present data on the purification and characterization of a new IRE-BP, BP2, from rat liver and rat hepatoma cells. Partial amino acid sequence analysis of rat BP2 indicates that it is homologous to the predicted amino acid sequence of an IRE-BP-like cDNA isolated from a human liver library (15). BP2 has similar biochemical properties to rat BP1 in that it binds the ferritin and TR IREs with similar relative affinities, functions as a translational repressor of IRE-containing RNAs, and loses RNA binding activity in extracts of cells treated with iron. Iron administration to cells results in a decrease in BP2 RNA binding activity via a decrease in BP2 levels; this differs from BP1, in which iron treatment produces a decrease in RNA binding activity via the conversion of high affinity to low affinity forms, without decreased BP1 levels. Finally, unlike BP1, BP2 does not exhibit aconitase activity. These data suggest that aconitase activity is not required for BP2 to function as an IRE-BP and that BP2 may be regulated by iron by mechanisms different from those of BP1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The rat hepatoma cell line FTO2B was used in the iron studies and in the purification of BP2. The cells were grown at 37°C in a 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. For experiments, FTO2B cells were grown in 25-cm² flasks and treated with 100 μg/ml ferric ammonium citrate for 1, 4, 8, 16, and 24 h or with 200 μM desferrioxamine mesylate (Desfera) for 16 h. For purification of BP2, 15 fluted roller bottles each containing 2 x 10⁷ cells were grown at 37°C in a 5% CO₂ atmosphere.

**RNA Band-shift Assays and Competition Experiments**—RNA band-shift assays were carried out as described previously (8). For competition experiments, purified rat BP1 (5 ng) or partially purified BP2 from a rat liver CM-Sepharose fraction (500 ng) was incubated with a 28-nucleotide ⁵²P-labeled ferritin IRE RNA (0.05 pmol) and increasing molecular amounts of unlabeled or trace-labeled ⁵²P-labeled ferritin IRE RNA or unlabeled IRE RNA transcribed by T7 RNA polymerase according to the procedure of Milligan and Uhlenbeck (28). Tracer amounts (10 μCi) of ⁵²P-UTP were added to the transcription reaction (100 μl) to quantitate the amount of competitor IRE RNA synthesized. [²²P]GTP (50 μCi) was added to the transcription reaction (40 μl) for the synthesis of ²²P-label ferritin IRE RNA. IRE RNA were purified on 15% urea-polyacrylamide gels. The bands were excised from the gels, and the RNA was eluted overnight in 0.1 M ammonium acetate, ethanol-precipitated, and quantitated. Competition experiments were carried out in duplicate and quantitated by densitometry.

**Purification of BP2—BP2 was purified from rat liver and FTO2B cells.** Ion-exchange chromatography was used in the initial purification step (15). Rat FTO2B cells, followed by FTO2B cells, were lysed with 100 mM Tris, pH 7.5, 1 mM EDTA, 5% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 mM benzamidine and centrifuged at 100,000 x g for 1 h. The supernatant was centrifuged at 100,000 x g for 1 h. The supernatant was dialyzed overnight to remove iron. Iron was added to the supernatant and centrifuged at 100,000 x g for 1 h. The supernatant was dialyzed against Buffer C (20 mM Hepes, 1 mM DTT, 5% glycerol) plus 25 mM KC1 and 1 mM phenylmethylsulfonyl fluoride and loaded on a DEAE-Sepharose (Amersham; 200 ml) column equilibrated in Buffer B (0.1 M Hepes, pH 7.5, 1.4 M KC1, 0.05 mM MgCl₂) was added to the supernatant and centrifuged at 100,000 x g for 1 h. The supernatant was centrifuged at 100,000 x g for 1 h. The supernatant was dialyzed against Buffer C plus 25 mM KC1 and was eluted in the 25-500 mM KC1 gradient. BP2-containing fractions were loaded on CM-Sepharose (50 ml) in Buffer C, pH 6.0, plus 25 mM KC1. Fractions containing BP2 were identified by silver staining or Western blotting using rabbit anti-BP2 antisera (see below). For the purification of BP2 from FTO2B cells, 15 roller bottles each containing 2 x 10⁷ cells were rinsed with washing with 10 mM EDTA in phosphate-buffered saline. The cells were washed with phosphate-buffered saline. The cells were rewarshed with phosphate-buffered saline and lysed in Buffer A plus 1.0% Nonident P40 and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 150,000 x g for 30 min. 0.01 volume of Buffer B was added to the supernatant and centrifuged at 100,000 x g for 1 h. The supernatant was dialyzed against Buffer B plus 25 mM KC1 and was eluted in the 25-500 KC1 gradient. BP2-containing fractions were cloned into the Hi-Load Q anion-exchange column (Pharmacia Biotech Inc.). Purification of BP2 was carried out using the protocol of Neupert et al. (29). A DNA fragment containing the rat liver ferritin IRE was cloned into the XbaI site of a pSP64 poly(A) vector containing 30 nucleotides of poly(A) (Promega). The IRE poly(A) DNA was linearized with EcoRI and used to synthesize RNA according to the protocols from Ambion Inc. (Austin, TX). Approximately 15 μg of IRE RNA was bound to 200 μl of poly(U)-agarose. BP2-containing fractions from the CM-Sepharose column (rat liver) were first applied to a poly(U) control column to remove nonspecific proteins and then were applied three times to the IRE column.

