Comprehensive analyses of the cysteine thiol oxidation of PKM2 reveal the effects of multiple oxidation on cellular oxidative stress response

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Abstract

Redox regulation of proteins via cysteine residue oxidation is involved in the control of various cellular signal pathways. Pyruvate kinase M2 (PKM2), a rate-limiting enzyme in glycolysis, is critical for the metabolic shift from glycolysis to the pentose phosphate pathway under oxidative stress in cancer cell growth. The PKM2 tetramer is required for optimal pyruvate kinase (PK) activity, whereas the inhibition of inter-subunit interaction of PKM2 induced by Cys358 oxidation has reduced PK activity. In the present study, we identified three oxidation-sensitive cysteine residues (Cys358, Cys423 and Cys424) responsible for four oxidation forms via the thiol oxidant diamide and/or hydrogen peroxide (H₂O₂). Possibly due to obstruction of the dimer-dimer interface, H₂O₂-induced sulfonylation (-SOH) and diamide-induced modification at Cys424 inhibited tetramer formation and PK activity. Cys423 is responsible for intermolecular disulphide bonds with heterologous proteins via diamide. Additionally, intramolecular polysulphide linkage (–Sn–, n≥3) between Cys358 and an unidentified PKM2 Cys could be induced by diamide. We observed that cells expressing the oxidation-resistant PKM2 (PKM2<sup>C358,424A</sup>) produced more intracellular reactive oxygen species (ROS) and exhibited greater sensitivity to ROS-generating reagents and ROS-inducible anti-cancer drugs compared to cells expressing wildtype PKM2. These results highlight the possibility that PKM2 inhibition via Cys358 and Cys424 oxidation contributes to eliminating excess ROS and oxidative stress.

Introduction

Oxidative modifications of protein cysteine residues represent post-translational modifications (PTMs). Notably, cysteine (Cys) residues can react with electrophilic compounds such as reactive oxygen species (ROS) and result in various chemical modifications. Hydrogen peroxide (H₂O₂), which is the most abundant ROS in aerobic organisms, reacts to Cys thiol (Cys-SH) to form various types of oxidised cysteine residues such as disulphide, sulfenic acid and sulfenic acid[1]. Such oxidations alter the function of target proteins and, in many cases, are involved in a signalling cascade for defence mechanisms against oxidative stress[2–7].

The reduction of glutaredoxins and peroxiredoxins, which are major antioxidant systems, requires NADPH as a proton donor[8,9]. The major pathway for NADPH production is the pentose phosphate pathway (PPP), which is a branch of glycolysis. Therefore, under oxidative stress conditions, the increased metabolic flux in the PPP via glycolysis inhibition serves an important role in the detoxification of H₂O₂[10–12]. This metabolic alteration is induced by the ROS-induced inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PK)[13]. GAPDH has an oxidation-sensitive (redox-active) cysteine residue at the enzymatic active site. The cysteine residues are evolutionarily conserved and oxidised by various SH-oxidising reagents and intracellular levels of H₂O₂, resulting in many modifications, including disulphide bond formation[14–20]. However, much of the PK redox regulation mechanism remains unknown. In the present study, we identified multiple redox-active cysteine residues and oxidation forms that affect PK activity.

As a rate-limiting enzyme for glycolysis the PK catalyses the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP (PK activity). This reaction is irreversible and represents the final step in glycolysis. In the yeast model system, inhibition of PK activity induces metabolic change under oxidative stress[21,22]. In mammals, there are four isoforms and two genes, with each isoform expressing in different tissues. The expression of PKL, PKR and PKM1 is tissue-specific[23], whereas that of PKM2 is ubiquitous. PKM1 and PKM2 are encoded by the PKM gene and depend on alternative splicing using two mutually exclusive exons[24]: PKM1 (includes exon 9 and is expressed in limited tissues such as heart, muscle, brain) and PKM2 (includes exon 10 and is ubiquitously expressed in many tissues and cells). Notably, the regulation of PKM2 enzymatic activity plays a critical role in cancer cell metabolism[25,26]. As such, the appropriate regulation of PKM2 might confer metabolic advantages for tumour growth and progression since reduced PK activity enhances metabolites from
pentose phosphate pathway and amino acid synthesis[25,26]. This is called the Warburg effect[27,28]. Tetrameric PKM2 and dimeric/monomeric PKM2 exhibit higher and lower PK activity, respectively. Allosteric activators of PKM2 such as fructose-1,6-bisphosphate (FBP) and serine increase PK activity by promoting the tetrameric form[29,30], while the PTMs of PKM2 (e.g., oxidation, phosphorylation, acetylation and glycosylation) decrease PK activity by blocking the formation of the tetramer. Decreased PKM2 activity via PTMs provides a metabolic advantage for the Warburg effect and facilitates cancer cell growth[31–34]. Thus, the appropriate inhibition of PK activity might induce accumulation of the intermediate metabolites of glycolysis and promote the synthesis of several amino acids and nucleotides, including NADPH. As a result, this change fulfills the metabolic requirement for cancer cells[35]. Moreover, dimeric PKM2—which is induced by several specific PTMs—is responsible for the regulation of gene transcription via interacting transcription factors in the nucleus[36,37]. Several studies have reported that the moonlighting (non-canonical) functions of PKM2 serve a critical role in tumorigenesis[38]. Taken together, the tetramer-to-dimer transition of PKM2 provides two benefits in cancer cell growth: the downregulation of PK activity and activation of moonlighting functions.

Cysteine modifications (oxidations) are important PTMs in PKM2 that control cell metabolism. An initial report indicated that Cys358 oxidation was crucial for oxidative stress-induced downregulation and the loss of inter-subunit interactions of PKM2[39]. However, the molecular basis of PKM2 oxidation has not been elucidated. Recently, two reports revealed that cysteine residues at positions 152, 326, 358, 423 and 424 were involved in S-nitrosylation induced by nitric oxide[40,41]. The nitric oxide-induced modification of PKM2 Cys residues by a balance of endothelial nitric oxide synthase (eNOS) and S-nitroso-coenzymeA reductase is responsible acute kidney injury by reprogramming of glycolysis and enhance NADPH production in PPP that is responsible for protection acute kidney injury[41]. These results suggested oxidation of multiple cysteine residues of PKM2 by an abundant ROS H2O2 and electrophiles has important roles to maintain cellular redox homeostasis in response to the environmental changes. Thus, comprehensive observation of the ROS-induced Cys oxidation of PKM2 provides insight into the role of PKM2 in the cellular oxidative stress response. In this study, we identified four oxidation forms of three PKM2 Cys: sulfenylation and diamide-induced oxidation (Cys424), intramolecular polysulphide linkage (Cys358) and intermolecular disulphide bonds with other proteins (Cys423). Moreover, we demonstrated that substitution of Cys358 and Cys424 to alanine (Ala) suppresses the oxidation-dependent suppression of PK activity and induces a change in cell metabolism and oxidative stress response.

