Iron regulatory protein 2 (IRP2) controls the synthesis of many proteins involved in iron metabolism, and the level of IRP2 itself is regulated by varying the rate of its degradation. The proteasome is known to mediate degradation, with specificity conferred by an iron-sensing E3 ligase. Most studies on the degradation of IRP2 have employed cells overexpressing IRP2 and also rendered iron deficient to further increase IRP2 levels. We utilized a sensitive, quantitative assay for IRP2, which allowed study of endogenous IRP2 degradation in HEK293A cells under more physiologic conditions. We found that under these conditions, the proteasome plays only a minor role in the degradation of IRP2, with almost all the IRP2 being degraded by a nonproteasomal pathway. This new pathway is calcium-dependent but is not mediated by calpain. Elevating the cellular level of IRP2 by inducing iron deficiency or by transfection causes the proteasomal pathway to account for the major fraction of IRP2 degradation. We conclude that under physiological, iron-sufficient conditions, the steady-state level of IRP2 in HEK293A cells is regulated by the nonproteasomal pathway.

Iron metabolism is exquisitely regulated by all organisms, from bacteria to humans. In mammals, the iron-regulatory proteins (IRPs) mediate the coordinate expression of proteins that participate in iron metabolism (1–3). When iron stores are low, the IRPs bind to an RNA stem-loop structure known as an iron-responsive element (IRE) and inhibit translation, leading to an increase in the level of the protein encoded by the mRNA. If the iron-responsive element is close to the cap site, binding of the IRP blocks initiation of translation, causing a decrease in the level of the protein encoded by that mRNA. Conversely, when the iron-responsive element is located in the 3′-untranslated region, binding of the IRP stabilizes the mRNA by decreasing susceptibility to nuclease attack, causing an increase in the level of the protein encoded by the mRNA.

Mammals have two known IRPs, IRP1 and IRP2. The two IRPs are regulated by different mechanisms (3). When cellular iron stores are low, IRP1 lacks a functional iron-sulfur center and binds to its iron-responsive element targets. When iron stores are sufficient, IRP1 regains its full iron-sulfur center, loses the ability to bind to iron-responsive element, and functions as a cytosolic aconitase (4, 5). The cellular levels of IRP1 are unaffected by iron status in most cell types.

In contrast, IRP2 protein and iron responsive element binding activity are readily detected when iron stores are limited but are low or absent when iron stores are sufficient (6, 7). The decrease in IRP2 protein occurs as a consequence of rapid degradation; synthesis of the protein is constitutive and generally does not vary substantially with iron status (6, 8). The proteasome was implicated in this degradation soon after IRP2 was described in 1994 (6–9). Identifying the E3 ligase specific for IRP2 took much longer and was reported in 2009 (10, 11).

Quantitation of physiological levels of IRP2 is problematic because of its low abundance in the cytoplasm. Most studies of IRP2 have rendered cells iron deficient by treatment with chelators such as deferoxamine, thus greatly increasing the IRP2 level and allowing more confident quantitation. Addition of a high concentration of iron, usually as ferric ammonium citrate (FAC), triggers rapid degradation of IRP2 by the proteasome. As Dycke and co-workers (12) pointed out, the turnover of IRP2 in cells not perturbed by these manipulations has not been well characterized. Furthermore, most studies have been carried out with transfected cells overexpressing IRP2, often with an epitope tag to facilitate detection. These manipulations can also alter the kinetics and pathways of IRP2 turnover (13).

These considerations led us to investigate the turnover of IRP2 in nontransfected cells grown under standard culture conditions without manipulation of their iron status by chelators. Under these conditions, we found that the turnover of IRP2 is primarily mediated by a nonproteasomal pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK293A cells were a kind gift from Dr. Ruiping Xiao (National Institute on Aging) and are also commercially available from Invitrogen. Cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), in a 37 °C humidified incubator (Thermo Fisher Scientific) with carbon dioxide controlled at 5%. Cells were grown on BioCoat™ collagen I-coated Petri dishes (BD Biosciences) and routinely passaged (~1:5) using citrate saline (135 mm potassium chloride, 15 mm sodium citrate, pH 7.4). Cells were washed with citrate saline twice and then incubated with 1.5 ml of citrate saline (for a 100-mm dish) for 10 min at 37 °C. Then, the dish was gently tapped to detach cells.
**Chemicals and Antibodies**—Proteasome inhibitors used in this study included the irreversible inhibitors epoxomicin (Peprotein International, Louisville, KY), lactacystin (Sigma), and the reversible inhibitor MG-132 (Sigma). Holo-transferrin was from Millipore. Calpain inhibitors PD150606, acetyl-i-leucyl-i-leucyl-norleucinal, calpeptin, calpain inhibitor XI, and calpastatin peptide were from Calbiochem. Caspase inhibitors Z-VAD-fmk, Ac-YVAD-cmk, and Z-YVAD-fmk were from Calbiochem. Thapsigargin was from Invitrogen. Calmodulin inhibitors W-7 and calmidazolium were from Calbiochem. Matrix metalloproteinase inhibitors GM6001, XG076, and TAPI-1 were from Calbiochem. An inhibitor of tripeptidyl peptidase, AAF-CMK, was from Biomol International. All other chemicals were from Sigma. Mouse 7H6 monoclonal anti-IRP2 antibody was kindly provided by Dr. Wolff Kirsch and Wayne Kelin (Loma Linda University, Loma Linda, CA). It is now available from Santa Cruz Biotechnology. Commercially available antibodies were 6C1 monoclonal anti-ubiquitin (Sigma), 1F7E3D10 monoclonal anti-calpastatin (Calbiochem), AA6 monoclonal anti-fodrin α (Millipore), EP1720Y rabbit monoclonal anti-ERK3 (ABcam), AC-15 mouse monoclonal anti-β-actin (Sigma), 13D3 mouse monoclonal anti-Hsc70 (Novus Biologicals), and affinity-purified rabbit polyclonal anti-HA (Sigma).

