PGAM5, a Bcl-XL-interacting protein, is a novel substrate for the redox-regulated Keap1-dependent ubiquitin ligase complex

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Keap1 is a BTB-Kelch substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex that functions as a sensor for thiol-reactive chemopreventive compounds and oxidative stress. Inhibition of Keap1-dependent ubiquitination of the bZIP transcription factor Nrf2 enables Nrf2 to activate a cytoprotective transcriptional program that counters the damaging effects of oxidative stress. In this report, we have identified a member of the phosphoglycerate mutase family, PGAM5, as a novel substrate for Keap1. The N-terminus of the PGAM5 protein contains a conserved NxESGE motif that binds to the substrate-binding pocket in the Kelch domain of Keap1, while the C-terminal PGAM domain binds Bcl-XL. Keap1-dependent ubiquitination of PGAM5 results in proteosome-dependent degradation of PGAM5. Quinone-induced oxidative stress and the chemopreventive agent sulforaphane inhibit Keap1-dependent ubiquitination of PGAM5. The identification of PGAM5 as a novel substrate of Keap1 suggests that Keap1 regulates both transcriptional and post-transcriptional responses of mammalian cells to oxidative stress.

Diverse metabolic activities of eukaryote cells generate reactive oxygen species, which can damage biological macromolecules, including DNA, proteins, and lipids (1). Oxidative damage to biological macromolecules can alter multiple cellular functions and has been implicated in cancer, inflammation, cardiovascular and neurodegenerative diseases, and aging (2-6). Consequently, eukaryote cells have evolved multiple protective mechanisms against reactive molecules and oxidative stress. A comprehensive analysis of oxidative stress resistance in yeast has identified several core functions, including transcription, protein trafficking and vacuolar formation that are broadly required for oxidative stress resistance (7).

In mammals, the Keap1 protein has emerged as a major sensor for oxidative stress and electrophilic molecules. Keap1 is a BTB-Kelch protein that functions as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex. Under basal conditions of cellular redox homeostasis, the N-terminal BTB domain of Keap1 binds to Cul3 while the C-terminal Kelch domain of Keap1 binds to the bZIP transcription factor Nrf2 (8-10). This ubiquitin ligase complex conjugates ubiquitin onto specific lysine residues within the N-terminal Neh2 domain of Nrf2 and targets Nrf2 for degradation by the 26S proteosome. Cyclical association and dissociation of this E3 ubiquitin ligase complex is required for efficient ubiquitination of Nrf2 and repression of Nrf2-dependent gene expression (11). Reactive electrophilic chemicals and oxidative stress modify one or more cysteine residues located in the N-terminal BTB and central linker domains of Keap1 and disrupt formation of a functional E3 ubiquitin ligase complex (12-17). Inhibition of Keap1-mediated ubiquitination of Nrf2 results in a marked increase in the steady-state level of Nrf2 and transcription of Nrf2-dependent genes. Induction of Nrf2-dependent genes represents a cytoprotective response to oxidative stress that neutralizes reactive molecules, eliminates...
damaged macromolecules, and restores cellular redox homeostasis (18-22).

Structural and functional analyses of Keap1 and Nrf2 have identified an evolutionarily conserved DxETGE motif in the N-terminal Neh2 domain of Nrf2 that binds in a shallow binding pocket on the top face of the Kelch domain of Keap1 (23-25). Although Nrf2 is the only known substrate for Keap1, the Kelch domain of Keap1 has been reported to bind several other proteins, including Nrf1, prothymosin alpha, and fetal Alz clone 1 (FAC1) (26-28). In general, substrate adaptors for cullin-dependent ubiquitin ligases often bind several substrates via a common recognition motif (29-32). For example, β-TrCP, a well-characterized substrate adaptor for Cul1, recognizes two substrate proteins, IκBα and β-catenin, after these proteins are phosphorylated on two serine residues embedded within a conserved sequence motif of DSGφXS (32,33).

In this report, we have identified a novel substrate of Keap1 encoded by the PGAM5 gene. The PGAM5 gene encodes two protein isoforms, PGAM5-L and PGAM5-S, which result from alternative splicing. Both PGAM5 isoforms contain an N-terminal region of approximately 100 amino acids, which includes a conserved NxESGE motif that is required for binding to Keap1, and a C-terminal phosphoglycerate mutase (PGAM) domain, which binds to Bcl-XL (34). PGAM5 is ubiquitinated by a Keap1-dependent E3 ubiquitin ligase complex, which targets PGAM5 for proteasome-mediated degradation. Both quinone-induced oxidative stress and sulforaphane inhibit Keap1-dependent ubiquitination and subsequent degradation of PGAM5. Our results establish PGAM5 as a novel substrate of Keap1 and support the notion that Keap1 regulates diverse cellular functions as part of a global response to oxidative stress.

Experimental Procedures

Construction of recombinant DNA molecules - The PGAM5-L cDNA (Accession number BG030775) and the PGAM5-S cDNA (Accession number BC008196) were obtained from ATCC and Open Biosystems, respectively. The PGAM5 expression vectors were constructed by cloning PCR-generated fragments containing the entire open reading frame of either PGAM5-L and PGAM5-S into the BamHI/EcoRI sites of pcDNA3 (Invitrogen), and the NotI/SmaI sites of pFLAG-CMV2 (Sigma). The mutant PGAM5-L cDNA containing alanines in place of Glu 79 and Ser 80 codons were generated by site-specific mutagenesis with standard overlap-extension techniques. Plasmids expressing wild type and the mutant Keap1, CBD-tagged Keap1, and Cul3 proteins have been previously described (10,11). The myc-Rbx1 expression vector was a gift from Dr. Joan Conaway (35). The Bxl-XL expression vector was a gift from Dr. Richard Youle (36). All the genes used in this study were sequenced in the context of the expression vectors used for the experiments.

Cell culture, transfections, and chemical reagents - COS1, HEK-293T, and Hela cells were purchased from ATCC. Cells were maintained in either Dulbecco’s modified Eagle’s medium (DMEM) or Eagle’s minimal essential medium (EMEM) in the presence of 10% fetal bovine serum (FBS). Plasmid DNA transfections were performed with Lipofectamine Plus (Gibco BRL) according to the manufacturer’s instructions. Lactacystin (clasto-lactacystin β-lactone) was purchased from Boston Biochem. Sulforaphane and tert-butylhydroquinone (tBHQ) were purchased from Sigma.

