Converse Modulation of IRP1 and IRP2 by Immunological Stimuli in Murine RAW 264.7 Macrophages*

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Iron regulatory proteins (IRP1 and IRP2) are two cytoplasmic RNA-binding proteins that control iron metabolism in mammalian cells. Both IRPs bind to specific sequences called iron-responsive elements (IREs) located in the 3′ or 5′ untranslated regions of several mRNAs, in particular mRNA encoding ferritin and transferrin receptor receptor. In this study, we followed in parallel the in vivo regulation of the two IRPs in physiologically stimulated macrophages. We show that stimulation of mouse RAW 264.7 macrophage-like cells increased IRP1 IRE binding activity 4-fold, whereas IRP2 activity decreased 2-fold 8 h after interferon-γ lipopolysaccharide treatment. Decrease in IRP2 was not due to nitric oxide (NO) production and did not require de novo protein synthesis. Our data therefore indicate that the two IRPs can be inversely regulated in response to the same stimulus. In addition, the effect of endogenously produced NO on IRP1 was further characterized in an activated macrophage/target cell system. We show that NO acts as an intercellular signal to increase IRP1 activity in adjacent cells. As the effect was detectable within 1 h and did not require de novo protein synthesis, this result supports a direct action of NO on IRP1.

Iron regulatory proteins (IRP1 and IRP2) are cytoplasmic trans-regulators that modulate expression of several mRNA containing one or several regulatory sequences in their untranslated regions termed iron-responsive elements (IREs) (1, 2). An IRE has been located at the 5′ end of the mRNA of ferritin H- and L-chain and erythroid 5-aminolevulinate synthase and, as revealed more recently, on mitochondrial aconitase and subunit b of Drosophila melanogaster succinate dehydrogenase mRNA (3–8). IRE/IRP interaction in the 5′ untranslated region inhibits mRNA translation (9–14). Five IRE sequences have also been located in the 3′ untranslated region of transferrin receptor mRNA and, in that case, IRE/IRP interaction confers stability against endonucleolytic cleavage (15, 16). Thus, IRPs control uptake, storage, and intracellular metabolism of iron through their IRE binding activity.

Two IRPs called IRP1 and IRP2 have been characterized and cloned in several cell types (12, 17, 18). IRP1 exhibits considerable sequence homology with mitochondrial aconitase and has been identified as the cytoplasmic aconitase (19–21). The two activities of this protein are mutually exclusive. The form that presents aconitase activity converting citrate into isocitrate in the cytosol possesses an intact 4Fe-4S cluster, whereas the IRE-binding form lacks it (22, 23). Thus, the status of the Fe-S cluster is crucial to determination of IRP1 function. In iron-repleted cells, holoirP1 predominates and exhibits aconitase activity. Conversely, apoIRP1, which binds IRE with high affinity, is the major form in iron-depleted cells. This first discovered regulation of IRP1 by iron led to the suggestion that a switch between the holo- and apoprotein without any change in IRP1 protein levels explains the regulation (2). In vitro, IRP2 binds IRE sequences of ferritin and transferrin receptor (TF-R) mRNA with similar affinity to IRP1 (17, 24). This second IRP shares 61% amino acid identity with IRP1, but despite conservation of the cluster-ligating cysteines at the active site, IRP2 is unable to assemble an Fe-S cluster in vitro and therefore is unable to exhibit aconitase activity (25). The primary sequences of IRPs principally differ from each other by the insertion of a 73-amino acid sequence in the N terminus domain of IRP2. It has been shown that this cysteine-rich sequence is required to regulate IRP2 expression by iron (26). Unlike the regulation of IRP1 by iron, loss of IRE binding of IRP2 is due to its own degradation via the proteasome pathway (26, 27). Despite the rising interest in IRP2 displayed for the last few years, the questions as to why two IRPs exist and about their respective roles are still puzzling.