**SDS-PAGE and Quantitative Western Blot**—Cells were briefly washed twice with phosphate buffered saline from Sigma (1 mM KH2PO4, 3 mM Na2HPO4·7H2O, 155 mM NaCl, pH 7.4, supplemented with 1 mM diethylenetriamine penta-acetic acid) followed by the addition of radioimmuno precipitation assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% deoxycholic acid, freshly supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM diethylenetriamine pentaacetic acid, and a protease inhibitor mixture (Sigma)). Then, the dishes were frozen on dry ice for 10 min, followed by a 30-min incubation on ice. Cells were scraped and transferred into microcentrifuge tubes and centrifuged at 16,000 × g at 4 °C for 10 min. The supernatant was transferred to fresh microcentrifuge tubes, and protein concentration was determined by the BCA method (Thermo Fisher Scientific). Protein samples were mixed with 2× Novex Tris-glycine SDS sample buffer (Invitrogen) supplemented with 1 ml 2-mercaptoethanol and heated for 10 min at 100 °C. The concentration of endogenous IRP2 was calculated from the cell number and a cell volume of 2.0 picoliters (16, 17).

**Western blot**—NucleoCounter (New Brunswick Scientific). One ml of cell suspension was centrifuged, and the cell pellet was dissolved by heating in 80 μl of 1× SDS-PAGE sample loading buffer (Invitrogen). Twenty μl of cell lysate was analyzed on the gel with other lanes containing known amounts of recombinant IRP2 to provide a standard curve. Proteins were transferred to a nitrocellulose membrane followed by quantitative immunodetection. The concentration of endogenous IRP2 was calculated from the cell number and a cell volume of 2.0 picoliters (16, 17).

**Overexpression of Proteins**—Cells were plated on 60-mm dishes 24 h before transfection. At the time of transfection, cells were at 70–80% confluency. The culture medium was changed to 3 ml of prewarmed medium 1 h before transfection.

An IRP2 vector with an amino-terminal FLAG tag and a carboxyl-terminal HA tag, designed for expression in Escherichia coli, was kindly provided by Yi He (National Heart, Lung, and Blood Institute). A forward primer (5’-TCTAGATCTAGGATGGACTATAAAGACGATGAT) and a reverse primer (5’-GGGCCCGGGCCCCTAAGCGTAATCTGGAACATC) were employed to clone FLAG-IRP2-HA from the vector. The PCR product was inserted between the XbaI and Apal sites of the mammalian expression vector pRC-CMV. The fidelity of the resulting plasmid pRC-CMV-FLAG-IRP2-HA was verified by sequencing. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

The calpastatin expression plasmid was a generous gift from Dr. David A. Potter (University of Minnesota, Minneapolis, MN) and Dr. Masatoshi Maki (Nagoya University, Nagoya, Japan). For each plate, 2–8 μg of plasmid DNA was added to 400 μl of 2.5 mM CaCl2, and the tube was gently tapped to facilitate mixing. The solution was added drop-by-drop to 400 μl of HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM dextrose, 50 mM KCl, 0.7 mM Na2HPO4·7H2O, pH 7.4) with agitation to facilitate formation of a DNA-calcium phosphate complex.
IRP2 Turnover

complex. After 5 min without agitation, the transfection solution was added drop-by-drop, and the plates were rocked back and forth to facilitate mixing. Cells were incubated for 6 h in the incubator before changing to fresh growth medium. The cells were further incubated for 24–48 h for experimental treatments, or the cells were split and allowed to grow until appropriate confluency was reached for experimental use.

