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Iron regulatory factor expressed from recombinant baculovirus: conversion between the RNA-binding apoprotein and Fe-S cluster containing aconitase

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ABSTRACT

Iron regulatory factor (IRF) is a cytoplasmic mRNA-binding protein that coordinates post-transcriptionally the expression of several important proteins in iron metabolism. Binding of IRF to iron-responsive elements (IRE) in the 5′ untranslated region (UTR) of ferritin and erythroid 5-aminolevulinic acid-synthase mRNAs inhibits their translation, whereas binding to IREs in the 3′ UTR of transferrin receptor (TfR) mRNA prevents the degradation of this mRNA. IRF binds RNA strongly after iron deprivation, but is inactive, yet present, under conditions of high cellular iron supply. Recently, IRF was also shown to have aconitase activity indicating the existence of an Fe-S cluster in the protein. In the current study we expressed human IRF in insect cells from recombinant baculovirus and analysed IRE-binding and aconitase activities under various culture conditions. Newly made apoprotein, synthesized in the absence of iron, was fully active in IRE-binding, but showed no aconitase activity. In contrast, IRF made by cells grown in high iron medium bound RNA poorly, but exhibited high aconitase activity with a K_m of 9.2 μM for *cis*-aconitate. Apo-IRF was converted *in vitro* to active aconitase by Fe-S cluster-generating conditions, and under the same conditions lost its RNA-binding capacity. These results indicate that the two activities are mutually exclusive and controlled through formation of the Fe-S cluster.

INTRODUCTION

Iron uptake by transferrin receptor (TfR) and iron storage in ferritin are coordinately controlled in response to cellular iron levels (1,2). Low iron supply induces transferrin receptor, but inhibits ferritin expression, whereas high-iron medium has opposite effects. This feedback control of iron homeostasis depends on post-transcriptional mechanisms of gene regulation. It involves iron regulatory factor (IRF) (3,4), also named IRE-BP (5) or FRP (6), a cytoplasmic mRNA-binding protein which acts as a sensor of iron levels and binds at low-iron conditions with high affinity to specific mRNA-hairpin structures, the iron-responsive elements (IRE) (3–5,7). Binding of IRF to an IRE present in the 5′ UTR of ferritin mRNA causes inhibition of ferritin translation (3,6,8), while multiple IREs in the 3′ UTR of transferrin receptor mRNA are required for stabilization of this mRNA (4,9–11). IREs have also been found in the 5′ UTR of erythroid 5-aminolevulinic acid-synthase mRNA (12,13) and mitochondrial aconitase mRNA (13). Thus, *trans*-acting effects of IRF on translation are likely to coordinate protoporphyrin synthesis with iron uptake in erythroid cells, and could also influence mitochondrial citrate metabolism.

IRF exists in at least three pools in the cell: an mRNA-bound fraction, a pool of free molecules with high affinity for a radioactive IRE probe, and a pool of molecules with low RNA-binding affinity (14,15). The fraction of free endogenous IRF molecules showing high IRE-binding affinity varies inversely to the extent of cellular iron supply (4,16). However the total level of IRF per cell does not seem to vary, since 'inactive' IRF molecules can be converted to the high affinity form by *in vitro* incubation with 2-mercaptoethanol (16). Recent evidence strongly suggests that iron-dependent inactivation of IRE-binding may depend on the presence of an Fe-S cluster in IRF (17–19). cDNA clones for human (20,21), mouse (22), rat (23) and rabbit IRF (24) have revealed interesting structural homology with mitochondrial and bacterial aconitases (17,25–27), which are [4Fe-4S]-cluster containing isomerases of the Krebs-cycle that convert citrate to isocitrate via *cis*-aconitate (28). The prediction that IRF may also be an aconitase has recently been confirmed experimentally (19,29). Since one iron atom of the Fe-S cluster of mitochondrial aconitase can readily be exchanged *in vitro* and thereby affect the aconitase activity (28,30), it has been speculated that iron may modulate IRF *in vivo* through the same mechanism, that is by switching a high affinity IRE-binding [3Fe-4S]-cluster form into a low affinity [4Fe-4S]-cluster form with aconitase activity (18,19,29).

