Iron regulatory proteins (IRP1 and IRP2) are redox-sensitive RNA-binding proteins that modulate the expression of several genes encoding key proteins of iron metabolism. IRP1 can also exist as an acatase containing a [4Fe-4S] cluster bound to three cysteines at the active site. We previously showed that biosynthesis of nitric oxide (NO) induces the transition of IRP1 from acatase to apoprotein able to bind RNA. This switch is also observed when cytosolic extracts are exposed to NO donors. However, the activation of IRP1 under these conditions is far from maximal. In this study we examined the capacity of physiological reducing systems to cooperate with NO in the activation of IRP1. Cytosolic extracts from the macrophage cell line RAW 264.7 or purified IRP1 were incubated with NO donors and subsequently exposed to glutathione or to thioredoxin (Trx), a strong protein disulfide reductase. Trx was the most effective, inducing a 2-6-fold enhancement of the RNA binding activity of NO-treated IRP1. Furthermore, the effect of NO on IRP1 from cytosolic extracts was abolished in the presence of anti-Trx antibodies. We also studied the combined effect of NO and Trx on IRP2, which exhibits constitutive RNA binding activity. We observed an inhibition of IRP2 activity following exposure to NO donors which was restored by Trx. Collectively, these results point to a crucial role of Trx as a modulator of IRP activity in situations of NO production.

Iron regulatory proteins (IRP1 and IRP2) are trans-regulators that post-transcriptionally control the expression of key proteins of iron metabolism by binding to hairpin loop structures named iron-responsive elements (IREs) on their mRNA (1–3). IRP binding to IREs in the 5′-untranslated region of ferritin and erythroid aminolevulinate synthase results in inhibition of translation (4–8), whereas binding of IRPs to multiple IREs in the 3′-untranslated region of transferrin receptor mRNA confers stability against endonucleolytic degradation (9, 10).

IRP1/IRE binding activity is regulated by cellular iron levels. In iron-depleted cells, high affinity of IRP1 for IREs diminishes iron storage in ferritin and induces transferrin receptor synthesis, thereby enhancing iron uptake via receptor-mediated endocytosis of serum transferrin. Conversely, in iron-loaded cells IRP1 fails to bind RNA but functions as an acatase, converting citrate into isocitrate in the cytosol (1, 2, 11). IRP1 is thus a bifunctional protein whose activities appear to depend on the presence or absence of an intact [4Fe-4S] cluster (12) which is ligated by three cysteines at the active site (13, 14). The switch of IRP1 from acatase to IRE-binding protein has been explained by the removal of the Fe-S cluster (2, 12, 13), but the mechanisms that underlie the insertion and extrusion of the cluster need to be delineated. The second IRP discovered, IRP2, shares 61% amino acid identity with IRP1, differing by the insertion of a 73-amino acid sequence rich in cysteine residues (15, 16). IRP2 is also regulated by iron, but unlike IRP1, it is rapidly degraded in cells that are iron-replete. Although IRP2 contains the three conserved cysteine ligands for the [4Fe-4S] cluster of IRP1, it seems not to have such a cluster, and it lacks acatase activity (17–19).

