Iron-responsive element-binding protein. Phosphorylation by protein kinase C.

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The iron-responsive element-binding protein (IRE-BP) is a cytosolic RNA-binding protein that functions in the maintenance of iron homeostasis by post-transcriptionally regulating transferrin receptor and ferritin synthesis. Little is known concerning how factors other than iron may modulate the activity of this central regulator of cellular iron utilization. We present evidence indicating that phosphorylation of the IRE-BP by protein kinase C (PKC) could provide a mechanism for regulation of IRE-BP function. Purified rat liver IRE-BP was phosphorylated by PKC up to 1.3 mol of phosphate/mol of protein with Ser the modified amino acid. Ser was also the phosphoacceptor in the IRE-BP in intact cells. The Km of PKC for the IRE-BP was 0.4 μM. Tryptic phosphopeptide mapping identified one major phosphopeptide plus several other peptides with lesser amounts of phosphate. Synthetic peptides of the IRE-BP containing Ser 138 (site A) and Ser 711 (site B) were phosphorylated by PKC.

In HL 60 cells, addition of phorbol 12-myristate 13-acetate (PMA) stimulated IRE-BP phosphorylation within 30 min and increased high affinity IRE RNA binding activity 2-fold. After 90 min, the level of phosphorylation had increased further, and high affinity IRE RNA binding activity had increased 3-fold above the control. Incorporation of [32P]Met into immunoprecipitable IRE-BP was not altered in cells treated with PMA for 30 or 90 min. PMA also stimulated IRE-BP phosphorylation in rat fibroblasts. Taken together, our studies begin to define a novel mechanism by which hormones, growth factors, and other agents may regulate cellular iron utilization through specific phosphorylation of the IRE-BP.

As an essential constituent of proteins involved in many cellular processes, iron performs an important role in the growth and viability of almost all organisms (1-4). In contrast, when present in excess, iron can be toxic due to its ability to induce oxidation of lipids and other cellular constituents (1-4). Vertebrates possess several proteins that serve to maintain cellular iron homeostasis. The transferrin receptor (TfR) and ferritin function in the transport and storage, respectively, of this essential nutrient (1-4). The TfR is a membrane-bound protein present in almost all cell types of vertebrates (5). On the cell surface, TfR binds diferric transferrin (Tf), the serum iron transport protein, and, together, the Tf-TfR complex is internalized resulting in cytosolic delivery of iron (6). Ferritin, a cytosolic macromolecule, containing varying proportions of 24 subunits of two types, H (heavy) and L (light), that can store up to 4,500 iron atoms (2). The synthesis rate of Tf and ferritin is coordinately but divergently regulated at the post-transcriptional level by cellular iron status.

Ferritin and Tf mRNAs contain common conserved cis-acting sequence(s) termed iron-responsive elements (IREs) that regulate their translation (ferritin) or stability (Tf) in an iron-dependent manner (1-4). Synthesis of these two proteins is coordinated by the iron-dependent binding of a cytosolic protein, the iron-responsive element-binding protein (IRE-BP), to the IREs in ferritin and Tf mRNAs (1-4). There appear to be at least two different IRE-BPs, IRE-BP 1 and IRE-BP 2, but little is known of any potential differences in their function or regulation (6). Ferritin mRNAs contain an IRE in their 5'-untranslated region (UTR), where binding of the IRE-BP is believed to interfere with the initiation of translation (7). In addition, the mRNAs encoding erythroid 5-aminolevulinate synthase and mitochondrial aconitate possess an IRE in their 5'-untranslated region (8-10). The synthesis of erythroid 5-aminolevulinate synthase appears to be translationally regulated by iron (11). In contrast to these mRNAs, Tf mRNA contains multiple IREs in its 3'-untranslated region, where they function to modulate its stability (12, 13). Because of its role in regulating synthesis of Tf, ferritin, and apparently erythroid 5-aminolevulinate synthase, it appears that the IRE-BP is a central regulator of iron homeostasis.

The evidence indicates that non-heme "chelatable" iron is capable of directly regulating ferritin and Tf synthesis and the RNA binding activity of the IRE-BP (14-17). Iron can

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1 The abbreviations used are: TfR, transferrin receptor; IRE, iron-responsive element; IRE-BP, iron-responsive element-binding protein; PMS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; MeSO, dimethyl sulfoxide; PKC, protein kinase C; 2-ME, 2-mercaptoethanol; RF2, rat 2 fibroblasts; m-, mitochondrial.

2 IRE-BP 1 refers to the IRE-binding protein first isolated from either human, rabbit, rat, or mouse by several groups (6, 18, 45, 46, 55, 56), and IRE-BP 2 refers to a second IRE-binding protein, approximately 70% identical to IRE-BP 1, isolated from a human T-cell cDNA library (45). Additional names for the IRE-BP are FRP for ferritin repressor protein (56), IRF for iron regulatory factor (46), or P-90 for 90,000 M, protein.

