

Involvement of Heme in the Degradation of Iron-regulatory Protein 2*

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Iron-regulatory proteins (IRPs) recognize and bind to specific RNA structures called iron-responsive elements. Mediation of these binding interactions by iron and iron-containing compounds regulates several post-transcriptional events relevant to iron metabolism. There are two known IRPs, IRP1 and IRP2, both of which can respond to iron fluxes in the cell. There is ample evidence that IRP1 is converted by iron to cytoplasmic aconitase *in vivo*. It has also been shown that, under certain conditions, a significant fraction of IRP1 is degraded in cells exposed to iron or heme. Studies have shown that the degradation of IRP1 that is induced by iron can be inhibited by either desferrioxamine mesylate (an iron chelator) or succinyl acetone (an inhibitor of heme synthesis), whereas the degradation induced by heme cannot. This suggests that heme rather than iron is responsible for this degradation.

Several laboratories have shown that IRP2 is also degraded in cells treated with iron salts. We now show evidence suggesting that this IRP2 degradation may be mediated by heme. Thus, in experiments analogous to those used previously to study IRP1, we find that IRP2 is degraded in rabbit fibroblast cells exposed to heme or iron salts. However, as shown earlier with IRP1, both desferrioxamine mesylate and succinyl acetone will inhibit the degradation of IRP2 induced by iron but not that induced by heme.

Several genes that are required for iron homeostasis are regulated via post-transcriptional events. One of the best understood examples is the regulation of ferritin synthesis. Near the 5'-cap of the ferritin mRNA is an iron-responsive element (IRE)¹ which acts as a docking site for a member of a family of proteins known as iron-regulatory proteins (IRPs). There are two related IRPs, IRP1 and IRP2, both of which recognize wild-type IREs with nearly equivalent binding affinity (1–5). However, the two IRPs have overlapping but distinct preferences for a variety of IRE variants generated *in vitro* (6, 7). Although these variant IREs have not been found naturally, such studies reveal subtle differences in how each IRP recognizes the natural IRE.

Regulation of IRE-IRP interactions *in vivo* is crucial to the expression of several gene products. Incubation of cells with iron or heme leads to dissociation of the IRP from the IRE. This

dissociation leads to increased translation of genes that contain an IRE in their 5'-untranslated region. Examples of mRNAs that are regulated by this mechanism are ferritin (8–11), erythroid δ -aminolevulinic acid synthase (12, 13), mitochondrial aconitase (14), and *Drosophila melanogaster* succinate dehydrogenase (15, 16). Transferrin receptor mRNA contains several functional IREs in its 3'-untranslated region (17, 18). Binding of IRPs to these IREs stabilizes the transferrin receptor mRNA (19–21). Dissociation of IRPs from the transferrin receptor IREs results in rapid degradation of transferrin receptor mRNA.

The form of IRP1 that binds with high affinity to IREs *in vivo* is most likely apo-IRP1 (9, 22–24). A primary response to cellular iron influx is conversion of apo-IRP1 to a holo-protein that contains a 4Fe-4S iron-sulfur cluster. This iron loaded IRP1 has a reduced affinity for IREs, and concomitantly gains aconitase activity (8, 9).

However it has been shown that, under some conditions, IRP1 can be degraded when iron enters the cell (25–27). The degradation of IRP1 induced by iron *in vivo* is stimulated by δ -aminolevulinic acid (a heme precursor) and inhibited by both succinyl acetone (SA; an inhibitor of heme synthesis) and desferrioxamine mesylate (Desferal; an iron chelator). By contrast heme-induced degradation of IRP1 is not affected by these agents. This suggests that heme rather than free iron is the causative agent of the degradation (25). Heme has also been shown to bind directly to IRP1 *in vitro* (28) and *in vivo* (27). The mechanism for heme-induced IRP1 breakdown is not known; however, some evidence suggests that a high molecular weight intermediate may be involved (25, 26).

