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SEPS1 protects RAW264.7 cells from pharmacological ER stress agent-induced apoptosis

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Abstract

Selenoprotein S (SEPS1) is a novel endoplasmic reticulum (ER) resident protein and it is known to play an important role in production of inflammatory cytokines. Here, we show evidence that SEPS1 is stimulated by pharmacological ER stress agents in RAW264.7 macrophages as well as other cell types. Overexpression studies reveal a protective action of SEPS1 in macrophages against ER stress-induced cytotoxicity and apoptosis, resulting in promoting cell survival during ER stress. The protective action of SEPS1 is largely dependent on ER stress-mediated cell death signal with less effect on non-ER stress component cell death signals. Conversely, suppression of *SEPS1* in macrophages results in sensitization of cells to ER stress-induced cell death. These findings suggest that SEPS1 could be a new ER stress-dependent survival factor that protects macrophage against ER stress-induced cellular dysfunction.

Keywords: Selenoprotein S; Tanis; VIMP; Endoplasmic reticulum stress; Macrophages; Apoptosis; Tunicamycin; Thapsigargin

SEPS1 (SelS/Tanis/VIMP) has been previously identified as a putative ER stress response protein that is likely to be associated with an inflammatory response [1–4]. A genetic variation in the human *SEPS1* promoter region that is strongly associated with substantial increase in circulating levels of pro-inflammatory cytokine is located in the center of a putative ER stress–response element (ERSE) [5], suggesting a possibility of ER stress-dependent regulation of *SEPS1* transcription.

The ER is the major site for protein folding and maturation, cellular response to stress, and maintaining Ca^{2+} homeostasis [6]. Accumulation of misfolded proteins and alteration of Ca^{2+} homeostasis in the ER generate ER stress that triggers various cellular dysfunctions including apoptosis and inflammation [7–11]. ER stress is primarily sensed by three ER-bound proteins: PKR-like ER-associated kinase (PERK), a kinase and endonuclease inositol requiring enzyme 1 (IRE1), and a basic leucine-zipper transcription factor activation of transcription factor 6 (ATF6) [8–12]. Activation of these proteins and their associated signaling pathways triggers attenuation of general protein synthesis and an increase in transcription of genes that are essential for molecular chaperones, protein folding and protein degradation in order to adapt to temporal ER stress [8,12]. Under the condition of prolonged and/or severe ER stress, the cell activates intracellular pathways that lead to programmed cell death [8,13,14] via several pathways, including caspase-12 and PERK-mediated activation of a transcriptional factor CHOP/GADD153 [8,13–18]. Elevated ER stress and its associated apoptosis are evidenced in many cell types including macrophages, pancreatic β-cells,

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neurons and endothelial cells with implication for various human diseases, including atherosclerosis, Parkinson's, Alzheimer's, and prion protein disease [15–17]. However, the protective mechanisms against ER stress-induced apoptosis have not yet been fully understood.

In this study, we have demonstrated an ER stress-dependent SEPS1 expression in macrophages as well as in various cell types. The overexpression and suppression studies of *SEPS1* suggest a survival role of SEPS1 in macrophages during ER stress and its potential role in controlling ER stress-associated signaling pathway.

Materials and methods

Materials and cell culture. Tunicamycin, thapsigargin, dithiothreitol (DTT), cycloheximide and staurosporine were purchased from Fisher Scientific. Homocysteine was purchased from Sigma. Anti-Fas was purchased from eBioscience. RAW264.7, HepG2 and HEK293 cells were obtained from American Tissue Culture Collection (ATCC) and all cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and antibiotics (penicillin, 75 μg/ml; streptomycin, 50 μg/ml) in 5% CO_2 at 37 °C.

Construction of plasmid and transient transfection. A N-terminal flag-tagged open reading frame plus 3′-untranslated region of human *SEPS1* mRNA was amplified by PCR with the 5′ primer, 5′-GCCACCA TGGATTACAAGGATGACGACGATAAGGAACGCCAAGA-3′ and 3′ primer, 5′-GAAGTCCATAAATCTCCTTG-3′. The resultant PCR product was ligated into pTARGET vector (Promega) and then subcloned into pcDNA3.1 vector (Invitrogen). The pcDNA3.1 expression vector was transiently transfected into RAW 264.7 cells using Lipofectamine 2000 (Invitrogen) by following the manufacturer's instructions.

siRNA transfection. SEPS1 and scrambled siRNAs were synthesized *in vitro* using a kit from Ambion. Primer sequences used in this study and the condition of transient transfection of siRNA into RAW264.7 cells were described previously [5].

