Granuphilin is activated by SREBP-1c and involved in impaired insulin secretion in diabetic mice

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Summary

Granuphilin is a crucial component of the docking machinery of insulin-containing vesicles to the plasma membrane. Here, we show that the granuphilin promoter is a target of SREBP-1c, a transcription factor that controls fatty acid synthesis, and MafA, a β cell differentiation factor. Potassium-stimulated insulin secretion (KSIS) was suppressed in islets with adenoviral-mediated overexpression of granuphilin and enhanced in islets with knockdown of granuphilin (in which granuphilin had been knocked down). SREBP-1c and granuphilin were activated in islets from β cell-specific SREBP-1c transgenic mice, as well as in several diabetic mouse models and normal islets treated with palmitate, accompanied by a corresponding reduction in insulin secretion. Knockdown- or knockout-mediated ablation of granuphilin or SREBP-1c restored KSIS in these islets. Collectively, our data provide evidence that activation of the SREBP-1c/granuphilin pathway is a potential mechanism for impaired insulin secretion in diabetes, contributing to β cell lipotoxicity.

Introduction

Insulin secretion by pancreatic β cells involves sequential intracellular events. Initially, glucose oxidation results in ATP production as controlled by intracellular energy-sensing mechanisms (Bratanova-Tochkova et al., 2002). This is followed by closure of ATP-dependent K^+ channels leading to a change in membrane voltage (depolarization), Ca^{2+} influx through voltage-dependent Ca^{2+} channels, and vesicular transport of insulin-containing granules, culminating in their exocytosis and release of insulin (Rizzoli and Betz, 2005; Rorsman and Renstrom, 2003). Theoretically, impaired insulin secretion could be caused by disturbances in any stage of this highly regulated process (Weir and Bonner-Weir, 2004). Current literature supports the concept that disturbances of energy metabolism in β cells leading to impaired ATP production are the primary pathological cause of impaired insulin secretion following peripheral insulin resistance in type 2 diabetes mellitus (Kahn, 2003). This pathophysiologic process can be observed in isolated islets from diabetic animal models and is characterized by impairment of glucose-stimulated insulin secretion (GSIS) (Diani et al., 2004). Meanwhile, potential perturbations in insulin secretion at steps following ATP production in islets from the diabetic models as estimated by K^+-stimulated insulin secretion (KSIS) have not been fully investigated.

Studies to unveil the molecular basis for exocytosis of secretory granules have been focused on the interactions between components of the fusion machinery (Burgoyne and Morgan, 2003; Rizzoli and Betz, 2005; Rorsman and Renstrom, 2003), including soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs: Vamp2/synaptobrevin, Syntaxin-1a, and SNAP25) and SNARE-associated proteins (Munc13-1 and Munc18-1) on the plasma membrane, and small Rab GTPase family members, Rab3 and Rab27a, on the vesicles. Granuphilin is a Rab27a effecter specific to pancreatic β cells and the pituitary gland. It has two isoforms, granuphilin-a and -b, which derive from alterative splicing (Coppola et al., 2002; Wang et al., 1999). Granuphilin directly binds to Rab27a, and also to syntaxin-1a and Munc18-1, assisting in the docking of insulin granules to the plasma membrane (Coppola et al., 2002; Torii et al., 2002, 2004; Yi et al., 2002). Contrary to expectations, overexpression of granuphilin in a β cell line inhibited insulin secretion (Coppola et al., 2002; Torii et al., 2002). In addition, granuphilin-null islets exhibited inhibition of vesicle docking, yet insulin secretion was increased (Gomi et al., 2005). These gain- and loss-of-function experiments implicated granuphilin as playing a regulatory role in the exocytosis of insulin granules. To date, the regulation of these vesicle proteins and the potential role of granuphilin in β cell lipotoxicity and diabetes are largely unknown.

SREBP-1c is a transcription factor established as a regulator for biosynthesis of fatty acids and triglycerides (Shimano et al., 1997a, 1997b). In addition to nutritional regulation of lipogenic enzymes (Horton et al., 1998; Hasty et al., 2000; Matsuzaka et al., 2004), SREBP-1c is found to play a role in insulin signaling

by inhibiting IRS-2, the major insulin-signaling mediator in the liver and in β cells (Ide et al., 2004; Takahashi et al., 2005). As a model for lipotoxicity by endogenous fatty acids in pancreatic β cells, we previously developed transgenic mice overexpressing the active form of SREBP-1c under the insulin promoter. These mice exhibited impaired insulin secretion in vivo due to ATP depletion caused by enhanced lipogenesis and direct UCP2 induction. In addition, these mice demonstrated loss of β cell mass, presumably due to suppression of IRS-2 and PDX-1 in islets from the transgenic mice (Takahashi et al., 2005). In addition to inhibition of GSIS, SREBP-1c-overexpressing islets exhibited decreased KSIS, which could indicate dysfunction in insulin secretion at a phase following ATP production. Our current study demonstrates that granuphilin is an SREBP-1c target exhibiting a molecular link between β cell lipotoxicity and impaired insulin secretion in the pathogenesis of diabetes development. These data provide a rational to study granuphilin as a potential therapeutic target for treatment of diabetes.

Results

Granuphilin is activated by SREBP-1c in islets

We have previously demonstrated that insulin secretion is blunted in islets isolated from nuclear SREBP-1c transgenic mice under the control of rat insulin promoter I, whereas SREBP-1-null islets demonstrated enhanced insulin secretion compared to wild-type controls (Takahashi et al., 2005). Expression of the transgene was barely detectable in the hypothalamus and was essentially β cell-specific in this transgenic line (Figure S1A in the Supplemental Data available with this article online). In the current study, we found that the negative effect of SREBP-1c on insulin secretion encompassed not only GSIS but also KSIS and induction by arginine (Figure 1A), suggesting that insulin secretion defects caused by SREBP-1c occurred post-ATP production. In an attempt to determine the molecular mechanisms responsible, we explored the expression of genes involved in vesicular transport of insulin granules in islets from these mice. Among many different vesicle factors involved in exocytosis of insulin granules, granuphilin was singly upregulated by overexpression of SREBP-1c and suppressed in the absence of SREBP-1c, as evidenced by real-time PCR (Figure 1B). Immunoblot analysis of islet proteins demonstrated that wild-type islets mainly express granuphilin-a (Figure 1C), and the ratio of granuphilin-a/-b mRNA was roughly 8 (data not shown). Consistent with changes at the mRNA level, both granuphilin-a and -b proteins were robustly induced by overexpression of SREBP-1c and reduced in SREBP-1c-null islets (Figure 1C). SREBP-1c slightly reduced Syntaxin-1a and Munc18-1 proteins but did not affect other related vesicle proteins such as Rab27a, Rab3, Vamp2, and SNAP25 (Figure 1C). Induction of granuphilin mRNA and proteins by acute overexpression of adenoviral SREBP-1c was also observed in a murine insulinoma cell line Min6 (Figure 1D) and islets (Figure 1E), respectively.

Granuphilin promoter is an SREBP target

We next sought to determine whether the granuphilin promoter is a potential direct target of SREBP. Analysis of the GeneBank database revealed that the promoter region of the murine granuphilin gene contains two potential binding sites for SREBP (SREs) and a single site for MafA, which was recently established as an important factor for β cell-differentiation and function (MARE) (Hagman et al., 2005; Kajihara et al., 2003) (Figure S2). Activation of the granuphilin promoter by transcription factors such as SREBPs, MafA, and others was tested in Min6 cells using a luciferase reporter gene fused to the 1.2 kb promoter region of mouse granuphilin (Granu-1200-Luc) (Figure 1F). SREBP-1a and -1c robustly and SREBP-2 moderately stimulated granuphilin promoter activity. MafA was found to be the strongest activator of the granuphilin promoter. A similar pattern of transactivation by these factors was also observed in non-β cells (HEK 293 cells), suggesting that SREBP activation does not require other β cell-specific factors (data not shown). Human granuphilin promoter (0.5 kb) luciferase reporter was also activated by SREBPs and MafA (data not shown).

This region of the mouse granuphilin promoter (Figure 2A) was further analyzed as an SREBP target. Activation of the granuphilin promoter was completely attenuated when SREBPs were mutated (YR mutants) to abolish their binding abilities to authentic SREs (Figure 2B). Deletion studies suggested that the proximal SRE is responsible for SREBP activation of granuphilin promoter (Figure 2C). EMSA assays demonstrated direct and specific binding of SREBPs to this SRE-1 (hereafter referred to as Granuphilin SRE) and not SRE-2 (Figure 2E). Mutation of Granuphilin SRE (Figure 2A) completely abolished the SREBP activation of Granuphilin-Luc (Figure 2D). To determine the physiological relevance of SREBP regulation of granuphilin, MIN6 cells were treated with delipidated serum (DLS) and 25-hydroxycholesterol (25-OH) to activate and suppress endogenous SREBPs, respectively, via modulation of the sterol-regulated SERBP cleavage system. The cells were also treated with T0901317, an LXR agonist, to induce endogenous SREBP-1c. Granu-1200-Luc activity was consistently up- and downregulated by these manipulations, respectively, demonstrating that endogenous SREBPs can also regulate granuphilin promoter activity (Figures 2F and 2G). Chromatin immunoprecipitation assays using MIN6 cells confirmed direct binding of SREBP-1c to Granuphilin SRE in vivo. The signal was increased by incubation with DLS and T0901317 (Figure 2H). Collectively, these data demonstrate that the granuphilin promoter is an SREBP target and that SREBPs regulate granuphilin expression.