Materials and methods

Cell lines, cell culture and the establishment of PKM2 knockdown

A human non-small cell lung carcinoma cell line (H1299) and a hepatocellular carcinoma (HepG2) were obtained from the American Type Culture Collection. Human embryonic kidney 293 (HEK293T) cells were obtained from Thermo Fisher Scientific (Waltham, MA, USA). These cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Nissui, Tokyo, Japan or Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biosera, Kansas, MO, USA), 3% glutamine (Nacalai Tesque), penicillin/streptomycin (FUJIFILM Wako, Osaka, Japan) and 7.5% NaHCO3 (Nacalai Tesque) at 37°C and 5% CO2 concentration.

Stable knockdown of endogenous PKM2 was performed using a lentivirus expressing a high PK knockdown efficiency shRNA (Supplemental sequence file 1) [42]. The shPKM2 sequence was cloned into the AgeI-EcoRI sites of a pLKO.1 puro vector (pLKO-shPKM). The lentivirus vectors were produced in HEK293FT cells by the co-transfection of plasmids pMD2.G and pCMV.R8.74 with pLKO.1-sh PKM2 using Lipofectamine 2000 (Thermo Fisher Scientific). To produce H1299 shPKM2 cells, H1299 cells were transduced by the lentivirus vectors and selected with DMEM containing 5 μg/ml puromycin (InvivoGen, San Diego, CA, USA).
pMD2.G (Addgene plasmid #12259; http://n2t.net/addgene:12259; RRID: Addgene_12259) and pCMVR8.74 (Addgene plasmid #22036; http://n2t.net/addgene:22036; RRID: Addgene_22036) were provided by Didier Trono. pLKO.1 neo was provided by Sheila Stewart (Addgene plasmid #13425; http://n2t.net/addgene: 13425; RRID: Addgene_13425).

Antibodies and reagents
The primary antibodies (ab) used in this study were rabbit anti-PKM2 (SAB4200095, Merck, Darmstadt, Germany), mouse anti-FLAG M2 (F1804, Merck), rabbit anti-SOH (07-2139, Merck) (for the detection of dimedone-linked protein), mouse anti-GAPDH (Merck), rabbit anti-His tag (PM032, MBL) and rabbit anti-actin (Santa Cruz, Dallas, TX, USA). Secondary antibodies included polyclonal goat anti-rabbit horseradish peroxidase (HRP, Dako, Agilent, Santa Clara, CA, USA) and polyclonal goat anti-mouse IgG HRP (Dako, Agilent). The oxidants used in this study included 30% hydrogen peroxide (H2O2, Nacalai Tesque), diamide (MP Biomedicals, Irvine, CA, USA), tert-butyl hydroperoxide, (tBHP, Merck) and glucose oxidase (GO, Merck). The cysteine residue modification reagents used were N-ethyl maleimide (NEM, Nacalai Tesque), iodoacetamide (IAA, FUJIFILM Wako), PEGylated maleimide (PEGM, MW 2,000, SUNBRIGHT®, ME-020MA, NOF Corporation, Tokyo, Japan) and dimedone (Nacalai Tesque). DTT (Nacalai Tesque) was used for the reduction of oxidised cysteine residues. Additional chemical reagents included sodium tetrasulfide (Na2S4, Dijinudo, Kumamoto, Japan), cisplatin (CDDP, FUJIFILM Wako) and doxorubicin (DOX, FUJIFILM Wako).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting
The proteins were quantified with a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) using BSA (Nacalai Tesque) as the standard. The proteins prepared in each experiment included mixed SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) supplemented with 50 mM DTT and boiled at 95°C for 5 min. We performed SDS-PAGE in running buffer (25 mM Tris-HCl, pH 7.5, 250 mM glycine, 0.1% SDS). Proteins in the gel were then transferred to a poly-1,1-difluoroethane (PVDF) membrane (Merck) in transfer buffer (25 mM Tris-HCl, pH 7.5, 250 mM glycine, 0.1% SDS, 5% methanol) using a trans-blot system (Bio-Rad). Blocking of the PVDF membrane was performed by shaking in TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk for 1 hr at room temperature. After the primary- and secondary-antibody reactions, we obtained chemiluminescent images using Immobilon Western Chemiluminescent HRP Substrate (Merck) and the VersaDoc imaging system (Bio-Rad) or ChemDoc imaging system (Bio-Rad).

Expression of PKM2 in H1299 shPKM2 cells
In human PKM2 cDNA (NM_002654.6), a FLAG-tag was fused to the corresponding C-terminus of the PKM2 sequences. FLAG-PKM2 was then cloned into a pEBMulti-Hyg vector (FUJIFILM Wako) between KpnI and NotI. The PKM2 coding sequence corresponding to codons 106–112 was modified to 5'-GCcGTcGcCCTg-3' (the lowercase letters indicate substitutions) to perform shRNA-resistant expression (Supplemental sequence file 2). To construct PKM2 cysteine mutants, the corresponding cysteine codon was mutated to Ala (GCC), Asp (GAC) or Ser (AGC) using specific oligo nucleotides (Additional materials and methods) by PCR. These plasmids were then transfected into H1299 shPKM2 cells using the FuGENE HD transfection reagent (Promega, Madison, WI, USA) and cultured in DMEM for 48 hr. We reproduced comparable expression levels of PKMs and its mutants in transfected cells (Fig. S1B). Cell viability was determined by AlamarBlue assay (Thermo Fisher Scientific) according to the manufacturer’s protocol.

Purification of recombinant PKM2 (rPKM2)
Recombinant PKM2 wildtype (rPKM2WT) or its mutants fused with a His-tag on the amino-terminus—were expressed in Escherichia coli BL21 (DE3) using pET15b vector (Merck) (Supplemental sequence file 3). A single colony was inoculated in 2 mL Luria-Bertani broth (LB) supplemented with ampicillin (LB Amp) and was incubated overnight at 37°C with shaking. Overnight cultures were diluted in 400 mL LB Amp and grown to an OD600 of 0.4–0.6 at 37°C with shaking. Then, 1 mM isopropyl-β-D-thiogalactopyranoside (Nacalai Tesque) was added to these cultures to induce the
expression of His-PKM2, followed by incubation at 25°C for 6 hr with shaking. Cells were then harvested by centrifugation (4,000 g, 4°C, 10 min) and pellets were stored at -20°C.