**Affinity Purification**—IRE affinity chromatography of BP2 was carried out using the protocol of Neupert et al. (29). A DNA fragment containing the rat liver ferritin IRE was cloned into the XbaI site of a pSP64 poly(A) vector containing 30 nucleotides of poly(A) (Promega). The IRE poly(A) DNA was linearized with EcoRI and used to synthesize RNA according to the protocols from Ambion Inc. (Austin, TX). Approximately 15 μg of IRE RNA was bound to 200 μl of poly(U)-agarose. BP2-containing fractions from the CM-Sepharose column (rat liver) were first applied to a poly(U) control column to remove nonspecific proteins and then were applied three times to the IRE column.

**Purification of BP2 from FTO2B cells.** For the purification of BP2 from FTO2B cells, 15 roller bottles each containing 2 x 10⁷ cells were rinsed with washing with 10 mM EDTA in phosphate-buffered saline. The cells were washed with phosphate-buffered saline. The cells were rewarshed with phosphate-buffered saline and lysed in Buffer A plus 1.0% Nonident P40 and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 150,000 x g for 30 min. 0.01 volume of Buffer B was added to the supernatant and centrifuged at 100,000 x g for 1 h. The supernatant was dialyzed against Buffer B plus 25 mM KC1 and was eluted in the 25-500 mM KC1 gradient. BP2-containing fractions were cloned into the Hi-Load Q anion-exchange column (Pharmacia Biotech Inc.) equilibrated in Buffer C plus 25 mM KC1 and was eluted in the 25-500 KC1 gradient and dialyzed against Buffer D plus 40 mM KC1 before binding to the IRE affinity column (see below).

**Western Blot Analysis**—Western blot analysis was performed using the IRE affinity-purified BP1 and BP2 antibodies. The membranes were washed and incubated with alkaline phosphatase-conjugated goat anti-chicken or anti-rabbit IgG, and the membranes were developed with bromochloroindolyl phosphate/nitro blue tetrazolium substrate. The signals obtained for BP1 and BP2 were compared with their respective standards. Both methods yielded similar concentrations of BP1 and BP2. Approximately 17 μg of BP2 and 45 μg of BP1 were isolated from 3 x 10⁷ FTO2B cells. For BP1, 10 μg of BP1 was isolated from 10⁷ cells. The low levels of BP2 purified from liver reflect degradation of IRE RNA from the affinity column and protein elution during purification.
N-terminal sequence was obtained for the rat liver IRE affinity-purified 83,000-Da protein. Approximately 10–15 pmol of protein was concentrated on a polyvinylidene difluoride membrane by centrifugation. Amino acid sequencing was carried out on an Applied Biosystems Model 477A Protein Sequencer by the Protein and DNA Core Facility of the Utah Cancer Center at the University of Utah.

- In Vitro Translation Assay-IRE affinity-purified BP1 or BP2 (3.0, 9.0, and 18 ng of each) purified from FTO2B cells was incubated in a wheat germ translation system (Promega) programmed with 10 ng of (+)-IRE RNA, 10 ng of globin mRNA (Life Technologies, Inc.), or 25 ng of brome mosaic virus RNA. (+)-IRE transcripts contain the entire 5’- and 3'-untranslated regions of the rat ferritin I-subunit mRNA fused to the bacterial chloramphenicol acetyltransferase gene. Globin and brome mosaic virus RNAs were used as controls to determine specificity of BP1 and BP2 translational repression and to monitor the translational efficiency of the wheat germ extract. (+)-IRE transcripts were capped in vitro using m7G'ppp'5'G (Pharmacia Biotech Inc.). The transcription/capping reaction was carried out according to Promega protocols, except that [5,6-3H]UTP (10 μCi) was included in the reaction to quantitate the amount of RNA synthesized. The translation reactions (25 μl) contained 12.5 μCi of Tran32S-label (ICN Biomedicals) and were carried out according to Promega protocols. Translation products were analyzed on 15% SDS-polyacrylamide gels, and the 32S-labeled proteins were electroblotted onto a nitrocellulose membrane and subjected to autoradiography.