Apart from the regulation mediated by iron availability, NO was the first physiological molecule found to be able to convert IRP1 from aconitase to the IRE-binding form in macrophages and non-macrophage cells (28, 29). It has also been established that endogenous NO production was able to repress ferritin mRNA translation and to stabilize transferrin receptor mRNA (30). IRP1 activation has also been reported in fibroblasts (Ltk-) exposed to H2O2 and chemicals able to release NO (31). The effect of H2O2 is fast and indirect and, in contrast to our previous proposal (32) it was stated that the effect of NO is slow and analogous to that exhibited by iron chelators (31). In the same set of experiments, it was shown that IRP2 of fibroblasts Ltk- also exhibited higher IRE binding activity after treatment by NO-releasing drugs (31). These results conflicted with those published by another group showing that neither endogenous NO production by the hepatoma cell line FTO2B nor its exposure to exogenous NO influences the IRE binding activity of IRP2 (33).

To clarify the regulation of the two IRPs by NO (or congener), we have first studied the modulation of their IRE binding activity in RAW 264.7 macrophages immunologically stimulated for NO production. We show that endogenous TNF is required for NO-dependent activation of IRP1 RNA binding activity. Further, we report that unlike IRP1, IRP2 loses its IRE binding activity independently of the production of NO. We have also carefully investigated the time course of the effect of
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**EXPERIMENTAL PROCEDURES**

**Reagents**—Murine recombinant interferon-γ (specific activity: 2 × 10⁷ units/mg) was produced by Genentech and provided by Dr. G. R. Adolf (Boehringer Ingelheim, Vienna, Austria). Rabbit anti-TNF-α antibodies (neutralizing titer was 1 to 25,000) were produced in our laboratory by repeated inoculations of rabbits with pure Mu-rTNF. N⁵-monomethyl-l-arginine, Escherichia coli lipopolysaccharide (serotype 0111: B4), cycloheximide, and desferrioxamine were purchased from Sigma.

**Macrophage and C58 Pre-T Cell Lines**—The macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection and cultured at 37 °C in a 5% CO₂ atmosphere in high glucose Dulbecco’s modified Eagle’s medium supplemented with 5% endotoxin low fetal calf serum. RAW 264.7 macrophages were stimulated for 16 h with 10 units/ml IFN-γ and 50 ng/ml LPS for NO synthase 2 (NOS2) induction. Cells were exhaustively washed with phosphate-buffered saline to remove the stimulating agents. Under these conditions, it has been shown that macrophages are able to produce NO for 24 h without further stimuli. Pre-T C58 target cells were added to the NO-producing macrophage monolayers at an effector to target ratio of 1. When required, release of the NOs2 products was blocked by addition of 1 mM G-monomethyl-L-arginine, and 100 μM desferrioxamine, or a rabbit anti-TNF-α antibody was added to the culture medium.

**Cell Cocultures**—RAW 264.7 macrophages were stimulated for 16 h with 10 units/ml IFN-γ and 50 ng/ml LPS for NO synthase 2 (NOS2) induction. Cells were exhaustively washed with phosphate-buffered saline to remove the stimulating agents. Under these conditions, it has been shown that macrophages are able to produce NO for 24 h without further stimuli. Pre-T C58 target cells were added to the NO-producing macrophage monolayers at an effector to target ratio of 1. When required, release of the NOS2 products was blocked by addition of 1 mM N⁵-monomethyl-l-arginine, and de novo protein synthesis was inhibited by 800 ng/ml cycloheximide.

**Preparation of Cell Extracts**—Cells (1 × 10⁶/ml) were treated with 0.007% digoxin for 10 min at 4 °C in 0.25 M sucrose, 100 mM HEPES, pH 7.2, to lyse cells without damaging mitochondria. The resulting lysate was then centrifuged at 75,000 rpm for 20 min in a Beckman TL 100 ultracentrifuge to spin down any particulate material. Cytosolic extracts (0.5 mg/ml) were aliquoted and kept at −80 °C until use for gel mobility shift assay and aconitase measurement (34). In some experiments, cells (1 × 10⁶/ml) were also treated with 0.5% Nonidet-P40 for 5 min at room temperature. The lysates were then centrifuged at 10,000 × g for 15 min at 4 °C. Supernatants were directly analyzed for gel mobility shift assay or kept at −80 °C.

