Regulation of the selenoprotein SelS by glucose deprivation and endoplasmic reticulum stress – SelS is a novel glucose-regulated protein

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Abstract SelS is a newly identified selenoprotein and its gene expression is up-regulated in the liver of *Psammomys obesus* after fasting. We have examined whether SelS is regulated by glucose deprivation and endoplasmic reticulum (ER) stress in HepG2 cells. Glucose deprivation and the ER stress inducers tunicamycin and thapsigargin increased SelS gene expression and protein content several-fold in parallel with glucose-regulated protein 78. The overexpression of SelS increased Min6 cell resistance to oxidative stress-induced toxicity. These results indicate that SelS is a novel member of the glucose-regulated protein family and its function is related to the regulation of cellular redox balance.

Key words: Selenoprotein; Glucose-regulated protein; Glucose deprivation; Endoplasmic reticulum stress; Gene expression

1. Introduction

Glucose-regulated proteins (GRPs) were first identified as being highly induced when cultured mammalian cells were depleted of glucose [1]. Subsequently, it was discovered that a variety of other agents, such as the protein glycosylation inhibitor tunicamycin and the endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor thapsigargin, also up-regulate the expression of these genes [2]. These agents commonly cause disturbance in the ER by the accumulation of misfolded proteins or disruption of ER Ca^{2+} homeostasis. Consequently, the cellular response to such agents or glucose starvation has been termed the ER stress response or unfolded protein response [3].

The GRP family so far comprises a diverse group of over 10 proteins including GRP78, GRP94 and calreticulin [4], and the list is still growing. The majority of GRPs are localized in the ER where they act as molecular chaperones assisting in protein folding and assembly [4]. Studies using overexpression or antisense oligonucleotides in tissue culture show that GRPs protect cells from stress-induced cell death or apoptosis [5–8], suggesting their induction under stress conditions is beneficial to cell survival.

The induction mechanism by ER stress and glucose starvation may differ depending on the particular gene or cell type. While both tunicamycin and thapsigargin induce GRP expression in many cell types, WEHI72 lymphoma cells only respond to tunicamycin but not to thapsigargin or the calcium ionophore A23187 [9]. Furthermore, the heme oxygenase 1 (HO-1) gene was only induced by glucose deprivation through the generation of reactive oxygen species, but not by tunicamycin or thapsigargin in hepatoma HepG2 cells [10,11]. These studies suggest that three separate signaling pathways, one by the accumulation of misfolded or underglycosylated proteins, one elicited by ER Ca^{2+} depletion, and one by production of reactive oxygen species, can induce GRP expression.

Selenoproteins characteristically contain the amino acid selenocysteine, which is considered the 21st amino acid in the genetic code, in their primary structures. In the universal genetic code, 61 codons encode 20 amino acids while three codons are terminators. The UGA codon has generally been regarded as a stop codon for protein synthesis. However, in the presence of a special secondary structure in the 3′-untranslated region of the mRNA, i.e. a stem loop called SECIS (*se*lenocysteine *i*nserting *s*equence), UGA can designate the incorporation of selenocysteine into the polypeptide so that protein synthesis continues through this codon until the next in-frame stop codon is encountered [12]. Only 26 selenoproteins are predicted and/or demonstrated in mammals [13].

We previously identified a novel protein, Tanis, in the liver of the obese/diabetic animal model *Psammomys obesus* [14]. Tanis, a type II transmembrane protein, is predominantly localized in the ER but also found on the cell surface [15]. Tanis is also the homologue of a newly identified selenoprotein, SelS [13]. The hepatic expression of Tanis was inversely correlated with plasma glucose concentration in *P. obesus* after fasting [14,15]. Its promoter sequence also contains features that are typical of many GRP genes. These have prompted us to examine whether SelS is regulated by ER stress. In this report, we have characterized the induction of the SelS gene in HepG2 cells and examined its function as an antioxidant.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERSE, ER stress response element; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GRP, glucose-regulated protein; HO-1, heme oxygenase 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RT, reverse transcription; Tm, tunicamycin; Tg, thapsigargin