The pellets were lysed in 10 mL buffer A (PBS, 20% glycerol (Nacalai Tesque), 10 mM imidazole (Nacalai Tesque), 0.1% Tween 20 (Nacalai Tesque), 1 mg/ml lysozyme chloride (Nacalai Tesque)) via sonication using Vibra-Cell™ (VC505, Sonics & Materials, CT, USA). After the lysates were centrifuged, the supernatants were mixed with Ni-NTA (Qiagen, Germantown, MD, USA), which was pre-equilibrated by buffer A before use with rotation for 3 hr at 4°C in Econo-Pac Chromatography Columns (20 mL, Bio-Rad). After the unbounded fraction was discarded, Ni-NTA was washed twice with 4 mL buffer B (PBS, 20% glycerol, 20 mM imidazole, 0.1% Tween 20). His-tagged PKM2 protein was eluted by buffer C (PBS, 20% glycerol, 250 mM imidazole, 0.1% Tween 20) and dialysed at 4°C overnight in dialysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 20% glycerol, 20% ammonium sulfate, 0.05% Tween 20). The purified proteins were then checked and quantified using Coomassie Brilliant Blue (CBB) staining (Quick-CBB, FUJIFILM Wako) with BSA as the standard (Fig. S1).

**Maleimide-based gel shift assay (NEM-DTT-PEGM assay)**

After cells were treated with each oxidant at the indicated time, they were incubated for 5 min in 100 mM NEM in PBS (Nacalai Tesque) to block free thiols. Cells were then lysed in PK lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Igepal-630 (Merck)) supplemented with 50 mM DTT and protease inhibitors (eComplete™ protease inhibitor cocktail EDTA-free, Roche, Basel, Switzerland). Lysates were then centrifuged (13,800 g, 5 min, 4°C). Supernatants were collected in new tubes and then incubated for 10 min at room temperature to reduce the oxidised thiols. To remove DTT, protein in these lysates were precipitated in 5% trichloroacetic acid (TCA)-75% acetone for 10 min on ice. The proteins were precipitated by centrifugation (13,800 g, 2 min, 4°C). The pellets were then washed with 500 µL acetone and collected by centrifugation. They were then mixed with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue) supplemented with 50 mM DTT. SDS-PAGE was then performed within 30 min of sample preparation.

**Non-reduced SDS-PAGE**

After cells were treated with each oxidant for the indicated time, they were incubated for 5 min in 100 mM IAA in PBS to block free thiols. Cells were then lysed in PK lysis buffer supplemented with 50 mM IAA and protease inhibitors. Lysates were then centrifuged (13,800 g, 5 min, 4°C) and the supernatants were collected in new tubes. These samples were mixed with SDS sample buffer (without DTT) and SDS-PAGE was performed. For the detection of a faster-migrating rPKM2 band, rPKM2 and its mutants were pre-treated with 100 mM DTT to reduce all cysteine residues and DTT was then removed using a NAP5 column (Cytiva, Marlborough, MA, USA). These proteins were treated with oxidants and combined with a sample buffer with IAA or NEM but not DTT.

**Detection of rPKM2 sulfonylation**

rPKM2 WT and its mutants (0.5 µg) were diluted in PBS. These proteins were treated with H₂O₂ for 10 min. Then, dimedone was added to the protein sample for a final concentration of 0.25 mM and incubated at room temperature for 30 min. The reaction mixtures were combined with a sample buffer supplemented with 2-mercaptoethanol (Nacalai Tesque) and analysed by SDS-PAGE and western blotting, as previously described.

**Detection of PKM2 sulfonylation in cultured cells**

H1299 shPKM2 cells transfected with pEB FLAG-PKM2 were treated with oxidants. To block free thiols, cells were incubated with 100 mM NEM in PBS at 37°C. Thereafter, cells were washed with PBS and lysed in a dimedone-containing lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM dimedone (Nacalai Tesque), 1% Igepal, protease inhibitors). After removal of the unresolved fraction
by centrifugation, the supernatants (as whole-cell lysates (WCLs)) were quantified using a DC Protein Assay Kit (Bio-Rad). Then, 600 μg WCL was mixed with anti-FLAG antibody beads (Merck) at 4°C for 3 hr with rotation. The beads were then washed three times with the lysis buffer. Bound proteins were eluted from the beads with 90 μL of sample buffer containing 50 mM DTT at 95°C for 5 min. The immunoprecipitates were analysed by SDS-PAGE and western blotting using the anti-PKM2 antibody and rabbit anti-SOH (anti-dimedone) antibody, which specifically reacts with dimedone-bound cysteine residues.

**Detection of rPKM2 multimer**
rPKM2 WT or its mutants (0.5 μg) were diluted in PBS and pre-incubated with 1 mM FBP and 5 mM DTT at 37°C for 30 min. Glutaraldehyde (GA) (Nacalai Tesque) was added to the protein samples for a final concentration of 0.025% and incubated at 37°C for 3 min. The reaction mixtures were then mixed with a sample buffer supplemented with 50 mM DTT and analysed by SDS-PAGE and western blotting using the anti-PKM2 antibody.

**Detection of PKM2 multimer in cultured cells**
Cells were lysed in PBS by adding 1% Igepal and protease inhibitors. GA was added to the cell lysate for a final concentration of 0.025% and incubated at 37°C for 3 min. The reaction mixtures were then mixed with a sample buffer supplemented with 50 mM DTT and analysed by SDS-PAGE and western blotting using the anti-FLAG antibody.

**Measurement of pyruvate kinase activity**
PK activity was measured by monitoring the change in absorbance at 340 nm due to the oxidation of NADH. This was a coupled reaction with lactate dehydrogenase (LDH). Recombinant proteins (0.12 μg) and cell lysates (20 μg) were diluted in PK reaction buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂). The final reaction mixture was created by adding 1.9 mM PEP (FUJIFILM Wako), 0.6 mM ADP (Merck), 0.3 mM NADH (Merck), 1.1 units LDH (Merck), 1 mM FBP (Merck) (where applicable) and DTT (where applicable). The final reaction volume was 150 μl in a 96-well plate (Greiner, Austria) and the change at 340 nm was measured by VariosKan Flash (Thermo Fisher Scientific).

**Measurement of NADPH ratio and intracellular ROS**
Measurement of NADPH ratio was performed using an NADP/NADPH-Glo™ assay kit (Promega), according to the manufacturer’s protocol. We used CellROX Orange Reagent (Thermo Fisher Scientific) and ROS-Glo (Promega) to measure total H₂O₂ level in the cell culture (medium and cells). For CellROX Orange, cells were incubated with oxidants at the indicated time in a 96-well plate (Perkin Elmer, Waltham, MT, USA). After removing the medium and washing with PBS, the cells were stained by adding CellROX orange and Hoechst (Thermo Fisher Scientific) at 37°C for 30 min in the dark. The cells were then washed three times with PBS. Fluorescence imaging analysis was then performed using the Operetta CLS High-content Analysis System (Perkin Elmer). For ROS-Glo, the cells were incubated in 384 white plates (Thermo Fisher Scientific) and the measurement of ROS was performed according to the manufacturer’s protocol (Promega).

