Inhibitor of Nrf2 (INrf2 or Keap1) Protein Degrades Bcl-xL via Phosphoglycerate Mutase 5 and Controls Cellular Apoptosis

INrf2 (Keap1) is an adapter protein that facilitates INrf2-Cul3-Rbx1-mediated ubiquitination/degradation of Nrf2, a master regulator of cytoprotective gene expression. Here, we present evidence that members of the phosphoglycerate mutase family 5 (PGAM5) proteins are involved in the INrf2-mediated ubiquitination/degradation of anti-apoptotic factor Bcl-xL. Mass spectrometry and co-immunoprecipitation assays revealed that INrf2, through its DGR domain, interacts with PGAM5, which in turn interacts with anti-apoptotic Bcl-xL protein. INrf2-Cul3-Rbx1 complex facilitates ubiquitination and degradation of both PGAM5 and Bcl-xL. Overexpression of PGAM5 protein increased INrf2-mediated degradation of Bcl-xL, whereas knocking down PGAM5 by siRNA decreased the degradation of Bcl-xL, resulting in increased stability. Mutation of PGAMA5-E79A/S80A abolished INrf2/PGAM5/Bcl-xL interaction. Therefore, PGAM5 bridged INrf2 and Bcl-xL interaction, and knockdown of PGAM5-E79A/S80A abolished the INrf2-Bcl-xL interaction. Moreover, PGAM5 overexpression increased INrf2-mediated degradation of Bcl-xL, whereas knocking down PGAM5 by siRNA decreased the degradation of Bcl-xL, resulting in increased stability. These data provide the first evidence that INrf2 controls Bcl-xL via PGAM5 and controls cellular apoptosis.

INrf2 (Keap1)-Nrf2 complex serves as a sensor of chemical- and radiation-induced oxidative and electrophilic stress (reviewed in Refs. 1 and 2). INrf2 (inhibitor of Nrf2) or Keap1 (Kelch-like ECH-associated protein 1) functions as a substrate adaptor protein for a Cul3-Rbx1-dependent E3 ubiquitin ligase complex to ubiquitinate and degrade Nrf2 (NF-E2-related factor), thus maintaining steady-state levels of Nrf2 (1, 2). Nrf2 is a nuclear transcription factor that responds to stress and is released from INrf2 (1, 2). Chemical modification of INrf2C151 or/and protein kinase Cδ phosphorylation of INrf2540 leads to the release of Nrf2 from INrf2 (2). Nrf2 is stabilized, translocates into the nucleus, binds with antioxidant response element, and activates a battery of cytoprotective gene expression. This provides protection against oxidative and electrophilic stress and promotes cell survival. Nrf2-null mice are prone to acute damages induced by 4-hydroxybutylamino, pentachlorophenol, tert-butylhydroquinone, and 4-vinylcyclohexene oxide (3–6). Nrf2-null mice have increased pulmonary DNA adducts and bladder and N-nitrososobutyl incidence of Nrf2-null mice demonstrated persistent accumulation of oxidized DNA and increased susceptibility to chemically induced tumors when exposed to diesel exhaust and radiation (7–9).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Fig. 1.

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In Nrf2 Degradation of PGAM5-Bcl-xL

Bcl-xL share regions of sequence similarity as well as a C-terminal hydrophobic region required for membrane localization (27). Bcl-2 and Bcl-xL appear to function in the same apoptotic pathway, and both confer resistance to multiple chemotherapy agents. Overexpression of either protein is usually associated with poor prognosis in many human cancers. However, in some cancer types, multiple anti-apoptotic proteins are expressed (28, 23) and have opposite effects on prognosis, indicating that there may be subtle but clinically and biologically relevant functional differences between family members. Experiments in mice with deletion of individual anti-apoptotic genes indicate that the phenotypes are not identical, presumably because of differential tissue expression of the various members (29). The mechanisms of action of Bcl-2 and Bcl-xL are complex, with many postulated interactions with other proteins, and the role of any single interaction in the final phenotype at the cellular level remains unknown.

Recently, INrf2 is shown to target anti-apoptotic Bcl-2 protein for degradation and control cellular apoptosis (30). In the present report, we investigated the novel role of INrf2 in the regulation of anti-apoptotic factor Bcl-xL. INrf2, through its DGR domain, interacts with PGAM5 proteins, which interact with Bcl-xL. Interestingly, we show that INrf2-Cul3-Rbx1 complex facilitates both PGAM5 and Bcl-xL ubiquitination and degradation. The data also revealed that PGAM5 protein acts as a bridge between INrf2 and Bcl-xL. Further, studies showed that overexpression of INrf2 degrades both PGAM5 and Bcl-xL proteins, which increases/activates protein for degradation and control cellular apoptosis (30). In the regulation of anti-apoptotic factor Bcl-xL. INrf2, through its DGR domain, interacts with PGAM5 proteins, which interact with Bcl-xL. Interestingly, we show that INrf2-Cul3-Rbx1 complex facilitates both PGAM5 and Bcl-xL ubiquitination and degradation. The data also revealed that PGAM5 protein acts as a bridge between INrf2 and Bcl-xL. Further, studies showed that overexpression of INrf2 degrades both PGAM5 and Bcl-xL proteins, which increases/activates protein for degradation and control cellular apoptosis.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—PGAM5 exists in two forms (31). Long form (PGAM5L) is 34 amino acids longer at the C terminus than short form (PGAM5S). PGAM5L cDNA was obtained from Origene, and coding sequence was amplified using forward (5'-AACCCCATGGGCTCTTCCGCACGTTGATCAGAGACAGTGG-3') and reverse (5'-GGATCGAGTGATCTTGTCGGGAGGCAT-3') primers, and PCR product was cloned in pcDNA3.1-V5-tagged vector by TA cloning, and the construct was designated as PGAM5L-V5. We generated PGAM5L-E79A/S80A-V5 double mutant plasmid by the site-directed mutagen GeneTailor kit (Invitrogen) using forward primer (5'-CGGAAGAGGAGACGTGGGTGCTGGGAGAGAGAGCTGG-3') and reverse primer (5'-CGATTTCTTCTTCGACGTCAGTACGAGACAGTGGG-3') to determine the role of mutated residues in PGAM5 interaction with INrf2. Mouse Bcl-xL plasmid was obtained from Addgene (ID 8772), and coding sequence was amplified by using forward primer (5'-ATAGGCTCTAGAATGTCATCGAGCAA-