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2. Materials and methods

2.1. Reagents

All tissue culture media, supplements and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). Tunicamycin, thapsigargin and actinomycin D were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Hepatoma HepG2 cells were cultured in monolayer in Dulbecco's modified Eagle's medium (4.5 g/l glucose) supplemented with 2 mM glutamine, 1 mM pyruvate, 10% (v/v) heat-inactivated FBS. Min6 cells, a mouse β-cell line [16], were grown in monolayer in DMEM (4.5 g/l glucose), 15% (v/v) heat-inactivated FBS, 60 μM 2-mercaptoethanol. Cells were grown in 5% CO_2/95% air at 37°C in medium that contained antibiotics (penicillin, 75 μg/ml; streptomycin, 50 μg/ml).

One day before treatment, HepG2 cells were split in growth medium without antibiotics in six-well plates. Cells at approximately 80% confluence were washed twice in phosphate-buffered saline (PBS) and incubated with serum-free DMEM containing different carbohydrates or ER stress agents (tunicamycin, 10 μg/ml; thapsigargin, 5 μM) for 24 h before cell harvest for analyses. For RNA stability studies, cells were pretreated with glucose-free DMEM, 25 mM glucose DMEM supplemented with or without tunicamycin or thapsigargin for 24 h. Cells were then treated with the same media containing actinomycin D (4 μM) for indicated time points and harvested for RNA analysis.

2.3. Immunoblot

After treatment with appropriate agents, HepG2 or Min6 cells were washed twice with ice-cold PBS and lysed in 250 μl of lysis buffer per well (20 mM Tris, pH 7.5, 1% NP-40, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and a cocktail of protease inhibitors). Cells were scraped and transferred to microcentrifuge tubes, incubated on ice for 30 min with intermittent mixing, and centrifuged at $13\,000 \times g$ for 15 min at 4°C. The supernatant was used for protein concentration determination using bovine serum albumin as a standard. 50 μg of protein was loaded on 12.5% polyacrylamide gels, and transferred to polyvinylidene difluoride for Western blots using standard protocols. The primary anti-SelS antibody was described previously [15] and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon (Victoria, Australia). Immunoreactive proteins were visualized using an enhanced chemiluminescent detection system from Amersham Biosciences (NSW, Australia).

2.4. Quantitative real time reverse transcription polymerase chain reaction (RT-PCR)

Following treatment of HepG2 cells, total RNA was isolated using Trizol (Invitrogen) and concentration was determined using an Agilent 2100 Bioanalyzer. cDNA was synthesized using a reverse transcription system (Promega, Madison, WI, USA). SelS, GRP78, and cyclophilin transcripts were quantified using real time RT-PCR on an ABI Prism 7700 sequence detector. Primer sequences for SelS were 5′-GTTGCGTTGAATGATGTCTTCCT-3′ (forward) and 5′-AGAAACAAACCCCATCAACTGT-3′ (reverse), for GRP78 were 5′-GGTGACCTGGTACTGCTTGATG-3′ (forward) and 5′-CCTTGGATTCAGTTTGGTCATG-3′ (reverse) and for cyclophilin were 5′-CCCACCGTGTTCTTCGACA-3′ (forward) and 5′-CCAGTGCTCAGAGCTCGAAA-3′ (reverse). PCR conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Under the conditions used, the PCR reactions were linear with the amount of cDNA input for all three genes (data not shown). All gene expression data are expressed as arbitrary units.

2.5. Plasmid construction, transient transfection and promoter activity assays

To make a reporter construct containing the SelS promoter, the SelS promoter region, i.e. −1073 bp to +39 bp, was amplified using the GC-2 PCR kit (BD Biosciences, NSW, Australia) using human genomic DNA as template. The PCR forward and reverse primers incorporated the restriction sites *Mlu*I and *Xho*I, respectively. The PCR products were ligated into the pGL3 basic firefly luciferase vector (Promega) and the sequence was confirmed by DNA sequencing. The plasmid pRL-SV40 (Promega), which contains the *Renilla* luciferase gene, was used as an internal control for co-transfections. HepG2 cells were grown in 12-well plates to ∼70% confluence for co-transfection with 0.7 μg of the reporter construct and 0.07 μg of the control plasmid per well using LipofectAMINE Plus (Invitrogen). Twenty-four hours post transfection, cells were washed twice in PBS and treated with serum-free DMEM containing 25 mM glucose supplemented with tunicamycin (10 μg/ml) or thapsigargin (5 μM) for 24 h. The cells were lysed in the passive lysis buffer for firefly luciferase and *Renilla* luciferase activity assays using the dual-luciferase system (Promega). Assays were done in duplicate with a luminometer and high reproducibility was obtained. Promoter activity was expressed as the ratio of firefly luciferase to *Renilla* luciferase activities with the ratio in glucose treatment being arbitrarily defined as 1.