**Data reproduction and statistical analyses**
Experiments were performed at least three times. The data are presented as the mean +/- the standard error of the mean (N = 3). Statistical comparisons were performed using the Student’s t-test or Dunnett’s post-hoc test.

**Results**

**Identification of novel oxidation-sensitive cysteine residues of PKM2**
A previous report indicated that a specific cysteine residue (Cys358) of PKM2 was involved in its redox regulation using dimaide as an oxidant and PKM2 with a loss of the Cys358 mutation (Cys358 to...
Ser)[39]. Recent global analyses on the oxidative modification of protein cysteine residues indicated that another cysteine residue (Cys424) in PKM2 was modified under oxidative stress induced by H₂O₂.[43,44] As shown in Fig. S2, the PK activity of H1299 cells expressing PKM2 with a loss of the Cys358 mutation (PKM2C358A) continued to be downregulated in the presence of H₂O₂ (see the “Redox regulation of PKM2 contributes to oxidative stress response” section).

Thus, multiple cysteine residues might be responsible for the redox downregulation of PK activity. To identify cysteine residues that might be oxidation-sensitive, we performed a PEGM-based gel shift assay. Sulphydryl residues of whole-cell proteins were first blocked with NEM. Then, the addition reaction of PEGM (MW 2,333) to NEM-free oxidised Cys (NEM-DTT-PEGM assay) was performed after reduction of pre-oxidised Cys by DTT treatment (see Fig. 1A). As shown in Fig. 1B, the mobility of PKM2 (63 kDa) in SDS-PAGE shifted to a higher molecular size (100 kDa) when all 10 PKM2 Cys were assumed to be modified by PEGM under the condition without NEM. In contrast, the 63 kDa band shifted to one band (66 kDa) and two bands (66 kDa and 70 kDa) when HEK293T and HepG2 cells were treated with H₂O₂ and diamide, respectively. However, tBHP treatment did not affect the mobility of PKM2 (Fig. 1B). Notably, lipophilic and bulky peroxides may not react with PKM2.

To identify the cysteine residues responsible for the shift (oxidation) in PKM2, we created 10 PKM2 mutants, in which each of 10 cysteine residues was replaced with Ala. H1299 shPKM2 cells expressing each of the PKM2 mutants were treated with oxidants. As shown in Fig. 1C, the appearance of the two molecular weight shifts by diadime were almost completely abolished by a Cys424 mutation (PKM2C424A), while only the upper-shift band (70 kDa) was absent when Cys423 was mutated (PKM2C423A), indicated as **; Fig. 1C). In contrast, a mutation on Cys358 (PKM2C358A and PKM2C358S) did not affect PKM2 oxidation (Fig. 1C). These results suggest that NEM-DTT-PEGM assays identified Cys424 and Cys423—but not Cys358—as sensitive to oxidation induced by diamide. Next, we focused on the oxidation induced by H₂O₂. The H₂O₂-induced 66 kDa shift was decreased by the mutation of either Cys424 (PKM2C424A), Cys423 (PKM2C423A) or both (PKM2C423,424A) (Fig. 1D), which suggests that these cysteines contribute to H₂O₂-induced oxidation. Notably, Cys424 is a unique Cys mutant present in PKM2 but not in PKM1. We determined that PKM1 was not oxidised by H₂O₂; instead, it was only partially oxidised by diamide (Fig. 1E). As expected, the Cys residues identified using diamide were also sensitive to H₂O₂. Taken together, we identified Cys423 and Cys424 as novel oxidation-sensitive cysteine residues.

The mutation of Cys31 (PKM2C31A) enhanced the oxidation levels of several Cys residues of PKM2 in the absence of oxidants (Fig. 1C). Moreover, the C31A mutation may result in PKM2 structural alterations that induce the oxidation of several other PKM2 Cys residues. However, we failed to identify such Cys residues through experiments employing simultaneous Cys mutations at points 152, 358, 423, and 424 with C31A (Fig. S3).

Formation of intramolecular polysulphide and disulphide bonds in PKM2
In certain proteins, the formation of an intramolecular disulphide bond enhances protein migration in SDS-PAGE under non-reducing conditions[3,45,46] (Fig. 2A). A previous study indicated that faster-migrating PKM2 bands appearing in response to diadime were completely abolished by the mutation of Cys358 to serine (Ser). We observed that faster-migrating PKM2 appeared in response to 0.4 mM diadime, but not in response to H₂O₂ (Fig. 2B). Treatment with DTT (Fig. 2B) and the substitution of Cys358 to Ala—but not Cys424 to Ala—(Fig. 2C) abolished the formation. These results are in strong agreement with previous reports[39]. Notably, we observed that this faster-migrating band was sensitive to NEM treatment, but resistant to iodoacetamide (IAA) treatment (Fig. 2D). Generally, a disulphide bond is not replaced by NEM. Therefore, the DTT-sensitive and faster-migrating band of PKM2 may be a NEM-sensitive linkage between Cys358 and other Cys. Recent findings have indicated that a polysulphide bond can link two cysteine residues (–Sn–, n≧3) and that the linkage is cleaved by NEM, but not by IAA[47]. Therefore, we investigated the possibility of polysulphide formation using Na₂S₄, a generator of reactive sulphur species (RSS). As shown in Fig. 2D, the faster-migrating band of PKM2 was strongly induced by Na₂S₄ and disappeared with the
addition of DTT (lower panel) or treatment with NEM instead of IAA (+NEM). Moreover, the in vitro oxidation assay of recombinant PKM2 protein (rPKM2) demonstrated that the faster-migrating band was also induced by Na₂S₂ and diamide. It disappeared with the addition of DTT or treatment with NEM (Fig. 2E). Furthermore, the faster-migrating band induced by Na₂S₂ was not detected in the recombinant PKM2C358A mutant (Fig. 2F). These results indicated that Cys358—not Cys424—is essential for the formation of the faster-migrating band. Based on these results, we concluded that the diamide- or Na₂S₂-induced faster-migrating PKM2 could be due to an intramolecular linkage via polysulphide bond, but not a disulphide bond between Cys358 and other Cys within the same PKM2 molecule.