CCGGGAGCTGGT-3') and reverse primer (5'-TTCAGGCTCGAAGTCTCCGACAGTAGACCCAGAAAACACC-3') and cloned into pc mxFLAG-2X vector using XbaI and XhoI restriction sites. The resultant plasmid was designated as FLAG-Bcl-xL. The construction of FLAG-INrf2, INrf2-V5, and FLAG-INrf2 and HA-Cul3, Myc-Rbx1, and HA-ubiquitin was described previously (30). Subcellular Western Blotting—Hepa-1 cells were seeded in 100-mm plates and transfected/treated as described previously. The cell lysates, the cell lysates, and the isolated mitochondrial fraction were used for Western blots. The isolated cell lysates and the isolated mitochondrial fraction were separated on SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. The membranes were probed with anti-INrf2 (E-20) (1:1000), anti-Nrf2 (H-300) (1:1000), anti-Bcl-xL (H5) (1:1000), anti-Bax (N20) (1:1000), anti-Bcl-2 (P4D1) (1:1000), anti-Mcl-1(S-19) (1:1000), anti-ubiquitin (HRP, and anti-

rabbits (Pacific Immunology) and purified. The membranes were washed three times with TBST, and immunoreactive bands were visualized using a chemiluminescence ECL system (Amersham Biosciences). The intensity of protein bands after treatment with anti-INrf2 and anti-Nrf2 antibodies was determined by densitometry. Anti-INrf2 and anti-Nrf2 antibodies were obtained from Sigma and used in 1:10,000 dilutions to probe the Western blots. Anti-V5 antibody and anti-V5-HRP antibody were obtained from Invitrogen, and anti-caspase-3 antibody was purchased from Cell Signaling. To confirm the purity of cytoplasmic and mitochondrial protein fractionation, the membranes were reprobed with cytoplasm-specific, anti-lactate dehydrogenase (Chemicon) and mitochondria-specific anti-cytochrome c or CoxI IV antibodies (Cell Signaling). Jurkat cells were treated with 100 ng/ml Killer TRAIL soluble human recombinant protein (Enzo Life Sciences, catalog no. ALX-201073). We generated and purified bacterial PGAM5L-His-tagged protein. The polyclonal antibodies against the PGAM5L form were generated in rabbits (Pacific Immunology) and purified. The membranes were washed three times with TBST, and immunoreactive bands were visualized using a chemiluminescence ECL system (Amersham Biosciences). The intensity of protein bands after Western blotting were quantified by using QuantityOne version 4.6.3 image software (ChemiDoc XRS, Bio-Rad) and normalized against proper loading controls. In related experi-

2 The abbreviations used are: t-BHQ, tert-butylhydroquinone; RIPA, radioimmunoprecipitation assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQO1, NAD(P)H:quinone oxidoreductase; IP, immunoprecipitation; WB, Western blot; TET, tetracycline.
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ments, the cells were treated with 50 $\mu$M t-BHQ or DMSO as a vehicle for different time intervals.

**Immunoprecipitation**—For immunoprecipitation, 1 mg of whole cell extracts or 300 $\mu$g of mitochondrial lysates were equilibrated in RIPA buffer and precleared with Protein AG Plus-agarose (Santa Cruz Biotechnology, Inc.), and then extracts were incubated with respective antibodies (1 $\mu$g) at 4°C overnight. Immune complexes were collected by the addition of Protein AG-agarose and centrifugation. The immune complexes were washed three times with 0.25% Nonidet P-40, and proteins were resolved by 10–12% reducing SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 3% nonfat dry milk and incubated with their respective primary and secondary antibodies. Immunoreactive bands were visualized using a chemiluminescence ECL system (Amersham Biosciences).

**Ubiquitination Assay**—INrf2-FRT293, INrf2DGFR-FRT293, and INrf2DGFR-FRT293 cells were co-transfected with PGAM5-V5 and HA-ubiquitin, treated with tetracycline, and analyzed for PGAM5 ubiquitination. The effect of overexpression of INrf2 in INrf2-293 and Hepa-1 cells or the effect of INrf2 siRNA on endogenous ubiquitination of Bcl-xL was also analyzed.

Cell pellets were lysed in RIPA buffer containing 1% SDS. One mg of the lysate (~100 $\mu$l) was diluted to 10-fold with RIPA buffer. After precleanning, samples were immunoprecipitated with 2 $\mu$g of antibody or anti-FLAG beads (15 $\mu$l) as indicated in the figures. Immune complexes were analyzed for endogenous ubiquitination of Protein AG-agarose. Immunoreactive bands were visualized using a chemiluminescence ECL system (Amersham Biosciences).

**Transient Transfection**—Hepa-1 cells were transfected with 100–200 $\mu$g of the indicated plasmids using Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. Thirty-two hours after transfection, cells were harvested, and INrf2, PGAM5L, and Bcl-xL siRNA were used to inhibit INrf2, PGAM5L, and Bcl-xL proteins, respectively. Control GAPDH siRNA and Bcl-xL siRNA were purchased from Dharmacon. PGAM5L and Bcl-xL siRNA were obtained from Ambion. In most cases, Hepa-1 cells were transfected with 25, 50, and 75 nM INrf2, PGAM5L, and Bcl-xL siRNA separately using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Thirty-two hours after transfection, cells were harvested, and INrf2, PGAM5L, Bcl-xL, and actin proteins were analyzed by Western blotting. In related experiments, Jurkat cells were transfected with 25 and 50 nM INrf2 siRNA. siRNA was mixed with Lipofectamine RNAiMAX reagent in Opti-MEM medium (1 ml), and the mixture was incubated at room temperature for 15 min. Then the reaction mixture was coated onto 100-mm tissue culture plates for 10 min. Exponentially grown Jurkat cell suspensions in RPMI medium without antibiotics (4 ml; 10$^6$ cells) were added into the plates, and cells were incubated at 37°C for 24 h followed by treatment with TRAIL protein (100 ng/ml) for 30 h. Cells were harvested, lysed, and immunoblotted.