3 The term "chelatable iron" refers to intracellular non-heme iron that is accessible to chelating agents such as desferrioxamine.
modulate the affinity of interaction between the IRE-BP and the IRE without changing the steady state amount of IRE-BP protein (14, 17–19). However, under some conditions, it appears that heme may stimulate turnover of newly synthesized IRE-BP, suggesting that there are multiple mechanisms by which cellular iron status may affect the RNA binding activity (20, 21). In relation to its regulation by iron, the IRE-BP was found to share significant amino acid sequence identity with the Fe-S protein aconitase (22–24). Since the fourth iron of the cubane 4Fe-4S cluster of mitochondrial aconitase was shown to be labile, at least in vitro, it was initially postulated that conversion of a putative 4Fe-4S cluster in the IRE-BP to the 3Fe-4S form might activate the RNA binding function (22, 23).

Several observations support the general relevance of the “aconitase model” as a mechanism for iron regulation of IRE-BP RNA binding activity. First, based on biochemical and biophysical evidence, the IRE-BP and the purified and characterized cytosolic aconitase have been shown to be the same protein (25). Second, when expressed in heterologous cell systems, the human IRE-BP displays aconitase activity in an iron-dependent manner (17, 26, 27). Third, the aconitase and RNA binding activity of the IRE-BP were observed to be reciprocally regulated by cellular iron status (17, 27, 28). Thus, the presence of the Fe-S cluster reduces the affinity between the IRE-BP and its RNA ligand. These and other observations led to the hypothesis that conversion between the apo- and 4Fe-4S (holoprotein) forms, not between the 3Fe-4S and 4Fe-4S forms, was one mechanism that regulated RNA binding (1, 25, 28). However, while the presence of the 4Fe-4S cluster prevents high affinity RNA binding by the IRE-BP, it is not yet clear that formation of the intact cluster is the primary mechanism by which non-heme iron regulates the RNA binding function in intact cells.

Since the IRE-BP is a central regulator of iron homeostasis, it is a likely focal point for the action of growth factors, hormones, and other agents to program changes in cellular iron metabolism. Since activation of specific protein kinase(s) is a common signal transduction mechanism in a variety of physiological or pathological processes, we have begun to investigate the extent to which phosphorylation of the IRE-BP may provide a basis for programmed changes in iron metabolism. Since activation of specific protein kinase(s) is a common signal transduction mechanism in a variety of physiological or pathological processes, we have begun to investigate the extent to which phosphorylation of the IRE-BP may provide a basis for programmed changes in iron metabolism (29–35). Our results demonstrate that the IRE-BP function and of cellular iron metabolism.

**MATERIALS AND METHODS**

**Reagents—**Materials were obtained from the following sources: cell culture reagents, Life Technologies, Inc.; fetal bovine serum (FBS), Hyclone Laboratories; all radioactive compounds, DuPont NEN; purified as described except that the DEAE flow-through fraction of rat liver cytosol was used directly in the RNA affinity procedure. Activation of the IRE-BP with 2-mercaptoethanol (2-ME) was shortened to 5 min prior to use of the affinity procedure. The identity of the purified protein was confirmed by demonstration of its ability to reversibly repress ferritin mRNA translation in vitro and by the fact that the first 47 N-terminal amino acids were identical to that encoded by the cDNA for the rat liver IRE-BP (19) (results not shown).

In Vitro Phosphorylation Assays—PKC was purified from bovine brain (36), protease-activated kinase I and casein kinase II were from bovine brain microsomes, casein kinase I was from calf thymus (39), and multipotential SS kinase was from rabbit liver (40). IRE-BP (0.5–1.0 µg) was phosphorylated in 50 µl of reaction mixtures containing buffer P (20 mM Tris, pH 7.4, 10 mM MgCl₂, 0.4 mM EGTA, 10 mM 2-ME, 0.2 mM g-ATP (2,000 dpm/pmol)) and protein kinase. Reactions with PKC contained 0.9 mM CaCl₂, 10 µg/ml phosphatidylethanolamine, 10 µg/ml phosphatidylinositol, and 2 µg/ml diolein, as described (41). Reactions with casein kinases I and II contained 50 mM KCl. Reactions were incubated at 30 °C for 30 min and terminated on ice with the addition of ATP to a final concentration of 20 mM. Reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis (42). Proteins were detected by staining with Perkin-Elmer’s Merck Brilliant Blue R; phosphor incorporation was quantified first by autoradiography and then by excising the phosphorylated band and Cerenkov counting in a scintillation counter. Two-dimensional tryptic phosphopeptide mapping (41) and phosphoamino acid analysis (43) were carried out as described.

