Mitochondrial phosphoglycerate mutase 5 uses alternate catalytic activity as a protein serine/threonine phosphatase to activate ASK1

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Phosphoglycerate mutase (PGAM) is an enzyme of intermediary metabolism that converts 3-phosphoglycerate to 2-phosphoglycerate in glycolysis. Here, we discovered PGAM5 that is anchored in the mitochondrial membrane lacks PGAM activity and instead associates with the MAP kinase kinase kinase ASK1 and acts as a specific protein Ser/Thr phosphatase that activates ASK1 by dephosphorylation of inhibitory sites. Mutation of an active site His-105 in PGAM5 abolished phosphatase activity with ASK1 and phospho-Thr peptides as substrates. The *Drosophila* and *Caenorhabditis elegans* orthologs of PGAM5 also exhibit specific Ser/Thr phosphatase activity and activate the corresponding *Drosophila* and *C. elegans* ASK1 kinases. PGAM5 is unrelated to the other known Ser/Thr phosphatases of the PPP, MPP, and FCP families, and our results suggest that this member of the PGAM family has crossed over from small molecules to protein substrates and been adapted to serve as a specialized activator of ASK1.

MAP kinase | protein phosphatase

Phosphoglycerate mutase (PGAM) is an evolutionarily conserved enzyme of intermediary metabolism that converts 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) in glycolysis and the founder member of the PGAM protein family (1). Members of this family share a common catalytic domain, designated as the PGAM domain, and function as phosphotransferases and/or phosphohydrolases with small molecule substrates. For instance, 1,3- and 1,2-bisphosphoglycerate and fructose-2,6-bisphosphate are the substrates of well-characterized members of this family, the bisphosphoglycerate mutase (BPGM) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB), respectively. The catalytic core of the PGAM domain centers on a histidine residue that acts as a phospho accepter during catalysis. These biochemical and structural features are also shared with the histidine acid phosphatase family of enzymes, and it has recently been proposed that these 2 families can be integrated into a histidine phosphatase superfamily (2). Whereas members of this superfamily predominantly target small molecule substrates, prostatic acid phosphatase has been shown to catalyze hydrolysis of high energy Phospho-Tyr bonds in substrates such as ErbB2 and EGF receptor (3, 4). In addition, Sts-1/TULA-2 in the PGAM family has been shown to function as a protein tyrosine phosphatase, dephosphorylating tyrosine kinases ZAP-70, Syk, and EGF receptor (5–7). These results open the possibility that enzymes in the PGAM family might regulate cellular functions beyond those in glucose metabolism.

PGAM5 is a member of the PGAM family that has been shown to interact with Bcl-X_L, an apoptosis regulator (8), and Keap1, a redox-regulated substrate adaptor for a cullin-dependent ubiquitin ligase complex (9). Although it has been suggested that the latter interaction is involved in the regulation of gene expression by the transcription factor Nrf2 (10), molecular functions of PGAM5 have not been elucidated. In this study, we identified PGAM5 as an interacting protein of apoptosis signal-regulating kinase 1 (ASK1), a mitogen-activated protein kinase (MAPK) kinase kinase that regulates the JNK and p38 MAPK pathways (11). ASK1 is preferentially activated by specific stressors and plays pivotal roles in a wide variety of cellular responses to these stressors (12). Here, we found that PGAM5 acted as a specific protein Ser/Thr phosphatase that activated ASK1 by dephosphorylation of inhibitory sites reacted with synthetic phosphopeptide substrates. This function of PGAM5 was conserved among species including nematode and fruit fly. Our results show that PGAM5 is a protein Ser/Thr phosphatase unrelated to the other known families of Ser/Thr phosphatases.

