SDF2L1 interacts with the ER-associated degradation machinery and retards the degradation of mutant proinsulin in pancreatic β-cells

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Summary

Stromal cell-derived factor 2-like 1 (SDF2L1) is an endoplasmic reticulum (ER)-localized protein whose function is undefined. Here we show that SDF2L1 protein levels are increased in response to ER stress-inducing compounds, but not other cell stressors that we tested in insulinoma cell lines. SDF2L1 protein levels were also induced by expression of misfolded proinsulin in insulinoma cells and in islets from diabetic mice. Immunoprecipitation and binding assays demonstrated that SDF2L1 interacts with the ER chaperone GRP78/BiP, the ER-associated degradation (ERAD) machinery and with misfolded proinsulin. Unexpectedly, knockdown of SDF2L1 in INS-1 (insulin 2 C96Y-GFP) cells increased the degradation kinetics of mutant proinsulin, suggesting that SDF2L1 regulates substrate availability for the ERAD system. We suggest that SDF2L1 increases the time that misfolded proteins have to achieve a correctly folded conformation and thus that SDF2L1 can act as a buffer for substrate availability for ERAD in pancreatic β-cells.

Key words: SDF2L1, ER stress, ER associated degradation, ERAD, Insulin, Proinsulin, Pancreatic β-cells

Introduction

Secretory and membrane protein biosynthesis is mediated by cytosolic ribosomes bound to the endoplasmic reticulum (ER) membrane. Newly synthesized proteins are translocated across the ER membrane, where they encounter chaperone proteins that assist in protein folding and enzymes that produce modifications such as N-linked glycosylation or disulfide bond formation (Braakman and Bulleid, 2011). Once membrane and secretory proteins achieve a normal, correctly folded state they are packaged into COPII vesicles that bud from transitional sites of the ER for transport along the secretory pathway. If, however, the proteins are unable to fold correctly they are targeted for degradation by the ER-associated degradation (ERAD) system and retrotranslocated out of the ER for ubiquitin proteasome mediated degradation (Vembar and Brodsky, 2008).

The ER of mammalian cells has a certain capacity to fold client proteins, which can be overwhelmed and various factors can perturb ER homeostasis such that unfolded, misfolded and aggregated proteins accumulate. Under such a situation eukaryotic cells activate the unfolded protein response (UPR) that serves to counteract the build up of misfolded proteins by transiently attenuating protein translation, followed by induction of a transcriptional response that increases the levels of genes involved in ER and secretory pathway function (Walter and Ron, 2011). Indeed, the most prominent genes induced by the UPR encode for ER chaperones and associated proteins. However, there is wide variability in the expression and relative abundance of various ER chaperone and co-chaperone proteins in different eukaryotic cells (Powers et al., 2009), likely due to the nature of the protein products produced by different cell types. Thus, highly specialized cells such as insulin-secreting pancreatic β-cells have a unique chaperone expression profile compared to other cell types (Powers et al., 2009).

To elucidate the UPR in pancreatic β-cells we recently identified gene expression changes resulting from the production of mutant proinsulin in an insulinoma cell culture model (Hartley et al., 2010). Expression of Akita mutant insulin 2 (C96Y) resulted in induction of various chaperones, co-chaperones, oxidoreductases, ERAD components and various other genes. One of the most highly induced genes at the mRNA level encoded for the protein stromal cell-derived factor 2-like 1 (SDF2L1). SDF2L1 has previously been shown to be an ER localized, ER stress-inducible protein found associated with ER chaperone complexes in mammalian cells (Bies et al., 2004; Fukuda et al., 2001; Meunier et al., 2002). The expression and function of this protein in islets and pancreatic β-cells has not been reported. Here we show that SDF2L1 is induced by ER stress caused by various conditions and plays a role in ERAD of misfolded proinsulin in pancreatic β-cells.

Results

SDF2L1 is induced by ER stress in insulinoma cells and in islets from diabetic mice

We examined SDF2L1 expression in pancreatic β-cell lines and rodent islets. As shown in Fig. 1A, SDF2L1 protein was detected

in human HEK293 kidney cells and in rat INS-1 832/13 insulinoma cells. In response to tunicamycin treatment, an N-linked glycosylation inhibitor that induces ER stress, the levels of SDF2L1 were increased, as was GRP78/BiP a marker of the UPR. We also tested the effect of other cellular stressors including heat shock, oxidative stress (H_2O_2), arsenic (Ars), and aminotriazole (ATZ), but none of these agents increased SDF2L1 expression (Fig. 1B). However, SDF2L1 expression was also induced by chronic exposure to the saturated free fatty acid palmitate that has previously been shown to induce ER stress in β-cells (Fig. 1C). *SDF2L1* mRNA and protein levels also increased in an INS-1 insulinoma cell line with doxycyline-inducible expression of a misfolded proinsulin molecule (insulin 2 C96Y-GFP) (Hartley et al., 2010) (Fig. 1D,E).

We also examined *SDF2L1* mRNA and SDF2L1 protein expression in islets isolated from control and diabetic mice. Islets from 8-week-old MKR mice, a model of non-obese diabetes associated with insulin resistance (Asghar et al., 2006; Fernández et al., 2001), had markedly higher *SDF2L1* mRNA (Fig. 1F) and protein expression (Fig. 1G) compared to islets from control mice. The MKR mouse expresses a dominant negative insulin-like growth factor receptor in skeletal muscle (Fernandez et al., 2001) and develops hyperglycemia by ~8 weeks of age (fed blood glucose ~8 mM in control mice versus ~15 mM in MKR mice) (Asghar et al., 2006). Proteomic analysis has revealed that various components of the ER stress response are increased in diabetic islets (Lu et al., 2008), indicating that islets in these mice are experiencing ER stress.

