Intraislet SLIT–ROBO signaling is required for beta-cell survival and potentiates insulin secretion

Yu Hsuan Carol Yang[a], Jocelyn E. Manning Fox[b], Kevin L. Zhang[a], Patrick E. MacDonald[b], and James D. Johnson[a,1]

[a]Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z3; and [b]Department of Pharmacology and Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada T6G 2E1

We previously cataloged putative autocrine/paracrine signaling loops in pancreatic islets, including factors best known for their roles in axon guidance. Emerging evidence points to nonneuronal roles for these factors, including the Slit–Roundabout receptor (Robo) family, in cell growth, migration, and survival. We found SLIT1 and SLIT3 in both beta cells and alpha cells, whereas SLIT2 was predominantly expressed in beta cells. ROBO1 and ROBO2 receptors were detected in beta and alpha cells. Remarkably, even modest knockdown of Slit production resulted in significant beta-cell death, demonstrating a critical autocrine/paracrine survival role for this pathway. Indeed, recombinant SLIT1, SLIT2, and SLIT3 decreased serum deprivation, cytokine, and thapsigargin-induced cell death under hyperglycemic conditions. SLIT treatment also induced a gradual release of endoplasmic reticulum luminal Ca^{2+}, suggesting a unique molecular mechanism capable of protecting beta cells from endoplasmic reticulum stress-induced apoptosis. SLIT treatment was also associated with rapid actin remodeling. SLITs potentiated glucose-stimulated insulin secretion and increased the frequency of glucose-induced Ca^{2+} oscillations. These observations point to unexpected roles for local Slit secretion in the survival and function of pancreatic beta cells. Because diabetes results from a deficiency in functional beta-cell mass, these studies may contribute to therapeutic approaches for improving beta-cell survival and function.

programmed cell death | glucose metabolism | intracellular calcium signaling

Emerging evidence highlights the important role of locally released pancreatic islet peptide factors on beta-cell mass growth, maintenance, and survival (1–12). We have published a list of 233 ligands and 234 receptors expressed in islets and/or beta cells (12). Although our list is undoubtedly not comprehensive, it provides a starting point for the investigation of factors in adult islets that had previously only been reported in other cell types or in fetal pancreas (12). We identified a group of secreted molecules known to provide axonal guidance cues during neuronal development, comprising members of the netrin, slit, semaphorin, and ephrin families (13). The parallels between cell fate decisions in neurons and the endocrine pancreas prompted us to examine some factors in detail and discover that netrin treatment modulates beta-cell survival signaling (12).

The Slit ligands and their Roundabout receptors (Robo) were discovered in *Drosophila* as regulators of axon guidance during development (14–17). Mammalian homologs of Slit and Robo with functions outside of axon guidance have since been identified (18, 19). Slit ligands have been implicated in liver, kidney, lung, and mammary development by modulating cell adhesion, migration, differentiation, and death (18, 20, 21). It was not known whether Slit–Robo signaling functions in beta cells. Here, we report that Slit expression can be regulated by stress and that local Slit production is required for beta-cell survival and optimal function via a mechanism involving endoplasmic reticulum (ER) Ca^{2+} homeostasis and actin remodeling. Our work provides examples of local guidance factors that are required for beta-cell survival and suggests avenues for protecting functional beta-cell mass.

Results

Slits Are Expressed in Adult Mouse and Human Islets. The mammalian genome contains three Slit ligands and four Robo receptors. Our bioinformatic studies identified the expression of several Slit and Robo family members in adult human and rodent pancreatic islet cells (12), and *Robo1* was identified by others as a transcript enriched in pancreatic endocrine cells during development (22). Nevertheless, no in-depth studies of these proteins have been reported. We detected *Slit1*, *Slit2*, and *Slit3* transcripts in 6- and 30 wk-old mouse islets, with higher expression of *Slit2* and *Slit3* (Fig. 1A). In human islets, *SLIT1*, *SLIT2*, and *SLIT3* expression was similar. *Robo1* and *Robo2* were expressed in MIN6 cells, mouse islets, and human islets (Fig. 1A). SLIT1, SLIT2, SLIT3, ROBO1, and ROBO2 were confirmed at the protein level (Fig. 1 B–F). Secretion of SLIT2 and SLIT3 from mouse islets was detected following incubation under 3 mM and 15 mM glucose conditions (Fig. 1B). Although SLIT1 secretion from islets and SLIT1 protein content within islets fell below the detection threshold of the ELISA kit, the protein could be detected by immunoblotting and immunostaining (Fig. 1 B–F). SLIT2 immunoreactivity was more predominant in beta cells, whereas SLIT1 and SLIT3 were detected in both beta and alpha cells with the same intensity (Fig. 1 E and F). Deconvolution microscopy illustrated that SLIT2 colocalized with insulin-positive granules, whereas SLIT1 and SLIT3 were present in distinct granules (Fig. 1F). These data suggest that SLIT2 may act in an autocrine manner on beta cells, whereas SLIT1 and SLIT3 may play both autocrine and paracrine roles.