**Immunofluorescence**—Hepa-1 cells were grown in Lab-Tek II chamber slides. Cells were fixed in 2% formaldehyde and permeabilized by the treatment of 0.25% Triton X-100. Cells were washed twice with PBS and incubated with a 1:1000 dilution of sheep cytochrome c antibody along with goat INrf2, rabbit PGAM5, and mouse Bcl-xL antibody separately at 4°C for 12 h. Then cells were washed twice with PBS and incubated with anti-sheep FITC-conjugated second antibody or Alexa Fluor-594-conjugated anti-goat, anti-rabbit, and anti-mouse second antibodies (Invitrogen). After immunostaining, cells were washed twice with PBS, stained with Vectashield containing nuclear DAPI stain, and mounted. Cells were observed under a Nikon fluorescence microscope and photographed.

**Real-time PCR**—Hepa-1 cells were transfected with increasing concentrations of FLAG-INrf2, or endogenous INrf2 expression was knocked down by siRNA (25–75 nM) for 30 h, and cells were harvested. Total RNA was isolated using the RNeasy minikit (Qiagen). One mg of total RNA was subjected to reverse transcription using a high capacity cDNA transcription kit (Applied Biosystems). After synthesis of cDNA, quantitative PCR was performed using the 7500 real-time PCR system (Applied Biosystems) and the manufacturer’s gene expression TaqMan mRNA detection kit (Hs00402923_m1) and control 18S TaqMan probe (Hs99999901_s1). Each reaction was performed in triplicate. The data were analyzed and plotted.

**MTT Cell Survival Assay**—For the MTT assay, Hepa-1 cells were transfected with the indicated plasmids treated with etoposide or etoposide plus t-BHQ (50 $\mu$M) for 48 h. The Dead End Fluorometric TUNEL assay kit (Promega) was used as per the manufacturer’s protocol. TUNEL-positive cells were counted from three independent experiments and plotted.
with fresh MTT solution (200 μl/well; stock 5 mg/ml in PBS) at 37 °C for 2 h, and absorbance at 570 nm was measured. Each combination of cell line and drug concentration was set up in eight replicate wells, and the experiment was repeated three times. Each data point represents a mean ± S.D. and is normalized to the value of the corresponding control cells.

Statistical Analyses—The data real-time PCR and immunoblotting band intensities were analyzed using a two-tailed Student’s t test. Data are expressed as mean ± S.D. of three independent experiments. The error bars indicate S.E. of triplicate samples, and comparisons were made using the two-tailed Student’s t test for repeated measures. Differences between means were accepted as statistically significant at the 95% level (p < 0.04).

RESULTS

**INrf2-mediated Degradation of PGAM5 and Anti-apoptotic Bcl-xL Protein**—Control 293 and INrf2-293 cells were treated with tetracycline for the indicated time periods, and the concentration of cell line and drug was adjusted according to the number of eight replicate wells, and the experiment was repeated three times. Each data point represents a mean ± S.D. and is normalized to the value of the corresponding control cells.

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**FIGURE 1.** Overexpression of INrf2 leads to PGAM5 and Bcl-xL degradation. A, overexpression of FLAG-INrf2 degrades endogenous/transfected PGAM5 and endogenous Bcl-xL. Control 293 or INrf2-293 cells were either untransfected (top left) or transfected with PGAM5L-V5 plasmid (top right). The cells were treated with tetracycline (0.5 μg/ml) for different time periods and immunoblotted (top panels). In similar experiments, Hepa-1 cells were transfected with FLAG-INrf2 or FLAG-INrf2 with PGAM5L-V5 plasmids and immunoblotted (middle panels). The bands were quantified from three independent experiments and plotted (bottom panels). B, siRNA inhibition of INrf2 stabilized PGAM5L and Bcl-xL protein. Hepa-1 cells were transfected with control (75 nM) or INrf2 siRNA (25–75 nM) for 36 h and immunoblotted (bottom) for 30 h. Cells were harvested and RNA was extracted. In related experiments, an siRNA-dependent decrease in INrf2 resulted in increased PGAM5L-V5 and Bcl-xL (Fig. 1A, middle left panels) and transfected PGAM5L-V5 and Bcl-xL proteins in transfected INrf2-293 cells. In another experiment, Hepa-1 cells showed similar results as INrf2-293 cells. The increase in INrf2 led to an INrf2 concentration-dependent decrease in endogenous PGAM5 and Bcl-xL. In related experiments, control 293 and INrf2-293 cells were transfected with PGAM5L-V5, and the experiments were repeated (Fig. 1A, top right panels). Results similar to those described above were also observed for transfected PGAM5L-V5. The increase in INrf2 showed a concentration-dependent decrease in transfected PGAM5L-V5 and Bcl-xL proteins in transfected INrf2-293 cells. In another experiment, Hepa-1 cells showed similar results as INrf2-293 cells. The increase in INrf2 led to an INrf2 concentration-dependent decrease in endogenous PGAM5 and Bcl-xL (Fig. 1A, middle left panels) and transfected PGAM5L-V5 and Bcl-xL (Fig. 1A, middle right panels). In Fig. 1A, the bottom panels demonstrate densitometry analysis of the INrf2-mediated decrease in Bcl-xL in the top and middle panels. The data clearly demonstrate an INrf2-dependent decrease in Bcl-xL. In related experiments, an siRNA-dependent decrease in INrf2 resulted in increased PGAM5L-V5 and Bcl-xL (Fig. 1B). Further experiments demonstrated marginal decreases in Bcl-xL mRNA with increasing INrf2 (Fig. 1C, top). Similar experiments
showed siRNA inhibition of INrf2 and marginal increases in Bcl-xL mRNA (Fig. 1C, bottom). The results together suggested that INrf2 mediated degradation of PGAM5-Bcl-xL.