Overall, these results show that the SDF2L1 protein is expressed in insulinoma cells and rodent islets at relatively low levels in control cells and that expression levels are markedly induced by ER stress and by diabetes in the MKR mouse model.

SDF2L1 interacts with GRP78 and the ERAD machinery and inhibits degradation of mutant proinsulin

To identify a function for SDF2L1 in pancreatic β-cells, we utilized the inducible INS-1 (insulin 2 C96Y-GFP) insulinoma cell line previously characterized (Hartley et al., 2010), where induction of SDF2L1 is observed in response to expression of misfolded proinsulin (Fig. 1D,E). Since the mutant proinsulin C96Y-GFP is largely retained in the ER and SDF2L1 has been shown to associate with a large chaperone complex (Meunier et al., 2002), we hypothesized that SDF2L1 may interact with the mutant proinsulin. Immunoprecipitation of the mutant proinsulin from Triton X-100 detergent lysates failed to co-precipitate SDF2L1 (Fig. 2A). However, with pre-treatment of the cells with the crosslinker DSP prior to immunoprecipitation, an association was identified (Fig. 2B, lane 6). We also observed that the ER chaperone GRP78/BiP co-precipitated with mutant proinsulin, which increased with DSP pre-treatment (Fig. 2C). SDF2L1 interaction with mutant proinsulin and GRP78/BiP was also examined by binding experiments using purified protein A-tagged SDF2L1. Protein A-tagged SDF2L1 bound to agarose beads pulled down GRP78, but not mutant proinsulin in lysates from the mutant insulin expressing cells (Fig. 2D). Thus, SDF2L1 interacts with GRP78/BiP and interacts weakly with mutant proinsulin as identified by crosslinking experiments. This

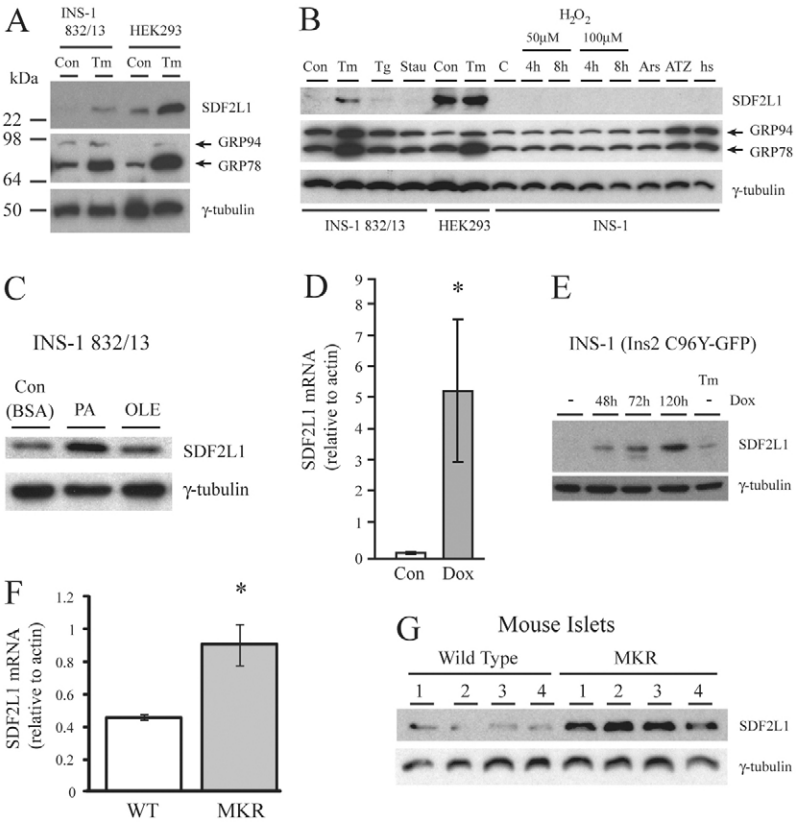

Fig. 1. SDF2L1 protein expression is induced by ER stress in pancreatic β-cell lines and rodent islets. (**A**) INS-1 832/13 and HEK293 cells were treated with tunicamycin (Tm, 2 μg/ml, 16 hours) or not (Con) and immunoblotted for the indicated proteins. (**B**) INS-1 insulinoma cells were treated with various stress-inducing compounds: tunicamycin (Tm, 2 μg/ml, 16 hours); thapsigargin (Tg, 1 μM, 4 hours); staurosporine (Stau, 0.3 mM, 16 hours); arsenate (Ars, 1 mM, 8 hours); aminotriazole (ATZ, 1 mM, 8 hours); heat shock protein (hs, 42°C, 24 hours); H_2O_2 (50 or 100 μM for 4 or 8 hours) and then immunoblotted for the indicated proteins. (**C**) INS-1 832/13 cells were treated with 1% BSA (Con, control) or 0.5 mM fatty acid (PA, palmitate; OLE, oleate) complexed to 1% BSA for 16 hours in serum-free media. Cell lysates were immunoblotted for SDF2L1 and a loading control protein (γ-tubulin). Result is representative of three independent experiments. (**D**) INS-1 clonal cell line expressing mutant proinsulin (insulin 2 C96Y-GFP) was treated with doxycyline (Dox, 2 μg/ml) for 24 hours or not (Con) and *SDF2L1* mRNA expression analyzed by real-time PCR. (n=3). (**E**) INS-1 (insulin 2 C96Y-GFP) cells were treated or not with doxycyline (2 μg/ml) for the times indicated and immunoblotted for the indicated proteins. (**F**) Islets were isolated from 8-week-old wild type (WT) mice or 8-week-old MKR mice and *SDF2L1* mRNA expression analyzed by real-time PCR. Result is from n=4 independent mice. (**G**) Islets were isolated from four individual 8-week-old wild-type mice or from four 8-week-old MKR mice and analyzed for SDF2L1 protein expression. *P<0.05, t-test.