Results and Discussion

PGAM5 Activates ASK1 by Enhancing Activation Loop Phosphorylation. We expressed Flag-tagged ASK1 (Flag-ASK1) in HEK293 cells and identified proteins coimmunoprecipitated by mass spectrometry proteomic analysis (13). We focused on PGAM5, because it is conserved from *Caenorhabditis elegans* and *Drosophila melanogaster* to mammals (Fig. S1) and the orthologs of mammalian ASK1 function in stress and immune responses in *C. elegans* and *Drosophila* (12). Therefore, we expected that PGAM5 might be an evolutionarily conserved partner of ASK1. Flag-PGAM5 coprecipitated HA-ASK1 from extracts of transiently transfected HEK293 cells (Fig. S2). More important, endogenous PGAM5 was coimmunoprecipitated with endogenous ASK1 from mouse B16 melanoma cells (Fig. 1*A*). PGAM5 is targeted to mitochondria by a transmembrane (TM) domain in the N terminus (10), and endogenous PGAM5 was found exclusively in the 10,000 × *g* pellet (P) fraction including mitochondria but not in the 10,000 × *g* soluble (S) fraction (Fig. 1*A*). ASK1 was in both the P and the S fractions, and only the ASK1 in the P fraction coprecipitated PGAM5.

Overexpression of PGAM5 activated ASK1 and the JNK and p38 kinases. We expressed different levels of HA-ASK1 in HEK293 cells with and without coexpression of a constant amount of PGAM5. The activation state of ASK1 was monitored by immunoblotting using a phospho-ASK1 antibody that recog-

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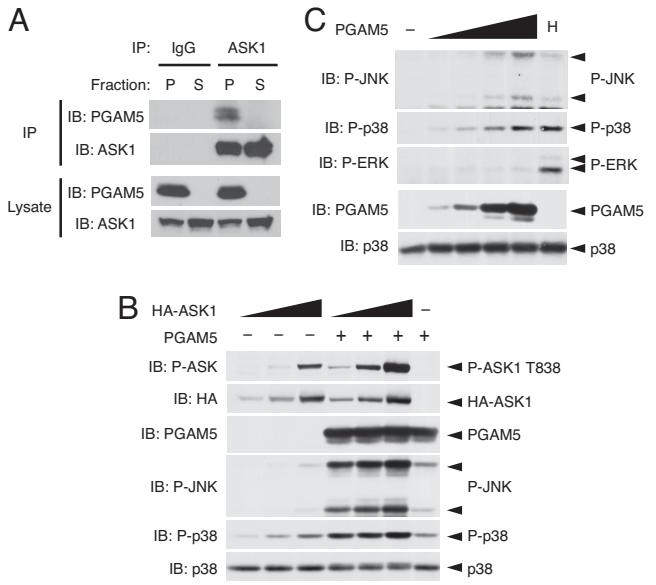

Fig. 1. PGAM5 associates with and activates ASK1. (*A*) Endogenous interaction between PGAM5 and ASK1. ASK1 was immunoprecipitated (IP) from the 10,000× *g* pellet (P) and supernatant (S) fractions of B16 cells, and coimmunoprecipitated PGAM5 was detected by immunoblotting (IB). (*B*) PGAM5 activates ASK1. HA-ASK1 and/or PGAM5 were expressed in HEK293 cells. Cell lysates were subjected to IB. P-ASK antibody recognizes phosphorylation of human ASK1 Thr-838. (*C*) PGAM5 activates JNK and p38 but not ERK. PGAM5 were expressed in HEK293 cells. For a positive control for ERK activation, cells were treated with 1 mM H_2O_2 for 15 min (indicated as H). Cell lysates were subjected to IB.

nizes a specific phosphorylation site within the activation loop of ASK1 (Thr-838, Thr-845, Thr-747, and Thr-815 of human, mouse, *Drosophila*, and *C. elegans* ASK1, respectively) (12, 14). Phosphorylation of Thr-838 in ASK1 was increased by coexpression of PGAM5 at each level of HA-ASK1 expression (Fig. 1*B*). This phosphorylation corresponded to activation of ASK1 by PGAM5, as seen in the robust phosphorylation and activation of endogenous JNK and p38 in cotransfected compared with singly transfected cells (Fig. 1*B*). We found that dose-dependent expression of PGAM5 alone induced activation of endogenous JNK and p38, but not of ERK, in HEK293 cells (Fig. 1*C*).

