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Silencing of the human microsomal glucose-6-phosphate translocase induces glioma cell death: Potential new anticancer target for curcumin

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Abstract **G6P translocase (G6PT) is thought to play a crucial role in transducing intracellular signaling events in brain tumor-derived cancer cells. In this report, we investigated the contribution of G6PT to the control of U-87 brain tumor-derived glioma cell survival using small interfering RNA (siRNA)-mediated suppression of G6PT. Three siRNA constructs were generated and found to suppress up to 91% G6PT gene expression. Flow cytometry analysis of propidium iodide/Annexin-V-stained cells indicated that silencing the G6PT gene induced necrosis and late apoptosis. The anticancer agent curcumin, also inhibited G6PT gene expression by more than 90% and triggered U-87 glioma cells death. Overexpression of recombinant G6PT rescued the cells from curcumin-induced cell death. Targeting G6PT expression may provide a new mechanistic rationale for the action of chemopreventive drugs and lead to the development of new anti-cancer strategies.**

Keywords: Glioma; Glucose-6-phosphate translocase; Curcumin; Cell death

1. Introduction

The incidence of malignant brain tumors is increasing in both children and adults, and this type of cancer is often unmanageable due to its diffuse infiltrating nature [1,2]. Although the prognosis is very grim, the standard therapies for malignant gliomas, i.e., surgical resection and radiation only retard glioma growth for a short period and, paradoxically, can facilitate recurrence in the long run [3]. Hence, new approaches are needed to target the very infiltrating nature of this cancer and prevent recurrence. Lately, many dietary polyphenols have been shown to have anti-cancer properties due to their chemopreventive and anti-tumor activities [4,5]. Among these, we have recently demonstrated that both the green tea polyphenol epigallocatechin-3-gallate (EGCg) [6,7], as well as chlorogenic acid (CHA) [8], efficiently inhibited several glioblastoma cell invasive processes. Interestingly, glucose-lowering properties have also been attributed to EGCg and CHA, which also make them valuable anti-diabetic agents [9,10]. Both the modulation of blood glucose levels and the chemopreventive properties of EGCg and CHA could be potentially linked to a common intracellular target, the microsomal glucose-6-phosphatase (G6Pase) system [11,12].

The G6Pase system catalyses the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate as a final step in both glucose-producing pathways in the liver: gluconeogenesis and glycogenolysis [13]. G6Pase is a multicomponent endoplasmic reticulum (ER) enzyme which rate-limiting step in G6P hydrolysis is thought to be catalyzed by a G6P translocase (G6PT). Whereas only a low number of tissues do express the G6Pase catalytic subunit and are gluconeogenic, G6PT's ubiquitous expression and functionality in non-gluconeogenic tissues such as brain remains poorly characterized [14]. Recent evidence, however, suggest that CHA, the most potent functional inhibitor of G6PT, triggers a host of cellular events including apoptosis in neutrophils and differentiated promyelocytic HL-60 cells [15], and inhibition of matrix metalloproteinase (MMP) secretion in the human Hep3B hepatocellular carcinoma cell line [16]. CHA also inhibits glioma cell migration, response to chemotactic growth factors, and secretion of MMP [8], all prerequisite processes needed for tumor growth. Whether G6PT is involved in the survival of brain tumor-derived cancer cells is currently unknown.

Aside from regulating the rate limiting step of G6P transport through the ER membrane, alternate G6PT roles include adenosine triphosphate (ATP)-mediated calcium sequestration in the ER lumen [17], and function as a G6P receptor/sensor [18]. Such underestimated G6PT-mediated ER functions may collectively be responsible for crucial survival processes such as cell proliferation, cell cycle division, extracellular matrix (ECM) degradation, and response to growth factors during brain tumor development [19]. Moreover, enhanced glucose utilization in vitro, as well as in vivo, is correlated with the degree of malignancy, but also with poor prognosis for patients with glioma tumors [20,21]. Selective interference with G6PT functions may thus be an attractive therapeutic approach to metabolic control of glioma cell growth. Interestingly, glioma cell proliferation and survival have recently been shown to

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Abbreviations: ATP, adenosine triphosphate; CHA, chlorogenic acid; 2-DG, 2-deoxy-D-glucose; ECM, extracellular matrix; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; G6PT, G6P translocase; GSD, glycogen storage disease; MMP, matrix metalloproteinase; PI, propidium iodide; siRNA, small interfering ribonucleic acid

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be affected by curcumin (diferuloyl-methane), the yellow pigment found in the spice turmeric [22,23]. Because curcumin regulates key enzymes involved in carbohydrate metabolism [24,25] and shows chemopreventive properties [26,27], we investigated the effects of curcumin on G6PT gene expression and U-87 glioma cells survival.