Fluorescent Calcium Imaging and Measurement—For calcium imaging, cells were grown on a 60-mm dish until ~80% confluent before treatment with a proteasome inhibitor. After the treatment, the cells were washed briefly with prewarmed PBS and then incubated in Dulbecco’s modified Eagle’s medium containing 10 μM Fluo-4, 0.02% Pluronic F-127, and 1 mM probenecid (Invitrogen) for 30 min. Calcium indicator loading medium was replaced with fresh, prewarmed medium supplemented with 1 mM probenecid, and the cells were further incubated for 30 min. Fluorescent calcium images were captured on a Zeiss LSM 5 Pascal confocal microscope.

For quantitative calcium measurement, cells were grown on BioCoat™ 96-well collagen I-coated black wall/clear bottom cell culture plates (BD Biosciences) until fully confluent. Cells were then pretreated with 60 μM PD150606 for 1 h and then with 1 μM epoxomicin for 1, 2, 4, or 6 h. The calcium indicator was loaded using a Fluo-4 NW calcium assay kit (Invitrogen), and fluorescence was measured in well scan mode using a SpectraMax Gemini EM fluorescent microplate reader (Molecular Devices) with excitation at 494 nm, emission at 516 nm, and a cutoff at 515 nm.

RNA Isolation—Cells were grown on 100-mm plates. After experimental treatment they were washed twice with 1× PBS pH 7.4, total RNA was extracted using an RNeasy® Mini Kit (Qiagen), and any DNA contamination was removed by an RNase-free DNase set (Qiagen). RNA concentration was measured at 260 nm on an Agilent 8453 spectrophotometer.

Real-time Quantitative RT-PCR—A primer pair was designed to specifically amplify a 623-base pair section near the 5’ end of the human IRP2 gene region. A BLAST search revealed less than a 50% match to any other gene. The forward primer was 5’-GCATGGACGGCCTGAAAAGCAAGGATACGCC-3’, and the reverse primer was 5’-CCTTGGAGAA-ＡＧＧＡＣＡＣＡＧＧＧＴＧＧＧＧＧT-3’. The human heat shock 70-kDa protein 6 (Hsp70B) forward primer was 5’-AAGGACACAGGATGGGTG-3’.

Quantitation of endogenous IRP2 in HEK293A cells. A, cells were treated with 300 μM FAC for 6 h or 100 μM DFO for 22 h. The same amount of total cellular protein was analyzed by anti-IRP2 Western blots. 5 ng of recombinant IRP2 was analyzed alone or mixed with control (Ctrl) lysate. β-Actin was used as loading control. B, quantitation of endogenous IRP2 in HEK293A cells was performed as described under “Experimental Procedures.”

Target Preparation and Hybridization to GeneChips—T7-based RNA amplification was carried out on 1 μg of the isolated total RNA as suggested by the manufacturer (Affymetrix). Total RNA was incubated with oligo(dT/T7) primers and reverse-transcribed into double-stranded cDNA. In vitro transcription and biotin labeling of the purified cDNA was performed using T7 RNA polymerase at 37 °C for 16 h using the Affymetrix in vitro transcription labeling kit. The yield of biotin labeled cRNA was determined spectrophotometrically with a Nanodrop ND-1000 spectrophotometer and integrity with the Agilent 2100 bioanalyzer. 20 μg of biotin-labeled RNA was fragmented to an ~200-bp size by incubating in buffer containing 200 mM Tris acetate, pH 8.2, 500 mM potassium acetate, and 500 mM magnesium acetate for 35 min at 94 °C prior to hybridization. Fragmented RNA was assessed for fragment size on the bioanalyzer and then hybridized to Affymetrix U133 plus 2.0 chips for 16 h, washed, and stained on an Affymetrix fluidics station.
Microarray Data Processing and Analysis—Affymetrix GCOS (version 1.4) was used to calculate the signal intensity and the percent present calls on the hybridized Affymetrix chip. The signal intensity values obtained for probe sets in the microarrays were transformed using an adaptive variance-stabilizing, quantile-normalizing transformation. Transformed data from all of the chips were subjected to principal component analysis to detect outliers. To correct for multiple comparisons, fold-cutoff filters and false discovery rate analysis filters were applied. Two-way hierarchical clustering was used to bring together sets of samples and genes with similar expression patterns. The hierarchical clustering was performed with the JMP5.1 statistical software package (SAS Institute, Cary, NC) using the ward method. Pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood City, CA).

RESULTS

Quantitation of IRP2—To study the regulation of IRP2 protein levels in untransfected cells, we established a robust, sensitive, and quantitative assay. We screened available polyclonal and monoclonal antibodies and found that the mouse monoclonal 7H6 provided the best signal:noise ratio. Quantitation was accomplished by use of a fluorescent tagged secondary antibody, which was excited by an infrared laser, minimizing interference from autofluorescence of cellular components. Fluorescence was detected and quantitated with an infrared scanner. Fig. 1 shows the determination of endogenous IRP2 in