For synthetic peptides, kinetic assays were performed with increasing amounts of peptide (1.3–14.0 µg) in 25-µl reaction mixtures in buffer P plus protein kinase C. Reaction mixtures were incubated and terminated as described above. Incorporation of 32P into the peptide was determined either by thin layer electrophoresis (44) or by binding the peptide to phosphocellulose (Whatman P-81) filter paper (45).

Antibody Production and Peptide Synthesis—Peptides of site A (VDFNRRAIASLQKNQDFLEFERNRC; residues 130–151 of the human IRE-BP) (46, 47) and site B (NSYGS^KRRGNDAVMARC; residues 177–201) were synthesized with a carboxyl terminus, purified by C-18 reverse phase high performance liquid chromatography, and coupled to maleimide-activated ovalbumin (site A) or keyhole limpet hemocyanin (site B) through the Cys using a kit from Pierce Chemical Co. Relative antibody titer was assessed by enzyme-linked immunosorbent assay using peptide cross-linked to 96-well mic rotor plates or using semi-pure or pure rat liver IRE-BP.

Antibodies against the rat liver IRE-BP were produced in New Zealand rabbits by injection of antigen directly into the lymph nodes of the hind leg. The first injection used 100 µg of peptide, and boosts (every 6–8 weeks) used 50 µg. Antibodies were not detected until after the second boost. Antipeptide antibodies were obtained using the multiple intradermal injection procedure of 100–200 µg of peptide/protein conjugate (48). Freund’s complete adjuvant was used for all primary injections, and incomplete Freund’s was used in the boost.

IgG was purified from serum using protein A-Sepharose.

Cell Culture and Metabolic Labeling—HL 60 cells, obtained from ATCC, were grown at a concentration of 0.5–2.0 x 10⁶ cells/ml in RPMI 1640 plus 20% heat-inactivated FBS with penicillin-streptomycin. For 32P or 35S labeling, cells were cultured at a concentration of 1–2 x 10⁶ cells/ml. For [32P]orthophosphate labeling, cells were washed in RPMI 1640 lacking phosphate plus 5% dialyzed heat-inactivated FBS plus penicillin and streptomycin. Cells were incubated in the same medium containing 2.0–5.0 µCi/ml of [32P]orthophosphate for 2 h to prelabel the intracellular nucleotide pool. PMA (dissolved in Me₂SO) was then added at a final concentration of 0.02–1 µM. Me₂SO was added to the uninduced cells at the same concentration as the PMA-treated cells received (0.1%). After the appropriate period of time, labeled cells were pelleted, washed in ice-cold phosphate-buffered saline, and lysed in buffer A (20 mM Hepes pH 7.4, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM sodium orthovanadate, 5 mM EDTA, 1 mM GTP, 1 mM sodium orthovanadate, 2 mM benzamidine, 100 µg/ml of leupeptin and pepstatin, 250 µg/ml of soybean trypsin inhibitor, 0.2 mM phenylmeth-

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Use of animals at the University of Wisconsin was approved by the University of Wisconsin Research Animal Resource Center.
ysulfonyl fluoride, 25 µg/ml of p-nitroquinolinobenzoate, and 0.5% Nonidet P-40). After centrifugation for 15 min at 15,000 rpm, the supernatants were used immediately or were stored at −70 °C. For [%][SS]Met labeling, the media was Dulbecco's modified Eagle's medium minus Met plus 5% dialyzed heat-inactivated FBS, and [%][32P]Met was added at 100–200 µCi/ml. Cells were incubated with labeled Met for 30 min prior to the time point of interest in the presence or absence of PMMA.

Rat 2 fibroblasts (RF2) were grown as described (15). For labeling with [%][SS]Met or [%][32P]orthophosphate, cells were incubated in the appropriate Met or phosphate-free media plus 4–5% dialyzed heat-inactivated FBS in T75 flasks at a concentration of 1–2 × 10⁶ cells/flask. For phosphate labeling, cells were pre-labeled for 2 h before addition of Met. For Met labeling, cells were incubated 1 h with labeled Met in the presence or absence of PMMA. Labeled Met or phosphate were added at the same concentrations used for the HL 60 cells. After labeling, RF2 cells were harvested with 0.5% trypsin in Hank's' balanced salt solution lacking calcium and magnesium. After dilution into phosphate-buffered saline, the cells were pelleted, resuspended once, and repelleted before lysis in buffer A.

