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The role of endogenous heme synthesis and degradation domain cysteines in cellular iron-dependent degradation of IRP2

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Abstract

Iron regulatory protein 2 (IRP2) is a mammalian cytosolic iron-sensing protein that regulates expression of iron metabolism proteins, including ferritin and transferrin receptor 1. IRP2 is ubiquitinated and degraded by the proteasome in iron-replete cells but is relatively stable in iron-depleted cells. Recent work has shown that IRP2 contains a unique 73-amino-acid domain that binds iron *in vitro* and undergoes iron-dependent oxidation and cleavage (*J. Biol. Chem.* 278 (2003), 14857). Several cysteines in the 73-amino-acid domain function as an *in vitro* iron-binding site. To assess the role of these cysteines in cellular iron-dependent degradation of IRP2, we mutagenized these cysteines in various combinations in the context of full-length protein and generated cell lines in which recombinant IRP2 expression was inducible. Iron-dependent degradation of IRP2 mutagenized at any or all of the cysteines of the putative degradation domain in cells was comparable to wild-type (WT). Both WT and cysteine mutant protein were stabilized in 3% oxygen. Treatment with sodium nitroprusside (SNP), an NO⁺ donor, caused a decrease in cellular IRP2 concentrations, but the SNP effect was abrogated by simultaneous addition of the iron chelator desferal and was not affected by cysteine mutations. Inhibition of endogenous heme synthesis with succinylacetone significantly inhibited iron-dependent degradation of IRP2. Addition of cobalt chloride inhibited degradation of both WT and mutagenized IRP2. Thus, we could not discern a role for the recently defined *in vitro* cysteine-dependent iron-binding site of IRP2 in cellular physiology. The early molecular events in iron-dependent degradation of IRP2 remain to be elucidated.

Introduction

Iron is essential for the function of numerous proteins in cells, including those involved in oxygen transport and electron transfer. Mammalian cells regulate expression of proteins involved in iron uptake and sequestration to ensure that iron supplies are sufficient to meet metabolic needs

while minimizing iron-mediated toxic damage. A major sensing system for mammalian cells consists of two iron-sensing proteins, IRP1⁴ and IRP2, which bind to RNA stem-loops known as iron-responsive elements (IREs) in transcripts that encode several iron metabolism proteins [reviewed in 1,2]. The two proteins are 58% identical to one another and are members of the aconitase gene family [1]. Each IRP senses levels of cytosolic iron and binds to IREs when cells are depleted of iron. Binding of IRPs to IREs in the 5'-UTR of ferritin transcripts and in the 3'-UTR of transferrin receptor (TfR) leads to repression of ferritin

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⁴ Abbreviations used: desferal, desferrioxamine mesylate; IRE, iron-responsive element; IRP, iron regulatory protein; NAP, *N*-acetyl D,L penicillamine disulfide; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; SNP, sodium nitroprusside; TfR, transferrin receptor; WT, wild type.

synthesis and stabilization of TfR mRNA, respectively. Both proteins are expressed in virtually all tissues and their functions overlap. However, genetic ablation studies of these two proteins in mice reveal that IRP2 plays a more important role than IRP1 in animal iron homeostasis [2]. It was a surprise that IRP2 proved to be more important in iron homeostasis than IRP1 in animals, since IRP2 has generally been more difficult to detect and was thought to be significantly less abundant than IRP1.

IRP1 and IRP2 differ significantly in their mechanism of iron sensing. IRP1 is a bifunctional protein: it is a functional cytosolic aconitase in iron-replete cells when it contains an iron–sulfur cluster at the enzymatic site, whereas in iron-depleted cells, the iron–sulfur cluster is absent, and the apoprotein is the form that binds IREs [reviewed in 1,2]. In iron-replete cells, IRP2 is ubiquitinated and degraded by proteasomes [1,3–5]. The molecular mechanisms by which IRP2 is targeted for ubiquitination are not yet known. We have previously shown that an early event in iron-dependent degradation of IRP2 is an iron-dependent oxidation [6]. We identified a 73-amino-acid portion of IRP2, encoded by a single exon that was unique to IRP2 and that appeared to play an important role in iron-dependent degradation of IRP2. There are five cysteines within this region, which we referred to as the “iron-dependent degradation domain,” and we hypothesized that these cysteines could ligate free iron, which would then mediate oxidation of IRP2 and create a recognition signal for ubiquitination [3,5]. These ideas were supported by our observation that purified IRP2 is subject to iron-dependent oxidations *in vitro* [5,7]. We recently extensively analyzed a 63-amino-acid peptide corresponding to residues 138–200 of IRP2 and found that addition of iron and a reducing agent led to cysteine oxidation. When these same cysteines were mutagenized to alanine, metal-catalyzed oxidation of the peptide was no longer observed [8].