**FIGURE 2. Epoxomicin decreases endogenous IRP2 protein levels unless cells are pretreated with DFO or chloroquine.** A, cells were pretreated with 100 μM deferoxamine overnight (16 h) before the 6-h exposure to 300 μM FAC and/or 1 μM epoxomicin (Epox). B, cells were treated with 300 μM FAC and/or 1 μM epoxomicin for 6 h. C, cells were treated with 1 μM epoxomicin, lactacystin, or MG-132 for 6 h. D, cells were treated with 25 μM holotransferrin (Holo-Tf) with and without 1 μM epoxomicin for 6 h. E, cells were treated with 1 μM epoxomicin or the same volume of dimethyl sulfoxide (DMSO) as a vehicle control. Hsc70 was used as the loading control. F, cells were pretreated with 50 μM chloroquine for 16 h before the addition of 300 μM FAC or 1 μM epoxomicin for 6 h. In all panels, IRP2 is shown as a percentage of the control level, and error bars show the S.E. from at least two experiments.
HEK293A cells, confirming that we can measure IRP2 levels without transfection and without inducing iron deficiency. That the monoclonal antibody is specifically recognizing IRP2 is demonstrated by (1) the response of the detected band to iron status and (2) co-migration with purified, full-length recombinant IRP2 (Fig. 1A). The concentration of IRP2 is ~3.2 nM corresponding to ~3,800 molecules per cell (Fig. 1B).

Effect of Proteasome Inhibition on IRP2 Levels—A common protocol employed in the study of the regulation of IRP2 is to induce iron deficiency by an overnight treatment with the iron chelator deferoxamine (DFO). In HEK293A cells, DFO treatment increases IRP2 levels by ~7–10×, up to ~30 nM (Fig. 1B). As expected, addition of FAC relieves the iron deficiency and causes a dramatic drop in IRP2 levels (Fig. 2A). This drop is blunted by proteasome inhibitors such as epoxomicin, lactacystin, and MG-132. In DFO-pretreated cells that were not exposed to FAC, epoxomicin had no effect (Fig. 2A).

In the absence of DFO, a steady-state level of IRP2 is maintained by constitutive synthesis of IRP2 balanced by continuous degradation. Modulation of the rate of degradation allows for rapid changes in IRP2 levels coincident with alterations in iron availability (18). Not surprisingly, addition of FAC decreased this steady state level by about half in 6 h (Figs. 1B and 2B). What was surprising was the finding that epoxomicin could not block this decrease (Fig. 2B, lane 3). Moreover, epoxomicin itself caused a decrease in IRP2 level without FAC treatment (Fig. 2B, lane 4). The effect was not a peculiarity of epoxomicin as two other proteasome inhibitors, lactacystin and MG-132, had the same effect (Fig. 2C). Transferrin is the physiological carrier of iron, with uptake via receptor-mediated endocytosis. Addition of iron-loaded transferrin (holotransferrin) caused a decrease in IRP2 levels, which also was not blocked by epoxomicin (Fig. 2D). Thus, the lack of effect of proteasome inhibitors was not due to the addition of ionic iron as FAC, nor was it due to failure to effectively inhibit the proteasome, as addition of epoxomicin caused marked accumulation of ubiquitinylated proteins (Fig. 3).

Without DFO pretreatment, exposure of HEK293A cells to epoxomicin reproducibly caused a decrease in IRP2 levels of ~50% at 6 h as shown in Fig. 2E. In addition, we noted that the levels of IRP2 increased by 10–20% at 1–2 h, with the marked decrease occurring after 4 h, also shown in Fig. 2E. The initial small increase followed by a decrease is consistent with two pathways for degradation of IRP2, one via the proteasome and the other via a nonproteasomal pathway. The former appears to dominate in DFO-treated cells, whereas the latter predominates in cells not treated with DFO.

DFO is trapped in the lysosome after uptake by endocytosis (19, 20). Lysosomal localization is to be expected because DFO has four amines with pKₐ values ranging from 8.3 to 10.8 (21). It is thus a lysosomotropic amine that becomes protonated and then trapped in the lysosome. Such lysosomotropic amines are well known to alkalinize endosomes and lysosomes, thereby interfering with their functions, including receptor-mediated endocytosis and protease activity. Ammonium chloride and chloroquine are well known lysosomotropic amines, which cause intracellular iron deficiency by inhibiting the release of iron from transferrin after receptor-mediated endocytosis of iron-loaded transferrin (22). The latter effects are consistent with the observed effects of DFO on IRP2, which is consistent with these points.

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holotransferrin (22–24). Chloroquine is not an iron chelator so that comparing its effects on IRP2 metabolism with those of DFO would allow assessment of whether the iron chelating property of DFO is required to observe proteasomal degradation of IRP2. Overnight treatment with chloroquine markedly increased IRP2, which was readily degraded by the proteasome upon addition of FAC; epoxomicin alone did not cause degradation of IRP2 in chloroquine-treated cells (Fig. 2F). Because the effects of chloroquine on IRP2 are the same as those observed with DFO, we conclude that chelation of iron is not required for degradation of IRP2 by the proteasomal pathway. Alkalinization of the endosome/lysosome suffices to greatly increase IRP2 levels and direct IRP2 degradation to the proteasome, as also observed by Dycke and colleagues (12). They suggested that the nonproteasomal degradation was mediated by a lysosomal pathway, but we do not think a lysosomal protease can be implicated by that observation because of the confounding effect caused by the simultaneous inhibition of receptor-mediated endocytosis of transferrin.