Immunofluorescence assays - Hela cells were grown on glass coverslips on 35 mm plates. Cells were transfected with 1.0 μg of the empty vector or the expression vectors for the FLAG-PGAM5 proteins. Cells were fixed with 100% methanol at -20°C for 10 min. Fixed cells were incubated for 40 min with mouse anti-FLAG M2 antibodies in phosphate buffered saline (10 mM sodium phosphate [pH 8.0] and 150 mM NaCl) containing 10% (vol/vol) FBS. Coverslips were washed and incubated with fluorescein isothiocyanate-conjugated anti-mouse antibodies (Jackson Laboratories) in phosphate buffered saline (10 mM sodium phosphate [pH 8.0] and 150 mM NaCl) containing 10% (vol/vol) FBS. Coverslips were washed and mounted on glass slides. Images were obtained with a Bio-Rad Radiance 2000 confocal system coupled to an Olympus IX70 inverted microscope and a digital camera. The images were captured with ImagePro.
Antibodies, immunoprecipitation, and immunoblot analysis - The anti-Keap1 antibody has been described (17). Antibodies against tubulin (Santa Cruz), the chitin binding domain (New England Biolabs), ubiquitin (Sigma), the FLAG epitope (Sigma), the myc epitope (Santa Cruz Biotechnology), the HA epitope (Covance) and Bcl-X\textsubscript{L} (L-19; Santa Cruz Biotechnology) were purchased from commercial sources. For detection of protein expression in total cell lysates, cells were lysed in sample buffer (50 mM Tris-HCl [pH 8.0], 2% SDS, 10% Glycerol, 100 mM DTT, 0.1% bromophenol blue) at 24 to 48 hr post-transfection. For immunoprecipitation assays, cell extracts were prepared in RIPA buffer (10 mM sodium phosphate [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma). Soluble cell lysates were incubated either with 2 µg of anti-FLAG M2 coupled to agarose (Sigma), or with 2 µg of affinity-purified antibodies for 2 hr at 4°C, followed by incubation at 4°C with protein A-agarose beads (Sigma) for 2 hr. Unbound proteins were removed by washing four times with lysis buffer. The immunoprecipitated proteins were eluted in sample buffer by boiling for 5 minutes, electrophoresed through SDS-polyacrylamide gels, transferred to nitrocellulose membranes and subjected to immunoblot analysis.

In vivo ubiquitination assay - For detection of ubiquitinated PGAM5-L proteins in vitro, cells were transfected with expression vectors for FLAG-PGAM5-L, Keap1-CBD, myc-Rbx1, and HA-Cul3 proteins. Cells were lysed in buffer B (15 mM Tris-HCl pH [7.5], 500 mM NaCl, 0.25% NP-40) containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma). The lysates were pre-cleared with protein A-agarose beads prior to incubation with chitin beads (New England Biolabs) for four hours at 4°C. Chitin beads were washed twice with buffer B, twice with buffer A (25 mM Tris-HCl [pH 7.5], 10% [v/v] glycerol, 1 mM EDTA, 0.01% NP-40 and 100 mM NaCl), and twice with reaction buffer (50 mM Tris-HCl [pH 7.5], 5 mM MgCl\textsubscript{2}, 2 mM NaF, 0.6 mM DTT). The pellets were incubated with ubiquitin (300 pmol), E1 (2 pmol), E2-UbcH5a (10 pmol), and ATP (2 mM) in 1 x reaction buffer in a total volume of 30 µl for 1 hr at 37°C. Ubiquitin, E1 and E2-UbcH5a were purchased from Boston Biochem. The chitin beads were pelleted by centrifugation (3,000 x g) and resuspended in 2% SDS, 150 mM NaCl, 10 mM Tris-HCl [pH 8.0] and 1 mM DTT and boiled for 5 min to release bound proteins, inactivate any contaminating ubiquitin hydrolases and disrupt protein-protein interactions. The supernatant was diluted five-fold with buffer lacking SDS prior to immunoprecipitation with anti-FLAG M2 agarose. Immunoprecipitated proteins were subjected to immunoblot analysis with anti-ubiquitin antibodies (Sigma).

Mass spectrometry - Gel slices containing bands “A”, “B” and “C” as indicated as in Fig. 1A were reduced, alkylated, and digested with trypsin. Tryptic peptides were desalted and subjected to MALDI-TOF mass spectrometry by the MU Proteomics Core using a Voyager DEPro with a 20Hz 337-nm nitrogen laser (Applied Biosystems, Foster City, CA). The Mascot software package...
was used to match the mass of the peptides with predicted tryptic peptides generated from the translated human genome. Mowse scores of 74 and 172 were obtained for PGAM5 (band B) in two independent experiments. The cut-off values for statistical significance (p<0.05) in these two experiments were 59 and 63, respectively. The matched peptides from the second experiment are shown in Table 1. A Mowse score of 59 was obtained for CH3L1 (Band A) in the first experiment (cut-off value at p<0.05 was 59 in this experiment). Details of the mass spectrometry experiments are available upon request.

RESULTS

Identification of PGAM5 as an interaction partner of Keap1 - To identify new Keap1-interacting proteins, the human Keap1 protein containing a C-terminal chitin-binding domain (CBD) was stably expressed in MDA-MB-231 cells, a human breast carcinoma-derived cell line. The Keap1-CBD protein was isolated from cell lysates using chitin beads and Keap1-associated proteins were visualized on silver-stained SDS-polyacrylamide gels. Three proteins, ranging in size from 30 to 39 kDa were found to co-purify with the Keap1-CBD protein in a specific and reproducible manner (Fig. 1A, lanes 3 and 4). Tryptic peptides from these three proteins were subjected to MALDI-TOF mass spectrometry. Band A (39 kDa) was tentatively identified as Chitinase-3-like protein 1 (CH3L1; NCBI accession number NP_001267), with a borderline Mowse score of 59 in one experiment (cut-off value for p<0.05 was 59; data not shown). Bands B and C (32 kDa and 30 kDa, respectively) were identified as phosphoglycerate mutase 5 (PGAM5; NCBI accession number NP_612642) in two independent experiments. The Mowse scores for Band B were 74 and 172, well above the cut-off values for statistical significance of 59 and 63, respectively. The position and sequence of the PGAM5-derived peptides identified by MALDI-TOF MS are given in Table 1.

A database search revealed the presence of two mRNA transcripts that originate from the PGAM5 gene located on chromosome 12. The proteins encoded by these mRNAs are identical from amino acid 1 to 239, with the shorter form (PGAM5-S) containing 16 additional C-terminal amino acids while the longer isoform (PGAM5-L) containing 50 additional C-terminal amino acids (Fig. 2A). Both isoforms of human PGAM5 contain a PGAM domain (pfam00300), which begins at amino acid 98 and extends to the C-terminal end of the longer isoform. The PGAM5 isoforms are distant members of the phosphoglycerate mutase family, with their closest relatives being two proteins, termed STS-1 and STS-2, that do not have phosphoglycerate mutase activity but participate in receptor-mediated signal transduction pathways (37,38) (Fig. 2B). In particular, the phosphohistidine signature motif that is characteristic of PGAM domains with enzymatic activity is poorly conserved in PGAM5 (39)(Fig. 2D). A region within the PGAM domain of PGAM5 has previously been shown to bind to Bcl-X₇ (34), and the ability of both isoforms of PGAM5 to associate with Bcl-X₇ was confirmed by co-immunoprecipitation (Fig. 2E and data not shown).

To confirm association between Keap1 and the two PGAM5 isoforms, untagged or FLAG-tagged versions of both PGAM5-S and PGAM5-L were expressed in HEK-293T cells along with the Keap1-CBD protein. Proteins that bound to chitin beads were analyzed by SDS-PAGE and silver stain. Both PGAM5 isoforms bound to the Keap1-CBD protein (Fig. 1B, lanes 3-6). The presence of the N-terminal FLAG tag decreased the mobility of both PGAM5 isoforms, but did not perturb their ability to associate with Keap1-CBD (Fig. 1B, lanes 7 and 8). The ectopic untagged PGAM5-L protein co-migrated exactly with the endogenous 32 kDa Keap1-associated protein (Fig. 1B, compare lanes 1 and 3). In contrast, the mobility of the ectopic untagged PGAM5-S protein was slightly faster than the endogenous Keap1-associated protein of 30 kDa (Figure 1B, compare lanes 1 and 5). This result supports the identification of the Keap1-associated protein of 32 kDa as the PGAM5-L isoform and suggests that the Keap1-associated protein of 30 kDa is not the PGAM5-S isoform but is derived from the 32 kDa protein by proteolysis during purification.