2.6. SelS overexpression and cell viability assays in Min6 cells

To make a construct for SelS overexpression in mammalian cells, the open reading frame plus the 3′-untranslated region of SelS mRNA was amplified from HepG2 cell cDNA using the forward primer 5′-CGGC*GGATCC*CATGGAACGCCAAGA-3′ and reverse primer 5′-AATT*GAATTC*CATAAATCTCCTTG-3′. The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the vector pCMV-Tag2a (Stratagene, La Jolla, CA, USA). The insert in the plasmid was confirmed by DNA sequencing. Min6 cells were transiently transfected using LipofectAMINE Plus (Invitrogen) with the SelS construct or a control plasmid that encoded green fluorescent protein (GFP). To ensure the supply of selenium for the synthesis of selenoproteins in the cells, culture medium was supplemented with 1 μM sodium selenite after transfection. Twenty-four hours post transfection, cells were treated with H_2O_2 for 18 h. Cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method [17]. Briefly, cells were incubated in serum-free DMEM containing 0.5 mg/ml MTT for 45 min at 37°C. During this period MTT was cleaved in the mitochondria of viable cells. The blue MTT formazan formed was dissolved in 0.04 M HCl in isopropanol, and the absorbance was determined at 570 nm (test wavelength) and 630 nm (reference wavelength) using a spectrophotometer. The results were graphed as percentage of viable cells with cells treated with vehicle only set at 100%.

2.7. Statistical analysis

All experiments were done at least twice each with three replicates. Data were expressed as mean ± S.E.M. and were analyzed by analysis of variance. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Induction of SelS protein and mRNA by glucose deprivation

The distinct feature of glucose-regulated proteins is their induction by glucose deprivation in cell culture [4]. To investigate the possibility that SelS was a novel member of the glucose-regulated protein family, we treated HepG2 cells with a range of glucose concentrations in serum-free DMEM for 24 h. In low glucose media (0.5 or 2 mM), SelS protein content was increased several-fold as compared with cells treated with high glucose media (15 or 25 mM) (Fig. 1a). A time course study indicated that the induction of SelS protein by low glucose was a slow process with a lag phase of 8–12 h (Fig. 1b). Consistent with previous reports [10,11], the level of the housekeeping enzyme GAPDH was not affected by glucose concentrations in the medium (Fig. 1a,b).

The increase in SelS protein content upon low glucose treatment could potentially be due to an increase in protein translation or mRNA amount. To differentiate these two processes, we measured SelS mRNA content. SelS mRNA increased with decreasing glucose in a concentration-dependent manner between 0.5 and 15 mM (Fig. 1c). Compared with 25 mM glucose, cells treated with 0.5, 2 mM and 5 mM glucose had 4.4, 3.0, and 1.7 times the amount of SelS mRNA, respectively. These changes in mRNA levels were similar to those in pro-