**INrf2 Interacts with PGAM5-Bcl-xL Complex**

In order to identify INrf2-interacting partners, we performed mass spectrometric analysis. The cell lysates from tetracycline-treated control 293 cells were immunoprecipitated with anti-FLAG antibodies, and the immune complexes were digested with trypsin. Tryptic peptides were desalted and subjected to LC-MS/MS analysis. The Mascot software was used to match the mass of the predicted tryptic peptides generated from the translated human genome. The LC-MS/MS data clearly suggested that INrf2 interacted with PGAM5. It is noteworthy that mass spectrometry analysis did not reveal Bcl-xL peptide, which is known to interact with PGAM5 (32). We believe this is due to overexpression of FLAG-INrf2 in INrf2-293 cells that might have degraded Bcl-xL protein, thus escaping its detection. Furthermore, in order to analyze INrf2 and PGAM5-Bcl-xL interaction, control 293 and INrf2-293 cells were transfected with PGAM5-V5 and treated with tetracycline for 12 h. Cell lysates (1 mg) were immunoprecipitated with anti-FLAG or anti-V5 antibody and immunoblotted. The results clearly showed that FLAG-INrf2 and PGAM5-V5 were pulled down with it (Fig. 2C, top and middle panels). Similarly, in reverse IP, anti-PGAM5 antibodies immunoprecipitated INrf2 and Bcl-xL proteins (Fig. 2C, middle bottom panels), and anti-Bcl-xL antibodies immunoprecipitated PGAM5 proteins (Fig. 2C, bottom three panels). Collectively, these results demonstrated that INrf2 interacts with PGAM5-Bcl-xL complex.

**INrf2-Cul3-Rbx1 Complex Ubiquitinates and Degrades both PGAM5 and Bcl-xL**

Therefore, we investigated INrf2-mediated ubiquitination and degradation of PGAM5-Bcl-xL. FLAG-INrf2-293 cells were transfected with PGAM5-V5 followed by treatment with tetracycline for the indicated times to examine whether INrf2 ubiquitinates and degrades PGAM5-Bcl-xL (Fig. 3A). The cells were analyzed for PGAM5-L-V5 and Bcl-xL ubiquitination with anti-FLAG or anti-V5 antibodies and immunoblotted. The results strongly showed a time-dependent increase in INrf2 and decrease in PGAM5 and Bcl-xL (Fig. 3A, left). Dose-dependent overexpression of FLAG-INrf2 also increased PGAM5-L-V5 and Bcl-xL ubiquitination (Fig. 3A, right four panels), suggesting that INrf2 degrades both PGAM5 and Bcl-xL by ubiquitination. Furthermore, to examine whether the INrf2-Cul3-Rbx1 complex is involved in the ubiquitination and...
degradation of PGAM5-V5 and FLAG-Bcl-xL, we overexpressed INrf2, Cul3, and Rbx1 proteins in Hepa-1 cells by transient transfection, and ubiquitination and degradation of PGAM5-V5 and FLAG-Bcl-xL was examined (Fig. 3B). Hepa-1 cells transfected with INrf2-Cul3-Rbx1 plasmids showed a higher magnitude of degradation of PGAM5-V5 and FLAG-Bcl-xL as compared with INrf2 alone or pcDNA-transfected cells (Fig. 3B, left). The results also demonstrated that overexpression of INrf2-Cul3-Rbx1 increased PGAM5-V5 and FLAG-Bcl-xL ubiquitination (Fig. 3B, right panels). Furthermore, siRNA-mediated dose-dependent knockdown of INrf2 significantly reduced ubiquitination of endogenous PGAM5 and Bcl-xL, leading to stabilization of both PGAM5 and Bcl-xL proteins (Fig. 3C). This suggested that the INrf2-Cul3-Rbx1 complex is involved in the ubiquitination and degradation of PGAM5 and Bcl-xL proteins.

**DGR Domain of INrf2 Is Required for Interaction and Ubiquitination/Degradation of PGAM5-Bcl-xL** — INrf2DGR domain is known to interact with Nrf2, leading to ubiquitination and degradation of Nrf2 (21). We generated two additional stable FRT-Hek-293 cells that upon exposure to tetracycline express FLAG-INrf2DGR and FLAG-INrf2/H9004DGR (Fig. 4A) for use in examining the requirement of the DGR domain for interaction with PGAM5-Bcl-xL. Control 293, INrf2-293, INrf2DGR-293, and INrf2/H9004DGR-293 cells were treated with tetracycline for 24 h. Ten mg of cell lysates were immunoprecipitated with anti-FLAG antibodies, and immune complexes were immunoblotted with anti-HA-HRP antibodies (left). For visualization of ubiquitination of PGAM5 and Bcl-xL, the same one mg of lysates was immunoprecipitated with anti-HA antibodies, respectively, and immunoblotted with anti-HA-HRP antibody (right panels). All experiments were repeated three times.
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**FIGURE 4.** INrf2-DGR domain is required for interaction and ubiquitination/degradation of PGAM5-Bcl-xL.  
**A**, tetracycline-induced expression of FLAG-INrf2, FLAG-INrf2DGR, and FLAG-INrf2ΔDGR in 293 cells. The cells were treated with tetracycline, and 10 μg of cell lysates were immunoprecipitated with anti-FLAG antibodies, and the immune complexes were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. INrf2, INrf2DGR, and INrf2ΔDGR protein bands are labeled with asterisks, and interacting PGAM5 proteins are shown by arrows.  
**B**, INrf2DGR domain is required for interaction with PGAM5L-Bcl-xL. FLAG-INrf2-293, FLAG-INrf2DGR, and FLAG-INrf2ΔDGR cells were transfected with PGAM5-V5 plasmid and treated with tetracycline. One mg of lysates was immunoprecipitated with anti-V5 antibody (left) or anti-FLAG antibodies (right) and immunoblotted with anti-FLAG or anti-V5 or anti-Bcl-xL antibodies.  
**C**, INrf2DGR domain is essential for ubiquitination and degradation of PGAM5 and Bcl-xL protein. FLAG-INrf2, FLAG-INrf2DGR, and FLAG-INrf2ΔDGR-293 cells were co-transfected with PGAM5-V5 and HA-UB plasmids, treated with tetracycline, and immunoblotted with anti-FLAG, anti-V5, and anti-Bcl-xL antibodies (left). One mg of lysates was immunoprecipitated with anti-V5 or anti-Bcl-xL antibodies and immunoblotted with HA-HRP antibodies (right panels).