Several investigators have shown that IRP2 is degraded in the presence of iron (4, 5, 29, 30). We were interested to know if heme, rather than free iron, may be responsible for this degradation as it appears to be for IRP1. In order to determine this, we studied the breakdown of IRP2 using experiments similar to those used to analyze IRP1.

EXPERIMENTAL PROCEDURES

Cell Culture—A normal rabbit skin fibroblast line, RAB-9, was used for all experiments reported. These cells were seeded at low density in Earle's minimal essential medium supplemented with 2% fetal calf serum and nonessential amino acids (alanine, aspartate, glutamate, glycine, proline, and serine, all at 0.1 mM), and 1 mM sodium pyruvate. Cells were allowed to grow for 2 days by which time they had reached from two-thirds to three-fourths confluency. On the day of each experiment, additions were made to the spent growth medium unless otherwise stated. At the end of the incubations, cells were washed twice with cold phosphate-buffered saline and were lysed by the addition of band shift lysis buffer (25 mM Tris-HCl, pH 7.4, 40 mM KCl, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 0.7 μ g/ml pepstatin A, and 7 mM 2-mercaptoethanol).

Analysis of Total Protein Amounts by Immunoblotting—After lysis in bandshift buffer, equal quantities of protein were analyzed by SDS-polyacrylamide gel electrophoresis and then transferred by electroblotting for 20–24 h at 100 mA onto nitrocellulose (Schleicher &

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¹ The abbreviations used are: IRE, iron-responsive element; Desferal, desferrioxamine mesylate; FAC, ferric ammonium citrate; IRP, iron-regulatory protein; SA, succinyl acetone.

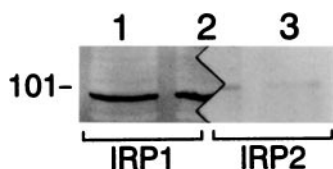


FIG. 1. Antibodies against IRP1 and IRP2 detect distinct proteins. Three aliquots of an untreated RAB-9 cell lysate were run on an SDS-polyacrylamide gel electrophoresis gel and blotted onto nitrocellulose as usual. The blot was then cut vertically into two halves down the middle lane with pink shears. Immunoblotting was performed as usual using anti-IRP1 on the left half and anti-IRP2 on the right half. Different secondary antibodies were used for each half (see "Experimental Procedures"). The 101-kDa molecular mass marker is indicated.

Schuell, Protran BA83) membranes. IRP2 was then detected using anti-IRP2 primary antibody (provided by Dr. E. Leibold, Salt Lake City, UT) and alkaline phosphatase conjugated to goat anti-rabbit IgG secondary antibody (36). IRP1 was detected using anti-IRP1 primary antibody and alkaline phosphatase conjugated to goat anti-rat IgG secondary antibody. Stained bands were quantitated by densitometry.

Reproducibility of Results—Most experiments were performed in duplicate or triplicate, and the results were averaged. Reproducibility of results was high. For example, the effect of iron on IRP2 was quantitated in 12 different individual reactions; the average reduction in IRP2 relative to the same number of untreated control reactions was 72%. The average deviation was $\pm 6.3\%$ of the untreated control value. The comparable values (reported in parenthesis) for other important experiments were: the reduction of IRP2 in the presence of Desferal plus iron (done 4 times, $\pm 8.5\%$ deviation); the reduction of IRP2 in the presence of SA plus iron (done 8 times, $\pm 13\%$); the reduction of IRP2 in the presence of heme (done 13 times, $\pm 8.5\%$); the reduction of IRP2 in the presence of SA plus 20 μM heme (done 5 times, $\pm 7.9\%$).

RESULTS

To test the ability to distinguish IRP1 from IRP2, Western analysis was performed on untreated RAB-9 lysates. The nitrocellulose membranes bearing transferred proteins were probed with antibodies specific for IRP1 and IRP2. Fig. 1 shows that antibodies raised against IRP2 do not cross-react with IRP1, and *vice versa*. The mobility of IRP2 in SDS-polyacrylamide gel electrophoresis is slightly slower than that of IRP1, in accordance with its higher molecular weight. In all cases, IRP1 is more abundant than IRP2 in RAB-9 cells, although IRP2 was still readily detectable.