2. Materials and methods

2.1. Cell culture and transfection method

The U-87 glioma cell line was purchased from American Type Culture Collection and cultured in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C under a humidified atmosphere containing 5% CO_2. The rabbit polyclonal antiserum against human G6PT (p46) was a kind gift from Dr. Gerald van de Werve (Centre de Recherche du CHUM, University of Montreal, Que.) [28]. The G6PT plasmid was generously provided by Dr. Christopher Newgard (University of Texas Southwestern Medical Center, Dallas, TX) and recombinant protein expression validated [28]. U-87 glioma cells were transiently transfected with the cDNA construct or with 20 nM small interfering ribonucleic acid (siRNA) (see below) using Lipofectamine 2000 (Invitrogen, Burlington, Ont.). The occurrence of G6PT specific gene knockdown as well as G6PT overexpression was also evaluated by semi-quantitative RT-PCR. All experiments involving these cells were performed 36 h following transfection. Mock transfections of U-87 cultures with pcDNA (3.1+) expression vector alone were used as controls.

2.2. RNA interference

RNA interference experiments were performed using Lipofectamine 2000. Three siRNA oligonucleotides for human G6PT (gene ID: NM_001467) and mismatch siRNA were synthesized by EZBiolab Inc. (Westfield, IN), and annealed to form duplexes. The sequences of the three siRNA used in this study are as follows: siG6PT #1: 5′-GCACUACAGUUGGAGCACAdTdT-3′ (sense) and 5′-UGUGCUCCAACUGUAGUGCdTdT-3′ (antisense), siG6PT #2: 5′-CUGUGAUCUUCUCAGCCAUdTdT-3′ (sense) and 5′-AUGGCUGAGAAGAUCACAGdTdT-3′ (antisense); siG6PT #3: 5′-CGAAACAUCCGCACCAAGAdTdT-3′ (sense) and 5′-UCUUGGUGCGGAUGUUUCGdTdT-3′ (antisense).

2.3. Semi-quantitative and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cultured monolayers of U-87 cells using TRIzol reagent (Life Technologies, Gaithersburg, MD). One microgram of total RNA was used for first strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR kit (Invitrogen) for semi-quantitative PCR products abundance analysis. Primers for G6Pase-α (forward: 5′-TTCAGCCACATCCACAGCATC-3′, reverse: 5′-GGGGTTTCAAGGAGTCAAAGACG-3′), for G6Pase-β (forward: 5′-ACTCTTCCTGACTTCTTGTGTGCC-3′, reverse: 5′-TTGCCTTTGCTCTTTGGGGG-3′) and for G6PT (forward: 5′-CAGGGCTATGGCTATTATCGCAC-3′, reverse: 5′-ATGGCTCAAACCACTTCCGCAG-3′) were all derived from human sequences. β-actin cDNA amplification was used as an internal house-keeping gene control. PCR conditions were optimized so that the gene products were examined at the exponential phase of their amplification [8] and the products were resolved on 1.8% agarose gels containing 1 μg/ml ethidium bromide. For quantitative RT-PCR, cDNA synthesis was performed by using 2 μg of total RNA, random hexamers and MULV reverse transcriptase reagents (ROCHE) as instructed by the manufacturer. Real-time PCR was performed with the SybrGreen Universal Master Mix (Invitrogen) according to the manufacturer's protocol, in which 50 ng of cDNA was amplified for G6PT gene and 5 ng amplified for 18S ribosomal RNA using specific primers at a final concentration of 200 nM in 2× SybrGreen Master Mix in a total volume of 50 μl. The thermocycler parameters for the real-time PCR consisted of two initial steps (50 °C for 2 min, followed by 95 °C for 10 min), 40 cycles of DNA amplification (95 °C for 15 s, 58 °C for 15 s, 72 °C for 20 s). At the end of the PCR a melting curve (disassociation curve) was run to ensure that only a single specific product was amplified. Relative transcript quantities were calculated as ΔCT values, as recommended by the manufacturer with 18S ribosomal RNA as the endogenous reference amplified from the samples.

