Excess Capacity of the Iron Regulatory Protein System*

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Iron regulatory proteins (IRP1 and IRP2) are master regulators of cellular iron metabolism. IRPs bind to iron-responsive elements (IREs) present in the untranslated regions of mRNAs encoding proteins of iron storage, uptake, transport, and export. Because simultaneous knockout of IRP1 and IRP2 is embryonically lethal, it has not been possible to use dual knockouts to explore the consequences of loss of both IRP1 and IRP2 in mammalian cells. In this report, we describe the use of small interfering RNA to assess the relative contributions of IRP1 and IRP2 in epithelial cells. Stable cell lines were created in which either IRP1, IRP2, or both were knocked down. Knockdown of IRP1 decreased IRE binding activity but did not affect ferritin H and transferrin receptor 1 (TfR1) expression, whereas knockdown of IRP2 marginally affected IRE binding activity but caused an increase in ferritin H and a decrease in TfR1. Knockdown of both IRPs resulted in a greater reduction of IRE binding activity and more severe perturbation of ferritin H and TfR1 expression compared with single IRP knockdown. Even though the knockdown of IRP-1, IRP-2, or both was efficient, resulting in nondetectable protein and under 5% of wild type levels of mRNA, all stable knockdowns retained an ability to modulate ferritin H and TfR1 appropriately in response to iron challenge. However, further knockdown of IRPs accomplished by transient transfection of small interfering RNA in stable knockdown cells completely abolished the response of ferritin H and TfR1 to iron challenge, demonstrating an extensive excess capacity of the IRP system.

Iron is an essential element required for the function of numerous critical enzymes and is essential for cell survival. However, because of the ability of iron to catalyze the formation of reactive oxygen species, excess iron is harmful. Consequently, levels of intracellular iron must be tightly controlled (1). Iron-responsive elements (IREs)² play a central role in the regulation of iron homeostasis. IREs are mRNA hairpin elements present in the untranslated region of mRNAs encoding proteins of iron storage (ferritin) (2–4), iron uptake (TfR1) (5, 6), iron transport (DMT1) (7, 8), and iron export (ferroportin) (9–11).

The role of IRE elements in ferritin and TfR1 has been particularly well studied. In ferritin H and L, an IRE is present in the 5′-UTR of the mRNA, whereas in TfR1, five IRE elements are present in the 3′-UTR. In both cases, the IRE can be bound by IRE-binding proteins. When IRE-binding proteins bind to the IRE in the 5′-UTR of ferritin, they inhibit translation (3, 4). When IRE-binding proteins bind to the 3′-UTR of TfR1, they stabilize the mRNA (6).

Two IRE-binding proteins have been identified, IRP1 (12, 13) and IRP2 (14). They are highly homologous but respond to iron challenge via different mechanisms. IRP1 is a bifunctional protein; its conformational and functional changes depend on cellular iron status. When iron levels are high, IRP1 forms a 4Fe-4S cluster and functions as a cytosolic aconitase. Under these conditions, IRP1 cannot bind the IRE. Conversely, when iron levels are low, the Fe-S cluster disassembles, and IRP1 binds to IREs. Recently, it has been suggested that iron-dependent regulation of IRP1 abundance represents an additional mechanism of control of IRP1 (15). IRP2 differs from IRP1 by the presence of an additional 73-amino acids in the N terminus. When iron levels are high, IRP2 undergoes iron-dependent degradation (16, 17). The consequence of this coordinate regulation of IRP proteins is that in iron-replete conditions, there is less IRP1 and IRP2 available to bind to IREs, leading to an increase in ferritin and a decrease in TfR1. Conversely, under conditions of iron limitation, increases in the activity and level of IRP1 and IRP2 lead to decreased ferritin and increased TfR1. Because increased ferritin increases iron storage, whereas decreased TfR1 decreases iron transport, these IRP-dependent events act in concert to maintain iron homeostasis. IRPs are also regulated by other physiological stimuli, including reactive oxidative species (18, 19), nitric oxide (19, 20), hypoxia (21), and phosphorylation (22, 23). For example, H2O2 rapidly activates IRP1, although it does not affect the activity of IRP2 in murine B6 fibroblasts (24). NO (reduced form) enhances IRE binding activity of IRP1 (25–28), leading to increased translational repression of ferritin synthesis. NO+ (oxidized form) increases IRP1 binding activity while decreasing IRP2 binding activity in murine RAW 264.7 macrophages (29). Hypoxia enhances IRP1 binding activity in human hepatoma cells (30) and IRP2 binding activity in rat FTO2B and human 293 cells (21).

