Expanded View for Li et al.

BIG3 inhibits insulin granule biogenesis and insulin secretion

EXPERIMENTAL PROCEDURES

Animal studies

All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of A*STAR (Agency for Science, Technology and Research). All mice used in this study were bred and housed in our animal facility. For tissue analysis, islet isolation and histochemistry, mice were killed by cervical dislocation. Refer to Supplemental Materials for details on the generation of BIG3 knockout (BKO) mouse and stable BIG3 knockdown (BKD) MIN6 cells, physiology measurements, islet isolation, prohormone processing and hormone secretion assays, microscopic imaging and electrophysiology studies.

Generation of BIG3 knockout mouse

The coding region of mouse BIG3 was derived from 34 exons. A BIG3 gene-targeting construct was created by inserting the Neo-cassette into the 12th exon (1351-2249 bp) encoding the majority of the Sec7 domain (1735-2376 bp). A SalI site was first introduced into this exon by site-directed mutagenesis and a targeting vector was generated by inserting the Neo-cassette into this site with a 3.3 kb right arm and 2.8 kb left arm flanking the insertion. The targeting vector pBluescript SK+ (Stratagene) was linearized and transfected into embryonic stem cells (E-14) via electroporation followed by G418 (Invitrogen) selection. After 7-9 days of selection, the surviving colonies were picked and screened by long-range PCR. The correctly targeted ES cells were used for blastocyst microinjection. Chimeras were used for

crossing with 129/Sv females to obtain F1 generation mice. Crosses between F1 mice gave rise to wildtype ($BIG3^{+/+}$), heterozygous knockout ($BIG3^{+/-}$) and homozygous knockout ($BIG3^{-/-}$) mice as assessed by a PCR-based method. Heterozygous knockout mice were back-crossed with wildtype 129/Sv mice for another eight times to purify the genetic background. Male littermates were used for studies.

Physiology measurements and tests

For glucose and insulin measurements, blood samples were collected from ~3-month-old male littermates at various conditions: free access to food, 2h fasting, 16h fasting, 1h post-refeeding after 16h fasting. Glucose levels were measured by handheld glucose meters (accu-check). Serum insulin was measured by ELISA (Mercodia). For glucose tolerance test, mice were fasted for 16h, weighed and injected intraperitoneal with 20% glucose at 2mg per gram body weight [1, 2]. Glucose levels were measured 15, 30, 60 and 120 minutes after injection. For refeeding test, glucose levels were measured from 16h fasted mice and following 0.5, 1, 2 and 3 hours after giving food. Hyperinsulinemic-euglycemic clamp analysis was performed as previously described [3].

Metabolic analysis

Littermates at 3 months of age were individually housed and maintained at 24 °C under a 12:12-hour light-dark cycle in a 12-cage equal flow Oxymax Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments) [4]. Mice were acclimatized to the metabolic cages for 2 days before beginning of data acquisition.

Tissue preparation for immunoblots, histochemistry, immunofluorescence and electron microscopy

Mouse tissues were snap frozen and lysed in RIPA buffer with protease inhibitor (Roche). Cultured cell lysates were prepared on ice with the same buffer. Cell lines were obtained from ATCC if not specified. Protein concentration of lysates was determined by BCA (Pierce) and subjected to SDS-PAGE and western blot, using iBlot (Invitrogen) for transfer, primary antibodies as below used at 1:1,000 dilution, HRP-conjugated secondary antibodies used at 1:3,000-5,000 dilution (Jacksons, Santa Cruz), and ECL Prime (Amersham) for developing. Primary antibodies used for Western blot included anti-SgIII (Santa Cruz), anti-CPE (BD), anti-BIP (BD), anti-γ-adaptin (BD), anti-chromograninA (Epitomics), anti-β-granin (BD), and anti-actin (Sigma-Aldrich). BIG3 polyclonal antibody was made by immunizing rabbits with a GST-tagged fragment of BIG3 (codons 608-877) and affinity purified with the immune sera. For histological analysis, mouse tissues were fixed in 4% PFA at 4 °C before paraffin or frozen embedding, followed by H&E or immunostaining. For morphological electron microscopy (EM), pancreatic islets were fixed with 2.5% glutaraldehyde (Agar Scientific) and 1% OsO_4 in PB (pH 7.4) at 4 °C for 2 hours.