Granuphilin promoter is a MafA target

Next, we focused the MARE downstream of the Granuphilin SRE in the granuphilin promoter as diagramed in Figure 3A. Activation of the granuphilin promoter by MafA was completely abolished by deletion of the acidic domain of MafA (Figure 3B). Deletion or mutation of the MARE dramatically suppressed the promoter activity (Figures 3C and 3D). Direct, specific, and in vivo binding of MafA to this MARE was confirmed by EMSA and ChIP assay (Figures 3E and 3F). Considering the two neighboring sites, potential synergism between SREBP-1c and MafA was estimated. Both factors exhibited dose-dependent activation of granuphilin promoter, although MafA has a curve slightly steeper and more saturable than SREBP-1c. Cotransfection of both SREBP-1c and MafA caused only additive activation of the granuphilin promoter (Figures 3G and 3H). In immunoprecipitation experiments, no significant protein-protein interactions between SREBP-1c and MafA were detected (data not shown).

Granuphilin negatively regulates insulin secretion

To determine whether granuphilin could be directly involved in insulin secretion, its expression was manipulated in islets

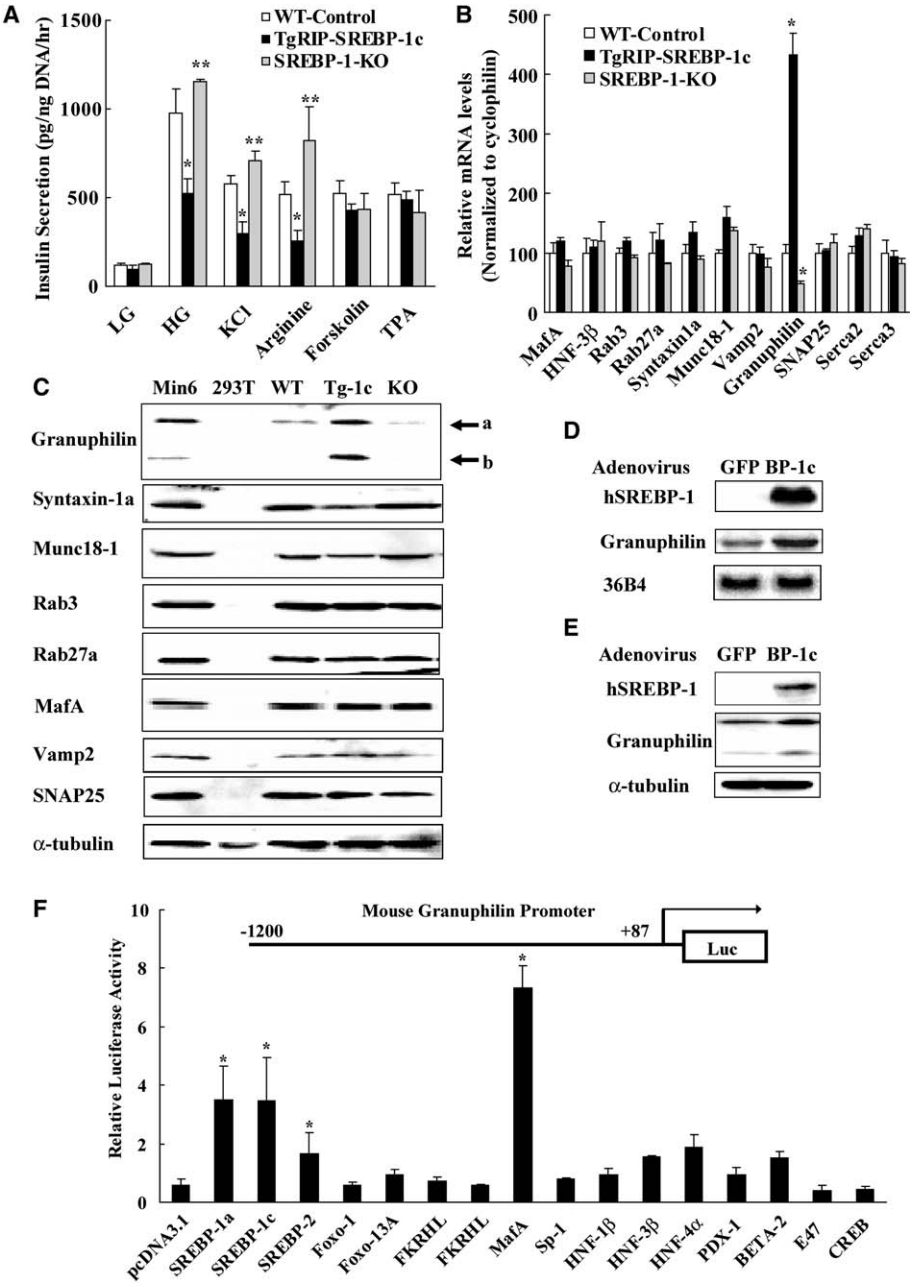

Figure 1. Influence of SREBP-1c on granuphilin expression

Granuphilin and other fusion machinery proteins and insulin secretion were estimated in islets from wild-type (WT-Control), β cell-specific nuclear SREBP-1c transgenic (TgRIP-SREBP-1c), and SREBP-1-null mice (SREBP-1-KO) mice.

A) Basal (low glucose LG; 2.8 mM), high glucose (HG; 20 mM), KCl- (30 mM), arginine- (3 mM), forskolin- (10 μM), or TPA (0.5 μM)-stimulated insulin secretion in isolated islets from age-matched (14- to 17-week-old) male WT-Control (white bars), TgRIP-SREBP-1c (black bars), and SREBP-1-KO (gray bars) mice. Results were normalized to cellular DNA content. *$p < 0.01$ and **$p < 0.05$ (versus WT-Control).

B) mRNA levels of the fusion machinery for exocytosis of insulin granules in the indicated islets as quantified by real-time PCR. Values were normalized to the cyclophilin expression level. *$p < 0.01$ (versus WT-Control).

C) Immunoblot analysis on total proteins (50 μg/lane) of the fusion machinery for exocytosis of insulin granules in the islets from indicated mice.

D) Northern blot analysis of granuphilin and hSREBP-1 gene expression in mouse insulinoma cell line, Min6. Cells were infected by adenoviral-GFP or -SREBP-1c (500 MOI) for 48 hr. Total RNA (15 μg/lane) was isolated and subjected to blot hybridization with the indicated ^{32}P-labeled probe.

E) Immunoblot analysis on nuclear or total proteins of SREBP-1 and mouse granuphilin in the islets from C57BL/6 mice with α-tubulin as a loading control. Islets were infected with adenoviral-GFP or -SREBP-1c (500 MOI) for 48 hr.

F) Activation of the granuphilin promoter by SREBPs and MafA. The mouse granuphilin promoter region (1.2 kb) was fused to a luciferase reporter gene (Granu-1200-Luc). Min6 cells were cotransfected with Granu-1200-Luc as a reporter gene, pSV-40-*Renilla* as a reference, and the indicated expression plasmids or an empty vector. The luciferase activity was normalized to *Renilla* luciferase activity and assays were performed in triplicate. *$p < 0.01$ (versus pcDNA3.1 group).

Results are expressed by mean ± SEM. Studies were performed in triplicate with sets of islets pooled from 3–4 mice per replicate (**A**–**C** and **E**).

isolated from C57BL/6 mice using adenoviral overexpression and RNA interference (Figures S3A and S3B). Adenoviral overexpression of granuphilin-a suppressed GSIS substantially and KSIS completely but not basal secretion at low glucose concentration, consistent with previous report in a β cell line (Coppola et al., 2002; Torii et al., 2002) (Figure S3C). Adenoviral RNAi (RNAi-784) completely inhibited expression of both granuphilin-a and-b. This knockdown of granuphilin resulted in a trend toward increased GSIS, and significantly increased KSIS, which is also consistent with a recent report describing granuphilin-null mice (Gomi et al., 2005) (Figure S3C). Granuphilin-a overexpression and knockdown did not significantly change either insulin content or ATP/ADP ratio in infected islets, demonstrating that the modulation of insulin secretion by granuphilin occurred in the process post-ATP production (Figures S3D and S3E).