- Rat BP1 and BP2 Antibody Production—Antibodies to rat liver BP1 were generated in chickens. The entire coding region of BP1 was amplified from the rat liver cDNA clone pSL8 (17) corresponding to the first 18 nucleotides of the coding region (ATGAAAGCTTCAT) and a second primer corresponding to the last 11 nucleotides of the coding region (CTACTGGCCATCTTGGCAT). Both primers contained a BamHI site at their 5'-ends. The polymerase chain reaction fragment was subcloned into the BamHI site of the glutathione transferase expression vector pGEX2T (Pharmacia Biotech Inc.). Approximately 1 mg of BP1-glutathione transferase fusion protein was purified from 1 liter of bacterial culture. Since the BP1-glutathione transferase fusion protein was insoluble and did not bind to a glutathione resin, it was purified by SDS-polyacrylamide gel electrophoresis. The purified fusion protein was used to inject chickens, and antibodies were isolated from the eggs (30). The titer and specificity of the chicken anti-rat BP1 antibodies were assayed by enzyme-linked immunosorbent assay and Western blotting.

Antibodies to rat liver BP2 were generated in New Zealand White rabbits. A 225-nucleotide fragment unique to BP2 was amplified from rat liver mRNA using two oligonucleotides corresponding to nucleotides 475–699 (ATACGAAAGCTTCAACAAT and 682–698 (GTCGGCTGAC-CTGAAAGTTCT). The 225-nucleotide fragment was subcloned into the NdeI BamHI site of the pET-16b expression vector (Novagen). The BP2-His fusion protein was purified by metal chelate chromatography on a nickel-activated metal chelate column according to the manufacturer’s protocols (Novagen). Approximately 350 μg of BP2-His fusion protein was isolated from 1 liter of bacterial culture after induction with 1 mM isopropyl-1-thio-b-D-galactopyranoside. The fusion protein was conjugated to keyhole limpet hemocyanin and injected into rabbits. Antibody preparation was carried out by Hazelton Research Laboratories. The titer and specificity of rabbit anti-rat BP2 antibodies were assayed by enzyme-linked immunosorbent assay and Western blotting.

- Western Blotting and Aconitase Assays—S100 extracts were prepared from 2 × 75-cm2 flasks of FTO2B cells. The protein was loaded on a Mono-Q column in Buffer C plus 25 mM KCI. BP1 and mitochondrial aconitase eluted in the flow-through fractions, whereas BP2 bound to the column and was eluted in a 25–550 mM KCl gradient. To ensure that BP2 was separated from BP1 and mitochondrial aconitase, Western blotting was carried out using 10 μg of protein from the S100 extract and the bound and flow-through Mono-Q fractions. The protein was fractionated on 8% SDS-polyacrylamide gels, and the protein was transferred to a polyvinylidene difluoride membrane. The membrane was incubated with chicken anti-rat BP1 antibody, rabbit anti-rat BP2 antibody, or chicken anti-porcine mitochondrial aconitase antibody and purified mitochondrial aconitase were gifts from Dr. Claire Kennedy). After 1 h, the membranes were washed and incubated with alkaline phosphatase-conjugated goat anti-chicken or anti-rabbit IgG. The membranes were developed with bromochloroindolyl phosphate/nitro blue tetrazolium substrate.

- Aconitase activity was measured in the Mono-Q-fractionated extracts using an assay based on the disappearance of cis-aconitate as measured by absorbance at 240 nm (31). The reactions were started by the addition of 0.2 mM cis-aconitate to the fractionated extracts (100 μg), and the activity was determined from the initial reaction rate. BP1- and mitochondrial aconitase-containing fractions from the Mono-Q column and BP2 were reconstituted anaerobically with iron by the procedure of Kennedy and Beinert (32). Ferrous ammonium sulfate and DTT were added to 100 μg of fractionated protein in 20 mM Hepes, pH 7.5, 1 mM DTT, and 5% glycerol such that the final concentrations of iron and DTT were 100 μs and 10 μM, respectively. After 1 h, the samples were assayed for aconitase activity using the 240 nm assay. Three independent experiments were carried out in duplicate.