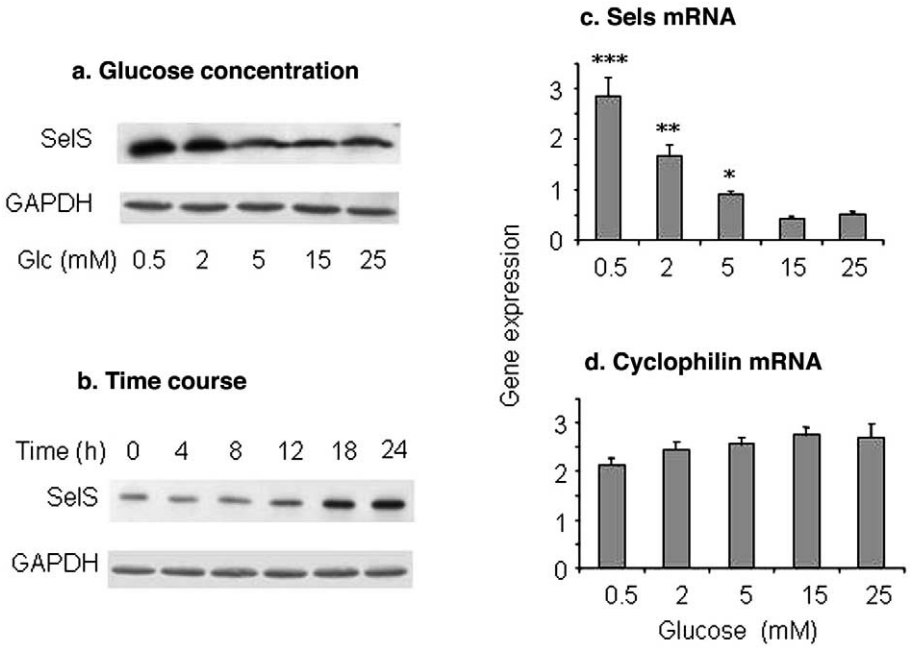

Fig. 1. Induction of SelS protein and mRNA by glucose deprivation. HepG2 cells were treated with serum-free DMEM containing different concentrations of glucose for 24 h (a,c,d) or treated with DMEM containing 0.5 mM glucose for the indicated length of time (b). Cells were analyzed for SelS and GAPDH proteins (a,b) or for SelS (c) and cyclophilin (d) transcripts. $^{*,**,***}P < 0.001$ when compared with 25 mM glucose.

tein content, and demonstrated enhanced mRNA content was responsible for the increased SelS protein levels during low glucose treatment. The threshold of glucose concentration for the induction was 2–5 mM. As expected, the housekeeping gene cyclophilin mRNA was not affected by glucose concentrations (Fig. 1d).

3.2. Carbohydrate specificity in SelS induction

Mammalian cells have the ability to use alternative carbohydrates as their energy source. To investigate SelS gene induction by other carbohydrates, we treated HepG2 cells for 24 h in serum- and glucose-free DMEM supplemented with various monosaccharides (25 mM). Glucose deprivation increased SelS protein content several-fold, and this induction was abolished by fructose, galactose and man-nose (Fig. 2a). In contrast, the addition of non-metabolizable 3-*O*-methylglucose did not block the induction by glucose starvation. The level of GAPDH protein was not affected by these treatments (Fig. 2a). SelS mRNA was four- to five-fold higher in cells treated without carbohydrate or with 3-*O*-methylglucose when compared with cells treated with glucose, fructose and mannose (Fig. 2b). Galactose appeared to be slightly less effective in suppressing SelS expression, indicating that it may be a less preferred carbohydrate for these cells. GRP78, which has been shown to be strongly induced by glucose starvation in HepG2 and many other cell types, was used as a positive control for this experiment. The response of GRP78 to carbohydrate treatments was almost in quantitative parallel with that of SelS (Fig. 2b). On the other hand, the expression of cyclophilin was decreased slightly by glucose starvation and 3-*O*-methylglucose (24–33%) (Fig. 2b).

3.3. Induction of SelS expression by ER stress

As shown above, SelS expression was highly up-regulated by glucose deprivation in HepG2 cells. Glucose starvation can activate the unfolded protein response by the accumulation of underglycosylated proteins in the ER. However, recent work has demonstrated that some genes can also be activated by glucose deprivation independent of the unfolded protein response [10,11]. To further establish the induction of SelS, we treated HepG2 cells with the ER stress agents tunicamycin and thapsigargin, which inhibit protein glycosylation in the ER and deplete ER calcium stores, respectively. Western blot showed that these agents increased SelS protein levels while having no effect on GAPDH protein content (Fig. 3a). Tunicamycin and thapsigargin increased SelS and GRP78 mRNA by three- to four- and four- to five-fold, respectively. However, these agents had a minimal effect on cyclophilin mRNA content, decreasing it by at most 30% (Fig. 3b).