These recent developments have prompted us to test the assumption that structural changes in an Fe-S cluster in IRF may provide the basis for its iron-regulated activation/inactivation as a *trans*-acting RNA-binding protein. We expressed human IRF

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from its full-length cDNA (21) in Sf9 insect cells by using recombinant baculovirus (31,32), and analysed both its IRE-binding and aconitase activities after biosynthesis in the absence or presence of iron. In addition, we reconstituted *in vitro* the Fe-S cluster in each batch of IRF by a method that has previously been successful for bovine mitochondrial aconitase (33) and measured the effect of cluster formation on the above parameters. The results indicate that IRF exists in two mutually exclusive conformations in the cell, the apoprotein which shows high affinity IRE-binding and an Fe-S containing form with aconitase activity.

MATERIALS AND METHODS

Subcloning and isolation of recombinant virus

An XbaI−ClaI fragment from pGEM-hIRF (21) containing the full-length human IRF cDNA was ligated into XbaI and BglII sites behind the strong viral polyhydrin promoter (31) of pVL1393 (Invitrogene, San Diego, CA). The plasmid (2 μg), referred to as pVL-hIRF, was transfected into Sf9 cells together with 1 μg of wild-type AcMNPV virus DNA in 25 mM Hepes pH 7.1, 140 mM NaCl, 125 mM $CaCl_2$ as described by Summers and Smith (32). Pure recombinant human IRF virus (rec-hIRF virus) was obtained after three rounds of purification by plaque assay. Clones were verified by slot blots for human IRF DNA and hybridization with a random-primed XbaI−HindIII probe of pGEM-hIRF, and by the gel-retardation assay measuring IRE-binding activity.

Cell culture and baculovirus infection

Sf9 cells (from *Spodoptera frugiperda*) (32) were maintained in TC-100 medium (Gibco-BRL) with 10 μg/ml gentamycin and 10% fetal calf serum at 27°C in air. In high iron-medium, 20 μg/ml ferric ammonium citrate was included, and low iron-medium contained 50 μM desferrioxamine (gift of Ciba-Geigy, Basel, Switzerland). For time-course experiments, infections were carried out in 24 well-plates using 3×10^5 cells per well and a multiplicity of infection (MOI) of 5. For aconitase assays, infections were done in 100 ml spinner cultures at a cell density of 1×10^6 cells/ml and a MOI of 7.5 (32). The typical infection lasted for 48 h when the peak of recombinant human IRF expression was observed.

Preparation of rabbit anti-IRF antisera and Western blot analysis

The peptide sequence corresponding to the 13 NH_2-terminal residues of human IRF (21) was synthesized as a branched peptide (34). This peptide (50 μg) was used to immunise and boost twice a New Zealand rabbit. Proteins for Western-blot analysis were either purified IRF from human placenta (35) or from total cytoplasmic extract of rec-hIRF virus infected Sf9 cells. The samples were separated electrophoretically in an 8% SDS-polyacrylamide gel and transferred to nitrocellulose filters using a BioRad Transblot transfer cell. Filters were air-dried, blocked in 1% BSA and incubated with rabbit anti-IRF serum or preimmune serum, followed by colour development using an anti-rabbit IgG/alkaline phosphatase conjugate (Promega, WI).