To verify the association of both isoforms of human PGAM5 with Keap1, reciprocal pull-down analyses of the proteins in transfected cells were performed. COS1 cells were transfected...
with expression vectors for untagged Keap and either of the FLAG-tagged PGAM5 isoforms. Cell lysates were immunoprecipitated with anti-FLAG antibodies and the resultant immunoprecipitates were probed for the presence of Keap1. The Keap1 protein was present in anti-FLAG immunoprecipitates of both PGAM5 isoforms (Fig. 1C, lanes 2 and 4).

The subcellular localization of both PGAM5 isoforms was determined by indirect immunofluorescence of Hela cells expressing the FLAG-tagged proteins. The PGAM5-L protein displayed diffuse cytoplasmic staining and a punctate nuclear staining, while the PGAM5-S protein displayed a punctate pattern in the cytoplasm (Fig. 3). A similar pattern was observed in COS1 cells (data not shown). Both PGAM isoforms co-localized with the Keap1 protein in the cytoplasm (data not shown). No nuclear staining of Keap1 was observed in our experiments. When co-expressed with Keap1, the cytoplasmic localization of both PGAM5-L and Keap1 shifted from a diffuse pattern to a punctate pattern (data not shown). Keap1 also displayed a punctate cytoplasmatic pattern when co-expressed with PGAM5-S (data not shown). In subsequent experiments, we focused on the PGAM5-L isoform, as this isoform co-migrated with the 32 kDa protein identified as PGAM5 by mass spectrometry of tryptic peptides and is expressed at higher levels than the PGAM5-S isoform (Fig. 1B).

Identification of the interaction interface between Keap1 and pGAM5-L - The Keap1 protein possesses five discrete domains: the N-terminal region (N); an N-terminal Broad complex, Tramtrack, and Bric-a-Brac domain (BTB); a central linker domain (Linker); a C-terminal Kelch repeat domain (Kelch); and a C-terminal short tail (C). To determine which domain(s) in Keap1 are responsible for binding to PGAM5, a series of mutant Keap1 proteins containing a specific deletion of each individual domain were expressed in HEK-293T cells along with FLAG-PGAM5-L proteins. Association of the mutant Keap1 proteins and PGAM5-L was assessed by coimmunoprecipitation. Only the Keap1-ΔKelch protein did not associate with PGAM5-L (Fig. 4A, lane 5), indicating that the Kelch domain of Keap1 is required for binding to PGAM5-L.

Previous structural and mutational studies have demonstrated that the Kelch domain of Keap1 is a six-bladed β-propeller structure that contains a shallow pocket on its top face that binds Nrf2 (23,25,40). The shallow pocket of Keap1 binds an Nrf2-derived peptide containing a highly conserved DxETGE motif. Our previous analysis of more than 30 Keap1 mutant proteins identified seven amino acids in Keap1 whose side chains participate in direct contacts with the Nrf2-derived peptide and for which individual alanine substitutions are sufficient to disrupt binding of Nrf2 to Keap1 (23). These residues are: Tyr334, Arg380, Asn382, Arg415, Arg483, Tyr525, and Tyr572. The charged residues are located at the base of the binding pocket while the hydrophobic residues line the sides of the binding pocket. A set of mutant CBD-tagged Keap1 proteins were characterized for their ability to bind pGAM5-L. Association of the endogenous PGAM5-L protein with these mutant Keap1 proteins was determined by co-purification on chitin beads followed by SDS-PAGE and visualization by silver stain. This analysis revealed four amino acid residues, including Tyr334, Arg415, Arg483, and Tyr572, for which individual alanine substitutions significantly disrupted the ability of Keap1 to bind PGAM5-L (Fig. 4B, lanes 2 and 3; Fig. 4C, lanes 5 and 7).

The highly conserved DxETGE motif in Nrf2 is required for the association of Nrf2 with Keap1 (23-25). The side chains of the glutamate residues contact multiple residues in Keap1, including Arg380, Asn382, Arg415 and Arg483. The side chains of the aspartate and threonine residues, on the other hand, interact with the backbone of the Nrf2-derived peptide and stabilize a β-turn region that enables the Nrf2-derived peptide to fit into the binding pocket on Keap1. Alanine substitution of the threonine residue within the DxETGE motif markedly reduces binding of Nrf2 to Keap1 and enables the mutant Nrf2 protein to escape Keap1-mediated repression (23). In contrast, serine substitution of this threonine residue does not disrupt the binding of Nrf2 to Keap1 and enables the mutant Nrf2 protein to escape Keap1-mediated repression (23). In contrast, serine substitution of this threonine residue does not disrupt the binding of Nrf2 to Keap1, suggesting that the hydroxyl group of the serine residue is an effective substitute for the hydroxyl group of the threonine residue. A NxE(S/T)GE motif, amino acids 77-82 in the human PGAM5 isoforms, is strongly conserved in mammals (Fig. 2C). To determine if the NxESGE
motif in PGAM5-L is required for binding to Keap1, a mutant PGAM5-L protein was constructed in which two alanine substitutions were introduced in place of Glu79 and Ser80. FLAG-tagged versions of the wild-type and PGAM5-L-E79A/S80A mutant proteins were expressed in HEK-293T cells along with Keap1-CBD and association with Keap1 was assessed by immunoblot following affinity purification with chitin beads. The mutant PGAM5-L-E79A/S80A protein did not bind to Keap1-CBD (Fig. 4D, lane 3). The cytoplasmic localization (Fig. 3M) and association of PGAM5-L with Bel-XL (Fig. 4D) were not altered by the E79A/S80A mutation. Taken together, these results indicate that four amino acids within the substrate binding pocket on the top face of the Kelch domain of Keap1 form critical contacts with the NxESGE motif in PGAM5-L and support the notion that Keap1 binds PGAM-L in a manner that is very similar to the way in which Keap1 binds Nrf2.

Keap1 targets PGAM5-L for ubiquitin-dependent degradation - Binding of Nrf2 to Keap1 results in the ubiquitination of specific lysine residues in Nrf2 by a Cul3-dependent ubiquitin ligase complex, leading to the proteosome-dependent degradation of Nrf2 (8-10). Several experimental approaches were used to determine if PGAM5-L is also a substrate for Keap1-dependent ubiquitination. In one set of experiments, an in vivo ubiquitination analysis was performed, in which expression vectors for FLAG-PGAM5-L, Keap1 and HA-ubiquitin were transfected into COS1 cells. Cell lysates were collected under strongly denaturing conditions to eliminate non-covalent protein-protein interactions and the presence of HA-ubiquitin conjugation onto the FLAG-PGAM5-L protein was measured by anti-HA immunoblot analysis of anti-FLAG immunoprecipitates. A low level of ubiquitin conjugation was observed in the absence of co-expressed Keap1 (Fig. 5A, lane 2). Coexpression of Keap1 resulted in a marked increase in ubiquitin conjugation onto PGAM5-L protein was measured by anti-FLAG immunoprecipitates. A low level of ubiquitin conjugation was observed in the absence of co-expressed Keap1 (Fig. 5A, lane 2). Coexpression of Keap1 resulted in a marked increase in ubiquitin conjugation onto PGAM5-L, including a distinct band with apparent molecular weight of 40 kDa and a prominent ladder comprised of multiple bands of higher apparent molecular weight (Fig. 5A, lane 3). Ubiquitin conjugation onto the mutant PGAM5-L-E79A/S80A protein was not observed (Fig. 5A, lane 4), consistent with the inability of the mutant PGAM5-L protein to associate with Keap1 (Fig. 4A, lane 3). Mutant Keap1 proteins defective in binding to PGAM5-L were likewise defective in directing ubiquitin conjugation of PGAM5-L (data not shown).