To evaluate the role of these cysteines in normal cellular degradation of IRP2, we mutagenized all five cysteines of the degradation domain to alanines and assessed iron-dependent degradation of recombinant full-length protein expressed from an inducible promoter. We discovered that recombinant IRP2 that lacks these cysteines is iron-dependently degraded as efficiently as wild-type protein. We also evaluated the effects of oxygen tension, SNP, SNAP, cobalt, exogenous heme, and the heme synthesis inhibitor succinylacetone on levels of WT and mutagenized IRP2. We observed that cysteine mutagenesis did not interfere with the effects of SNP, cobalt, or heme. Our results suggest that SNP and exogenous heme contribute to IRP2 degradation by supplying iron to cells and that cobalt interferes with iron-dependent degradation of IRP2 by an unknown mechanism that does not involve competing for the cysteine-dependent iron binding site in the unique 73-amino-acid exon of IRP2. Our experiments imply that endogenous heme synthesis may play an important role in regulation of cellular IRP2 levels and therefore in regulation of mammalian iron homeostasis.

Materials and methods

IRP2 wild-type and mutants construction and expression

The 73-amino-acid putative degradation domain of IRP2 contains five cysteines: Cys137, Cys168, Cys174, Cys178, and Cys201. Site-directed mutagenesis of these cysteines into serine or alanine were generated from myc tagged-IRP2 by two-step PCR. IRP2 constructs were cut out of the pXS-IRP2 [3] vector with *Xho*I and *Eco* RV; put into *Sall* and *Eco* RV sites of pTET-on vector (Clontech). To generate stable cell lines with cysteines 168, 174, and 178 mutagenized to serines, we transfected with the LCS plasmid [3]. HEK 293 Tet-on cell lines (Clontech) were transfected using the Fugene 6 (Pharmacia-Roche) reagent according to the manufacturer's instructions: in a 2063 Falcon tube, 3 μ l of Fugene reagent was added to 100 μ l DMEM medium with alpha modification and incubated 5 min at room temperature. One microgram of pTET-on vector and 0.05 μ g hygromycin resistance vector (1:20 ratio) were added and, after a 15-min incubation, the solution was transferred into a six-well plate containing HEK 293 Tet-on cells at 50% confluency in 2 ml DMEM medium with alpha modification supplemented with glutamine and antibiotics. After 8 h incubation (37°C, 5% CO₂), fetal calf serum was added to a final concentration of 10%. Twenty-four hours later, the cells were split into a 10-cm-diam culture plate and allowed to grow in DMEM containing 10% FCS, 0.1 mg/ml geneticin, glutamine, and antibiotics. When the cells reached 20 to 30% confluency, hygromycin was added to the medium at a final concentration of 0.05 mg/ml. This media was changed every week and the first colonies appeared after 4 to 5 weeks. Stable cell lines with cysteines 137, 168, 174, 178, and 201, named C1, C2, C3, C4, and C5, respectively, mutagenized to alanines were also generated using the same strategy for making stable cell lines transfected with the LCS plasmid. After colony selection using 5-mm-diam cylinders, the cells were tested for specific doxycycline-induced IRP2 expression by Western blotting using antibody directed against the C-terminal myc epitope. DNA from each positive colony was extracted in PCR lysis solution containing 67 mM Tris–HCl (pH 8.8), 16.6 mM ammonium sulfate, 5 mM β -mercaptoethanol, 6.7 μ M EDTA (pH 8.0), 1.7 μ M SDS, and 500 μ g/ml proteinase K. The sequence that encodes the degradation domain of the recombinant colonies was checked using specific PCR primers derived from two separate exons that flank the degradation domain part of the transcript. The product was sequenced to verify the presence of mutations in each stable cell line with the Thermo Sequenase dye terminator (Amersham).

Cell incubations and treatments

Cells were cultured in 10-cm-diam tissue culture plates in DMEM with alpha modification (Sigma) containing 10%

FCS (Clontech), 0.05 mg/ml hygromycin (Calbiochem), 0.1 mg/ml geneticin (Gibco BRL), glutamine (Gibco BRL), and antibiotics (Gibco BRL). Forty hours prior to harvest of cells, doxycycline (Sigma) to a final concentration of 2 $\mu\text{g/ml}$ was added to the cells to induce expression of recombinant IRP2. Twenty-four hours later, the following treatments were performed: ferric ammonium citrate (75–450 μM Fisher Scientific); desferal (deferoxamine mesylate, 0.1 mM unless otherwise mentioned, Sigma); SNAP (*S*-nitroso-*N*-acetyl-D,L-penicillamine, 50–150 μM , Alexis); SNP (sodium nitroprusside, 100 μM , Alexis); NAP (*N*-acetyl D,L penicillamine disulfide, 150 μM , Alexis); succinylacetone (5 or 10 mM, Sigma), Hemin (40–60 μM , Sigma), and cobalt(II) chloride (100 or 500 μM , Sigma) was added to the cells. To compare directly with the concentrations of the other reagents used, we described iron concentration in μM instead of $\mu\text{g/ml}$ ferric ammonium citrate. The conversion factor (1 $\mu\text{g/ml}$ is equivalent to 3 μM) used in our calculation is based on 16% iron content of this green form of ferric ammonium citrate. After 16 h incubation, cells were washed with cold PBS containing 0.1 mM desferal and lysed with IRP2 lysis buffer containing 25 mM Tris (Biofluid) (pH 8.3), 10 mM KCl (Sigma), 1 mM DTT (Sigma), 0.1 mM desferal, 25 μM NPGb (Sigma), 25 μM AEBSF, (ICN) 10 $\mu\text{g/ml}$ leupeptin (Boehringer Mannheim), and 1% Triton (Boehringer Mannheim). Cells debris was removed by a 14,000 rpm spin (10 min) and protein was quantified by the Bradford technique.