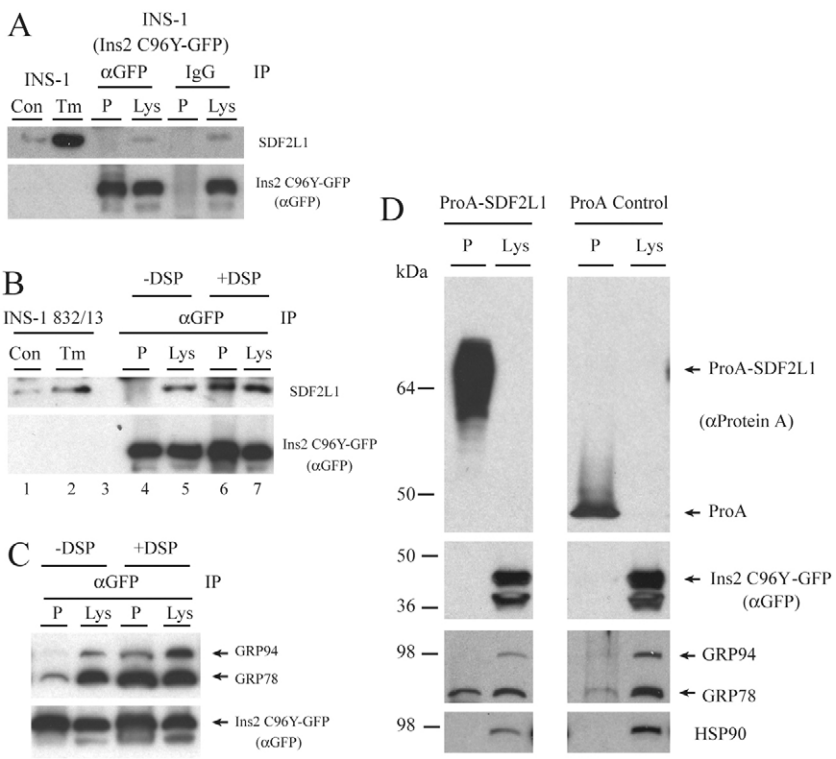

Fig. 2. SDF2L1 interacts with GRP78/BiP and with mutant proinsulin under crosslinking conditions. (**A**) INS-1 (insulin 2 C96Y-GFP) cells were induced with doxycyline for 48 hours then lysed and mutant proinsulin immunoprecipitated (IP) using mouse monoclonal anti-GFP antibodies. The pull-down (P) and lysate (Lys) fractions post IP (100% and 10% of total, respectively) were immunoblotted with GFP (rabbit polyclonal) and SDF2L1 antibodies. INS-1 cells treated with 2 μg/ml tunicamycin (Tm) for 16 hours were used as a positive control for SDF2L1 detection. (**B**) INS-1(insulin 2 C96Y-GFP) cells were treated or not with 1 mM DSP for 30 minutes prior to lysis. Lysates were immunoprecipitated using GFP antibodies; pull-down and 10% of the lysate fractions post IP were immunoblotted for the indicated proteins. INS-1 832/13 cells treated with 2 μg/ml Tm for 16 hours were used as a positive control for SDF2L1 detection. (**C**) Similar experiment as in B, although fractions were immunoblotted with an anti-KDEL antibody to detect GRP78/BiP and GRP94. (**D**) Agarose beads bound to protein-A-tagged SDF2L1 Δss, ΔHDEL (ProA-SDF2L1) or protein A (ProA Control) were incubated with lysates from INS-1 (insulin 2 C96Y-GFP) cells. The bound pull-down fraction and ~10% of the lysate fraction were resolved by SDS-PAGE and immunoblotted for the indicated proteins. Note that ProA-SDF2L1 binds GRP78, but not the other proteins examined.

is consistent with previous observations showing that SDF2L1 is found in large chaperone complexes and can interact with the GRP78/BiP co-chaperone ERj3p (Bies et al., 2004; Meunier et al., 2002) and consistent with studies showing that the Akita mutant proinsulin in mouse islets is able to interact with GRP78/BiP (Wang et al., 1999).

To identify novel SDF2L1 interacting proteins we used the protein A-tagged SDF2L1 as bait in binding studies with cell and tissue lysates followed by proteomic analysis. Using liver tissue lysate we found a specific protein interacting with protein A-tagged SDF2L1, but not protein A alone in Coomassie stained SDS-PAGE gels (not shown). By mass spectrometry we identified this protein as the AAA-type ATPase p97/VCP, a cytosolic protein known to be required in the ERAD process (Jentsch and Rumpf, 2007; Ye et al., 2005). Given that in intact cells SDF2L1 is in the lumen of the ER and p97/VCP is in the cytosol we verified the interaction by western blot analysis. Indeed, p97/VCP was pulled down by protein-A-tagged SDF2L1 from both liver and insulinoma cell lysates (Fig. 3A,D). Although the relative amount of p97/VCP pulled down was only a small fraction of the total, the pull-down is specific as abundant proteins such as GM130, tubulin (Fig. 3C) or proinsulin (Fig. 3D) were not detected in the protein A-tagged SDF2L1 pull-down. Importantly, we also identified small amounts of other ERAD components, such as Herp and Derlin 2 (Fig. 3A,B), which are membrane-associated proteins of the ERAD machinery (Oda et al., 2006; Okuda-Shimizu and Hendershot, 2007). We also examined SDF2L1 localization by cell fractionation. Similar to GRP78, SDF2L1 is primarily in the membrane fraction when cells are homogenized in sucrose buffer and total membranes are pelleted, while significant amounts of p97/VCP is in the cytosol as expected (Fig. 3E). By sucrose density fractionation of insulinoma cells we observed that p97/VCP partitioned in the lighter fractions, while the ER chaperone GRP78 partitioned in heavier fractions further down the sucrose gradient (Fig. 3F). SDF2L1 was found in the same fractions as GRP78, suggesting ER localization. Interestingly, there were a couple of fractions where all three proteins were detected, as would be expected if a subset of these molecules are interacting in the cell. Thus, SDF2L1 is able to pull-down small amounts of components of the ERAD machinery from Triton X-100 lysates from both liver and insulinoma cells and a portion of SDF2L1 co-fractionates with p97/VCP by sucrose gradient fractionation.