In a second set of experiments, the ability of Keap1 to recruit PGAM5-L into a Cul3-dependent ubiquitin ligase complex capable of targeting PGAM5-L for ubiquitination was determined. Expression vectors for FLAG-tagged PGAM5-L, Keap1, HA-Cul3, and Myc-Rbx1 were cotransfected into COS1 cells. Cell lysates were immunoprecipitated with anti-FLAG antibodies and analyzed for the presence of Keap1, Cul3 and Rbx1 by immunoblotting with specific antibodies. Coimmunoprecipitation of either Cul3 and Rbx1 was markedly enhanced in the presence of co-expressed Keap1 (Fig. 5B, compare lanes 2 and 3), consistent with the notion that Keap1 bridges the interaction between PGAM5-L and the Cul3:Rbx1 complex. Furthermore, co-expression of Cul3 and Rbx1 markedly increased Keap1-dependent ubiquitination of PGAM5-L (Fig. 5C, compare lanes 2 - 4). Finally, the quaternary PGAM5-L:Keap1-CBD:Cul3:Rbx1 complex was purified from transfected cells using chitin beads and the ability of this complex to support ubiquitin conjugation onto PGAM5-L was determined. Ubiquitin conjugation onto PGAM5-L was readily observed in the presence of E1 activating enzyme, the UbcH5 E2 conjugating enzyme, ubiquitin and ATP (Fig. 5D, lane 2). Ubiquitin conjugation onto PGAM5-L was not observed with reactions carried out in the absence of the E1 ubiquitin-activating enzyme (Fig. 5D, lane 1), confirming that conjugation of ubiquitin onto PGAM5-L occurred in vitro. A low level of ubiquitin conjugation onto PGAM5-L was observed in the absence of the Cul3/Rbx1 subcomplex (Fig. 5D, lane 3), presumably due to copurification of trace amount of the endogenous Cul3:Rbx1 subcomplex with ectopically expressed Keap1-CBD protein.

To determine if the ubiquitination of pGAM5-L by Keap1 alters the stability of the PGAM5-L protein, the steady-state levels of the wild-type and E79A/S80A mutant PGAM5-L proteins in the absence and presence of Keap1 were compared. Both proteins were expressed at comparable levels in HeLa cells in the absence of Keap1 (Fig. 6A, compare lanes 1 and 4). However, co-expression of Keap1 markedly
decreased steady-state levels of the wild-type but not the mutant PGAM5-L protein (Fig. 6A, compare lanes 2 and 3 with lanes 5 and 6). The half-life of the PGAM5-L protein in the absence or presence of Keap1 was determined in a cycloheximide pulse-chase experiment. Transfected HeLa cells were treated with cycloheximide to block protein synthesis and the level of the PGAM5-L protein was determined at time points following addition of cycloheximide. The half-life of the ectopically expressed PGAM5-L protein was 75 hours in the absence of co-expressed Keap1, but was reduced to 6 hours in the presence of co-expressed Keap1 (Figs. 6B and 6C).

To confirm that Keap1 targets PGAM5-L for degradation by the 26S proteasome, the ability of the proteasome-specific inhibitor, clasto-lactacystin β-lactone (lactacystin), to block Keap1-dependent degradation and increase steady-state levels of PGAM5-L was determined. HeLa cells transfected with expression vectors for PGAM5-L and Keap1 were exposed to either DMSO or lactacystin, followed by cycloheximide to block protein synthesis. The level of PGAM5-L was determined at 0, 2, 6 and 8 hours following cycloheximide treatment. As before, the level of PGAM5-L was reduced in the presence of lactacystin (Fig. 6D, lanes 1-4). However, levels of PGAM5-L did not decrease in the presence of lactacystin (Fig. 6D, lanes 5-8). Taken together, these experiments demonstrate that Keap1-mediated ubiquitination of PGAM5-L protein targets the PGAM5-L protein for degradation by the proteasome.

**Keap1-dependent ubiquitination of PGAM5-L is blocked by sulforaphane and quinone-induced oxidative stress** - Several reports have demonstrated that oxidative stress and thiol-reactive chemopreventive compounds block the ability of Keap1 to target Nrf2 for ubiquitin-dependent degradation (10,17,41). As a result, steady-state levels of Nrf2 are increased and transcription of Nrf2-dependent target genes is increased. As Keap1 binds the PGAM5-L protein and targets the PGAM5-L protein for ubiquitin-dependent degradation, the ability of both quinone-induced oxidative stress and sulforaphane, a chemopreventive isothiocyanate compound, to inhibit Keap1-dependent ubiquitination of PGAM5-L was determined. Both quinone-induced oxidative stress and sulforaphane markedly diminished Keap1-dependent ubiquitination of PGAM5-L (Fig. 7A, lanes 3 and 4) and increased steady-state levels of PGAM5-L (Fig. 7B, lanes 3-8). Treatment of cells with either lactacystin or sulforaphane also increased the amount of the endogenous PGAM5 that was associated with Keap1-CBD (Fig. 7C).

**Inhibition of Keap1-dependent ubiquitination of Nrf2 by electrophilic molecules and oxidative stress has been suggested to occur through modification of one or more cysteine residues in Keap1 (10,12,14-17).** In previous work, we have identified a single cysteine residue, C151, located in the BTB domain of Keap1 that is required for inhibition of Keap1-dependent ubiquitination of Nrf2 by both quinone-induced oxidative stress and sulforaphane (10,17). Direct modification of C151 in Keap1 by iodoacetamide in vitro has been demonstrated (15). In agreement with importance of Cys 151 for inhibition of Keap1-dependent substrate ubiquitination, the ability of the Keap1-C151S mutant protein to target PGAM5-L for ubiquitin conjugation (Fig. 7A, lanes 5-7) was resistant to inhibition by either oxidative stress or sulforaphane.

**DISCUSSION**

The ability of Keap1 to function as a redox-sensitive substrate adaptor protein for a Cul3-based E3 ubiquitin ligase has emerged as a major regulatory mechanism that governs downstream cellular responses to oxidative stress. In the present work, we have identified a member of the phosphoglycerate mutase family, PGAM5, as an interaction partner for Keap1. Human cells express two isoforms of human PGAM5 proteins, PGAM5-L and PGAM5-S, both of which bind to Keap1. We provide several lines of evidence that the PGAM5-L protein is a substrate of a Keap1-dependent ubiquitin ligase complex. First, PGAM5-L contains a conserved NxES(T)GE motif that is required for binding to Keap1. As PGAM5-L and PGAM5-S only differ at their C-termini, it is likely that PGAM5-S also uses this motif to bind Keap1. Second, co-expression of Keap1 with PGAM-L in cells markedly increases ubiquitin conjugation onto PGAM5-L and Keap1-
dependent ubiquitination of PGAM5-L by a Cul3:Rbx1-dependent E3 ubiquitin ligase complex can be reconstituted in vitro. Third, co-expression of Keap1 decreases steady-state levels of PGAM5-L, which are recovered by inhibition of the 26S proteosome. Finally, Keap1-dependent ubiquitination of PGAM5-L is impaired by both quinone-induced oxidative stress and sulforaphane, resulting in elevated levels of PGAM5-L. Mutation of a single cysteine residue in the BTB-domain of Keap1, C151, confers resistance to inhibition by oxidative stress and sulforaphane of Keap1-dependent ubiquitination of PGAM5-L. Taken together, these results indicate that PGAM5-L joins Nrf2 on a short list of bona fide substrates for a Keap1-dependent ubiquitin ligase complex.