To identify a putative partner Cys for the Cys358-polysulphide linkage, we examined the faster-migrating band formation by using cells expressing each of the Cys mutants of PKM2. As shown in Fig. 2G, levels of the faster-migrating band decreased when Cys152 or Cys317 was mutated. Therefore, an intramolecular polysulphide linkage might be formed between Cys358 and multiple Cys residues (e.g., Cys152 or Cys317). The distance in 3D structure from Cys358 to Cys152 and Cys317 are approximately 36 Å and 11 Å, respectively (PDB ID: 4B2D). While the distance of a disulphide bond is 2.05 Å, a polysulphide formation may be required to form the linkage. Notably, C152A mutation has been shown to decrease PKM2 S-nitrosylation [41].

Additionally, we detected multiple bands with high molecular weight (MW130–180 proteins, as indicated by ‘#’ in Figs. 2B and 2G) that were sensitive to DTT treatment (Fig. 2B) were induced in response to oxidative stress by both diamide and H₂O₂ (Fig. 2B). Since these bands did not appear in oxidised recombinant PKM2 (Fig. S4), this suggests that the multiple bands with high molecular weight might be mixed disulphide bonds formed between PKM2 and unidentified cellular protein(s), and were unlikely to be PKM2 homopolymer. Since PKM2C423A failed to form multiple bands with high molecular weight (Fig. 2G), Cys423 could be responsible for the intermolecular disulphide bond between PKM2 and unknown protein(s).

**Sulphenylation and diamide induced modification of Cys424 and the effect on the tetramer formation of PKM2**

Hydrogen peroxide (H₂O₂) reacts with Cys-thiol (Cys-SH) and forms Cys-sulphenic acid (Cys-SOH) through a process known as protein sulphenylation. The Cys-SOH proceeds to undergo various modifications such as the formation of a disulphide bond or Cys-sulfinic acid (Cys-SOOH) [48,49]. Recent reports have indicated that protein sulphenylation is involved in the redox-dependent regulation of many biological pathways [50,51]. Our results demonstrate that PKM2 Cys424 is oxidised by H₂O₂ (Fig. 1D). Thus, Cys424 is a candidate sulphenylation site of PKM2. We used dimedone—a Cys-SOH reactive compound—to detect PKM2 sulphenylation. Dimedone-conjugated protein was detected by the anti-dimedone (anti-SOH) antibody. Although we failed to identify dimedone-reactive PKM2 in immunoprecipitates of H₂O₂-treated cell lysates (Fig. 3A), dimedone-reactive rPKM2 was found to increase in an H₂O₂ concentration-dependent manner (Fig. 3B). The possible sulphenylation of rPKM2 was specific to Cys424, but not Cys358 (Fig. 3B). These results suggest that Cys424 might be a sulphenylation-sensitive Cys in PKM2.

Since diamide appeared to induce intracellular H₂O₂ levels (Fig. S5), we examined the effect of diamide on PKM2 sulphenylation. We identified the dimedone-reactive PKM2 in lysates prepared from diamide-treated cells (Fig. 3C, Fig. S6). Additionally, we found that diamide was the most effective oxidant among H₂O₂, Na₂S₂, diamide and tBHP to stimulate dimedone-reactive rPKM2 (Fig. 3D). Diamide is a diazenedicarbonyl derivative, widely utilised thiol oxidant and oxidative stress inducer. It reacts directly and electrophilically with Cys thiolate anions to form a sulfenylhydrazine (diamide adduct) and then ultimately induces inter- and/or intramolecular disulphide bonds when a partner thiolate anion is available [52]. Thus, we assume that specific-Cys thiois of PKM2 form diamide adducts or disulphide bonds when cells are treated with diamide. Since diamide induced dimedone-reactive rPKM2 in the absence of H₂O₂ in the in vitro system, we speculated that rather than sulfenic acid, Cys424 formed a diamide adduct that might be displaced by dimedone.
Taken together, our results demonstrate that three cysteine residues of PKM2 (Cys358, Cys423 and Cys424) are responsible for four different oxidative modifications. These might include selenylation and diamide adduct formation on Cys424, intramolecular polysulphide formation on Cys358 and intermolecular disulphide formation on Cys423 in PKM2 under oxidant-dependent oxidative stress (Fig. 3E).

Next, we investigated the effects of Cys424 oxidation on the multimer formation of PKM2. The PK activity of PKM2 is activated by tetramer formation, whereas dimers and monomers of PKM2 are less active[53,54]. We treated cell lysate with glutaraldehyde (GA) to crosslink multimer proteins, which are separated by SDS-PAGE. As previously indicated[55], the tetramer formation of PKM2 was abrogated by S437Y mutation (Fig. 4A). We observed that the level of tetramer in cells (-diamide) was significantly increased in PKM2 with a C424A mutation (PKM2C424A) (Fig. 4A), while the tetramer levels of DTT-treated (reduced) rPKM2s were not strongly affected by these mutations (C358A, C358,424A and C424A) in vitro (Fig. 4B). In addition, tetramer of the PKM2 and the mutants decreased via treatment with diamide (Fig. 4A). Thus, the inhibition of tetramer formation induced by diamide may be due to the oxidation of multiple Cys, including Cys358 and Cys424. In contrast, we did not detect H2O2-induced inhibition of tetramer formation of PKM2C424A (Fig. 4C) and PKM2C358,424A (Fig. S7) in cultured cells. To clarify the effect of selenylation Cys424 on multimer formation, rPKM2s were used for further investigation. The tetramer formation of rPKM2WT markedly decreased after treatment with H2O2, while its formation of rPKM2C424A was constitutive and unaffected by H2O2 treatment (Fig. 4D). These results suggested that PKM2 tetramer formation in cultured cells is strongly inhibited by possible oxidation of Cys424 due to endogenous ROS (H2O2) produced in standard culture condition without imposition of oxidative stress.

Notably, Cys424 is located on the surface of dimer-dimer interaction and considered a crucial residue for tetramer formation[56]. Furthermore, a previous study demonstrated that the mutation of Cys424 to a hydrophobic residue (e.g., leucine, C424L) increases tetramer ratio and PK activity, whereas this mutation decreases tetramer ratio and PK activity in the case of hydrophilic residues (e.g., serine, C424S)[40]. As shown in Figs. S8A and S8B, the tetramer ratio of rPKM2C424D is not affected by its mutation (tetramer ratio: WT = 0.6, C424A = 0.63), while the tetramer ratio of the hydrophilic mutations rPKM2C424D (aspartate, C424D) and rPKM2C424S—which might mimic Cys424 selenylation—were lower than that of rPKM2WT (tetramer ratio: C424D = 0.37, C424S = 0.32, Fig. S8B). Furthermore, the PK activity of these mutants (rPKM2C424D and rPKM2C424S) was lower than that of rPKM2WT (Fig. S8C). Taken together, it is possible that selenylation of Cys424—which increases the hydrophilicity of Cys-SH—disrupts tetramer formation by increasing hydrophilicity on the dimer-dimer interface and contributes to the inhibition of PK activity. Additionally, the residual level of tetrameric PKM2 in cells expressing PKM2C424A and PKM2C358,424A treated with diamide (Fig. 4A) suggested that the oxidation of Cys424 (diamide adduct) might also inhibit tetrameric PKM2 and that multiple Cys including Cys358 and Cys424 might also be responsible for the diamide induced inhibition of tetrameric PKM2.