**Mechanism of Change in IRP2 Levels**—Previous investigators established that the primary mechanism of regulation of IRP2 levels is via modulation of the rate of its degradation and not of its mRNA level (6, 8). However, given our unexpected finding that treatment with a proteasome inhibitor decreased the level of IRP2 in nontransfected cells (Fig. 2B), we measured the IRP2 mRNA levels by quantitative RT-PCR to determine whether epoxomicin treatment induced a change. Fig. 4A shows that it does not. Hsp70B mRNA levels are known to increase markedly upon epoxomicin treatment, so we also measured those levels as a positive control for epoxomicin action (Fig. 4B).

Because the mRNA levels of IRP2 were unchanged, we then determined whether the rate of IRP2 protein degradation was

![FIGURE 5. Epoxomicin accelerates IRP2 degradation.](image)

**FIGURE 5.** *Epoxomicin accelerates IRP2 degradation.* *A,* cells were treated with dimethyl sulfoxide (DMSO) as a vehicle control, 40 μM cycloheximide (CHX), 40 μM cycloheximide with 1 μM epoxomicin (CHX + Epox), or pretreated with 1 μM epoxomicin for 3 h before the addition of 40 μM cycloheximide (Epox → CHX). Cells were lysed, and quantitative Western blotting for IRP2 and ERK3 was performed. *B,* half-life of IRP2. The level of IRP2 at 0 h was set to 100%. The y-axis is logarithmic because degradation is first order. ▲, without epoxomicin; ●, epoxomicin added at 0 h with cycloheximide; ▲, 3-h pretreatment with epoxomicin before addition of cycloheximide at 0 h.

![FIGURE 6. Epoxomicin does not stimulate degradation of IRP2 when its level is increased by transfection.](image)

**FIGURE 6.** *Epoxomicin does not stimulate degradation of IRP2 when its level is increased by transfection.* Cells were transfected with the FLAG-IRP2-HA plasmid using Lipofectamine and treated with or without 1 μM epoxomicin for 6 h. Endogenous and recombinant IRP2 was detected by anti-IRP2 monoclonal antibody, and recombinant IRP2 alone was detected by anti-HA antibody. *Inset,* the fractional degradation of IRP2 triggered by epoxomicin decreased as IRP2 levels increased.
altered. Treatment of cells with cycloheximide blocks protein synthesis, allowing the rate of protein degradation to be measured. To validate the experimental approach, we also quantitated levels of ERK3, a protein known to be degraded by the proteasome (25). Treatment of HEK293A cells with cycloheximide allowed the observation of ERK3 degradation, which was blocked by epoxomicin (Fig. 5A). Thus, both cycloheximide and epoxomicin were pharmacologically effective. Treatment of the cells with cycloheximide also caused a decrease in IRP2 levels, but addition of epoxomicin increased the rate of loss of IRP2. A first order plot of IRP2 levels (Fig. 5B) allows determination of the half-life of IRP2. In the absence of epoxomicin, the half-life was 3.0 h. With a 3-h pretreatment of epoxomicin, the half-life decreased to 1.7 h. We conclude that epoxomicin lowers cellular levels of IRP2 by increasing its rate of degradation.

Characterization of Nonproteasomal Pathway of IRP2 Degradation—We noted above that DFO and chloroquine both alkalinize the lysosome and thus inhibit lysosomal proteases and induce iron deficiency by blocking unloading of iron from holotransferrin. Either effect could cause the observed increase in IRP2 level. We considered the possibility that lysosomal dysfunction was not required to observe proteasomal degradation and that an increased IRP2 level alone would suffice. We therefore transfected HEK293A cells with a full-length construct of IRP2 carrying an epitope tag so that we could compare the behavior of endogenous and transfected IRP2. We used increasing amounts of vector DNA to assure that the increase in IRP2 would encompass levels achieved by DFO, that is, 5–10-fold. Fig. 6 shows that increasing the level of cellular IRP2 by transfection also eliminates the epoxomicin stimulated degradation of IRP2. Epitope-tagged IRP2 exhibited the same degradation behavior, indicating that the presence of an epitope tag did not perturb the characteristics of degradation. This observation is consistent with the non-proteasomal pathway having a high affinity for IRP2 but relatively low capacity. Only the activity of the proteasomal pathway is observed upon epoxomicin exposure at higher levels of IRP2 when the nonproteasomal system becomes saturated.