**Immunoprecipitation**—All immunoprecipitations used antibodies raised against the purified rat liver IRE-BP. For HL 60 cells, the extract was brought to 5% non-fat dry milk and 25 µg/ml bovine serum albumin, each dissolved in buffer B, (80 mM (g)-glycerophosphate, 50 mM sodium fluoride, 1 mM ATP, 5 mM sodium pyrophosphate, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and incubated at 4 °C with mixing for 1 h. Protein A-Sepharose (700 µg) was added and the incubation continued for 1 h. Sepharose beads were removed by centrifugation, and the precleared lysate was incubated with 30–60 µg of either preimmune or immune IgG. After overnight incubation at 4 °C, the antigen-antibody complexes were collected with protein A-Sepharose and washed extensively with buffer B and finally twice with buffer C (buffer lacking detergents).

Fibroblast lysates were brought to 1.0% SDS and boiled for 5 min. The denatured extract was centrifuged at 100,000 × g for 60 min to remove insoluble material. After boiling, the extract was diluted to a final SDS concentration of 0.1%. Immunoprecipitations were then performed as described above. This procedure greatly lowered the background in the immunoprecipitations of RF2 cells.

Washed immunoprecipitates were heated to 65 °C in reducing sample buffer (49) for 10 min before loading onto a 20-cm 7.5–15% linear gradient SDS-polyacrylamide gel that was run at 8 mA overnight. Gels were fixed in 10% trichloroacetic acid plus 40% ethanol and stained with one part Coomassie Blue R-250 in 50% methanol and 10% acetic acid and plus three parts 20% ethanol and 10% acetic acid. The denatured 73-nucleotide 32P-labeled RNA, denoted R4 RNA, was produced using T7 RNA polymerase and purified through a 10% acrylamide 8 M urea gel. The specific radioactivity of the RNA was 6,000–7,000 dpm/fmol.

**Gel Shift Analysis**—IRE RNA binding activity was quantitated as described previously with modifications (51). The vector p16Bgl diluted into binding buffer (5% glycerol, 1 mM magnesium acetate, 0.1 mM dithiothreitol, 0.5% bovine serum albumin, each dissolved in buffer B, (80 mM (g)-glycerophosphate, 5 mM magnesium chloride, 0.5 µM ATP, 5 mM sodium pyrophosphate, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and incubated at 4 °C with mixing for 1 h. Protein A-Sepharose (700 µg) was added and the incubation continued for 1 h. Sepharose beads were removed by centrifugation, and the precleared lysate was incubated with 30–60 µg of either preimmune or immune IgG. After overnight incubation at 4 °C, the antigen-antibody complexes were collected with protein A-Sepharose and washed extensively with buffer B and finally twice with buffer C (buffer lacking detergents).

**Results**

**The IRE-BP as a Substrate for Different Protein Kinases**—To investigate the potential role of phosphorylation in the regulation of IRE-BP RNA binding activity, we examined the ability of several protein kinases to phosphorylate this regulatory RNA-binding protein. Of the kinases tested, PKC and protease-activated kinase I were able to phosphorylate the IRE-BP to the greatest extent (Fig. 1, A and B). The molar incorporation of phosphate into the IRE-BP catalyzed by PKC ranged from 0.5 to 1.3 mol of phosphate/mol of IRE-BP. The Km of PKC for the IRE-BP was 0.4 µM. The action of PKC was stimulated by agents that mimic physiological activation of the kinase (Fig. 1A, compare lanes 5 and 6). Casein kinase I and multipotent S6 kinase phosphorylated the IRE-BP to a limited extent (Fig. 1A, lanes 2 and 7), but the IRE-BP at a concentration of 10 nM, whereas mutants of the IRE (5′M and 3′M (52)) only partially competed at a concentration of 1 µM. Binding data was curve-fit by computer (GraphPAD Software, San Diego, CA). The coefficient of variation for RNA binding determinations for a given sample was less than 10%.

**FIG. 1.** The IRE-BP as a substrate for different protein kinases. A, purified rat liver IRE-BP (0.5 µg) was incubated with various protein kinases for 30 min in the presence of [%][32P]ATP and appropriate buffers and/or activators (see "Materials and Methods"). Lane 1, no kinase added; lane 2, casein kinase I (60 units); lane 3, casein kinase II (100 units); lane 4, casein kinase II plus polylysine (0.1 µM) to stimulate the kinase; lane 5, bovine brain protein kinase C (20 units); lane 6, PKC plus diolien (0.8 µg/ml); lane 7, PKC plus diolphin (0.5 µg/ml); lane 8, 0.9 µM, phosphatidyl-serine (10 µg/ml), and phosphatidylinositol (10 µg/ml); lane 7, multipotent S6 kinase (1.0 unit); lane 8, protease-activated kinase I (4.0 units). B, purified PKC was incubated without IRE-BP (lane 1), with purified rat liver IRE-BP (lane 2), or the IRE-BP catalyzed by [%][32P]ATP (lane 3) with the activators and time as indicated in A, lane 6. These results are representative of those obtained with multiple experiments and with multiple preparations of IRE-BP.
the IRE-BP was not phosphorylated by casein kinase II (Fig. 1A, lanes 3 and 4).