**Redox regulation of PKM2 contributes to oxidative stress response**

To assess the functional significance of the redox regulation of PKM2, we examined the importance of the redox-sensitive cysteine residues to PK activity and the oxidative stress sensitivity of cells. The aforementioned results indicate that a mutation on neither Cys358 nor Cys424 affects the oxidative stress-induced suppression of PK activity (Fig. S2). Thus, we created a PKM2 mutant with a simultaneous mutation on Cys358 and Cys424 (PKM2C358,424A). FLAG-PKM2WT and its Cys mutants were introduced in H1299 cells, of which endogenous PKM2 was stably knocked down using a lentivirus-based shRNA expression vector (H1299 shPKM2 cells; Figs. S9 and S10). The oxidation of PKM2 treated with H2O2 or diamide was strongly abrogated in cultured cells expressing PKM2C358,424A (Figs. 1C and 1D). Moreover, the induction of the faster-migrating band (the intramolecular polysulphide) by Na2S4 (Fig. S11) and selenylation by H2O2 (Fig. 3B) were not detected in rPKM2C358,424A in vitro. Thus, we investigated the effect of cysteine oxidation on PK activity using this mutant. Basal PK activity of in the cells expressing PKM2C358,424A was same level as that of PKM2WT.
(Fig. S12). Although PK activity in the lysate of H1299 cells expressing PKM2WT significantly increased with DTT treatment, the PK activity of PKM2C358,424A did not increase as much as that of PKM2WT (Fig. 5A). Similarly, the decreased level of PK activity in cells expressing PKM2WT in response to H2O2 and diamide was lower in the cells expressing PKM2C358,424A (Fig. 5A). PK activity in the cells expressing PKM2C358,424A was higher than that of PKM2WT (Fig. S12). Thus, these results indicate that the oxidation of two Cys residues (Cys358 and Cys424) is responsible for the suppression of PKM2 activity.

The inhibition of PKM2 enzymatic activity alters the flow of metabolites into the PPP and contributes to NADPH generation and oxidative stress response[39,57]. Therefore, we investigated the intracellular level of NADPH and the accumulation of intracellular ROS under oxidative stress conditions. Notably, the NADPH ratios (NADPH/(NADPH+NADP)) in cells decreased in response to the diamide treatment. These decreases were observed in cells expressing PKM2C358,424A (Fig. 5B). Additionally, intracellular ROS levels were elevated in response to oxidative stress (H2O2 and diamide). Again, ROS levels in response to H2O2 and diamide were higher in cells expressing PKM2C358,424A than in those expressing PKM2WT (Fig. 5C). Next, to examine the effect of continuous oxidative stress on cell viability, we added GO to the culture medium to generate H2O2 (GO catalyses medium glucose and oxygen to produce gluconate and H2O2[58]). Cells expressing PKM2C358,424A were more sensitive than those expressing PKM2WT (Fig. 5D). These results highlight the possibility that PKM2 oxidation via Cys358 and Cys424 oxidative stress contributes to eliminating excess ROS and oxidative stress.

Some anti-cancer drugs induce intracellular ROS, which gives rise to cytotoxic cancer cells[59,60]. Therefore, the ability to eliminate ROS is an important factor for chemosensitivity. We tested whether the oxidation of PKM2 inhibits the chemosensitivity of CDDP and DOX. As shown in Fig. 5E, the cells expressing PKM2C358,424A were more sensitive than those expressing PKM2WT. These results suggest that the oxidative stress response via the Cys358 and Cys424 oxidation in PKM2 might be involved in the sensitivity of CDDP and DOX. Collectively, our findings indicate a mechanism for oxidative stress resistance via the downregulation of PKM2 by redox-based modification of Cys358 and Cys424 to enhance NADPH levels through the potential activation of PPP under oxidative stress conditions.

Discussion

In the present study, we demonstrated the existence of four different oxidation statuses of PKM2 (Cys424-sulphenylation, Cys424-diamide adduct, Cys423-disulphide and Cys358-polysulphide).

Using NEM-DTT-PEGM assays, we identified Cys423 and Cys424 as oxidation-sensitive cysteine residues by diamide (Figs. 1A, 1B and 1C). Diamide intracellularly induces disulphide bonds in proteins and glutathione [52] as well as protein glutathiolation [61]. It is likely that PKM2 Cys424 forms a diamide adduct but is not glutathionylated because dimedone could not be replaced with a disulphide bond (glutathiolated-Cys-protein) [62]. However, dimedone could react with a putative sulphenyl hydrazine bond between PKM2 Cys424 and diamide to form dimedone-conjugates. The diamide adduct could be reduced with DTT and reacted with PEGM in a NEM-DTT-PEGM assay. Furthermore, we revealed that Cys424 is the sulphenylation site on PKM2 using H2O2 in vitro. This is the first report to identify the oxidative modification of Cys424 induced by H2O2. In addition to the previous findings[63–65], our findings suggest that—as a redox-active cysteine residue—Cys424 might tend to be a thiolate anion. Cys424 is located at the interface of the dimer-dimer interaction of the tetramer PKM2. Thus, sulphenylation and the electrophilic addition of Cys424-SH might induce structural changes on the dimer-dimer interface. Previous studies have revealed that electrophilic compounds such as 4-hydroxy-2-nonenal and 4-oxo-2-nonenal can react with Cys424 and form Michael adducts[63–65].

We also demonstrated that PKM2 Cys423 is involved in intermolecular disulphide bonding with other proteins (Fig. 2G). However, since the PKM2C423A mutant was enzymatically inactive (Fig. S12), we could not pursue further analysis to understand the effect of Cys423 oxidation on PK activity. Since the
levels of Cys423-dependent disulphide complex in diamide-treated cells was low, we speculated that the contribution of this oxidation to PK activity may be minor.