2.4. Analysis of cell death by flow cytometry

Cell death was assessed by flow cytometry in cells treated with curcumin (Sigma, Oakville, ON), as well as in untransfected (mock) cells or cells transfected with the G6PT cDNA or with siG6PT #3 oligonucleotides. Adherent and floating cells were harvested by trypsin digestion and gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). Then, 2×10^5 cells were pelleted and suspended in 200 μL of buffer solution and stained with annexin-V–fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's protocol (BD Biosciences, Mississauga, Ont.). The cells were diluted by adding 300 μL of buffer solution and processed for data acquisition and analysis on a Becton–Dickinson FACS Calibur flow cytometer using CellQuest Pro software. The *X*- and *Y*-axes indicate the fluorescence of annexin-V and PI, respectively. It was possible to detect and quantitatively compare the percentages of gated populations in all of the four regions delineated. In the early stages of apoptosis, phosphatidylserine is well known to translocate to the outer surface of the plasma membrane, which still remains physically intact. As annexin-V binds to phosphatidylserine but not to PI, and the dye is incapable of passing the plasma membrane, it is excluded in early apoptosis (annexin-V^+/PI^-). Cells in late apoptosis are stained with annexin-V and PI (annexin-V^+/PI^+). Necrotic cells have lost the integrity of their plasma membrane and are predominantly stained with PI (annexin-V^-/PI^+).

2.5. Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using non-parametric one-way ANOVA with GraphPad Prism Version 4.0. Probability values of less than 0.05 were considered significant, and an asterisk (∗) identifies such significance in each figure.

3. Results

3.1. Specific G6PT gene silencing in U-87 glioma cells

We first assessed microsomal G6PT gene expression, as well as the expression of glucose-6-phosphatase (G6Pase) isoforms α and β isoforms. Total RNA was extracted from HepG2 hepatoma and U-87 glioma cells, and then gene expression levels were analyzed by RT-PCR. As it would be expected for a cell line derived from a gluconeogenic tissue, HepG2 cells expressed all three components of the G6Pase system, with a higher expression of G6Pase-β (Fig. 1A). In contrast to HepG2, only G6PT and G6Pase-β transcripts were significantly expressed in U-87 glioma cells, with very low to undetectable levels of G6Pase-α (Fig. 1A). This is in agreement with previous reports demonstrating a lack of G6Pase-α expression in brain-derived cells [14]. Because previous evidence demonstrated that functional inhibition of the microsomal G6PT with CHA abrogates the cell migration and chemotactic response of U-87 cells to growth factors [8], we have generated siRNA constructs designed to specifically downregulate G6PT gene expression in U-87 glioma cells. Three siRNA constructs were designed and cell transfection performed as described in Section 2. Semi-quantitative RT-PCR analysis showed that G6PT transcription was specifically downregulated by all three constructs, while G6Pase-β gene expression remained unaffected by any of the constructs (Fig. 1B). Relative PCR product abundance was quantified