3.4. Glucose deprivation and ER stress did not affect SelS mRNA stability

Increased SelS mRNA content by glucose starvation and ER stress may be due to an increase in its gene transcription rate and/or to an increase in its stability. To study whether there were any changes in SelS mRNA stability, actinomycin D, a general inhibitor of RNA transcription [10,18], was used to block gene expression. Decay of SelS mRNA was followed as a function of time after the addition of actinomycin D. The half-life of SelS mRNA was 5.7 h for high glucose, 6.7 h for glucose-free, 6.1 h for tunicamycin and 6.8 h for thapsigargin treatments (Fig. 4). There was no statistical significance between these four treatments at any of the time points, indicating that increased SelS mRNA content following glucose deprivation and ER stress was not due to enhanced mRNA stability.

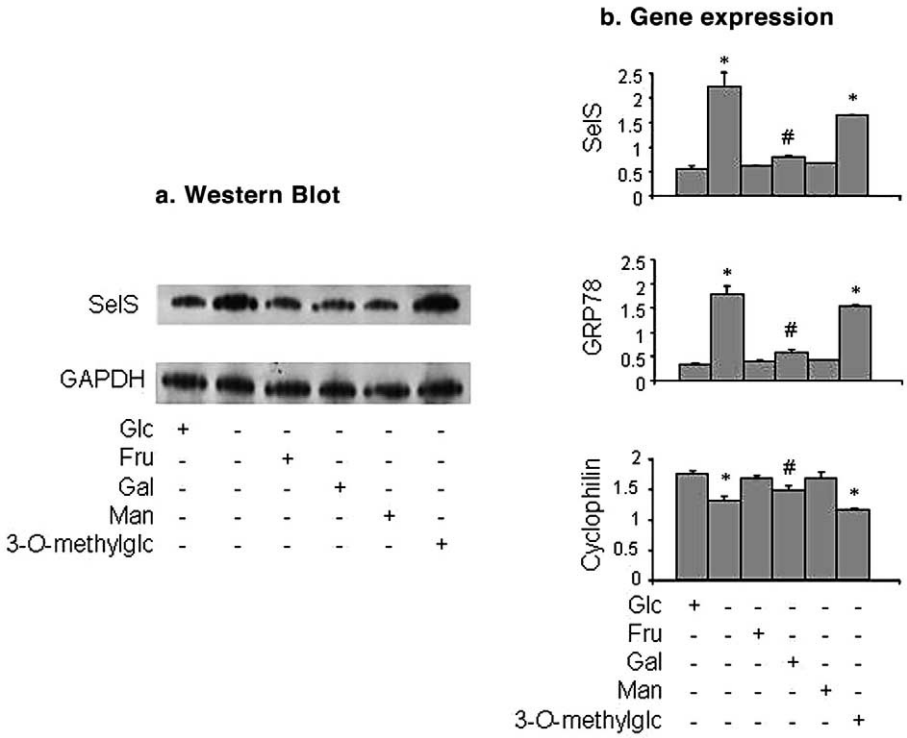

Fig. 2. Effect of monosaccharides on SelS expression. HepG2 cells were treated with serum-free DMEM containing the indicated monosaccharide (25 mM) for 24 h. a: SelS and GAPDH protein levels. b: SelS, GRP78 and cyclophilin mRNA levels. $^{*}P < 0.01$, $^{\#}P < 0.05$ when compared with glucose DMEM.

3.5. *The SelS promoter contains an ER stress response element (ERSE) and is activated by ER stress*

One feature shared by GRP promoters is a GC-rich region immediately upstream of the transcription start site [4]. We identified the SelS upstream sequence from GenBank (accession number AC023024.6) and the −196 to +66 region is shown in Fig. 5a. This region is indeed highly GC-rich. For many GRP genes, there exist up to three copies of the ERSE within this GC-rich region. This motif, while not strictly conserved, has the consensus sequence CCAAT(N9)CCACG (where N9 is nine often GC-rich nucleotides) and is critical for GRP induction under ER stress conditions [19,20]. The SelS promoter contains the sequence GGATT(N9)CCACG which is similar to the consensus GRP ERSE motif in vertebrates and almost identical to the ERSE found in GRP78 of *Spinacia oleracea* (Fig. 5b) [19].