Protein expression and immunoprecipitation

Total protein (200 μg) was incubated in the presence of 30 μl anti-myc monoclonal antibody agarose beads (Clontech) for 1 h at 4°C in 1 ml PBS containing 0.1 mM Desferal. After three washes with PBS containing 0.05% Tween 20 and 0.1 mM desferal, bead-associated proteins were denatured at 95°C during 5 min in sample buffer and loaded on a 8% acrylamide gel. Western blots were performed with a rabbit polyclonal antibody to IRP2 [2] followed by incubation with a biotin-bound anti-rabbit antibody (Amersham Pharmacia). Blots were then incubated with Streptavidin- I^{125} , exposed to film, and quantified with Image Quant software. To detect ferritin, Western blots were performed using a goat polyclonal antibody (Boehringer).

RNA gel mobility shift

Gel mobility shift studies were performed as previously described [4] using 10 μg lysate diluted in 12.5 μl of Band Shift Buffer (BSB) consisting of 40 mM KCl and 25 mM Tris-HCl (pH 7.5). An equal volume of reaction mix containing 800,000 cpm/ml ^{32}P -labeled IRE, 171 units/ml super RNAsin, 0.6 mg/ml tRNA, 10% glycerol, and 0.2 mM DTT in BSB was added. After a 5-min reaction at 4°C, an 8% acrylamide gel was loaded with 20 μl of the reaction

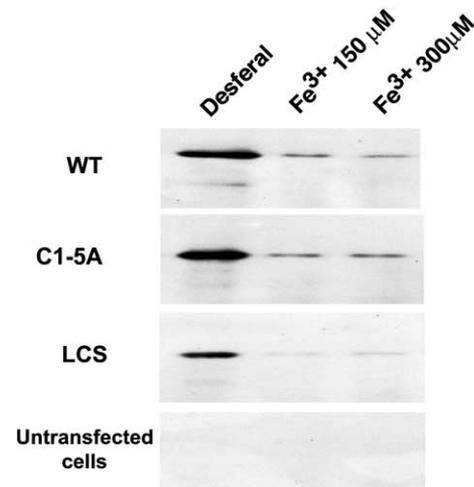


Fig. 1. IRP2 is iron-dependently degraded in wild-type, LCS, and C1-5A-inducible mutant cell lines. Doxycycline-induced cells were incubated 16 h in the presence of 10 μM desferal, 150 or 300 μM of iron. After incubation of 400 μg of total proteins with 30 μl of myc-beads, immunoprecipitated materials were Western blotted with rabbit polyclonal anti-IRP2 antibody.

solution and run at 200 V for 2 h. After drying, the gel was exposed overnight on a phosphorimager cassette (Molecular Dynamics) and the image was analyzed using the Image Quant software.

Results

Since our *in vitro* work demonstrated that simultaneous mutagenesis of all the cysteines from the cysteine-rich peptide of IRP2 protects from metal-catalyzed oxidation, we generated cell lines in which all five cysteines of the degradation domain were simultaneously mutagenized to alanine in full-length IRP2 and other cell lines in which cysteines 168, 174, and 178 were mutagenized to serines, to reproduce a transfection experiment that was performed previously [5]. Multiple independent cell lines that expressed each mutant were generated. The higher-molecular-weight recombinant epitope-tagged IRP2 was readily distinguished from endogenous IRP2 in immunoprecipitation experiments using antibody to the recombinant epitope tag followed by Western blot analysis (Fig. 1). Iron-dependent degradation of WT IRP2 was demonstrated and, in addition, degradation of C1-5 and LCS (cysteine to serine mutations of C168, 174, and 178) was almost complete when cells were treated with iron. Because we had numerous colonies, expression levels of IRP2 in cell lines that overexpressed WT IRP2 could be compared with cell lines in which mutagenized IRP2 was similarly overexpressed, thus allowing us to evaluate regulation without having to correct for large differences in expression. Notably, C1-5 and LCS were iron-dependently degraded even in cell lines that expressed high amounts of recombinant protein. These results differ from those reported in a previous experiment [5],