It is now clear that signals other than iron can regulate IRP1 and IRP2 activities and thus modulate cellular iron metabolism. Indeed, previous studies showed that biosynthesis of NO, oxidative stress, and phosphorylation increase IRP1/IRE binding in different cell types (20–26). As regards stimulation by NO, previous results from our laboratory showed that IRE binding by IRP1 is almost maximal when NO synthase 2 is induced in macrophages by stimulation with both interferon-γ and lipopolysaccharide (20). The conversion of IRP1 from apoprotein can also be elicited in vitro by exposing cell cytoplasmic extracts to NO-releasing chemicals. However, despite high concentrations of NO donor, we consistently observed that IRP1 activation under these conditions was far from that observed in intact cells (27). Moreover, exposure of purified recombinant IRP1 to NO donors or to NO gas resulted in an even weaker IRP1 activation (20). It seems therefore that simplification of the experimental system results in the loss of a cellular component that plays a part in NO-mediated IRP1 activation. Moreover, it is also worth recalling that IRP1 and IRP2 are redox-sensitive proteins. Indeed, it has long been known that high concentrations of 2-mercaptoethanol (2-ME) fully activate IRP1 in vitro (28). Conversely, IRP1 is inhibited in its ability to bind IREs following treatment with the sulfhydryl-modifying oxidant diamide or by alklylation (13, 14, 28, 29). Furthermore, site-directed mutagenesis studies have shown that Cys457, one of the three cysteines that hold the iron-sulfur cluster, must be reduced to allow IRP1 to bind IREs (13, 14). As regards IRP2, its regulation mainly proceeds through protease-dependent degradation (16, 30), but recent evidence indicates that it is also activable to some extent by reducing agents (26, 31–33). It is thus likely that endogenous redox systems may play a part in the process of IRP activation. Here, we first investigated the capacity of endogenous reducing systems to cooperate with NO in IRP1 activation. We focused on two major physiological reducing systems, i.e. reduced gluta-
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thione and the thioredoxin (Trx/Trx reductase system. Trx is a 12-kDa protein present in many species from plants to mammals which functions as a major protein disulfide reductase within cells (34). We found that reduced Trx strongly enhances the RNA binding activity of IRP1 exposed to NO donors. In addition, we observed an inhibition of IRP2 activity after exposure to NO in vitro, which was restored by the Trx system.

EXPERIMENTAL PROCEDURES

Materials—3-Morpholinosydnonimine hydrochloride (SIN-1) was synthesized by Cassella AG (Frankfurt, Germany) and kindly provided by J. Winicki, Hoechst, France. S-Nitroso-L-glutathione (GSNO) and diethylamine NONOate (DEANO) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Bovine thioredoxin reductase (TR), Escherichia coli thioredoxin (Trx), and goat anti-human Trx antibody were from IMCO Corp. (Stockholm, Sweden). Bovine erythrocyte Cu,Zn-superoxide dismutase (SOD), insulin, and all other chemicals were from Sigma.

Cell Culture—The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Paisley, UK) supplemented with 5% low endotoxin fetal calf serum.

Treatment of IRPs—Mitochondria-free cytosolic extracts were prepared as described previously (35). In some experiments, cytosol was concentrated by ultrafiltration on microconcentrators (Microsep, Ultrafiltron, Northborough, MA, molecular mass cut-off, 30 kDa). Cytosolic extract (0.5 mg/ml) was incubated with NO-generating compounds at 37 °C for different times in 100 mM HEPES, pH 7.4. In the case of SIN-1, experiments were always performed in the presence of 3000 units/ml SOD (referred to as SIN-1/SOD). Under these conditions the production of peroxynitrite, the coupling product of the reaction between NO and O2, was reduced by 95–98%, as testified by the rhodamine assay (36). Production of nitrite, one of the end products of NO, was followed to test the efficacy of the NO donor. Samples treated with NO donors were routinely desalted on P-6 Bio-Spin chromatography columns (Bio-Rad) prior to exposure to Trx. Reactions with the Trx system, containing various concentrations of Trx (oxidized form), 1 mM TR, and 0.4 mM NADPH, were performed at 37 °C for 20 min. These reaction conditions were sufficient to reduce oxidized Trx fully, as indicated by the insulin disulfide reduction assay.

Disulfide Reduction Assay—Disulfide reduction by thioredoxin was evaluated by the insulin reduction assay (37). Briefly, 0.4 mM NADPH, 1 mM TR, and different concentrations of Trx were incubated with 9 units/ml insulin in 2 mM EDTA, 80 mM HEPES, pH 7.6, for 20 min at 37 °C in a final volume of 120 μl. The reaction was terminated by addition of 0.5 ml of 0.4 mg/ml 5,5′-dithio-bis-(2-nitrobenzoic acid), 6 mM guanidine hydrochloride in 50 mM Tris-HCl, pH 8.0, and the absorbance at 412 nm was measured.