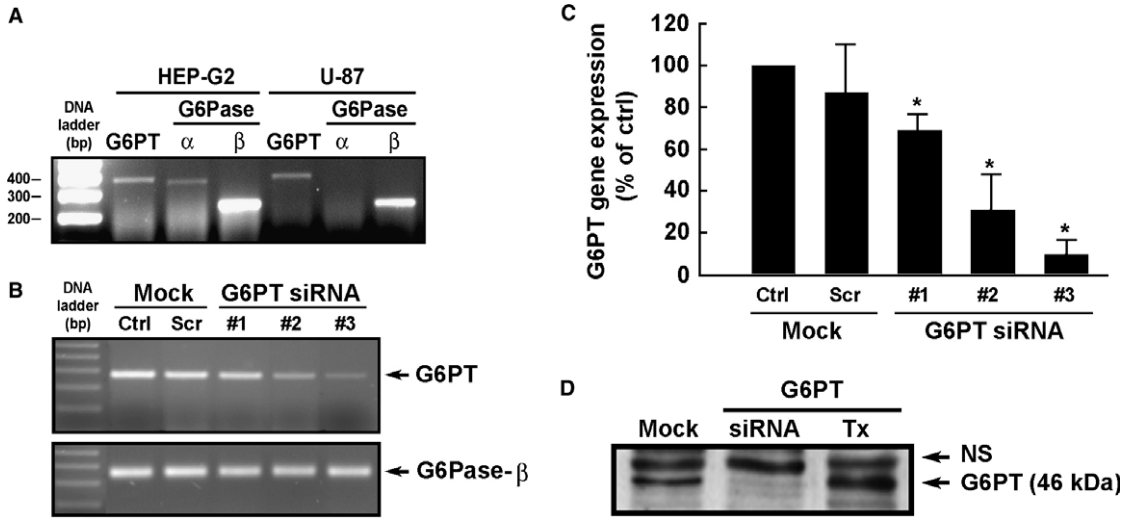

Fig. 1. Specific G6PT gene silencing in U-87 glioma cells. (A) Brain tumor-derived U-87 glioma cells were cultured until they reached approximately 90% confluency. Total RNA was extracted and RT-PCR performed in order to generate the cDNA reflecting gene expression levels of G6PT (380 bp), G6Pase-α (360 bp), and G6Pase-β (236 bp) as described in Section 2. HepG2 hepatoma cells were used as positive controls for the presence of all three genes. (B) Three siRNA constructs were generated to knock-down the human G6PT gene in U-87 glioma cells. Cells were either transfected with the siRNA constructs, mock-transfected or transfected with a scrambled negative control construct (Neg. Ctrl) as described in Section 2. Total RNA was isolated 36 h post-transfection and the cDNA of G6PT or G6Pase-β amplified as described in Section 2. A representative ethidium bromide-stained agarose gel of the levels of the respective amplicons is shown. (C) Scanning densitometry of the cDNA products was performed on each gel and the average values ± S.E.M. of four independent experiments are shown. (D) Total membrane-enriched fraction was isolated from mock, cells transfected with siG6PT construct #3 or with G6PT cDNA (Tx) and immunodetection for G6PT performed (NS, non-specific immunoreactive band).

in cells transfected with siG6PT constructs #1, #2, and #3, and G6PT gene expression was inhibited by 32%, 55% and 91%, respectively (Fig. 1C). Quantitative analysis using real-time RT-PCR was also performed and confirmed the maximum decrease observed in the siG6PT #3 condition by a factor of 9.65 ± 0.18. Although treatment with a combination of the respective constructs was not tested, subsequent experiments were performed using siG6PT construct #3. Total membrane-enriched fraction was isolated from mock, cells transfected with siG6PT construct #3 or with G6PT cDNA. Immunodetection for the G6PT protein was performed as previously described and validated [28], and shows that both the gene silencing as well as the overexpression significantly modulated G6PT protein expression (Fig. 1D).

3.2. G6PT gene silencing triggers U-87 glioma cell death

To investigate the specific contribution of G6PT to cell survival processes, we used siG6PT construct #3 to downregulate G6PT gene expression. This construct specifically silenced the G6PT gene and not that of G6Pase-β and MT1-MMP, a membrane-bound matrix metalloproteinase that we have previously shown to regulate, in part, the invasiveness of U-87 glioma cells [7,29] (Fig. 2A). We next assessed cell survival using flow cytometry with propidium iodide and annexin-V staining. Our results show that, in siG6PT-transfected cells, there was an increase in overall cell death as demonstrated by a significant shift in fluorescence in cells that stained positive for necrosis (Fig. 2B, upper left quadrant) as well as in late phase of apoptosis (Fig. 2B, upper right quadrant). Quantification of these data shows that G6PT gene downregulation triggered a 1.5-fold increase in cell necrosis and a 2.4-fold increase in cells undergoing the late stage of apoptosis (Fig. 2C). Altogether, these results suggest that G6PT is an important pro-survival protein and that any alteration in its expression could be deleterious to the cell.