We cloned the −1073 bp to +39 bp region of the SelS promoter into a firefly luciferase reporter construct for promoter activity studies. Reporter assays indicated that the promoter was activated three- to four-fold by tunicamycin and

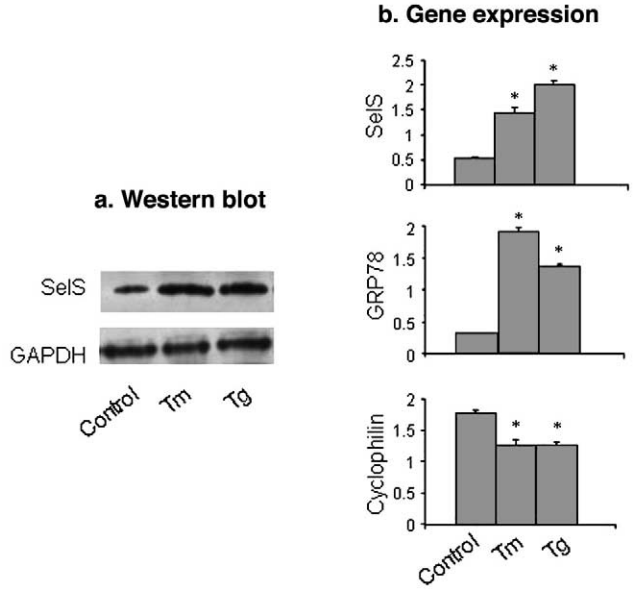

Fig. 3. Induction of SelS expression by ER stress. HepG2 cells were treated with serum-free DMEM containing 25 mM glucose, or 25 mM glucose supplemented with tunicamycin or thapsigargin for 24 h. a: SelS and GAPDH Western blots. b: SelS, GRP78 and cyclophilin mRNA. $^{*}P < 0.01$ when compared with glucose DMEM.

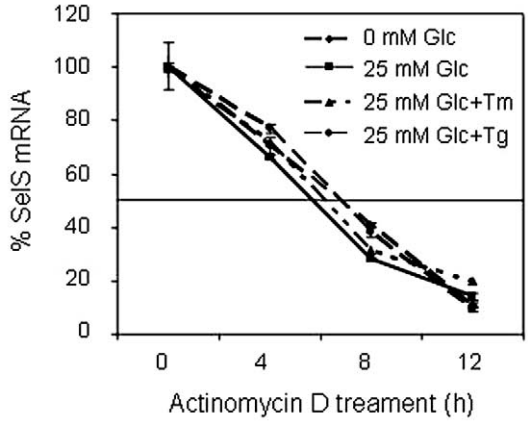

Fig. 4. Glucose deprivation and ER stress did not affect SelS mRNA stability. $P > 0.05$ for all time points.