We performed DNA microarray analysis in an attempt to detect mRNA of protease(s) that were up-regulated via proteasome inhibition by epoxomicin. We used the Affymetrix U133 human genome array, which interrogates 33,000 human genes, but no up-regulated protease genes were detected. The microarray data, which may also be of interest to those studying the effects of proteasome inhibition, have been deposited in the GEO database (National Center for Biotechnology Information) under accession no. GSE14429. We then tested chemical inhibitors of various classes of proteases for their ability to

| Target | Compounda |
|--------|-----------|
| Calpain | ALLN (20 μM), calpeptin (2 μM), calpain inhibitor XII (80 μM), calpastatin peptide (40 μM), calpastatin transfection |
| Calmodulin | W-7 (40 μM), calmidazolium (10 μM) |
| Caspase | Z-VAD-fmk (40 μM), Ac-YVAD-CMK (40 μM), Z-YVAD-fmk (80 μM) |
| Lysosomal proteases | E-64-D (100 μM), pepstatin A (100 μM), leupeptin (100 μM) |
| Macroautophagy | 3-Methyladenine (2 mM) |
| Matrix metalloproteinases | GM6001 (100 μM), XG076 (50 μM), TAPI-1 (50 μM) |
| Tripeptidyl peptidase II | AAF-CMK (40 μM) |

a Compounds were added to HEK293A cells for 6 h. None inhibited the epoxomicin-induced (1 μM) IRP2 degradation.

FIGURE 7. Calpain does not degrade IRP2 in epoxomicin-treated cells. A, cells were treated with 1 μM epoxomicin and/or PD150606 for 6 h. The level of IRP2 is shown as a percentage of control. B, IRP2 mRNA from cells treated with 60 μM PD150606 was analyzed by QRT-PCR. The IRP2 mRNA was normalized to β-actin mRNA and shown as the fraction relative to 0 h. C, cells were transfected using calcium phosphate with an empty vector or the human calpastatin expression vector for 6 h. The medium was replaced, and cells were incubated for another 16 h. Cells were then treated with 1 μM epoxomicin for 6 h before lysis and quantitative Western blot analysis. IRP2 and fodrin levels are shown as a percentage of control. The error bars in each panel give the S.E. from at least two experiments.
FIGURE 8. Calcium is required for epoxomicin-induced IRP2 degradation. A, intracellular calcium visualization. Cells were treated with 1 μM epoxomicin for 6 h before loading with Fluo-4 AM calcium indicator. Phase contrast and fluorescent images were taken with a Zeiss LSM 5 Pascal confocal microscope at room temperature. B, quantitation of intracellular calcium. Cells were grown in 96-well plates in triplicate until confluent. Cells were pretreated with 60 μM PD150606 for 1 h before the addition of 1 μM epoxomicin (Epox). The calcium indicator was then loaded into the cells, and its signal was measured with a fluorescent plate reader. The results shown are the average and S.D. from three experiments. C, thapsigargin triggers degradation of IRP2. Cells were treated with 1 μM thapsigargin or the same volume of dimethyl sulfoxide (DMSO). D, inhibition of the proteasome does not prevent thapsigargin-induced degradation of IRP2. Cells were treated with 1 μM thapsigargin and/or 1 μM epoxomicin for 6 h. E, thapsigargin-induced degradation is inhibited by PD150606. Cells were treated with 5 μM thapsigargin and the indicated concentrations of PD150606 for 6 h. F, the degradation rate of endogenous and transfected IRP2 are the same. Cells grown in a 60-mm dish were transfected with 0.05 μg pRC-CMV-FLAG-IRP2-HA plasmid for 24 h before 1 μM thapsigargin treatment. Endogenous and transfected IRP2 were detected by anti-IRP2 antibody, quantitated separately, and shown as a percentage of the 0 h value. G, quantitative RT-PCR of IRP2 mRNA levels. Cells were treated with 1 μM thapsigargin (TG), followed by isolation of total RNA and RT-PCR. IRP2 levels are shown as a percentage of the control (Ctrl) level, and error bars give the S.E. from at least two experiments.
block the epoxomicin-triggered degradation of IRP2. Table 1 lists those that had no effect on IRP2 degradation.

**Calcium Is Required for Epoxomicin-induced Nonproteasomal Degradation of IRP2**—The one compound that did blunt the epoxomicin-triggered degradation was PD150606 (3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid), a calpain inhibitor (Fig. 7A). Without exposure to epoxomicin, treatment with PD150606 increased IRP2 levels above those in untreated cells, without altering the mRNA level of IRP2 (Fig. 7B). This observation suggests that the non-proteasomal pathway of IRP2 degradation operates normally even in cells not perturbed by epoxomicin. However, ~60 μM PD150606 was required to see a clear effect (Fig. 7A), whereas effective inhibition of calpain within cells has been observed at 10 μM PD150606 (27). Moreover, other calpain inhibitors did not have the ability to block the degradation of IRP2 triggered by epoxomicin (Table 1).