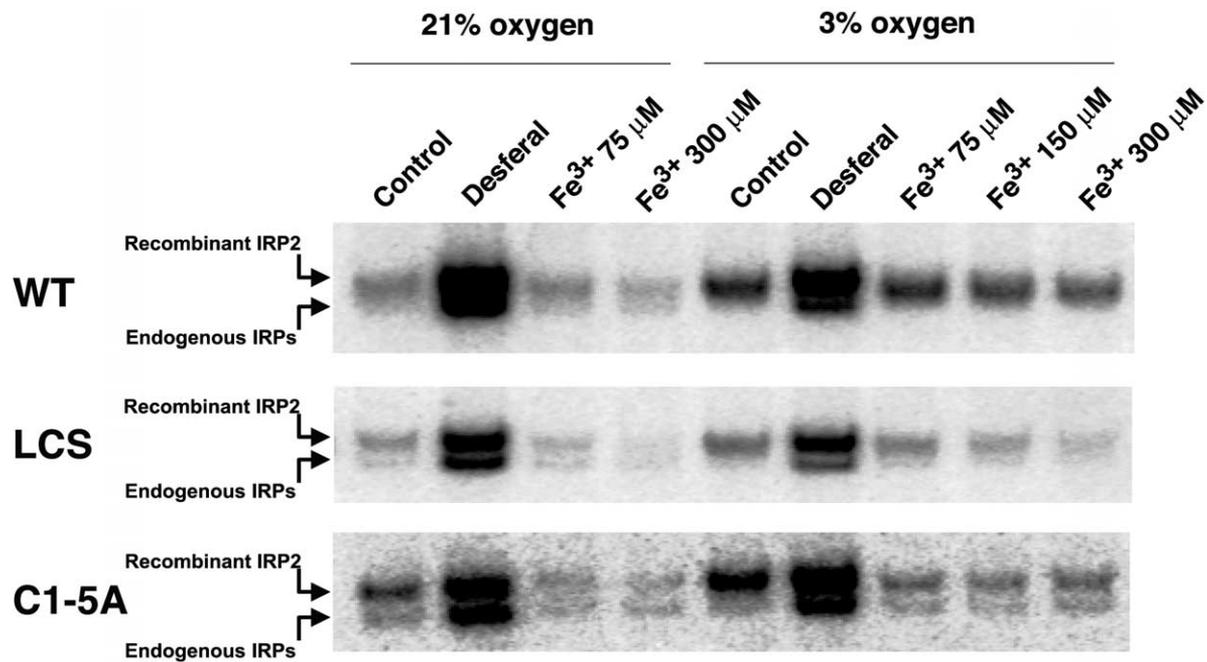


Fig. 2. IRE-binding activity of recombinant WT and C1-5A mutant IRP2 decreases in iron-replete cells and is sensitive to ambient oxygen concentrations. Stable transformants (293 cells) expressing IRP2 wild-type, LCS, or C1-5A, were cultured during 16 h in regular medium (control) or in the presence of 100 μM desferal or 75, 150, or 300 μM iron. Gel-mobility shift studies were performed as described under Materials and methods. In each case, the top band is recombinant IRP2 and the bottom band is comprised of comigrating endogenous IRP1 and IRP2. Cells were grown in room air (left) or 3% oxygen (right). In the latter case, cells were preincubated in reduced oxygen concentrations 3 h prior the addition of the treatment to the media.

but the previous results were based on results in a single cell line with low expression levels of IRP2, whereas the present results were obtained in multiple cell lines in which we could definitively distinguish the IRP2 band from other nonspecific bands.

We compared IRE binding activity of recombinant wild-type vs C1-5A and LCS and observed that iron-dependent regulation of IRE binding activity was intact (Fig. 2), as would be expected from the results of the IP/Western blot. In human cells, endogenous IRP1 and IRP2 cannot be separated from one another in gel-shift assays, but recombinant epitope-tagged IRP2 is easily distinguished from endogenous IRPs in our experimental system because it runs at a higher position in the gel. To evaluate degradation of IRP2 under more physiologically relevant conditions, we exposed cell lines to 3% oxygen, a concentration that approximates the oxygen concentration of normal mammalian tissues [7]. We observed that WT and mutant IRP2 binding activities were stabilized at lower oxygen concentrations, as assessed by gel-shift (Fig 2) or IP Western blot (Fig. 3a and b) in agreement with previous reports [9].

Much has been written about the sensitivity of IRP2 to nitric oxide [10,11]. Most assessments of the effects of various reagents on IRP2 have utilized the gel-mobility shift assay after treatment of rodent cells with pharmacologic NO donors, but interpretation of these assays is complicated by the difficulty of completely separating IRP1 from IRP2 for quantification. In our experimental system quantitation of overexpressed recombinant IRP2 using Western blots re-

fects the information gained from gel-shift assays (see Figs. 1 and 2) and we find that the results are robust and reproducible because recombinant IRP2 is easily distinguished from endogenous IRPs in Western blots as well as in gel-shift assays. Because the literature regarding the effect of NO donors is somewhat contradictory [10], we decided to treat our IRP2 WT and C1-5A cell lines with sodium nitroprusside (SNP), a donor of NO⁺, to evaluate the effect of this reagent on IRP2 levels using our experimental system. Recombinant WT IRP2 levels decreased in cells treated with SNP, as has been observed previously in RNA gel-shift studies [12,13]; IRP2 levels also decreased comparably in the C1-5A mutant cell line, suggesting that none of the five cysteines of the IRP2-specific exon is required for SNP-dependent degradation (Fig. 4). When cells were treated with both SNP and desferal, we found that IRP2 levels were high, as would be expected for desferal treatment alone. These results are not in agreement with a similar previously reported experiment [14]. SNP is believed to be involved in S-nitrosylation of various proteins [10]. However, SNP (sodium nitroferricyanide) is also an iron-containing compound ($\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]$) capable of donating iron [15], and its effect on IRP2 levels could potentially be mediated by its associated iron. The fact that desferal substantially reverses the effect of SNP (Fig. 4) is consistent with the possibility that SNP functions as a donor of iron rather than as a donor of nitrosonium ion. When we evaluate the effect of SNP treatment on ferritin synthesis (Fig. 4), it is apparent that decreased IRP2 correlates with increased ferritin syn-

