siah-1 Protein Is Necessary for High Glucose-induced Glyceraldehyde-3-phosphate Dehydrogenase Nuclear Accumulation and Cell Death in Müller Cells*

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The translocation and accumulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the nucleus has closely been associated with cell death induction. However, the mechanism of this process has not been completely understood. The E3 ubiquitin ligase siah-1 (seven in absentia homolog 1) has been associated with cell death induction. However, the mechanism of movement of GAPDH from the cytosol to the nucleus is unclear. GAPDH lacks a common nuclear localization signal (NLS) and therefore cannot enter the nucleus by itself because of its size. A recent study has identified the E3 ubiquitin ligase siah-1 (seven in absentia homolog 1) as a potential carrier/shuttle protein (13). According to this study, GAPDH binds the NLS-bearing siah-1, forming a complex subsequently promoting translocation of GAPDH from the cytosol to the nucleus. It is postulated that the NLS on siah-1 facilitates the nuclear movement of the complex.

siah-1 proteins are human homologs of the evolutionarily conserved Drosophila, E3 ubiquitin ligase sina (seven in absentia) protein (17). sina was first identified as a key protein during R7 photoreceptor development in the Drosophila fruit fly whereby it facilitates the degradation of the transcriptional repressor of neuronal fate tramtrack (TTK88) (18–20). Follow-up studies have identified functions for siah-1 in various cellular processes including mitosis, neuronal plasticity, development, angiogenesis, inflammation, and cell death (13, 17, 20–29). Two siah genes, siah-1 and siah-2 are present in humans and rats, whereas mice have three siah genes: siah-1a, siah-1b, and siah-2 (17, 30, 31). siah-1a and siah-1b have 98% sequence homology. Structurally, siah-1 contains an N-terminal RING domain that facilitates the ligase function, two cysteine-rich zinc finger domains, and a substrate-binding domain, which is localized on the C-terminal of the protein (27, 32, 33). The last 12 amino acids on the C-terminal in the substrate-binding domain are necessary for GAPDH-siah-1 interaction (13). The NLS motif is also located in the substrate-binding domain.

We have recently identified that elevated glucose levels act as a stimulus for GAPDH nuclear accumulation in retinal Müller (gila) cells via high glucose-induced activation of a caspase-1/interleukin-1β signaling pathway (16). We have demonstrated that hyperglycemia-induced GAPDH nuclear accumulation not only occurs in retinal Müller cells in vitro but also in vivo during the development of diabetic retinopathy in rodents (4). Because Müller cells maintain the retinal environment (34–39), death processes within these cells can potentially compromise their function leading to disease. A better understanding
siah-1 and GAPDH Nuclear Accumulation

of mechanisms leading to GAPDH translocation from the cytosol to the nucleus is needed to develop more target-specific treatments. Some studies have already indicated the potential of inhibiting this event by showing increased cell survival in vivo and in vitro (4, 9, 14, 40). Therefore, the purpose of this study was to determine whether high glucose regulates siah-1 in Müller cells and to examine the potential of this protein to bind and regulate GAPDH nuclear movement and cell death under hyperglycemic conditions.

