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Molecules and Cells

Glyceraldehyde-3-Phosphate, a Glycolytic Intermediate, Plays a Key Role in Controlling Cell Fate Via Inhibition of Caspase Activity

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Glyceraldehyde-3-phosphate is a key intermediate in several central metabolic pathways of all organisms. Aldolase and glyceraldehyde-3-phosphate dehydrogenase are involved in the production or elimination of glyceraldehyde-3-phosphate during glycolysis or gluconeogenesis, and are differentially expressed under various physiological conditions, including cancer, hypoxia, and apoptosis. In this study, we examine the effects of glyceraldehyde-3-phosphate on cell survival and apoptosis. Overexpression of aldolase protected cells against apoptosis, and addition of glyceraldehyde-3-phosphate to cells delayed apoptosis. Additionally, delayed apoptotic phenomena were observed when glyceraldehyde-3-phosphate was added to a cell-free system, in which artificial apoptotic process was induced by adding dATP and cytochrome *c*. Surprisingly, glyceraldehyde-3-phosphate directly suppressed caspase-3 activity in a reversible noncompetitive mode, preventing caspase-dependent proteolysis. Based on these results, we suggest that glyceraldehyde-3-phosphate, a key molecule in several central metabolic pathways, functions as a molecule switch between cell survival and apoptosis.

INTRODUCTION

Cellular energy metabolism and apoptosis are two major determinants of survival. Many growth and survival factors stimulate energy metabolism, including glycolysis, and inhibit apoptosis (Garland et al., 1997; Plas et al., 2002; Vander Heiden et al., 2001). Growth factor withdrawal consequently induces a metabolic decline marked by decreased glycolytic rate, lowered oxygen consumption, decreased ATP levels, and reduced protein synthesis. Unless reversed, these metabolic changes ultimately lead to apoptosis. Conversely, recent studies show that an ATP-dependent step is required for apoptotic signal transduction, including apoptosome formation, chromatin condensation, and phosphorylation of pro-apoptotic proteins. The ATP level is regulated by glycolytic flux or the mitochondrial system. Earlier studies by other groups suggest that the intracellular ATP concentration is responsible for the switch between apoptosis and survival (Jiang et al., 2000; Kass et al., 1996; Leist et al., 1997; Priault et al., 1999). However, the mechanism underlying the involvement of glucose metabolism, the main source of ATP, in the apoptotic pathway remains to be established.

Glyceraldehyde-3-phosphate (G-3-P), also known as triose phosphate or 3-phosphoglyceraldehyde, is a key intermediate in several central metabolic pathways, including glycolysis and gluconeogenesis. G-3-P is formed from the splitting of fructose 1,6-bisphosphate (FBP) by aldolase, isomerization of dihydroxyacetone phosphate (DHAP) by triose phosphate isomerase, and reversible reaction catalyzed by glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Aldolases are ubiquitous enzymes that have attracted considerable interest due to their ability to catalyze carbon-carbon bond formation. Aldolase A (ALDOA) is found in skeletal muscle and red blood cells, and functions in glycolysis, promoting the reversible cleavage of FBP to triose phosphate, G-3-P and DHAP. ALDOA is upregulated in various cancers, including human lung squamous and renal cell carcinomas (Li et al., 2006; Unwin et al., 2003). GAPDH, another key glycolytic enzyme, is involved in apoptotic processes, and may participate in neuronal death in certain neurodegenerative diseases. GAPDH is overexpressed in the nucleus during apoptosis in diverse cell types (Chuang et al., 2005; Ishitani et al., 1996). Based on these findings, we suggest that the G-3-P concentration alters continuously during the course of various cellular processes, including hypoxia, apoptosis, and cancer.

In this study, we demonstrate that G-3-P, an enzymatic product or substrate of ALDOA and GAPDH, directly lowers caspase activity, hindering the apoptotic process. Based on these findings, we suggest that the G-3-P level is a key link between apoptosis and glucose metabolism.

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MATERIALS AND METHODS

Cells and transfection

The human leukemia cell line, HL-60, was cultured in RPMI 1640 containing 10% FBS. The human embryonic kidney cell line, BOSC 23, was cultured in DMEM containing 10% FBS. BOSC 23 cells were transfected with Lipofectamine reagent, according to manufacturer's instructions (Invitrogen).