IRP knockout mice have provided important insights into the function of IRPs in vivo (31–35). IRP1 knockout mice display normal iron metabolism in most tissues, except for brown fat and kidney. In contrast, mice with deletion of IRP2 show high ferritin and low TfR1 levels in multiple tissues (32). IRP2 defi-
ciency also leads to iron misdistribution and microcytosis (34, 35). One group reported that IRP2−/− mice developed progressive adult onset neurodegeneration (31), although another group observed microcytosis and no overt signs of neuropathology in an independently generated IRP2−/− mouse (35, 36). The different phenotypes observed in IRP1 and IRP2 knockout mice have led to the suggestion that these proteins are partially nonredundant. Based on the comparative phenotype of IRP1 and IRP2 knockout mice, it has been further suggested that IRP2 may be the dominant regulator of iron homeostasis in vivo (32).

Because combined inactivation of both IRP1 and IRP2 results in embryonic lethality in the mouse (35, 37), IRP function that would have been elucidated with such models remain unexplored. We undertook to understand and measure the cellular effects of deficiency of IRP1 and IRP2, both singly and in combination, in epithelial cells using siRNA technology to efficiently knock down IRP1 and IRP2.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Cell Cultures**—Ferri-citrate (FAC), deferoxamine (DFO), H2O2, and puromycin were purchased from Sigma. HeLa cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated in a humidified atmosphere of 5% CO2 at 37 °C.

**Establishment of Stable Clones**—The pSilencer 2.1-U6 puro vector and pSilencer2.1-U6 neo vector were obtained from Ambion (Austin, TX). Vectors that express hairpin siRNA were constructed by inserting pairs of annealed DNA oligonucleotides into pSilencer 2.1-U6 between BamHI and HindIII restriction sites according to the manufacturer's instructions. The siRNA target sequences were as follows: for IRP1, 5′-CTAGAACCCCTATCATCAT-3′, and for IRP2, 5′-GTTGGTTGGATGTGGAGTT-3′. A nontargeting sequence, 5′-ACTA-CCGGTTTTAGGTGTA-3′, was used as a control. To make single knockdown clones, HeLa cells were transfected with pSilencer2.1-U6-IRP1 siRNA (puro) or pSilencer2.1-U6 IRP2 siRNA (puro). 24 h after transfection, 3 μg/ml puromycin was added to medium for selection. After selection, the stable clones were maintained in medium containing 1.5 μg/ml puromycin. Stable clones were analyzed by real-time RT-PCR and Western blotting to confirm IRPs down-regulation. To make transient knockdown efficient, we designed new siRNA sequences that are different from the ones used in making stable knockdown cells. The siRNA target sequences were as follows: for IRP1, 5′-GGGCAAGAAAGCATATCTA-GTA′, and for IRP2, 5′-GGGAAACATGTGGTGTAGTTAGGA′. To assess IRE function in different cells, 2 × 105 cells were seeded into each well of 6-well plates and incubated overnight. pGL3−1201 (no IRE) or pGL3−1236 (wild type IRE) (0.5 μg) was transfected into each well. pRL-TK (Promega) or pRL-actin (38) (a generous gift of Dr. Kazuo Yamamoto) was cotransfected as an internal control for transfection efficiency. Luciferase activity was measured 24 h after transfection using a dual luciferase assay kit (Promega). For transient transfection of siRNA oligonucleotide into cells, siPortTM NeoFXTM (Ambion) was used for transfection following the manufacturer’s protocol. 10–30 nM siRNA was transfected. To make transient knockdown efficient, we designed new siRNA sequences that are different from the ones used in making stable knockdown cells. The siRNA target sequences were as follows: for IRP1, 5′-GGGCAAGAAAGCATATCTA-3′, and for IRP2, 5′-GGGAAACATGTGGTGTAGTTAGGA-3′. To assess IRE function in different cells, 2 × 105 cells were seeded into each well of 6-well plates and incubated overnight. pGL3−1201 (no IRE) or pGL3−1236 (wild type IRE) (0.5 μg) was transfected into each well. pRL-TK (Promega) or pRL-actin (38) (a generous gift of Dr. Kazuo Yamamoto) was cotransfected as an internal control for transfection efficiency. Luciferase activity was measured 24 h after transfection using a dual luciferase assay kit (Promega). For transient transfection of siRNA oligonucleotide into cells, siPortTM NeoFXTM (Ambion) was used for transfection following the manufacturer’s protocol. 10–30 nM siRNA was transfected. To make transient knockdown efficient, we designed new siRNA sequences that are different from the ones used in making stable knockdown cells. The siRNA target sequences were as follows: for IRP1, 5′-GGGCAAGAAAGCATATCTA-3′, and for IRP2, 5′-GGGAAACATGTGGTGTAGTTAGGA-3′.