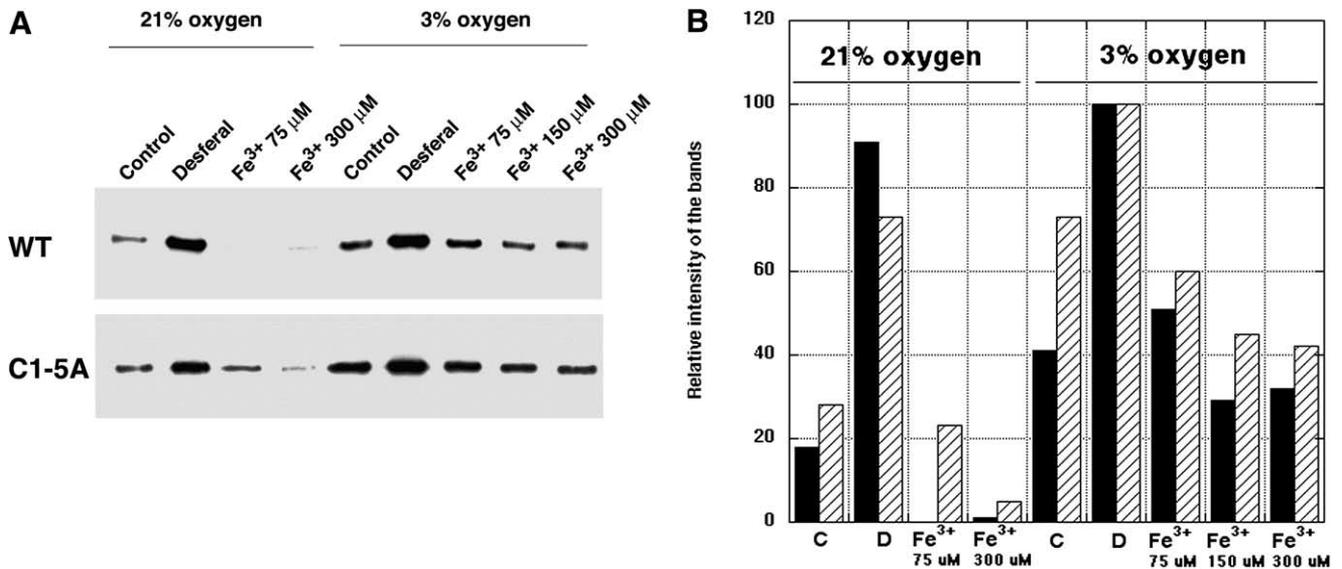


Fig. 3. Both recombinant WT and C1-5A mutant IRP2 are stabilized by lowered oxygen tension. (A) Lysates from cells in Fig 2 were immunoprecipitated with anti-myc antibody and proteins were separated by SDS-PAGE and blotted with rabbit anti-IRP2 polyclonal antibodies. (B) The relative intensity of the bands in A, calculated using the NIH image program, was plotted against the corresponding treatments received by the cells, revealing comparable iron regulation of WT vs C1-5A IRP2 in room air and at low oxygen growth conditions. Notably, iron-dependent degradation is attenuated in both WT and C1-5A IRP2 in 3% oxygen growth conditions. Calculations were done by taking the intensity of the bands for desferal lanes in 3% oxygen as 100 for both WT and C1-5A cells. Filled bars represent the WT cells, and striped bars correspond to C1-5A cells.

thesis in the WT recombinant cell line and the C1-5A cell line. These results imply, as expected, that overexpressed IRP2 can repress ferritin synthesis, and degradation of overexpressed IRP2 can relieve translational repression.

The NO donor SNAP has previously been shown to activate IRP2 [16]. Accordingly, we evaluated the effect of SNAP on IRP2 but found no effects under our experimental conditions (Fig. 5), in agreement with others [17,18].