**Aconitase Activity**—Aconitase activity was measured spectrophotometrically by following the disappearance of cis-aconitate at 240 nm as described previously (34, 36). Briefly, the reaction volume (900 μl) contains 60 μg of cytoplasmic extract in 100 mM Tris-HCl pH 7.4, and kinetics was started by the addition of 15 μl of 20 mM cis-aconitate. Units represent nanomoles of substrate consumed/min at 37 °C (ε = 3.6 mm⁻¹ cm⁻¹).

**Measurement of Nitrite**—The formation of nitrite, one of the end products of nitric oxide, was determined spectrophotometrically in the culture medium at 540 nm, using the Griess reagent containing 0.5% sulphanilamide and 0.05% N-(1-naphthyl)ethylenediamine hydrochloride in 45% acetic acid.

**Measurement of Protein Synthesis**—Cells were cultivated with various concentrations of cycloheximide in leucine-starved RPMI medium for 24 h. Then, cells were pulse-labeled with 4 μCi/ml of L-[3H]leucine for 2 h. After incorporation of leucine, cells were washed ten times and

![Fig. 1. Endogenous TNF participates in IFN-γ-mediated activation of IRP1.](image1)

**Fig. 1.** Endogenous TNF participates in IFN-γ-mediated activation of IRP1. RAW 264.7 cells were stimulated with 400 units/ml IFN-γ alone or with anti-TNF-α antibodies. After 18 h, nitrite production was measured in the culture medium of control and treated cells using Griess reagent. Cell cytosols were tested for IRP/IRE binding by electromobility shift assay. In a parallel experiment, cell extracts were treated with 2% 2-ME before binding to a ³²P-IRE probe.

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IRP1 and IRP2 Are Conversely Regulated in Activated RAW 264.7 Macrophages—As shown previously, IFN-γ-treated macrophages express NOS2, and release of endogenous NO can be followed by nitrite accumulation in culture medium. As a consequence, IRP1 exhibits high affinity for IRE (28, 29) (Fig. 1, lane 2). Here we show that incubation of cells with anti-TNF-α antibodies before treatment with IFN-γ maintains IRE binding activity of IRP1 at a basal level (Fig. 1, compare lanes 1–3). It is likely that production of endogenous TNF-α, by boosting NOS2 expression, allows activation of IRP1 in concert with IFN-γ. In unstimulated RAW 264.7 cells, IRP2 expresses constitutive basal IRE binding activity as high as that of IRP1 (Fig. 2A, lane 1) and, in agreement with previous data, is markedly enhanced after treatment with 2% 2-ME (39). As expected, in response to overnight stimulation by IFN-γ, IRE binding activity of IRP1 was increased, but in striking contrast, that of IRP2 decreased and was not fully restored by 2-ME (Fig. 2A, lane 2). Furthermore, when associated with IFN-γ, LPS, which maximally induces NOS2 in murine macrophages, enhanced IRE binding activity of IRP1 and amplified the loss of IRE binding activity of IRP2 (Fig. 2A, lane 3). As evidenced by PhosphorImaging analysis, activation of IRP1 and inactivation of IRP2 correlated with NO synthesis as measured by nitrite release in the culture medium (Fig. 2B). Despite loading of an identical amount of protein on the gel, we consistently observed that IRE binding activity of IRP1 after addition 2% 2-ME was less in stimulated cells as compared with control cells (Fig. 2A, lower panel, compare lanes 2 and 3 to lane 1).