Next, we assessed the mechanism of local Slit signaling by investigating the expression and localization of Slit receptors. ROBO1 and ROBO2 staining was detected in both beta and alpha
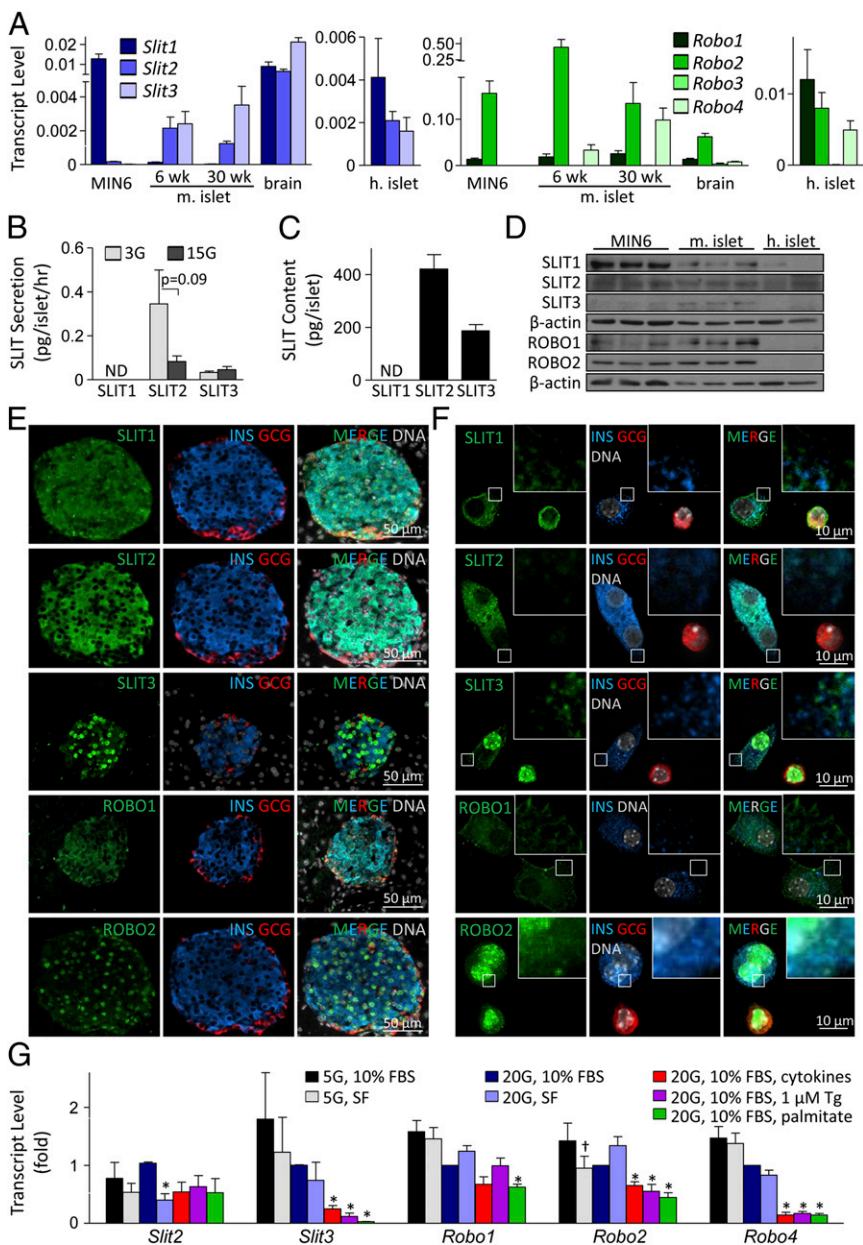
Fig. 1. Slit–Robo autocrine/paracrine network in pancreatic islet cells. (*A*) qRT-PCR of Slit and Robo expression in mouse islets, human islets, and MIN6 cells ($2^{-\Delta C_t}$ relative to actin). (*B*) SLIT1–3 secretion from mouse islets under 3 mM and 15 mM glucose ($n = 11$) (ND, not detected). (*C*) SLIT1–3 protein content in mouse islets ($n = 11$). (*D*) SLIT and ROBO immunoblot in MIN6, mouse islet, and human islet lysates. (*E* and *F*) SLIT and ROBO staining in mouse pancreas sections and mouse islet cells. (*G*) Slit and Robo expression in mouse islet cells treated with cytokine mixture (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ), 1 μM thapsigargin (Tg), 1.5 mM palmitate, or 5 mM or 20 mM glucose containing medium supplemented with 10% FBS or under serum-free (SF) conditions ($2^{-\Delta\Delta C_t}$; $n = 4$, mean ± SEM, *$P < 0.05$ compared with 20 mM glucose, 10% FBS; †$P < 0.05$ compared with 5 mM glucose, 10% FBS).

cells (Fig. 1*E*). However, although ROBO1 could be found in the plasma membrane and cytosolic compartments typical for receptors of soluble ligands, ROBO2 displayed nuclear localization (Fig. 1*F*). Further biochemical assays, such as nuclear fractionation, will be required to support this observation. Although ROBO1 has been reported to be nuclear in some cell types (23), we are unaware of reports of nuclear ROBO2. Our data suggest that SLIT ligands act via ROBO1 receptors on islet cell plasma membranes.