EXPERIMENTAL PROCEDURES

Materials

Goat anti-siah-1 antibody and goat anti-mouse antibody conjugated to horseradish peroxidase was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), whereas rabbit anti-goat conjugated to horseradish peroxidase was from R & D Systems (Minneapolis, MN). Mouse anti-GAPDH antibody was purchased from Chemicon International (Temecula, CA). Mouse anti-histone 2B was from MBL laboratories (Naka-ku Nagoya, Japan). Rabbit anti-lactate dehydrogenase (LDH) was from Abcam (Cambridge, MA). Rabbit anti-Phospho-p53-S15 and rabbit anti-p53 were purchased from Cell Signaling (Danvers, MA). Rabbit anti-Bax was from BD Biosciences (San Jose, CA). Rabbit anti-Phospho-p53-S15 and rabbit anti-p53 were purchased from Abcam (Cambridge, MA). Rabbit anti-goat antibody conjugated to Alexa 594 and goat anti-mouse IgG conjugated to Texas Red were from Invitrogen. Rabbit anti-goat antibody conjugated to Alexa 594 and goat anti-mouse IgG conjugated to Texas Red were from Invitrogen. Rabbit anti-actin, GAPDH, phospho-p53-S15, bax, p53) and incubated for 24 h with the goat polyclonal antibody against either siah-1 (1:500), mouse monoclonal antibody against GAPDH (1:5000), mouse monoclonal against β-actin (1:10,000), rabbit polyclonal against phospho-p53-S15(1:1000), rabbit polyclonal against bax (1:1000), or rabbit polyclonal against total p53 (1:1000). The antibodies were diluted in respective blocking solutions. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminescence horseradish peroxidase detection reagent (Pierce). The bands were quantified using densitometry analysis using the Bio-Rad Quantity One program and expressed as the ratio between proteins of interest to actin. Phospho-p53-S15 bands were normalized to total p53. Histone 2B (1:1000) and lactate dehydrogenase (1:1000) were used to demonstrate purity of nuclear or cytosolic fractions, respectively.

Reverse Transcription and Real Time Quantitative PCR

Total RNA from treated cells was isolated using TRIzol reagent (Invitrogen) and treated with PureLink DNase treatment (Invitrogen) to digest DNA. RNA (2 μg) was reverse transcribed using Applied Biosystem high capacity cDNA reverse transcription kit (Foster City, CA). siah-1 mRNA levels were determined through quantitative real time PCR assays using the TaqMan® gene expression assays and the Applied Biosystems PRISM 7900HT sequence detection system. 18 S RNA was used to normalize for the starting amount of cDNA, and the assays were performed in triplicate. Fold changes relative to control treatment were quantified. Context sequences provided by the manufacturer for amplified genes are follows: rat siah-1, CTTCCACAGAGATAAAGGCACCCATC; rat 18s, TGG-AGGGCAAGTCTGGTGCCAGCAG; human siah-1, CTCTC-CGCCCAAGAATGAGCGT; and human 18s, TGG-AGGGCAAGTCTGGTGCCAGCAG.

Immunofluorescence Analysis of siah-1 and GAPDH—rMC-1 (5.0 × 10⁶) or hMC (3.0 × 10⁶) plated on glass coverslips were treated with normal or high glucose for 24 (rMC-1) or 48 (hMC) h. Following treatment, the cells were fixed in a solution of freshly prepared 4% paraformaldehyde for 10 min at room temperature and rinsed twice with PBS. The cells were permeabilized with ice-cold acetone for 10 min, blocked with 1% bovine serum albumin in PBS-T and incubated overnight at 4 °C with commercially available antibodies against GAPDH (1:800 dilution). For siah-1 primary antibody (1:200 dilution), the cells were incubated at 4 °C for at least 48 h. The cells were
then rinsed twice with PBS and incubated in either 5% rabbit serum (siah-1) or 5% goat serum (GAPDH) in 1% bovine serum albumin/PBS for 30 min, followed by 1 h of incubation with the appropriate secondary antibody (anti-goat secondary antibody conjugated to Alexa Fluor 594—1:1000 dilution for siah-1-hMC or anti-mouse secondary antibody conjugated to Texas Red-1: 200 dilution for GAPDH-rMC-1) at room temperature. Coverslips were rinsed extensively in PBS-T and mounted on glass slides using Vectashield anti-fade fluorescence mounting medium (Vector Laboratories, Burlingame, CA). Blinded samples were then unblinded, and the average values of several GAPDH in four different fields/sample was established. The percentage of cells that were positive for nuclear siah-1 and GAPDH was determined via Bio-Rad protein assay.

Subcellular Fractionation—rMC-1 (2.5 × 10⁶) were treated as described above with normal or high glucose for 24 h. Following treatment, the nuclear fractions were generated as previously described (16). Briefly, treated cells were rinsed twice with ice-cold Hanks’ buffered saline solution, scraped in ice-cold homogenization buffer, and resuspended in 200 μl of homogenization buffer. Suspension was homogenized with 10 strokes of a homogenizer, and nuclear fractions were collected by a low speed spin (1000 × g) for 5 min. Nuclear fractions were washed in homogenization buffer twice and resuspended in low stringency ice-cold radio immunoprecipitation buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin) for co-immunoprecipitation analysis. Cytosolic fraction-containing supernatant was retained for analysis of fraction purity. The protein concentrations were determined via Bio-Rad protein assay.