PGAM5 Is a Protein Ser/Thr Phosphatase That Activates ASK1. We sought to identify enzymatic activity of PGAM5 that would account for its activation of ASK1. We assayed PGAM5 for conversion of 3PG to 2PG but were unable to detect any PGAM activity (Fig. 2*A*). Some members of the PGAM family hydrolyze phospho-Tyr in proteins, so we examined phosphatase activity of PGAM5. Recombinant GST-tagged PGAM5 lacking the TM domain (GST-PGAM5) was purified from bacteria and incubated with various phosphorylated peptides to assay for release of inorganic phosphate. GST-PGAM5 did not dephosphorylate phospho-Tyr peptides, compared with the Tyr-specific phosphatase PTP1B used as a positive control. However, GST-PGAM5 effectively dephosphorylated both phospho-Ser and -Thr peptides (Fig. 2*B*). These results showed that PGAM5 had phospho-Ser/Thr-specific phosphatase activity. This is surprising because the known protein Ser/Thr phosphatases are metalloenzymes (PPP and MPP families) or Asp-based phosphohydrolases (FCP family) that have entirely different primary structure from PGAM (15). Recently, Sts-1, another member of the PGAM family, was shown to hydrolyze phospho-Tyr peptides (5–7). We confirmed that Flag-Sts-1 dephosphorylated phospho-Tyr but not phospho-Thr peptides, whereas Flag-PGAM5 immunoprecipitated from HEK293 cells showed the reverse specificity, reacting with phospho-Thr but not phospho-Tyr peptides (Fig. 2*C*). Phospho-Tyr is a high energy phosphoester (16), making it a more likely substrate for nonspecific phosphohydrolases, including members of the PGAM family. The substrate specificity of PGAM5 suggests it functions as a unique protein Ser/Thr phosphatase.

Among the invariant 4 residues of the catalytic core of the PGAM domain, one of the histidine residues (corresponding to His-105 of human PGAM5) functions as a phospho-acceptor during the course of the reaction (2). Thus, we introduced mutations at His-105 and examined the requirement of this residue for protein Ser/Thr phosphatase activity of PGAM5. GST-PGAM5 with His-105 (WT) exhibited increasing phosphatase activity with 0 to 400 μM phospho-Thr peptide, whereas in contrast GST-PGAM5, in that His-105 was substituted by Ala or Phe, did not exhibit any activity (Fig. 2*D*). Flag-PGAM5-WT immunoprecipitated from HEK293 cells exhibited phosphatase activity toward phospho-Thr peptides, but neither the H105A nor H105F forms of the protein were active (Fig. 2*E*). These results demonstrated that PGAM5 is a unique Ser/Thr-specific phosphatase that requires His-105 in the PGAM domain for activity.

The PGAM5 protein phosphatase activity was required for activation of ASK1. For this assay we used the ASK1-Δcoil mutant that exhibited lower basal activity, due to deletion of the C-terminal coiled-coil domain (17). ASK1-Δcoil became phosphorylated in the activation loop, based on immunoblotting with the phospho-specific antibody, when coexpressed with PGAM5-WT, but not by coexpression with PGAM5 H105A or H105F, indicating that phosphatase activity of PGAM5 was required for ASK1 activation (Fig. 3*A*). PGAM5-induced activation of endogenous JNK and p38 was seen in response to PGAM5-WT but to a much lesser extent to the H105A or H105F mutants (Fig. 3*B*). These results support our proposal that Ser/Thr dephosphorylation by PGAM5 caused activation of ASK1 kinase through Thr-838 phosphorylation, allowing activation of JNK and p38 kinases. Nevertheless, there also remains a possibility that PGAM5 activates JNK and p38 independently of phosphatase activity of PGAM5 by an unknown mechanism, because the H105A and H105F mutants still weakly activated JNK and p38 (Fig. 3*B*).