RESULTS

Two IRE-BPs Are Found in Rat Tissues and in Rat Hepatoma Cells—We have previously demonstrated that cytoplasmic extracts from rat liver and heart and from the rat hepatoma cell line FTO2B contain two IRE-BPs that form complexes with the ferritin IRE RNA (8). The slower migrating complex contains BP1, which is the previously characterized IRE-BP (Fig. 1). The faster migrating complex contains a protein that we have termed BP2. This IRE-BP, BP2, is a specific IRE-binding protein since its binding to the IRE can be competed against by specific IRE RNA, but not by nonspecific RNAs (8). To determine the effect of intracellular iron on the RNA binding activity of BP2, FTO2B cells were treated with ferric ammonium citrate for 1, 4, 8, 16, and 24 h. BP2 RNA binding activity was quantitated in cytoplasmic extracts by RNA protein band-shift assays using [3P]-labeled IRE RNA, followed by analysis of the complexes using native polyacrylamide gels (Fig. 1). BP1 activity decreased 2-fold after 1 h of iron treatment and remained at this level for up to 8 h (Fig. 1, lanes 1–6). Similarly, BP2 activity also decreased after 1 h of iron treatment, but was undetectable after 4 h of iron treatment (lane 3). After 8 h of iron treatment, BP2 activity was restored to ~20% of BP2 levels in untreated cells (lane 4). Incubation of the cells with either desferrioxamine alone or desferrioxamine plus iron for 16 h increased BP1 activity 5-fold and BP2 activity 2-fold (lanes 7 and 8). The extracts were also pretreated with β-mercaptoethanol before the addition of the IRE RNA. Reducing agents have been demonstrated to increase BP1 activity; however, the mechanism by which this occurs is unknown (33). BP1 activity increased 10-fold (lanes 9–14). In contrast, BP2 activity was not affected by β-mercaptoethanol at any time after iron treatment (lanes 9–14). These data indicate that in hepatoma cells, while iron treatment causes a reduction in the RNA binding
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Fig. 2. Purification of BP2 from rat liver. A, RNA band-shift assays were performed on fractions obtained from the purification of BP2. 32P-labeled ferritin IRE RNA was incubated with aliquots of protein from each column step. 10 μg of protein was used in the S100 (lane 1), DEAE (lane 2), heparin (HEP) (lane 3), CM (lane 4), and control (CON) poly(U) flow-through (FT) fraction (lane 5) steps. Protein in the control poly(U)-bound (BD) fraction (lane 6), the IRE affinity-bound fraction (lane 7), and the IRE affinity flow-through fraction (lane 8) was not determined. As a control, 10 ng (lane 9) and 50 ng (lane 10) of IRE affinity-purified BP1 were used in the assay. Arrows indicate BP1, BP2, and free RNA (lane 11). B, aliquots of protein from each column step (A) were separated on an 8% SDS-polyacrylamide gel and visualized by silver staining. All fractions contained 3 μg of protein, except for the control poly(U)-bound fraction (lane 7) and the IRE affinity-bound fraction (lane 8), which were not determined. Purified BP1 (150 ng (lane 10) and bovine serum albumin (BSA) (500 ng) (lane 11)) are indicated. Arrows indicate BP1, BP2, and bovine serum albumin. Std, standards.

activity of both BP1 and BP2, BP1 and BP2 differ in the extent to which reducing agents restore RNA binding activity.

Purification and Characterization of BP2 from Rat Liver and Rat Hepatoma Cells—To study the function of BP2, BP2 was purified from rat liver using ion-exchange chromatography followed by IRE affinity chromatography (see “Experimental Procedures”). BP1 and BP2 were separated in the first step of the purification procedure using DEAE-Sepharose. BP1 was found in the flow-through fractions, while BP2 bound and was eluted with 200 mM KCl. BP2 levels were monitored during purification by SDS-polyacrylamide gel electrophoresis, and activity was measured by RNA band-shift assays (Fig. 2, A and B). Quantitation of BP2 activity by RNA band-shift assays indicated that BP2 activity was barely detectable in liver S100 extracts, but was enriched ~8-fold after passage over DEAE (Fig. 2A, lanes 1 and 2). After IRE affinity chromatography, one major band was observed on a SDS-polyacrylamide gel that migrated with a molecular mass of 83,000 Da (Fig. 2B, lane 8). Albumin was added to purified BP2 to stabilize the protein and to prevent protein loss (Fig. 2B, lanes 8 and 11). Affinity-purified BP1 was loaded in an adjacent lane as a control and migrated as a doublet with a molecular mass of 98,000 Da (Fig. 2B, lane 10). The appearance of an IRE-BP doublet on SDS-polyacrylamide gels has been previously reported and is thought to be due to incomplete reduction of the protein (22). We estimate that ~5 μg of purified BP2 was obtained from 192 g of rat liver.

Sequence was obtained for the amino terminus of the 83,000-Da BP2 protein. Nine amino acids were sequenced, yielding the sequence IENTPVLPFP. Comparison of these 9 amino acids with data base sequences showed that 8 out of the 9 amino acids of the BP2 sequence matched the sequence of a human cDNA clone termed 10.1 (18). This cDNA was isolated from a human library using oligonucleotides derived from the amino acid sequence of human BP1. The sequence encoded by clone 10.1 exhibits ~57% amino acid identity to the human BP1 sequence. Although the amino terminus of the protein encoded by clone 10.1 has not been identified, inspection of the human 10.1 cDNA sequence identified a putative open reading frame that could code for a protein with a molecular mass of 101,000 Da. The sequence obtained for rat liver BP2 was located 194 amino acids downstream from the putative initiator methionine codon in the human BP2 open reading frame. The discrepancy in size between rat BP2 and the human 10.1 protein suggests that the 83,000-Da protein we identified may be a proteolytic fragment of rat BP2.