**Real Time RT-PCR**—Real time PCR was carried out on the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The standard curve method was used for quantification. Total RNA was isolated using TRizol reagent, according to the manufacturer’s instructions. 30 μg of RNA was treated with 35 units of DNase I (Promega) for 30 min at 37 °C. After DNase I digestion, RNA was purified using an RNeasy kit (Qiagen). cDNA was synthesized from 2 μg of total RNA by RT (Superscript III; Invitrogen). PCR was performed in a reaction mixture containing 125 μM of each dNTP, 1× SYBR® Green PCR Master Mix (Applied Biosystems) and 200–400 nM primers. Absence of DNA contamination was verified by performing amplification from cDNA without reverse transcriptase. The primers for PCR were designed with IDT PrimerQuest software (Integrated DNA Technologies, Inc.); for IRP1, forward, 5′-TGTGCTCCTGACCATGCTGCT-3′, and reverse, 5′-TAGCTCGGTGACCATGCTGCT-3′; for IRP2, forward, 5′-ACCCAGGAACTGGTGTGTGTGTTAG-3′, and reverse, 5′-ACTCCTACTTGCCTGGA-3′.
GGTGCTTT-3'; for β-actin, forward, TTGCCGACAGGACTGAGATTAAGGA-3'; for firefly luciferase, forward, 5'-GGGCAATCAGGCAAATC-3'; for Renilla luciferase, forward, 5'-AGTGGACAGCAGCAG-3'; and for TfR1, forward, 5'-AGTTGAACAAAGTGGCACGAGCAG-3', and reverse, 5'-AGCAAGTTGGCTGTTGTACCTCA-3'.

Western Blotting—Western blot analysis was performed as described (39). IRP1 and IRP2 antibodies were generous gifts of Dr. M. Hentze (Heidelberg, Germany) and Dr. R. Eisenstein (Madison, WI). Anti-TfR1 was purchased from Zymed Laboratories Inc. Anti-ferritin H is a polyclonal rabbit antibody (39).

RNA Bandshift Assay—RNA bandshift assays were performed as described (40). When supershift assays were performed, antibodies were added 1 h before the addition of IRE probe. The ferritin H IRE probe was prepared from in vivo transcription of BamHI linearized pST18 plasmid (a generous gift of Dr. P. Ponka). To quantify the bands in supershift assay, the UN-SCANIT Automatic digitizing system Version 5.1 was used.

RESULTS

Efficient Knockdown of IRP1 and IRP2 in HeLa Cells—To assess the cellular effects of deficiency of IRP1 and IRP2, we used siRNA technology to stably knock down IRP1 and IRP2 in HeLa cells (commonly used to study IRPs and iron metabolism (21, 41–48)). At the mRNA level, IRP1 was knocked down to <2% (Fig. 1A, left panel, two IRP1 KD clones), and IRP2 was knocked down to <5% (Fig. 1A, middle panel, two IRP2 KD clones), whereas both IRP1 and IRP2 were knocked down to <5% (Fig. 1A, right panel, two IRP1/2 double KD clones). At the protein level, IRP1 was undetectable in IRP1 knockdown cells (Fig. 1B, top panel, lanes 3 and 4). IRP2 was not detected in IRP2 knockdown cells (Fig. 1B, middle panel, lanes 5 and 6). Neither
IRP1 or IRP2 shRNA were transiently transfected with IRP1 or IRP2 knockdown cells. We also anticipated that IRP knockdown should cause an increase in TfR1 mRNA was consistent with the change of TfR1 measured TfR1 mRNA level in different knockdown cells. The observed in double IRP1/IRP2 knockdowns. Because the bind- ing of IRP to the IRE in TfR1 regulates mRNA stability, we also measured TfR1 mRNA level in different knockdown cells. The change of TfR1 mRNA was consistent with the change of TfR1 protein in knockdown cells: levels of TfR1 mRNA were 119 ± 28% of control (means ± S.D., n = 4) in IRP1 knockdown cells, 57 ± 4% of control (means ± S.D., n = 4) in IRP2 knockdown cells, and 35 ± 7% of control (means ± S.D., n = 4) in double knockdown cells.

Because unanticipated genetic alterations might have taken place during the selection of stable transfectants, we also assessed changes in iron-regulated genes in cells transiently transfected with shRNAs to IRP1 and IRP2. Both HeLa cells and 293 cells were transiently transfected with IRP1 or IRP2 shRNA vectors, and levels of ferritin H were measured by Western blotting. Similar results were observed in transient transfectants: knockdown of IRP2 but not IRP1 caused an increase in ferritin H (data not shown).