Reagents and antibodies

Ac-DEVD-*fmk* was obtained from Enzyme System Products, Ac-DEVD-*p*NA from Anaspec, etoposide, G-3-P, DHAP, and FBP from Sigma, and ECL solution from Pierce or Millipore. The following antibodies were used: anti-caspase-3 (Calbiochem), anti-PARP (Sigma), anti-FLAG (Sigma), HRP-conjugated anti-rabbit IgG (Sigma), HRP-conjugated anti-mouse IgG (Sigma), and HRP-conjugated anti-goat IgG (Santa Cruz Biotechnology).

Apoptotic stimuli

Apoptosis was induced in HL-60 and BOSC 23 cells by treatment with 100 μM etoposide, as previously described (Jang et al., 2008; 2009). Where necessary, cells were pretreated for 1 h with 100 μM z-DEVD-*fmk*, a pan caspase inhibitor.

Western blotting

Cells were harvested and lysed in NP-40 lysis buffer (137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, and 1% NP-40). Cell extracts (10-40 μg) were separated on SDS gels, and transferred to PVDF membranes. After blocking in TBST buffer (20 mM Tris-HCl, pH 7.6, 0.1369 M NaCl, and 0.1% Triton X-100) containing 5% skim milk at room temperature for 1 h, membranes were probed with the appropriate antibodies. The membrane was incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h, and washed in TBST. Detection was performed with an enhanced chemiluminescence (ECL) system (Pierce, USA).

Cell death analysis

Cell viability was determined with the MTS assay using the CellTiter 96R Aqueous Non-radioactive Cell Proliferation Assay kit, as described by the manufacturer (Promega). The MTS/PMS assay was successfully employed to detect viable cells using cellular capacity to convert MTS tetrazolium salt to formazan. For visualization of chromatin fragmentation, cells were seeded onto glass slides. After treatment with test compounds, cells were fixed with ethanol and stained with the nuclear dye, Hoechst 33258 (5 μmol/ml). The percentage of apoptotic cells was determined by counting nuclei with condensed and fragmented chromatin under an immunofluorescence microscope. Cells were extensively washed in PBS. Slides were mounted with mounting solution containing 10% glycerol. Stained cells were observed under a laser confocal fluorescence microscope (Carl Zeiss). Three different experiments were performed, and at least 300 cells were analyzed per condition.

Measurement of the intracellular G-3-P concentration

G-3-P levels were calculated from a calibration curve of GAPDH activity (Shimizu et al., 1988). The reaction mixture (total of 1 ml) consisting of 0.18 M hydrazine, 0.45 M glycine buffer (pH 9.5), 2.5 mM NAD^+, 60 μg of GAPDH (Lab frontier, Korea) and 0.2 ml of cell extracts was incubated at 25°C for 2 min, and the A_{340} value was measured.

Cell-free apoptosis assay

Cell extracts were generated from HL-60 cells as described previously with minor modifications (Jang et al., 2007). Cells ($2\text{-}5 \times 10^8$) were pelleted, and washed twice with PBS and once with ice-cold cell extract buffer (CEB; 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 100 μM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Next, cells were transferred to a homogenizer, and two volumes of ice-cold CEB added to the packed cell pellet. Following incubation under hypotonic conditions for 15 min on ice, cells were disrupted with a homogenizer. Cell extracts were centrifuged at 15,000 × *g* for 15 min at 4°C. Aliquots of the supernatants fractions were frozen at -70°C until required. Caspase activation in cell extracts (10-15 mg/ml) was induced by incubation at 37°C for various times with 2 mM dATP/$MgCl_2$ plus 50 μg/ml bovine heart cytochrome *c*.