It is well known that IRP1 and IRP2 activities are enhanced following treatment of cells with the iron chelator, desferrioxamine. To see whether IFN-γ-mediated down-regulation of IRP2 can influence the iron-dependent pathway, cells were cultured in the presence of both IFN-γ and desferrioxamine. As shown in Fig. 3, IRP2 of RAW 264.7 cells stimulated by IFN-γ and LPS, Cells were grown in the presence (lanes 8–14) or absence (lanes 1–7) of 10 units/ml IFN-γ and 50 ng/ml LPS for 1–24 h. A, NO production was measured by assaying nitrite in culture supernatants of stimulated and unstimulated cells. B, cell extracts were prepared as described under “Experimental Procedures,” and equal amounts of protein (3 µg) were treated with or without 2% 2-ME before incubation with a 32P-labeled IRE probe and electrophoresis of the RNA-protein complexes on a 6% native polyacrylamide gel. As signals given by IRP2 are weaker than those of IRP1 in RAW 264.7 cells, they were amplified 7.5 times relative to those of IRP1.

To clarify the mechanism by which immunological stimuli modulate IRP2, we investigated the kinetics of IRP2 modulation in IFN-γ/LPS-treated cells in parallel to that of IRP1. When IRF binding of IRP1 exhibited nearly a 4-fold increase after 8 h of treatment, IRP2 activity decreased 2-fold. Converse modulation of both IRPs occurred as soon as NOS2 was expressed as testified by nitrite production. In parallel experiments, we routinely treated cytoplasmic extracts with 2% 2-ME prior to the binding step to fully express the IRE binding activity of both IRPs. We observed that full binding activity of both IRPs of IFN-γ/LPS-treated cells decreased from the first hour (Fig. 4, compare lanes 1–7 with 8–14). To determine if decrease in IRP2 binding activity was due to NO production, IFN-γ- or IFN-γ/LPS-treated RAW 264.7 macrophages were stimulated in the presence of L-NMA, a NOS inhibitor. As shown in Fig. 5, reduction of NO production led to a decrease in IRP1 binding to IRE but had no effect on IRP2 activity. However, as a competitive inhibitor, L-NMA was unable to com-
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Two cytoplasmic proteins, IRP1 and IRP2, have a key role in the regulation of iron metabolism in mammals, by binding specifically to stem-loop structures of several mRNAs. IRP1 is an Fe-S protein whose RNA-binding activity is regulated post-translationally in response to environmental signals: intracellular iron level, nitric oxide synthesis, oxidative stress, and degree of phosphorylation (1, 2, 40, 41). Recent evidence indicates that IRP2 is also regulated by iron but by a different mechanism. Indeed, in response to iron accumulation, IRP2 is degraded via the proteasome pathway (26, 42). In this paper, we report that physiological stimulation of macrophages modulates RNA binding activity of IRP1 and IRP2 conversely. As shown earlier, the pathway that allows cytokines to modulate IRP1 activity relies on NO synthesis. IFN-γ is a major positive

Figure 5. IRE binding activities of IRP1 and IRP2 in RAW 264.7 macrophages stimulated in the presence of a NOS inhibitor. RAW 264.7 macrophages were exposed for 16 h to 400 units/ml IFN-γ (left panel) or to 10 units/ml IFN-γ and 50 ng/ml LPS (right panel) in the presence or absence of 1 mM L-NMA. NO production was measured by assaying nitrite in cell supernatants of stimulated and unstimulated cells. Cytoplasmic extracts were prepared as described under “Experimental Procedures” and tested for IRE binding activities of IRP1 and IRP2 by electromobility shift assay in the presence or absence of 2% 2-ME.