Granuphilin mediates SREBP-1 inhibition of KSIS

We also evaluated the effect of granuphilin knockdown by RNA interference in SREBP-1c-overexpressing islets (Figures 4 and S4). Granuphilin mRNA and protein levels were upregulated by SREBP-1c in these islets in the basal state; however, expression was completely abolished by granuphilin RNAi (Figures 4A and 4B). This suppression of granuphilin did not affect the protein level of endogenous membrane SREBP-1c, transgenic nuclear SREBP-1c, or MafA (Figure 4B). Other vesicle and related molecules were also unchanged at the protein and mRNA levels (Figures 4C and S4A). However, the granuphilin knockdown substantially ameliorated the decreased GSIS caused by SREBP-1c overexpression and completely restored the suppressed KSIS without changes in decreased ATP/ADP ratio and increased triglyceride content (Figures 4D–4F). These data

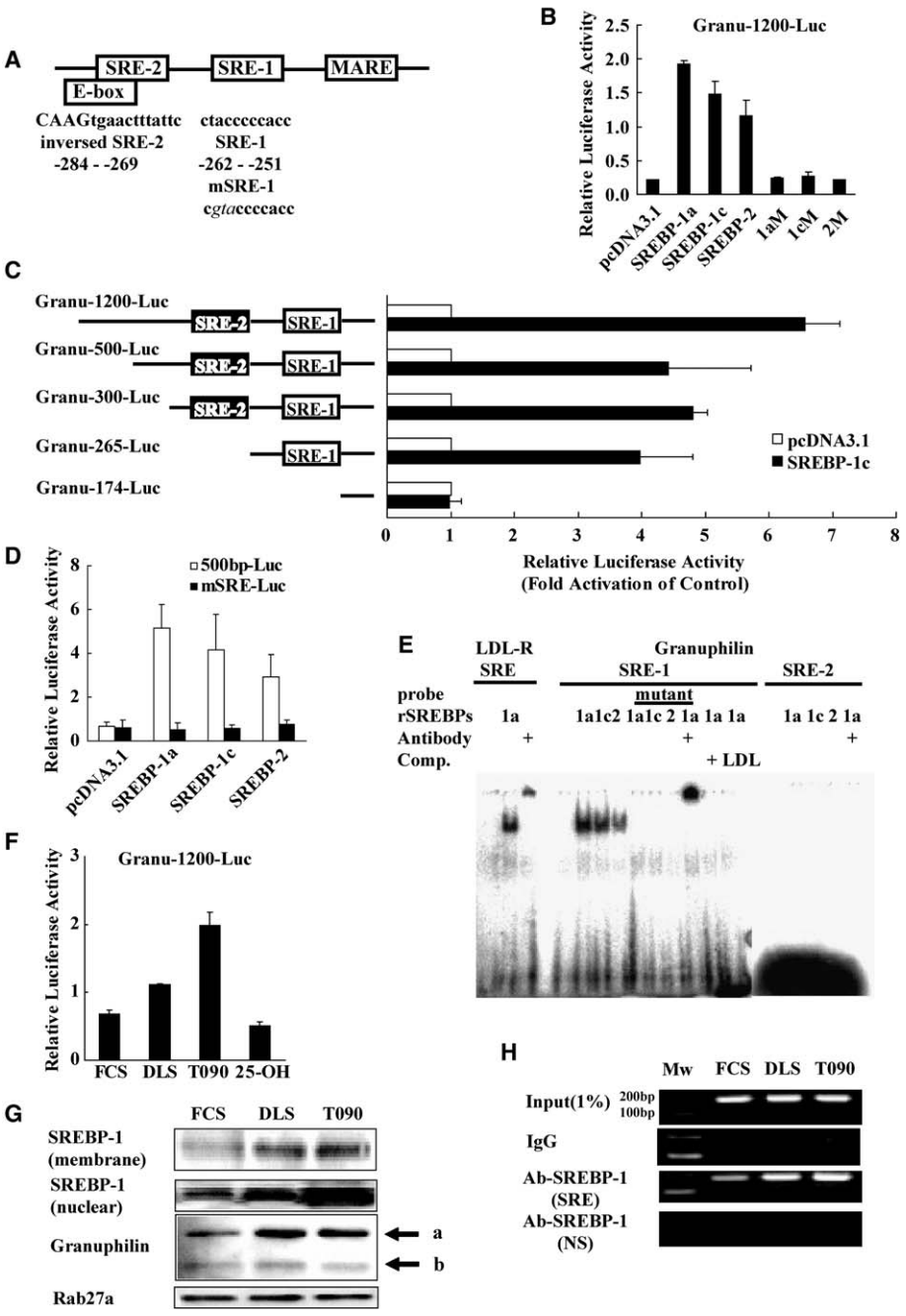

Figure 2. Mouse granuphilin promoter analysis as an SREBP target

A) Schematic representation of the mouse granuphilin promoter with the sequence and location of potential SREBP binding sites (SRE-1 and SRE-2). The SRE-2 element has an inverted consensus sequence and partially overlaps with an E-box.

B) Transcriptional activities of nuclear SREBP-1a, -1c, and -2 and their respective Tyr-Arg mutants on Granu-1200-Luc in Min6 cells.

C) Effects of deletion of SREBP-1c activation in the granuphilin promoter. Sequentially deleted luciferase constructs of the granuphilin promoter, as indicated, were used for transfection studies in Min6 cells.

D) Effects of a mutation in SRE-1 on SREBP activation of granuphilin promoter. Native (Granu-500-Luc) or mutant-SRE-1 (mSRE-Luc) reporter gene plasmids were cotransfected into Min6 cells with pSV40-*Renilla* and expression plasmids for SREBPs.

E) EMSA with radio-labeled probes for the authentic human LDL receptor SRE and granuphilin SRE-1 and SRE-2. In vitro translated SREBP-1a, -1c, and -2 proteins were incubated with these labeled probes. Specific binding was confirmed by super-shift using their respective antibody.

F) Effect of endogenous SREBPs in Min6 cells on Granu-1200-Luc. Cells were incubated with DMEM in 7.5% FCS, in 7.5% DLS, an LXR agonist: T0901317 (1 μM), and 25-hydroxycholesterol (25-OH, 0.1 μg/ml).

G) Immunoblot analysis of endogenous SREBP-1 and granuphilin proteins in Min6 cells. Cells were incubated with DMEM in 7.5% FCS, in 7.5% DLS, and T0901317 (1 μM) for 48 hr.

H) In vivo binding of SREBP-1 to Granuphilin-SRE. Chromatin prepared from the Min6 cells were subjected to the ChIP assay with anti-mouse SREBP-1 antibody and rabbit IgG as a negative control. Immunoprecipitated samples were subjected to PCR using primers to amplify the SRE-element in the granuphilin promoter and for the nonspecific-element (NS). Mw, molecular weight.

The luciferase activity was normalized to *Renilla* luciferase activity, and assays were performed in triplicate (**B**–**D** and **F**). Results are expressed as mean ± SEM.

indicate that SREBP-1c-mediated inhibition of insulin secretion can be accounted for, in part, by upregulation of granuphilin.

Activation of SREBP-1c/granuphilin inhibits KSIS in islets from diabetic mice

The inhibitory effect of the SREBP-1c/granuphilin pathway on insulin secretion was tested in islets from murine models of diabetes (Figure 5A). Along with consistent upregulation of SREBP-1c, both granuphilin mRNA and protein levels were increased in islets from genetically obese diabetic mice such as ob/ob, KK-Ay, and db/db, as compared to control C57BL/6 mice. In addition, mice made obese with high-fat or high-fat/high-sucrose diets also displayed increased SREBP-1c and granuphilin expression. We next sought to determine the effect of granuphilin knockdown on gene expression in islets from KK-Ay mice (Figures 5B–5E). Comparison of LacZi-Ad-infected islets from C57BL/6 and KK-Ay mice also allowed us to determine basal difference in gene expression under control and diabetic conditions. mRNA levels of SREBP-1c and granuphilin were elevated in the KK-Ay mice compared to C57BL/6 controls. Granuphilin knockdown resulted in essential abolishment of both granuphilin mRNA and protein levels without impacting SREBP-1c levels (Figure 5B). Islets from KK-Ay mice had higher basal nuclear SREBP-1 protein with concomitant increases in SREBP-1c target genes FAS and UCP-2 with significant increases in triglyceride content (Figures 5E and S4B). Protein levels of Syntaxin-1a and Munc18-1 were slightly decreased; however, levels of other vesicle proteins remained unchanged by either the increase in