**Direct Effects of IRP Knockdowns on IRE Function**—The target of the IRPs is the IRE. In the case of ferritin, IRPs bind to this sequence in the 5’-UTR of the mRNA to block translation. To directly assess the effect of IRP knockdown on repression of the ferritin IRE, we prepared two ferritin H promoter-luciferase constructs; pGL3–1201 contains no IRE sequence, whereas pGL3–1236 contains full-length IRE (Fig. 3A). When IRE is present (pGL3–1236), IRPs will bind to the IRE to inhibit luciferase mRNA translation. The ratio of luciferase activity in reporter genes pGL3–1201 (no IRE) and pGL3–1236 (full-length IRE) represents a direct measure of the contribution of the IRE to repression of the luciferase reporter construct. The cells were transfected with these plasmids, and luciferase activity was measured. In control cells, luciferase activity was decreased about 5.5-fold by wild type IRE (Fig. 3). In IRP1 knockdown cells, IRE-mediated repression of luciferase activity was not reduced and in fact increased to about 8.5-fold (p < 0.0001 versus control). Although surprising, this result is consistent with results seen with endogenous IRE-regulated genes (see Fig. 2 and “Discussion”). However, in IRP2 knockdown cells, IRE-mediated repression of luciferase decreased to about 3.7-fold (p < 0.0001 versus control and IRP1 knockdown cells). Knockdown of both IRPs led to a greater reduction in translational repression of a luciferase reporter than knockdown of either IRP alone, reducing IRE-dependent repression to 2.8-fold (p < 0.0001 versus control, IRP1 knockdown cells, and IRP2 knockdown cells). Similar results were observed when wild type IRE (pGL3–1236) was compared with a nonfunctional IRE point mutant with a deletion of a single “C” in the loop (Ref. 12 and data not shown). To confirm that knockdown of IRPs affected translation of luciferase rather than luciferase mRNA levels, we also measured luciferase mRNA in transient transfections. As expected, knockdown of IRP had no effect on luciferase mRNA as measured by real time RT-PCR (Fig. 3C). Thus, IRP2 knockdown blocked repression of an IRE-dependent luciferase reporter, whereas knockdown of IRP1 did not.

**Effects of IRP Knockdown on IRE Binding**—We then tested the effect of IRP knockdown on IRE binding activity. Equal cytosolic extracts from control cells (Fig. 4, lanes 1 and 2), IRP1 knockdown cells (Fig. 4, lanes 3 and 4), IRP2 knockdown cells (Fig. 4, lanes 5, 6), and double knockdown cells (Fig. 4, lanes 7 and 8) were incubated with 32P-labeled IRE, and RNA bandshift assays were performed as described under “Experimental Procedures.” Knockdown of IRP1 decreased IRE binding activity substantially (Fig. 4, compare lanes 3 and 4 with lanes 1 and 2), whereas knockdown of IRP2 marginally affected IRE binding activity (Fig. 4, compare lanes 5 and 6 with lanes 1 and 2). Double knockdown caused the greatest reduction of IRE binding activity (Fig. 4, lanes 7 and 8).

**Knockdown of One IRP Cause a Compensatory Increase in the Other IRP**—To assess the relative contribution of IRP1 and IRP2 to IRE binding activity, as well as to determine the effect of knockdown of one IRP on the IRE binding activity of the other IRP, we used RNA bandshift assays in control, IRP1, and IRP2 knockdown cells (Fig. 5). Because complexes of IRE-IRP1 cannot be distinguished from IRE-IPR2 complexes in human cells...
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IRP1 knockdown cells, more IRP2 was bound to the IRE compared with control cells (Fig. 5, compare lane 6 with lane 3). Thus, in IRP1 knockdown cells, IRP2 accounted for 50% of total activity (compare lane 6 (IRP2) with lane 4 (total IRE binding activity)), versus 27% in controls (compare lane 3 (IRP2) with lane 1 (total IRE binding activity)). Conversely, in IRP2 knockdown cells, more IRP1 was bound to the IRE (Fig. 5, compare lane 6 with lane 3); IRP1 accounted for 93% of total IRE binding activity in IRP2 knockdown cells (compare lane 8 (IRP1) with lane 7 (total IRE binding activity)) versus 73% in controls (compare lane 2 (IRP1) to lane 1 (total IRE binding activity)).

These supershift analyses support the Western blot results (Fig. 1B) and suggest that knockdown of either IRP1 or 2 leads to a compensatory increase in the remaining IRP. They also demonstrate that the increased IRP is functionally competent to bind to the IRE.