Treatment of Recombinant IRP1—The expression vector for recombinant IRP1 (rIRP1) has been constructed in the laboratory of Dr. Lukas Kuhn (Epalinges, Switzerland). rIRP1 was expressed as a glutathione S-transferase fusion protein in E. coli and purified on a glutathione-Sepharose column as described (13). Purified rIRP1 was incubated with NO donors in 10 mM HEPES, pH 7.6, 40 mM KCl, 3 mM MgCl2, and 5% glycerol. IRP1 activity was little enhanced by high (not shown). The modulation of IRP1 activity after exposure to NO donors was measured in the presence of 50 μM of (32P)CTP (NEN Life Science Products).

Electrophoretic Mobility Shift Assay—The IRP-IRE interactions were analyzed as described previously (4, 9) by incubating 2 μg of cytoplasminic protein with a molar excess (0.1 ng = 40,000 cpm) of 32P-labeled ferritin IRE probe in 20 μl of 10 mM HEPES, pH 7.6, 40 mM KCl, 3 mM MgCl2, and 5% glycerol. IRP-IRE complexes were resolved on 6% non-denaturing polyacrylamide gels. In parallel experiments, samples were treated with 2-ME (a final concentration of 2% prior to the addition of the RNA probe). The IRP-IRE complexes were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Electrophoretic mobility shift experiments were performed at least three times, and one representative experiment is shown.

Aconitase Activity Determination—The aconitase activity was measured spectrophotometrically by following the disappearance of cis-aconitate at 240 nm at 37 °C as described (35).

RESULTS

Activation of NO-treated IRP1 by Low Molecular Weight Cytosolic Factor(s)—We consistently observed that the IRE binding activity of IRP1 in cytosolic lysates was little enhanced by treatment with NO donors like SIN-1/SOD when lysates had been previously concentrated on a membrane with an Mf cut-off of 30,000 (Fig. 1, lane 2). Furthermore, when filtrate containing low molecular weight fractions (LF) was added back to the retentate (HF), the capacity to bind IREs after NO treatment was largely increased (lane 4). LF had little, if any, effect on HF from control cell extracts (lane 3). We therefore considered the hypothesis that LF contained a constitutive component with an Mf <30,000 able to activate an NO-primed pool of IRP1.

Sensitivity of IRP1 to GSH or Trx after Exposure to NO Donors—RAW 264.7 cell cytosol was first incubated with SIN-1 together with SOD. Cytosolic extracts were subsequently exposed to increasing concentrations of GSH or Trx in the presence of TR and NADPH and analyzed for IRP1 IRE binding activity. As expected from previous experiments (27), the incubation with SIN-1/SOD for 1 h induced an approximately 2-fold increase in the IRE binding activity of IRP1 relative to the control (Fig. 2, A and B, compare lanes 5 and 1). Addition of up to 1 mM reduced glutathione to cell lysate had only a marginal effect on IRP1 IRE binding. When lysate had been previously exposed to an NO-generating system, GSH had a slight but consistent effect at high concentration (≥1 mM) (Fig. 2A). In contrast, a strong enhancement of IRP1 activity (up to 6 times the control value) was observed when SIN-1/SOD-pretreated cytosolic extracts were exposed to increasing concentrations of Trx for 20 min (Fig. 2B, compare lanes 6 and 1). Under these conditions, control IRP1 kept its IRE binding capacity low (lanes 1–4) and aconitase activity high (not shown). The modulation of IRP1 activity by Trx after exposure to SIN-1/SOD required electron transfer from NADPH through TR, since omission of one com-
ponent of the thioredoxin system, either NADPH, or TR, or Trx itself, did not induce any further increase in IRP1/IRE binding (not shown). When Trx was added before or together with the NO donor, its contribution was not significant. The level of nitrite released was diminished, suggesting that added Trx partially scavenged NO.