3.3. The anticancer molecule curcumin inhibits G6PT gene expression in U-87 glioma cells

Since curcumin (diferuloyl-methane) has recently been attributed chemopreventive properties [26,27], and appears to affect glioma cell proliferation and survival [22,23], we investigated whether it would affect G6PT gene expression in U-87 glioma cells. Cells were treated with increasing concentrations of curcumin and then total RNA was isolated to assess G6PT and G6Pase-β gene expression using RT-PCR (Fig. 3A). While G6Pase-β and actin relative gene expression levels were not altered, 35 μM curcumin downregulated G6PT transcript levels by more than 90% (Fig. 3B) and by a factor of 10.22 ± 0.18 as quantified by real-time RT-PCR. These effects of curcumin suggest that, among its many intracellular protein targets, G6PT may be crucial for U-87 glioma cell survival.

3.4. The overexpression of recombinant G6PT rescues U-87 glioma cells from curcumin-induced cell death

We next addressed whether G6PT possesses any pro-survival functions in U-87 glioma cells. Untransfected (mock) cells or cells transfected with an expression vector for G6PT [8] were exposed to increasing concentrations of curcumin and cell death was evaluated by flow cytometry as in Fig. 2. Overexpression of recombinant G6PT had no effect on cell survival in untreated cells (Fig. 4A). However, curcumin dose-dependently triggered an increase in cell death (combined necrosis, early and late apoptosis) that reached an optimal effect at 25 μM (Fig. 4B), and concomitantly reduced cell viability in untransfected mock cells (Fig. 4B). When transiently transfected in U-87 glioma cells, the newly expressed G6PT prevented