Response of Knockdown Cells to Oxidative Stress—Iron contributes to oxidative stress by catalyzing the formation of reactive oxygen species via Fenton chemistry. The cells that overexpress ferritin are more resistant to oxidative stress, because iron sequestered in ferritin is unavailable for participation in Fenton chemistry (46, 50). Because IRP2 knockdown and double knockdown cells exhibited an increase in ferritin and a decrease in TfR1, we asked whether these cells were more resistant to H2O2 when compared with IRP1 knockdown cells. The cells were treated with H2O2, and viability was assessed after 48 h using an methyltetrazolium assay (Fig. 6). In control and IRP1 knockdown cells, H2O2 reduced viability to 18% of that observed in untreated cells. IRP2 knockdown cells exhibited a slightly enhanced resistance to H2O2 when compared with IRP1 knockdown cells, consistent with the increased expression of ferritin seen in IRP2 knockdown cells when compared with IRP1 knockdown or controls (Fig. 2). In double knockdown cells, which exhibited the highest ferritin expression and lowest TfR1 expression, cell viability was 36%, or 2-fold greater than in controls or single knockdown cells. These results

(49), supershift assays were performed to determine the contribution of each IRP to IRE binding activity. Equal cytosolic extracts from control, IRP1, and IRP2 knockdown cells were incubated with 32P-labeled IRE in the presence of IRP1 (Fig. 5, lanes 2, 5, and 8) or IRP2 antibody (Fig. 5, lanes 3, 6, and 9). Normal rabbit IgG was used as a control (Fig. 5, lanes 1, 4, and 7). In control cells, IRP1 antibody shifted most of the IRE binding activity (compare lane 2 with lane 1). This indicates that IRP1 is the predominant IRE-binding protein in control cells. In H2O2 reduced viability to 18% of that observed in untreated cells. IRP2 knockdown cells exhibited a slightly enhanced resistance to H2O2 when compared with IRP1 knockdown cells, consistent with the increased expression of ferritin seen in IRP2 knockdown cells when compared with IRP1 knockdown or controls (Fig. 2). In double knockdown cells, which exhibited the highest ferritin expression and lowest TfR1 expression, cell viability was ~36%, or 2-fold greater than in controls or single knockdown cells. These results

FIGURE 3. Effects of IRP knockdown on IRE repressive function. A, cartoon of IRE constructs used in these assays. A 1.2-kb human ferritin H promoter sequence was inserted into pGL3-basic vector. pGL3–1201 contains no IRE sequence, whereas pGL3–1236 contains full-length IRE sequence. B, pGL3–1201 and pGL3–1236 were transfected into control cells and different stable knockdown cells; pRL-TK was cotransfected to monitor transfection efficiency. Luciferase (Luc) activity was determined by dual luciferase assay. Lucifase activity from cells transfected with pGL3–1201 was defined as 100. IRE-dependent reduction was calculated as a ratio of luciferase activity obtained from transfection of pGL3–1201 to pGL3–1236. The means and standard deviations of five independent experiments are shown. When compared with controls, IRE-dependent reduction of luciferase activity was increased in IRP1 knockdown cells (8.5-fold reduction in IRP1 knockdown versus 5.5-fold in controls, p < 0.0001) and decreased in IRP2 knockdown cells (3.7-fold versus 5.5-fold, p < 0.0001). Knockdown of both IRP1 and IRP2 further decreased IRE-dependent reduction of luciferase activity when compared with IRP2 knockdown alone (2.8-fold reduction versus 3.7-fold, p < 0.0001). C, transfections were performed as in B. The luciferase mRNA level was measured by real time RT-PCR. The ratio of luciferase mRNA in —IRE/IRE constructs (pGL3–1201/pGL3–1236) in control cells was defined as 1. The means and standard deviations of four independent experiments are shown.

| group          | mean ± S.D. |
|----------------|-------------|
| C              | 1           |
| IRP1 KD        | 1.06 ± 0.18 |
| IRP2 KD        | 0.98 ± 0.13 |
| IRP1/2 KD      | 0.95 ± 0.03 |

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282 • NUMBER 34 • AUGUST 24, 2007

24654
suggest that double IRP knockdown protects cells from oxidative stress.

**IRP Knockdown Cells Respond Appropriately to Iron Challenge**—To assess whether knockdown of IRP1 and IRP2 would compromise the ability of cells to modulate ferritin H and TfR1 in the face of changes in exogenous iron, control and knockdown cells were challenged with iron (FAC) or iron chelator (DFO). TfR1 and ferritin H were determined by Western blotting. As shown in Fig. 7, control cells demonstrated an increase in ferritin H protein and a decrease in TfR1 protein in response to iron, as anticipated; the converse was observed in cells treated with an iron chelator. Unexpectedly, in IRP knockdown cells, the response of ferritin H and TfR1 to iron was not impaired. In all cells, regardless of IRP status, iron repressed TfR1 protein and induced ferritin H protein. Similarly, in all cells, DFO induced TfR1 and repressed ferritin H. Thus, despite effective knockdown as measured by differences in basal levels of ferritin H and TfR1 (also see Fig. 2), these cells preserved an ability to respond to iron challenge.