It is not well understood in any cell type whether the Slit–Robo system can be dynamically regulated, for example by stresses (24, 25). Quantitative RT-PCR (qRT-PCR) revealed that cytokines, thapsigargin, and palmitate down-regulated *Slit3*, and serum deprivation down-regulated *Slit2* (Fig. 1*G*). *Slit1* expression could not be consistently detected under all of the treatment conditions or up-regulated under stress. In contrast to the situation in primary islets, thapsigargin and palmitate up-regulated *Slit1* and *Slit2* in MIN6 cells (Fig. S1). *Slit3*, which is typically absent in MIN6 cells, was robustly induced by ER stress (Fig. S1). These data suggest that the production of Slit ligands

can be regulated in response to specific cellular stresses, with differential effects observed between primary islet cells and MIN6 insulinoma cells. We also examined the effects of stress on the Robo genes, and observed significant regulation (Fig. 1*G*).

Knockdown of Endogenous Slits Decreases Beta-Cell Survival. The regulation of Slit expression by stress suggests that Slit–Robo signaling play a role in beta-cell survival. A loss-of-function approach was used to determine the role of endogenous Slit ligands in beta-cell survival. In mouse islet cells, simultaneous siRNA targeting of all Slit ligands (to circumvent isoform compensation) resulted in a 48% knockdown of *Slit1*, 46% of *Slit2*, and 49% of *Slit3* mRNA (Fig. 2*A*). Remarkably, even this incomplete reduction in endogenous Slit production significantly reduced beta-cell survival in serum-free conditions (Fig. 2*B*). Similarly, knockdown of *Slit1/2/3* in MIN6 cells had significant negative effects on beta-cell survival (Fig. S2). These studies demonstrate that the local production of Slit ligands is required for optimal beta-cell survival.
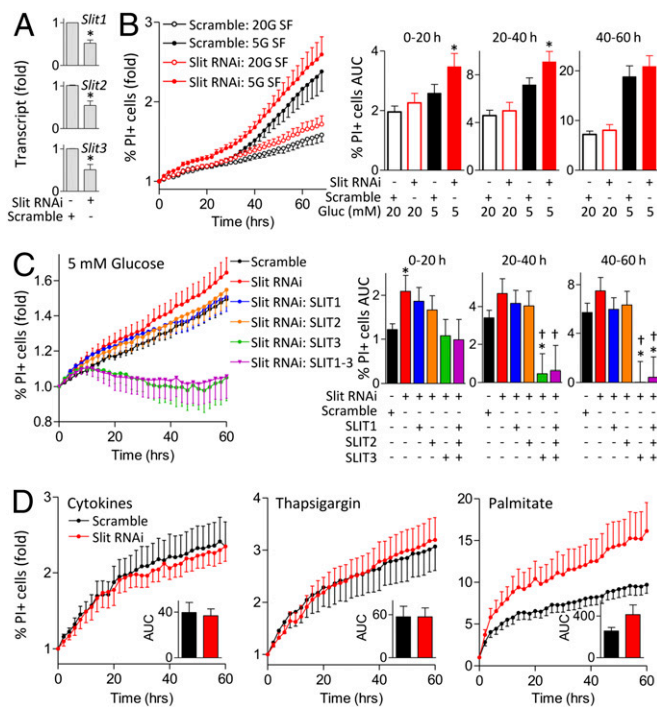
Fig. 2. Knockdown of endogenous Slits increases cell death. (*A*) Dispersed mouse islet cells were transfected with siRNA for *Slit1*, *Slit2*, and *Slit3* or scramble siRNA as control, and examined by qRT-PCR after 72 h ($2^{-\Delta\Delta C_t}$; $n = 6$, *$P < 0.05$ compared with control). (*B*) Mouse islet cells transfected with Slit siRNAs were stained with 0.05 μg/mL Hoechst and 0.5 μg/mL propidium iodide (PI) 48 h following transfection and imaged. Cells were in SF conditions with 20 mM and 5 mM glucose. Percent PI-positive cells and area under the curve (AUC) were calculated for indicated time intervals ($n = 12$, *$P < 0.05$ compared with control). (*C*) Mouse islet cells transfected with Slit siRNAs were incubated in 5 mM glucose SF conditions supplemented with 10 nM SLIT1-3 ($n = 10$, *$P < 0.05$ compared with scramble control, †$P < 0.05$ compared with Slit knockdown without SLIT supplement). (*D*) Mouse islet cells transfected with Slit siRNAs were stained and treated with cytokine mixture (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ), 1 μM thapsigargin, or 1.5 mM palmitate in 20 mM glucose. (*Inset*) AUC from 0 to 60 h ($n = 8$–10).