In previous work we showed that the mutant proinsulin fusion protein is degraded via a proteasome-dependent ERAD mechanism (Hartley et al., 2010), which is also the case for untagged Akita mutant proinsulin (Allen et al., 2004). Given that SDF2L1 associates with the ERAD system we hypothesized that SDF2L1 may play a role in the degradation of misfolded proteins in the ER. Therefore, we used the inducible mutant insulin expressing cell line INS-1 (insulin 2 C96Y-GFP) to examine the requirement of SDF2L1 in the degradation of mutant proinsulin. We first identified siRNAs that effectively knocked down SDF2L1 expression greater than 80% (Fig. 4A). We then examined mutant proinsulin expression using antibodies to the GFP tag. Steady-state levels of mutant proinsulin tended to be reduced in uninduced cells (−Dox) treated with SDF2L1 siRNA compared to control siRNA-treated cells (Fig. 4B, left side). With Dox induction (+Dox) there is a large increase in mutant proinsulin fusion protein expression, although there was no apparent difference in the levels between control and SDF2L1 siRNA-treated cells (Fig. 4B, right side). We therefore examined degradation kinetics of the mutant proinsulin in uninduced cells so as not to overwhelm the ERAD system to determine if SDF2L1 knockdown affects mutant proinsulin degradation. First, a cycloheximide degradation assay was used to monitor mutant proinsulin levels following inhibition of protein

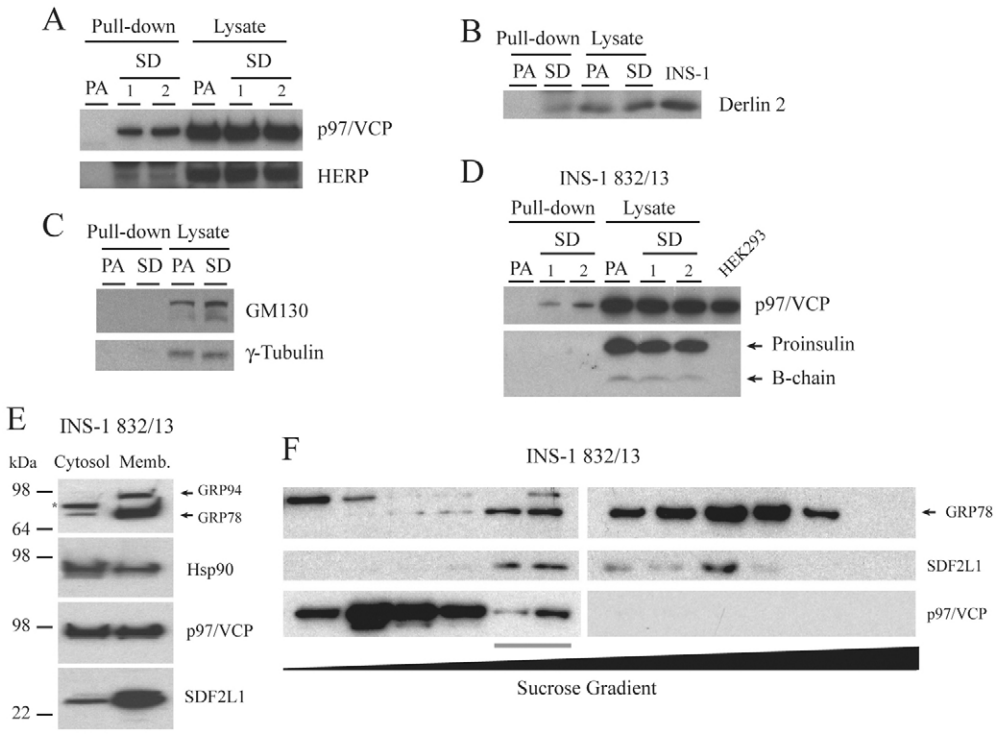

Fig. 3. SDF2L1 interacts with the ERAD machinery. (**A–C**) Agarose beads bound to protein A-tagged SDF2L1 Δss,ΔHDEL (SD) or protein A (PA) were incubated with lysate from rat liver tissue. The bound pull-down fractions and lysate fractions were resolved by SDS-PAGE and immunoblotted for p97/VCP and Herp (A), Derlin 2 (B) and two control proteins, the Golgi matrix protein GM130 and γ-tubulin (C). (**D**) Agarose beads bound to protein-A-tagged SDF2L1 Δss,ΔHDEL (SD) or protein A (PA) were incubated with protein lysate from INS-1 832/13 cells. The bound pull-down fraction and lysate fraction were resolved by SDS-PAGE and immunoblotted for p97/VCP and insulin. In each case, 100% of the pull-down fraction and ~10% of the lysate fraction was loaded. (**E**) INS-1 832/13 cells were treated with 2 μg/ml tunicamycin for 16 hours to induce SDF2L1 expression. The cells were then washed and homogenized in sucrose buffer as described in Materials and Methods. The homogenate was fractionated into total membrane (Memb) and cytosol fractions and equivalent protein amounts were immunoblotted for the indicated proteins. The KDEL antibody was used to detect GRP94, GRP78 and an unidentified protein appearing in the cytosol (asterisk). (**F**) Cells were treated and homogenized as for E. The homogenate was fractionated on a linear sucrose density gradient and equal volumes of fractions from the gradient were immunoblotted for the indicated proteins. Black bar indicates the sucrose gradient from low to high sucrose concentration and the grey bar indicates fractions where all three proteins were detected.