Next, we revealed that Cys358 is essential for the formation of a faster-migrating band under non-reduced SDS-PAGE (Figs. 2C and 2F). The band disappeared following treatment with NEM, an SH-alkylation reagent (Figs. 2D and 2E). An intramolecular disulphide bond—induced by H$_2$O$_2$ in many cases—is not reactive to NEM. Recently, Ida et al. reported protein S-polythiolation, which is more unstable and highly reactive[66]. Moreover, we observed that an intracellular generator of RSS induced a faster-migrating band (Figs. 2D and 2E). These findings suggest that an intramolecular polysulphide linkage between Cys358 and other Cys residues (probably Cys152 and Cys317) enhances the faster-migrating band. Although diamide induced the polysulphide linkage of PKM2, it was unknown whether intracellular RSS was induced by diamide, and its oxidation mechanism remains unclear.

We showed that C358A mutation did not affect tetramer formation in cultured cells (-diamide, Fig. 4A) and reduced rPKM2 in vitro (Fig. 4B). In contrast, Mitchell et al. showed C358S mutation stabilizes the tetramer of PKM2[40]. Interestingly, C358A might result in PKM2 to be prone to oxidation, since the mutation was likely to induce sensitivity of possible Cys424 oxidation by diamide (Fig. 1C and Fig. 3D). Therefore, our results suggested that substitution at Cys358 to Ala but not Ser might induce PKM2 structural alteration that shifts PKM2 towards oxidation sensitivity. Overall, the results of the present study suggest the intramolecular polysulphide linkage on Cys358 and oxidation of Cys424 at the dimer-dimer interface leads to decreased tetrameter formation and the suppression of PK activity. As a result, this modification might be involved in oxidative stress resistance (Fig. 5E).

We found that a slower-migrating PKM2 band (sm-PKM2) above the major PKM2 band (63k) in non-reduced SDS-PAGE, which was sensitive to DTT treatment, appeared when the cells are treated with dimaide and H$_2$O$_2$ (Fig. 2B). Formation of sm-PKM2 was also induced in rPKM2 by dimaide and H$_2$O$_2$ but not by Na$_2$S$_2$O$_5$ (Fig. 2F). Furthermore, sm-PKM2 was resistant to NEM treatment (Fig. 2E). Interestingly, sm-PKM2 formation was inhibited by C31A and C424A mutation (Fig. 2G). These results suggested that the mobility shift might be due to oxidative modification that affect the mobility in SDS-PAGE, e.g., an intramolecular disulphide bond possibly between Cys31/Cys424 and other unidentified cysteine residues.

Diamide is widely used as an oxidative stressor. We demonstrated that intracellular H$_2$O$_2$ increased in response to diamide, which might be due to a decreased intracellular GSH/GSSG ratio. Our results indicate that diamide induced disulphide bond formation, an intramolecular polysulphide bond and putative diamide adduct in PKM2. Thus, the outcome of diamide-induced oxidative modification of proteins and related mechanisms for the stress response should be carefully explained.

The inhibition of PKM2 by oxidants contributes to the metabolic change required to enhance metabolites in PPP[39]. Our findings indicate that cells expressing the oxidation-resistant form of PKM2 (PKM2$^{C358A, C424A}$) increased intracellular ROS and that they were more sensitive to ROS-generating reagents such as GO and ROS-inducible anti-cancer drugs (e.g., cisplatin and doxorubicin) (Figs. 4D–4F). Thus, the inhibition of PKM2 via Cys358 and Cys424 oxidation is important to the oxidative stress response.

In the tumour progression process, cancer cells are exposed to higher levels of oxidative stress[67]. As such, compounds that increase intracellular ROS are considered anti-cancer agents that enhance cell death[68, 69]. Since PK activity may be repressed due to the oxidation of PKM2 in the oxidative tumour environment, cancer cells can eliminate excess ROS. Thus, if we can inhibit the oxidation of PKM2, cancer cells cannot eliminate excess ROS, which may lead to cell death. Anastasiou et al. reported that PKM2 activators successfully repress tumour growth[70]. Thus, our findings suggest that PKM2 activators capable of activating PKM2$^{C342D}$ and PKM2$^{C424D}$, which can mimic Cys424
oxidation-induced repression of PKM2, may represent an ideal option for anti-cancer therapy.
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Data Availability
All supporting data, materials and sequence information are included within the main article and its supplementary files.

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Footnotes
Abbreviation used:

ab antibody
Ala alanine
CDDP cisplatin
Cys cysteine
DOX doxorubicin
DMEM Dulbecco’s Modified Eagle’s Medium
FBP fructose-1,6-bisphosphate
GA glutaraldehyde
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GO glucose oxidase
IAA iodoacetamide
LB Luria-Bertani broth
NEM N-ethyl maleimide
PEGM polyethylene glycol maleimide
PEP phosphoenolpyruvate
PK pyruvate kinase
PKM2 pyruvate kinase M2
| Abbreviation | Description               |
|--------------|---------------------------|
| PPP          | pentose phosphate pathway |
| PTMs         | post-transcriptional modifications |
| PVDF         | poly-1,1-difluoroethane   |
| ROS          | reactive oxygen species   |
| Ser          | serine                    |
| tBHP         | tert-butyl hydroperoxide  |
| WCL          | whole-cell lysate         |
| WT           | wildtype                  |
Figure legends

Figure 1
Identification of oxidation-sensitive cysteine residues of PKM2 via NEM-DTT-PEGM assay. A. Schematic representation of the NEM-DTT-PEGM assay. B. Left panel: molecular weight shift of PKM2 in H1299 cells. Each of the three steps was omitted (lanes 1 to 3) and all steps were performed (lane 4) as indicated. Right panels: molecular weight shift (oxidation) of PKM2 in HEK293T and HepG2 cells in response to the indicated concentration (mM) of diamide, tBHT and H₂O₂ in DMEM for 10 min. C–E. Oxidation of the PKM2 cysteine mutant was detected by NEM-DTT-PEGM assay in 0.4 mM diamide-treated H1299 shPKM2 cells expressing FLAG-PKM2WT (WT) or each of the 11 Cys (C) mutants (indicated as CxxxA, where xxx is the number of cysteine residues mutated to Ala or as FLAG-PKM2CxxxA mutants) in 0–1.0 mM H₂O₂-treated H1299 shPKM2 cells expressing either FLAG-PKM2WT, FLAG-PKM2C424A or FLAG-PKM2C424A (D) in 0–1.0 mM H₂O₂ or 0.4 mM diamide-treated H1299 shPKM2 cells expressing FLAG-PKM2WT and FLAG-PKM1 (E). Cells expressing FLAG-PKM2 with the simultaneous mutation of Cys358 and Cys424 (PKM2C358,424A) were also examined (1C and 1D).