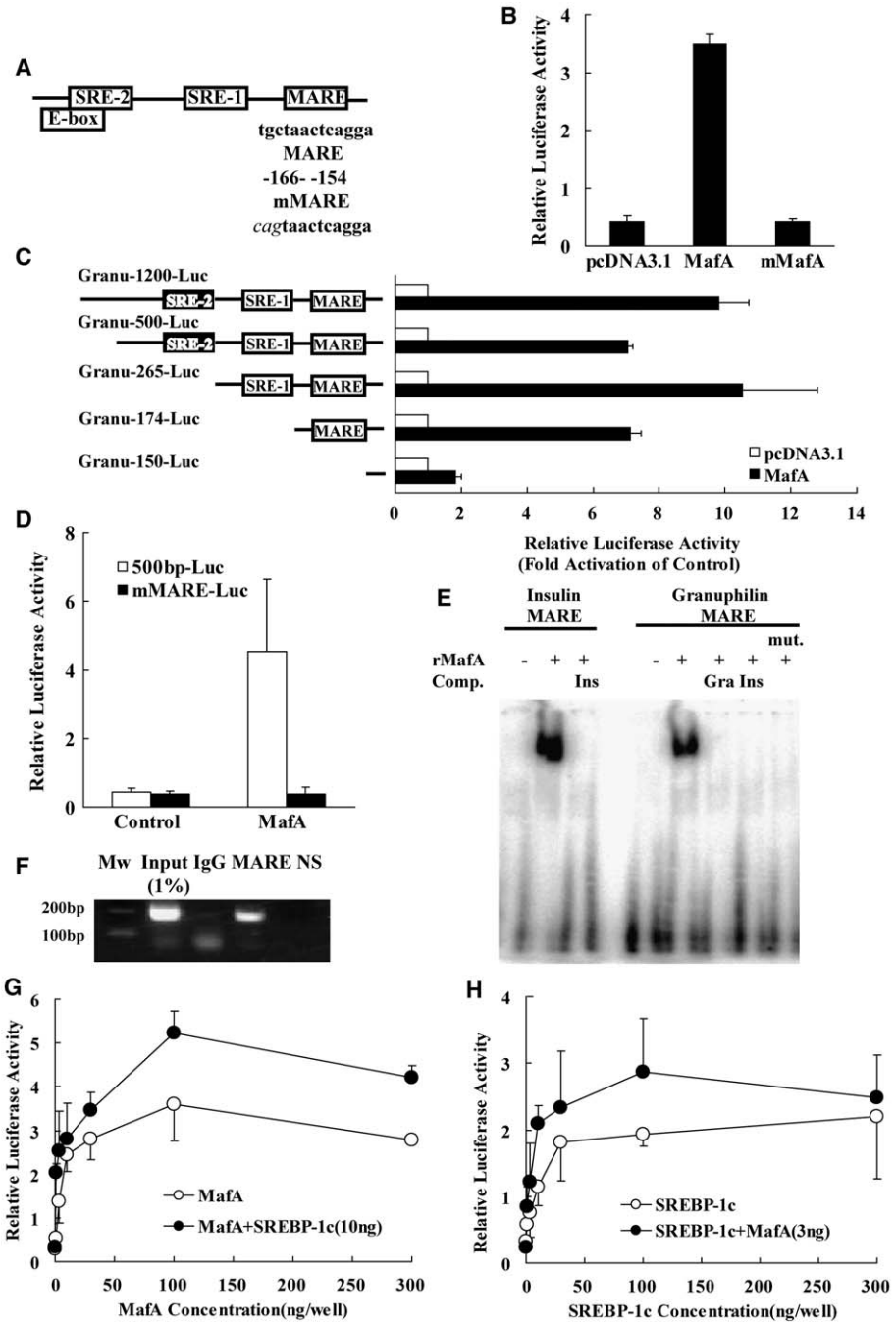

Figure 3. Mouse granuphilin promoter analysis as a MafA target

A) Schematic representation of the granuphilin promoter with sequence and location of a potential MafA binding site (MARE).

B) Transcriptional activities of wild-type MafA and mutant MafA (deletion of transactivation domain, amino acid 229–359) on Granu-1200-Luc in Min6 cells.

C) Deletion studies in MafA activation of granuphilin promoter.

D) Effect of mutation in the MARE on MafA activation of granuphilin. Native (Granu-500-Luc) or mutant-MARE (mMARE-Luc) reporter gene plasmids were cotransfected into Min6 cells with pSV40-*Renilla* and MafA.

E) EMSA with radio-labeled probes for rat insulin II MARE and granuphilin MARE-elements. In vitro translated MafA protein was incubated with these labeled probes. Specific binding was confirmed by an excess of nonlabeled probe.

F) In vivo binding to Granuphilin-MARE. Chromatin prepared from the Min6 cells were subjected to the ChIP assay with anti-rabbit MafA antibody and rabbit IgG as a negative control. Immunoprecipitated samples were subjected to PCR using primers to amplify the granuphilin promoter containing the MARE-element and of a nonspecific-element (NS). Mw, molecular weight.

G) Dose-dependent effects of MafA with cotransfection of SREBP-1c on Granu-Luc-1200 in Min6 cells.

H) Dose-dependent effects of SREBP-1c with cotransfection of MafA on Granu-Luc-1200 in Min6 cells.

The luciferase activity was normalized to *Renilla* luciferase activity and performed in triplicate (**B**–**D**, **G**, and **H**). Results are expressed as mean ± SEM.

SREBP-1c in the diabetic KK-Ay mice or by knockdown of granuphilin (data not shown). Thus, knockdown of granuphilin does not appear to alter mRNA or protein levels of any other proteins involved in insulin-containing granule transport in islets. Both GSIS and KSIS were reduced in KK-Ay islets with a reduction in the ATP/ADP ratio and an increase in the content of triglycerides (Figures 5C–5E). Knockdown of granuphilin completely restored KSIS but not GSIS without modulating ATP/ADP ratio or triglyceride content. In addition, restoration of decreased KSIS by granuphilin knockdown was also observed in islets from ob/ob and diet-induced obese (DIO) mice (Figures 5F, 5G, S4C, and S4D).

Knockdown of SREBP-1c by adenoviral RNAi was performed in islets from KK-Ay and ob/ob mice that exhibit upregulation of SREBP-1c and granuphilin (Figure S5). Although acute inhibition of SREBP-1c was not complete in these islets, reduction of granuphilin and restoration of decreased GSIS and KSIS were partially but consistently observed to support the concept of the contribution of the SREBP-1c/granuphilin pathway to impaired insulin secretion. Combined, these data implicate the specific involvement of granuphilin in impaired insulin secretion in obesity-related diabetic mice.

Palmitate lipotoxicity involves inhibition of KSIS through granuphilin

Saturated fatty acids are known to impair insulin secretion, supporting the theory that lipotoxicity plays a role in impaired β–cell

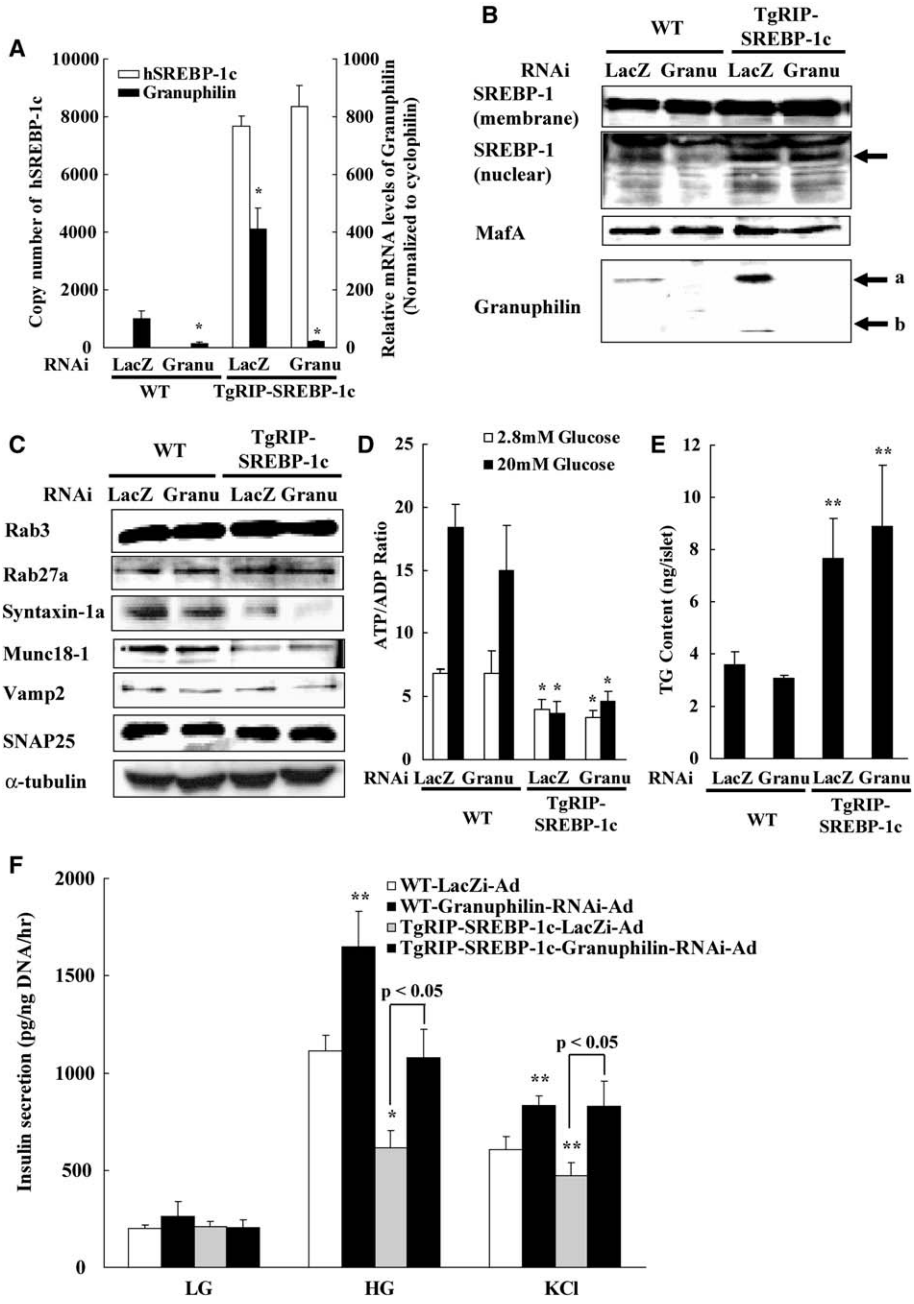

Figure 4. Effects of knockdown of granuphilin on insulin secretion in pancreatic islets from β cell-specific nuclear SREBP-1c transgenic mice

Islets were isolated from male age-matched (13- to 18-week-old) Wild-type-Control or TgRIP-SREBP-1c mice and were infected adenoviral-LacZ-RNAi or -granuphilin (Granu)-RNAi (500 MOI) for 48 hr.