We asked whether supplementing islet cell cultures with recombinant SLIT would be sufficient to rescue the effects of *Slit1/2/3* knockdown. Indeed, although SLIT1 and SLIT2 alone could not rescue the elevated level of cell death observed under 5 mM glucose serum-free condition (Fig. 2*C*), SLIT3 and a combination of all SLITs reversed the effects of SLIT knockdown on islet cell death (Fig. 2*C*). We did not observe significant differences in cell survival between control and Slit knockdown in primary islet cells and MIN6 treated with cytokines, thapsigargin, or palmitate (Fig. 2*D* and Fig. S3). Collectively, our data indicate that SLIT ligands have acute protective effects on islet cells.

Exogenous SLITs Increase Beta-Cell Survival During Stress and Hyperglycemia. Next, we tested whether exogenous SLIT1, SLIT2, and SLIT3 could protect beta cells from multiple forms of death. We first sought to determine whether the glucose milieu altered the protective effects of Slit treatment, as we have observed with netrin and Notch signaling (12, 26). Indeed, treatment with SLIT1 and SLIT2 recombinant proteins significantly reduced thapsigargin-induced death in MIN6 cells under high-, but not low-glucose conditions (Fig. S4 *A* and *B*). In mouse islet cells, SLIT1, SLIT2, and SLIT3 also reduced islet cell death in response to serum deprivation alone and in combination with cytokines (Fig. 3 *A* and *B*). These effects were only seen in the context of high-glucose and never in low-glucose conditions, suggesting a context-dependent switch. Cell

death induced by exposure to thapsigargin under serum deprivation was rescued with combination treatment of SLIT1-3 (Fig. 3*C*). SLIT1 and SLIT2 also reduced the level of cell death induced by thapsigargin treatment alone (Fig. S4*C*). Palmitate-induced cell death was not significantly reduced with SLITs (Fig. 3*D*). These data show that exogenous SLIT ligands, especially in combination, exert significant protection against several harsh stresses.

Slits Protect Beta Cells by Suppressing Apoptosis and ER Stress. To assess the molecular mechanisms associated with Slit action in beta cells, we examined markers of ER stress and apoptosis. Significant increases in annexin V-positive cells were observed in *Slit1/2/3* knockdown cells compared with control (Fig. 4 *A*–*C*), pointing to an effect on apoptotic cell death. Treatment of mouse islets with exogenous SLIT decreased expression of *Chop*, *Gadd34*, and s*Xbp1* mRNA, but only in high glucose (Fig. 4*D*). In low glucose, SLIT increased the expression of *Bip*, a protective chaperone (Fig. 4*D* and Fig. S5). Consistent with the down-regulation of *Chop* observed in mouse islet cells, we also found a decrease in thapsigargin-induced CHOP protein upon treatment with SLIT2 in high glucose (Fig. S6*A*). Treating mouse islets with SLITs reduced cleaved caspase-3 and cleaved PARP (Fig. 4*E*).