Phosphopeptide and Phosphoamino Acid Analysis of the Phosphorylated IRE-BP—After phosphorylation by PKC, the IRE-BP was digested to completion with trypsin and the phosphopeptides separated by electrophoresis and chromatography. IRE-BP phosphorylated by PKC displayed one major phosphopeptide and several species with lesser amounts of phosphate (Fig. 2). PKC-phosphorylated IRE-BP was hydrolyzed and the phosphoamino acid composition of the protein examined. For the IRE-BP treated with PKC, only phosphoserine was detected (Fig. 3). Phosphoserine was also obtained after phosphoamino acid analysis of the IRE-BP immunoprecipitated from PMA-treated fibroblasts (results not shown).

Phosphorylation of Peptides of the Human IRE-BP by PKC—We examined the deduced amino acid sequence of the human IRE-BP (6, 46, 47) and observed five Ser that we speculated were putative PKC phosphorylation sites. These included Ser 38, 138, 470, 690, and 711 (see Table I for sequences). These sites fit consensus sequences for PKC phosphorylation sites of either (S/T)XR, (K/R)XX(S/T), or (Y/F)X(S/T) (53-55). Peptide fragments of each of these regions were synthesized and tested as substrates for PKC. PKC-phosphorylated peptides containing Ser 138 (site A peptide, residues 130-151) and especially Ser 711 (site B peptide, residues 707-721) (Table I). The K<sub>a</sub> for site A peptide was between 1.2 and 1.8 mM, whereas for site B peptide, it was 0.6 mM (Table I). A peptide containing an earlier version of the IRE-BP sequence around Ser 711 (46) was also phos-

![Image of Tryptic phosphopeptide maps of IRE-BP phosphorylated by PKC.](image)

**Fig. 2.** Tryptic phosphopeptide maps of IRE-BP phosphorylated by PKC. Purified rat liver IRE-BP was phosphorylated by PKC as indicated under "Materials and Methods." After fractionation by SDS-polyacrylamide gel electrophoresis, the IRE-BP was excised and digested with trypsin overnight, and the peptide digests were separated by chromatography and electrophoresis. The origin is indicated by the dot. These results are representative of those obtained in multiple experiments.

![Image of Phosphoamino acid analysis of IRE-BP phosphorylated by PKC.](image)

**Fig. 3.** Phosphoamino acid analysis of IRE-BP phosphorylated by PKC. The IRE-BP was phosphorylated by PKC, gel-purified, and hydrolyzed, and the phosphoamino acids were separated by thin layer electrophoresis as described. *Lane A* depicts an autoradiogram of the phosphoamino acids, and *lane B* is a representation of the data shown in *lane A* but showing the outline of the spots obtained after ninhydrin staining. The origin is indicated by the dot. These results are representative of those obtained in multiple experiments.

| Peptide* | Relative rate b | K<sub>a</sub> (mM) |
|----------|----------------|------------------|
| GRLPFS<sup>38</sup>IRYVLEAAC | 0 | ND |
| VDFNRRADS<sup>138</sup>LQKNNQDLEFERNR | 0.2 | 1.2-1.8 |
| PYIKTS<sup>796</sup>LSPG | 0 | ND |
| AGNIARNS<sup>800</sup>PAARYLC | 0 | ND |
| NSYGS<sup>711</sup>RRPN<sup>713</sup> | 2.0 | 3.0-3.2 |
| NSYGS<sup>711</sup>RRGNDAVMRC<sup>721</sup> | 1.0 | 0.5-0.7 |

* The sequences described are from the human IRE-BP (6, 46, 47).

b Rates were obtained as described under "Experimental Procedures" using 15 nmoL of peptide in 0.025-mL reaction mixtures.