A) mRNA levels of the human SREBP-1c transgene and endogenous granuphilin from the indicated islets as estimated by real-time PCR. hSREBP-1c levels were calculated as copy numbers.

B) Immunoblot analysis of SREBP-1, MafA, and granuphilin from the indicated islets.

C) Immunoblot analysis of the fusion machinery for exocytosis of insulin granules from the indicated islets with α-tubulin as a loading control.

D) Cellular ATP/ADP ratio from the indicated islets.

E) Cellular TG content from the indicated islets.

F) LG-, HG-, and KCl-stimulated insulin secretion in the indicated islets: WT-LacZ-RNAi (white bars), WT-Granuphilin-RNAi (black bars), TgRIP-SREBP-1c-LacZ-RNAi (light gray bars), and TgRIP-SREBP-1c-Granuphilin-RNAi (dark gray bars).

Results were normalized to cellular DNA content (**F**) and are expressed as mean ± SEM. *p< 0.01 and **p < 0.05 (versus WT-LacZi). Studies were performed in triplicate with sets of islets pooled from 3–4 mice per replicate.

function (Dubois et al., 2004; Joseph et al., 2004). To test whether granuphilin expression might contribute to the pathophysiologic consequences of lipotoxicity, we incubated isolated islets with palmitate (PA) for 48 hr. PA induced both SREBP-1c and granuphilin in normal islets at both mRNA and protein levels (Figures 6A, 6B, and S4E). These inductions were blunted in the absence of SREBP-1c, indicating that SREBP-1c plays a dominant role in PA-induced granuphilin expression. PA reduced KSIS as well as GSIS in SREBP-1c-normal mice; however, the PA-suppressed GSIS and KSIS were almost completely restored in SREBP-1-null mice (Figure 6C). These results suggested that PA-induced insulin secretion impairment is mediated through SREBP-1c. Furthermore, we used adenoviral-granuphilin RNAi treatment to completely suppress granuphilin expression in control and PA-treated islets (Figures 6D and S4F). The absence of granuphilin significantly ameliorated the suppression of both GSIS and KSIS induced by PA (Figure 6E), indicating that granuphilin upregulation is involved in PA-induced impairment of insulin secretion. PA induction of SREBP-1c/granuphilin was investigated at the transcriptional level in MIN6 cells. Both Granu-1200-Luc (Figure 6F) and SRE-Luc (data not shown) were activated by incubation with PA, indicating that induction of nuclear forms of endogenous SREBPs and granuphilin was at the transcriptional level. Knockdown of SREBP-1c led to a strong reduction in PA induction of the granuphilin promoter, but dominant-negative mutant MafA did not. These data demonstrated that incubation of isolated islets with PA, a model for lipotoxicity in β cells, caused impaired insulin secretion at least partially through induction of SREBP-1c and granuphilin.

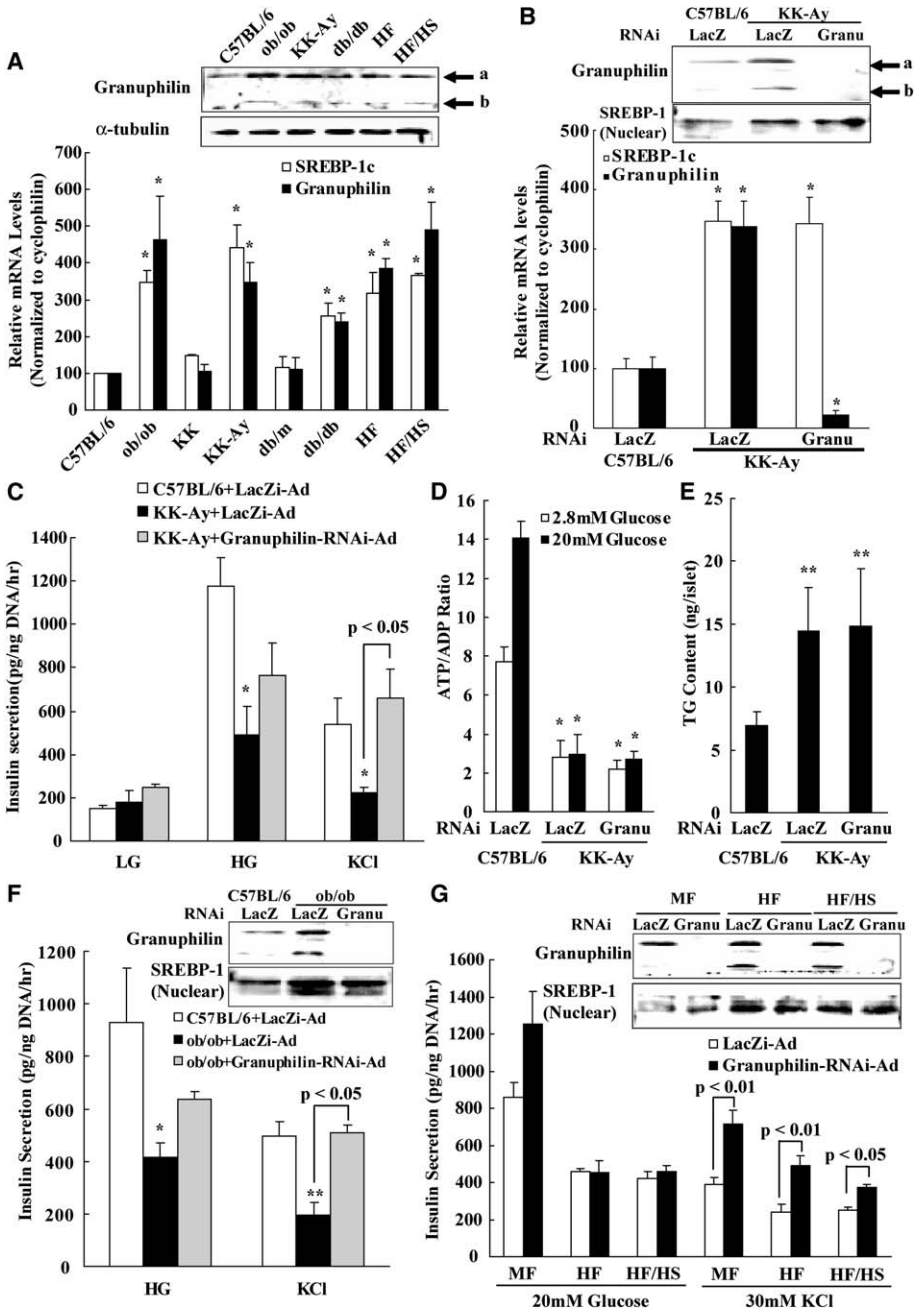

Figure 5. Activation of SREBP-1c and granuphilin in pancreatic islets from different diabetic model mice and effects of granuphilin knockdown on insulin secretion

Islets were isolated from male C57BL/6, ob/ob, KK, KK-Ay (10 weeks old), db/misty, and db/db mice (6 weeks old). C57BL/6, KK-Ay, and ob/ob mice islets were infected adenoviral-LacZ-RNAi or -granuphilin(Granu)-RNAi (500 MOI) for 48 hr.

A) Upper panel: immunoblot analysis of granuphilin from the indicated mice with α-tubulin as a loading control. Lower panel: mRNA levels of SREBP-1c (white bars) and granuphilin (black bars) in the islets from the indicated mice as estimated by real-time PCR. HF: high-fat diet, HF/HS: high-fat and high-sucrose diet. *$p < 0.01$ (versus C57BL/6).

B) Upper panel: immunoblot analysis of granuphilin and SREBP-1 from the indicated islets. Lower panel: mRNA levels of SREBP-1c (white bars) and granuphilin (black bars), from the indicated islets.

C) LG-, HG-, and KCl-stimulated insulin secretion in the indicated islets: C57BL/6-LacZ-RNAi (white bars), KK-Ay-LacZ-RNAi (black bars), and KK-Ay-Granuphilin-RNAi (gray bars).

D) Cellular ATP/ADP ratio from the indicated islets.

E) Cellular TG content from the indicated islets.

F) Upper panel: immunoblot analysis of granuphilin and SREBP-1 from the indicated islets. Lower panel: LG-, HG-, and KCl-stimulated insulin secretion in the indicated islets: C57BL/6-LacZ-RNAi (white bars), ob/ob-LacZ-RNAi (black bars), and ob/ob-Granuphilin-RNAi (gray bars).

G) Upper panel: immunoblot analysis of granuphilin and SREBP-1 from indicated islets. Lower panel: LG-, HG-, and KCl-stimulated insulin secretion in the indicated islets: LacZ-RNAi (white bars), Granuphilin-RNAi (black bars).

Results were normalized to cellular DNA content (**C**, **F**, and **G**) and are expressed as mean ± SEM. *$p < 0.01$ and **$p < 0.05$ (versus C57BL/6-LacZ, B-E). Studies were performed in triplicate with sets of islets pooled from 3–4 mice per replicate.