To determine the size of native rat liver BP2, rat BP2 antibodies were generated that were used to assay BP2 from FTO2B cells. FTO2B cells were chosen for these studies since these cells contain higher levels of BP2 activity compared with rat liver. Anti-rat BP2 antibodies were generated against a unique 75-amino acid polypeptide in the protein encoded by the human 10.1 cDNA that is not present in human or rat BP1. The 225-nucleotide region in 10.1 was amplified from rat liver mRNA using two oligonucleotides corresponding to sequences flanking the unique 10.1 region. The polymerase chain reaction fragment was cloned into the pET-16b His expression vector, and the 75-amino acid-His fusion protein was used to generate antibodies in rabbits (see “Experimental Procedures”). The antibodies were tested in a RNA band-shift assay to determine if they were capable of interacting with rat liver BP2 (Fig. 3). Partially fractionated rat liver BP1 and BP2 preparations were preincubated with either rabbit preimmune antisera (lanes 3 and 4) or anti-BP2 antisera (lanes 5–8), followed by the addition of 32P-labeled IRE RNA to the extracts. The complexes were resolved on native polyacrylamide gels. Anti-BP2 antibodies supershifted only the BP2 IRE complex (lanes 5 and 7) and had no effect on the BP1 IRE complex (lanes 6 and 8), confirming that the anti-rat BP2 antibodies are specific for BP2, and not for BP1.

Fractions from the IRE affinity column and a control poly(U) column were analyzed by SDS-polyacrylamide gel electrophoresis, and the protein was visualized by silver staining and immunoblotting using anti-rat BP2 antibody (Fig. 4, A and B). SDS-polyacrylamide gels stained with silver showed that a 104,000-Da protein was detected in the bound IRE affinity fraction in addition to the 83,000-Da protein (Fig. 4A, lane 8). These proteins were not present in the bound fraction from the control poly(U) column (Fig. 4A, lane 6). Western blotting of the FTO2B fractions using anti-rat BP2 antibody showed that the 104,000- and 83,000-Da proteins were detected by the anti-rat BP2 antibodies in the bound fraction from the IRE affinity column (Fig. 4B, lane 6) and were not present in the bound fractions from the poly(U) control column (lane 4). Finally, molecular sizing of a partially purified mixture of BP1 and BP2
by gel filtration on a Superdex G-200 column demonstrated that BP2 eluted with an apparent $M_r$ of $\sim 112,000$ (data not shown). These data indicate that rat BP2 has an apparent $M_r$ of 104,000 and may be homologous to the protein encoded by the human 10.1 cDNA.

**BP2 Does Not Contain Aconitase Activity**—BP1 has been shown to contain aconitase activity, which is believed to be important in its role as a iron sensor (10). To determine if BP2 contains aconitase activity, FTO2B extracts were fractionated on a Mono-Q anion-exchange column so that BP1 and mitochondrial aconitase were separated from BP2, and the fractions were assayed for aconitase activity. Mono-Q-fractionated BP1 and BP2 were used to assay for aconitase activity since only small amounts BP2 could be isolated from the IRE affinity column, and these amounts were not sufficient for aconitase assays. To confirm that BP2 was separated from BP1 and mitochondrial aconitase, fractions from the Mono-Q column and from the S100 extract were immunoblotted using anti-BP1, anti-BP2, or anti-mitochondrial aconitase antibodies (Fig. 5).

Immunoblots showed that BP1 and mitochondrial aconitase coeluted in the flow-through fractions, whereas BP2 bound to Mono-Q. Aliquots from the Mono-Q flow-through and bound fractions and the total S100 extract were assayed for aconitase activity by measuring the disappearance of $\text{cis}$-aconitate at 240 nm. Table I shows that aconitase activity was detected in the S100 extract and in the flow-through fractions. No detectable aconitase activity was found in the bound fraction containing BP2. To ensure that the bound-containing fractions did not contain an aconitase inhibitor, flow-through and bound fractions were mixed, and the mixture was assayed for aconitase activity. Aconitase levels in the mixed extract were identical to those of the flow-through fraction alone. Since aconitase activity requires the presence of a 4Fe-4S cluster, we thought that the lack of aconitase activity in BP2 may be due to the lack of iron in the protein. Mono-Q flow-through and bound fractions and the S100 extract were therefore reconstituted with iron in vitro, and the extracts were assayed for aconitase activity. Aconitase activity was increased in the BP1 and mitochondrial aconitase fractions, but not in the BP2-containing fraction. These data suggest that unlike BP1 and mitochondrial aconitase, BP2 does not contain detectable aconitase activity and can not be reconstituted in vitro by iron.