The NxE(S/T)GE motif present in PGAM5 is similar in sequence to the conserved DxETGE motif in Nrf2 that binds Keap1 (24). The crystal structure of a Nrf2-derived peptide containing the DxETGE motif bound to the Kelch domain of Keap1 reveals that the hydroxyl residue of the threonine residue stabilizes a type I β-turn that enables the two glutamate side chains to contact multiple residues in Keap1 (23,25). A serine residue, but not an alanine residue, can substitute for the threonine residue in the Nrf2-DxETGE motif (23). It is likely that the serine residue in the NxESGE motif in PGAM5-L forms a similar type I β-turn that fits into the binding pocket in Keap1 and enables the side-chains of the flanking glutamate residues to contact specific residues in Keap1. In support of this notion, two arginine residues, Arg415 and Arg483, which participate in direct contacts with the corresponding glutamate residues in Nrf2 (23,25), are required for binding of PGAM5-L. Taken together, our mutational data indicate that PGAM5-L binds to Keap1 in a manner that is similar, though not identical, to the way in which Nrf2 binds to Keap1. In particular, the number of critical contacts formed between Nrf2 and Keap1 appear to be greater than the number of critical contacts formed between PGAM5-L and Keap1. Two other proteins, prothymosin alpha and FAC1, also interact with Keap1 in a way that suggests these two proteins may also be substrates of Keap1 (27,42). However, neither prothymosin alpha nor FAC1 have a D/NxE(S/T)GE-like motif, suggesting that binding pocket on the Kelch domain of Keap1 may recognize several different binding motifs.

Although both Nrf2 and PGAM5-L are substrates for Keap1-dependent ubiquitination and are subsequently degraded by the 26S proteosome, PGAM5-L is a more abundant protein with longer half-life than Nrf2. Keap1-dependent ubiquitination of Nrf2 results in a rapid turnover of Nrf2, with a half-life of approximately 30 minutes under basal conditions (17). In contrast, PGAM5-L has a half-life under basal conditions of approximately 6 hours when co-expressed with Keap1. This marked difference in the rate of protein turnover between Nrf2 and PGAM5-L likely reflects the rate at which multi-ubiquitination chains are added to Nrf2 or PGAM5-L. Several factors, including the affinity of the respective substrate proteins for Keap1 and the spatial orientation of the targeted lysine residues in the substrate, will contribute to the rate at which a given substrate acquires multi-ubiquitination chains. The importance of processivity of ubiquitin ligase complexes for determining the relative rates of substrate degradation has been suggested by a recent study of the anaphase-promoting complex (APC). The APC has several different substrates that are degraded in a temporal manner during progression through mitosis and G1, and the processivity of APC towards the different substrates has been suggested to determine the temporal order of substrate degradation (43).

PGAM5 is a member of the phosphoglycerate mutase superfamily. In humans, the phosphoglycerate mutase superfamily consists of at least ten distinct protein-encoding genes defined by the presence of the evolutionarily conserved PGAM domain (pfam00300). These genes can be divided into three groups based on sequence similarities and known functions (Fig. 2B). One group includes two genes that encode enzymes with known phosphoglycerate mutase activity (PGAM1 and PGAM2) and one gene that encodes an enzyme with diphosphoglycerate mutase activity (BPGM). This group also includes several pseudogenes, at least one of which is expressed (44). A second group of four genes encode isozenzymes of the bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase enzyme (PFKFB). This group includes the TIGAR protein, encoded by p53-
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inducible gene, that may contribute to dysregulation of glycolysis and apoptosis in cancer cells (45). A third group of proteins includes two related proteins, termed STS-1 and STS-2, and PGAM5. STS-1 and STS-2, which associate with the Cbl ubiquitin ligase, contain a ubiquitin-associated motif and an SH3 domain in addition to a C-terminal PGAM domain and have been implicated in regulation of T-cell receptor signaling and endocytosis of the receptor tyrosine kinases (37,38).

A common feature of phosphoglycerate mutase, diphosphoglycerate mutase and the 6-phosphofructo-2-kinase /fructose-2,6-bisphosphatase enzymes is the use of a phosphohistidine intermediate to mediate the transfer of phosphate to carbon atoms of their respective substrates (39). This catalytic histidine residue is embedded within a highly conserved signature motif of [LIVM]xRHG[EQ]x(Y)xN (Prosite PS00175) (39). The catalytic histidine residue is present in the proteins encoded by the third group of PGAM-domain containing proteins, although the C-terminal Asn residue in this signature motif, which is part of the active site of PGAM1 (39), has not been conserved in STS-1, STS-2 and PGAM5 (Fig. 2D). The PGAM domains in STS-1 and STS-2 do not appear to have phosphotransfer activity towards phosphoglycerate (43,46). Rather, the PGAM domains in STS-1 and STS-2 function as protein-protein interaction domains that enable the STS proteins to form dimers (38). Preliminary results suggest that the PGAM5 isoforms also form dimers (unpublished data). As the phosphohistidine signature motif of PGAM5 is the most divergent of all PGAM-domain containing proteins, it is likely that the PGAM5 proteins, like STS-1 and STS-2, will function as a mediators of protein-protein interactions rather than as a metabolic enzymes.

Our results confirm a previous report that identified PGAM5 as a Bcl-XL-binding protein using in vitro mRNA display and peptide binding analysis (34). Bcl-XL is a member of the Bcl-2 protein family that contains both anti-apoptotic (Bcl-2, Bcl-XL, etc.) and pro-apoptotic members (Bax, Bak, Bad, etc.). A balance between the actions of anti-apoptotic and pro-apoptotic members is essential to maintain tissue homeostasis in multicellular organisms. The formation of homodimeric and heterodimeric complexes between Bcl-2 family members, mediated in large part by the conserved BH3 domain, regulates the activity of the Bcl-2 proteins. For example, BH3-derived peptides from the BH3-only proteins BID or BIM are able to induce oligomerization of the pro-apoptotic BAX or BAK proteins, with subsequent induction of apoptosis (47). These BH3-derived peptides bind in a hydrophobic cleft located on the surface of the Bcl-2 family proteins. A PGAM5-derived peptide, amino acids 125 to 156, has been shown to bind Bcl-XL (34). However, binding of the PGAM5-derived peptide to Bcl-XL was not competed by a peptide derived from the BH3 domain of Bak, although a Bim-derived BH3 peptide was a very effective competitor for the Bak-derived BH3 peptide (34). This result suggests that the PGAM5 proteins bind outside of the groove in Bcl-XL that binds BH3 domains and are not likely to directly compete with BH3-only proteins for binding to Bcl-XL. Although it remains to be determined if binding of PGAM5 to Bcl-XL will modulate the balance between cell survival and death in response to changes in redox homeostasis, the PGAM5 proteins provide an intriguing molecular link between oxidative stress and apoptosis.