Co-immunoprecipitation Assays—Equal amounts of protein (1000 μg) were pre-cleared using 30 μl of protein G plus agarose beads at 4 °C for 3 h. The beads were pelleted, and supernatant was transferred to a fresh microcentrifuge tube. 7.5 μg of anti-siah-1 antibody was added to the supernatant and incubated for 2 h with constant rotation. Protein G plus agarose beads (30 μl) were then added to the supernatant and incubated overnight at 4 °C with constant rotation. Antibody and protein bound beads as well as the bead only controls were pelleted by a 14,000 × g spin (1 min). Supernatants lacking siah-1 because of siah-1 protein pulldown by immunoprecipitation were retained. These siah-1-depleted supernatants were subjected to siah-1 Western blot analysis alongside a siah-1 positive control to ascertain efficient siah-1 pulldown. Immunoprecipitates were washed five times in ice-cold high stringency radio immunoprecipitation buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, 500 mM sodium chloride, 10 mM sodium fluoride, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin), resuspended in 20 μl sample buffer, vortexed, boiled at 100 °C for 5 min, and subjected to Western blot analysis as described above. The membrane was then stripped and reprobed with antibody against GAPDH to determine complex formation. As control, LDH was used to demonstrate that siah-1/GAPDH binding is specific.

siah-1 siRNA Transfection—To knock down siah-1, rMC-1 were transfected with siah-1-specific SMART pool siRNA, which contains a pool of four siRNA duplexes (Table 1) using Amaxa nucleofection electroporation system (Cologne, Germany). For control, the cells were subjected to electroporation only or transfected with 50 nm nonspecific scrambled siRNA or risk-free siRNA. Sequences for control siRNA are patent-protected. Transfection was performed according to the manufacturer’s instructions. Briefly, rMC-1 were harvested by centrifugation and resuspended at 3.0 × 10⁶ cells/100 μl in solution L (Amaza kit). 100 μl of cell suspensions with or without siRNA were dispensed into electroporation cuvettes. The final concentration of siRNA was either 20 or 50 nM. Transfection efficiency by this method was 65%. Following electroporation, the cells were plated on tissue culture plates containing regular growth medium. For experiments, the cells were switched to experimental medium 12 or 24 h post-transfection and treated as described above.

siah-1 Truncation Mutation—siah-1 C-terminal truncation of the last 12 amino acids was generated by site-directed mutagenesis on a siah-1 cDNA, using PCR with stop codon-containing primers. Wild type siah-1 construct was also generated for control. A Myc epitope tag (EQKLI SEEDL) was engineered into the N terminus of both proteins. The primers sequences used for construct generation were as follows: siah-1 wt, 5′-TGATGAAATTCTCATGGAAA AACTCATCTCATGAGAAGAGATCTGAGCCGT-CTAGCGGCCGCTCAGTGTCTGCTACGACGATC3′ (outer forward primer); siah-1 wt, 5′-TGCAACGCGGCGCTC- AACACATGGAATAGTATTACATTGATG-3′ (outer reverse primer); siah-1Δ amino acids 1–270, 5′-TGATGAAATTCT- GGAACAAAAACTCATCTCATGAAAGAGATCTGCGCCGCTACGACGATC3′ (outer forward primer); siah-1Δ amino acids 1–270, 5′-GACTCGCGCCTCATTCTATTTT- TCTGCAAAGAGCTGTGCAATGCTCTGAGACGCGCCGCTCTTGCTACAGACGATC3′ (outer reverse primer). The primer sequences used for sequencing confirmation were as follows: siah-1 wt, ATAGCCGATGGCGAGACGGAATAGTATTACATTGATGCTGAGCGCCGCTGCAGACGATC3′ (outer forward primer), and siah-1Δ amino acids 1–270, CTCAAGATGTCACACCATCCTGACGCGCCGCTCTTGCTACAGACGATC3′ (outer reverse primer).