DISCUSSION

Two cytoplasmic proteins, IRP1 and IRP2, have a key role in the regulation of iron metabolism in mammals, by binding specifically to stem-loop structures of several mRNAs. IRP1 is an Fe-S protein whose RNA-binding activity is regulated post-translationally in response to environmental signals: intracellular iron level, nitric oxide synthesis, oxidative stress, and degree of phosphorylation (1, 2, 40, 41). Recent evidence indicates that IRP2 is also regulated by iron but by a different mechanism. Indeed, in response to iron accumulation, IRP2 is degraded via the proteasome pathway (26, 42). In this paper, we report that physiological stimulation of macrophages modulates RNA binding activity of IRP1 and IRP2 conversely. As shown earlier, the pathway that allows cytokines to modulate IRP1 activity relies on NO synthesis. IFN-γ is a major positive

Complete inhibition of expression of NOS2 as indicated by residual nitrite production. Because IRP2, at least in RAW 264.7 cells, is more sensitive to post-translational redox modulation than IRP1 (39), we cannot exclude the possibility at this stage that low output of NO is sufficient to down-regulate IRP2. To see whether modulation of IRP2 activity requires de novo protein synthesis, we assessed IRP modulation in IFN-γ/LPS-treated cells in the presence of cycloheximide, a translation inhibitor. In the presence of cycloheximide, stimulated cells did not produce significant amounts of nitrite before 24 h (Fig. 6A). Under these conditions, IRP1 IRE binding activity was not increased whereas that of IRP2 decreased to the same extent as in cells stimulated in the absence of cycloheximide (Fig. 6B). From these two sets of experiments, we conclude that IRP2 expression or activity is affected in response to immunological stimuli, i.e. IFN-γ and LPS, independently of NO.

Activation of IRP1 by NO Proceeds by a Fast Response Mechanism—In a previous paper (28), we showed that kinetics of IRP1 activation in primary macrophages is superimposable on that of endogenous NO production, which suggested that NO (or some species derived from NO) reacts rapidly and directly with IRP1. Yet results of another study (31) indicated that NO exerts a delayed response on IRP1 activity and may therefore affect iron availability rather than targeting the protein. Accordingly, to shed light on this controversial matter, we reinvestigated this question in greater detail. To avoid the lag necessary to induce NOS2 activity, we performed a coculture study. First, macrophages were maximally activated for NO production for 18 h and after exhaustive washings C58 lymphoma target cells, which are unable to produce NO, were added to the macrophage monolayer. RNA binding activity of C58 target cells was then measured versus time. Significant induction of IRE binding activity of IRP1 appeared in C58 cells within 1 h (Fig. 7A) and was correlated with release of nitrite from effector cells (Fig. 7B, upper panel). Meanwhile aconitase activity of IRP1 rapidly decreased in response to NO production (data not shown). In fact, IRP2 expression, which was low at the beginning of cell culture, progressively increased and was maximal after 8 h (see Fig. 7A and B, lower panel). Interestingly, we noted that IRP2 expression in C58 cells cocultured with NO-releasing macrophages, but in the absence of immunological stimuli, was unchanged (Fig. 7B, lower panel). As expected, IRE binding by IRP1 in target cells was prevented when the macrophage monolayer was cultured in the presence of L-NMA, and notable aconitase activity was measured (Fig. 8). However, induction of IRP1 IRE binding activity was unaffected in the presence of cycloheximide, showing that modulation of IRP1 did not require de novo protein synthesis (Fig. 8).

Figure 6. Effect of cycloheximide on IRE binding activities of IRP1 and IRP2 in IFN-γ/LPS-stimulated RAW 264.7 cells. Cells were stimulated for 1–24 h with 10 units/ml IFN-γ and 50 ng/ml LPS in the presence or absence of 800 ng/ml cycloheximide (CHX). A, NO production was measured by assaying nitrite in the culture medium of stimulated and control cells using the Griess reagent. B, cell extracts were prepared as described under “Experimental Procedures,” and equal amounts of protein (3 μg) were treated with or without 2% 2-ME before performing an RNA binding assay. Signals of IRP2 were 10-fold amplified relative to those of IRP1.
regulator (28, 29), whereas interleukin-4 and interleukin-13 down-regulate IRP1 RNA binding activity (40). In this report, we show that endogenous production of TNF, an important autocrine inducer of NO synthase, is crucial for IFN-γ-mediated activation of IRP1. It is likely that the part played by inflammatory cytokines in iron availability has far reaching implications for cell-mediated responses to microbial infections or to oxidative stress.