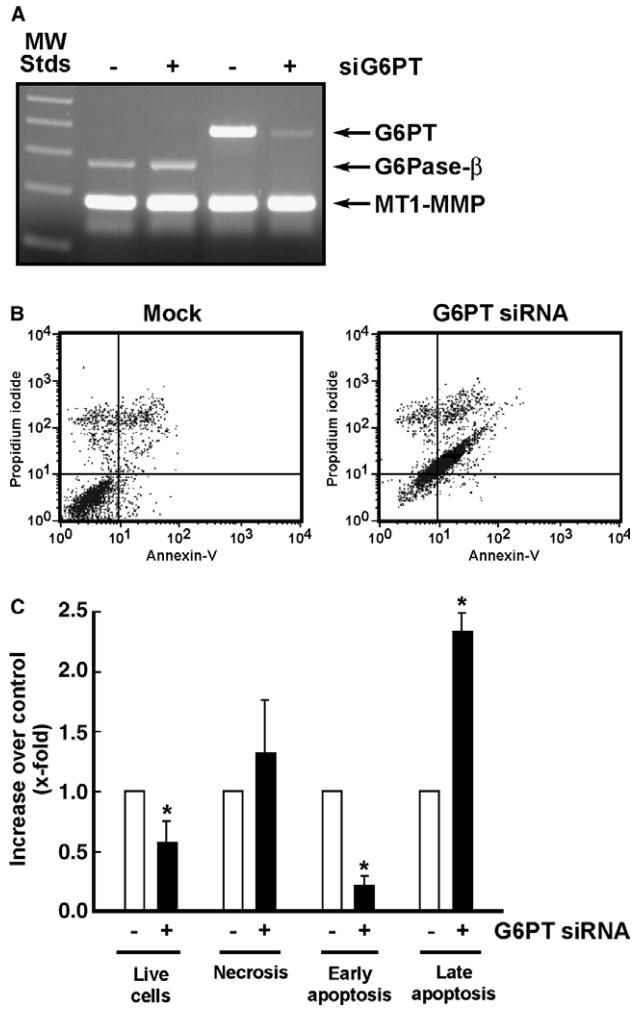

Fig. 2. G6PT gene silencing triggers U-87 glioma cell death. (A) SiRNA construct #3 was used to transfect U-87 glioma cells in order to decrease G6PT gene expression. Total RNA was isolated and the gene expression of G6PT, G6Pase-β, and MT1-MMP evaluated by RT-PCR as described in Section 2. (B) Cell apoptosis/necrosis was evaluated by flow cytometry in cells that were stained with propidium iodide (FL2-H) and annexin-V (FL1-H) as described in Section 2. (C) A representative quantification is shown for each of the quadrants in (B). The results are presented as follows: lower left quadrant, live cells from untransfected (mock) or cells transfected with the G6PT siRNA; upper left quadrant, cells undergoing necrosis; lower right quadrant, cells in the early phase of apoptosis; and upper right quadrant, cells in late phase of apoptosis.

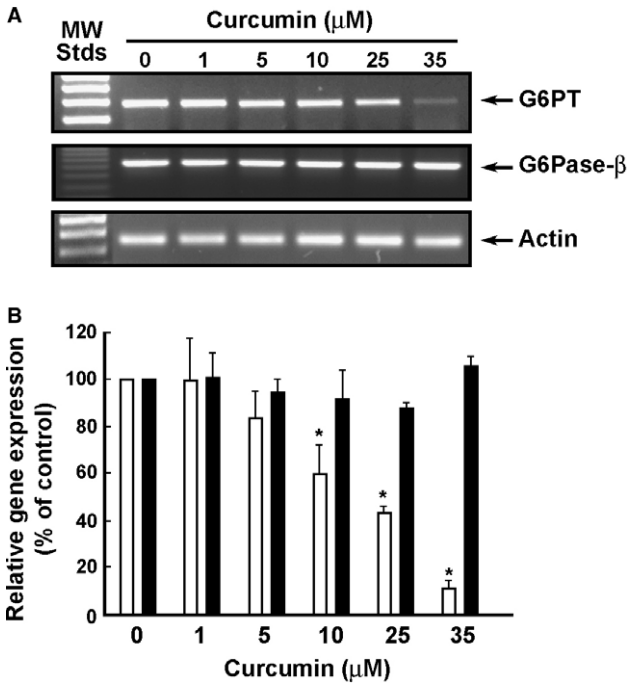

Fig. 3. The anti-cancer molecule curcumin inhibits G6PT gene expression in U-87 glioma cells. U-87 glioma cells were serum-starved and treated with different concentrations of curcumin for 18 h. (A) Total RNA was isolated and G6PT, G6Pase-β, and β-actin mRNA levels were evaluated by RT-PCR as described in Section 2. A representative ethidium bromide-stained agarose gel of the observed amplicons is shown. (B) Scanning densitometry was used to evaluate the extent of the effect of curcumin on G6PT (white bars) and G6Pase-β (black bars) gene expression. Values were normalized with reference to the values for the β-actin gene. The results represent the mean values ± S.E.M. of three experiments.

cells from undergoing apoptosis (Fig. 4A). In fact, 40–55% of cells treated with 25–35 μM of curcumin were rescued from entering apoptosis (Fig. 4B). Altogether, these data strongly suggest that G6PT regulates crucial pro-survival processes in U-87 glioma cells. Our results also suggest that targeting G6PT function or gene/protein expression may permit the development of new anticancer strategies.

4. Discussion

Glucose is absolutely essential for the survival and function of the brain since, in this tissue, there is no endogenous glucose production. Glucose availability thus remains exclusively dependent upon blood supply which is generated in the postprandial state by the hydrolysis of G6P through the hepatic and renal G6Pase system. Although the coupling of the G6Pase-β, the catalytic subunit isoform expressed in astrocytes [14], with G6PT activities enabled the formation of an active G6Pase complex, the physiological significance of this finding remains uncertain since brain G6Pase-β has only about 12% of the activity of hepatic G6Pase-α [30]. Moreover, when co-expressed with recombinant G6PT, the G6Paseβ-G6PT complex showed only ~25% of the maximal G6P accumulation activity of the liver complex [30]. Thus, the physiological roles of the native G6Pase-β remain to be confirmed as initial reports ascribed very low to undetectable activity for this protein [31].