Further Knockdown of IRPs in Stable Knockdown Cells Abolishes Cell Response to Iron Challenge—The finding that all IRP knockdown cells responded appropriately to iron challenge suggested either that IRP1 and IRP2 are present in excess or that additional IRE-binding proteins can mediate a cell response to iron challenge. Although IRP1 and IRP2 mRNA were knocked down to under 5% of wild type levels, and there was no detectable IRP protein in stable double knockdown cells, residual IRE binding activity still exists after both IRP1 and IRP2 are knocked down (Fig. 4, lanes 7 and 8). The residual IRE binding activity is due to either residual IRP1 and IRP2 left after knockdown or unidentified IRE-binding proteins besides IRP1 and IRP2.

To test whether the normal response of cells to iron challenge is mediated by residual IRP1 and IRP2, we further reduced IRP1 and IRP2 using transient transfection with siRNA in stable double knockdown cells. Stable knockdown cells in which IRP1 and IRP2 were already knocked down to less than 5% were transiently transfected with scrambled siRNA (Fig. 8A, lane 2), IRP1 siRNA (Fig. 8A, lane 3), IRP2 siRNA (Fig. 8A, lane 4), or both IRP1 and IRP2 siRNA (Fig. 8A, lane 5). Real time
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FIGURE 8. Further knockdown of IRPs in stable knockdown cells abolishes cell response to iron challenge. A, stable double knockdown cells were transiently transfected with scrambled siRNA, IRP1 siRNA, IRP2 siRNA, or both IRP1 and IRP2 siRNA. Real time RT-PCR was performed to determine IRP mRNA after transient transfection. The means and standard deviations of three independent experiments are shown. Relative to stable knockdown cells (lane 2), IRP1 levels were further decreased by transient knockdown of either IRP1 or IRP1 + 2 (p < 0.01); IRP2 levels were further decreased by transient knockdown of either IRP2 or IRP1 + 2 (p < 0.01). B, RNA bandshift assay was performed as described for Fig. 4 using stable knockdown cells with transient transfection of siRNA (corresponding to A, lanes 2–5). A representative experiment of three independent experiments is shown. C, ferritin H and TfR1 were assessed by Western blot as described for Fig. 2. A representative experiment of three independent experiments is shown. D, 24 h after transient transfection of siRNA, control cells (lanes 1–3) and double knockdown cells (lanes 4–6) were divided into three dishes. After 24 h, the cells were left untreated or treated with 500 µM FAC or 250 µM DFO. A Western blot was performed 24 h after the treatment to determine ferritin H and TfR1 levels. A representative experiment of three independent experiments is shown. In B–D, – indicates stable double knockdown cells with transient transfection of scrambled siRNA (corresponding to A, lane 2), IRP1 indicates stable double knockdown cells with transient transfection of IRP1 siRNA (corresponding to A, lane 3). IRP2 indicates stable double knockdown cells with transient transfection of IRP2 siRNA (corresponding to A, lane 4). IRP1 + 2 indicates stable double knockdown cells with transient transfection of IRP1 and IRP2 siRNA (corresponding to A, lane 5). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

RT-PCR was performed to determine levels of IRP mRNA after transient knockdown (Fig. 8A). IRP1 and IRP2 mRNA levels in stable double knockdown cells transiently transfected with scrambled siRNA was 2.3 and 3.2% of wild type, respectively (Fig. 8A, lane 2, also as shown in Fig. 1A, right panel). Transfection of IRP1 siRNA further decreased IRP1 mRNA to 1.2% of wild type (Fig. 8A, lane 3). Transfection of IRP2 siRNA decreased IRP2 to 2.0% of wild type (Fig. 8A, lane 4). Transfection of both IRP1 siRNA and IRP2 siRNA decreased IRP1 mRNA and IRP2 mRNA to 1.3 and 2.1% of wild type, respectively (Fig. 8A, lane 5). Thus, transient transfection with siRNAs further lowered IRP expression in stable knockdown cells to ~50–60% of their already reduced levels.

Following transient knockdown of IRPs in stable knockdown cells, RNA bandshift assays were performed to assess IRE binding activity. The results of the combined transient and stable knockdowns were similar to the stable knockdowns described in Fig. 4; knockdown of IRP1 had the most dramatic effect on IRE binding activity. Transient knockdown of IRP1 decreased IRE binding activity, whereas knockdown of IRP2 did not. Double knockdown of IRP1 and IRP2 had the least IRE binding activity (Fig. 8B).

DISCUSSION

In this report we used siRNA technology to explore the roles of IRP1 and IRP2 in maintenance of iron homeostasis in epithelial cells. Knockdown of both IRP1 and IRP2 was efficient and led to a >95% reduction of both IRPs at the mRNA level and undetectable protein levels. There was no effect of these knockdowns on cell growth.

The use of a model system that allows for the sequential or simultaneous knockdown of the IRPs allowed us to probe the extent and the capacity of the iron-regulatory system in cells. It also allowed the careful, simultaneous analysis of IRP protein abundance and protein function, which can be inferred but not directly tested in mouse models. Finally, because double knockdowns are lethal at an early stage of embryogenesis, this model system allowed us to explore the effects of simultaneous knockdown of IRP1 and IRP2 and its consequences for cellular iron homeostasis.