**BP2 Levels Are Decreased in Iron-treated Cells**—As mentioned above, iron treatment of cells resulted in a reduction in the protein. Mono-Q flow-through and bound fractions and the S100 extract were therefore reconstituted with iron in vitro, and the extracts were assayed for aconitase activity. Aconitase activity was increased in the BP1 and mitochondrial aconitase fractions, but not in the BP2-containing fraction. These data suggest that unlike BP1 and mitochondrial aconitase, BP2 does not contain detectable aconitase activity and can not be reconstituted in vitro by iron.
after 1 h of iron treatment and were undetectable after 4 h of iron treatment (Fig. 6, lanes 2 and 3). BP2 levels began to increase slightly after 8 h of iron treatment and remained at this level for up to 24 h (Fig. 6, lanes 4–5). Incubation of the cells with desferrioxamine or desferrioxamine plus iron showed that BP2 levels increased ~2-fold when compared with BP2 levels in untreated cells (lanes 7 and 8). The change in BP2 levels with iron and iron chelation treatment correlates with the changes in RNA binding activity during the same time course (Fig. 1). These data show that the decrease in BP2 RNA binding activity in the extracts of cells treated with iron is due to decreased levels of BP2.

**BP1 and BP2 Bind Ferritin and Transferrin Receptor IREs with Similar Relative Affinities**—One possible explanation for the function of BP2 is that it may have a differential affinity for either the ferritin IRE or the TR-IREs. Variation between mRNAs in IRE binding affinity might permit differential regulation of expression. The ferritin and TR-IREs contain limited sequence homology, except for a conserved six-member loop and a bulge nucleotide in the stem (6). BP1 has been shown to bind with similar affinities to the ferritin and TR-IREs despite differences in the stem sequences (34). To compare the relative binding affinities of BP1 and BP2 for the ferritin and TR-IREs, competition experiments were performed by incubating partially purified BP1 or BP2 with the [32P]-labeled ferritin IRE in the presence of increasing molar amounts (x0.1–1000) of either ferritin IRE or TR-B and TR-C IREs (Fig. 7, A and B). The sequences of TR-B and TR-C IREs differ by two nucleotide substitutions, one in the six-membered loop and the other in the stem (6). The RNA-protein complexes were analyzed on RNA band-shift gels (data not shown), and the amount of radioactivity in each complex was quantitated by densitometry. No significant differences were seen between the relative binding affinities of BP1 and BP2 for the ferritin and TR IREs. For each competition experiment, the concentration of unlabeled competitor RNA that inhibits the binding of BP1 or BP2 to the [32P]-labeled ferritin IRE by 50% (IC50) was calculated. The IC50 values for BP1 and BP2 binding to the ferritin IRE were both 0.3 nM. The IC50 values for BP1 binding to the TR-B and TR-C IREs were 0.2 and 0.1 nM, respectively, and the IC50 values for BP2 binding to the TR-B and TR-C IREs were 0.3 and 0.4 nM, respectively. These data suggest that BP1 and BP2 bind to the ferritin and TR IREs with similar affinities in vitro.

**BP1 and BP2 Repress Synthesis of IRE-containing RNA in an In Vitro Translation Extract**—BP1 has been shown to repress ferritin synthesis in an in vitro translation system (12). To determine if BP2 represses the translation of an IRE-containing RNA in vitro, equal amounts of BP1 or BP2 from one round of IRE affinity purification were added to a wheat germ translation extract programmed with a (+) IRE RNA that contains the entire 5’- and 3’-untranslated regions of the ferritin subunit mRNA including the IRE fused to the bacterial chloramphenicol acetyltransferase coding region. To determine the specificity of translational repression by BP1 or BP2, globin mRNA and brome mosaic virus RNA, which do not contain IREs, were added to wheat germ translation extracts containing either BP1 or BP2. The [35S]methionine-labeled products from the translation reaction were analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 8 shows that increasing amounts of BP1 (lanes 2–4) or BP2 (lanes 5–7) in extracts resulted in increasing levels of translational inhibition of (+) IRE RNA. In contrast, neither BP1 or BP2 affected the translation of globin mRNA (lanes 8–14) or brome mosaic virus RNA (lanes 15–17) significantly, indicating that BP1 and BP2 inhibition of (+) IRE RNA translation is specific for IRE-containing RNAs. Quantitation of the blots by densitometry indicated that 18 ng of either BP1 and BP2 inhibited translation of the (+) IRE RNA 8-fold compared with controls with no added protein, demonstrating that both proteins are equally competent to...
curves were plotted. The data represent two independent experiments. Experimental data were quantitated by densitometry, and competition experiments. RNA-protein complexes were separated by biochemical characterization of a new IRE-BP from rat tissues.