In summary, we have identified PGAM5 as a novel substrate for a redox-regulated Keap1-dependent ubiquitin ligase. The possibility that PGAM5 proteins may modulate oxidative stress-induced apoptosis is under active investigation by our laboratory.
REFERENCES

1. Imlay, J. A. (2003) *Annu Rev Microbiol* 57, 395-418
2. Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E. K., Jones, P. K., Ghanbari, H., Wataya, T., Shimohama, S., Chiba, S., Atwood, C. S., Petersen, R. B., and Smith, M. A. (2001) *J Neuropathol Exp Neurol* 60(8), 759-767
3. Andreassi, M. G. (2003) *Mutat Res* 543(1), 67-86
4. Nathan, C. (2002) *Nature* 420(6917), 846-852
5. Jackson, A. L., and Loeb, L. A. (2001) *Mutat Res* 477(1-2), 7-21
6. Ames, B. N., and Shigenaga, M. K. (1993) DNA and Free Radicals. In: Halliwell, B., and Aruoma, O. I. (eds). *Oxidants are a major contributor to cancer and aging*, Ellis Horwood, New York, NY
7. Ahmad, S., Anderson, W. L., and Kitchin, K. T. (1999) *Cancer Lett* 139(2), 129-135
8. Cullinan, S. B., Gordan, J. D., Jin, J., Harper, J. W., and Diehl, J. A. (2004) *Mol Cell Biol* 24(19), 8477-8486
9. Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., and Yamamoto, M. (2004) *Mol Cell Biol* 24(16), 7130-7139
10. Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., and Hannink, M. (2004) *Mol Cell Biol* 24(24), 10941-10953
11. Lo, S. C., and Hannink, M. (2006) *Mol Cell Biol* 26(4), 1235-1244
12. Hong, F., Freeman, M. L., and Liebler, D. C. (2005) *Chem Res Toxicol* 18(12), 1917-1926
13. Hong, F., Sekhar, K. R., Freeman, M. L., and Liebler, D. C. (2005) *J Biol Chem* 280(36), 31768-31775
14. Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., and Talalay, P. (2002) *Proc Natl Acad Sci U S A* 99(18), 11908-11913
15. Eggler, A. L., Liu, G., Pezzuto, J. M., van Breemen, R. B., and Mesecar, A. D. (2005) *Proc Natl Acad Sci U S A* 102(29), 10070-10075
16. Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., and Talalay, P. (2004) *Proc Natl Acad Sci U S A* 101(7), 2040-2045
17. Zhang, D. D., and Hannink, M. (2003) *Mol Cell Biol* 23(22), 8137-8151
18. Shen, G., Xu, C., Hu, R., Jain, M. R., Nair, S., Lin, W., Yang, C. S., Chan, J. Y., and Kong, A. N. (2005) *Pharm Res*
19. Lee, J. M., Calkins, M. J., Chan, K., Kan, Y. W., and Johnson, J. A. (2003) *J Biol Chem* 278(14), 12029-12038
20. Thimmulappa, R. K., Mai, K. H., Sriruma, S., Kensler, T. W., Yamamoto, M., and Biswal, S. (2002) *Cancer Res* 62(18), 5196-5203
21. Mathers, J., Fraser, J. A., McMahon, M., Saunders, R. D., Hayes, J. D., and McLellan, L. I. (2004) *Biochem Soc Symp* (71), 157-176
22. Kwak, M. K., Wakabayashi, N., Itoh, K., Motohashi, H., Yamamoto, M., and Kensler, T. W. (2003) *J Biol Chem* 278(10), 8135-8145
PGAM5: A novel substrate for Keap1

23. Lo, S. C., Li, X., Henzl, M. T., Beamer, L. J., and Hannink, M. (2006) *Embo J* 25(15), 3605-3617
24. Kobayashi, M., Itoh, K., Suzuki, T., Osanai, H., Nishikawa, K., Katoh, Y., Takagi, Y., and Yamamoto, M. (2002) *Genes Cells* 7(8), 807-820
25. Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsuji, M., Kang, M. I., Kobayashi, A., Yokoyama, S., and Yamamoto, M. (2006) *Mol Cell* 21(5), 689-700
26. Strachan, G. D., Morgan, K. L., Otis, L. L., Caltagarone, J., Gittis, A., Bowser, R., and Jordan-Sciutto, K. L. (2004) *Biochemistry* 43(38), 12113-12122
27. Karapetian, R. N., Efstatiouva, A. G., Abaeva, I. S., Chichkova, N. V., Filonov, G. S., Rubtsov, Y. P., Sukhacheva, E. A., Melnikov, S. V., Schneider, U., Wanker, E. E., and Vartapetian, A. B. (2005) *Mol Cell Biol* 25(3), 1089-1099
28. Wang, W., and Chan, J. Y. (2006) *J Biol Chem*
29. Orlicky, S., Tang, X., Willems, A., Tyers, M., and Sicheri, F. (2003) *Cell* 112(2), 243-256
30. Petroski, M. D., and Deshaies, R. J. (2005) *Nat Rev Mol Cell Biol* 6(1), 9-20
31. Sakamoto, K. M., Kim, K. B., Verma, R., Ransick, A., Stein, B., Crews, C. M., and Deshaies, R. J. (2003) *Mol Cell Proteomics* 2(12), 1350-1358
32. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) *Genes Dev* 13(3), 270-283
33. Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003) *Mol Cell* 11(6), 1445-1456
34. Hammond, P. W., Alpin, J., Rise, C. E., Wright, M., and Kreider, B. L. (2001) *J Biol Chem* 276(24), 20898-20906
35. Kamura, T., Koepp, D. M., Conrad, M. N., Skowyra, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. (1999) *Science* 284(5414), 657-661
36. Jeong, S. Y., Gaume, B., Lee, Y. J., Hsu, Y. T., Ryu, S. W., Yoon, S. H., and Youle, R. J. (2004) *Embo J* 23(10), 2146-2155
37. Carpino, N., Turner, S., Mekala, D., Takahashi, Y., Zang, H., Geiger, T. L., Doherty, P., and Ihle, J. N. (2004) *Immunity* 20(1), 37-46
38. Kowanetz, K., Crosetto, N., Haglund, K., Schmidt, M. H., Heldin, C. H., and Dikic, I. (2004) *J Biol Chem* 279(31), 32786-32795
39. Jedrzejas, M. J. (2000) *Prog Biophys Mol Biol* 73(2-4), 263-287
40. Li, X., Zhang, D., and Beamer, L. J. (2004) *J Biol Chem* 279(52), 54750-54758
41. Kobayashi, A., Kang, M. I., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., and Yamamoto, M. (2006) *Mol Cell Biol* 26(1), 221-229
42. Strachan, G. D., Ostrow, L. A., and Jordan-Sciutto, K. L. (2005) *Biochem Biophys Res Comm* 336(2), 490-495
43. Rape, M., Reddy, S. K., and Kirschner, M. W. (2006) *Cell* 124(1), 89-103
44. Betran, E., Wang, W., Jin, L., and Long, M. (2002) *Mol Biol Evol* 19(5), 654-663
45. Bensaad, K., Tsuruta, A., Selak, M. A., Vidal, M. N., Nakano, K., Bartrons, R., Gottlieb, E., and Vousden, K. H. (2006) *Cell* 126(1), 107-120
46. Carpino, N., Kobayashi, R., Zang, H., Takahashi, Y., Jou, S. T., Feng, J., Nakajima, H., and Ihle, J. N. (2002) *Mol Cell Biol* 22(21), 7491-7500
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47. Letai, A., Bassik, M. C., Walensky, L. D., Sorcinelli, M. D., Weiler, S., and Korsmeyer, S. J. (2002) Cancer Cell 2(3), 183-192