We observed that knockdown of IRP1 led to a compensatory increase in IRP2 protein; similarly, knockdown of IRP2 led to a compensatory increase in IRP1 protein, as measured by Western blotting (Fig. 1B) and bandshift assays (Fig. 4). Consistent with these observations, IRP1−/− mice also display a higher
IRP2 level compared with normal mice (33). Although we did not explore the mechanism underlying this response in detail, it is likely post-transcriptional, because the levels of IRP mRNA did not change (Fig. 1A) in the knockdown cells.

Although both IRPs bind to the IRE, IRP2 knockdown had more robust functional consequences for iron homeostasis. Our experimental system allowed us to explore the functional consequences of IRP knockdowns in two ways: (1) a biological assay that examined the consequences of IRP binding on endogenous ferritin and transferrin receptor proteins and (2) a direct assay for repressive function of the IRP on the ferritin IRE using a luciferase reporter.

The first assay examined the effects of the knockdown on ferritin and TfR1. As anticipated, the IRP2 knockdown exhibited an increased level of ferritin H and a decreased level of TfR1 relative to control (Fig. 2). Surprisingly, however, the role of IRP1 in iron regulation was not apparent in IRP1 knockdowns, which exhibited similar levels of ferritin and TfR1 as controls. However, a role for IRP1 was unmasked in cells in which IRP2 was also knocked down; these double knockdown cells exhibited a higher level of ferritin and lower level of TfR1 relative to knockdown of IRP2 alone (Fig. 2, compare lanes 7 and 8 with lanes 5 and 6), indicating that IRP1 also contributes to iron homeostasis.

In the second assay of IRP function, we measured the ability of IRP proteins to regulate IRE-dependent events using an IRE-dependent luciferase assay (Fig. 3). The results of this assay were concordant with results shown in Fig. 2; there was a loss of IRE-dependent repressive activity in IRP2 knockdown cells compared with normal cells, and a contribution of IRP1 to IRE repression was only observed in the context of a simultaneous knockdown of IRP2. These luciferase assays also exposed an unexpected effect of altering the balance of IRPs that was not evident from the measurements of ferritin and the transferrin receptor; knockdown of IRP1 alone did not reduce repression, but in fact increased repression. We speculate that the more effective repression of the IRE in an IRP1 knockdown than in control cells may be due to the compensatory increase in IRP2 protein induced by IRP1 knockdown, as discussed above.

We expected that the observed primacy of IRP2 in the functional regulation of the proteins of iron metabolism would be explained by a reduced IRE binding activity in these cells. That is, we expected to find changes in IRE binding activity proportional to the changes in IRE activity. To our surprise, we found no such correlation. Instead, IRE binding was unchanged in the IRP2 knockdown (Fig. 4), although repressive function was altered as measured by luciferase assays (Fig. 3) and levels of endogenous IRE-regulated genes (Fig. 2). Conversely, in the IRP1 knockdown, where there was no change in function (Fig. 2), the binding activity dramatically decreased. This suggests that there is a discord between IRE activity as measured by in vitro assays of IRE binding, and IRP activity as measured by functional assays in cells. Although oxygen tension can affect IRE binding activity (21, 33), we conducted all our experiments (both binding assays and in vivo activity assays) at the same oxygen tension. Thus, the discord between in vitro binding assays and in vivo activity in our experiments cannot be attributed to differences in oxygen tension. We conclude that in vitro bandshift assays should be used cautiously to predict IRE function.

Based on our binding assays (Fig. 4) and functional assays (Figs. 2 and 3), we postulate that IRP2 is a more potent regulator of the IRE than IRP1. Although IRP2 is much less abundant than IRP1 in IRE binding activity (Fig. 5), IRP2 knockdown blocked repression of IRE-dependent translation of a luciferase reporter, whereas knockdown of IRP1 had no effect and in fact resulted in increased repression (Fig. 3), possibly because of a compensatory increase in IRP2, as discussed above. The observation of the dominance of IRP2 in IRE regulation was not limited to reporter constructs; knockdown of IRP2 also exerted a greater effect on expression of endogenous IRE-responsive genes than knockdown of IRP1. Thus, endogenous levels of ferritin H and TfR1 were unaffected in IRP1 knockdown cells, whereas in IRP2 knockdown cells, endogenous ferritin H levels increased, and TfR1 levels decreased. Overall, compared with IRP1, IRP2 has less IRE binding but more function, indicating that IRP2 is more potent than IRP1. This conclusion differs from those obtained using in vitro translation assays, in which IRP1 and IRP2 were demonstrated to have equivalent repressive activity in terms of inhibiting ferritin H translation, i.e. IRP2 inhibited ferritin H mRNA translation with a molar efficiency equal to that of IRP1 in vitro (51). The discrepancy between these in vitro results and our in vivo experiments suggests the existence of pathways that enhance IRP2 activity that are present in vivo but absent in vitro, such as post-translational modifications of IRP2 or proteins that interact with IRP2 to modulate its activity. This discrepancy between in vitro and in vivo results may also suggest a contribution of subcellular localization of IRPs to their function in vivo. Even though IRP1 is predominant in IRE binding in vitro, there may be less IRP1 accessible to the IRE in vivo. The view of IRP2 as a more potent regulator of the IRE than IRP1 is consistent with results obtained in knockout mice: IRP1 knockout mice display normal iron metabolism in most tissues, whereas IRP2 knockout mice show high ferritin and low TfR1 levels in multiple tissues (32). One explanation that has been offered to explain these results is the heightened binding activity of IRP2 relative to IRP1 at physiological oxygen tension (33); however, an inherently greater activity of IRP2 relative to IRP1 in iron homeostasis suggested by the results in this manuscript may also contribute to the enhanced iron misregulation observed in an IRP2 versus IRP1 knockout mouse.