Whether or not immunological stimuli like cytokines can also drive IRP2 is still an open question. Indeed, conflicting results have been reported concerning the effect of NO on IRP2 (31, 33). It has been reported that exposure of fibroblasts to NO-releasing drugs increases RNA binding activity of IRP2, whereas an independent study concluded that endogenous production of NO by hepatoma cells or their treatment by exogenous NO does not have any effect on IRP2 (31, 33). To perform this study, we deliberately chose two cell lines that express significant basal IRP2 activity, i.e. murine RAW 264.7 macrophages and rat lymphoma C58 cells. In RAW 264.7 cells, we observed that in contrast to IRP1, IRP2 activity dropped upon exposure to IFN-γ. It is noteworthy that this effect was strong enough to counteract the up-regulation of IRP2 classically observed in cells depleted of iron after desferrioxamine treatment.

As IRP2 possesses redox-active cysteines (25, 39, 44), which, as previously pointed out by our group and others, are crucial for its activity (39, 40, 45), it was expected that NO would be the effector molecule responsible for the down-regulation. However, our coculture system clearly demonstrated that IRP2 is controlled independently of NO endogenously produced by macrophages. Overall, these results are reminiscent of data reported by Phillips et al. (33) showing that endogenous NO released by IFN-γ/LPS-stimulated hepatoma cells does not activate IRP2. However, this is the first report that IRP1 and IRP2 are regulated in opposite ways.

As reported by Schalinske and Eisenstein (40), RNA binding capacity of IRP2 may depend on phosphorylation. Indeed, in HL-60 promonocytic cells, phosphorylation of IRP2 stabilizes a reduced (i.e. active) form, and as a consequence, Tf-R mRNA level is increased (40). It is therefore possible that a modification of the kinase/phosphatase balance within cells could favor formation of a latent form of IRP2 under our experimental conditions. Alternatively, the down-regulation of IRP2 in activated macrophages described in this paper may be due to accelerated degradation of the protein. We also point out that translation inhibition, in IFN-γ/LPS-stimulated cells, prevents IRP1 activation without altering loss of IRP2 activity. This result stresses an intrinsic role of IRP2 in cells and suggests that IFN-γ/LPS regulation is not mediated by iron because iron-dependent degradation of IRP2 generally requires de novo protein synthesis (12, 26, 46). Altogether, these results are in keeping with a recent observation showing that in cells lacking IRP1, IRP2 can mediate IRE-dependent regulation of cellular iron by itself (47).

Our observation that immunological/inflammatory stimuli decrease RNA binding by IRP2 may reconcile some published divergent data. Indeed, cells exposed to NO-releasing drugs or overexpressing NO synthase logically exhibit higher expres-
tension of T-R mRNA (31, 33, 48, 49). Yet it was also reported in the same papers that cells stimulated by IFN-γ and LPS, despite production of endogenous NO and subsequent activation of IRP1, unexpectedly exhibited a low T-R mRNA level (31, 33). The most likely interpretation of these data was that some ill-defined negative effect on T-R mRNA expression resulting from immunological stimulation, overcomes the effect of the NO/IRP1 pathway. Besides, Cairo and Pietrangolo (50) using an experimental model of inflammation have reported that IRP2 rather than IRP1 is responsible for modulation of T-R mRNA stabilization (50). At this juncture, it is difficult to understand how IRP2 could out-compete IRP1 in binding T-R IREs. One simple explanation is that an active IRE-binding form of IRP2 is more abundant in some tissues. If NOS2 is not induced in these tissues, IRP2 activity may prevail. Response would thus be tissue-specific. An alternative interpretation is that IRP2 expresses greater affinity for T-R IREs. A previous report (12) addressed this question and revealed that both IRPs are utilized in vivo, one which can. This means that the biochemical NO-dependent process that activates the protein was completed. Whether it one which can. This means that the biochemical NO-dependent process that activates the protein was...
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