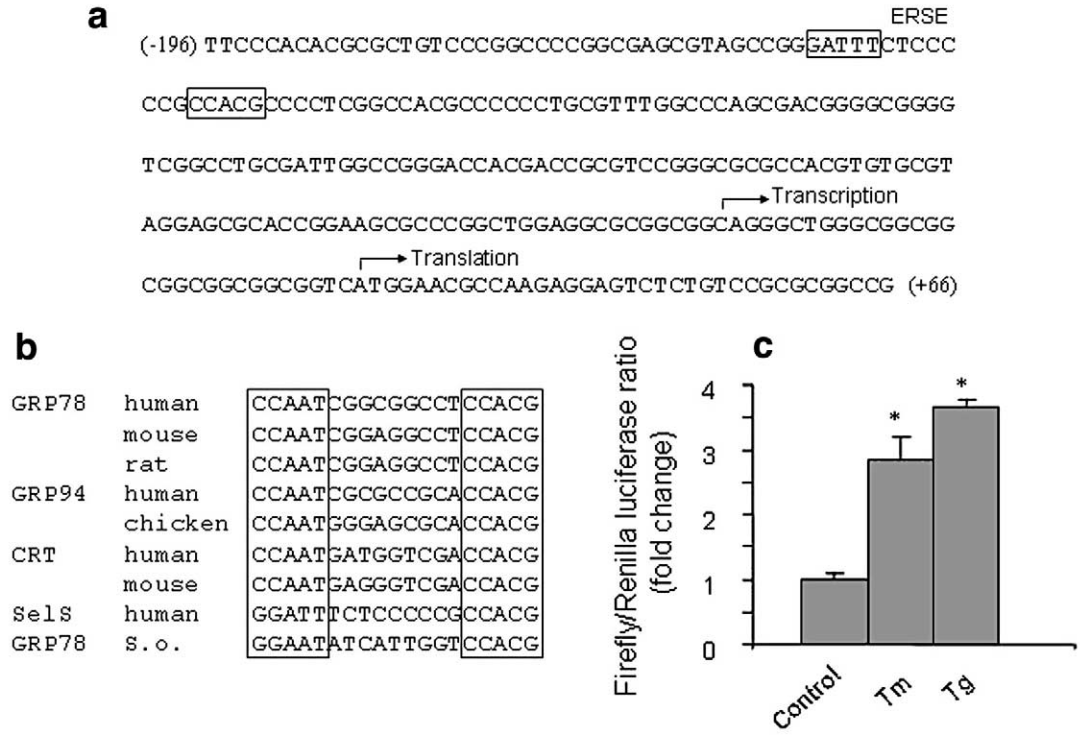

Fig. 5. a: The proximate SelS promoter is highly GC-rich and contains a conserved ERSE. b: Alignment of SelS ERSE with the consensus ERSE of other glucose-regulated protein genes in vertebrates and in *Spinacia oleracea* (S.o.). CRT: calreticulin. c: SelS promoter-driven gene expression under ER stress conditions. A 1-kb promoter sequence was cloned into a firefly luciferase reporter construct for promoter activity studies. $^{*}P < 0.01$ when compared with DMEM containing 25 mM glucose only (control).

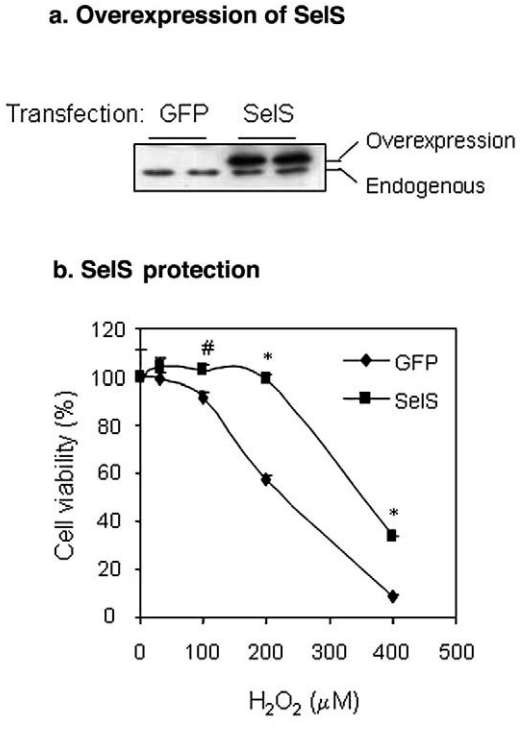

Fig. 6. SelS overexpression protected Min6 cells from oxidative stress-induced Min6 cell death. a: Overexpression of SelS. The overexpressed SelS carried a Flag tag at the N-terminus and was therefore larger than the endogenous protein on the gel. b: Cell viability. $^{\#}P < 0.05$, $^{*}P < 0.01$.

thapsigargin (Fig. 5c). This magnitude was comparable to the induction of the endogenous SelS gene by these agents (Fig. 3). Further deletion of the promoter indicated that the 300-bp upstream sequence was sufficient for ER stress induction (data not shown).