FOOTNOTES
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FIGURE LEGENDS
Fig. 1. (A) MDA-MB-231 cells that stably express the wild type Keap1-CBD proteins were generated by co-transfection of pBABE-puro and subsequent selection for puromycin resistance. MDA-MB-231 cells stably transfected with pBABE-puro alone were generated in parallel as a control cell line. Lysates from these cells were subjected to affinity purification using chitin beads. Proteins that remained associated with the chitin beads after extensive washing were analyzed by SDS-PAGE and visualized by silver stain. (B) Sixty-millimeter-diameter dishes of MDA-MB-231 cells were transfected with 2.0 µg of either an empty vector (lane 2) or an expression vector for Keap1-CBD (lane 1). Thirty-five-millimeter-diameter dishes of HEK-293T cells (lanes 3-8) were singly or co-transfected with 0.5 µg each of expression vectors for Keap1-CBD and FLAG-tagged or untagged versions of both PGAM5-L and PGAM5-S as indicated. Keap1-CBD proteins were purified from cell lysates from the singly transfected MDA-MB-231 cells (lanes 1 and 2) or the co-transfected HEK-293T cells (lanes 3-8) with chitin beads. Proteins remained bound to the chitin beads after extensive washing were analyzed by SDS-PAGE and visualized by silver stain. (C) Thirty-five-millimeter-diameter dishes of COS1 cells were transfected with 0.5 µg each of expression vectors for Keap1 and FLAG-tagged version of either PGAM5-S or PGAM5-L as indicated. Total cell lysates were analyzed by immunoblotting with anti-FLAG and anti-Keap1 antibodies (bottom two panels). Anti-FLAG immunoprecipitates (IP) were subjected to immunoblot analysis using anti-Keap1 antibodies (top panel).

Fig. 2. (A) The two isoforms of PGAM5 are depicted, with relevant regions of the proteins indicated. The two isoforms are identical up to amino acid 239 and have different C-termini as the result of alternative splicing. The NxESGE motif is located between amino acids 77-82 and the Bcl-XL interaction region is located between amino acids 125 and 156. The PGAM domain extends from amino acid 98 to the C-terminus (amino acid 289) of the PGAM5-L isoform. (B) The relationship between ten human proteins that contain a PGAM domain is depicted by a cladogram generated using the ClustalW program at European Bioinformatics Institute website (http://www.ebi.ac.uk/). The protein sequences were obtained from the Swiss Institute of Bioinformatics website (http://ca.expasy.org/) using the following accession numbers in parentheses: PGAM1 (P18669); PGAM2 (P15259); BPGM (P07738); STS-1 (Q96IG9); STS-2 (P57075); PGAM5 (Q96HS1); PFKFB1 (P16118); PFKFB2 (O60825); PFKFB3 (Q16875); PFKFB4 (Q16877). Several PGAM1-derived pseudogenes, including an expressed pseudogene termed PGAM4 (Q8N0Y7), were not included in this analysis (44). (C) Mammalian PGAM5 proteins contain a conserved NxE(S/T)GE motif that is similar to the conserved DxETGE motif in the human Nrf2 protein (Q16236). The NCBI accession numbers for the indicated PGAM5 proteins are in parentheses. Hs., Homo sapiens (NP_612642); Pt., Pan troglodytes (XP_509498); Mm., Mus musculus (NP_082549); Bt., Bos Taurus (XP_585405). The numbers on the right indicate the number of the last amino acid shown. (D) Alignment of ten PGAM domain-containing proteins showing the conservation of residues surrounding the phosphohistidine signature motif ([LIVM]xRHG[EQ]x(Y)xN; Prosite PS00175) for the phosphoglycerate mutase family, as described on the Swiss Institute of Bioinformatics website. A simplified version of signature motif (LxRHGxxxN) is shown in the Figure, with the conserved residues in bold and the histidine that forms a phosphohistidine intermediate underlined (39). The numbers on the right indicate the number of the last amino acid shown in each
protein. (E) Thirty-five-millimeter-diameter dishes of HEK-293T cells were cotransfected with expression vectors for FLAG-PGAM5-L and Bcl-X\textsubscript{L}. Total cell lysates were analyzed by immunoblotting with anti-FLAG and anti-Bcl-X\textsubscript{L} antibodies (bottom two panels). Anti-FLAG immunoprecipitates (IP) were subjected to immunoblot analysis using anti-Bcl-X\textsubscript{L} and anti-FLAG antibodies (top two panels).

Fig. 3. (A-I) Thirty-five-millimeter-diameter dishes of Hela cells growing on coverslips were transfected with an empty expression vector (panels A, B, and C) or expression vectors for FLAG-PGAM5-L (panels D, E, and F) or FLAG-PGAM5-S (panels G, H, and I). The cellular localization of the PGAM5 proteins was determined by indirect immunofluorescence with anti-FLAG antibodies (panels A, D, and G). Nuclei were stained with Topro3 (panels B, E, and H). Merged images were shown on the right (panels C, F, and I). (J-O) Thirty-five-millimeter-diameter dishes of COS1 cells growing on coverslips were transfected with expression vectors for the wild type (panels J, K, and L) or mutant (panels M, N, and O) FLAG-PGAM5-L. The cellular localization of the PGAM5 proteins was determined by indirect immunofluorescence with anti-FLAG antibodies (panels J and M). Nuclei were stained with propidium iodide (panels K and N). Merged images were shown on the right (panels L and O).

Fig. 4. (A) Thirty-five-millimeter-diameter dishes of HEK-293T cells were cotransfected with expression vectors for FLAG-PGAM5-L and the full-length (F.L.) or mutant Keap1 proteins containing a specific deletion of each individual domain in Keap1 as indicated. Total cell lysates were analyzed by immunoblotting with anti-FLAG and anti-Keap1 antibodies (bottom two panels). Anti-Keap1 immunoprecipitates (IP) were subjected to immunoblot analysis using anti-FLAG antibodies (top panel). (B) Thirty-five-millimeter-diameter dishes of Hela cells were transfected with expression vectors for the wild type or mutant Keap1-CBD proteins as indicated. Cell lysates were incubated with chitin beads and proteins that remained bound after extensive washing were analyzed by SDS-PAGE and visualized by silver stain. (C) Pull-down assays were performed for the indicated Keap1 proteins as described for panel B. (D) Thirty-five-millimeter-diameter dishes of COS1 cells were cotransfected with expression vectors for Bcl-X\textsubscript{L} (0.4 \(\mu\)g), Keap1 (0.4 \(\mu\)g) and the wild type or mutant FLAG-PGAM5-L (0.2 \(\mu\)g) proteins as indicated. Total cell lysates were analyzed by immunoblotting with anti-FLAG, anti-Keap1 and anti-Bcl-X\textsubscript{L} (L-19) antibodies (bottom three panels). Anti-FLAG immunoprecipitates (IP) were analyzed by immunoblotting with anti-Bcl-X\textsubscript{L} (L-19) and anti-Keap1 antibodies (top two panels).