Gel retardation assay

Cytoplasmic extracts were prepared from virus-infected cells lysed for 5 min on ice in 0.2% NP-40 and buffer A (5% glycerol, 40 mM KCl, 3 mM $MgCl_2$, 10 mM Hepes pH 7.5) and spun at $12,000\times g$ for 1 min. Protein concentration was determined by using a commercial assay (BioRad, Richmond, CA). Unless indicated differently, equal aliquots of protein (20 ng) were incubated with an excess of ^{32}P-labelled RNA-transcript from plasmid pSPT-fer (4), containing the human ferritin H chain IRE, and analysed by a gel retardation assay as previously described (36). In some cases, cell extracts were pretreated with 1.5 mM azidodicarboxylic acid bis[dimethylamide] (Sigma St Louis, MO) (diamide) or 2% 2-mercaptoethanol (2-ME) (16,35). To test the specificity of binding, an excess of unlabelled specific competitor (pSPT-fer RNA) or non-specific competitor (4F2 heavy chain RNA (37)) was added.

Aconitase assay

Cytosolic extracts were made by adding 1 ml of 0.2% NP-40 in buffer A per 5×10^7 cells and passed over a Heparin-Sepharose CL-6B column (35). Bound proteins were eluted at 250 mM KCl. Aconitase activity of these fractions was tested according to Drapier and Hibbs (38). Aliquots of 5−50 μg total protein were incubated at 37°C with 200 *m*M *cis*-aconitate in 50 mM Tris−HCl pH 7.2, 100 mM NaCl and 0.02% BSA in a reaction volume of 2 ml. The transformation of substrate was monitored by decreasing absorbance at 240 nm. The specific activity in fractions was determined with the extinction coefficient $\epsilon_{240} = 3.41\ cm^{-1}mM^{-1}$ for *cis*-aconitate (39).

For the conversion of apo-IRF to [4Fe-4S]-IRF, an aliquot of each fraction was treated anaerobically under argon with 100 μM $FeSO_4$, 100 μM Na_2S and 10 mM DTT (33) for 1 h at room-temperature prior to aconitase measurements.

RESULTS AND DISCUSSION

Expression of functional recombinant human IRF in insect cells

To investigate the proposed switch between different forms of IRF with IRE-binding and aconitase activity, we have over-expressed recombinant human IRF (rec-hIRF) by the baculovirus expression system (31,32). The full-length human IRF cDNA (21) in plasmid pVL1393 was transfected into Sf9 cells, and recombinant virus obtained by homologous recombination after infection with wild-type AcMNPV virus (32). As shown in Fig.1A, expressed rec-hIRF gave a gel-retardation pattern with a human ferritin H chain IRE-probe (lane 1) identical to that of human IRF from placenta (lane 8), but clearly distinct from endogenous insect IRF (lane 7). Like placental IRF, rec-hIRF was inactivated by diamide (16,35), a sulfhydryl-oxidizing agent (lanes 2 and 9), and enhanced in its activity by 2% 2-ME (lanes 3 and 10). The rec-hIRF−IRE interaction was specifically inhibited by excess unlabelled IRE, but not by unrelated RNA (lanes 5 and 6). After 48 h of virus infection, rec-hIRF reached its highest intracellular level and was about 100-fold over-expressed compared to endogenous insect IRF (lane 4). Presumably due to cell lysis, however, 30−50% rec-hIRF were lost to the medium, and this may explain a low level of insect IRF in virus infected cells (lanes 3,4 and 6). Based on radioactivity in RNA−protein complexes, we estimate optimal expression at 1−3 μg IRF/ml cell culture or $3-10\times10^6$ rec-hIRF molecules per insect cell. The identity of rec-hIRF was further verified by Western-blot analysis using a specific antibody raised against an NH_2-terminal peptide of human IRF. Rec-hIRF resolved, exactly like human placental IRF (35), into two bands at about 100 kDa in an 8% SDS-polyacrylamide gel