In a set of time course experiments, cytosolic extracts were incubated with SIN-1/SOD for increasing periods before exposure to Trx. As can be seen in Fig. 3, IRE binding activity of SIN-1/SOD-treated IRP1, which reached 50% of the full activity expressed in the presence of 2% 2-ME within 30 min, was almost maximal (90%) when cell cytosol was subsequently exposed to 5 μM Trx. This enhancement of IRP1 activity induced by Trx in SIN-1/SOD-treated cytosolic extracts correlated with NO production which was assessed spectrophotometrically by measuring nitrite production (Fig. 3, bottom).

To investigate further if NO is a prerequisite in rendering IRP1 sensitive to Trx, we tested two other types of NO donors that do not release oxygen-derived reactive molecules: the fast NO-releasing compound diethylamine NONOate (DEANO), and S-nitrosoglutathione (GSNO), a product derived from a physiologically relevant thiol. As shown in Fig. 4A, the IRE binding activity of IRP1 was moderately increased after incu-
ference from other cytosolic components in the activation of IRP1 by Trx, recombinant IRP1 (rIRP1) was purified using a bacterial expression system and exposed to the Trx system after preincubation with GSNO or SIN-1/SOD. As shown in Fig. 5, incubation of rIRP1 with GSNO or SIN-1/SOD slightly increased rIRP1/IRE binding. However, when GSNO- or SIN-1/SOD-pretreated rIRP1 was further exposed to Trx, IRE binding activity was dramatically enhanced in a concentration-dependent manner. Increase in IRE binding after treatment with Trx did not occur with control rIRP1. These experiments demonstrate that electrons provided by the Trx system directly activate the RNA binding of IRP1 previously exposed to NO generators.

Endogenous Trx Participates in NO-mediated IRP1 Activation—To gain further insights into the role of endogenous Trx in NO-dependent IRP1 activation, we treated cytosolic lysates with anti-Trx antibodies before exposure to SIN-1/SOD. As shown in Fig. 6, incubation of cell cytosols with anti-Trx antibodies abolished the increase of RNA binding activity of IRP1 routinely observed after treatment with SIN-1/SOD (Fig. 6, compare lanes 3 and 4). It is therefore clear from this result that endogenous Trx allows activation of IRP1 in concert with NO.

Thioredoxin Reverses IRP2 Inactivation by NO—RNA binding activity of IRP2 can be inactivated by treatment with sulfhydryl-modifying compounds and oxidants (26, 31–33). Therefore, we investigated whether IRP2 activity could also be affected by NO and Trx. As shown in Fig. 7, treatment of RAW 264.7 cell cytosol with DEANO decreased IRE binding activity of IRP2 in a concentration-dependent manner. Neither incubation of cell cytosol with 500 μM diethylamine nor exposure to stable end products of DEANO decomposition inhibited IRE binding activity of IRP2 (not shown). The lower activity of IRP2 upon DEANO treatment was not a consequence of protein degradation as it could be restored after incubation with 0.5% 2-ME, which reveals maximal IRP2/IRE binding (32). Interestingly, IRP2 activity was also restored after exposure of DEANO-treated cell cytosol to 5 μM Trx in the presence of TR and NADPH. Similar results were obtained with other NO donors like GSNO (data not shown). It is worth noting that, contrary to what was observed for IRP1, Trx enhanced RNA binding activity of control IRP2.