However, when comparing the IRE-BP among these species, the degree of identity of amino acid sequence for the entire protein is high (92-94%), indicating that the regions around sites A and B are essentially as well conserved as is the entire protein sequence. A second IRE-BP has been cloned from human cells (6, 46). The region from residues 774 to 796 in IRE-BP 2 shares nearly complete identity with the 699-721 region in IRE-BP 1 with the notable exception of the Ser to Ala substitution at residue 786 of IRE-BP 2 (Fig. 4B).
IRE-BP Phosphorylation by PKC

The human IRE-BP has significant identity to mitochondrial aconitase (about 30% identity) and is even more identical to the aconitase of Escherichia coli (53% identity) (22-24). Recent evidence indicates that the IRE-BP is the heretofore partially characterized “cytosolic aconitase,” and formation of an Fe-S cluster in the IRE-BP affects its RNA binding function (1, 25-28). The sequences surrounding and including Ser 138 and 711 of the IRE-BP are not well conserved in mitochondrial aconitases for which sequence information is available (Fig. 4, A and B). However, the region from residue 699 to 721 of the human IRE-BP is 65% identical and 78% similar with the 696-718 region of E. coli aconitase (Fig. 4B). It is of interest that based on computer predictions both Ser 711 and 138 map to the surface of the IRE-BP, suggesting that they would be able to serve as substrates for a kinase. Furthermore, antipeptide antibodies against sites 1 and 2 recognize semi-purified and purified rat liver IRE-BP by enzyme-linked immunosorbent assay (results not shown), again suggesting that these regions are surface-exposed in the native protein.

Phorbol Esters Stimulate IRE-BP Phosphorylation and RNA Binding Activity in Intact Cells—To evaluate the physiological relevance of our observations, we examined the phosphorylation state of the IRE-BP in human HL 60 or RF2 cells treated with and without PMA. HL 60 cells are a human promyelocytic cell line, which can be induced to differentiate into monocytes/macrophages by PMA. Short-term treatment with PMA has been shown to stimulate TTR mRNA accumulation in HL 60 cells (58). Phosphorylation of the IRE-BP was stimulated within 30 min after addition of 0.2 µM PMA, as shown by immunoprecipitation with antibodies raised against the purified rat liver IRE-BP (Fig. 5, lane 2). The level of phosphorylation of the binding protein was further enhanced after 90 min (Fig. 5, lane 3). After 24 h in the presence of PMA, most of the cells had attached to the dish and exhibited macrophage-like characteristics. There was little difference in the phosphorylation state of the IRE-BP in HL 60 cells treated with PMA for 90 min or 24 h (results not shown).

We wished to determine if PMA could rapidly affect the synthesis rate of the IRE-BP in HL 60 cells. HL 60 cells were incubated with 0.2 µM PMA or Me2SO (0.1%) for 30 or 90 min, were then pulsed with [35S]Met for the final 30 min of their incubation, and the relative rate of incorporation of Met into the IRE-BP was determined by immunoprecipitation. We observed no significant effect of PMA on the incorporation of [35S]Met into the immunoprecipitable IRE-BP (results not shown).

Treatment of HL 60 cells with 0.2 µM PMA rapidly increased the amount of IRE-BP that bound RNA with high affinity in the absence of 2-mercaptoethanol (Fig. 6, A and B). PMA stimulated IRE-BP RNA binding activity at concentrations as low as 20 nM (results not shown). After 90 min, PMA stimulated IRE RNA binding activity by 2-fold, and, after 90 min, the RNA binding activity had increased by 3-fold over control (Fig. 6, A and B). Computer curve fitting of the binding data obtained in the presence of increasing amounts of IRE-containing RNA revealed a 2-fold increase in B0 for the high affinity site in cells treated with PMA for 30 min with no significant change in K0. After a 24-h treatment with PMA, at which time most of the cells were differentiated, there was a slight increase in the level of RNA binding over that observed after 90 min (Fig. 6, A and B).

Treatment of cell extracts with high levels of 2-ME converts the IRE-BP from a low to a high affinity state for RNA binding (18). Comparing the RNA binding activity in the presence and absence of 2-ME indicates that in the absence of PMA, about 25% of the IRE-BP was in a high affinity state, whereas 90 min after addition of PMA, approximately 70% of the protein could bind RNA with high affinity (Fig. 6B). These observations suggest the possibility that phosphorylation of the binding protein converts it from a low to a high affinity state with respect to RNA binding. Taken together, our results demonstrate that the IRE-BP is a phosphoprotein in intact cells and that in HL 60 cells, phosphorylation of the binding protein is associated with an increase in IRE RNA binding activity.

We observed stimulation of IRE-BP phosphorylation by PMA in another cell type. In rat fibroblasts labeled with [32P]orthophosphate, the IRE-BP was found to be phosphorylated at a low level (Fig. 7, lane 2). Addition of 1 µM PMA to rat fibroblasts stimulated IRE-BP phosphorylation by 4-fold within 30 min (Fig. 7, lane 4), and this level of protein phosphorylation was unchanged after 1 (Fig. 7, lane 8) and 2 h but decreased to 2-fold after 4 h (results not shown).