We next wanted to corroborate findings that IRP2 is degraded in cells exposed to iron salts. RAB-9 cells were incubated with control medium or medium containing 100 μM ferric ammonium citrate (FAC) for 3.5 h and then lysed (see "Experimental Procedures"). Results of this experiment are shown in Fig. 2A. Lane 2 shows that significantly less IRP2 is present in cells exposed to 100 μM FAC than in cells grown in control medium (lane 1). These results agree quite well with data shown by Samaniego *et al.* (4).

To assess the effect of limiting porphyrin synthesis, cells were also treated with both FAC and 7.5 mM SA (Fig. 2A, lane 3). Simultaneous incubation of SA with FAC reduces the extent of IRP2 degradation attributable to iron.

Administration of 40 μM heme to RAB-9 cells also induces the degradation of IRP2, as shown in Fig. 2A, lane 4. Addition of Desferal after 30 min of heme treatment does not inhibit this degradation (Fig. 2B, lane 6).

Quantitation of the effects of iron, heme, SA, and Desferal is shown in Table I. In Experiment 1, nearly 80% of IRP2 is degraded in the presence of iron. This effect is largely prevented by the addition of Desferal 30 min after iron addition. By contrast, the degradation induced by heme is not prevented by Desferal. In Experiment 2, the effect of SA in inhibiting the iron-induced degradation of IRP2 is seen; it is quantitatively similar to that of Desferal. A lower concentration of heme than that used in Experiment 1 produces less IRP2 degradation;

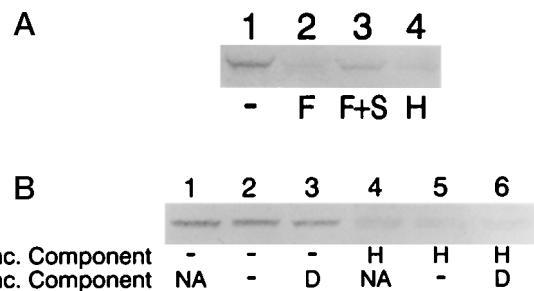


FIG. 2. Effects of iron and heme on IRP2. Panel A, rabbit fibroblasts (RAB-9) were untreated (–, lane 1) or treated with 100 μM ferric ammonium citrate (F, lane 2), or 100 μM ferric ammonium citrate plus 7.5 mM succinyl acetone (F + S, lane 3), or 40 μM heme (H, lane 4) for 3.5 h then lysed as described under "Experimental Procedures." Samples were analyzed by immunoblotting using anti-IRP2 antibody. Panel B, RAB-9 cells were preincubated in serum free medium lacking methionine and cysteine for 30 min. This medium was removed, and spent medium was added containing 50 μM heme (H) where indicated for 4 h (1st incubation). Cells were then either lysed (lanes 1 and 4) or washed twice with serum free Earle's minimal essential medium and incubated for 3 h in spent medium containing 200 μM Desferal (D) as indicated (2nd incubation). Cells were lysed as described under "Experimental Procedures," and samples were analyzed by immunoblotting using anti-IRP2 antibody.

TABLE I

Succinyl acetone and Desferal inhibit the effect of iron but not heme on IRP2

For all experiments, cells were lysed as described under "Experimental Procedures" and samples were analyzed by immunoblotting with anti-IRP2 antibody. Residual IRP2 levels were quantitated by densitometry and are reported as a percentage of the indicated control.