Trypan Blue Cell Death Assay—Following treatment with 5 or 25 mM glucose for 96 h, rMC-1 (1 × 10⁶) transfected with either control scrambled (50 nM) or siah-1(50 nM) siRNA were trypsinized (0.25% trypsin, 2 min). Equal amounts (100 μl) of cell suspension and trypan blue solutions were mixed. Blinded

**TABLE 1**

| ON-TARGET plus SMART pool siRNA | Target sequence |
|--------------------------------|----------------|
| j-094413-09, siah-1             | CAACAAADGACUUGCCGGAUU |
| j-094413-10, siah-1             | GGCGUCUGGDCCAACGCCTUU |
| j-094413-11, siah-1             | CCAGAAAGGCAGAAGACGAG |
| j-094413-12, siah-1             | GAGAUACUCUCUGCCGACA |
samples were counted using hemocytometer, and cell death was quantified as the number of blue cells/total cell number. The samples were then unblinded, and the values from independent experiments were averaged.

**Caspase Activity Assay**—Caspase activities were measured as described previously (4, 44–47). Briefly, equal amounts of cytosolic lysates (15 μg) from rMC-1 cells treated for 96 h were incubated in lysate buffer containing the fluorogenic caspase substrate (2.5 μM) in a total volume of 100 μl at 32 °C for 1 h. Cleavage of the substrate emits a fluorescence signal that was quantified by Tecan Spectra FluorPlus fluorescence plate reader (excitation, 400 nm; emission, 505 nm). Caspase activities were calculated against an 7-amino-4-trifluoromethylcoumarin standard curve and expressed as pmol of 7-amino-4-trifluoromethylcoumarin/mg of protein/min.

**Statistical Analysis**—The data were analyzed using one-way analysis of variance (correlated samples, p < 0.05) followed by Tukey’s post-analysis to determine statistical significance among groups. The ordinal data were analyzed using a Kruskal-Wallis test (p < 0.05) followed by Dunn’s post analysis to determine the statistical significance among groups. For details in statistical analysis see the VasserStats statistical computation web site.

**RESULTS**

**Effect of High Glucose on siah-1 Expression in rMC-1**—We have recently shown that hyperglycemia leads to cell death of retinal Müller cells via GAPDH nuclear accumulation (4, 16). However, the mechanism of GAPDH translocation from the cytosol to the nucleus is unknown. Because a recent report has suggested that siah-1 can act as a carrier protein during this process (13), we determined whether high glucose can regulate expression of this protein. High (25 mM) glucose significantly increased siah-1 mRNA levels within 12 h by 1.9 ± 0.1-fold in rMC-1 compared with control cells cultured under normal (5 mM) glucose conditions (Fig. 1A). In addition, a significant, 1.4 ± 0.1-fold increase in siah-1 protein was detected after 12 h of treatment (Fig. 1B). Increased siah-1 protein levels were sustained throughout 24 h, the time point at which GAPDH nuclear accumulation can significantly be determined (16).
Determination of siah-1 Protein Levels and Localization in hMCs—To confirm that high glucose-induced changes in siah-1 were not the result of transformation in the rMC-1 cell line, the effect of elevated glucose levels on isolated human Müller cells (hMC) was determined. High glucose (25 mM) treatment induced a modest but significant increase in siah-1 mRNA levels by 1.4 ± 0.03-fold compared with control (Fig. 2A) and a significant 1.7 ± 0.2-fold increase in siah-1 protein levels as determined by Western blot analysis (Fig. 2B). The antibody against human siah-1 allowed for determination of siah-1 localization within hMC under normal and high glucose conditions. A significant increase in the number of cells positive for nuclear siah-1 was observed in cells cultured under high glucose (69.7 ± 9.4%) conditions compared with control cells cultured in normal glucose (24.5 ± 4.0%) (Fig. 2C).