Our current study supports the possibility of alternate functions of the ubiquitously expressed G6PT in non-gluconeogenic tissues distinct from the classical G6Pase system. For instance, translocation of G6P by G6PT within the ER may serve to feed a luminal hexose-6-phosphate dehydrogenase, which ubiquitous expression is consistent with that of G6PT [13]. In fact, this enzyme serves to provide the reducing equivalents needed for several important reductases that protect the ER against damage by reactive oxygen species. Lack of protection may result in premature cell death through apoptosis. Our findings thus provide a molecular mechanism accounting for the function and pro-survival effects of G6PT in brain tumor-derived cancer cells. The involvement of G6PT in brain tumor-derived cell survival processes could also be of major

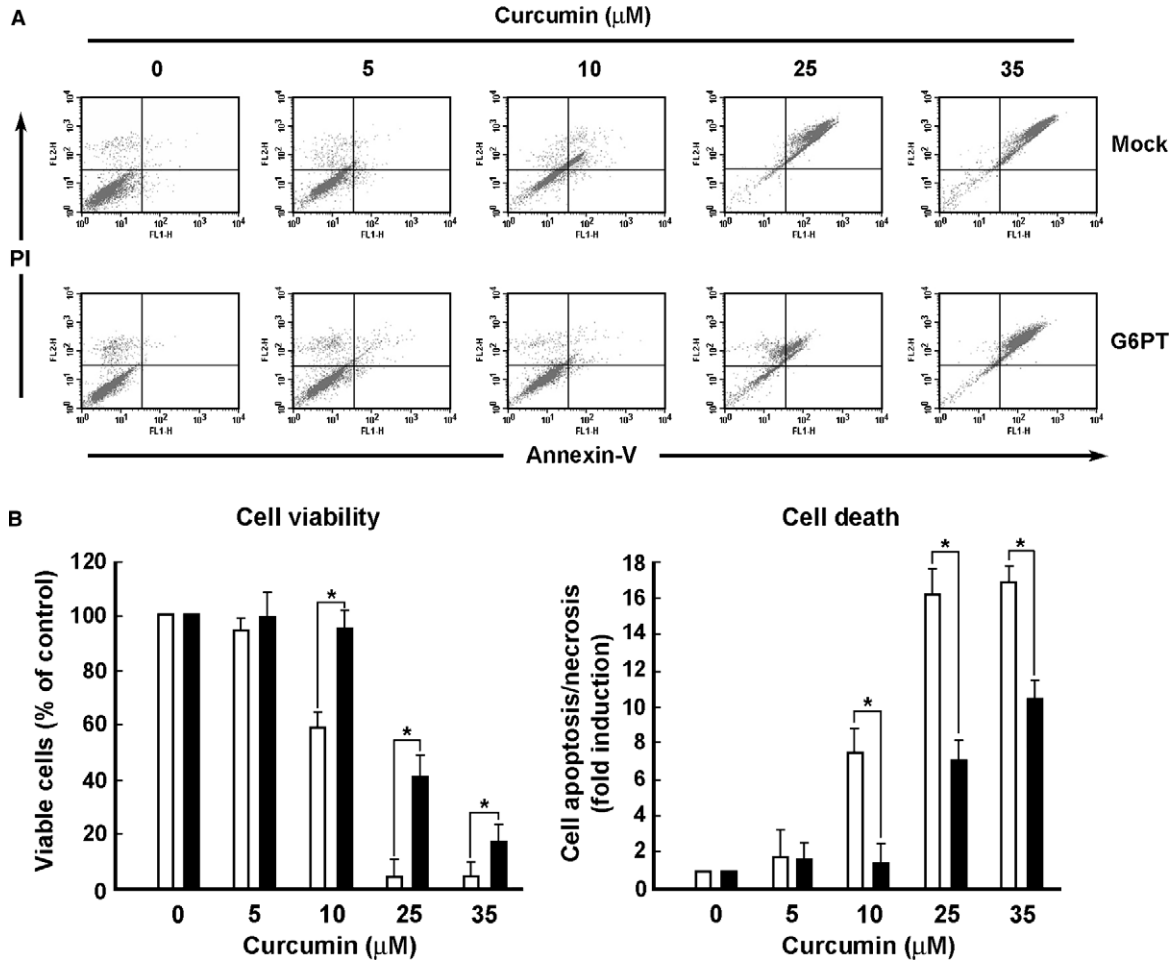

Fig. 4. The overexpression of recombinant G6PT rescues U-87 glioma cells from curcumin-induced cell death. (A) Untransfected (mock) U-87 glioma cells or cells transfected with the G6PT expression vector were serum-starved and treated with different concentrations of curcumin for 18 h. To evaluate cell death, we used flow cytometry of propidium iodide and annexin-V-stained cells as described in Section 2. (B) Quantification was performed as in the legend to Fig. 2. Cell viability values come from the lower, left quadrant, while cell death represents the combined values of necrosis, early, and late apoptosis. White bars: mock cells; black bars: G6PT-transfected cells.

physiological significance. Consequently, specific interference with G6PT functions [8,15] or expression [this study] becomes an attractive strategy for therapeutic control of glioma cell growth, and selective inhibition of G6PT may provide an ideal approach for the metabolic regulation of brain tumor cells. Several inhibitors of G6PT have been reported [32–36] and include complex natural products such as ilicicolinic acid B, hericenal C, mumbaistatin, kodaistatins [37], and derivatives of CHA [11]. Aside from CHA, few of these documented G6PT inhibitors have been systematically tested for their anti-cancer properties. Our study provides a further molecular-level explanation for the chemopreventive properties of curcumin [22,23] by targeting the pro-survival functions of G6PT in U-87 glioma cells.