Upon induction of ER stress, IRE1 activation can lead to the downstream activation of NF-κB and ASK1-p38MAPK/JNK signaling cascades. Treatment with SLITs significantly reduced phospho-JNK and phospho-p38MAPK, indicative of the down-regulation of these signaling cascades (Fig. 4*E*). Downstream mediators of p38MAPK pathway, p53 and HSP27, were also down-regulated. Treatment of MIN6 cells with SLIT2 following ER-stress induction reduced ASK1 activation (Fig. S6*B*). Thapsigargin-induced cleaved caspase-3 and cleaved caspase-12 were also down-regulated by SLIT2 (Fig. S6 *C*–*E*). Serum deprivation-induced cleaved caspase-3 levels were also down-regulated upon SLIT1 and SLIT2 treatments under high-glucose, but not
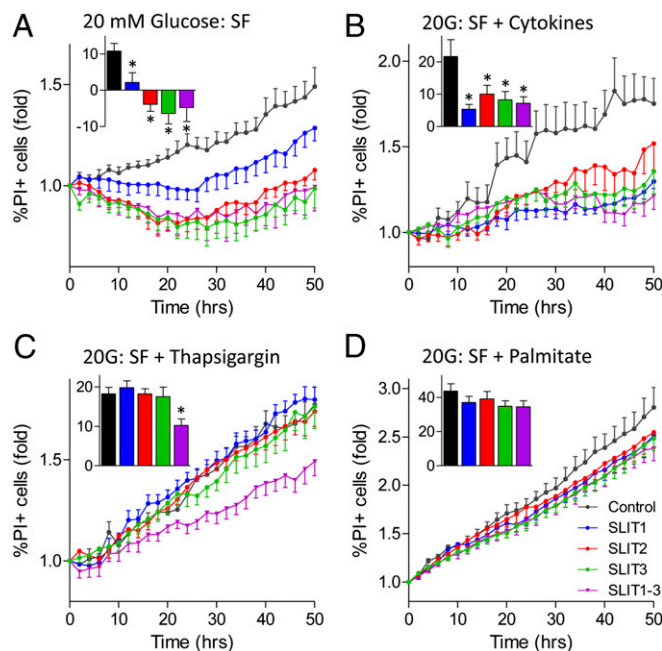
Fig. 3. Slits reduce stress-induced islet cell death under high glucose conditions. Dispersed mouse islet cells were stained with Hoechst and PI and imaged. The percentage of PI-positive cells was determined following treatments with 10 nM SLIT1, SLIT2, and/or SLIT3 in 20 mM glucose SF (*A*), 20 mM glucose SF with cytokine mixture (*B*), 20 mM glucose SF with 1 μM thapsigargin (*C*), and 20 mM glucose SF with 1.5 mM palmitate (*D*). (*Inset*) Area under the curve from 0 to 50 h ($n = 8$, *$P < 0.05$ compared with untreated control).

Fig. 4. Slits down-regulate proapoptotic and ER-stress signaling. MIN6 cells transfected with *Slit1*, *Slit2*, and *Sli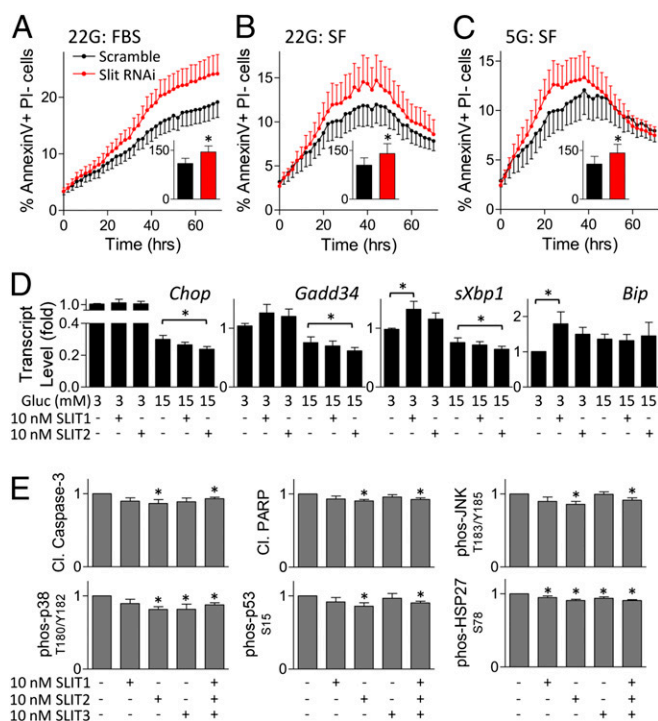t3* siRNAs were stained with Hoechst, PI, and Alexa Fluor 647-conjugated Annexin V 48 h following transfection. Cells were cultured in 22 mM (*A* and *B*) and 5 mM (*C*) glucose in the presence or absence of FBS and imaged. (*Inset*) Area under the curve from 20 to 40 h (*n* = 10, *P* < 0.05 compared with scramble siRNA control). (*D*) Mouse islet cells were treated with SLIT1 or SLIT2 for 4 h before RNA isolation and qRT-PCR analysis (2$^{-\Delta\Delta C_t}$; *n* = 8, *P* < 0.05). (*E*) Mouse islet cells were treated with SLIT1-3 under 20 mM glucose SF condition. Immunoassay for cleaved caspase-3, cleaved PARP, phospho-JNK, phospho-p38, phospho-p53, and phospho-HSP27 (*n* = 6–8, *P* < 0.05).

low-glucose conditions (Fig. S6 *F* and *G*). Cleaved caspase-7 and cleaved caspase-12 levels were also significantly decreased in cells treated with SLIT1 under hyperglycemic serum-free conditions (Fig. S6 *H* and *I*). Together, these experiments indicate that SLIT protects beta cells by broad suppression of the ER stress-induced apoptosis pathway. Because we have previously shown that the CHOP–caspase axis in beta cells can be controlled by luminal Ca^{2+} (24, 25), we predicted that the effects of SLIT–ROBO signaling on cell death signaling were downstream of the modulation of ER Ca^{2+}.