3.6. SelS protects cells from oxidative stress

Many selenoproteins, including the glutathione peroxidases and thioredoxin reductases [12], are enzymes that regulate cellular redox balance. Selenoproteins W and P, which have no enzymatic activities, also show antioxidant properties [21,22]. We examined whether SelS had a similar role in cytoprotection in Min6 pancreatic β-cells, which were sensitive to oxidative stress-induced apoptosis. Transient transfection of Min6 cells resulted in the expression of the recombinant SelS protein several-fold above the endogenous level (Fig. 6a); and the expression of the control protein GFP was verified under a fluorescence microscope (data not shown). When the cells transfected with SelS or GFP were challenged with oxidative stress, cells expressing SelS were resistant to H_2O_2 up to 200 μM. In contrast, cells expressing GFP lost 42.3% of their viability at this concentration. At 400 μM H_2O_2, viability in cells expressing SelS was 3.7 times that of the cells expressing GFP (Fig. 6b).

4. Discussion

In this report, we have demonstrated that the expression of the selenoprotein SelS is highly inducible by glucose deprivation and ER stress. We add SelS as a novel member to the GRP family. Secondly, the overexpression of SelS gives cells

resistance to oxidative stress, suggesting its function is related to the regulation of cellular redox state. This study represents the first report that selenoprotein can be induced by ER stress and glucose starvation.

SelS gene expression was highly induced by glucose starvation and this was completely offset by fructose and mannose, but not by the non-metabolizable 3-*O*-methylglucose. The induction had a slow response, requiring 8–12 h for accumulation of SelS protein in the cells. The sugar specificity and time course of induction are similar to those reported for other GRPs such as GRP78, HO-1 and asparagine synthetase [10,23], and support that it is the concentration of a common sugar metabolite, perhaps glucose-6-phosphate, that is a critical regulator of gene expression. Furthermore, SelS was induced by the ER stress inducers tunicamycin and thapsigargin, indicating the induction involved the ER stress pathway. Noticeably, the response of SelS to ER stress and glucose starvation paralleled that of GRP78, one of the most extensively studied GRPs (Figs. 2 and 3).

Most studies on GRP gene induction have been conducted with cells in culture but the induction has rarely been observed in vivo. Limited evidence shows the expression of GRP78 is enhanced in vivo in embryonic mouse heart following hypoglycemic stress or induced in fast growing tumor cells as a result of glucose depletion and/or hypoxia [4]. It is noteworthy that SelS expression was enhanced in the livers of *P. obesus* after a 24-h fast [14,15]. The threshold glucose concentration (2–5 mM) for SelS induction in vitro appears to be much higher than those for other GRPs (<1 mM) [10,23]. This concentration is comparable to blood glucose levels in *P. obesus* in the fasted state and suggests the in vivo induction is the result of a modest decrease in blood glucose. Such sensitivity in induction makes SelS an ideal model system in the studies of GRPs and selenoproteins.

Many members of both the selenoprotein and GRP families function to protect cells from stress conditions. Several selenoproteins, including the four glutathione peroxidases and three thioredoxin reductases, are enzymes that regulate cellular redox balance and therefore have antioxidant properties [12]. Knockout or reduction in their activity is either lethal in mice or produces increased susceptibility to oxidative damage [24–27]. SelW (unknown function) and SelP (a proposed selenium transport protein) also exert protection against oxidative stress [21,22]. Similarly, typical members of the GRP family, such as GRP78, GRP94 and calreticulin, have been shown to protect cells from ER stress in numerous studies [5–8]. It is therefore possible that SelS, being a selenoprotein and a GRP, may have a similar protective function against one or more assaults. Indeed, overexpression of SelS significantly increased Min6 cell tolerance to oxidative stress (Fig. 6). This result suggests that SelS, like many other selenoproteins, may also regulate intracellular reactive oxygen species levels.

Hyperglycemia in diabetes generates elevated levels of reactive oxygen species which induce tissue damage and aggravate insulin resistance [28]. The induction of SelS in the obese/diabetic animal model *P. obesus* after fasting [14] and its ability to protect cells against oxidative stress suggest it may be implicated in the development of diabetes. The role of SelS in the protection of pancreatic β-cells from apoptosis and in the improvement of insulin sensitivity in the liver and peripheral tissues through the regulation of intracellular redox state warrants further investigation.

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