Kinetic analysis

Human caspase-3 was prepared as described previously (Lee et al., 2004; Na et al., 2007). Caspase-3 activity was determined using a chromogenic caspase-3 substrate, Ac-DEVD-*p*NA, following previous protocols (Fang et al., 2006; Shim et al., 2007). Caspase-3 was activated by incubation in assay buffer (50 mM HEPES pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT) at room temperature for 20 min before use. Ac-DEVD-*p*NA (0.7, 1.4, 2.8, and 5.6 mM; 25 μl) in assay buffer was added to caspase-3 (30 nM of the final concentration) pretreated with G-3-P (0, 2, 4, 6, and 8 mM of the final concentration), DHAP (20 mM of the final concentration), or FBP (200 mM of the final concentration) in assay buffer (475 μl) at room temperature for 20 min in a cuvette. Formation of *p*NA was measured at room temperature by monitoring the absorbance change at 410 nm on a DU 800 spectrophotometer (Beckman Coulter) for 5 min. K_m and V_{max} values were determined from a Lineweaver-Burk Plot. The inhibition constant (K_i) of G-3-P was established by nonlinear regression analysis using Equation (I), where V_{maxi} represents V_{max} in the presence of inhibitor (Segal, 1975).

$$V_{max}/V_{maxi} = [I]/K_i + 1 \qquad (I)$$

Graphs were generated and nonlinear regression analyses performed using KaleidaGraph 4.0™ from Synergy Software.

Statistical analysis

Data were presented as means ± S.E. (standard error) of three independent experiments performed in triplicate. Data were analyzed for statistical significance using Student's *t*-test. The minimum level of significance was set at $p < 0.05$.

RESULTS

ALDOA inhibits cell death during etoposide-induced apoptosis

To determine the effect of ALDOA on apoptosis, two constructs encoding wild-type and D33A (catalytically inactive mutant) ALDOA were generated, followed by transfection into BOSC 23 cells. To quantify the degree of cell death, the rate of apoptosis after etoposide treatment was analyzed using MTS analysis and Hoechst staining. As shown in Fig. 1, cells transfected with the wild-type ALDOA construct were more resistant to etoposide-induced apoptosis than the mock control. Approximately 84.5% of the mock control cells died, whereas only 42.5% of the wild-type-transfected cells underwent apoptosis (Fig. 1). On the other hand, D33A ALDOA-transfected cells displayed no significant differences in terms of apoptosis (76.4%), compared to mock-transfected cells (Fig. 1). To further confirm the activity

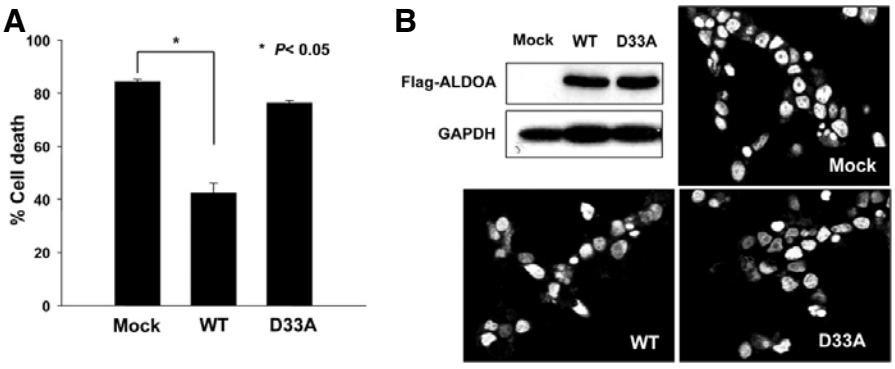

Fig. 1. Anti-apoptotic effect of ALDOA. (A) BOSC 23 cells were transiently transfected with Flag-CMV, wild-type, or D33A ALDOA-containing vectors, respectively. After treatment with etoposide for 24 h, cells were scored using the MTS assay. Data are presented as means ± S.D. from at least three independent determinations. (B) Percentage of condensed nuclei in BOSC 23 cells stained with Hoechst 33258. The image is a representation of three independent experiments.