SLITs Accelerate Ca^{2+} Oscillations and Modulate ER Luminal Ca^{2+}. Ca^{2+} homeostasis, especially within the ER, plays a key role in both beta-cell survival (24, 25, 27, 28) and glucose responsiveness (27, 29, 30). Given that SLIT2 signaling in neurons involves Ca^{2+} release from the ER (31–33), we examined this mechanism in beta cells. Interestingly, SLIT treatment increased the frequency of glucose-stimulated cytosolic Ca^{2+} oscillations (Fig. 5 *A* and *B*), a phenomenon that is known to be modulated by the ER Ca^{2+} filling state and associated with increased insulin secretion (27, 29, 30, 34). At basal glucose, SLIT treatment had little or no effect on cytosolic Ca^{2+} (Fig. 5*C*). However, in cells transfected with luminal ER Ca^{2+} sensor, D1ER (25, 35), we observed that SLIT induced a gradual release of Ca^{2+} from the ER filled after exposure to high glucose (Fig. 5*D* and Fig. S7). These effects correlate well with the conditions under which SLIT proteins protect beta cells from ER stress induced by cytokines and by thapsigargin, a drug that blocks ER Ca^{2+} refilling. This result fits with a model whereby ER stress-induced cell death is dependent on the rate at which Ca^{2+} is

depleted and the level of depletion (25). SLITs only partially depleted ER Ca^{2+} because thapsigargin treatment led to further depletion of ER Ca^{2+} (Fig. 5*D* and Fig. S7). The partial depletion of ER Ca^{2+} was maintained throughout a 6-h treatment with SLITs (Fig. S7, *Bottom*). These experiments demonstrate that Slit signaling has direct effects on ER luminal Ca^{2+}, a parameter known to modulate ER stress and insulin secretion (24, 25, 28).

Ca^{2+}-dependent actin remodeling is induced by SLIT proteins during axon guidance (31, 32). Treatment of mouse beta cells with recombinant SLIT1, SLIT2, or SLIT3 decreased cortical F-actin (Fig. 5*E*), an event expected to promote glucose-stimulated insulin secretion (36–38). Temporal analysis of F-actin levels revealed a biphasic response following SLIT1 and SLIT3 treatments (Fig. 5*E*).

SLITs Potentiate Glucose-Stimulated Insulin Secretion. Given the roles of Ca^{2+} and actin on insulin secretion, we investigated whether SLITs affect insulin release. Static incubation showed that mouse islets cultured in 15 mM glucose secreted more insulin in the presence of SLIT (control: 9.6 ± 2.8 ng/mL, SLIT1: 21.6 ± 8.2 ng/mL, SLIT2: 19.1 ± 8.4 ng/mL). This observation led us to conduct islet perifusions. Indeed, glucose-stimulated insulin secretion was significantly potentiated in the presence of SLIT1, SLIT2, or SLIT3 (Fig. 6*A*). SLIT treatment did not potentiate insulin secretion when beta cells were depolarized with 30 mM KCl (Fig. 6*A*), ruling out effects distal to the opening of voltage-gated Ca^{2+} channels and pointing to effects on glucose sensing/signaling. SLITs did not affect *Ins1* and *Ins2* transcription (Fig. S8). Thus, Slit proteins can both protect beta cells and increase insulin secretion, which itself is antiapoptotic (8, 9, 39).
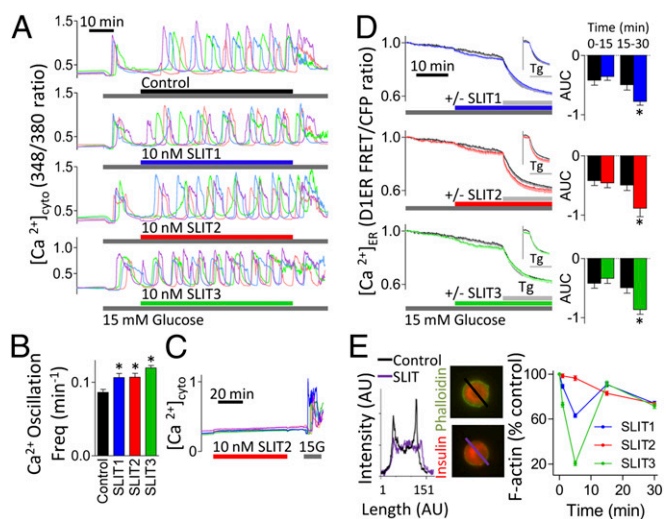