synthesis. Cycloheximide resulted in rapid degradation of the mutant proinsulin in control siRNA-treated cells, but did not affect expression levels of a control protein, the Golgi Matrix protein GM130 (Fig. 4C,D). Unexpectedly, in cells treated with SDF2L1 siRNAs the degradation of mutant proinsulin was faster, such that after 6 hours ~10% of the mutant proinsulin remained, compared to ~30% in control siRNA-treated cells. The effect of SDF2L1 depletion resulting in greater mutant proinsulin degradation was confirmed by pulse-chase experiments (Fig. 4E,F).

We examined p97/VCP and GRP78 expression in SDF2L1 knockdown cells to determine if expression of these proteins in the absence of SDF2L1 might account for the increased ERAD of mutant proinsulin, but no effect was observed (Fig. 4G). Finally, we tested if ER stress-induced cell apoptosis was affected by SDF2L1 knockdown (Fig. 4H). Indeed, knockdown of SDF2L1 in the insulin 2 C96Y-GFP cells modestly, but significantly, reduced apoptosis in response to mutant proinsulin expression.

Discussion

In this study we show that the ER localized soluble protein SDF2L1 is expressed in insulinoma cells and rodent islets and protein expression is induced by various conditions that cause ER stress, including pharmacological treatment (tunicamycin), misfolded proinsulin expression and diabetes in the MKR mouse islets. Although SDF2L1 is markedly induced the function of this small (~26 kDa) protein in pancreatic β-cells was unknown. Given that the protein was previously found associated with a large ER chaperone complexes (Meunier et al., 2002) we hypothesized that SDF2L1 may interact and play a role in the biosynthesis or degradation of misfolded proinsulin in pancreatic β-cells. Indeed, we found that SDF2L1 interacts with GRP78/BiP, mutant proinsulin as well as with the ERAD machinery, which is involved in the retrotranslocation and degradation of misfolded ER proteins.

However, contrary to expectations, depletion of SDF2L1 in the mutant insulin expressing insulinoma cell line resulted in enhanced mutant proinsulin degradation. Thus, it appears SDF2L1 may play a role in retarding, or limiting the accessibility of misfolded proinsulin for ERAD. We speculate that such a process may be employed in an attempt to increase the time the mutant proinsulin resides in the ER thereby allowing it a greater chance to fold correctly. Clearly this process is futile in the case of mutant proinsulin, since it is terminally misfolded and eventually results in the induction of ER stress-induced apoptosis. Interestingly, knockdown of SDF2L1 reduces cell apoptosis in the