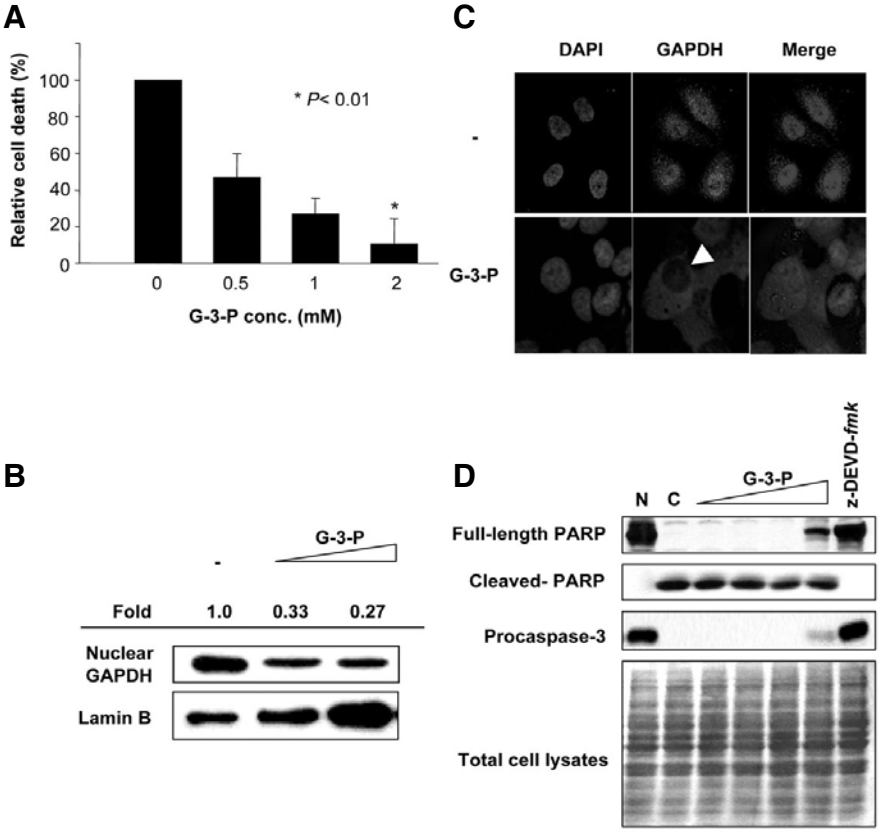

Fig. 2. Inhibitory effects of G-3-P on apoptotic cell death. (A) G-3-P protects cells from apoptosis in a dose-dependent manner. After pretreat-ment with G-3-P at the indicated con-centrations, cell viability against apoptosis induced by etoposide (100 μM, 24 h) was scored using the MTS assay. Data are presented as means ± S.D. from at least three independent determinations. (B) Decrease in the nuclear GAPDH level by G-3-P. The added G-3-P concentrations were 0.5 and 1 mM. The nuclear GAPDH level was monitored by Western blot analysis with anti-GAPDH antibody after subcellular fractionation. Lamin B served as a nuclear-specific marker. (C) Decrease in the nuclear GAPDH level by G-3-P (1 mM) was assessed by confocal microscopic analysis with anti-GAPDH antibody. (D) Effect of G-3-P on PARP cleavage and caspase-3 activation during apoptosis. To induce artificial apoptosis, HL-60 cell lysates were incubated with cytochrome *c*/dATP without (C) or with G-3-P (0.25, 0.5, 1 and 2 mM). Processing of caspase-3 and cleavage of PARP in celllys ates were assessed by immunoblot analysis. As a negative control, the caspase inhibitor, z-DEVD-*fmk*, was included in the reaction mixture.

of ALDOA, we generated bacterially expressed and purified recombinant proteins. Recombinant wild-type ALDOA displayed 8.5 U/mg specific activity, while the D33A mutant protein exhibited only marginal activity (data not shown). Our results imply that aldolase is involved in regulating apoptotic cell death.

G-3-P induces delayed cell death

We examined the effects of G-3-P, an enzymatic product of aldolase, on cell death. Changes in the intracellular concentration of G-3-P induced by exogenous addition of the compound to cell culture media were measured (Shimizu et al., 1988). The cellular G-3-P level was gradually increased upon the addition of increasing concentrations of G-3-P between 0 and 2 mM (data not shown). Next, we measured the rate of apoptosis in cells pretreated with G-3-P followed by 100 μM etoposide. As shown in Fig. 2A, cell death was inhibited in a dose-dependent manner upon pre-treatment with G-3-P. DHAP, another aldolase enzymatic product, also suppressed apoptosis (data not shown). It is speculated that that DHAP is rapidly converted to G-3-P by triose phosphate isomerase in the cell. In addition, GAPDH, another glycolytic enzyme that affects the G-3-P level, is overexpressed and translocates to the nucleus during apoptosis in diverse cell types (Chuang et al., 2005; Sen et al., 2008). To further examine this theory, we investigated the effects of G-3-P on GAPDH translocation during apoptosis. Intracellular localization of GAPDH was monitored by western blot analysis after subcellular fractionation (Fig. 2B) and confocal microscopy using an anti-GAPDH antibody (Fig. 2C) following treatment with G-3-P. Translocation of GAPDH into the nucleus was severely decreased in the presence of exogenous G-3-P (Figs. 2B and 2C).