FLAG-INrf2 and FLAG-INrf2DGR, as determined by mass spectrometry analysis (Fig. 4A). This suggested that the DGR domain of INrf2 was required for binding with the PGAM5-Bcl-xL complex. Interestingly, overexpression of INrf2 in INrf2-293 cells failed to ubiquitinate PGAM5L or Bcl-xL protein (Fig. 4C, right panels). These data clearly demonstrated that the DGR domain of INrf2 was required for binding with the PGAM5-Bcl-xL complex. However, the DGR domain of INrf2 was not sufficient for ubiquitination and degradation of PGAM5 and Bcl-xL protein because Cul3-Rbx1 ubiquitin E3 ligase complex binds with the BTB domain at the N terminus of INrf2 and mediates ubiquitination and degradation. Therefore, both the BTB and DGR domains of INrf2 are required for ubiquitination and degradation of the PGAM5-Bcl-xL complex.

**INrf2 Physically Interacts with PGAM5 but Not Bcl-xL**—Several experiments were performed to examine if INrf2 directly interacts only with PGAM5 or with both PGAM5 and Bcl-xL. To investigate, we knocked down PGAM5L protein by siRNA in Hepa-1 cells, and the levels of PGAM5, Bcl-xL, INrf2, and actin were analyzed by Western blotting (Fig. 5A). Transient transfection of PGAM5 siRNA (50 to 75 nM) decreased PGAM5 protein by 60–80%. However, Bcl-xL protein levels significantly increased (2–2.5-fold) upon PGAM5 knockdown (Fig. 5A, left). Using the same cell lysates, we analyzed INrf2-PGAM5 and INrf2-Bcl-xL interaction by immunoprecipitation and immunoblotting (Fig. 5A, right). Transfection of PGAM5 siRNA decreased the interaction between INrf2 and PGAM5,
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PGAM5L is Required for INrf2 and Bcl-xL Interaction

For this, we transfected control or PGAM5 siRNA for 36 h, lysed, and immunoblotted with anti-INrf2 and anti-Bcl-xL antibodies (Fig. 5A, left). Interactions of PGAM5-V5 with HA-INrf2 and FLAG-Bcl-xL constructs were analyzed by immunoprecipitation/immunoblotting (top panel). A, Western analysis/immunoprecipitation. Hepa-1 cells were transfected with control or PGAM5 siRNA for 36 h, lysed, and immunoblotted with antibodies as shown. B, Western analysis/immunoprecipitation. Hepa-1 cells were transfected with PGAM5 siRNA for 36 h, lysed, and immunoblotted with antibodies as shown. As expected, whereas it also decreased INrf2/Bcl-xL interaction to the same magnitude (Fig. 5A, right), suggesting that PGAM5L is required for INrf2/Bcl-xL interaction. PGAM5 at the N terminus is known to contain a motif, NXXESGE, which is similar to the Nrf2 motif DEETGE (17). Both of these motifs are binding sites for other proteins. To test whether the NXXESGE motif in PGAM5L is involved in the binding to INrf2, a mutant PGAM5L protein was generated in which two alanine substitutions were introduced in place of Glu-79 and Ser-80. V5-tagged plasmids of the wild-type PGAM5L and PGAM5L-E79A/S80A mutant proteins were transfected in Hepa-1 cells along with HA-INrf2 and FLAG-Bcl-xL constructs for 30 h, and cell lysates were immunoblotted (Fig. 5B, top). Immunoblotting data alone indicate that in mutant PGAM5L-transfected cells, the levels of mutant PGAM5 and Bcl-xL protein were ~1.5-fold more than in wild type PGAM5L-transfected cells, suggesting that the NXXESGE motif of PGAM5L is required for the binding to INrf2. To support this observation, we performed the forward and reverse immunoprecipitation and immunoblotting experiments. The mutant PGAM5L-E79A/S80A protein did not bind with INrf2 (Fig. 5B, bottom left), whereas Bcl-xL interaction was the same with wild type and mutant PGAM5. FLAG-Bcl-xL interaction with INrf2 was observed in wild type PGAM5-V5-transfected cells. However, FLAG-Bcl-xL and HA-INrf2 interaction was abolished in mutant PGAM5L-E79A/S80A-V5-transfected cells (Fig. 5B, bottom right). In addition, we further investigated whether

PGAM5 Is Required for Mitochondrial Localization of Nrf2-INrf2-PGAM5-Bcl-xL Complex—PGAM5L contains mitochondrial localization signal between amino acids 9 and 29 and is shown to localize in the mitochondria (31). Therefore, we performed immunohistochemistry analysis to investigate the co-localization of Nrf2-INrf2-PGAM5-Bcl-xL complex in the mitochondria. Immunohistochemistry analysis clearly showed the co-localization of Nrf2, PGAM5, and Bcl-xL proteins with mitochondrial cytochrome c protein (Fig. 6A). Interestingly, immunohistochemistry analysis of Nrf2 in the same experiment demonstrated that Nrf2 also co-localized with Nrf2-PGAM5-Bcl-xL complex in the mitochondria (Fig. 6A). These results together suggested that INrf2 physically interacts with PGAM5 but not Bcl-xL. In addition, INrf2 interaction with Bcl-xL required PGAM5.
INrf2 Degradation of PGAM5-Bcl-xL