Figure 2
Oxidation of Cys358 is responsible for the possible formation of an intramolecular polysulphide bond. A. The formation of an intramolecular bridge enhances the mobility of PKM2 in SDS-PAGE. B. Detection of DTT-sensitive faster-migrating PKM2 and multiple high molecular weight PKM2 bands. H1299 cells were treated with each oxidant (mM) for 10 min in DMEM. For the detection of an intramolecular bridge in PKM2, non-reduced SDS-PAGE (untreated DTT) and reduced SDS-PAGE (treated with 50 mM DTT) were performed. Multiple high molecular weight PKM2 bands (MW130–180) are indicated as “##”. C. H1299 shPKM2 cells expressing FLAG-PKM2WT and FLAG-PKM2 cysteine mutants were treated with 0.4 mM diamide for 10 min. Non-reduced SDS-PAGE was performed. D. The faster-migrating PKM2 was sensitive to NEM and induced by Na₂S₄. H1299 cells were treated with diamide or Na₂S₄ (mM) for 10 min in DMEM. After removing DMEM, 100 mM IAA or NEM in PBS was added and incubated for 10 min at 37 °C to block free thiols. To detect an intramolecular bridge in PKM2, non-reduced SDS-PAGE (untreated DTT) and reduced SDS-PAGE (treated 50 mM DTT) were performed. E. Recombinant proteins of PKM2 (rPKM2) were used for the detection of the faster-migrating band. rPKM2 were pre-treated with 100 mM DTT to reduce all cysteine residues and DTT was then removed using a NAP5 column. Then, rPKM2 (0.5 µg) were treated with each agent (mM) for 10 min in PBS. Following the treatment of each agent, 10 mM IAA or NEM were added to these reactions for 10 min and mixed with a sample buffer to perform non-reduced SDS-PAGE (-DTT) and reduced SDS-PAGE (+DTT). The full-length western image is shown in Fig. S4. F. rPKM2 WT, C358A and C424A were pre-treated with 100 mM DTT to reducing all cysteine residues and DTT was then removed using a NAP5 column. Thereafter, each rPKM2 (0.5 µg) was treated with Na₂S₄ (mM) for 10 min in PBS. After treatment with Na₂S₄, 10 mM IAA was added to these reactions for 10 min and mixed with a sample buffer to perform non-reduced SDS-PAGE. G. H1299 shPKM2 cells expressing FLAG-PKM2WT or FLAG-PKM2 cysteine mutants were treated with 0.4 mM diamide for 10 min. Non-reduced SDS-PAGE was performed.

Figure 3
Sulfenylation (-SOH) and diamide-induced modification at PKM2 Cys424 inhibit its tetramer formation. A and C. Detection of sulfenylated PKM2 in cultured cells. H1299 shPKM2 expressing FLAG-PKM2WT and FLAG-tagged PKM2 cysteine mutants were treated with 1 mM H₂O₂ (+) or without (-) for 10 min (A) as well as with 0.4 mM diamide (+) or without (-) for 10 min (C). Cells were lysed in lysis buffer containing dimedone. Immunoprecipitation was performed with the anti-FLAG antibody and western blotting was performed with the anti-dimedone antibody to detect dimedone-modified (sulfenylated) PKM2. B and D. Detection of dimedone-reactive rPKM2 in the oxidised condition in vitro. Under the indicated conditions, rPKM2WT and rPKM2 cysteine mutants were treated with 0.4 mM diamide for 10 min. Non-reduced SDS-PAGE was performed.
were treated with the indicated oxidants and dimedone in PBS for 30 min. Western blotting was performed to detect dimedone-modified PKM2. E Schematic illustration of three types of cysteine oxidation in PKM2. An open box indicates PKM2 and X indicates an unknown non-PKM2 protein.

**Figure 4**
Detection of monomers, dimers and tetramers of PKM2 cysteine mutants. A and C. Inhibition of PKM2 tetramer formation in cells treated with 0.4 mM diamide (A) and indicated concentration of H$_2$O$_2$ (C). Average of the tetramer levels (ratio to total) of PKM2 and its cysteine mutants in cultured cells treated with 0.4 mM diamide (closed bars) and untreated (open bars) were indicated (A). Results were normalised to the WT (-). B. Tetramer levels of reduced rPKM2$^{WT}$, rPKM2$^{C358A}$, rPKM2$^{C358,424A}$ and rPKM2$^{C424A}$ in vitro. Samples were treated with GA (GA(+)) or untreated (GA(-)). D. Inhibition of rPKM2 tetramer formation via H$_2$O$_2$ treatment in vitro. The indicated concentration of H$_2$O$_2$ was treated for 120 min before GA treatment.

**Figure 5**
Contribution of PKM2 redox regulation to the oxidative stress response. A. Effect of C358,424A mutation on PK activity in cells under reduction and oxidation conditions. H1299 shPKM2 cells expressing FLAG-PKM2$^{WT}$ (closed bars) or FLAG-PKM2$^{C358,424A}$ mutant (open bars) were treated with the indicated concentrations of H$_2$O$_2$ and diamide for 10 min, respectively. The cells were then lysed in lysis buffer and PK activity was observed. For DTT experiments, lysate prepared from cells without oxidant treatment was treated with the indicated concentrations of DTT 37°C for 10 min before activity was measured. Results were normalised to the untreated group. B. H1299 shPKM2 cells expressing FLAG-PKM2$^{WT}$ or FLAG-PKM2$^{C358,424A}$ mutants were treated with diamide for 30 min. The NADPH ratio was observed as described in the Materials and Methods section. C. H1299 shPKM2 cells expressing FLAG-PKM2$^{WT}$ (closed bars) or FLAG-PKM2$^{C358,424A}$ mutants (open bars) were treated with each oxidant for 30 min. To measure intracellular ROS, we used CellRox Orange Reagent, as described in the Materials and Methods section. The fluorescence intensity was normalised to WT without oxidant treatment. D and E. H1299 shPKM2 cells expressing FLAG-PKM2$^{WT}$ (closed bars) or FLAG-PKM2$^{C358,424A}$ mutant (open bars) were treated with GO or anti-cancer drugs overnight. Cell viability was determined by AlamarBlue assay. Results were normalised to the untreated group. (A–E) The data are presented as the mean +/- the standard error of the mean (N = 3). Statistical comparisons were performed using the Student’s t-test. Differences were considered significant at * p < 0.05 and ** p < 0.01, vs. WT.
Fig. 2

A

B

C

PKM2-Flag | WT | C358A | C424A
---|---|---|---
| diamide | 0 | 0.4 | 0 | 0.4 | 0 | 0.4

D

E

F

G

PKM2-Flag | WT | C31A | C49A | C152A | C165A | C317A
---|---|---|---|---|---|---
| diamide | - | + | - | - | - | +

PKM2-Flag | WT | C326A | C423A | C424A | C474A
---|---|---|---|---|---
| diamide | - | + | - | + | -