To reach a more confident assessment of whether calpain was the intracellular protease responsible for epoxomicin-triggered degradation of IRP2, we expressed calpastatin, the specific intracellular inhibitor of calpain. Fodrin α is known to be degraded by calpain, so the appearance of its 150-kDa cleavage product was also followed as a positive control for calpastatin activity. Fig. 7C demonstrates that epoxomicin causes the degradation of both fodrin α and IRP2, and calpastatin protects fodrin α but not IRP2.

Having established that PD150606 inhibited epoxomicin-triggered degradation of IRP2 by a mechanism other than inhibition of calpain, we considered the possibility that epoxomicin induced a change in intracellular calcium, which was required for IRP2 degradation, and, consistent with published studies (28), PD150606 was able to inhibit the change in calcium. Imaging of HEK293A cells with Fluo-4 AM demonstrated that a 6-h exposure to 1 μM epoxomicin did increase the intracellular calcium concentration (Fig. 8A). The time course of calcium increase and the inhibition by PD150606 are shown in Fig. 8B.

Thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase, raises intracellular calcium concentration. Exposure of HEK293A cells to 1 μM thapsigargin caused an even more rapid degradation of IRP2 than epoxomicin (compare Figs. 2E and 8C). This thapsigargin-induced degradation was not mediated by the proteasome (Fig. 8D), and PD150606 inhibited the effect (Fig. 8E). As with epoxomicin, the inhibition required 40–60 μM PD150606. A double-tagged IRP2 (FLAG-IRP2-HA) whose expression was carefully controlled to match the endogenous level was also subject to thapsigargin-induced degradation (Fig. 8F). The rate of degradation of endogenous and transfected IRP2 were comparable. This result also provides additional confirmation that the anti-IRP2 monoclonal antibody recognized endogenous IRP2. Thapsigargin did not alter the IRP2 mRNA level (Fig. 8G).

Calmodulin is required to mediate many calcium-dependent processes (29). However, treatment with the calmodulin inhibitor W-7 did not blunt the epoxomicin-triggered degradation of IRP2 (Fig. 9). We conclude that the epoxomicin-induced degradation of IRP2 requires an increase in intracellular calcium, but calmodulin may not mediate the process.

**DISCUSSION**

Utilizing a monoclonal primary antibody and a secondary antibody labeled with a chromophore that fluoresces in the near-infrared, we can readily quantitate physiological levels of IRP2. This methodology allowed characterization of the degradation of IRP2 in HEK293A cells without elevation of IRP2 levels by transfection or induction of iron deficiency. This quantitative, sensitive method demonstrated that the steady-state turnover of IRP2 in iron-replete cells is maintained by a nonproteasomal degradation system. Cells and tissues may vary in utilization of the two pathways (6), as we did not detect the nonproteasomal pathway in two other cell lines that we tested, HeLa and COS-7. We also observed that the activity of the nonproteasomal system varied among lineages of the HEK293 cell line. Although HEK293A cells utilized the nonproteasomal system under physiological conditions, Guo et al. demonstrated that a proteasome inhibitor blocked iron-induced IRP2 degradation in the rat FTO2B hepatoma cell line even without DFO pretreatment.

The proteasomal degradation pathway is elegantly regulated by an iron-sensing E3 ubiquitin ligase (10, 11). The pathway does not require the 73-amino acid "intervening domain" that is present in IRP2 but not IRP1 (30–32). We have not yet characterized the regulation of the nonproteasomal pathway, other than demonstrating that calcium is required to observe its action. Characterization will be facilitated when the protease is identified. Neither a battery of protease inhibitors nor microarray analysis identified candidate proteases, suggesting that it may have novel properties. The behavior of the nonproteasomal pathway toward IRP2 is that of a high affinity, low capacity system whose action can no longer be observed when IRP2 increases 5–10-fold above basal level. The proteasomal system comes into action when the cell requires degradation of elevated levels of IRP2, such as upon relief of chelator-induced iron deficiency.

We found that proteasome inhibitors increase cellular calcium levels, an effect that has not been studied in detail previously. In undifferentiated PC12 cells, MG-132 caused an increase of cytosolic calcium and cell death (33). In myeloma
cells, another proteasome inhibitor, bortezomib, caused an increase of cytosolic calcium, mitochondrial calcium loading, capacitative calcium influx from the extracellular space, activation of caspasases, and cell death (26). Although calcium is required for the non-proteasomal pathway, our experiments with calpain inhibitors, especially the specific calpastatin, establish that calpain is not the non-proteasomal protease.

Fifteen years passed between when the proteasome was shown capable of degrading IRP2 and the enabling E3 ligase was identified. Hopefully, the interval from recognizing the nonproteasomal pathway to identification of its protease will be much shorter.