Western blot analyses. Cells were lysed in a buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride for 10 min on ice. The protein content was determined by Bradford assay (Bio-Rad). Ten micrograms of protein were subjected to SDS–polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membrane, and immunodetected using primary antibodies and goat anti-mouse IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology) by using an enhanced chemiluminescence detection kit (Pierce). The primary antibodies used in this study are as follow: anti-SEPS1 antibody [4], anti-GRP78 and anti-GAPDH (Santa Cruz Biotechnology), anti-CHOP, anti-phospho-PERK and anti-phospho-eIF2α (Cell Signaling Technology).

DNA fragmentation assay. RAW 264.7 cells transfected with control or *SEPS1* expression vector were incubated with 10 μg/ml tunicamycin or 10 μM thapsigargin for 24 h in the absence of serum. Cells were scraped, pelleted, washed in ice-cold PBS, and gently resuspended in lysis buffer (1× TE, 0.5% SDS, 20 μg/ml RNAse) followed by incubation at 37 °C for 1 h. After incubation, the lysates were treated with proteinase K (100 μg/ml) at 50 °C for 1 h and DNA ladder formation was visualized by agarose gel electrophoresis.

Cell cytotoxicity, apoptosis and viability assays. RAW264.7 cells transfected with control or *SEPS1* expression vector were challenged with indicated concentration of tunicamycin or thapsigargin for 24 h in the absence of serum. After 24 h, lactate dehydrogenase (LDH) activity in the media was determined using a cytotoxicity assay kit (Biovision). Caspase-3 activity in cell lysates was determined using a colorimetric Caspase-3 Assay System (Biovision). Cell viability was determined using either CellTiter Aqueous Assay (Promega) or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method [19].

Statistical analysis. Student's *t*-test was used with $P < 0.05$ considered significant.

Results and discussion

Tunicamycin- and thapsigargin-induced SEPS1 expression

We previously identified that human *SEPS1* promoter contains a putative ER stress response element (ERSE) [20]. This result suggests that SEPS1 expression and function can be regulated by ER stress. We first examined the effect of pharmacological ER stress agents on SEPS1 expression in macrophages. RAW264.7 cells were challenged with various concentration of pharmacological ER stress agents tunicamycin (Tm), an inhibitor of *N*-glycosylation, and thapsigargin (Tg), an inhibitor of ER Ca^{2+} ATPase activity for 24 h [21–23]. RAW264.7 cells treated with Tm or Tg resulted in elevated SEPS1 protein levels in a dose-dependent manner with maximum induction at 10 μg/ml or 5 μM, respectively (Fig. 1A). Tm- or Tg-induced ER stress in RAW264.7 cells was evidenced by a dose-dependent phosphorylation of ER stress response proteins PERK and eukaryotic initiation factor 2α (eIF2α) (Fig. 1A). SEPS1 expression in RW264.7 cells was also dependent on the time of treatment with 10 μg/ml Tm or 5 μM Tg with maximum induction after 8 h of treatment (Fig. 1B). Time-dependent induction of ER stress in Tm or Tg treated RAW264.7 cells was evidenced by induction of the two ER stress–response proteins GRP78 and CHOP (Fig. 1B). *SEPS1* mRNA levels in RAW264.7 cells were also observed to be markedly induced by Tm or Tg treatment (data not shown). ER stress-dependent induction of SEPS1 in RAW264.7 cells was further confirmed by challenging cells with other ER stress agents, including dithiothreitol (DTT) and homocysteine (Hcy) for 24 h. Both 2 mM DTT and 0.1 mM Hcy also induced SEPS1 expression in RAW264.7 cells (Fig. 1C). DTT- and Hcy-induced ER stress in RAW264.7 cells was evidenced by elevated level of GRP78 (Fig. 1C). These results clearly show that SEPS1 expression is up-regulated by ER stress agents in macrophages. Since prolonged exposure of cells to ER stress is known to trigger apoptosis [8,13,14], we next questioned whether non-ER stress component apoptotic signals are able to induce SEPS1 expression. RAW264.7 cells were challenged with non-ER stress component apoptotic agents, including staurosporine (STS), a broad kinase inhibitor and potent apoptosis inducer, and anti-Fas, a mitochondrial-targeted apoptotic inducer, for 18 h. As expected, treatment of RAW264.7 cells with 5 μM Tg resulted in an induction of SEPS1 and GRP78 (Fig. 1D). However, we found that non-ER stress component apoptotic signals tested in this study had little or no effect on SEPS1 expression in RAW264.7 cells. Treatment of RAW264.7 cells with STS resulted in slightly elevated levels of SEPS1 and GRP78 compared with those induced by Tg treatment. Moreover, anti-Fas treatment did not show any change in both SEPS1 and GRP78 expression in