FIGURE 6. PGAM5 is required for localization of Nrf2-INrf2-PGAM5-Bcl-xL complex to the mitochondria. A, immunocytochemistry for localization of Nrf2-INrf2-PGAM5-Bcl-xL complex to the mitochondria. Hepa-1 cells were grown on coverslips, fixed, permeabilized, washed, and first incubated with a 1:500 dilution of anti-cytochrome c sheep antibody for 12 h. After washing, cells were incubated with 1:1000 dilution of anti-Nrf2 rabbit, anti-INrf2 goat, anti-Bcl-xL mouse, and anti-PGAM5 rabbit antibody for 12 h separately. After washing with PBS, cells were incubated with Alexa Fluor-594-conjugated anti-goat, anti-rabbit, and anti-mouse second antibody for 1 h. After immunostaining, cells were observed under a Nikon fluorescence microscope, and photographs were captured. B, Hepa-1 cells were transfected with 100 nM PGAM5 siRNA, and cytosolic and mitochondrial fractions were prepared. Sixty-μg extracts were immunoblotted with anti-PGAM5L, anti-Bcl-xL, anti-INrf2, and anti-Nrf2, anti-lactate dehydrogenase (LDH), and anti-CoxIV antibodies.

mitochondria (Fig. 6B). Hepa-1 cells were transfected with control and PGAM5 siRNA, and cytoplasmic and mitochondrial fractions were isolated and immunoblotted for PGAM5, Bcl-xL, INrf2, and Nrf2. The immunoblot was also probed for anti-lactate dehydrogenase (cytosolic marker) and anti-CoxIV (mitochondrial marker). Results revealed that PGAM5 and Bcl-xL protein levels were significantly reduced by siRNA targeting PGAM5 (Fig. 6C). They immunoprecipitated INrf2, PGAM5L-V5, and Bcl-xL. However, Bcl-xL immunoprecipitation data clearly showed that PGAM5 interacts with INrf2 and release of Nrf2 in cytosol as well as in mitochondria but not in cytoplasmic and mitochondrial fractions iso-

destabilization of the dimeric structure of INrf2, degradation of PGAM5-Bcl-xL protein (Fig. 1). This indicated that t-BHQ destabilized INrf2-PGAM5-Bcl-xL complex, leading to degradation of PGAM5 and stabilization of Bcl-xL.

Next we investigated the effect of t-BHQ on Nrf2, INrf2, PGAM5, Bcl-xL, and Bax interactions in cytosol and mitochondria (Fig. 6C). The various results indicated that t-BHQ destabilized INrf2-PGAM5-Bcl-xL complex, leading to degradation of PGAM5 and stabilization of Bcl-xL.

Antioxidant Treatment Led to the Release of Bcl-xL

FIGURE 7. Antioxidant Treatment Led to the Release of Bcl-xL. A, Hepa-1 cells were treated with DMSO or t-BHQ for different time periods (2–8 h), and cell lysates were immunoblotted for INrf2, PGAM5L-V5, and Bcl-xL (Fig. 7A). DMSO treatment showed virtually no significant effect on endogenous levels of INrf2, PGAM5L-V5, and Bcl-xL. However, cells upon treatment with t-BHQ (between 2 and 4 h) showed decreased levels of INrf2 and PGAM5-V5 and increased levels of Bcl-xL (Fig. 7A). Forward and reverse immunoprecipitation followed by immunoblotting in similar experiments also analyzed INrf2/PGAM5/Bcl-xL interactions (Fig. 7B). Forward IP results revealed t-BHQ exposure time-dependent loss in interaction of INrf2 with PGAM5 and Bcl-xL (Fig. 7B, right top three panels). Reverse IP in similar experiments also showed loss of interaction of PGAM5 with INrf2 and Bcl-xL (Fig. 7B, right bottom three panels). The various results indicated that t-BHQ destabilized INrf2-PGAM5-Bcl-xL complex, leading to degradation of PGAM5 and stabilization of Bcl-xL.

Interestingly, immunoprecipitation of Bcl-xL and Bax data clearly showed that the mitochondrial interaction of Bcl-xL and Bax was increased after t-BHQ treatment as compared with DMSO treatment (Fig. 7D) and no change in cytoplasmic Bcl-xL and Bax interaction. Collectively, the results demonstrated that antioxidant t-BHQ led to release of Nrf2 in the cytosol and Nrf2 and Bcl-xL in the mitochondria. The release of Bcl-xL in mitochondria led to increased interaction with Bax that is expected to contribute to altered apoptosis.

Overexpression of INrf2 Led to Degradation of PGAM5-Bcl-xL and Enhancement of Etoposide-induced Cytochrome c Release, Up-regulation of Pro-apoptotic Bax, and Increase in Activated Caspase-3/7—Our data suggested that INrf2 mediated degradation of PGAM5-Bcl-xL protein (Fig. 1). This indi-
cated that INrf2, through regulation of anti-apoptotic Bcl-xL protein, might influence apoptotic cell death/survival. Therefore, we examined the role of INrf2-mediated degradation of PGAM5 and Bcl-xL in cellular apoptosis. INrf2-293 cells were transfected with PGAM5-V5 and treated with tetracycline, and Hepa-1 cells transfected with INrf2 were treated with two different concentrations of etoposide and analyzed for PGAM5-Bcl-xL degradation, cytochrome C release, caspase-3/7 activity, and cleaved caspase-3 (Fig. 8). INrf2-293 and Hepa-1 cells overexpressing INrf2 showed degradation of PGAM5-Bcl-xL and an increase in Bax (Fig. 8A). Etoposide treatment marginally increased alterations in the various molecules. INrf2 overexpression followed by etoposide treatment in Hepa-1 cells also induced cytochrome c release from mitochondria to cytosol by 1.8-fold (Fig. 8B, compare lanes 2, 3, and 4; data shown only for Hepa-1), increased 1.5–2-fold caspase-3/7 activity (Fig. 8C), and...
and cleaved caspase-3, as compared with etoposide-treated cells expressing endogenous levels of INrf2. These data suggested that INrf2 overexpression degrades PGAM5 and Bcl-xL proteins, promotes etoposide-mediated increases in cellular Bax level, releases cytochrome from mitochondria, and activates caspase-3/7. In similar experiments, we also examined the role of INrf2 in the regulation of anti-apoptotic factors Bcl-2 and Mcl-1. Increasing INrf2-V5 levels in Hepa-1 cells by transient transfection degraded not only Bcl-xL as described above but also Bcl-2 and Mcl-1 (supplemental Fig. S1A). Moreover, a dose-dependent siRNA-mediated INrf2 knockdown stabilized anti-apoptotic proteins Bcl-xL, Bcl-2, and Mcl-1 (supplemental Fig. S1B). These results together indicate that INrf2 down-regulates these anti-apoptotic factors in addition to Bcl-xL to control apoptosis. It is noteworthy that we have recently reported INrf2 degradation of anti-apoptotic factor Bcl-2 (30).