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REFERENCES

1. Wallander, M. L., Leibold, E. A., and Eisenstein, R. S. (2006) Biochim. Biophys. Acta. 1763, 668–689
2. Hentze, M. W., and Kühn, L. C. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 8175–8182
3. Rouault, T., and Klausner, R. (1997) Curr. Top Cell Regul. 35, 1–19
4. Haile, D. J., Rouault, T. A., Tang, C. K., Chin, J., Harford, J. B., and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 7536–7540
5. Gray, N. K., Quick, S., Goossen, B., Constable, A., Hirling, H., Kühn, L. C., and Hentze, M. W. (1993) Eur. J. Biochem. 218, 657–667
6. Guo, B., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995) J. Biol. Chem. 270, 21645–21651
7. Iwai, K., Klausner, R. D., and Rouault, T. A. (1995) EMBO J. 14, 5350–5357
8. Samaniego, F., Chin, J., Iwai, K., Rouault, T. A., and Klausner, R. D. (1994) J. Biol. Chem. 269, 30904–30910
9. Guo, B., Yu, Y., and Leibold, E. A. (1994) J. Biol. Chem. 269, 24252–24260
10. Vashisht, A. A., Zumbrennen, K. B., Huang, X., Powers, D. N., Durazo, A., Sun, D., Bhaskaran, N., Persson, A., Uhlen, M., Sangfelt, O., Spruck, C., Leibold, E. A., and Wohlschlegel, J. A. (2009) Science 326, 718–721
11. Salahudeen, A. A., Thompson, J. W., Ruiz, J. C., Ma, H. W., Kinch, L. N., Li, Q., Grishin, N. V., and Bruijc, R. K. (2009) Science 326, 722–726
12. Dycke, C., Charbonnier, P., Pantopoulos, K., and Moulis, J. M. (2008) Int. J. Biochem. Cell Biol. 40, 2826–2832
13. Wang, J., Chen, G., Muckenthaler, M., Galy, B., Hentze, M. W., and Pantopoulos, K. (2004) Mol. Cell. Biol. 24, 954–965
14. Kim, G., Cole, N. B., Lim, J. C., Zhao, H., and Levine, R. L. (2010) J. Biol. Chem. 285, 18085–18094
15. Luo, S., Wehr, N. B., and Levine, R. L. (2006) Anal. Biochem. 350, 233–238
16. Zimmermann, D., Terpitz, U., Zhou, A., Reuss, R., Müller, K., Sukhorukov, V. L., Gessner, P., Nagel, G., Zimmermann, U., and Bamberg, E. (2006) Biochem. Biophys. Res. Commun. 348, 673–681
17. Thomas, P., and Smart, T. G. (2005) J. Pharmacol. Toxicol. Methods 51, 187–200
18. Rouault, T. A. (2006) Nat. Chem. Biol. 2, 406–414
19. Lloyd, J. B., Cable, H., and Rice-Evans, C. (1991) Biochem. Pharmacol 41, 1361–1363
20. Pernson, H. L., Yu, Z., Tirosh, O., Eaton, J. W., and Brunk, U. T. (2003) Free Radic. Biol. Med. 34, 1295–1305
21. Evers, A., Hancock, R. D., Martell, A. E., and Motekaitis, R. J. (1989) Inorg. Chem. 28, 2189–2195
22. Octave, J. N., Schneider, Y. J., Hoffmann, P., Trouet, A., and Crichton, R. R. (1979) FEBS Lett. 108, 127–130
23. Morgan, E. H. (1981) Biochim. Biophys. Acta. 642, 119–134
24. Karin, M., and Mintz, B. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6687–6692
25. Landowski, T. H., Megli, C. J., Nullmeyer, K. D., Lynch, R. M., and Dorr, R. T. (2005) Cancer Res. 65, 3828–3836
26. Wang, K. K., Nath, R., Posner, A., Raser, K. J., Buroker-Kilgore, M., Haji-mohammadiereza, l., Probert, A. W., Jr., Marcoux, F. W., Ye, Q., Takano, E., Hatanaka, M., Maki, M., Caner, H., Collins, J. L., Fergus, A., Lee, K. S., Lunney, E. A., Hays, S. J., and Yuen, P. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 6687–6692
27. Van den Bosch, L., Van Damme, P., Vlemminckx, V., Van Houtte, E., Lemmens, G., Missiaen, L., Callewaert, G., and Robberecht, W. (2002) Neurouropharmacology 42, 706–713
28. Klee, C. B., Ren, H., and Wang, X. (1998) J. Biol. Chem. 273, 13367–13370
29. Ishikawa, H., Kato, M., Hori, H., Ishimori, K., Kirisako, T., Tokunaga, F., and Iwai, K. (2005) Mol. Cell 19, 171–181
30. Wang, J., Chen, G., Lee, J., and Pantopoulos, K. (2008) BMC. Mol. Biol. 9, 15
31. Bourdon, E., Kang, D. K., Ghosh, M. C., Drake, S. K., Wey, J., Levine, R. L., and Rouault, T. A. (2003) Blood Cells Mol. Dis. 31, 247–255
32. Lee, C. S., Han, E. S., Han, Y. S., and Bang, H. (2005) Brain Res. Bull. 67, 225–234