FIG. 7. BP1 and BP2 bind ferritin and transferrin receptor IREs with similar relative affinities. Partially purified BP1 or BP2 was incubated with the $^{32}$P-labeled ferritin IRE with increasing amounts of unlabeled ferritin IRE (A) or TfR-B and TfR-C IREs (B) as competitors. RNA-protein complexes were separated by 5% native polyacrylamide gel electrophoresis and subjected to autoradiography. Experimental data were quantitated by densitometry, and competition curves were plotted. The data represent two independent experiments.

function as translational repressors of IRE-containing mRNAs in vitro.

DISCUSSION

The work presented here describes the identification and biochemical characterization of a new IRE-BP from rat tissues and cells. BP2 was previously identified using RNA band-shift assays as a minor IRE-protein complex in cytoplasmic extracts from rat heart, liver, and spleen (8). BP2 has many of the characteristics of an IRE-BP, but differs in two significant ways. First, BP2 does not contain detectable aconitase activity. Second, levels of BP2 are decreased in extracts of cells treated with iron. These data suggest that aconitase activity is not required for the functioning of an IRE-BP and that BP2 is regulated by different mechanisms compared with BP1.

Purification of rat BP2 demonstrates that it has an apparent molecular mass of 104,000 Da by SDS-polyacrylamide gel electrophoresis and 112,000 Da by gel filtration and that it is slightly larger in size than the 98,000-Da BP1 protein. The size of rat BP2 is similar to that of an IRE-BP, termed IRF$_{10.1}$, identified by RNA band-shift analysis and UV cross-linking studies in mouse tissues (26). Whether rat BP2 and mouse IRF$_{10.1}$ are identical proteins remains to be determined. However, sequence comparison of a 9-amino acid peptide from the 83,000-Da BP2 protein with the predicted sequence of the human 10.1 cDNA suggests that these proteins may be homologous. Further evidence for the identity of rat BP2 is that antibodies prepared against the unique 75-amino acid region in rat cause BP2 to be supershifted in a rat liver extract. The unique region in rat BP2 shares 98% homology with the similar region in the predicted sequence of the human 10.1 cDNA. Although the amino termini of the human and rat BP2 proteins have not been identified, a putative open reading frame that could encode for a protein of 101,000 Da is present in the human 10.1 cDNA. The size of rat BP2 estimated by SDS-polyacrylamide gel electrophoresis is consistent with the predicted size of the protein encoded by the human 10.1 open reading frame.

BP2 activity is present in rat liver at ~30-fold lower levels than BP1 activity. The BP2-IRE complex is generally not observed in S100 extracts from rat tissues by RNA band-shift assays unless at least 50 µg of crude protein extract is used. In contrast, the levels of BP2 activity in FTO2B cells are only 2-3-fold lower than those of BP1 activity. In addition to low levels of BP2 in liver, BP2 is susceptible to proteolytic degradation during purification. The generation of the 83,000-Da protein is suggestive of a defined site in the unique 75-amino acid region that is susceptible to cleavage. Our data suggest that preparations containing mainly the 83,000-Da protein show band-shift activity. These results were surprising in light of new data that have identified a serine residue in a peptide in human BP1 that cross-links to the IRE (35). This peptide (DLVIDHSLQV) is located at amino acids 121–130 in BP1. A similar sequence (DLTVHSLQV) is located in BP2 16 amino acids upstream from the unique region (Fig. 9). These data suggest that this peptide may also be important for the interaction of BP2 with the IRE and that if BP2 is cleaved in the unique 75-amino acid region, RNA binding would be eliminated. Therefore, one interpretation of our data is that although BP2 is cleaved in the unique region, the structure of BP2 remains intact due to interactions of the residues located amino-terminal to the cleavage site with the rest of the protein. Only under denaturing conditions such as SDS-polyacrylamide gel electrophoresis would the 83,000-Da protein be observed. An alternative explanation is that cleavage of BP2 results in the generation of a 83,000-Da protein and a 21,000-Da fragment that corresponds to the amino terminus. In this case, BP2 either would not require the peptide for IRE binding or perhaps could bind the IRE in the absence of this peptide, but with lower affinity.

While the exact cellular function of BP2 is unknown, biochemical analysis of BP2 shows that it can function as an authentic IRE-BP. First, BP2 functions as well as BP1 in translational repression of IRE-containing RNAs in an in vitro translation extract. Second, RNA band-shift assays demonstrate that BP2 binds the ferritin and TfR IREs with similar affinities. This was important to determine since the ferritin and TfR IREs share limited sequence homology and the possibility existed that BP2 had different affinities for the ferritin and TfR IREs. Furthermore, BP1 and BP2 have similar affinities for the IREs, suggesting that, at least in vitro, the amino acid sequence differences do not affect IRE binding. Third, like BP1, BP2 binding activity is decreased in cytoplasmic extracts from cells treated with iron. It is not known whether iron binds to BP2 or, if it does, what form of iron binds.