**Overexpression of INrf2 Increased and Treatment with Antioxidant t-BHQ Decreased Etoposide-mediated DNA Fragmentation and Cellular Apoptosis**—The biochemical significance of INrf2-mediated degradation of PGAM5-Bcl-xL complex and up-regulation of pro-apoptotic marker proteins by INrf2 raised an interesting question, whether INrf2 regulates cellular apoptosis. Hepa-1 cells transfected with pcDNA (vector control) or INrf2-V5 were treated with the various concentrations of etoposide. Similarly, control 293 and INrf2-293 cells were treated with tetracycline first for 12 h and then treated with various

![FIGURE 8. Overexpression of INrf2 leads to degradation of PGAM5-Bcl-xL and activation of pro-apoptotic proteins.](http://www.jbc.org/)

**A** Hepa-1 cells were transfected with pcDNA vector or FLAG-INrf2 and treated with etoposide (20 μM) as indicated for an additional 36 h. Cells were lysed, and 20 μg of cell lysates were immunoblotted with anti-caspase-3, anti-FLAG, and anti-actin antibodies (left and right). **B** Hepa-1 cells were transfected with pcDNA vector or FLAG-INrf2 and treated with etoposide (20 μM) as indicated for an additional 36 h. Cells were lysed, and 20 μg of cell lysates were immunoblotted with anti-caspase-3, anti-FLAG, and anti-actin antibodies (left and right). **C** Hepa-1 cells were transfected with pcDNA vector or FLAG-INrf2 and treated with etoposide (20 μM) as indicated for an additional 36 h. Cells were lysed, and 20 μg of cell lysates were immunoblotted with anti-caspase-3, anti-FLAG, and anti-actin antibodies (left and right). **D** Hepa-1 cells were transfected with pcDNA vector or FLAG-INrf2 and treated with etoposide (20 μM) as indicated for an additional 36 h. Cells were lysed, and 20 μg of cell lysates were immunoblotted with anti-caspase-3, anti-FLAG, and anti-actin antibodies (left and right).
survival. Hepa-1 cells were plated at a density of 5000 cells/well in 24-well plates and transfected with different concentrations of PGAM5L-V5 constructs (0.5, 1, and 2 μg) (left) or transfected with different concentrations of siRNA against PGAM5 (25, 50, and 100 nM) for 24 h (g) or transfected with different concentration of siRNA against INrf2 for 24 h and etoposide for 36 h. Cells were incubated with fresh MTT solution for 2 h at 37°C, and absorbance at 570 nm was measured. The experiment was repeated three times. Each data point represents the mean ± S.D. from two experiments.

DNA fragmentation/TUNEL assay. Hepa-1 cells were transfected with pcDNA or INrf2-V5 plasmids and treated with etoposide for 48 h. Cells were fixed and permeabilized, and the TUNEL assay was performed. TUNEL-positive cells were observed under a fluorescence microscope, quantified, and plotted. The data are represented as the mean ± S.D. from two experiments. A, overexpression of INrf2 results in increased cell survival by 20–25% compared with control siRNA-transfected cells (Fig. 10B, left and right). B, siRNA-mediated INrf2 knockdown decreased INrf2-mediated Bcl-xL ubiquitination compared by cell survival assays (Fig. 9D).

siRNA-mediated Knockdown of INrf2 Increased Etoposide- and TRAIL-mediated Cell Survival—We investigated the effect of siRNA-mediated inhibition of INrf2 on Bcl-xL stabilization and etoposide-mediated cell survival in three different cancer cell lines. Hepa-1, Hek-293, and HepG2 cells were transfected with different concentrations of either control siRNA or INrf2 siRNA for 24 h and treated with etoposide for 36 h, and cell survival analysis was performed by an MTT assay (Fig. 10A). A dose-dependent knockdown of INrf2 by siRNA in all three cell lines followed by etoposide treatment showed 20–40% increased cell survival compared with control siRNA-transfected cells (Fig. 10A, top, middle, and bottom). The reasons for increasing cell survival after knockdown of INrf2 were further examined in Hepa-1 cells (Fig. 10B). INrf2 knockdown and etoposide treatment stabilized cellular Bcl-xL and also increased cell survival by 20–40% compared with control siRNA-transfected cells (Fig. 10B, left and right). These results together suggested that knockdown of INrf2 stabilized Bcl-xL and increased cell survival. Interestingly, we also used cell killer TRAIL protein, which is known to activate the extrinsic apoptotic pathway in cells by the activation of caspase-8. For this we used Jurkat (T cell Lymphoma) cells. Cells were transfected with control siRNA or INrf2 siRNA and treated with TRAIL protein as indicated (Fig. 10C, bottom), and cell survival was measured. Treatment of cells with TRAIL decreased cell survival by 40% compared with untreated cells. However, knockdown of INrf2 followed by TRAIL treatment increased cell survival by 20–25% compared with control siRNA-transfected and TRAIL-treated cells (p < 0.01) (Fig. 10C, bottom). In addition, we also confirmed INrf2 knockdown and Bcl-xL stabilization in Jurkat cells by immunoblotting of the same cell lysates with INrf2, Bcl-xL, and actin antibodies (Fig. 10C, top). These data indicate that INrf2 knockdown led to increased level of Bcl-xL that contributed to resistance against extrinsic apoptotic pathway in Jurkat cells.