The addition of cytochrome *c* and dATP to cell-free extracts triggers a cascade of caspase activation events involving all cell

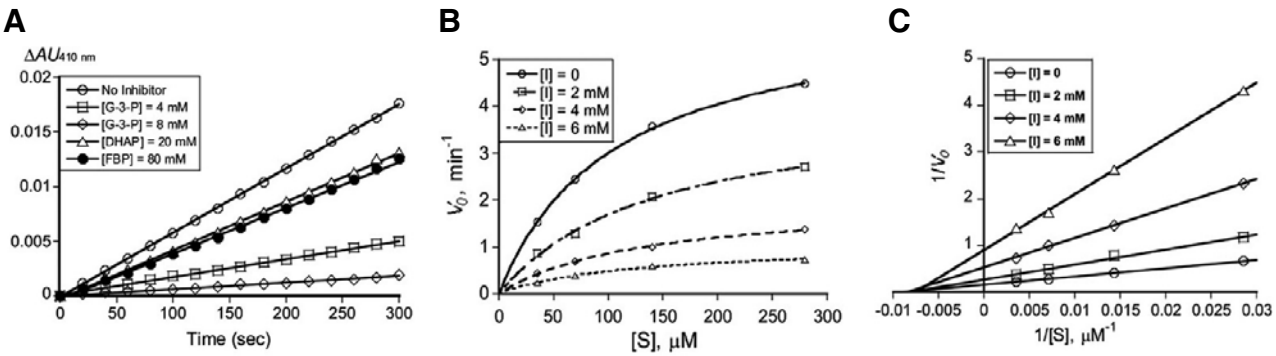

Fig. 3. Inhibition of caspase-3 activity by G-3-P. (A) Progress curves of Ac-DEVD-*p*NA hydrolysis by caspase-3 in the presence of 0, 4, 8 mM G-3-P, 20 mM DHAP, and 80 mM FBP. FBP and DHAP induce very weak inhibition of caspase-3 activity, even at significantly higher concentrations than G-3-P. (B) Kinetic analyses of caspase-3 inhibition by G-3-P using Michaelis-Menten and (C) Lineweaver-Burk plots.

death-associated caspases (Adrain et al., 2004; Jang et al., 2007). Using this system, we examined the effects of G-3-P on caspase-3 activation and PARP cleavage. As shown in Fig. 2D, the addition of cytochrome *c*/dATP to HL60 cell extracts induced cleavage of PARP and caspase-3 activation. The addition of G-3-P to HL-60 cell extracts prevented PARP cleavage (Fig. 2D), and slightly inhibited caspase-3 activation. These results strongly imply that G-3-P influences the pro-apoptotic pathway, thereby blocking cleavage of substrates by caspase-3.

G-3-P directly inhibits caspase-3 activity

Next, we examined whether G-3-P directly affects caspase activity. Surprisingly, caspase-3 activity was severely decreased at G-3-P concentration of 4 and 8 mM (Fig. 3A). In addition, FBP (final concentration, 80 mM) and DHAP (final concentration, 20 mM) induced a slight reduction in caspase-3 activity, but at significantly higher concentrations, compared to G-3-P. Inhibition of caspase-3 by G-3-P was further analyzed with the Michaelis-Menten (Fig. 3B) and Lineweaver-Burk plots (Fig. 3C). The maximum velocity (V_{max}) of enzymatic activity was decreased (Fig. 3B), but the K_M value for the substrate (*x*-intercepts in Fig. 3C) was not affected by G-3-P. Our data suggest that G-3-P is a noncompetitive inhibitor of caspase-3, supporting the possibility that G-3-P binds at a position other than the active site of the enzyme. The inhibition constant (K_i) was determined as 1.1 ± 0.12 mM by nonlinear regression analysis. Base on these findings, we propose that G-3-P is intimately involved in the regulation of apoptosis via direct inhibition of caspase activity.