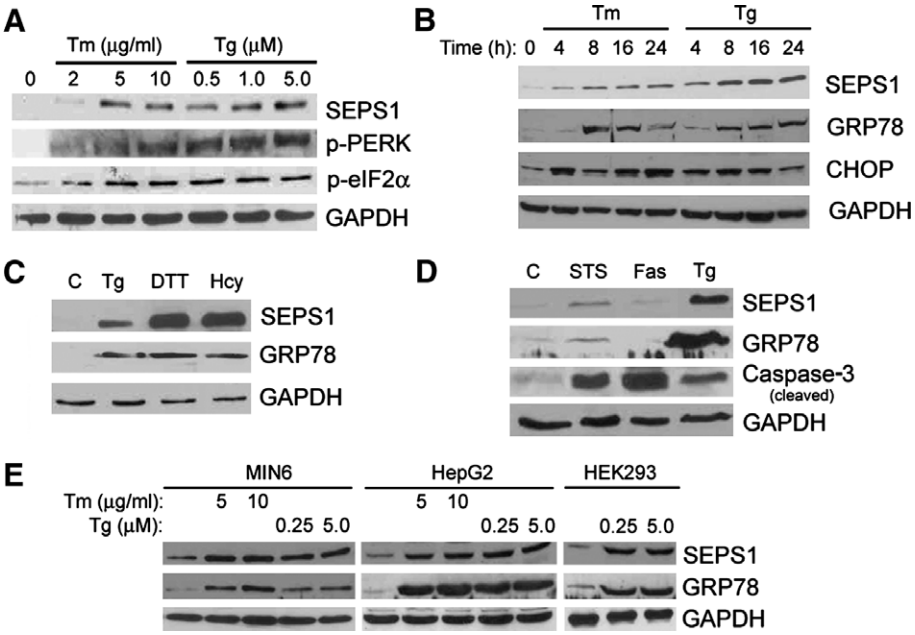

Fig. 1. Pharmacological ER stress agents-induced SEPS1 expression. RAW264.7 cells were exposed to the indicated concentrations of tunicamycin (Tm) and tunicamycin (Tm) for 24 h (A), Tm (10 μg/ml) or Tg (5 μM) for the indicated times (B), or Tm (10 μg/ml), Tg (5 μM), DTT (2.5 mM) or homocysteine (Hcy) (0.1 mM) for 24 h (C). (D) RAW264.7 cells were also exposed to staurosporine (STS) (1 μM), Fas-antibody (0.5 μg/ml) or Tg (5 μM), for 18 h. (E) MIN6, HepG2 and HEK293 cells were exposed to with indicated concentration of Tm or Tg for 24 h. Cell lysates isolated from these cells were probed with anti-SEPS1, anti-GRP78, anti-CHOP, anti-phospho-PERK, anti-phospho-eIF2α or anti-GAPDH antibodies.

RAW264.7 cells (Fig. 1D). Tg-, STS- and Fas-induced apoptosis was evidenced by the generation of cleaved caspase-3 (Fig. 1D). Although the underlying basis for ER stress agent dependent induction of SEPS1 is not known at this time, this result indicates that SEPS1 expression is largely dependent upon ER stress with less significant association with STS- and Fas-induced general apoptotic signals in macrophages. To further support this notion, it will be of interest to examine whether intrinsic ER stress signal triggered by accumulated misfolded proteins [7–12] or free cholesterol [24] in the ER could induce SEPS1 expression in macrophages.