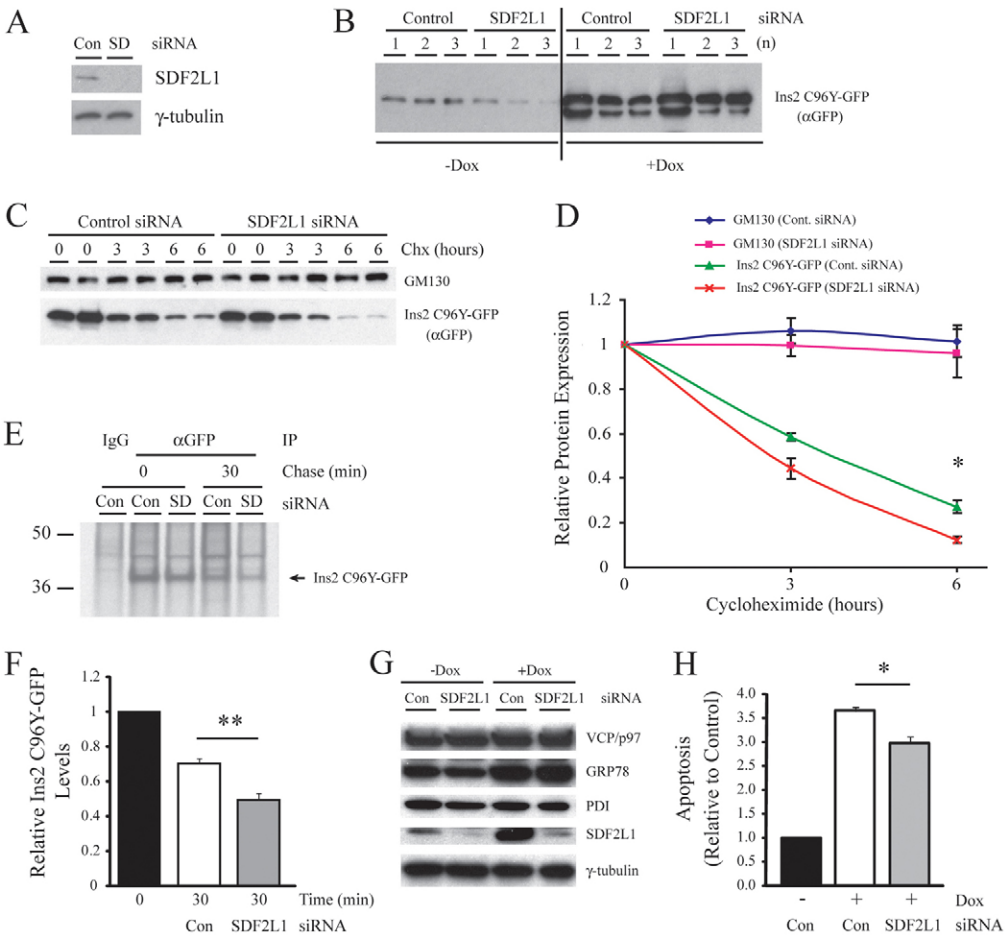

Fig. 4. SDF2L1 knockdown enhances misfolded proinsulin degradation. (**A**) INS-1 (insulin 2 C96Y-GFP) cells were transfected with siRNAs directed to SDF2L1 (SD) or with control siRNAs (directed to *LacZ*) (10 nM) for 48 hours. Following the treatment, cells were lysed and proteins resolved by SDS-PAGE and immunoblotted for the indicated proteins. (**B**) INS-1 (insulin 2 C96Y-GFP) cells were transfected with siRNAs directed to SDF2L1 or with control siRNAs (directed to *LacZ*) for 48 hours in the presence or absence of doxycycline (Dox). Lysates were immunoblotted with antibodies to GFP to detect the mutant proinsulin fusion protein. Results from three separate experiments are shown. (**C**) INS-1 (insulin 2 C96Y-GFP) cells were transfected with siRNAs directed to SDF2L1 or with control siRNAs (directed to the luciferase gene) for 48 hours. The cells were then untreated (zero time point) or treated with 100 μM cycloheximide (Chx) for 3 hours or 6 hours. The cells were subsequently lysed and immunoblotted with GFP antibodies (to detect the mutant proinsulin fusion protein) and GM130. Note the reduction in mutant proinsulin protein levels with time. (**D**) The GM130 and mutant proinsulin-GFP levels in the SDF2L1 siRNA and control siRNA conditions were quantified and the relative levels normalized to the starting amount at time zero (*$P<0.05$ for mutant proinsulin in SDF2L1 siRNA versus control siRNA, *t*-test; $n=3$). (**E**) INS-1 (insulin 2 C96Y-GFP) cells were transfected with siRNAs directed to SDF2L1 or with control siRNAs (directed to *LacZ*) for 48 hours. The cells were labelled with ^{35}[S]methionine/cysteine for 5 minutes and chased for 0 minutes or 30 minutes prior to immunoprecipitation with anti-GFP antibodies (representative of three experiments). (**F**) The mutant proinsulin-GFP protein levels in E were quantified and expressed relative to time zero. (**G**) INS-1 (insulin 2 C96Y-GFP) cells were transfected with siRNAs directed to SDF2L1 or with control siRNAs (directed to *Luc*) for 48 hours in the presence or absence of Dox. Cell lysates were prepared and immunoblotted for the indicated proteins. (**H**) INS-1 (insulin 2 C96Y-GFP) cells were transfected with siRNAs directed to SDF2L1 or with control siRNA (directed to *LacZ*) for 48 hours in the presence or absence of Dox. Relative cell apoptosis was monitored by the Cell Death ELISA kit as described in Materials and Methods. *$P<0.05$, **$P<0.01$; *t*-test.

cell population possibly due to enhanced ERAD of the misfolded proinsulin, which might spare some cells from apoptotic cell death. However, in normal β-cells induction of SDF2L1 as part of the ER stress response may be beneficial for proteins that have a difficult time folding and require additional time. Thus, *in vivo*, this mechanism may give partially misfolded or unfolded proteins a better chance in obtaining a proper fold and thus ER export instead of degradation via the ERAD pathway.

Such a mechanism may also explain the relatively low levels of SDF2L1 in basal insulinoma and islet cells and its marked upregulation in response to ER stress. Pancreatic β-cells produce relatively little proinsulin during basal (low glucose) conditions, but in response to high glucose proinsulin translation is stimulated >10-fold (Steiner et al., 2009; Uchizono et al., 2007). This increases the burden on ER chaperone capacity and physiological induction of the UPR (Elouil et al., 2007). The resulting increase in SDF2L1 may provide added time for misfolded proteins to fold correctly, or potentially time so that chaperone capacity increases to better handle protein load as part of the ER stress response.

Although studies on SDF2L1 are limited, recent reports have identified additional protein interactions. SDF2L1 has been found

to interact with defensins (Tongaonkar and Selsted, 2009). These small proteins are similar to proinsulin in that they are non-glycosylated, but contain disulfide bonds. They are produced in large amounts by various cell types including neutrophils and monocytes, especially when stimulated. Thus, SDF2L1 could provide protection for these abundant proteins in the ER from ERAD, particularly under stimulatory conditions. In addition, recent studies have shown that the plant homologue of SDF2L1 (SDF2) is also induced by ER stress and increased in fast growing and differentiating cells with high secretory protein capacity (Schott et al., 2010). Interestingly, loss of SDF2 expression results in lower protein expression, ER retention and degradation of the cell surface protein leucine-rich repeat receptor kinase EFR in *Arabidopsis* (Nekrasov et al., 2009). EFR is actively degraded in SDF2 null cells, suggesting that SDF2 is required to prevent EFR degradation. This is consistent with our results in mutant insulin expressing cells, where SDF2L1 depletion leads to faster mutant proinsulin degradation. However, the fact that EFR ER export is blocked in SDF2 null cells, suggests that the protein may also be required for normal EFR secretion.