Additions		Relative residual IRP2 % control
1st incubation	2nd incubation	
Experiment 1 ^a		
None	none	100
FAC	FAC	21
FAC	FAC + Desferal	84
Heme	heme	28
Heme	heme + Desferal	31
Experiment 2 ^b		
None	none	100
None	FAC	29
SA	FAC + SA	73
None	heme	51
SA	heme + SA	58
Experiment 3 ^c		
Desferal	Desferal	100
Desferal	Desferal + heme	52
Desferal + SA	Desferal + heme + SA	54

^a RAB-9 cells were pretreated for 30 min with 100 μM FAC or 30 μM heme (1st incubation). Fresh medium was then added containing 200 μM Desferal, 100 μM FAC or 30 μM heme as indicated, and cells were incubated for an additional 4.5 h (2nd incubation).

^b RAB-9 cells were preincubated with or without 8 mM SA for 80 min (1st incubation). 100 μM FAC or 20 μM heme was added as indicated, and cells were incubated for 5 h (2nd incubation).

^c RAB-9 cells were treated with 200 μM Desferal with or without 7.5 mM SA for 1 h (1st incubation). 20 μM heme was then added as indicated, and all were incubated an additional 5 h (2nd incubation).

however, SA has a slight effect on the heme-induced degradation of IRP2 in this experiment. We speculated that this might be due to a small amount of iron that was released from heme by the action of heme oxygenase. To test this possibility, cells were pretreated with Desferal before heme addition. The results are shown in Experiment 3: as anticipated, the influence of SA on the heme-induced degradation is negligible in the presence of Desferal. Moreover, comparison of the results in

Experiments 2 and 3 show that even pretreatment of cells with Desferal does not mitigate the degradation induced by heme.

The above data show that the degradation of IRP2 caused by heme addition is not inhibited by Desferal. This suggests that heme is not merely serving as a source of free iron. By contrast, the degradation caused by FAC is inhibited by both SA and Desferal. Together, these results suggest that iron must first be incorporated into heme (or some other compound in the porphyrin biosynthetic pathway) before it can influence IRP2 stability.

DISCUSSION

The results shown here confirm reports by others (4, 5, 29, 30) that IRP2 is degraded in the presence of iron or heme. The data presented also suggest that iron must be converted to heme, or a heme-like compound, before it can trigger IRP2 degradation. Previous studies on IRP1 degradation by iron or heme showed similar results (25, 26).

We also find that the rate and extent of IRP2 degradation are significantly greater than for IRP1 (indeed, under certain conditions, no degradation of IRP1 was seen (26)). These results are consistent with findings of others that a 73-amino acid insert present in IRP2 protein relative to IRP1 is responsible for the high rate of IRP2 degradation (31).

Another interesting difference between IRP1 and IRP2 is that the latter appears unable to form an iron-sulfur cluster (32). One interesting possibility is that the absence of a cluster in IRP2 (caused by the presence of the 73-amino acid insert?) might facilitate its rapid degradation. If this were true, then it might offer an explanation for why IRP1 degradation is so variable; perhaps only IRP1 that lacks an iron-sulfur cluster is degraded by heme. However, before this notion can be seriously pursued, more information about the roles of cysteine residues within the 73-amino acid insert is required. It should also be noted that the phosphorylation state of IRP1 might play a role in determining whether it will be degraded.

It is still not certain which of the two major iron-dependent pathways (iron-sulfur cluster formation or protein degradation) is primarily responsible for derepression of ferritin mRNA translation. It seems likely that both are involved, and that the relative importance of each may be determined by variables such as cell type (33) and cell physiological state (25, 26). However, the fact that the re-repression after iron treatment requires new protein synthesis is consistent with the degradation pathway being operational in the cells employed (26). Similarly, it is not yet clear which protein, IRP1 or IRP2, is primarily responsible for repression. Again, both may play roles that depend upon cell type and physiological circumstances. Alternatively, or in addition, the two IRP forms may recognize different versions of the IRE *in vivo* (32). If IRP2 cannot form an iron-sulfur cluster then it is presumably not a target for nitric oxide regulation (34, 35). This would constitute

an important functional distinction between the two forms of IRP.

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