Detection of High Glucose-induced siah-1-GAPDH Complex in the Nucleus of rMC-1—A recent study suggested that formation of a complex between siah-1 and GAPDH is essential in the process of GAPDH nuclear accumulation (13). Because we have demonstrated that high glucose up-regulates siah-1, we were interested in examining whether such a complex can be detected in the nucleus of Müller cells, further identifying a potential role for siah-1 as a shuttle protein for GAPDH to allow for translocation to the nucleus.

First, several immunoprecipitation control experiments were performed. Fig. 3A shows that immunoprecipitation using goat serum did not pull down siah-1 or GAPDH. Immunoprecipitation of beads only also did not show any unspecific pulldown of siah-1 and GAPDH (data not shown). The siah-1 antibody used for immunoprecipitation assays was specific for siah-1, because pulldown of siah-1 and subsequent analysis of the immunoprecipitates for siah-1 using Western blot analysis detected only the siah-1 protein. To confirm that GAPDH does not just bind to proteins in an unspecific manner, immunoprecipitation assays using LDH were performed, because retinal Müller cells highly express LDH. Fig. 3A shows that GAPDH binds to siah-1 but not to the abundant LDH protein. Input levels of siah-1 and GAPDH are presented as control. Finally, the siah-1 Western blot analysis in the right panel of Fig. 3A demonstrates that lysates subjected to siah-1 immunoprecipitation are devoid of the protein, indicating that pulldown of siah-1 was efficient and complete under our experimental conditions.

To demonstrate the presence of a siah-1-GAPDH complex in the nucleus of Müller cells, co-immunoprecipitation studies were done using equal amounts of nuclear fractions from rMC-1 cells subjected to normal or high glucose treatment. The 24-h treatment time point was chosen, because this is the time point at which GAPDH nuclear accumulation can significantly be detected (16). High glucose treatment led to a significant increase in the amounts of siah-1 proteins in the nucleus and increased levels of nuclear GAPDH bound to siah-1 (Fig. 3B). To determine whether increased levels of nuclear GAPDH bound to siah-1 were due to a potential increased binding of...
GAPDH to siah-1, ratios of GAPDH to siah-1 protein levels were calculated based on densitometry analysis of Western blots. High glucose treatment significantly increased the ratio of GAPDH:siah-1 binding by 1.9 ± 0.3-fold based on this analysis (Fig. 3B).

Effect of siah-1 Knockdown on GAPDH and siah-1 Protein Level—Although the co-immunoprecipitation assays indicated the formation of a complex between siah-1 and GAPDH under hyperglycemic conditions, which was detectable in the nucleus of Müller cells, we opted to use siRNA technology to knock down siah-1 to further confirm the necessity of siah-1 in the process of GAPDH translocation from the cytosol to the nucleus. 50 nm siah-1 siRNA significantly decreased siah-1 mRNA expression at 20 h by 63.9 ± 8.5% compared with electroporation only (Fig. 4A). Scrambled siRNA had no significant effect on siah-1 mRNA levels. In addition, 50 nm siah-1 siRNA decreased siah-1 protein levels by 48.3 ± 11.8% compared with electroporation only (Fig. 4B). Scrambled and risk-free siRNA had no apparent significant effect on siah-1 protein levels. siah-1 knockdown did not affect GAPDH protein levels (Fig. 4B).

Effect of siah-1 Knockdown on GAPDH Nuclear Accumulation—Because we had established that 50 nm siRNA significantly reduced siah-1 protein levels, this concentration was used for further experiments. To test whether siah-1 knockdown can prevent high glucose-induced GAPDH nuclear accumulation, the levels of nuclear GAPDH were determined following siah-1 knockdown in high glucose-treated retinal Müller cells. The number of rMC-1 cells positive for nuclear GAPDH following high glucose treatment significantly decreased from 60.7 ± 4.3% in scrambled siRNA-transfected control cells to 36.6 ± 5.2% in siah-1 siRNA-transfected cells, strongly indicating that siah-1 is necessary for GAPDH nuclear translocation (Fig. 4C). The number of GAPDH-positive cells in the normal scrambled control siRNA control cells was 31.1 ± 2.5%.