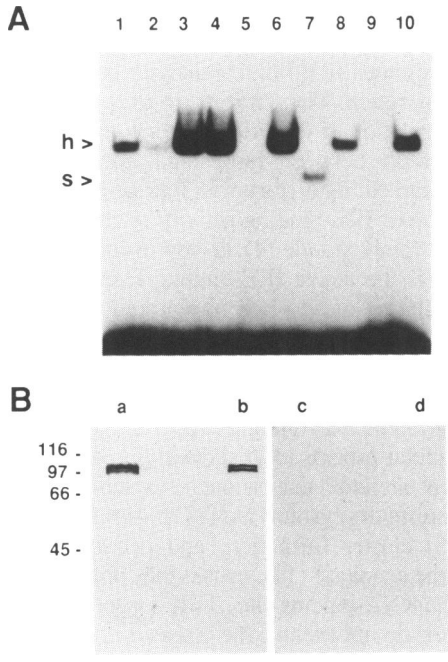

Figure 1. Expression of recombinant human IRF from baculovirus-vectors in Sf9 cells. **A.** IRE-binding activity was determined by a gel retardation assay with ^{32}P-labelled IRE of human ferritin H chain (transcript of pSPT-fer; ref. (4) and a 1:200 dilution of cell extracts from rec-hIRF virus-infected Sf9 cells (lanes 1–6), a 1:8 dilution of uninfected cells (lane 7) or IRF from human placenta (lanes 8–10). Lane 1: incubation of rec-hIRF virus-infected cell extract with 0.1 ng IRE RNA; lane 2: as in lane 1, but after preincubation of recombinant IRF with the sulfhydryl oxidizing reagent diamide at 1.5 mM; lane 3: as in lane 2, but followed by reduction of oxidized IRF with 2% 2-mercaptoethanol (2-ME); lane 4: as lane 1, after *in vitro* reduction with 2% 2-ME; lane 5: 2-ME treated IRF in the presence of 60 ng unlabelled competitor pSPT-fer RNA; lane 6: 2-ME treated IRF in the presence of 60 ng 4F2 antigen heavy chain RNA; lane 7: uninfected Sf9 extract incubated with the probe; lane 8: incubation of probe with 1 μg human placenta extract; lane 9; as in 8, but in the presence of 60 ng unlabelled pSPT-fer RNA; and lane 10: in the presence of 60 ng 4F2 antigen heavy chain RNA. **B.** Identification of recombinant IRF in Western blots. Extracts of rec-hIRF virus infected cells (10 *mg* protein) (lanes *a* and *c*) or 0.5 *mg* purified IRF from human placenta (lanes *b* and *d*) were resolved in an 8% SDS-polyacrylamide gel and analysed after blotting. In lanes *a* and *b*, the blot was incubated with a rabbit antiserum raised against the human IRF NH_2-terminal peptide. The control blot (lanes *c* and *d*) was incubated with preimmune serum from the same rabbit. IRF was revealed by binding of alkaline phophatase-conjugated anti-rabbit IgG antibody and a corresponding colour reaction. Based on molecular weight markers (indicated on the left), IRF migrated as a doublet at approximately 100 kDa.