Next, we tested whether induction of SEPS1 by ER stress agents can also be seen in other cell types. We employed MIN6 mouse pancreatic β-cells, HepG2 hepatoma cells and HEK 293 cells to test Tm- and Tg-dependent induction of SEPS1. Fig. 1E shows a dose-dependent effect of Tm and Tg on SEPS1 expression in various cell types tested in this study. This result implies that induction of SEPS1 could be a common indicator of cells under the condition of ER stress.

SEPS1 protects macrophages from Tm- and Tg-induced cytotoxicity and apoptosis

To understand the physiological function of SEP1 we determined the consequence of SEPS1 overexpression on pharmacological ER stress agent-induced cytotoxicity and apoptosis in RAW264.7 cells. Cells were transiently transfected with either control or flag-SEPS1 expression vector, followed by challenging to serum-free medium containing various concentration of Tm (0–10 μg/ml) or Tg (0–5 μM) for 24 h. Fig. 2A confirms transient expression of flag-tagged SEPS1 fusion protein in RAW264.7 cells. We also observed that overexpression of SEPS1 in RAW264.7 cells is associated with reduced levels of Tm- and Tg-mediated GRP78 expression compared with those in control vector transfected cells (Fig. 2A). We then assessed the Tm- and Tg-induced cytotoxicity of these cells by measuring the activity of lactate dehydrogenase (LDH) in the medium released from the cells. Consistent with previous reports on the cytotoxic action of Tm and Tg [25,26], treatment of control vector transfected RAW264.7 cells with Tm and Tg resulted in a dose-dependent increase in LDH activity in the cell culture media (Fig. 2B). However, Fig. 2B illustrates that cells transfected with flag-SEPS1 expression vector were resistant to both Tm- and Tg-induced cytotoxicity indicating a cytoprotective action of SEPS1 during ER stress. Moreover, the basal cytotoxicity of cells transfected with flag-SEPS1 expression vector was also lower than that of control vector transfected cells. This result implies that SEPS1 could also protect RAW264.7 cells against cytotoxicity caused by serum depletion.

Both pharmacological ER stress agents Tm and Tg have been known to promote apoptosis via activating ER stress signaling pathways [23]. Thus, we next further examined the consequence of SEPS1 overexpression on ER stress-induced apoptosis in RAW264.7 cells. We first determined the effect of SEPS1 overexpression on Tm- or Tg-induced