In summary, we show that SDF2L1 protein expression is induced by ER stress in insulinoma cell lines and rodent islets. Using a mutant proinsulin fusion protein as a model substrate we show that SDF2L1 depletion results in enhanced ERAD. Thus, SDF2L1 may increase the time misfolded proteins have to achieve a correctly folded conformation and therefore SDF2L1 may act as a buffer for substrate availability for ERAD in pancreatic β-cells.

Materials and Methods

Cell culture and rodent islet isolation

Rat INS-1 insulinoma cells were obtained from Dr Claus Wollheim (University of Geneva) (Asfari et al., 1992). INS1 832/13 insulinoma cells were obtained from Dr Chris Newgard (Duke University) (Hohmeier et al., 2000). INS-1 (insulin 2 C96Y-GFP) cells (clone number 4S2) were generated as described (Hartley et al., 2010). These cell lines were maintained as described in the references. Islets were isolated from rat pancreas as described previously (MacDonald et al., 2001). Islets from 8-week-old control mice and MKR mice were obtained as described previously (Asghar et al., 2006). Pellets of ~20 islets were lysed in RIPA buffer (+0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 nM okadaic acid, 2 mM sodium vanadate, 10 mM sodium fluoride) on ice for 1 hour, followed by a 10-minute centrifugation at 13,200 rpm at 4℃. A Pierce BCA protein assay was used for protein quantification.

Immunoprecipitation

INS-1 (insulin 2 C96Y-GFP) cells (Hartley et al., 2010) were treated with doxycycyline (2 μg/ml) for 48 hours followed by incubation with 1 mM dithiobis(succinimidyl propionate) (DSP) (Sigma) for 30 minutes (in DSP crosslinking experiments). The cells were washed in PBS and lysed in ice-cold lysis buffer (1% Triton X-100, 20 mM HEPES (pH 7.4), 100 mM KCl, 2 mM EDTA, 1 mM PMSF plus Roche complete protease inhibitors) for 15 minutes on ice, then centrifuged in a microfuge at 13,200 rpm for 15 minutes at 4℃. The Triton-soluble supernatant was used for immunoprecipitation of the mutant proinsulin fusion protein using ~100 μg of lysate and incubation with 5 μg of mouse mononclonal anti-GFP antibody (Clontech, 6323681) or control mouse IgG antibodies overnight at 4℃. Samples were subjected to immunoisolation with Protein G Dynabeads (Invitrogen) for 2 hours at 4℃. The immunoprecipitate pull-down fraction and a portion of the lysate fraction were analyzed by western blot analysis as indicated in the figure legends.

SDF2L1 cloning and generation of protein A-SDF2L1 (Δss;ΔHDEL) fusion protein

Full-length rat SDF2L1 (633 bp) was reverse transcribed from total RNA from INS-1 cells using Qiagen OneStep RT-PCR kit. The primers were: forward ATGTTGGGCGCGAGCCGC and reverse TCAGAGTTCATCGTGACCTGT. The cDNA fragment was gel purified using Qiagen gel Extraction kit and cloned into TOPO-TA vector (Invitrogen). To produce the protein A-SDF2L1 fusion protein lacking the SDF2L1 signal sequence (ss) and C-terminal HDEL retention sequence, SDF2L1 was amplified with primers encoding a 5′-*Fse*I site and a 3′-*Not*1 site: The primers used were: forward CCCACGAAGGCCGG-CCCTCGAAGGCCAGCGCCGGGCTA; reverse TTTTCCTTTTGCGGCCGC-CTAACCTGTGGAGGGATCTGCTCC. The PCR product was purified and cloned into the protein A expression vector (pA-IRES; obtained from Dr James Rini, University of Toronto) at the *Fse*1/*Not*1 sites and sequenced. This vector contains a transin ER targeting signal sequence upstream of the protein A. The SDF2L1 cDNA was inserted downstream of the protein A to generate the fusion protein protein A-SDF2L1 (Δss;ΔHDEL). To express the fusion protein for purification, HEK 293 cells were transiently transfected with pA-IRES-SDF2L1 (Δss;ΔHDEL) and stable clones were selected using puromycin resistance (10 μg/ml). The clone with highest expression and secretion (clone pA-SDF2L1#7) of the protein A-SDF2L1 (Δss;ΔHDEL) fusion protein was used for purification from either cell lysates or culture supernatant using rabbit IgG agarose beads.

Protein A-SDF2L1 binding assays and proteomic analysis

First, protein A-SDF2L1 (Δss;ΔHDEL) fusion protein was purified using rabbit IgG-agarose beads from supernatant of HEK293 (clone pA-SDF2L1#7) cells grown to confluence in a 10-cm dish. Control protein A-agarose beads were treated similarly. Beads bound with either protein A-SDF2L1 (Δss;ΔHDEL) or protein A alone were then used in binding assays by incubating the beads with lysates prepared from INS-1 (insulin 2 C96Y-GFP) cells treated with Dox for 48 hours. Cells were washed in PBS and lysed in ice-cold lysis buffer (1% Triton X-100, 20 mM HEPES (pH 7.4), 100 mM KCl, 2 mM EDTA, 1 mM PMSF plus Roche protease inhibitors). Beads were incubated with lysates overnight at 4℃, then washed with lysis buffer three times for 5 minutes at 4℃. The beads (pull-down fraction) were then boiled for 5 minutes in sample buffer. The pull-down fraction and a portion of the lysate fraction were analyzed by western blot analysis as indicated in the figure legends.