SREBP-1c inhibition of KSIS is abolished in granuphilin KO islets

Finally, the SREBP-1c effect on insulin secretion was investigated in islets from granuphilin-null mice (Figure 7). Adenoviral nuclear SREBP-1c caused changes in its target genes, ATP/ADP ratio, and triglyceride content in both wild-type and granuphilin KO islets in a similar manner to those in SREBP-1c transgenic islets (Figures 7A–7E). As compared to wild-type islets, both GSIS and KSIS were increased in the absence of granuphilin. Suppression of KSIS by SREBP-1c overexpression observed in granuphilin-normal islets was cancelled in granuphilin KO islets. Meanwhile, SREBP-1c-mediated inhibition of GSIS was partially ameliorated, but remained significantly reduced compared to controls (Figure 7F). Taken together with the data from granuphilin knockdown in SREBP-1c transgenic islets, these data demonstrate that granuphilin contributes to SREBP-1c-mediated KSIS inhibition.

Discussion

Granuphilin is a target gene of SREBP and MafA

Our current study clearly shows that mouse granuphilin is an SREBP target. Because SREBP-1c expression is highly regulated by nutrition (Horton et al., 1998; Hasty et al., 2000; Matsuzaka et al., 2004) and is upregulated in pathological states related to over-nutrition and insulin resistance (Biddinger et al., 2005), we propose that SREBP-1-induced granuphilin activation may contribute to β cell lipotoxicity and diabetes that can

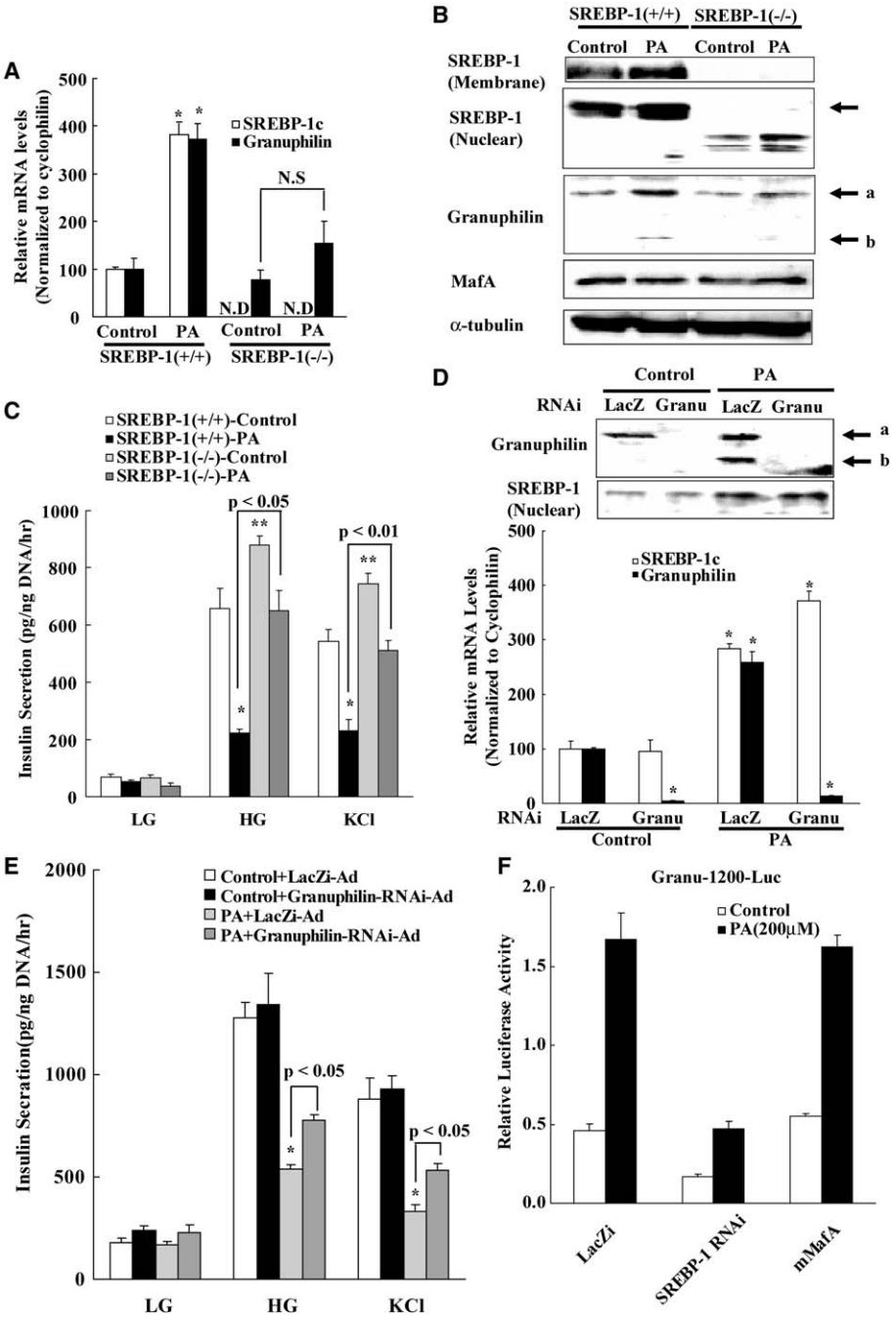

Figure 6. Involvement of granuphilin in insulin secretion from isolated murine islets treated with palmitate

Islets were isolated from male age-matched (13- to 17-week-old) SREBP-1-null, SREBP-1(−/−), and wild-type littermate, SREBP-1 (+/+), mice. The islets were incubated with 400 μM palmitate (PA) (**A**–**E**) and were infected with adenoviral-LacZ-RNAi or -granuphilin-RNAi (500 MOI) for 48 hr (**D** and **E**).

A) mRNA levels of SREBP-1c (white bars) and granuphilin (black bars) from the indicated islets as estimated by real-time RT-PCR. * $p < 0.01$ (versus SREBP-1(+/+)-Control). ND, not detectable.

B) Immunoblot analysis of SREBP-1, MafA, and mouse granuphilin from indicated islets with α-tubulin as a loading control.

C) LG-, HG-, and KCl-stimulated insulin secretion in SREBP-1(+/+)-control group (white bars), -PA group (black bars), SREBP-1(−/−)-control group (light gray bars), and -PA group (dark gray bars). *$p < 0.01$ and **$p < 0.05$ (versus SREBP-1(+/+)-Control).

D) Upper panel: immunoblot analysis of mouse granuphilin and SREBP-1. Lower panel: expression of SREBP-1c (white bars) and granuphilin (black bars) from the indicated islets as estimated by real-time RT-PCR. *$p < 0.01$ (versus Control-LacZ-RNAi).

E) LG-, HG-, and KCl-stimulated insulin secretion in islets untreated (Control) or treated with palmitate (PA), and infected with Adeno-LacZ-RNAi (LacZi-Ad) or Adeno-granuphilin RNAi (granuphilin RNAi-Ad). *$p < 0.01$ (versus Control-LacZi).

F) Effects of SREBP-1-RNAi and mutant MafA on Granu-1200-Luc in PA treated Min6 cells. The luciferase activity was normalized to *Renilla* luciferase activity and performed in triplicate.

Results were normalized to cellular DNA content (**C** and **E**) and are expressed by mean ± SEM. Studies were performed in triplicate with sets of islets pooled from three to four mice per replicate.

develop in these situations. We also show that the granuphilin promoter is activated by MafA. MafA has recently been established to be crucial for normal structure and function of β cells and insulin gene expression (Hagman et al., 2005; Kajihara et al., 2003) and thus is likely to be responsible for β cell-specific basal expression of granuphilin. Supportively, basal expression of granuphilin was severely diminished in islets from MafA knockout mice (S.T., unpublished data), while it was only 50% reduced in homozygous SREBP-1-null islets.

Granuphilin activation inhibits insulin secretion

Granuphilin has been thought to be a component of the fusion machinery for exocytosis of insulin granules. However, activation of granuphilin leads to inhibition of insulin secretion. Although the exact molecular mechanisms involved are yet unknown, various gain- and loss-of-function experiments from our current data as well as published reports strongly suggest that this Rab27a effector is a potent inhibitor of insulin secretion (Coppola et al., 2002; Gomi et al., 2005; Torii et al., 2002). SREBP-1c and thus granuphilin was activated in islets from SREBP-1c transgenic, KK-Ay, ob/ob, and DIO mice, as well as in islets treated with PA. Decreased KSIS in these islets were all restored by granuphilin knockdown, demonstrating that granuphilin plays a crucial role in the disturbance of insulin secretion post-ATP production. In combination with granuphilin activation, syntaxin-1a and Munc18-1, components of this docking machinery, were decreased by SREBP-1c activation and thus, may also be involved in the secretion impairment. Dysregulation