**DISCUSSION**

Phosphoregulation of IRE-BP function by PKC provides a potential basis for iron-independent modulation of the uptake and metabolic fate of this essential nutrient. PKC consists of a family of calcium and/or phospholipid-dependent protein kinases with at least 11 different isoforms (59). These iso-
were performed with a single concentration of RNA extracts in the absence of 2-ME was 0.1 pmol/mg of protein (1,521 dpm). The level of RNA binding activity in control cells did not change proliferation or differentiation (59). Some of these processes pathways of a variety of growth factors, hormones, and other agents and appears to participate in a variety of physiological functions including, in certain cell types, influencing cell proliferation or differentiation (59). Some of these processes in which PKC may function, such as lymphocyte proliferation or macrophage maturation, are associated with alterations in the uptake or metabolic fate of iron (29-31). Regulation of IRE-BP function by PKC or other kinases could provide a mechanism for the manipulation of cellular iron homeostasis by a diverse set of agents and under circumstances in which changes in intracellular iron levels do not precede, i.e. are not the signal-inducing, alterations in ferritin or TIR synthesis.

We have demonstrated that the IRE-BP is an efficient substrate for bovine brain PKC in vitro and that activation of PKC by PMA stimulates phosphorylation of the binding protein in different cell types. Phosphopeptide mapping coupled with the use of synthetic peptides of the human IRE-BP suggests that there are two PKC phosphorylation sites in the binding protein, Ser 138 (site A) and Ser 711 (site B). A peptide containing site B was the preferred substrate for the kinase, but it remains to be determined whether this Ser is a preferred site in the intact protein. Comparing the amino acid sequence of IRE-BPs of species from which cDNAs have been isolated reveals that both site A and site B are 91-100% identical. However, this level of identity is not greatly different from the 92-94% observed for the proteins as a whole. Site B, but not site A, is well conserved (65% identity, 78% similarity) when compared with the aconitase of E. coli, but neither is found in mitochondrial aconitases. When comparing IRE-BP 2 with IRE-BP 1, the amino acid sequence of phosphorylation site A is not present. In contrast, the region including site B is well conserved, with the notable exception that the Ser 711 of IRE-BP 1 is an Ala in IRE-BP 2. Thus, we speculate that PKC may differentially regulate the function of the two IRE-BPs. It remains to be determined whether other functional PKC sites exist in IRE-BP 2. For IRE-BP 1, it will be of interest to define the functional effects of phosphorylation at one or both of these sites as well as the extent to which the phosphorylation of these sites may be differentially regulated under specific physiological conditions.

Based on computer predictions using the crystal structure of mitochondrial aconitase (m-aconitase) as a basis, it appears that the IRE-BP and m-aconitase may have similar tertiary structures (22). m-Aconitase contains four domains (60). Domains 1-3 form a region that binds the Fe-S cluster and is separated from domain 4 by a "hinged linker" region (60).
The Fe/S cluster is solvent-exposed and is in a cleft formed between domains 1–3 and domain 4 (60). It has been hypothesized that movement of domain 4 relative to domains 1–3 alters the extent to which the putative cleft of the IRE-BP is accessible for RNA binding or in a conformation that permits cluster assembly (1). This hypothesis suggests that the amino acid residues important for Fe-S cluster formation are in the same region as those used for RNA binding. Based on the apparent similarity in tertiary structure between m-aconitase and the IRE-BP, it appears that Ser 138 is in domain 1 and Ser 711 is in domain 4. Ser 711, apparently the best substrate for PKC, is predicted to be located in the region of domain 4 near the entrance to the cleft. In close proximity to Ser 711 and 138 are amino acids presumably important for substrate recognition (Arg 899 in IRE-BP and Arg 580 in m-aconitase) or catalysis (Asp 125 and His 126 in IRE-BP and Asp 100 and His 101 of m-aconitase) (60, 60). It will be of interest to determine whether phosphorylation at Ser 711 or 138 alters these properties of the holo-IRE-BP.

Since the IRE-BP is a bifunctional protein with both RNA binding and aconitase activities, it will be important to determine how phosphorylation affects these activities. Phosphorylation may be a determinant of whether the IRE-BP functions as an RNA-binding protein or an aconitase, or it may affect other aspects of IRE-BP function, such as its sensitivity to iron regulation. The function of many RNA- and DNA-binding proteins, as well as metabolic enzymes, including iron-sulfur proteins, is affected by phosphorylation (61-63). The effects of phosphorylation include alterations in protein conformation, protein-protein interaction, subcellular localization, and substrate recognition and catalysis (64-66).