**DISCUSSION**

Relatively little is known about the cellular mechanism that drives IRPs to modulate translation and turnover of IRE-containing mRNA. As largely documented before, the mode of activation/inactivation of IRP1 can be explained, at least in
part, by the removal/insertion of a [4Fe-4S] cluster (38). Yet it is well established that IRP1 activation requires reduction of cysteine residues (13, 14, 28). Overall, it emerged from these studies that cluster removal may not be sufficient to give IRP1 the proper conformation required to fit the stem-loop-shaped IREs. We previously demonstrated that in response to NO synthesis, IRP1 loses aconitase activity but gains IRE binding capacity (20). To explain this change, we proposed that NO or a related nitrosating species directly destabilizes the Fe-S cluster of IRP1. As regards IRP2, in vitro experiments revealed that it is also activatable to some extent by reducing agents (26, 31–33) and inhibited by peroxynitrite, an NO-derived oxidant (32). In this study, we tested the capacity of two major endogenous reducing systems, i.e. GSH and Trx, to promote or at least participate in concert with NO in IRP activation. First, we found that addition of GSH to macrophage lysate had only a marginal effect on IRP1/IRE binding in vitro, thus confirming previous data (29). Moreover, when cell cytosols were pre-treated with NO donors, no significant effect of GSH on IRP1 activity was detected below the millimolar range. These results imply that modulation of the glutathione pool is not a major parameter which interferes with IRP1 activity. Rather, our data point to reduced thioredoxin as a potent modulator of IRP activity. Trx is a multifunctional enzyme involved in a vast array of biological functions ranging from hydrogen donor for ribonucleotide reductase activity to immunostimulatory effects (39). Yet its main function is a disulfide reductase activity. By so doing, it allows refolding of disulphide bridge-containing proteins (40) and modulation of the DNA binding activity of several redox-sensitive transcription factors including NF-kB, AP-1 via reduction of Ref-1, glucocorticoid receptor, or heat shock factor-1 (41–45).

Trx by itself had little direct effect on IRP1. The low amount of IRP1 activated directly by reduced Trx in some experiments probably corresponded to a fraction of apo-IRP1 which spontaneously underwent oxidation. Interestingly, addition of low concentrations of Trx to IRP1 markedly enhanced the effect of different classes of NO donors on IRE binding. It is noteworthy that GSNO had two effects on IRP1 as follows: (i) like the other NO donors, it predisposed IRP1 to IRE binding upon further reduction, and (ii) it yielded a modification in the protein characterized by a downward shift in the polyacrylamide gel migration. Migration returned to normal after exposure to 2% 2-ME or 10 µM Trx. It is tempting to speculate that trans-nitrosation mediated by GSNO promoted formation of two different disulfide bridges. One would mostly affect binding to IRE by oxidizing at least one allosteric thiol close or belonging to the IRE-binding domain. Another disulfide bond, selectively formed by GSNO, may affect vicinal thiols distal from the IRE-binding domain. The structural change that may result from the latter does not seem to be crucial for activity as it did not affect RNA binding capacity and may not be effective in the presence of reduced Trx. Besides, we observed that sequential exposure of IRP1 to NO and reduced Trx led to the largest increase in IRP1/IRE binding. This is probably due to the fact that Trx must be maintained reduced by TR and NADPH. Such a situation was previously encountered with H2O2-enhanced DNA binding of heat shock factor-1 (45). It is likely that combining an NO generator with exogenously added Trx can partially overcome the reducing capacity of the latter. Indeed, as pointed out by Nikitovic and Holmgren (46), reaction of GSNO with Trx leads to homolytic cleavage with release of NO and inhibition of the TR/Trx system. Further evidence for a physiological role of Trx in IRP1 activation was provided by the use of specific anti-Trx antibodies. Indeed, neutralization of endogenous Trx prevented NO-mediated activation of IRE binding by IRP1.