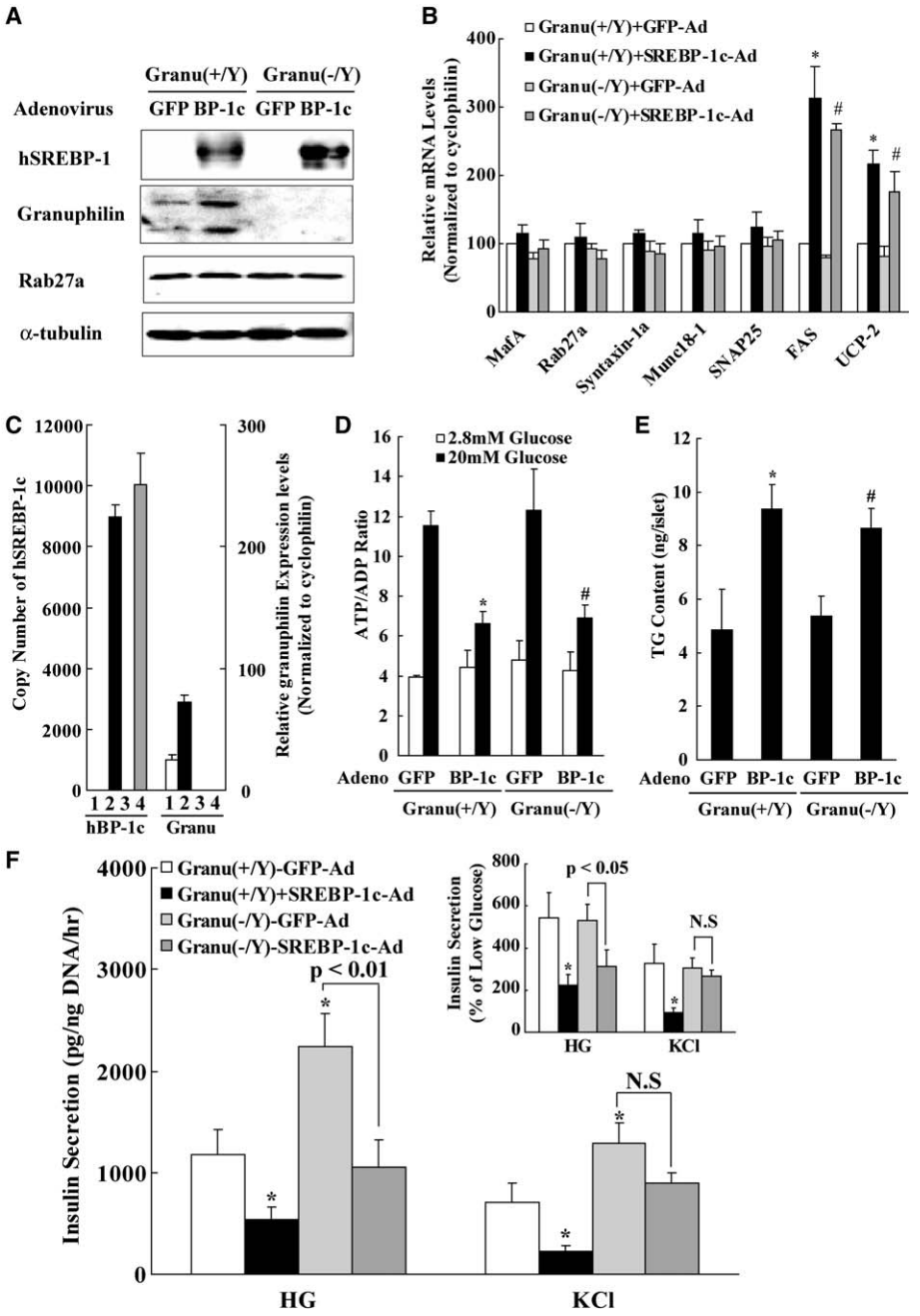

Figure 7. Effect of overexpression of nuclear SREBP-1c on insulin secretion in pancreatic islets from granuphilin-null mice

Islets were isolated from male age-matched (30- to 32-week-old) granuphilin-null (Granu(−/Y)) and wild-type littermate (Granu(+/Y)) mice. The islets were infected with adenoviral-GFP or -SREBP-1c (500 MOI) for 48 hr.

A) Immunoblot analysis of SREBP-1, granuphilin, and Rab27a from the indicated islets with α-tubulin as a loading control.

B) mRNA levels of indicated genes from the indicated islets as estimated by real-time RT-PCR. Granu(+/Y)-GFP (white bars), Granu(+/Y)-SREBP-1c (black bars), Granu(−/Y)-GFP (light gray bars), and Granu(−/Y)-SREBP-1c (dark gray bars).

C) mRNA levels of human SREBP-1c (hBP-1c) and granuphilin (Granu) from the indicated islets as estimated by real-time RT-PCR. Lane 1, Granu(+/Y)-GFP; lane 2, Granu(+/Y)-SREBP-1c; lane 3, Granu(−/Y)-GFP; lane 4, Granu(−/Y)-SREBP-1c.

D) Cellular ATP/ADP ratio from the indicated islets.

E) Cellular TG content from the indicated islets.

F) Upper panel: HG and KCl-stimulated insulin secretion ratio, normalized by LG-stimulated insulin secretion. Lower panel: HG- and KCl-stimulated insulin secretion in Granu(+/Y)-GFP (white bars), Granu(+/Y)-SREBP-1c (black bars), Granu(−/Y)-GFP (light gray bars), and Granu(−/Y)-SREBP-1c (dark gray bars).

Results were normalized to cellular DNA content (**F**) and are expressed by mean ± SEM. *p <0.01 (versus Granu(+/Y)-GFP) and #p < 0.01 (versus Granu(−/Y)-GFP). Studies were performed in triplicate with sets of islets from 3–4 mice per replicate.

of these three proteins may impair insulin secretion by disturbing the balance of vesicle transport component concentrations.

Potential involvement of SREBP-1c/granuphilin pathway in β cell lipotoxicity

We showed that upregulation of granuphilin is observed in β cells not only from transgenic mice overexpressing SREBP-1c but also from obesity-associated insulin resistant diabetic mice such as KK-Ay, ob/ob, and DIO mice, leading to a reduction of KSIS. It has been believed that impaired GSIS, due to deterioration of glucose metabolism characterized by decreased ATP/ADP ratio and/or insulin production in β-cells, is responsible for the insulin secretion defect in type 2 diabetes (Weir and Bonner-Weir, 2004). Our current data demonstrate that additional disturbances can also occur at the exocytosis stage of insulin secretion in diabetic models. Impaired KSIS was primarily mediated through activation of granuphilin, providing another mechanism for development of diabetes. Overexpression of granuphilin also inhibited GSIS in normal islets, but this was because the path to GSIS also includes the process of KSIS, and disturbance causing decreased KSIS should result in impaired GSIS as well. Supportively, granuphilin knockdown efficiently restored impaired KSIS in SREBP-1c transgenic and diabetic islets but only partially restored GSIS, indicating that the SREBP-1c/granuphilin effect was specific to the process post-ATP production.

Our data also provide further evidence that SREBP-1c is intimately involved in β cell dysfunction. In islets from the mice with lipotoxicity and diabetes, SREBP-1c is upregulated and contributes to disturbances in ATP production leading to impaired GSIS

and also to upregulation of granuphilin leading to attenuated KSIS. Various approaches using gene engineering and adenovirus consistently supported this hypothesis. Thus, the SREBP-1c/granuphilin pathway should be added to the list of potential mechanisms for β cell lipotoxicity in diabetes and could be a potential target for therapy of obesity-related diabetes. In contrast, it was reported that granuphilin as well as Rab27a and 3 were suppressed in a glucotoxic state of INS-1 cells through induction of ICER (Abderrahmani et al., 2006). Further studies are needed to elucidate the precise molecular role of granuphilin in insulin secretion and to determine the extent to which granuphilin contributes to diabetes in humans.

Experimental procedures

Materials

All chemical compounds were obtained from Sigma chemicals. Enhanced chemiluminescence Western blot detection kit and redivue [α-^{32}P] dCTP were purchased from Amersham Pharmacia and restriction enzymes were from Takara Bio Inc.

Animals

All animal studies were approved by the Animal Care Committee of University of Tsukuba. Male C57BL/6, ob/ob, db/m, and db/db mice were purchased from Charles River Laboratories. KK and KK-Ay were purchased from Clea. RIP-human nuclear SREBP-1c transgenic mice (Takahashi et al., 2005), SREBP-1-null mice (Shimano et al., 1997b), and Granuphilin-null mice (Gomi et al., 2005) were generated as previously described. Animals were adapted to environments for 1 week before isolation of pancreatic islets. The mice were housed in colony cages and maintained on a 12 hr light/12 hr dark cycle and given free access to water and a standard chow diet (MF, Oriental yeast).

Expression plasmids

All expression vectors were produced as previously described (Amemiya-Kudo et al., 2000, 2005; Ide et al., 2004; Kajihara et al., 2003; Yamamoto et al., 2004).