PGAM5 Dephosphorylates ASK1. We examined whether ASK1 is a substrate of PGAM5 protein phosphatase activity. We expressed deletion mutants of ASK1 with or without PGAM5 in HEK293 cells and detected a PGAM5-dependent mobility shift of proteins containing the ASK1 C-terminal (CT) region (ASK1-CT and -ΔN), but not ASK1 lacking the CT region (ASK1-ΔC) (Fig. 4*A*). This result suggested that ASK1-CT possessed phosphorylation site(s) targeted by PGAM5. Treatment of a cell lysate with λPPase induced a similar increase in mobility of ASK1-CT as seen with coexpression with PGAM5, indicating that the mobility change indeed represented dephosphorylation of ASK1 (Fig. 4*B*). Consistent with this finding, ASK1-CT immunoprecipitated from HEK293 cells showed an increase in mobility when incubated with purified recombinant WT-PGAM5 or λPPase, compared with controls (Fig. 4*C*). These results indicated that PGAM5 directly dephosphorylated the ASK1-CT region. We assayed for changes in ASK1 phosphorylation at the known sites of Ser-83 and Ser-966 (18, 19) using available phosphosite-specific antibodies, but did not observe decreases in phosphorylation in response to overexpression of PGAM5, even though ASK1 was activated (Fig. S3). Phosphorylation of Ser-1033 is an inviting possibility as the target for PGAM5 because this site is in the C term region of ASK1, and phosphorylation inactivates the kinase (20). However, PGAM5-dependent mobility shift of ASK1-CT was not affected by substitution of