Hyperinsulinemic-euglycemic clamp analysis

Mice were implanted with a catheter into the right jugular vein at least 5 days before the experiment. The mice were fasted for 5 hours and restrained for cut tail sampling. A 5 μCi bolus of [3-^{3}H] glucose (PerkinElmer) was given at time (t) = -90 minute, followed by a 0.05 μCi/min continuous infusion for 90 min to measure basal glucose turnover. After basal sampling, the insulin clamp was started at t = 0 with a bolus and continuous infusion of human insulin (300 mU/kg bolus followed by 4 mU/kg/min; Eli

Lilly Co.) coupled with a variable infusion of 20% glucose to maintain euglycemia (~150-160mg/dl). At the same time, the infusion rate of [3-^{3}H] glucose was increased to 0.1 μCi/min for the remaining experimental period. A 10 μCi bolus of 2-deoxy-D-[1-^{14}C] glucose (PerkinElmer) was administrated at t=88 min. Blood samples were collected every 10 minute from t = 90-120 min for analysis of plasma levels of [3-^{3}H] glucose and 2-deoxy-D-[1-^{14}C] glucose. At t = 120 min, mice were euthanized with sodium pentobarbital. White adipose tissue, soleus and extensor digitorum longus (EDL) muscle were rapidly isolated and stored at -80°C for further analysis.

Glucose uptake measurement

After deproteinization with barium hydroxide [0.3 N] and zinc sulfate [0.3 N], plasma samples were dried and resuspended in 1 ml distilled water. Plasma levels of [3-^{3}H] glucose and 2-deoxy-D-[1-^{14}C] glucose were assessed by a dual channel scintillation counter (Beckman Coulter). Tissue samples were homogenized in 0.5% perchloric acid, followed by centrifugation at 2,000 g at 4°C. The homogenates were neutralized with potassium hydroxide. An aliquot of the homogenates was used to measure radioactivity of 2-deoxy-D-[1-^{14}C] glucose and 2-deoxy-D-[1-^{14}C] glucose-6-phosphate. Another aliquot of homogenates were treated with barium hydroxide and zinc sulfate to remove 2-deoxy-D-[1-^{14}C] glucose-6-phosphate, and radioactivity of 2-deoxy-D-[1-^{14}C] glucose was measured. The level of 2-deoxy-D-[1-^{14}C] glucose is the difference between the two aliquots. Calculation of hepatic glucose production and tissue-specific glucose uptake was previously described [5].

Islet isolation and measurements

Islets from BKO and control mice were isolated after liberase digestion (1 mg/ml; Roche) and cultured for 24h at 11.1 mM glucose in hCell medium (hCell Technology). Subsequent experimental handling was performed with a Krebs-Ringer medium (KRH) containing (in mM) 130 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 2.56 $CaCl_2$, supplemented with 1 mg/ml BSA and 3 mM D-glucose, and buffered with 20 mM Hepes-NaOH to pH 7.4. For estimation of islet insulin/proinsulin content, islets were lysed by sonication in 200 ml of passive lysis buffer (Promega) after incubation in KRH medium for 2 hours. Insulin content in the buffer was measured by ELISA (Mercodia). For analysis of islet secretory responses, similar-sized islets from a single mouse (10 islets per dish) were first incubated at 37 °C for 60 min in a flow chamber under continuous perifusion with KRH medium. Islets were then stimulated with 16.7 mM glucose for 30 min. Fractions of the medium were collected every 3 min, starting at 3 min before stimulation. Insulin concentration in each fraction was measured as described above. Total protein of lysed islets were measured by BCA**.** Islet mRNA was extracted from freshly isolated islets with mRNA isolation kit (Qiagen), and converted to cDNA for quantitative real-time PCR with Taqman probe (ABI) following manufacturers' instructions.