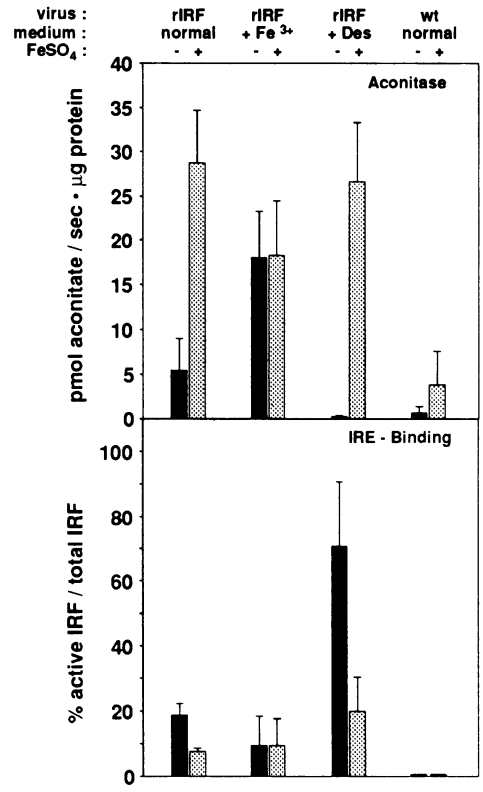

Figure 2. Aconitase and IRE-binding activities of recombinant human IRF. Sf9 cells were infected with rec-hIRF (rIRF) or wild-type (wt) virus and cultured for 48 h in normal TC100 medium, iron-rich medium (+Fe^{3+}) or low iron-medium (+Des) as indicated. Cytoplasmic cell extracts were passed over a Heparin-Sepharose column. For each condition, aliquots were analysed without any treatment (black bars), or after incubation *in vitro* with 100 μM $FeSO_4$, 100 μM Na_2S, and 10 mM dithiotreitol (dotted bars). Top panel: Aconitase activity was measured by using *cis*-aconitate as a substrate and following the change in absorbance at 240 nm (see Materials and Methods). Bottom panel: IRE-binding activity was determined by the gel retardation assay as described in Fig.1. The percentage of active IRF was defined as the ratio of shifted probe without and with preincubation of extracts in 2% 2-ME. Error bars indicate the standard deviation from at least 3 independent experiments.

(Fig.1B). This result supports our previous conclusion that post-transcriptional or post-translational processing most likely accounts for the IRF heterogeneity.

IRF synthesized in the absence of iron has a high IRE-binding activity

To study modulation of rec-hIRF by iron, virus-infected Sf9 cells were cultured under various conditions of iron in the medium and analysed at different time-points for rec-hIRF/IRE complex formation in the absence or presence of 2% 2-ME. Rec-hIRF was measurable after 24 h and maximal at 48 h independently of whether the culture medium was normal, or supplemented with 20 *mg*/ml ferric ammonium citrate or 50 μM desferrioxamine. The total amount of rec-hIRF did not vary significantly with different media indicating that rec-hIRF synthesis and stability were not markedly influenced by iron levels (results not shown).

Concerning the role of a putative Fe-S cluster in IRF, it is relevant that rec-hIRF was strongly modulated in its IRE-binding activity by the various culture conditions. As previously observed for cell lines (4,16), IRE-binding activity was high after iron-depletion, but low in the presence of iron salt-containing medium (Fig.2, *lower panel*). This paralleled the regulation of endogenous insect IRF (not shown). We can therefore infer that the RNA-binding activity of rec-hIRF was responding normally to iron levels. However, rec-hIRF, unlike endogenous IRF, was definitely synthesized *de novo*. We conclude that rec-hIRF made in the presence of desferrioxamine cannot have incorporated an Fe-S cluster, and that this apoprotein is constitutively active in IRE-binding. Indeed, as seen in Fig.2 (*lower panel*), additional treatment of the apoprotein with 2% 2-ME did not significantly increase the percentage of high affinity IRE-binding molecules. This observation supports our previous conclusion about *in vitro* transcribed/translated IRF. When made in the test-tube in the presence of air (conditions known to inhibit Fe-S cluster formation (28,33)), *in vitro* translated IRF was fully competent in RNA-binding and presumably lacked an Fe-S cluster (21).

In vitro conversion of IRE-binding apo-IRF to an Fe-S protein with aconitase activity

The iron-dependent modulatory switch inactivating the RNA-binding capacity of IRF must represent a post-translational modification, since the total amount of IRF (as measured by IRE-binding activity after 2% 2-ME treatment or by Western blotting (B.Henderson, unpublished results)) is virtually constant under different conditions of iron supply, both in normal proliferating cells (4,16) and in the present expression system. Thus, if the hypothesis of a switch between a high affinity IRE-binding conformation and a [4Fe-4S]-cluster aconitase form is correct (18,29), one would predict that the two IRF-associated activities might be mutually exclusive. In order to test this hypothesis, we compared aconitase activity in cytoplasmic extracts of virus-infected Sf9 cells. Under normal medium conditions only a small background aconitase activity was present in wild-type virus infected cells, but the amount was significantly (8-fold) higher in cells infected with the rec-hIRF virus (Fig.2, *upper panel*). This activity was further increased when infected Sf9 cells were cultured in medium with iron salts, but decreased after iron-chelation. Thus the results indicated a modulation of aconitase activity exactly opposite to the change in IRE-binding activity (Fig.2, *lower panel*).