Fig. 5. Slits modulate cytosolic Ca^{2+} and ER Ca^{2+} signaling and actin remodeling. Dispersed mouse islet cells loaded with Fura-2-AM. Representative single-cell traces following 10 nM SLIT1, SLIT2, or SLIT3 treatment in 15 mM glucose (*A*). Single-cell oscillation frequency of cytosolic Ca^{2+} (*n* = 91–104, *P* < 0.05) (*B*). Representative single-cell traces following exposure to 10 nM SLIT2 in 3 mM glucose (*C*). (*D*) Dispersed mouse islet cells were transfected with D1ER cameleon to image ER Ca^{2+}. Cells were exposed to 1 μM Tg 15 min following treatment with 10 nM SLIT1, SLIT2, or SLIT3 (colored lines) or untreated (black line) in 15 mM glucose (*n* = 13–14). (*Inset*) D1ER FRET/CFP ratios normalized to the time point following Tg addition. (*Right*) AUC for the 15-min preincubation with 15 mM glucose and 15-min treatment with SLIT1-3 (*n* = 13–14, *P* < 0.05 compared with untreated). (*E*) Mouse islet cells treated with 10 nM SLIT1, SLIT2, or SLIT3 in 11 mM glucose-containing RPMI. Beta cells stained for insulin and phalloidin and peak intensity of cortical F-actin staining was normalized to untreated cells at the same time point (*n* = 39–60 cells).

CELL BIOLOGY

Discussion

The present study was conducted to determine the expression pattern, regulation, and roles of the Slit family of secreted factors and their Robo receptors in pancreatic islet cells. Slit and Robo transcripts and protein were detected in adult mouse and human islets and regulated in stress conditions. We identified an important role for this local autocrine/paracrine network in beta-cell survival and function. We identified a unique anti-ER stress and antiapoptotic mechanism of action involving the controlled release of Ca^{2+} from the ER lumen (Fig. 6B).

The roles of Slit–Robo signaling in cell survival remain poorly understood. Knockout mouse studies, along with the observed loss of expression of ROBO1 in some human cancers, provide evidence that the loss of ROBO is tumorigenic. *SLIT1* and *SLIT3* are candidate tumor suppressor genes (40), although *SLIT1* is up-regulated in prostate tumors (41). In islet cells, a modest knockdown of Slits significantly increased cell death, suggesting that endogenous SLIT secretion plays an important role in cell survival. Conversely, SLIT1, SLIT2, and/or SLIT3 supplementation reduced stress-induced cell death. We observed significant decreases in both ER stress- and serum starvation-induced cell death, but only under hyperglycemic conditions. Our data suggest that Ca^{2+}-dependent mechanisms

are important for the protective effects of Slit, which is in line with a role for Ca^{2+} in Slit–Robo signaling in other cell types (24, 25, 27, 28, 31–33). In particular, our results implicate a controlled depletion in ER Ca^{2+} and an increase in the frequency of Ca^{2+} oscillations.

It is possible that Slit may protect beta cells via interaction with receptors for other guidance factors. DCC, UNC5, and Neogenin are dependence receptors that can increase cell survival in the presence of netrins and induce cell death in the absence of netrins (42, 43). Interaction between ROBO1 and DCC in a Slit-dependent manner decreases netrin-induced chemo-attraction in neurons (44, 45). Perhaps Slit–Robo signaling regulates beta-cell survival through down-regulation of netrin receptor-induced apoptosis. Additionally, because Slit–Robo signaling induces tumor angiogenesis by attracting endothelial cells, perhaps local production of SLITs could improve islet engraftment following transplantation (46). The stress-induced up-regulation of Slit expression, the survival effects of exogenous SLITs under high-glucose conditions, and the protective effects of endogenous SLITs suggest an autocrine/paracrine compensatory survival network.

In addition to the protective effects on beta cells, we uncovered effects of SLIT ligands on proximal glucose sensing/signaling, which were associated with changes in Ca^{2+} and actin. It was not surprising to find that SLITs potentiate glucose-stimulated insulin secretion given that Cdc42, Rac1, and RhoA play important roles in Slit-induced cellular migration (33, 47, 48). The down-regulation of cortical F-actin staining observed upon SLIT treatment is in agreement with the requirement for Cdc42 and RhoA inactivation via increasing Rho GTPase-activating proteins for the repulsive effects of SLITs in neurons and olfactory ensheathing cells (33, 47). Our current data do not allow us to distinguish whether the biphasic F-actin depletion following SLIT1 and SLIT3 treatments directly or indirectly mediates the potentiation of insulin secretion, but this could be a fruitful area for future investigation. In addition to the release of antiapoptotic insulin (8, 9, 39, 49–51), modulation of actin polymerization may directly promote survival (52–55). We have not determined whether the survival effects of SLITs are due to direct down-regulation of apoptotic pathways or indirect effects through up-regulation of insulin secretion (39, 56).

In conclusion, we provide detailed evidence that Slit ligands and their Robo receptors are present in pancreatic islet cells and define multiple roles for the Slit-secreted factors in beta-cell physiology. Our results reveal that Slit signaling depletes ER Ca^{2+} and protects beta cells from ER stress. Together, our results point to the Slit–Robo pathway and a novel area for investigations around beta-cell survival and function.