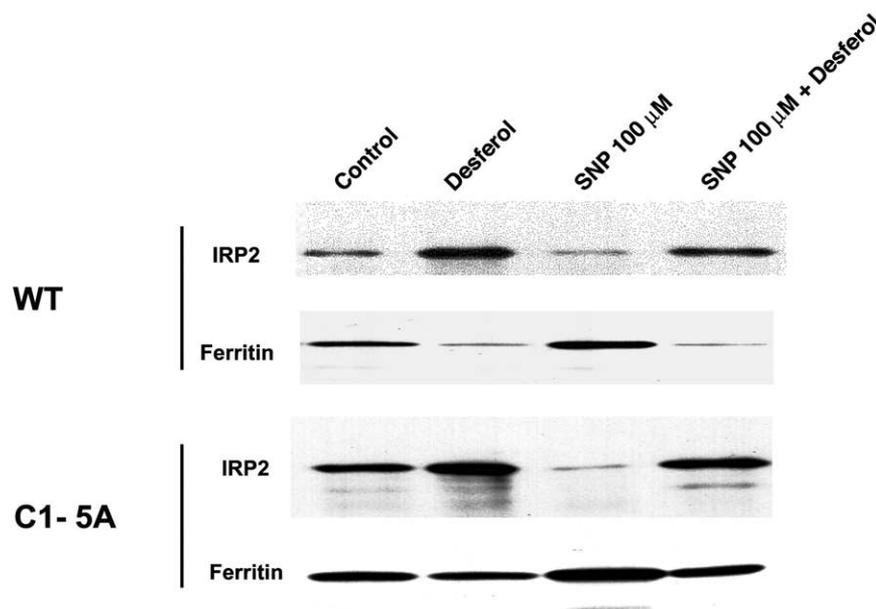


Fig. 4. The NO donor SNAP decreases IRP2 and increases ferritin levels. Doxycycline-induced wild-type or C1-5A cells were incubated 16 h in the absence of any treatment (control) or in the presence of 100 μ M of desferal, 100 μ M sodium nitroprusside alone, or 100 μ M sodium nitroprusside plus 100 μ M of desferal. After incubation of 200 μ g of total proteins with anti-myc beads, immunoprecipitated proteins were Western blotted with polyclonal rabbit anti-IRP2 antibody. To detect ferritin, 20 μ g of total proteins were Western blotted with antibodies directed against human ferritin as described under Materials and methods.

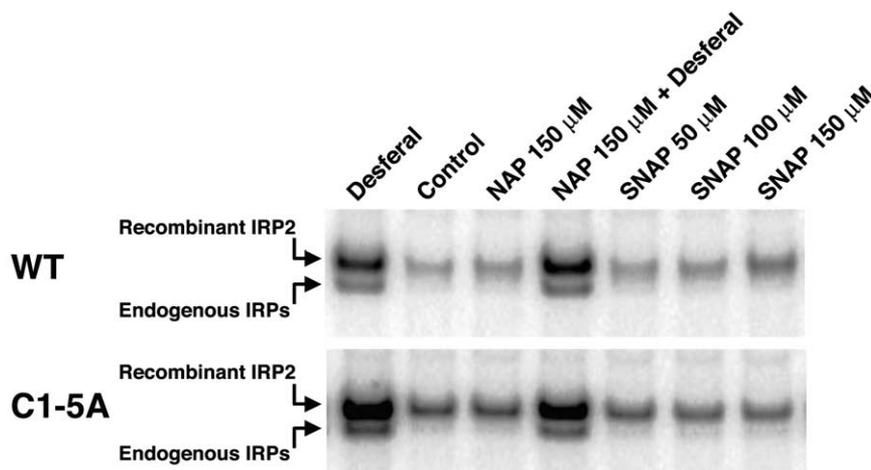


Fig. 5. IRP2 levels are not affected by treatment with the NO · donor SNAP. Stable transformants (293 cells) expressing IRP2 wild-type or C1-5A were cultured for 16 h in regular medium (control) or in the presence of 100 μM desferal, 150 μM *N*-acetyl-D,L-penicillamine disulfide (NAP-control for SNAP) in the absence or the presence of 100 μM desferal, 50–150 μM *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, an NO donor).

When we discovered that cysteine mutagenesis did not interfere with IRP2 degradation, we considered that perhaps a second efficient iron-dependent degradation pathway was still intact. Recently, it has been suggested that heme may bind to IRP2 and thereby promote its degradation [19,20], and we have observed that heme is at least as efficient as iron in mediating oxidation of the recombinant peptide derived from the putative degradation domain [8; J Jeong, unpublished observations]. To further evaluate the role of heme, we treated cells that overexpress IRP2 with hemin alone, or simultaneously with hemin and desferal. We found that heme treatment decreased IRP2 levels but that simultaneous treatment with desferal could abrogate the heme effect, suggesting that exogenous heme functions as an iron source in degradation of IRP2. Because the possibility that heme is involved in IRP2 degradation has been repeatedly

raised, we inhibited endogenous heme synthesis with succinylacetone to evaluate its effect on degradation of WT recombinant IRP2 and in C1-5 IRP2. Succinylacetone treatment significantly inhibited degradation of both WT and C1-5 IRP2 (Fig. 6). Moreover, succinylacetone treatment substantially antagonized the effect of exogenously added heme, suggesting that perhaps endogenous and exogenous heme affect IRP2 levels by different mechanisms (Fig. 7).

To further evaluate IRP2 degradation, we treated cells with cobalt chloride, a compound that has been frequently used as a hypoxia mimetic in studies of hypoxia inducible factor degradation [12]. We found that cobalt prevented degradation of WT (Fig. 8), in agreement with a previous report [9], and also interfered with degradation of C1-5 IRP2. Thus, cobalt dose not exert its effect by binding directly to cysteines of the IRP2 degradation domain [5].