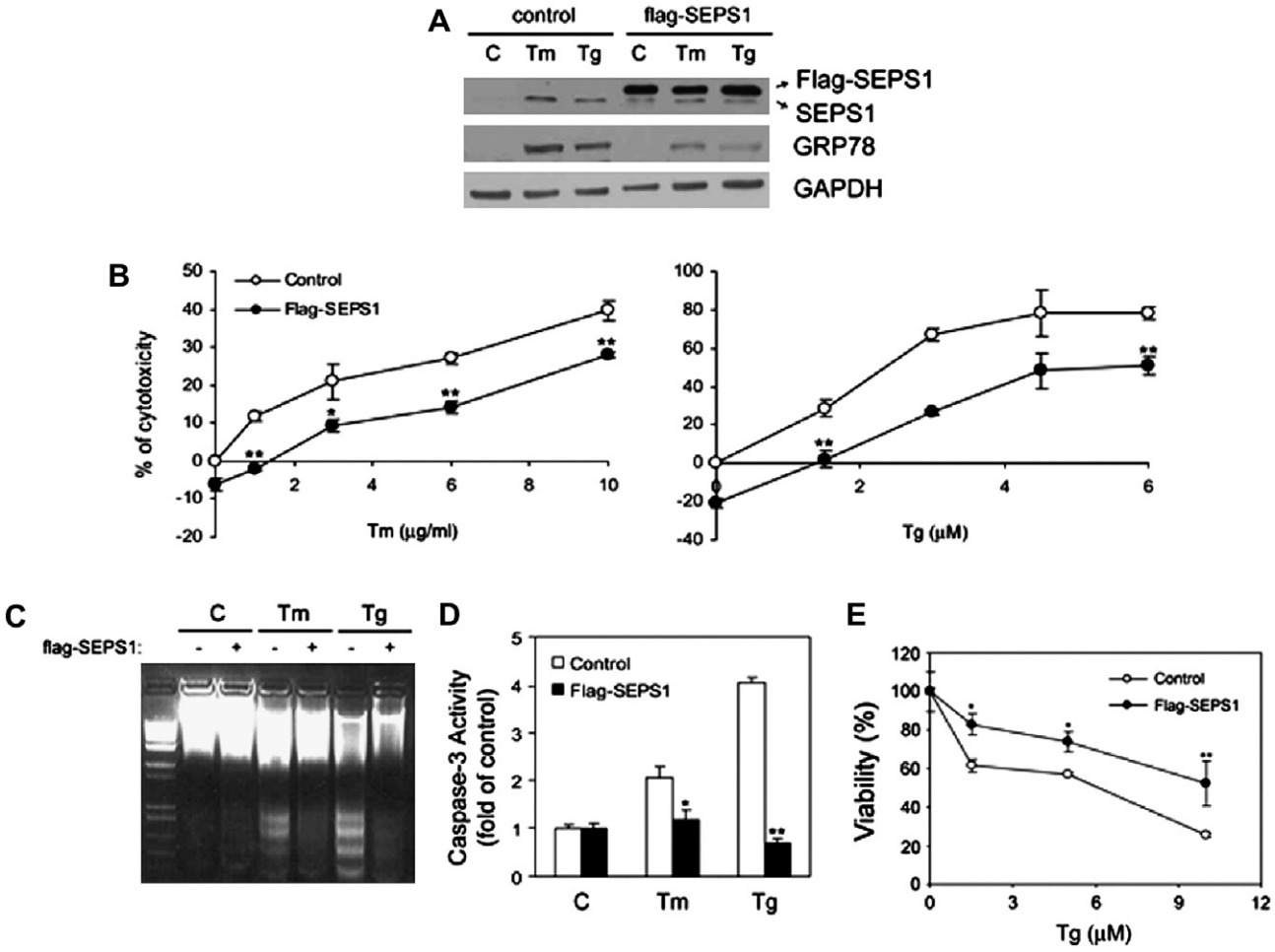

Fig. 2. Effect of SEPS1 overexpression on pharmacological ER stress agents-induced cytotoxicity and apoptosis. (A) RAW264.7 cells were transfected with pcDNA3.1 control vector (control) or expression vector containing flag-tagged human *SEPS1* cDNA sequence (flag-SEPS1) as described under Materials and methods. Cell lysates isolated from these cells were probed with anti-SEPS1, anti-GRP78 or anti-GAPDH antibodies. (B) RAW264.7 cells transfected with pcDNA3.1 control vector (control) or expression vector for flag-SEPS1 were exposed to increased concentrations of Tm or Tg for 24 h and the cellular cytotoxicity was determined by measuring lactate dehydrogenase (LDH) activity in the media released from cells. (C) The generation of fragmented DNA in expression vector transfected RAW264.7 cells treated with Tm (10 μg/ml) or Tg (5 μM) for 24 h was visualized by agarose gel electrophoresis. The first lane of the agarose gel contained 0.5 μg 1 kb plus DNA ladder (Invitrogen). (D) Caspase-3 activity was determined in the cell lysate as described under Materials and methods. (E) Expression vector transfected cells were challenged with indicated concentration of Tg for 24 h. Cell viability was determined in the cell lysates using CellTiter Aqueous Assay. Data are presented as means ± SEM. ($^{*}P < 0.05$; $^{**}P < 0.01$) and the experiment was repeated twice with similar results.