Materials and Methods

Detailed methods can be found in *SI Materials and Methods*.

Primary Islet Isolation, Cell Culture, and Perifusion. Islets were isolated from C57BL/6J mice using collagenase and filtration (12, 56). Human islets (>80% pure) were provided by Garth Warnock (University of British Columbia, Vancouver), collected via protocols approved by the University of British Columbia Research Ethics Board. Islets and MIN6 cells were cultured as described (8, 12, 57). Insulin secretion was measured by perifusion and RIA (26).

siRNA, RT-PCR, and Immunoblotting. MIN6 and dispersed islet cells were Neon (Invitrogen)-transfected with 100 nM (MIN6) or 200 nM (islet cell) Silencer Select siRNA (Ambion) targeting *Slit1*, *Slit2*, and *Slit3*, with scramble siRNA (Ambion) as a negative control. Total RNA was isolated and qRT-PCR was performed. Immunoblotting was performed as described (12).

Imaging. MIN6 and dispersed islet cells stained as described (12). For cell death assays, cells were seeded into 96-well plates and exposed to 0.05 μg/mL Hoechst 33342 (Invitrogen), 0.5 μg/mL propidium iodide (Sigma), and Alexa-Fluor647-conjugated Annexin V (1:250; Invitrogen) (25). Cells were imaged
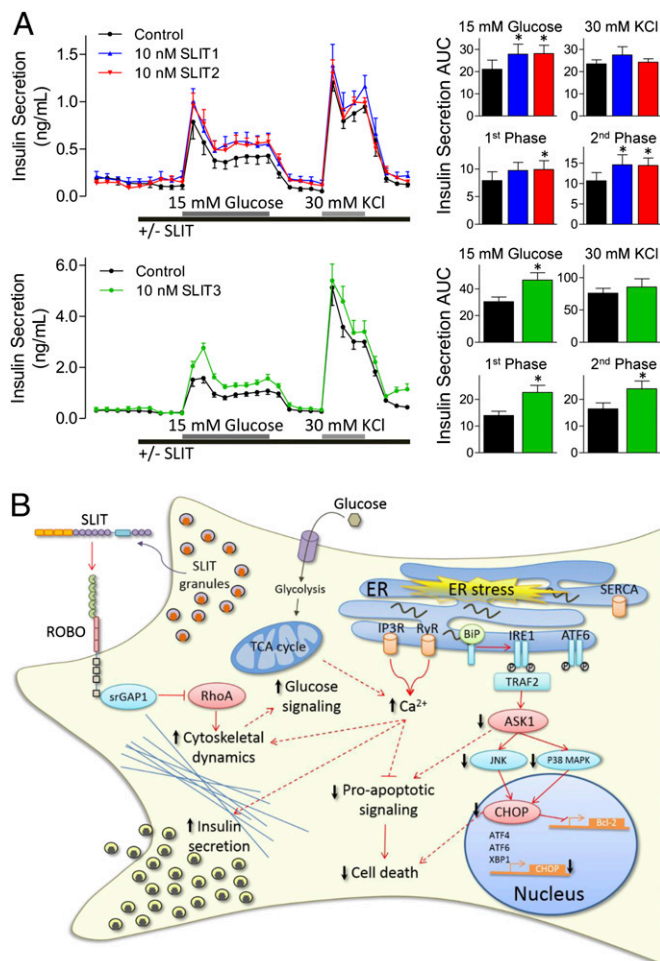

Fig. 6. Slits modulate insulin secretion. (A) Mouse islets perifused with Krebs–Ringer buffer containing 3 or 15 mM glucose in combination with 10 nM SLIT1, SLIT2, or SLIT3 (n = 5–6). (Right) Baseline subtracted AUC for the 15 mM glucose- and KCl-stimulated insulin secretion, along with first- and second-phase insulin secretion (n = 5–6, *P < 0.05 compared with control). (B) Model of Slit–Robo signaling in beta cells. TCA, tricarboxylic acid.

with ImageXpress^{MICRO} (Molecular Devices) every 1 or 2 h at 37 °C and 5% (vol/vol) CO_2 (12). Cytosolic Ca^{2+} was imaged using Fura-2-AM (Invitrogen) (57). To measure ER Ca^{2+}, mouse islet and MIN6 cells were transfected with the D1ER sensor (24, 25). To detect F-actin, mouse islet cells were fixed with Z-FIX (Anatech Ltd.) and stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen). Beta cells were identified by insulin staining (Santa Cruz Biotechnology). Line scans were performed using ImageJ (version 1.41o; National Institutes of Health) and mean peak-intensity calculated.

Statistics. Data are expressed as mean ± SEM unless otherwise indicated. Results were considered significant when $P < 0.05$ using Student t test or ANOVA, where appropriate (Prism; GraphPad).

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