The above results together suggested that overexpression of INrf2 degraded the PGAM5-Bcl-xL complex, activated pro-apoptotic factors, and promoted etoposide-mediated cellular death.
apoptosis. In contrast, the knockdown of INrf2, resulting in increased Nrf2, the overexpression of Nrf2, or t-BHQ treatment, leading to stabilization of Nrf2, all promoted cell survival. Therefore, INrf2 and Nrf2 play opposite roles in the regulation of cellular apoptosis.

DISCUSSION

Recently, we have shown that INrf2 interacts and degrades anti-apoptotic protein Bcl-2 and controls cellular apoptosis (30). In this report, we demonstrate INrf2 interaction and degradation of another anti-apoptotic protein Bcl-xL and its role in cellular apoptosis. INrf2 is known to directly interact with Bcl-2 to control apoptosis (30). However, unlike Bcl-2, INrf2 does not directly interact with Bcl-xL. INrf2 interacts with Bcl-xL through the PGAM5 protein. Results also revealed that the DGR region of INrf2 is required for interaction with PGAM5. PGAM5 protein not only mediated INrf2 interaction with Bcl-xL but also directed localization of Nrf2-INrf2-PGAM5-Bcl-xL complex to the mitochondria. These observations are also supported by previous studies (17, 31). Our data also agree with a previous report that the INrf2 dimer binds to Nrf2 on one strand and PGAM5 on the other strand. However, this clearly requires further investigation. Further studies revealed that INrf2 along with the E3 ubiquitin ligase complex Cul3-Rbx1 ubiquitinate and degrade PGAM5 and Bcl-xL. Antioxidant t-BHQ destabilizes Nrf2-INrf2-PGAM5-Bcl-xL complex especially in mitochondria.
to stabilize Bcl-2 that forms complex with Bax and contributes to decreased apoptosis and increased cell survival (30). Therefore, it is expected that antioxidant-induced stabilization of anti-apoptotic proteins Bcl-xL and Bcl-2 leads to reduced apoptotic cell death and increased cell survival.

PGAM5 exists in two isoforms, identical in the N-terminal 239 amino acids (17). The longer form (PGAM5L) contains 289 amino acids, and the shorter form (PGAM5S) contains 255 amino acids. The 16 C-terminal amino acids in PGAM5S are not similar to those of the PGAM5L isoform. The N terminus of the PGAM5 protein contains a conserved NXSXE82 motif (amino acid 77–82), similar to Nrf2, that binds to the DGR region of INrf2, whereas the C-terminal PGAM domain (amino acids 125–156) binds anti-apoptotic factor Bcl-xL. Interestingly, both isoforms of human PGAM5 contain a large PGAM domain, which begins at amino acid 98 and extends to the C-terminal end (17). INrf2 homodimers, PGAM5 proteins also possess an N-terminal mitochondrial localization signal (amino acids 9–29), which is involved in the mitochondrial localization of PGAM5 and its binding partners to the mitochondria. The present studies used the PGAM5L form. PGAML5-E79A/S80A failed to bind with INrf2 and ubiquitinate/degrade Bcl-xL. This indicated that PGAM5, through the NXSXE82 domain, binds to INrf2. Because this domain is present in both isoforms, we believe that PGAM5L and PGAM5S both function as a bridge between INrf2 and Bcl-xL.

A hypothetical model demonstrating the role of INrf2 control of Bcl-xL and apoptosis is depicted in Fig. 11. The INrf2 homodimer bound to Nrf2 on one monomer and PGAM5-Bcl-xL on the other monomer exists in the cytosol and mitochondria. Under physiological conditions, INrf2 homodimers promote a Cul3-Rbx1-mediated degradation of Nrf2 and PGAM5-Bcl-xL, thereby contributing to the maintenance of a normal level of Bcl-xL and apoptosis. Oxidative/electrophilic stress leads to the release of Nrf2 and PGAM5-Bcl-xL from INrf2 dimers in the cytosol. Nrf2 translocates to the nucleus, leading to activation of cytoprotective gene expression (2). PGAM5-Bcl-xL is directed to mitochondria, where PGAM5 is degraded to release Bcl-xL. In addition, oxidative/electrophilic stress also leads to stabilization of Bcl-2 in the cytosol that forms complex with Bax and contributes to reduced apoptosis and increased cell survival (30). It is noteworthy that the INrf2-INrf2-PGAM5-Bcl-xL complex in the cytosol might be in addition to the previously characterized INrf2-INrf2 complex (2) and remains to be further studied.

The stabilization of Bcl-xL (current study) and Bcl-2 (30) from INrf2 and prevention of apoptosis are presumably an important mechanism to save cells from dying in acute stress due to exposure to antioxidants, xenobiotics, drugs, and radiation. Once the exposure effect subsides, the levels of Bcl-xL, Bcl-2, and cytoprotective proteins are brought back to normal, and a normal level of apoptotic cell death is restored. Recent studies have reported increased stabilization/accumulation of Nrf2 due to mutations in INrf2, resulting in loss of function in lung and breast tumors (12, 13, 33, 20). Lung cancer cell line A549 contains INrf2G333C mutant protein that has lost its capacity to bind/degrade Nrf2, leading to accumulation of Nrf2 in the nucleus (13). It has been suggested that higher levels of
INrf2 Degradation of PGAM5-Bcl-xL

Nrf2 in A549 cells might have contributed to the survival of these cells in lung cancer. Similarly, our studies demonstrated that stress mediated loss of INrf2-PGAM5 interaction stabilized cellular Bcl-xL that resulted in decreased cellular apoptosis. In conclusion, we demonstrate that both INrf2 and PGAM5 protein contribute to the regulation of Bcl-xL protein and apoptosis.

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