GAPDH-siah-1 Interaction Is Necessary for Nuclear Accumulation under High Glucose Conditions—A previous study has shown that a 12-amino acid truncation of the siah-1 C terminus prevented complex formation with GAPDH (13). These 12 amino acids are part of the substrate-binding domain. Using a truncated form of siah-1, we confirmed the necessity of GAPDH-siah-1 interaction for high glucose-induced GAPDH nuclear accumulation. Cells transfected with truncated siah-1 lacking residues 271–282 (siah-1 Δ amino acids 1–270) did not
The Role of siah-1 Knockdown on p53 Phosphorylation under High Glucose Conditions—So far, we have demonstrated that siah-1 is necessary for GAPDH nuclear translocation. Although nuclear GAPDH has strongly been linked to cell death induction, the function of GAPDH in the nucleus is unknown and has only been speculated about. To date, only one study has proposed a mechanism for cell death induction by nuclear GAPDH. This recent report suggested that GAPDH plays an integral role in the acetylation of p53, a well known protein involved in a variety of cell death processes, connecting nuclear siah-1 and GAPDH to cell death induction (15). Because transcriptional activity of p53 is also heavily regulated by phosphorylation, we examined the effect of siah-1 knockdown on p53 phosphorylation at serine 15. To test whether changes in the phosphorylation state of p53 at serine 15 indeed induce transcriptional activity of p53 and whether siah-1 knockdown can prevent p53 transcriptional activity, we determined protein expression levels for the well known p53-driven target gene bax. A significant 21.2 \pm 0.6% increase in bax protein was observed following high glucose treatment compared with normal glucose in the control scrambled siRNA-treated cells (Fig. 6B), whereas siah-1 knockdown using siRNA reduced high glucose-induced bax levels by 75.2 \pm 3.8%.

Effect of siah-1 Knockdown on High Glucose-induced Cell Death in Müller Cells—To reinforce the idea that GAPDH nuclear translocation leads to cell death under high glucose conditions, the effect of siah-1 knockdown on high glucose-induced cell death was determined using caspase-6 activity and trypan blue viability assays. High glucose significantly increased the activity of caspase-6, an executioner caspase, to 166.8 \pm 4.2 pmol 7 amino-4-trifluoromethylcoumarin/mg/min from 118.3 \pm 11.5 in control cells. siah-1 siRNA knockdown reduced high glucose-induced caspase-6 activity to 123.6.\pm 13.6% (Fig. 7A). Further, high glucose treatment increased cell death by 4.5 \pm 2.0-fold. High glucose-induced cell death was diminished in cells subjected to siah-1 siRNA, indicating that inhibition of GAPDH nuclear translocation within the first 24 h strongly affects cell death detectable at 96 h (Fig. 7B).

DISCUSSION

Hyperglycemia causes GAPDH nuclear translocation and accumulation in retinal Müller cells in vivo and in vitro (4, 16). The mechanism underlying the movement of GAPDH from the cytosol to the nucleus under high glucose conditions in these cells is not completely understood. In the present study, we have demonstrated that the E3 ubiquitin ligase siah-1 facilitates GAPDH nuclear translocation via formation of a complex with further evidence that GAPDH siah-1 interaction is crucial for high glucose-induced GAPDH nuclear translocation in retinal Müller cells.
GAPDH. In contrast to GAPDH, siah-1 carries a NLS motif allowing for transport to the nucleus. More importantly, siah-1 knockdown studies and studies using a truncated form of siah-1 have confirmed that siah-1 is necessary and crucial for the processes of high glucose-induced GAPDH nuclear accumulation and subsequent cell death in Müller cells. Although the exact function of GAPDH in the nucleus is largely unknown, our results indicate that nuclear GAPDH seems to be involved in the regulation of p53 activation.