Reporter plasmid

The reporter plasmid Granu-1200-Luc contains a fragment of the granuphilin promoter from −1200 to +87 bp cloned into the MluI/XhoI sites of the pGL3 basic vector (Promega) containing the coding sequences of firefly luciferase cDNA. Other constructs were produced by PCR using this construct as a DNA template, and the PCR products were inserted into pGL3 basic vector. The primers used for PCR were as follows: 5′ primers Granu-1200-Luc 5′-taagcgttgagaaatgaatg-3′, Granu-500-Luc 5′-agggttgcttacagggcgat-3′, Granu-300-Luc 5′-tcgctgaacaaaaaggcaag-3′, Granu-265-Luc 5′-ttcctacccc caccccgac-3′, Granu-174-Luc 5′-tgctaactcaggaggaaattc-3′, Granu-150-Luc 5′-aattcctccagtgccttaag-3′, and 3′ primer 5′-ggtccgggtgccgaatg-3′. Restriction sites MluI and XhoI were added to each 5′ primer and 3′ primer, respectively. The site-directed mutagenesis constructs mSRE- and mMARE-Luc were produced by PCR with the following primers: mSRE-5′ 5′-attcccat tcc*gta*ccccaccccgacccc-3′, -3′ 5′-ggggtcgggggtgggg*tac*ggaatgggaat-3′, mMARE-5′ 5′-tcaaactct*cag*aactcaggaggaaattcc-3′, and -3′ 5′-ggaatttcctcc tgagttc*tga*gagtttga-3′. SRE-Luc was produced as previously described (Amemiya-Kudo et al., 2000).

Cell cultures, transfection, and luciferase assays

Min6 cells were cultured at 37°C in atmosphere of 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) with 25 mM glucose supplemented with 15% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and β-mercaptoethanol. Each expression plasmid (0–0.5μg), luciferase reporter plasmid (0.2 μg), and pSV40-*Renilla* plasmid (0.05 μg) were cotransfected using the LipofectAMINE reagent (Invitrogen) according to manufacture's instructions. Min6 cells were seeded in 24-well plates at a density of 3 × 10^5 cells/well. The total amount of DNA was adjusted to 0.5–1 μg/well with empty vector DNA. After 48 hr of transfection, cells were washed with phosphate-buffer saline (PBS) and harvested. Luciferase assays were carried out according to the manufacture's instructions (Promega), and luciferase activity was quantified by using ALOVA 1420 multilabel counter (Perkin Elmer Life Sciences). As the internal standard, SV40 *Renilla* luciferase control vector was also cotransfected to normalize for transfection efficiency.

Gel mobility shift assay

Gel mobility shift assays (EMSA) were performed as previously described (Amemiya-Kudo et al., 2005; Nakagawa et al., 2006; Yamamoto et al., 2004). In brief, recombinant SREBPs (SREBP-1a, -1c,and -2) and MafA proteins were produced using the T_NT T7 quick-coupled transcription/translation system (Promega). The reaction mixture were incubated with in vitro synthetic protein lysate and then analyzed on 4% polyacrylamide gels. Super-shift reactions were performed by adding antibody (anti-SREBP-1, sc-8984 Santa Cruz) to the reaction mixture. The DNA probes for EMSA are followed: granuphilin-SRE, aactttattcccattcctacccccacccccgaccccaatc atctaagaga, granuphilin-mSRE, aactttattcccattcc*gta*ccccaccccgaccccaat catctaagaga, granuphilin-MARE, cctcaaactctgctaactcaggaggaaattcctccagtg cc, and granuphilin-mMARE, cctcaaactc*cag*taactcaggaggaaattcctccagtgcc.

Isolation of mouse pancreatic islets and insulin secretion experiments

Isolation of islets from mice was carried out using the Ficol-Conray protocol as previously described(Scharp et al., 1973; Takahashi et al., 2005). Briefly, 4 mg/ml collagenase was injected into the pancreatic duct and incubated at 37°C for 20 min. The islets were subsequently purified by Ficol gradient and hand picking. The remaining islets were cultured for 2 hr at 37°C in a humidified atmosphere containing 5% CO_2 and in regular RPMI-1640 medium supplemented with 10% FCS (pH 7.4) prior to the experiments. Insulin release from islets was measured as previously described (Takahashi et al., 2005). In brief, groups of 10 islets of similar size for each condition were preincubated in Krebs-Ringer bicarbonate buffer (KRBH [pH 7.4]) containing 0.5% BSA at 2.8mM glucose for 30 min. Islets were then incubated with 1 ml of KRBH with 0.5% BSA at 2.8mM glucose (low glucose; LG), 20mM glucose (high glucose; HG), 30mM KCl with 2.8 mM glucose (KCl), 3mM Arginine with 2.8 mM glucose, forscolin (10μM) with 2.8 mM glucose, or TPA (0.5 μM) with 2.8 mM glucose, respectively. Subsequently, medium were replaced with fresh medium for insulin determination followed by total islet insulin extraction by 0.5ml of cold acid ethanol mixture (75% ethanol with 0.2M HCl). Hoechst-33258 staining of sonicated islets was performed to determine the islet DNA content.

Determination of ATP/ADP ratio and triglyceride contents of islets

ATP and ADP content in isolated islets were determined as previously described (Schultz et al., 1993; Takahashi et al., 2005). In brief, after preincubation at 37°C for 30 min in KRBH buffer containing 2.8 mM glucose, groups of 25 islets were incubated at 37°C for 30 min in KRBH buffer containing 20 mM glucose. The conversion from ADP to ATP was performed in the reaction buffer (20mM HEPES and 3mM $MgCl_2$ [pH 7.75]) containing 2.3 U/ml pyruvate kinase and 1.5mM phosphoenolpyruvate at room temperature for 15 min. ATP and ADP were extracted from islets with 100 μl of 5% trichloroacetic acid (TCA). After centrifugation, the supernatants were neutralized with NaOH. ATP content was measured using CellTiter-Glo luminescent cell viability assay kit (Promega).

Real-time PCR and immnoblot analysis

Comparative analysis of mRNA levels was performed with fluorescence-based real-time PCR. Total RNA extraction and first-strand cDNA synthesis were previously as described (Takahashi et al., 2005). Quantitative real-time PCR was performed using Sybr-Green Dye (Nihon Gene) in an ABI 7000 PCR instrument (Applied Biosystems). Relative abundance for each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of a cDNA sample and normalized to cyclophilin. Primer sequences are available upon request. For immnoblot blots, total or nuclear cell extracts of isolated islets (Hagman et al., 2005; Takahashi et al., 2005) and nuclear extracts from Min6 cells (Amemiya-Kudo et al., 2005) were prepared as described previously, separated by SDS-PAGE, transferred to immobilon-P membranes (Millipore), and were probed with polyclonal anti-SREBP-1 (sc-8984), anti-Rab27a (sc-22756), anti-α-tubulin (sc-5282, Santa Cruz), anti-MafA (A-300-611, Bethyl Lab. Inc.), anti-syntaxin-1a (S1172, Sigma), anti-Vamp2 (627724, Calbiochem), anti-Rab3 (R35520), anti-Munc18-1 (M32320), and

anti-SNAP25 (S35020, BD) antibodies. Detection was performed using an ECL advance Western blotting ECL detection kit and Hyperfilm (Amersham Biosciences). Anti-granuphilin-a/b antibody was as previously described (Gomi et al., 2005).

Northern blot analysis

Northern blot analysis was performed as previously described (Amemiya-Kudo et al., 2005; Matsuzaka et al., 2004). cDNA probe for granuphilin was prepared from reverse transcriptase for PCR of Min6 cells total RNA with the following primers: 5′-cgagatggaaagggatttga-3′ for sense and 5′-gtctgc tgaaggagggactg-3′ for antisense of granuphilin.

Preparation of recombinant adenovirus

The construct of granuphilin-a coding cDNA was generated by PCR amplification and into the pShuttle-CMV vector (Ide et al., 2004; Nakagawa et al., 2006). The following primers were used: 5′ primer, 5′-atgtcggagatactagacc tctctt-3′, and 3′ primer 5′-catacacccagcttctgcttgaccat-3′. Generation of recombinant adenoviral plasmid was produced by homologous recombination with the pAdEasy-1 plasmid. The siRNA construct for mouse granuphilin and SREBP-1 was generated in the coding sequence; Granuphilin-RNAi-784 5′-gagcgagagtctggatagctaca-3′, Granuphilin-RNAi-1955 5′-aggctggaagatct acaacaaat-3′, and SREBP-1-RNAi 5′-ggcaaaggaggcactacag-3′. Oligonucleotide containing this sequence was subcloned into U6/RNAi empty vector (Invitrogen). Granuphilin RNAi adenoviruses were generated using BLOCK-iT Adenoviral RNAi Expression System (Invitrogen). Production of recombinant adenoviruses was performed by CsCl gradient centrifugation as previously described (Ide et al., 2004).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Amemiya-Kudo et al., 2005; Nakagawa et al., 2006). To amplify the granuphilin promoter region containing SRE and MARE, the following primer sets were used: for SRE 5′-gtactttactattggacaac-3′ for sense, 5′-ccttaaggcactggaggaatttc-3′ for antisense, and for MARE 5′-gtgagcaaagata gactaga-3′ for sense, 5′-tggagcggggaggagctggactc-3′ for antisense. The PCR conditions were 5 min at 95°C and 30 cycles of 30 s at 94°C, 30 s at 57°C and 1 min 72°C for SRE region, and 10 min at 94°C and 30 cycles of 10 s at 98°C, 30 s at 54°C, and 1 min 72°C for MARE region.

Palmitate treatment

Palmitate was dissolved in ethanol as 100 mM stock solution with 0.5% fatty acid free BSA (Sigma). For using experiments, palmitate was diluted in DMEM supplemented with 0.5% fatty acid free BSA to a final concentration of 400 μM for islets study or 200 μM for Luc-assay.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using analysis of variance (ANOVA).

Supplemental data

Supplemental data include five figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/2/143/DC1/.

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