DISCUSSION

Glycolytic enzymes, which convert glucose to pyruvic acid, constitute the most ancient molecular metabolic network. Early investigations focus on the glycolytic functions of these enzymes. Recent studies provide evidence that specific glycolytic enzymes are more complicated multi-functional proteins, rather than simple components of the glycolytic pathway (Chuang et al., 2005; Sen et al., 2008). Several isoforms of hexokinase and GAPDH are involved in apoptosis (Chuang et al., 2005; Robey and Hay, 2006). Based on studies showing that glucose metabolism is implicated in cell death and survival, it is reasonable to speculate that apoptosis and glycolysis are linked. However, the detailed mechanisms underlying the regulation of cellular energetics during apoptosis remain to be elucidated (Majors et al., 2007).

Aldolase is a ubiquitous and constitutively expressed housekeeping enzyme that drives the glycolytic metabolic pathway in mammalian cells. While aldolase is overexpressed in several cancers, including lung, primary colorectal and cervical carcinomas (Altenberg and Greulich, 2004; Kilic et al., 2007; Lu et al., 2002), its role in the apoptotic pathway remains to be established. In this study, we show that aldolase prevents etoposide-induced apoptosis. Specifically, catalytically inactive mutant D33A aldolase exerted no significant inhibitory effects on cell death, while the wild-type protein clearly suppressed apoptosis. In contrast to aldolase, overexpression of GAPDH induced pro-apoptotic process (Chuang et al., 2005). Earlier studies show that GAPDH is highly expressed at the early phase of apoptosis (Sen et al., 2008). On the basis of these findings in conjunction with earlier reports, we speculate that the G-3-P level decreases during apoptosis, which, in turn promotes the pro-apoptotic process.

Surprisingly, G-3-P inhibited apoptosis in a dose-dependent manner (Fig. 2A), although the mechanism by which the compound penetrates the cell interior is unclear. However, G-3-P levels were increased upon exogenous addition of the compound. In addition, PARP, a well-known substrate of caspase-3, was poorly digested when G-3-P was added (Fig. 2D). An *in vitro* assay revealed that caspase-3 activity was severely reduced in the presence of G-3-P (Fig. 3A). XIAP also has the ability to directly inhibit caspase activity, preventing inappropriate caspase activation. However, this finding is controversial, since XIAP-KO mice do not display a significant phenotype (Harlin et al., 2001). The presence of a conserved back-up system is therefore feasible. Based on our data, we conclude that caspase inhibition by G-3-P is another defense mechanism against inappropriate caspase activation. However, details of the mechanism of G-3-P- induced inhibition of caspase function remain to be established.

Apoptosis requires energy, since it is a highly regulated process involving a number of ATP-dependent steps, including caspase activation, enzymatic hydrolysis of macromolecules, bleb formation, and apoptotic body formation (Bao and Shi, 2007; Kumar, 2007). Therefore, it is reasonable to assume the existence of a cell fate switch molecule that senses the intracellular energy level. Apoptosis occurs when energy levels fall below the threshold level. However, more depletion of energy triggers changes in the mode of cell death from apoptosis to necrosis (Leist et al., 1997). We propose that G-3-P is a key indicator of the cell energy level status. In case G-3-P drops below a certain level as a result of exposure to specific stimuli, the apoptosis system is turned on. On the other hand, in the resting state, G-3-P protects cells by inhibiting inadvertently activated caspase-3. The G-3-P concentration may be uni-

formly maintained by triose phosphate isomerase. The equilibrium constant (K_{eq}) for the triose phosphate isomerase reaction favors DHAP, but removal of G-3-P by a subsequent spontaneous reaction allows throughput. Accordingly, we suggest that DHAP buffers the G-3-P level in cells. The precise concentration of G-3-P within the cell has not been determined to data. However, the G-3-P concentration in *E. coli* and yeast is estimated as between the submillimolar and millimolar range (Hoque et al., 2005; Schaefer et al., 1999; Vazquez et al., 2008).

In view of these results, we strongly suggest that G-3-P, a key molecule of several central metabolic pathways, plays a crucial role as a cell fate decision factor. To our knowledge, this is the first report showing that activity of an effector caspase (caspase-3) is directly inhibited by metabolic intermediate.

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