Enhancement of IRP1 RNA binding activity in response to high concentrations of reducers such as dithiothreitol or 2-ME has long been known (28), but intriguingly neither low levels of these chemicals nor physiologically relevant reducers have been shown to reproduce this effect. The major obstacle to mild reduction is probably the presence of the Fe-S cluster which, in partnership with substrate (e.g. citrate), prevents access to the sensitive thiols, i.e. Cys437, Cys503 and Cys506 (13). Even at high concentrations, 2-ME is not able to remove the cluster since filtration on a G-25 column, IRP1 still needs a high concentration of 2-ME to bind IREs (13), which is characteristic of a cluster-containing form of IRP1. In fact, IRP1 becomes readily sensitive to low amounts of 2-ME only after cluster removal (13). Apart from the two classical forms of IRP1, i.e. the [4Fe-4S]-IRP1 (aconitase) and the apo-IRP1 (IRE-binding regulator), a [3Fe-4S] cluster-containing IRP1 was described (47, 48). In addition, recent evidence from several laboratories including ours has indicated that an oxidized form of apo-IRP1 exists in vitro (32, 49, 50) and in living cells (26). This form of IRP1 has been assigned to a cluster-free protein whose oxidation of sulphydryl groups at the active site confers on it neither aconitase activity nor IRE binding capacity. We recently showed that such an IRP1 also exists after exposure to peroxynitrite (32). In this form of IRP1, oxidation of Cys437 by peroxynitrite would prevent IRE binding. We proposed that this oxidized apo-IRP1 is a latent form rapidly available to bind IREs upon physiologic reduction (32). However, thus far the nature of this reduction has remained enigmatic. The data presented here suggest that reduced Trx acts in partnership with NO or NO-derived oxidizing species to activate IRP1 within cells. Moreover, the fact that Trx activates a pool of IRP1 previously exposed to NO provides an explanation for a somewhat paradoxical situation. Indeed, NO is a signaling molecule that, in the presence of oxygen, can yield oxidizing or nitrosating reactive species which form S-nitrosothiol adducts and/or disulfides on proteins. Higher oxides derived from NO, like peroxynitrite, may also yield sulfenic or sulfonic groups (51). It was therefore intriguing that NO or related products alone could activate IRP1, which needs to be reduced prior to binding IREs. Whether critical sulphydryls of IRP1 are S-nitrosoylated or oxidized to sulfenic or sulfonic acids in NO-producing cells remains to be determined, but since the disulfide-reducing Trx was able to generate full RNA binding activity, the most likely conclusion is that nitrogen oxides, in addition to disrupting the cluster, also interact with the cluster-coordinat-
cystines Cys\textsuperscript{503}, Cys\textsuperscript{506}, and Cys\textsuperscript{437} to promote a disulfide bridge. We anticipate that such a bridge should link Cys\textsuperscript{437} and either Cys\textsuperscript{503} or Cys\textsuperscript{506}. Our results point to the Trx system as the most effective reducing system ultimately to activate such an oxidized apo-IRP1.

IRP2 has the same specificity as IRP1 and, despite the fact that it is generally less abundant than IRP1 (52), it can regulate IRP function(s). A few examples already exist where inflammatory areas or secrete oxidants during phagocytosis or oxidizing species can be generated, both NO and large amounts of reactive oxygen species, it is in vivo insertion sequence (16). Its activity was enhanced in vitro after exposure to 2-ME or dithiothreitol (26, 31–33) and in vivo, in a model of acute inflammation (56). As macrophages can produce both NO and large amounts of reactive oxygen species, it is therefore relevant to consider carefully the effect of physiological reactive mediators such as NO on IRP2. Here, we showed an inhibition of RNA binding of IRP2 in macrophage cytosol following exposure to NO donors. Inhibition was reversed by reduced Trx but not by GSH. Trx/TR-dependent reduction of intracellular disulfide may therefore regenerate IRP2/IRE binding activity of macrophages exposed to situations where oxidizing species can be generated, e.g. when they migrate into inflammatory areas or secrete oxidants during phagocytosis or after stimulation by chemotactic factors.

In conclusion, these findings provide a decisive clue to the regulation of IRPs. We propose that NO and Trx cooperate to regulate IRP function(s). A few examples already exist where the NO/Trx tandem has been shown to modulate efficiently inflammatory areas or secrete oxidants during phagocytosis or oxidizing species, it is in vivo insertion sequence (16). Its activity was enhanced in vitro after exposure to 2-ME or dithiothreitol (26, 31–33) and in vivo, in a model of acute inflammation (56). As macrophages can produce both NO and large amounts of reactive oxygen species, it is therefore relevant to consider carefully the effect of physiological reactive mediators such as NO on IRP2. Here, we showed an inhibition of RNA binding of IRP2 in macrophage cytosol following exposure to NO donors. Inhibition was reversed by reduced Trx but not by GSH. Trx/TR-dependent reduction of intracellular disulfide may therefore regenerate IRP2/IRE binding activity of macrophages exposed to situations where oxidizing species can be generated, e.g. when they migrate into inflammatory areas or secrete oxidants during phagocytosis or after stimulation by chemotactic factors.

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