Our current studies provide evidence that siah-1 plays a critical role in the process of GAPDH nuclear translocation. However, it is somehow surprising that this type of protein is critically involved in this process. siah-1 is most commonly associated with its E3 ubiquitin ligase function during ubiquitin-dependent proteasomal degradation (24, 26, 28, 48–50). In diabetic retinopathy, proteins associated with ubiquitination and protein turnover are up-regulated during the development of the disease (51). The glucose transporter GLUT1 and angiotensin II type 1 receptor have been identified as specific targets for protein degradation through this pathway in the diabetic retina. In addition to tagging proteins for proteasomal degradation, post-translational modification via ubiquitination may also act as a signaling mechanism. Increased ubiquitination has been associated with the progression of neurodegenerative diseases. For example, ubiquitination by siah-2 has been shown to induce α-synuclein aggregation and cytotoxicity in neuronal cells during the development of Parkinson disease, and siah-1 auto-ubiquitination has been suggested to occur during complex formation between GAPDH and siah-1 (13, 29).

Other functions of siah-1 involve regulation of cell cycle and pro-survival proteins through protein degradation (22, 24, 49, 52). Whether inhibition of high glucose-induced cell death of Müller cells by siah-1 knockdown results from the inhibition of these alternative functions of siah-1 is not known. Future studies need to be done to determine the role of ubiquitination in this process. The focus of our study was to understand the role of siah-1 in the process of GAPDH nuclear translocation and subsequent cell death induction. Although humans and rats have two siah-1 homologs: siah-1 and siah-2 (17, 30, 31), our studies have focused on siah-1, because siah-2 does not have a NLS motif based on sequence prediction analysis.

Even though GAPDH does not contain a NLS, the protein contains an export signal for protein extrusion from the nucleus (53). It is possible that the absence of GAPDH in Müller cell nuclei under normal glucose conditions results from efficient nuclear to cytosolic export. Impaired protein export in high glucose conditions might also be responsible for accumulation of GAPDH in the nucleus. Conformational changes to GAPDH following siah-1 binding may also affect the ability of GAPDH to interact with the export machinery of the nucleus.

Although the translocation and accumulation of GAPDH has been linked to cell death induction, the function(s) of GAPDH within the nucleus have not fully been identified. Studies in neurons that made use of an NLS-tagged GAPDH construct...
known protein heavily involved in induction of several types of cell death (15). A recent study has suggested that the process involves activation of p53 via acetylation by p300/CBP subsequent to GAPDH-mediated activation of these acetylation enzymes (15). Our studies also suggest that in addition to acetylation of p53, nuclear GAPDH might also regulate phosphorylation of p53, which stabilizes p53. The role of siah-1 in both of these processes, acetylation and phosphorylation of p53, has not been determined and might just be related to transport of GAPDH to the nucleus. On the other hand, a previous study not focused on the interaction between GAPDH and siah-1 has demonstrated a role for siah-1 itself in the process of p53 activation (22). siah-1 and p53 seem to be connected and regulated via a positive feedback mechanism because siah-1 is also regulated by p53 (58). More studies are needed to clearly identify the role of nuclear siah-1 and GAPDH in the process of cell death induction.

Retinal Müller cells maintain the retina and its vasculature (34–39, 59). Therefore, Müller cell dysfunction and loss in the diabetic retina (60) possibly compromises the integrity of retinal tissue leading to disease. GAPDH nuclear accumulation might play an integral role in the induction of cell loss within the diabetic retina. A recent report has demonstrated that GAPDH nuclear accumulation persists when diabetic animals were brought back from poor to good control of blood glucose levels (11). The study also indicated that in these animals progression of diabetic retinopathy was still present. Although our studies have focused on identifying mechanisms underlying GAPDH nuclear accumulation in Müller cells in vitro and in vivo (4, 16), we do not want to exclude the possibility that other retinal cells are affected by hyperglycemia in a similar fashion. High glucose induces cell death in several other retinal cell types in the diabetic retina (10, 61–65). Inhibition of GAPDH nuclear translocation is clearly protective. We have previously shown that the mono amino oxidase inhibitor R-deprenyl prevents GAPDH nuclear accumulation and cell death in Müller cells cultured under high glucose conditions (4). Recently, a study has demonstrated that R-deprenyl acts by interfering with GAPDH-siah-1 interaction (9). Taken together, our results indicate the potential of compounds that target the GAPDH/siah-1 interaction as therapies to increase Müller cell survival under hyperglycemic conditions and potentially prevent the progression of diabetic retinopathy.

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