Fig. 5. (A) Sixty-millimeter-diameter dishes of COS1 cells were transfected with expression vectors for HA-Ub (0.4 \(\mu\)g), Keap1 (1.2 \(\mu\)g), and the wild type or mutant FLAG-PGAM5-L proteins (0.4 \(\mu\)g) as indicated. Anti-FLAG immunoprecipitates (IP) were analyzed by immunoblot with anti-HA antibodies. (B) Sixty-millimeter-diameter dishes of COS1 cells were transfected with expression vectors for Keap1 (0.8 \(\mu\)g), HA-Cul3 (0.8 \(\mu\)g), and myc-Rbx1 (0.4 \(\mu\)g) as indicated. An expression vector for FLAG-PGAM5-L (2.0 \(\mu\)g) was separately transfected into cells. Cell lysates were mixed prior to immunoblotting with the indicated antibodies (bottom four panels), or immunoprecipitation with anti-FLAG M2 agarose beads. Anti-FLAG immunoprecipitates (IP) were analyzed by immunoblot with the indicated antibodies (top three panels). This procedure enabled input cell lysates to be normalized to input levels of FLAG-PGAM5-L prior to the immunoprecipitation. (C) Sixty-millimeter-diameter dishes of COS1 cells were transfected with expression vectors for HA-Ub (0.4 \(\mu\)g), FLAG-PGAM5-L (0.4 \(\mu\)g), Keap1 (0.4 \(\mu\)g), Cul3 (0.4 \(\mu\)g), and Rbx1 (0.05 \(\mu\)g) as indicated. Total cell lysates were analyzed by immunoblotting with anti-FLAG antibodies (bottom panel). Anti-FLAG immunoprecipitates (IP) were analyzed by immunoblot with anti-HA antibodies (top panel). (D) Sixty-millimeter-diameter dishes of COS1 cells were transfected with expression vectors for FLAG-PGAM5-L (0.3 \(\mu\)g), Keap1-CBD (0.8 \(\mu\)g), HA-Cul3 (0.5 \(\mu\)g), and myc-Rbx1 (0.4 \(\mu\)g) as indicate. Lysates from four 60-mm-diameter dishes were pooled for each sample and incubated with chitin beads. After washing, the chitin beads were incubated with E1, E2-UbcH5a, ubiquitin, and ATP. The E1 enzyme was omitted from one sample (lane 1). Subsequently, the chitin beads were pelleted and washed, and proteins that were eluted from the beads under denaturing
conditions were split into two sets of samples. One set was immunoprecipitated with anti-FLAG M2 agarose beads and then analyzed by immunoblotting with anti-ubiquitin antibodies (top panel). The other set was subjected to immunoblot analysis with the indicated antibodies (bottom four panels).

Fig. 6. (A) Twenty-four-well plates of Hela cells were transfected with expression vectors for the wild type or mutant FLAG-PGAM5-L proteins (0.2 µg) along with an empty expression vector (lanes 1 and 4) or expression vectors for Keap1 (0.02 or 0.2 µg; lanes 2, 3, 5, and 6). Total cell lysates were subjected to immunoblot analysis with anti-FLAG and anti-Tubulin antibodies. (B) Thirty-five-millimeter-diameter dishes of Hela cells were transfected with an expression vector for FLAG-PGAM5-L (0.8 µg), along with an empty vector (lanes 1-6) or an expression vector for Keap1 (0.2 µg, lanes 7-12). Cells were treated with 50 µg/ml cycloheximide. Total cell lysates were collected at the indicated time points following cycloheximide treatment and subjected to immunoblot analysis with anti-FLAG and anti-Tubulin antibodies. (C) The relative intensities of FLAG-PGAM5-L were quantified by using the Multi Gauge software (FUJIFILM) and plotted on a semi-log graph. The amount of FLAG-PGAM5-L present at the beginning of cycloheximide treatment was set at 100. The half-life of PGAM5-L proteins in the presence (closed square) and absence (open triangle) of Keap1 are indicated in the inset. (D) Thirty-five-millimeter-diameter dishes of Hela cells were transfected with expression vectors for FLAG-PGAM5-L (0.68 µg) and Keap1 (0.34 µg). Cells were treated with solvent only (DMSO; 20 µl) (lanes 1-4) or 10 µM clasto-lactacystin β-lactone (Lactacystin) (lanes 5-8) 1 h prior to and during the cycloheximide treatment (50 µg/ml). Total cell lysates were collected at the indicated time points following cycloheximide treatment and subjected to immunoblot analysis with anti-FLAG and anti-Tubulin antibodies.

Fig. 7. (A) Sixty-millimeter-diameter dishes of COS1 cells were transfected with expression vectors for HA-Ub (0.6 µg), FLAG-PGAM5-L (0.7 µg), and the wild type or mutant Keap1 proteins (0.7 µg) as indicated. The cells were either untreated (lanes 1, 2 and 5) or treated with 25 µM sulforaphane (lanes 3 and 6) or 50 µM tBHQ (lanes 4 and 7) for 4.5 h prior to cell lysis under strongly denaturing conditions. Anti-FLAG immunoprecipitates (IP) were analyzed by immunoblot with anti-HA antibodies. (B) Thirty-five-millimeter-diameter dishes of Hela cells were transfected with expression vectors for FLAG-PGAM5-L (0.68 µg) and Keap1 (0.34 µg). The cells were either untreated (lanes 1 and 2) or treated with 25 µM sulforaphane (lanes 6-8) or 50 µM tBHQ (lanes 3-5) for the indicated time periods prior to cell lysis. Total cell lysates were analyzed by immunoblotting with anti-FLAG and anti-Tubulin antibodies. (C) One-hundred-millimeter-diameter dishes of MDA-MB-231 cells that stably express the wild type Keap1-CBD protein were treated with DMSO (lanes 1 and 2), or 5 µM Sulforaphane for 16 hours (lane 3), or 10 µM clasto-lactacystin β-lactone (Lactacystin, lane 4) for one hour as indicated. All the cells were further treated with cycloheximide (50 µg/ml) for six additional hours prior to lysis to block new protein synthesis. Cells were lysed in RIPA buffer and the lysates were incubated with chitin beads, pelleted by centrifugation (3,000 X g), and washed three times in the lysis buffer. Proteins that remained associated with the chitin beads were analyzed by SDS-PAGE and visualized by silver stain (top panel). Total cell lysates were analyzed by immunoblotting with anti-Tubulin antibodies (bottom panel).
### Table 1

| Position of PGAM5 peptides<sup>a</sup> | Mr (calculated)<sup>b</sup> | Mr (measured)<sup>c</sup> |
|--------------------------------------|-----------------|-----------------|
| 33-52 AGGDAEPRPAEPPAWAGGARD        | 1931.92         | 1931.90         |
| 53-64 PGPGVWDPNWDR<sup>d</sup>      | 1394.64         | 1395.64         |
| 65-74 REPLSLINVR                    | 1195.70         | 1195.68         |
| 66-74 EPLSLINVRK                    | 1039.60         | 1039.59         |
| 99-104 HIFLIR                      | 797.49          | 797.49          |
| 105-116 HSQYHDGSLEK                 | 1398.65         | 1398.65         |
| 105-118 HSQYHDGSLEKDR               | 1669.78         | 1669.76         |
| 119-125 TLTPLGR                     | 765.45          | 765.44          |
| 126-134 EQAELTGLR                   | 1015.53         | 1015.52         |
| 153-162 AIEETDIISR                  | 1117.69         | 1117.60         |
| 170-176 VSTDLLR                     | 802.45          | 802.44          |
| 177-203 EGAPIEPDPVSHWKPEAVQYYEDGARIEAAFR | 3036.43     | 3036.35         |
| 204-209 IEAAFR                      | 705.38          | 705.38          |
| 210-214 NYIHR                       | 701.36          | 701.36          |
| 219-235 QEEDSYEIFICHANVIR           | 2121.98         | 2121.98         |

<sup>a</sup>: The position and sequence of PGAM5-derived peptides identified by MALDI-TOF MS.

<sup>b</sup>: The calculated mass of the indicated PGAM5-derived peptides.

<sup>c</sup>: The observed mass of the indicated PGAM5-derived peptides by mass spectrometry.

<sup>d</sup>: The amino acid composition of the indicated peptides was confirmed by MS/MS. These two peptides were only observed in band B of Fig. 1A. All other peptides were observed in both bands B and C.
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Shih-Ching Lo and Mark Hannink

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