In some binding experiments lysates from INS-1 832/13 cells or rat liver tissue were used. Rat liver tissue lysates were prepared by homogenization of the minced tissue in 250 mM sucrose, 4 mM HEPES, 1 mM $MgCl_2$, pH 7.4 supplemented with 1 mM PMSF and protease inhibitors (Roche) using a Dounce homogenizer, followed by centrifugation in a microfuge (5 minutes, 10,000 rpm) to remove debris. The supernatant was mixed with 2× concentrated Triton X-100 lysis buffer for 1 hour at 4℃ and centrifuged again. The protein concentration was measured and the lysate was used in binding assays. To identify interacting proteins with SDF2L1, a binding assay was conducted using liver tissue lysate and samples were resolved by SDS-PAGE and Coomassie stained. A band present in protein A-SDF2L1 (Δss;ΔHDEL) pull-downs, but not protein A control was excised and the gel was subjected to trypsin digestion and mass spectrometry analysis at the Advanced Protein Technology Centre (the Hospital for Sick Children, Toronto). p97/VCP was identified from 12 unique peptides with 20% coverage.

Cell fractionation

For membrane and cytosol preparation INS-1 832/13 cells were placed on ice, washed with PBS then scraped in ice-cold homogenization buffer (255 mM sucrose, 20 mM HEPES, 1 mM EDTA, pH 7.4, 1 mM PMSF containing Roche protease inhibitor). Cells were transferred to Eppendorf tubes and homogenized by passing 10 times through an 18-gauge needle followed by 10 times through a 25-gauge needle. The homogenate was centrifuged at 5000×***g*** at 4℃ for 5 minutes to pellet nuclei and unbroken cells. The supernatant was centrifuged at 100,000×***g*** at 4℃ for 90 minutes. The supernatant was removed (cytosol fraction) and the pellet was resuspended in ice-cold homogenization buffer (total membrane fraction). Protein concentration in the fractions was measured using the BCA protein assay (Pierce).

Sucrose density fractionation was performed as described in (Hartley et al., 2010). Briefly, INS-1 832/13 cells were washed in PBS and scraped using a cell scraper in homogenization buffer (250 mM sucrose, 4 mM HEPES, 1 mM $MgCl_2$, 1.5 mM EDTA, pH 7.4 supplemented with 1 mM PMSF and protease inhibitors). The cells were homogenized on ice with a ball-bearing cell homogenizer and the cell homogenate was layered on a 0.45 M to 2 M continuous sucrose gradient and centrifuged for 18 hours at 30,000 rpm at 4℃. Following ultracentrifugation fractions were taken from the top of the gradient.

siRNA knockdown experiments

Control siRNAs directed to either β-galactosidase (*LacZ*) (cat. no. 12935-147) or luciferase (*Luc*) (cat. no. 12935-146) and siRNAs directed to rat SDF2L1 were obtained from Invitrogen and transfected into cells using RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Pulse-chase degradation assay

INS-1 (insulin 2 C96Y-GFP) cells were treated with *LacZ* or SDF2L1 siRNA (10 nM) for 48 hours. Cells were then labeled with Easytag Express Protein Labeling Mix; ^{35}[S]-methione/cysteine; PerkinElmer (NEG772) in methionine/cysteine-free media. After 5 minutes labeling cells were lysed or washed in PBS and incubated in RPMI1640 media for 30 minutes before lysis. Mutant proinsulin C96Y-GFP was immunoprecipitated using a mouse anti-GFP antibody from equal amounts of cell lysates. The immunoprecipitates were resolved by SDS-PAGE, the gel was dried and labelled bands detected and quantified by Phosphorimager analysis.

RNA isolation and real-time PCR analysis

Total RNA was isolated from rat INS-1 (insulin 2 C96Y-GFP) cells or mouse islets using TRIzol (Invitrogen) and real-time PCR analysis was performed using the TaqMan Gene Expression system (Life Technologies) as described previously (Zhang et al., 2009). Gene-specific primers and control β-actin primers were obtained from Life Technologies: rat SDF2L1 (Rn01404681_g1), mouse SDF2L1 (Mm00452079_m1).

Cell apoptosis

Cell apoptosis was measured using the cell death detection ELISA kit (Roche) according to the instructions provided in the kit and in reference (Hartley et al., 2010). The ELISA assay detects oligonucleosomes in the cytosol, as an indicator of apoptotic cells.

Western blot analysis

Proteins were resolved on 10% SDS-PAGE gels or 4–12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes as described in (Zhang et al., 2009). Antibodies used: from Sigma–Aldrich (SDF2L1, HPA005638), Protein A (P2921), γ-tubulin (T6557), Derlin 2 (D1194); from BD Biosciences (HSP 90, 610418), p97/VCP (612182), GM130, (G65120); GFP (Clontech, 632381); KDEL (StressGen, SPA-827); PDI (StressGen, SPA-890); insulin (Santa Cruz Biotechnology SC-9168); Herp (provided by Dr Linda Hendershot, St. Jude Children's Hospital, Memphis, TN). Western blot signals were detected by enhanced chemiluminescence (GE Healthcare) using photographic film (Kodak, Inc.). The films were scanned and band intensities quantified using Scion Image software. For quantitation of mutant proinsulin degradation (Fig. C,D) exposures were chosen where the signal was not saturated. Relative mutant proinsulin band intensities were normalized to a loading control protein (GM130).

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Author contributions

A.T. generated the majority of the experimental data, wrote and edited the manuscript. I.S. and L.Z. contributed experimental data and reviewed and edited the manuscript. E.M.A. and M.B.W. provided islets from the MKR mouse experiments in Fig. 1. Both also reviewed and edited the manuscript. A.V. contributed to the experimental plan, supervision of the project, interpretation of the data and writing of the manuscript.

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