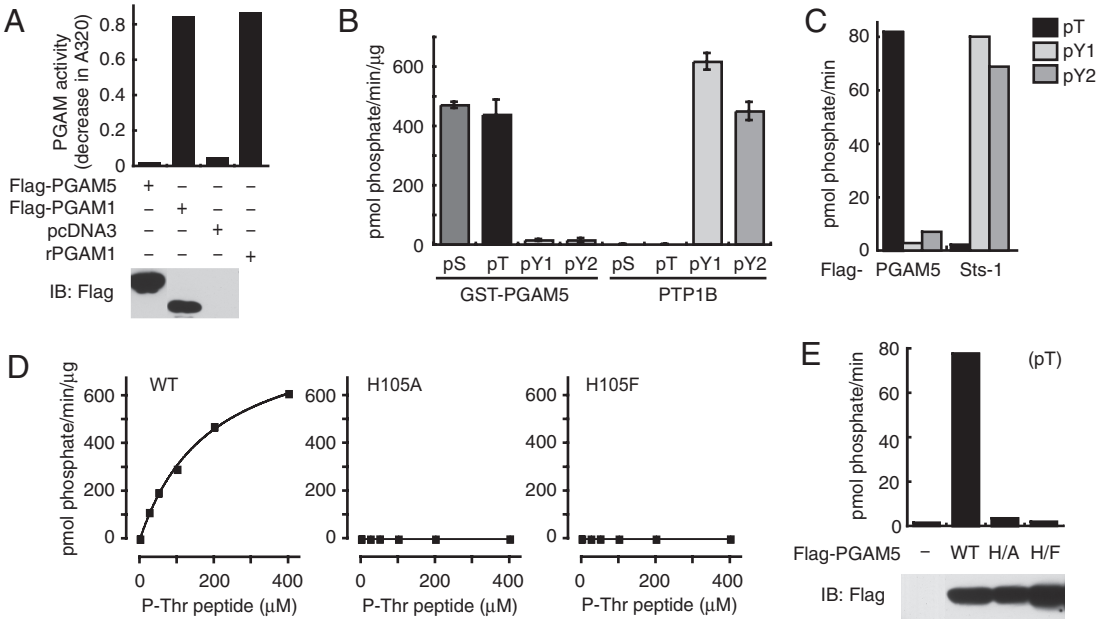

Fig. 2. PGAM5 is a protein Ser/Thr phosphatase that activates ASK1. (*A*) PGAM5 lacks PGAM activity. Flag-PGAM5 (amino acids 29–289) lacking the TM domain, Flag-PGAM1, and pcDNA3 were individually expressed in HEK293 cells and were subjected to immunoprecipitation with anti-Flag antibody, followed by phosphoglycerate mutase assay in vitro. As a positive control, recombinant human PGAM1 (rPGAM1) was used instead of immunoprecipitated proteins. An aliquot of cell lysate was subjected to immunoblotting (IB). (*B*) PGAM5 specifically dephosphorylates phospho-Ser and -Thr peptides. Bacterially generated GST-tagged PGAM5 lacking the TM domain (GST-PGAM5) and PTP1B were subjected to in vitro phosphatase assay using phosphopeptides. Results shown are the means of triplicate determinations ± SD. (*C*) PGAM5 and Sts-1 exhibit strict substrate specificity. Flag-PGAM5 and Flag-Sts-1 were individually expressed in HEK293 cells and were subjected to immunoprecipitation with anti-Flag antibody, followed by in vitro phosphatase assay. (*D*) His-105 is essential for phosphatase activity of PGAM5. GST-PGAM5 with intact His-105 (WT), His105Ala, and His105Phe were subjected to in vitro phosphatase assay using various concentrations of phospho-Thr peptides. Results shown are the means of triplicate determinations. (*E*) PGAM5 immunoprecipitated from HEK293 cells exhibits His-105-dependent phosphatase activity. Flag-PGAM5-WT, -His105Ala (H/A), and -His105Phe (H/F) were individually expressed in HEK293 cells and were subjected to immunoprecipitation with anti-Flag antibody, followed by in vitro phosphatase assay. An aliquot of cell lysate was subjected to IB.

Ser-1033 to Ala (Fig. S4), suggesting that Ser-1033 is neither the major phosphorylation site responsible for PGAM5-dependent dephosphorylation. We could not exclude the formal possibility that PGAM5 reacts with phosphorylation site(s) in other regions of ASK1, whose phosphorylation state was not reflected by a change in electrophoretic mobility. In any case, PGAM5 may activate ASK1 by direct dephosphorylation of phosphorylation site(s) of ASK1 that contributes to the suppression of its kinase activity. This dephosphorylation unleashes phosphorylation of Thr-838 in the kinase domain, with activation of ASK1.

PGAM5 Phosphatases Are Evolutionarily Conserved Activators of ASK1. Conservation in the primary structure of PGAM5 orthologs (Fig. S1) motivated us to examine whether the PGAM5 in *C. elegans* and *Drosophila* (CePGAM5 and DPGAM5, respectively) activate the corresponding ASK1. NSY-1 and DASK1 are the single gene orthologs of mammalian ASK1 in *C. elegans* and *Drosophila*, respectively (12). NSY-1 activity was increased by coexpression with CePGAM5 in HEK293 cells (Fig.

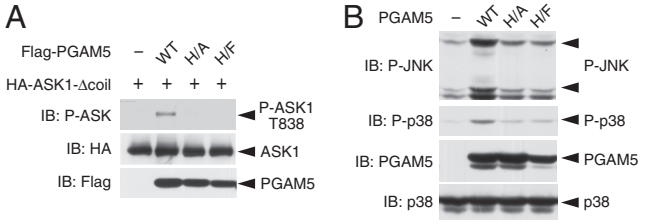

Fig. 3. Phosphatase activity of PGAM5 is required for activation of ASK1. (*A*) Phosphatase activity of PGAM5 is required for ASK1 activation. HA-ASK1-Δcoil was coexpressed with Flag-PGAM5-WT, -H/A, or -H/F in HEK293 cells. Cell lysates were subjected to immunoblotting (IB). P-ASK antibody recognizes phosphorylation of human ASK1 Thr-838. (*B*) Phosphatase activity of PGAM5 is required for activation of JNK and p38. PGAM5-WT, -H/A, and -H/F were individually expressed in HEK293 cells. Cell lysates were subjected to IB.