Perhaps IRE-BP phosphorylation could influence the formation or destruction of the Fe-S cluster through an effect on IRE-BP conformation or by altering the interaction of the binding protein with other proteins that influence cluster formation or destruction. For m-aconitase, the presence of substrate stabilizes the Fe-S center by binding directly to the cluster and forming a bridge between domains 1–3 and domain 4. (60, 67). Phosphorylation of the holo-IRE-BP at Ser 711 might destabilize the cluster by blocking the ability of substrate to interact with the binding protein. Some precedence for this is seen in the case of the isocitrate dehydrogenase of E. coli where phosphorylation at the active site blocks substrate binding (64). Alternatively, phosphorylation may divert the IRE-BP more toward its RNA-binding function by inhibiting the assembly of cluster in the apoprotein. Studies are currently underway to determine if both apo- and holo-IRE-BP are substrates for PKC and for other protein kinases and whether phosphorylation of the binding protein influences cluster assembly, stability, or function in vitro.

Phosphorylation by PKC may modify the sensitivity of the IRE-BP to iron regulation and thereby alter the uptake or metabolic fate of iron in a cell type-specific manner. This may reflect a specialized requirement for iron in supporting cellular proliferation or in the differentiated function of certain cell types. First, it is well documented that proliferating cells have significantly higher levels of TFs than quiescent cells (29, 30, 34, 35). In proliferating cells, phosphorylation may increase the RNA binding activity of the IRE-BP, resulting in stabilization of TFR mRNA and increases in iron uptake. Activation of IRE RNA binding activity should also repress ferritin synthesis, perhaps permitting the diversion of a greater proportion of iron away from storage and into pathways directed at synthesis of iron-containing proteins needed for cellular proliferation. An illustration of such alterations in TFR and ferritin synthesis occurs during T-cell proliferative activation (29, 30, 34). Furthermore, there is a significant increase in IRE RNA binding activity after T-cell activation, and it has been suggested that this may involve post-translational modification of the binding protein (30). Second, during avian or rodent erythroid development, there is a large demand for iron in order to support heme formation, and it appears that the synthesis of both ferritin and TFR is less sensitive to intracellular iron levels in erythroid cells than in other cell types (68, 69). Alterations in the phosphorylation state of the IRE-BP during erythroid development might alter the sensitivity of the binding protein to iron regulation and promote diversion of iron from deposition in ferritin into the formation of heme. Third, during monocyte/macrophage differentiation, synthesis of both ferritin and TFR is increased (70-72). This atypical response appears to be mediated in part by increases in the amount of high affinity IRE-BP coupled to a significant increase in ferritin mRNA levels (70, 72). It appears that activation of RNA binding activity of the IRE-BP promotes TFR mRNA accumulation, whereas the concurrent large increases in ferritin mRNA levels may partly overcome the expected repression of ferritin synthesis by increasing the level of ferritin mRNA in both the RNP and polysomal pools. In this manner, it appears possible to stimulate uptake of iron while simultaneously increasing iron storage in ferritin. It will be of interest to determine the extent to which phosphoregulation of IRE-BP function contributes to the specific modulation of the uptake and metabolic fate of iron in these and other cell types.

It is clear that multiple factors contribute to the maintenance of iron homeostasis and that iron uptake and utilization is modulated in a cell type-specific manner. Given the role of the IRE-BP as a focal point for modulating the uptake and metabolic fate of iron, it appears that phosphoregulation of IRE-BP function by PKC or other kinases can provide a novel mechanism for regulating iron metabolism in response to changes in cellular proliferation or differentiation. Phosphorylation may provide a mechanism by which the action of extracellular agents can override intracellular signals (e.g. iron) in regulating IRE-BP function, as has been suggested in the case of other phosphoproteins (73). Alternatively, it has been suggested that iron may indirectly influence the RNA binding function of the IRE-BP (4, 20); such an indirect mechanism may include regulation by phosphorylation. However, the extent to which phosphorylation- and iron-dependent changes in IRE-BP function represent components of separate or overlapping signal transduction pathways that may modulate IRE RNA binding activity remains to be elucidated.

Future investigations will focus on whether phosphorylation affects IRE-BP function by acting on the apoprotein specifically or whether it also affects the stability of the Fe-S cluster and/or aconitase activity of the holoprotein. Coupled with the potential ability of PKC activation to influence iron metabolism through phosphorylation of the IRE-BP are the actions of agents that reverse the effect of PKC on the binding protein. This could be accomplished through the action of specific phosphatases, perhaps including the tartrate-resistant iron-containing phosphatases; however, they appear to be lysosomally localised (74). Alternatively, phosphorylation of the IRE-BP may alter the protein's rate of degradation, thereby influencing the temporal nature of the effects of phosphorylation on RNA binding. By addressing these issues, it should be possible to further understand how alterations of IRE-BP function influence the uptake and utilization of iron in higher eucaryotes as well as elucidating one mechanism by
which the assembly or destruction of iron-sulfur clusters may be modulated in vivo.

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