Although BP1 and BP2 share similar characteristics, they differ in two significant ways. First, unlike BP1, BP2 does not exhibit aconitase activity. While we cannot rule out the possi-
FIG. 8. BP2 inhibits translation of IRE-containing RNAs. An in vitro synthesized RNA transcript containing the chloramphenicol acetyltransferase gene fused to the ferritin 5′-untranslated region containing the IRE (lanes 1–7), globin mRNA (lanes 8–14), or brome mosaic virus (BMV) RNA (lanes 15–17) was incubated in a wheat germ translation extract containing [35S]methionine in the absence (lanes 1, 8, and 15) or presence of increasing amounts of BP1 (lanes 2–4, 9–11, and 16) or BP2 (lanes 5–7, 12–14, and 17). The amounts of BP1 and BP2 are indicated above the lanes. The [35S]-labeled translation products were separated on 15% SDS-polyacrylamide gel. Chloramphenicol acetyltransferase (CAT) and globin proteins are indicated.

FIG. 9. Structural features of BP2. Shown is a schematic representation of the BP2 protein with structural features indicated. The black bar indicates the amino acid sequence encoded by the human 10.1 cDNA (18). The numbers used represent amino acid positions in the predicted sequence, with the first AUG codon in the 10.1 sequence as 1. The unique region is indicated by a hatched box, with the cleavage site marked as amino acid 194. The sequence obtained for the amino terminus of the rat 83,000-Da BP2 protein is indicated and is compared with the predicted human 16.1 sequence. The Q at the first position in the rat sequence indicates the first residue after the cleavage site. Since this is the first residue in the peptide, amino acid sequencing data were ambiguous. The black bar indicates the location of the homologous human BP1 peptide that cross-links to the IRE (35).

Acknowledgments—We thank Dr. Claire Kennedy for the mitochondrial aconitase antibody and purified mitochondrial aconitase and for the rabbit liver BP2 probe corresponding to the unique region; we isolated a BP2 cDNA from a rat liver library.2 Comparison of the predicted amino acid sequence of rat BP2 cDNA with that of human mitochondrial aconitase indicates that 16 of the 18 active-site residues, including the 3 cysteines that are ligands for the iron-sulfur cluster, are conserved in BP2. These data are in contrast to the published sequence of the human 10.1 cDNA, where only 1 of the 3 cysteines is conserved (18). We have also purified a human cDNA clone homologous to the 10.1 cDNA, and sequence analysis of this cDNA revealed that the 3 cysteines are conserved, suggesting that the 10.1 clone may contain a sequencing artifact.3 The active-site residues in mitochondrial aconitase that are changed in BP2 are Arg47 → Lys and Ser482 → Asn (mitochondrial aconitase positions). Mutational analysis of mitochondrial aconitase has shown that replacement of Arg47 with Lys results in a 10–230-fold decrease in aconitase activity depending on the substrate used in the assay and that replacement of Ser482 with any other amino acid results in a 6000-fold decrease in aconitase activity (36). These data suggest that although BP2 may contain an iron-sulfur cluster similar to those of BP1 and mitochondrial aconitase, the active-site residue substitutions found in BP2 may have deleterious effects on aconitase activity. The function of aconitase activity in BP1 is unknown, but one hypothesis is that it may be important in rapid turnover of the Fe-S cluster and in “sensing” iron levels (10, 37). The lack of aconitase activity in BP2 suggests that aconitase activity is not necessary for IRE-BP function.

Second, the levels of BP2 are undetectable in cells treated with iron for 4 h. The decrease in BP2 levels correlates well with the decrease in BP2 RNA binding activity in extracts of cells treated with iron. These data are in contrast to the post-translational regulation of BP1 in rat liver by iron, where the activity of BP1 decreases 50% while the levels of BP1 are not significantly decreased (17). Other studies in murine cells expressing BP1 have also shown that the activity of BP1 is regulated by iron without changes in the level of BP1 (38). One study, however, has demonstrated that the half-life of BP1 is decreased in extracts of iron- or hemin-treated cells (39). We do not know if hemin has a similar effect in decreasing BP2 levels. An interesting observation is that the levels of BP2 begin to increase after 8 h of iron treatment. The increase in BP2 levels after 8 h of iron treatment may be due to a decrease in intracellular iron caused by the sequestration of iron by newly synthesized ferritin. Finally, our preliminary studies indicate that BP2 mRNA levels remain constant during iron treatment, suggesting that either the synthesis or the degradation of BP2 may be affected by iron. Pathways by which iron may mediate the changes in the concentration of BP2 are at present under investigation. Expression and purification of larger quantities of BP2 should permit more detailed analysis of its iron binding characteristics. This in turn may offer an explanation of the cellular function of BP2 in the regulation of IRE-containing mRNAs.

2 B. Guo and E. A. Leibold, unpublished data.
3 F. Brown and E. A. Leibold, unpublished data.
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