DNA fragmentation in RAW264.7 cells. As described in Materials and methods, the genomic DNA was isolated from cells transfected with control or flag-SEPS1 expression vector followed by challenging with 10 μg/ml Tm or 5 μM Tg for 24 h. The fragmented DNA was then visualized in an ethidium bromide-stained agarose gel. Consistent with previous report [27], both treatment of RAW264.7 cells with Tm- and Tg resulted in generation of fragmented DNA with size of 1 kb or less, an indicative of apoptosis (Fig. 2C). However, DNA fragmentation was barely detectable in Tm- or Tg-treated SEPS1 overexpressing cells (Fig. 2C). Effect of SEPS1 overexpression on Tm- or Tg-induced apoptosis was further determined by measuring caspase-3 activity in cell lysate isolated from RAW264.7 cells treated with 10 μg/ml Tm or 5 μM Tg for 24 h. As expected, the levels of caspase-3 activity in control cells challenged with Tm or Tg were approximately 2- or 4-fold greater, respectively, when compared with non-treated control cells (Fig. 2D). However, caspase-3 activity in RAW264.7 cells transfected with flag-SEPS1 expression vector was not influenced by Tm and Tg treatment (Fig. 2D). We also assessed the consequence of SEPS1 overexpression on RAW264.7 cell death induced by Tg treatment. Cells transfected with control vector exhibited a dose-dependent decrease in cell viability after 24 h of treatment with increasing concentration of Tg (0–10 μM) resulting in approximately 30% of cell viability at 10 μM Tg. However, RAW264.7 cells transfected with flag-SEPS1 expression vector were resistant to Tg-induced cell death with approximately 60% of cell viability at 10 μM Tg (Fig. 2E). Taken together, the results presented above suggest that SEPS1 protects macrophages from

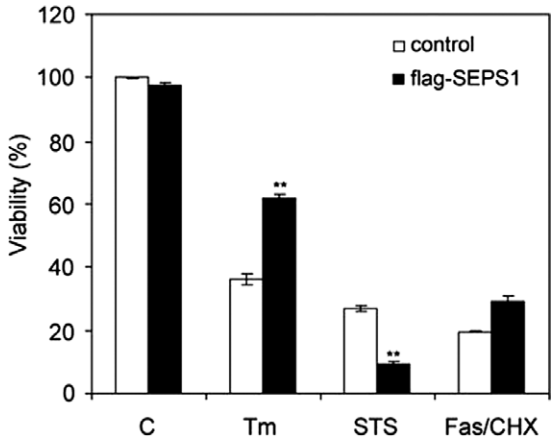

Fig. 3. Effect of SEPS1 on non-ER stress-induced cell death. RAW264.7 cells transfected with expression vectors for control pcDNA3.1 (control) or flag-SEPS1 were exposed to Tm (10 μg/ml), staurosporine (STS) (1 μM), or Fas-antibody (0.5 μg/ml) plus cycloheximide (CHX) (10 μg/ml) for 18 h. Cell viability was determined using CellTiter Aqueous Assay. Data are presented as means ± SEM. ($^{**}P < 0.01$) and the experiment was repeated twice with similar results.

pharmacological ER stress agent-induced apoptosis thereby promoting cell survival during ER stress.

Protective action of SEPS1 against non-ER stress-induced macrophage death

We next attempted to test whether SEPS1 overexpression also improves RAW264.7 cell viability against non-ER stress-induced cell death signals. RAW264.7 cells transfected with control vector or flag-SEPS1 expression vector were challenged with STS (1 μM) or Fas-specific antibody (0.5 μg/ml) plus cycloheximide (CHX) (10 μg/ml), as well as an ER stress agent Tm (10 μg/ml) for 18 h. As expected, RAW264.7 cells transfected with flag-SEPS1 expression vector exhibited improved cell viability compared with cells transfected with control vector against Tm-induced death (Fig. 3). SEPS1 overexpression, however, did not show significant protective effect against STS- and Fas-induced cell death (Fig. 3). Indeed, cells transfected with flag-SEPS1 expression vector exhibited increased sensitivity to STS-induced cell death. This result suggests that SEPS1 protects macrophages largely against pharmacological ER stress agent-induced apoptosis with less effect on non-ER stress-induced cell death signals.