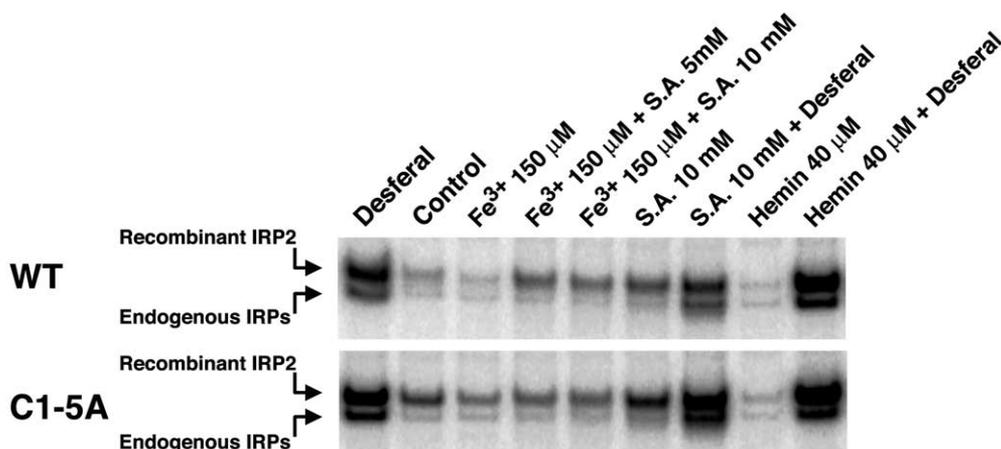


Fig. 6. Inhibition of heme synthesis with succinylacetone interferes with iron-dependent IRP2 degradation of WT and C1-5A IRP2. Wild-type IRP2- and C1-5A-expressing cells were incubated in the presence of 100 μM desferal, 150 μM iron, and 5 or 10 mM succinyl acetone in the absence or the presence of iron or 40 μM hemin with or without desferal for 16 h. IRP2 levels were evaluated by gel-shift.

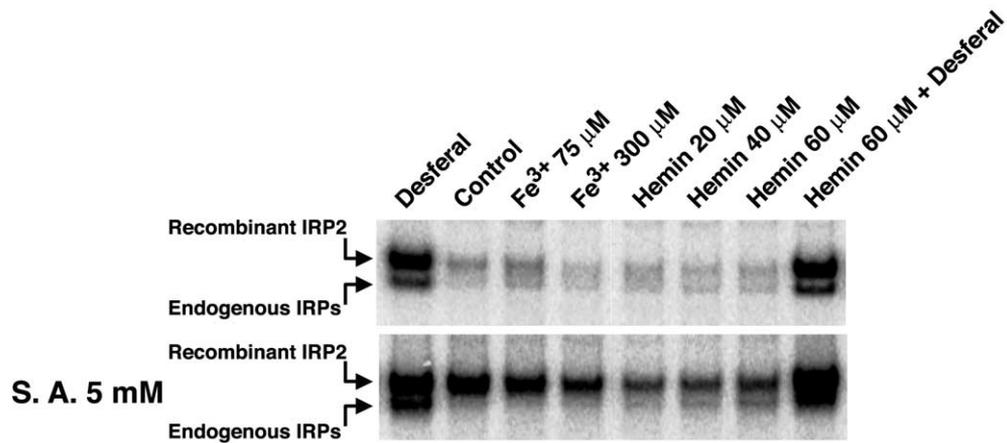


Fig. 7. Succinylacetone interferes with degradation of IRP2 when the iron source added to tissue culture medium is either ferric ammonium citrate or hemin. IRP2 from cells treated with 75 or 300 μM ferric ammonium citrate or 60 μM hemin with or without desferal were performed in the absence or presence of 5 mM succinylacetone, a heme synthesis inhibitor. IRP2 levels were assessed by gel-shift assays.

Discussion

IRP1 and IRP2 are both potentially important in post-transcriptional regulation of iron metabolism in mammals. IRP2 likely arose as the product of a gene duplication and is found only in mammals. Although it is 58% identical to IRP1, IRP2 lacks aconitase activity, and aconitase activity cannot be restored even when putative active site residues are mutagenized to match the active site residues of IRP1 [21,22]. In addition, IRP2 acquired an extra 73-amino-acid domain encoded by a single exon, likely as the result of an exon shuffle, and this domain has unusual sequence features and properties [5]. We have previously reported that this

IRP2-specific 73-amino-acid domain appears to be largely responsible for iron-dependent degradation of IRP2. Working with IRP2 is notoriously difficult because IRP2 is prone to iron-dependent oxidations and cleavages *in vitro*, and degassing of solutions and use of iron chelators is necessary to preserve the integrity of IRP2 in experiments. To further characterize the properties of the putative IRP2 degradation domain, we studied a 63-amino-acid peptide and discovered that exposure of this peptide to iron and oxygen led to oxidation of one cysteine. Mutagenesis of cysteines to alanines eliminated these *in vitro* iron-dependent oxidation and cleavage events. We hypothesized that this portion of IRP2 evolved to function as an iron-binding site and that iron