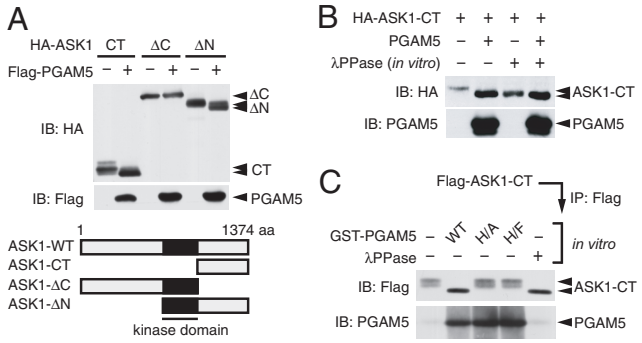

Fig. 4. PGAM5 dephosphorylates ASK1. (*A*) PGAM5 induces a mobility shift of the band of ASK1. Deletion mutants of ASK1 [CT, amino acids (aa) 956–1374; ΔC, amino acid 1–940; ΔN, amino acid 649–1374] with or without Flag-PGAM5 were expressed in HEK293 cells. Cell lysates were subjected to immunoblotting (IB). Schematic illustrations of ASK1 and its deletion mutants are also shown. (*B*) ASK1-CT possesses phosphorylation site(s) targeted by PGAM5. Cell lysates from HEK293 cells expressing HA-ASK1-CT with or without PGAM5 were left untreated or treated with λPPase and subjected to IB. (*C*) PGAM5 dephosphorylates ASK1-CT in vitro. Flag-ASK1-CT was expressed in HEK293 cells and immunoprecipitated with anti-Flag antibody. After incubation with GST-PGAM5-WT, -H/A, -H/F, or λPPase, the immunoprecipitates were subjected to IB.

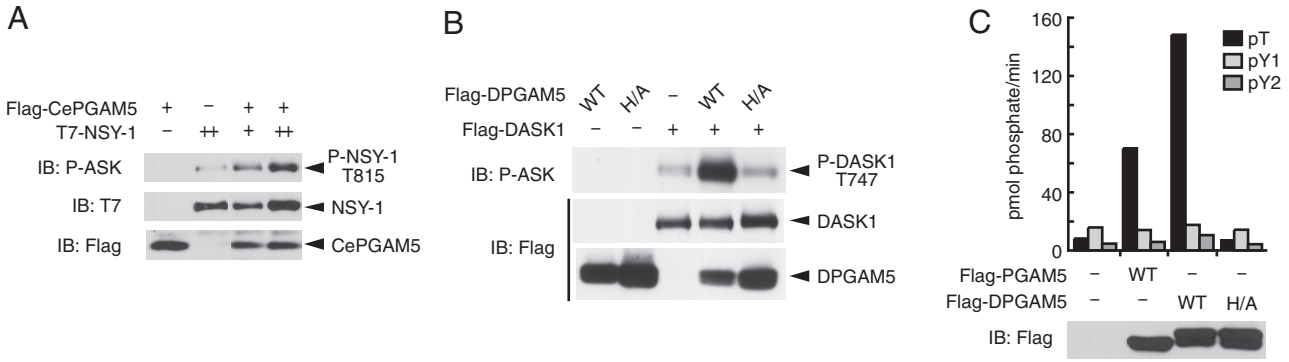

Fig. 5. PGAM5 phosphatases are evolutionarily conserved activators of ASK1. (*A*) CePGAM5 activates NSY-1 in HEK293 cells. Flag-CePGAM5 and/or T7-NSY-1 were expressed in HEK293 cells. Cell lysates were subjected to immunoblotting (IB). P-ASK antibody recognizes phosphorylation of NSY-1 Thr-815. (*B*) DPGAM5 activates DASK1 in a His-94-dependent fashion in S2 cells. Flag-DPGAM5 [WT or His94Ala (H/A)] and/or Flag-DASK1 were expressed in S2 cells. Cell lysates were subjected to IB. P-ASK antibody recognizes phosphorylation of DASK1 Thr-747. (*C*) DPGAM5 specifically dephosphorylates phospho-Thr peptides in a His-94-dependent fashion. Flag-PGAM5-WT and Flag-DPGAM5-WT and -H/A were individually expressed in HEK293 cells and were subjected to immunoprecipitation with anti-Flag antibody, followed by in vitro phosphatase assay. An aliquot of cell lysate was subjected to IB.

5*A*), and DASK1 activity was also increased by coexpression with DPGAM5 in *Drosophila* S2 cells (Fig. 5*B*), demonstrating that the potential of PGAM5 to activate ASK1 is highly conserved among species. Importantly, a mutant of DPGAM5, in which the His-94 that corresponds to the His-105 of human PGAM5 was substituted by Ala, did not induce DASK1 activation (Fig. 5*B*). DPGAM5 exhibited protein Ser/Thr phosphatase activity and specifically dephosphorylated phospho-Thr but not phospho-Tyr peptides in a His-94-dependent fashion (Fig. 5*C*). Activation of ASK1 appeared to employ a conserved mechanism that required phosphatase activity of PGAM5.