Role of endogenous SEPS1 in ER stress-induced cell death

We next determined the role of endogenous *SEPS1* mRNA in pharmacological ER stress agent-induced cell death in macrophages. We tested the consequence of siRNA-mediated *SEPS1* suppression on Tm-induced RAW264.7 cell death. After 18 h of *SEPS1* siRNA transfection into RAW264.7 cells, *SEPS1* mRNA level was suppressed approximately by 60% (Fig. 4A). We then challenged these cells with various concentration of Tm (0–2 μg/ml) for 18 h and cell viability was determined using MTT assay. Control scrambled siRNA transfected RAW264.7 cells showed a dose-dependent cell death upon increasing concentration of Tm with approximately 60% of cell viability at 1 μg/ml Tm (Fig. 4B). However, siRNA-mediated suppression of endogenous *SEPS1* mRNA resulted in sensitization of cells to Tm-induced cell death with approximately 40% of cell viability at 1 μg/ml Tm (Fig. 4B). This result suggests that endogenous *SEPS1* is required for macrophage survival against Tm-induced cell death. The remaining viability of *SEPS1* siRNA transfected cells exposed to Tm could be due to an incomplete *SEPS1* mRNA suppression or the presence of SEPS1-independent survival mechanisms. Nevertheless, this result together with the data shown in Fig. 2 clearly demonstrate that SEPS1 plays an important role in promoting macro-

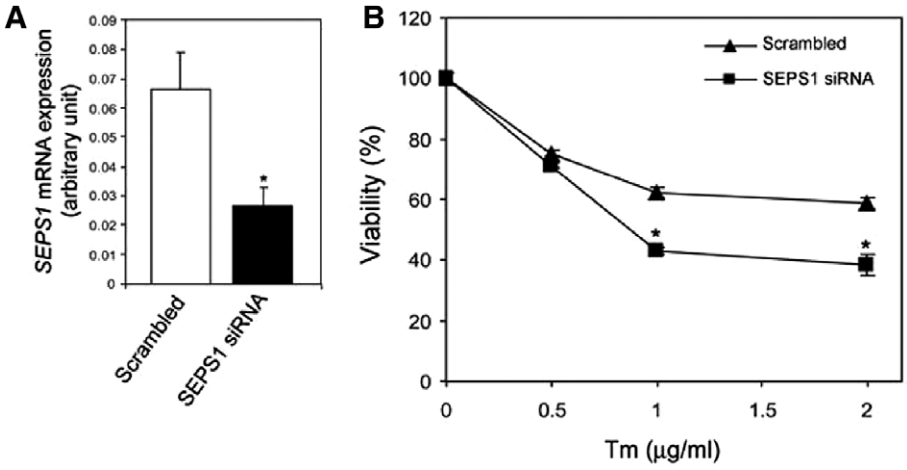

Fig. 4. Effect of *SEPS1* mRNA suppression on tunicamycin-induced cell death. RAW264.7 cells transfected with scrambled or *SEPS1* siRNA (20 nM) for 24 h were exposed to indicated concentration of Tm for 24 h. (A) *SEPS1* mRNA levels in RAW264.7 cells transfected with scrambled or *SEPS1* siRNA were determined by qRT-PCR and normalized against cyclophilin. (B) Cell viability of these cells was determined by MTT assay. Data are presented as means ± SEM. ($^{*}P < 0.05$) and the experiment was repeated twice with similar results.

phage survival during the condition of pharmacological agent-induced ER stress.

Recently, SEPS1/VIMP has been identified as a novel ER membrane protein interacting with VCP/p97 and Derlin-1, key protein components of the retrotranslocation/ER-associated degradation (ERAD) machinery [1–3]. Given that retrotranslocation/ERAD is a critical step in counteracting ER stress and maintaining the ER homeostasis, the protective action of SEPS1 against ER stress-induced cell death presented in this study could be, at least in part, a potential mechanism by which retrotranslocation/ERAD alleviates ER stress-associated dysfunction. It will be of great interest to study the role of SEPS1 in VCP/p97-mediated retrotranslocation/ERAD.

In summary, our results provide evidence that SEPS1 is a novel ER stress-induced protein that attenuates pharmacological ER stress-induced cytotoxicity and apoptosis, resulting in promotion of cell survival. These results suggest that modulation of SEPS1 expression could be a novel mechanism to control ER stress-induced cell apoptosis.

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