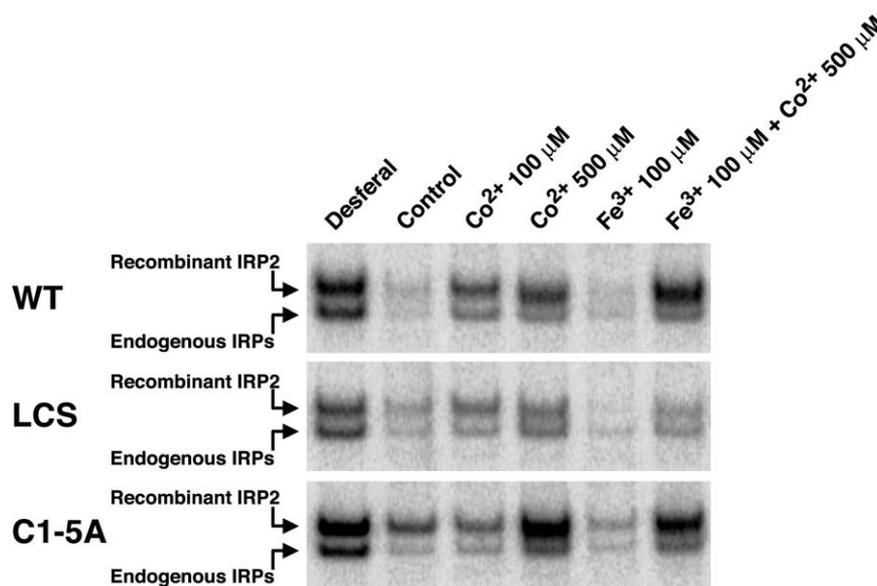


Fig. 8. Cobalt inhibits iron-dependent degradation of WT, LCS, and C1-5A IRP2. Cell lines that inducibly express wild-type, LCS, and C1-5A IRP2 were incubated with desferal, cobalt, and Fe³⁺ at the indicated concentrations, indicating that cysteines of the IRP2 degradation domain are not required for cobalt to interfere with degradation.

binding was the first step in iron-dependent degradation [8]. To pursue the relevance of these observations to cellular IRP2 degradation, we generated inducible cell lines and evaluated the role of these cysteines in iron-dependent degradation of recombinant cellular IRP2.

We discovered that IRP2 in which all of the cysteines of the putative degradation domain are replaced by alanines undergoes normal iron-dependent degradation. We then used our recombinant IRP2 cell lines to assess the effects of other reagents that have been reported to affect IRP2 degradation. We found that the enhanced IRP2 degradation caused by the NO⁺ donor SNP and exogenous heme (supplied as hemin) could be antagonized by addition of desferal. These results imply that SNP and hemin may act by increasing availability of cytosolic iron. SNP, also known as sodium nitroferricyanide, contains iron that it may deliver directly to cells. Our data with the C1-5A IRP2 show that SNP does not function by nitrosylating cysteines of the degradation domain.

Our experiments also distinguish between the effects of endogenous and exogenous heme. We demonstrate that the effects of exogenous heme can be abrogated by simultaneous treatment with desferal. Interestingly, we also find that exogenous heme does not reverse the effect of succinylacetone (Fig. 7, lane 5), as might be expected if exogenous heme remained intact and functional after cellular uptake. Perhaps heme synthesized by cells is specifically trafficked and accompanied by chaperone proteins to its various targets, as has been observed in bacteria [13]. It appears that IRP2 is sensitive to the contents of this pool of endogenous heme. Notably, animals treated with succinylacetone develop increased tissue iron and oxidative damage [15]. We suggest that hemin taken up by cells may be trafficked very differently from endogenously synthesized heme and may be more readily degraded by heme oxygenase to yield iron, biliverdin, and CO. The iron released by heme degradation could then exert its effect, perhaps by incorporating into endogenously synthesized heme or by participating in another iron-dependent degradation pathway. Both iron and hemin decrease WT and C1-5A IRP2 levels in succinylacetone-treated cells, raising the possibility that there is another pathway for IRP2 degradation that senses free iron levels independent of both heme and cysteines of the putative degradation domain. Recent experimental work suggests that heme may exert its effect by directly binding to the degradation domain of IRP2 [20].

Our data also confirm that IRP2 is relatively stabilized in low-oxygen environments. This observation is very relevant to normal physiology as tissue oxygen concentrations are normally much lower than air concentrations [11,23].

The stabilization of IRP2 by cobalt treatment is reminiscent of cobalt-induced stabilization of HIF- α . Cobalt may stabilize HIF- α by competing for an iron-binding site in the prolyl hydroxylase that oxidizes HIF- α , but recent data indicate that cobalt directly binds to the oxygen-dependent

degradation domain of HIF- α and sterically hinders VHL-dependent ubiquitination [17]. Our data show that the protective effect of cobalt does not involve competition for the cysteine-dependent iron-binding site we have previously identified in the putative IRP2 degradation domain [8]. Cobalt treatment could potentially cause intracellular heme levels to decrease by activating heme oxygenase [24,25]. Cobalt-induced degradation of heme and attendant stabilization of IRP2 would be consistent with the recent proposal that heme exerts its effect by directly binding and oxidizing IRP2 [20]. It will be important in the future to analyze IRP2 obtained from mammalian cells treated with proteasome inhibitors to look for evidence of heme-binding or specific mass changes in IRP2 that is destined for proteasomal degradation.

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