In *Drosophila* S2 cells activation of endogenous DASK1 could be readily detected using phospho-ASK1 antibody and highly effective RNAi-based knockdown operates in these cells (21). When S2 cells were treated with either of 2 double-stranded RNAs (dsRNAs) targeting different regions of the *DPGAM5* mRNA, mRNA levels of *DPGAM5* were successfully knocked down >95% compared with cells treated with control dsRNA. (Fig. S5). Levels of the DPGAM5 substrate *DASK1* were not different in either case, however, the activity of endogenous DASK1 was reduced in cells treated with *DPGAM5* dsRNAs (Fig. 6*A*). In mouse neuroblastoma Neuro2A cells, basal activity of ASK1 was reduced upon knockdown of PGAM5 using either of 2 independent siRNAs (Fig. 6*B*). Taken together, the results show that PGAM5 phosphatase is required for activation of endogenous ASK1 kinases in *Drosophila* and in mammalian cells. In human ASK1, phosphorylation of Ser-83, Ser-966, and Ser-1033 have been proposed to restrict kinase activity, but none of these are conserved among ASK1, DASK1, and NSY-1 (18–20). Because these ASK1 orthologs are activated by the corresponding PGAM5, which requires the phosphatase activity, we expect there is a conserved site for PGAM5 dephosphorylation. Thus, identification of such a phosphorylation site is a prime objective toward elucidation of the mechanism of the PGAM5 dephosphorylation-dependent activation of ASK1. Although we propose a role of PGAM5 in controlling basal activity of ASK1 in the present study, it is also important to elucidate whether PGAM5 is involved in stimulus-induced activation of ASK1.

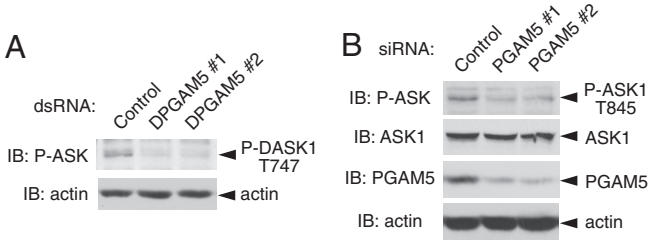

Fig. 6. PGAM5 is required for basal activity of ASK1. (*A*) DPGAM5 is required for basal activity of DASK1. Lysates of S2 cells treated with control double-stranded RNA (dsRNA) or either of 2 dsRNAs targeting different regions of the *DPGAM5* mRNA (#1 and #2) were subjected to immunoblotting (IB). P-ASK antibody recognizes phosphorylation of DASK1 Thr-747. (*B*) PGAM5 is required for basal activity of ASK1. Lysates of Neuro2A cells transfected with control siRNA or either of 2 siRNAs targeting different regions of the *PGAM5* mRNA (#1 and #2) were subjected to IB. P-ASK antibody recognizes phosphorylation of mouse ASK1 Thr-845.

Conclusion and Perspective. In this study, we have shown that PGAM5, a member of the phosphoglycerate mutase gene family, does not possess PGAM activity, but instead alternately functions as a protein phosphatase specific for Ser/Thr residues, which associates with, dephosphorylates, and activates ASK1 kinase. This pairing of a phosphatase with ASK1 kinase is conserved during evolution from *C. elegans* and *Drosophila* to mammals. Members of the PGAM family, including PGAM5, are unrelated in sequence or structure to the known Ser/Thr phosphatases of the PPP, MPP, and FCP families (15). Another member of the PGAM family Sts-1 has recently been shown to catalyze dephosphorylation of phospho-Tyr but not phospho-Ser/Thr substrates (5–7). This could represent a general propensity of enzymes that catalyze phosphoryl transfer reactions (such as PGAM) to exhibit phosphohydrolase activity, however, that would not account for the strict specificity observed with PGAM5. Structural analysis of PGAM5, by comparing with the recently reported crystal structure of Sts-1 (5), might reveal the molecular basis for substrate specificity exhibited by these unusual protein phosphatases. These discoveries of alternate activities will necessitate changing annotations of the PGAM family members in terms of their functions, which will alter integration of networks in systems biology (22).