Several approaches have been used to differentially modulate glucose flux and energy supply in cancer cells. We have recently shown that the glucose antimetabolite, 2-deoxy-D-glucose (2-DG), a competitive inhibitor of glucose transport and phosphorylation known to block glycolytic flux therefore modulating the synthesis of ATP [38], also inhibited the secretion of MMP by U-87 glioma cells. This compound inhibited intracellular transduction in response to sphingosine-1-phosphate [8], presumably by a mechanism involving the ATP-dependent calcium-sequestering activity of G6PT. Since the failure of radiotherapy in cerebral gliomas is primarily due to the diffuse infiltrating nature of the tumor, the abrupt changes in glycolytic energy demands of the brain tumor-derived cells may trigger growth arrest and/or cell death [39]. Thus, an important implication of the current study is the potential therapeutic impact of targeting G6PT functions as part of a radiotherapeutic regimen. Therefore, it is tempting to speculate that strategies aiming at the inhibition of G6PT would be beneficial in conjunction with radiotherapeutic modalities. In support of that hypothesis, in vitro studies performed in established glioma cell lines show that exposure to 2-DG for a few hours after irradiation significantly increased radiation-induced cellular damage [40], and that cancer radiotherapy was optimized in 2-DG dose escalation studies [41].

Deficiency in G6PT function has long been recognized to cause glycogen storage disease type 1b (GSD-1b) [42,43]. Indeed, at least 69 distinct mutations in the *G6PT* gene, which either greatly reduced or completely abolished G6PT function, have been identified and lead to premature death [42,44]. Over the last few years, other unrecognized functions of G6PT have been identified. For instance, polymorphonuclear leukocytes from GSD-1b patients exhibit impaired mobility, chemotaxis,

and Ca^{2+} flux responses [44]. In addition, their respiratory burst, pentose phosphate shunt, glycolytic activity and phagocytotic activity are also diminished. Inhibition of G6PT functions also resulted in dysfunctional apoptotic neutrophils from GSD-1b patients [15]. These observations strongly support a crucial role for G6PT in keeping optimal cellular functions. While the role of G6PT in carbohydrate metabolism is well understood, its roles in alternate mechanisms such as in immune deficiency or in cancer are relatively unknown. In conclusion, our data suggest that G6PT plays a central role in regulating glioblastoma cell survival and invasiveness. Strategies aiming at the inhibition of G6PT functions with anticancer agents, such as the naturally occurring curcumin, may provide a new mechanistic rationale for the action of chemopreventive drugs and lead to the development of new anticancer strategies.

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