Gene knockdown, overexpression and rescure

Short-hairpin RNAs against mouse BIG3 were designed with Oligoengine and Dharmacon online tools and screened for efficacy. The sequence 5'-ACA CCA ACC ACC CAC CTT A-3' was constructed into a pCMV-GEF-U3-NHE1 vector (kindly provided by Dr. Tergaonkar, IMCB, A-STAR, Singapore). Plasmids with target or control scrambled sequences were delivered into MIN6 cells (kindly provided by Dr. Junichi Miyazaki, Osaka University) by a lenti-viral system as previously described

[6]. Infected cells were sorted on a FACSAriaII (BD) and grown with G418 (invitrogen) selective media to isolate stable cell pools.

KIAA1244 clone, a generous gift from the Kazusa DNA Research Institute (Chiba, Japan), was cloned into pmCherry C-1(Clotech), and the resulting expression plasmid was transfected into MIN6 by TurboFect (Thermo) according to manufacturer's instructions.

Insulin secretion in MIN6 cells

MIN6 cells were cultured to ~80% confluence, rinsed and incubated with fresh pre-warmed serum and glucose-free DMEM, followed by the addition of D-glucose (final concentration 16.7 mmol/L) alone, or together with KCl and $CaCl_2$ (50 mM and 5 mM final concentration, respectively). 3% of medium was collected at 30 and 0 minutes before treatment, as well as 15, 30, 45 and 60 minutes after treatments, and quantified by ELISA (Mercodia) to determine secreted insulin. Cells were harvest after 60 minutes to determine total protein levels by BCA assay.

Imaging and analysis

For histological analysis, thirty serial sections (5 μm) from each sample were stained with H&E. Islet and total pancreas areas were measured. For EM, islets were dehydrated in an ethanol series and embedded in araldite epoxy resin (EMS). Ultrathin (90 nm) sections were stained with 2% uranyl acetate (Analar) for 5 min and examined by using a JEOL JEM-1220 electron microscope (JEOL Asia Pte). Randomly selected β-cells were imaged. Granules in every β-cell were examined, and cytoplasm area and dense core area were measured. In total, 80 cells from 4 animals per group were analyzed. For immune-fluorescent microscopy, 12-μm

cryosections were mounted on polylysine-coated slides and stained with primary antibodies followed by appropriate FITC- and Texas red-conjugated or Alexa Fluor 488- and Alexa Fluor 555-conjugated secondary antibodies (Invitrogen). MIN6 cells were cultured to ~80% confluency on cover slips and fixed with 2.7% PFA in PBS. Treated samples were refreshed with pre-warmed growth medium containing 50 mM KCl and 5 mM $CaCl_2$, or 50 µg/ml cycloheximide, and incubation continued for 1 hour before fixation. ImageJ (NIH) was used to analyze images. Antibodies used for immunostaining in this study included anti-insulin (Sigma-Aldrich), anti-chromogranin A (BD), anti-γ-adaptin (BD), anti-Vti1a (BD), anti-Stx6 (BD), anti-GS28 (BD), anti-GM130 (BD).

Electrophysiological measurements

Membrane capacitance was recorded from MIN6 cells cultured on glass slides, or β-cells in intact isolated islets using the standard whole-cell patch-clamp technique [7-10]. Exocytosis was elicited by a 50 or 500 ms depolarizing pulse from −70 to 0 mV. Single β-cells were identified by a discernible Na^+ current and TEA-resistant K^+ currents and/or by their electrical activity in the absence of glucose or low glucose that could be suppressed by the addition of glucose (20 mmol/l). Pipette resistance ranged between 3 and 6 MΩ when pipettes were filled with an intracellular solution containing (in mM): 125 potassium glutamate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 Hepes, 0.05 EGTA, 0.1 cAMP and 4 MgATP, pH 7.1. The extracellular solution contained (in mM) 118 NaCl, 20 TEA-Cl (tetraethylammonium chloride), 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, and 5 Hepes, pH 7.4. Cells were stimulated at low frequency (<0.05 Hz) to allow full recovery of exocytotic capacity between pulses. Measurements were performed using EPC10-2 patch clamp amplifier and Pulse software (HEKA

Elektronik, Lambrecht/Pfalz, Germany). Currents were compensated for capacitive transients and linear leak using a –P/4 protocol. Exocytosis was detected as changes in cell membrane capacitance (Cm), which was estimated by the Lindau–Neher technique implementing the 'Sine+DC' feature of the lock-in module [11]. The amplitude of the sine wave was 30 mV and the frequency was set at 1 kHz. All Cm measurements were performed at 28 °C.