The present findings are compatible with the hypothesis of an iron-induced switch from the apoprotein to an Fe-S cluster containing rec-hIRF with aconitase activity but lacking IRE-binding activity. If this were true, it seemed possible to generate an Fe-S cluster *in vitro* by methods that have previously been applied to reconstitute the [4Fe-4S] cluster of mitochondrial aconitase (33). We therefore treated each of the cytoplasmic extracts anaerobically under argon with 100 μM $FeSO_4$, 100 μM Na_2S and 10 mM DTT (33). Subsequently, both the aconitase and IRE-binding activities were measured. As shown in Fig.2, there was a clear enhancement in the aconitase activity of extracts with apoprotein (cells grown under limiting iron conditions), but no such change was found where iron had been supplied in excess during rec-hIRF biosynthesis. The change was particularly striking in extracts of cells grown in desferrioxamine. Here, formation of Fe-S clusters reversed almost entirely the endogenously acquired IRF activities: IRE-binding was reduced to less than 20%, and aconitase activity increased from background levels to a value similar to the one in cells grown in high iron-medium.

The apparent K_m of the recombinant aconitase for *cis*-aconitate was 9.2 μM, a value which is in the range of previously reported values for beef liver cytoplasmic aconitase (39), but somewhat lower than those for mitochondrial aconitases (40). We conclude that conditions known to reconstitute Fe-S clusters in mitochondrial aconitase are also effective for rec-hIRF and transform the apo-IRF into an enzymatic form with aconitase activity. Simultaneously, the formation of an Fe-S cluster in IRF is inhibitory to IRE-binding.

CONCLUSIONS

The data presented in this study extend previous observations that IRF can function both as an aconitase as well as an RNA-binding protein (19,29), and that these activities are mutually exclusive. The switch from the IRE-binding form to the aconitase form in our study corresponds to the *de novo* formation of an Fe-S cluster. This is supported by the finding that biosynthesis in high iron-medium results spontaneously in maximal aconitase activity. As a consequence we can suggest that normal cells might set their endogenous IRE-binding activity based on the relative amount of apoprotein. Thus, it remains to be seen whether iron-dependent regulation of the Fe-S cluster in cells corresponds in fact to the ratio between holo- and apo-IRF. A previous observation from our laboratory with murine L cells had indicated that acquisition of IRE-binding activity after iron-chelation was inhibited by cycloheximide (4). It now would seem compatible that apo-IRF is the active IRE-binding form in cells, and that *de novo* translation of apo-IRF is required to raise its relative abundance. The formation of the [4Fe-4S] cluster containing aconitase would occur only when iron is present in sufficient quantities and may appear as a maturation process of the protein. This inactivation of IRE-binding properties by iron is known to be a rapid cellular process (4) and clearly involves a spontaneous and permanent alteration that cannot be explained by the oxidation of sensitive sulfhydryl groups alone (16,41). It must be assumed that [4Fe-4S] cluster formation, and perhaps the binding of substrate to the aconitase (19), causes the conformational change that makes the RNA-binding site of IRF inaccessible. Our present data, however, do not exclude the possible existence of a cellular mechanism which converts the holo-IRF with its [4Fe-4S] cluster back to the apoprotein or a form with a different Fe-S cluster composition. If such a process exists, then we would have to predict that like induction of IRE-binding activity, the conversion is slow, suggesting rate-limiting steps that may require enzyme catalysis. Further biophysical and *in vitro* reconstitution experiments should provide clues to these questions.

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