These knockdowns of IRPs are of sufficient magnitude to have functional consequences for cells, as measured by enhanced resistance to oxidant stress (Fig. 6). Resistance was observed in cells that exhibited an increase in ferritin and decrease in TfR1, suggesting that the altered iron metabolism in these cells underlies their increased resistance to oxidant stress. Use of knockdowns also allowed us to explore the functional consequences of simultaneous knockdown of both IRP1 and IRP2. This has not been possible in mouse models, because constitutive double knockouts are not viable (35, 37). Knockdown of both IRPs led to a greater reduction in translational repression of a luciferase reporter than knockdown of either IRP alone (Fig. 4). We also investigated the effect of double knockdown on the endogenous IRE-responsive genes, ferritin...
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H and TfR1. Consistent with the luciferase assays, basal levels of these proteins were more severely affected in double knockdowns than in IRP2 or IRP1 single knockdowns (Figs. 2 and 6).

An experimental question approachable in this system is the extent to which IRP knockdown affects the ability of cells to respond to changes in levels of extracellular iron. Knockdown cells were challenged with iron or an iron chelator, and changes in levels of ferritin H and TfR1 were compared with controls. Control cells exhibited the expected increase in ferritin H and decrease in TfR1 in the presence of iron and a decrease in ferritin H and increase in TfR1 in the presence of an iron chelator. Unexpectedly, although basal levels of ferritin H and TfR1 were altered in IRP2 and IRP1/IRP2 double knockdown cells compared with controls, ferritin H and TfR1 were also regulated appropriately by iron supplementation or depletion in these cells (Fig. 7).

The ability of stable double knockdown cells to respond appropriately to changes in environmental iron suggested either that the IRP system has extensive excess capacity or that additional IRE-binding proteins exist. Several findings have suggested the existence of other, yet unidentified, IRE-binding proteins. For example, using Northwestern blotting, the IRE binding complex was recently resolved into four components, leading to the suggestion that novel IRE-binding proteins may exist (52). Similarly, Lin et al. (53) reported that a specific cytoplasmic protein bound to an IRE-like sequence in the transcript of the 75-kDa subunit of mitochondrial complex 1. We therefore tested whether further knockdown of IRP1 and IRP2 using siRNA in stable knockdown cells would affect the response to iron. If an additional IRP contributes to the response to iron, we would have expected that such cells would retain their ability to respond to iron. Instead, we observed that further knockdown of IRP1 and IRP2 was sufficient to completely abolish the response of ferritin H and TfR1 to iron challenge, suggesting that although other IRE-binding proteins may exist, they cannot substitute to maintain iron homeostasis when IRP1 and IRP2 are greatly diminished. The essential requirement for IRP1 or IRP2 is supported by the finding that double knockouts of IRP1 and IRP2 are not viable (35, 37), although results in mice may be complicated by requirements for IRP1 or IRP2 at particular phases of development or embryogenesis. Here, we demonstrate that in the context of a single cell type, an appropriate response to iron or iron deprivation cannot be mounted when levels of IRP1 and IRP2 fall below a certain threshold.

IRP proteins appear to be present in substantial excess. Although basal levels of IRP-responsive genes were altered by knockdown of IRP to less than 5% of wild type levels, the response to iron was preserved in these cells. Only by further knockdown of IRPs to about 2% of wild type levels using siRNA was the response to iron abolished. Thus, only at profoundly low levels of IRPs is regulation of iron homeostasis lost. This excess capacity of the IRP system likely reflects the importance of the cellular requirement to respond to alterations in ambient iron.

Acknowledgments—We thank M. Hentze, B. Galey, and R. Eisenstein for generous gifts of IRP antibodies.

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