Prohormone processing assay

For proinsulin processing assay, MIN6 cells were pre-stimulated with 50 mM KCl and 5 mM $CaCl_2$ for 1h in growth medium, then switched to fresh medium with 50 µg/ml cycloheximide. Cells were harvested to determine the proinsulin content by ELISA (Mercodia) and total protein at 0, 15, 30, 60 and 90 minutes. Duplicates or triplicates of individual wells at each time point were used in each experiment, and data represented the average of 3 experiments. For CGA processing assay, equal amount of MIN6 cells were pre-treated with 50 mM KCl and 5 mM $CaCl_2$ and without L-methionine for 60 minutes. Then the cells were labeled with 20 µCi of [^{35}S]Methionine (Perkin Elmer) for 15 min. Cells were washed for 5 times with DMEM without L-methionine and then incubated with DMEM containing 10 mM L-methionine. The cell lysates were collected at 0, 15, 30, 60 minutes and applied to 40 µl Protein A/G Sepharose beads and incubated for 1h at 4°C. The beads were pre-bound with 20 µg each of the mouse monoclonal (BD) and rabbit polyclonal (Epitomics) antibodies against CGA. After 5 times washing with lysis buffer, the beads were eluted with 2× Laemmli sample buffer. The samples collected were analyzed by SDS-PAGE followed by autoradiography.

REFERENCES

1. Gustavsson N *et al* (2008) Impaired insulin secretion and glucose intolerance in synaptotagmin-7 null mutant mice. *Proc Natl Acad Sci U S A* **105:** 3992-3997

2. Gustavsson N, Wei SH, Hoang DN, Lao Y, Zhang Q, Radda GK, Rorsman P, Sudhof TC, Han W (2009) Synaptotagmin-7 is a principal Ca2+ sensor for Ca2+ -induced glucagon exocytosis in pancreas. *The Journal of physiology* **587:** 1169-1178

3. Liu Y, Turdi S, Park T, Morris NJ, Deshaies Y, Xu A, Sweeney G (2013) Adiponectin corrects high-fat diet-induced disturbances in muscle metabolomic profile and whole-body glucose homeostasis. *Diabetes* **62:** 743-752

4. Lou PH, Gustavsson N, Wang Y, Radda GK, Han W (2011) Increased lipolysis and energy expenditure in a mouse model with severely impaired glucagon secretion. *PLoS One* **6:** e26671

5. Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, Wasserman DH, McGuinness OP (2010) Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Dis Model Mech* **3:** 525-534

6. Tergaonkar V, Correa RG, Ikawa M, Verma IM (2005) Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. *Nat Cell Biol* **7:** 921-923

7. Gopel SO, Kanno T, Barg S, Rorsman P (2000) Patch-clamp characterisation of somatostatin-secreting -cells in intact mouse pancreatic islets. *J Physiol* **528:** 497-507

8. Gopel S, Zhang Q, Eliasson L, Ma XS, Galvanovskis J, Kanno T, Salehi A, Rorsman P (2004) Capacitance measurements of exocytosis in mouse pancreatic alpha-, beta- and delta-cells within intact islets of Langerhans. *J Physiol* **556:** 711-726

9. Gustavsson N, Wei SH, Hoang DN, Lao Y, Zhang Q, Radda GK, Rorsman P, Sudhof TC, Han W (2009) Synaptotagmin-7 is a principal Ca2+ sensor for Ca2+ -induced glucagon exocytosis in pancreas. *J Physiol* **587:** 1169-1178

10. MacDonald PE, De Marinis YZ, Ramracheya R, Salehi A, Ma X, Johnson PR, Cox R, Eliasson L, Rorsman P (2007) A K ATP channel-dependent pathway within alpha cells regulates glucagon release from both rodent and human islets of Langerhans. *PLoS Biol* **5:** e143

11. Lindau M, Neher E (1988) Patch-clamp techniques for time-resolved capacitance measurements in single cells. *Pflugers Arch* **411:** 137-146