Materials and Methods

Cell Culture and Transfection. HEK293, B16, and Neuro2A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 4.5 mg/mL glucose, and 100 units/mL penicillin G in a 5% CO_2 atmosphere at 37 °C. S2 cells were cultured in Schneider's *Drosophila* Medium (Invitrogen) containing 10% FBS, 100 units/mL penicillin G, and 5 mg/mL Bacto Pepton (BD Difco) at 26 °C. Transfection of expression plasmids into HEK293 and S2 cells was

performed with FuGENE6 (Roche) and Cellfectin (Invitrogen), respectively, according to the manufacturers' instructions.

Expression Plasmids, Antibodies, and Reagents. See *SI Text*.

Protein Identification. Flag-tagged ASK1 was expressed in HEK293 cells and immunoprecipitated with Flag antibody gel (M2 gel; Sigma). Proteins coimmunoprecipitated with ASK1 were analyzed as described in ref. 13.

Immunoprecipitation. B16 cells were homogenized in the hypotonic buffer (50 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and 1.5 mM $MgCl_2$) and separated into pellet and supernatant fractions by centrifugation at 10,000 $\times$ *g*. The pellet fraction was solubilized with the IP lysis buffer. ASK1 was then immunoprecipitated by adding ASK1 antibody preabsorbed to ExactaCruz matrix (Santa Cruz Biotechnology) to the supernatant and solubilized pellet fractions. The matrix was washed 4 times with the IP lysis buffer and subsequently 2 times with PBS (−) before analysis by immunoblotting.

Immunoblotting. Cell extracts and immunoprecipitates were resolved on SDS/PAGE and electroblotted onto PVDF membranes. After blocking with 5% skim milk in TBS-T (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20), the membranes were probed with antibodies. The antibody-antigen complexes were detected using the ECL system (GE Healthcare).

Phosphoglycerate Mutase Assay. Flag-tagged proteins transiently expressed in HEK293 cells were immunoprecipitated with M2 gel. Immunoprecipitates were incubated in 800-μL reaction mixture containing 100 mM Tris·HCl, pH 8.0, 0.5 mM EDTA, 10 mM $MgCl_2$, 100 mM KCl, 0.2 mM NADH (Calbiochem), 3 mM ADP (Calbiochem), 0.1 mM 2,3-diphosphoglycerate (Sigma), 6 units/mL lactate dehydrogenase (Calbiochem), 5 units/mL pyruvate kinase (Calbiochem), and 1 units/mL enolase (Oriental Yeast) for 10 min at 37 °C and were further incubated for 10 min at 37 °C after the addition of 200 μL 10 mM 3-phosphoglycerate (Sigma). PGAM activity was measured as the oxidation of NADH to NAD by monitoring the decrease in absorbance at 320 nm (A_{320}). Recombinant human phosphoglycerate mutase (Oriental Yeast) was used as a positive control.

In Vitro Phosphatase Assay. Phosphatase activity was measured using the Serine/Threonine and Tyrosine Phosphatase Assay Systems (Promega) that determined the absorbance of a molybdate:malachite:phosphate complex. The following phospho-peptides were used as substrates: Phospho-Tyr, END-pYINASL (pY1) and DADEpYLIPQQG (pY2) (Promega); phospho-Thr, RRApTVA (pT) (Promega); and phospho-Ser, RRApSVA (Upstate Biotechnology).

Phosphatase Treatment. Cell lysate and immunoprecipitates were treated with 2 units/mL lambda protein phosphatase (λPPase; New England Biolabs) in a buffer containing 50 mM Hepes, pH 7.5, 5 mM DTT, 0.1 mM EDTA, 0.01% Brij 35, and 2 mM $MnCl_2$ for 30 min at 30 °C. Reactions were stopped by adding SDS sample buffer.

RNA Interference. See *SI Text*.

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