Munc18-1 Regulates First-Phase Insulin Release by Promoting Granule Docking to Multiple Syntaxin Isoforms

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Running title: Munc18-1 promotes SNARE assembly in β-cells

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Background: Munc18-1 is expressed in islet β-cells, but its functional requirement remains unknown.

Results: Munc18-1 knockout mice have impaired glucose tolerance and first-phase insulin release defects. Munc18-1 over-expression enhances human islet insulin release, and increases SNARE complex formation.

Conclusions: Munc18-1 is required in insulin exocytosis for facilitating SNARE assembly using multiple syntaxin isoforms.

Significance: Increased Munc18-1 in human islets enhances β-cell function.

SUMMARY

Attenuated levels of the Sec1/Munc18 (SM) protein Munc18-1 in human islet beta cells is coincident with Type 2 diabetes, although how Munc18-1 facilitates insulin secretion remains enigmatic. Herein, using conventional Munc18-1-/− and beta-cell specific Munc18-1−/− knockout mice, we establish that Munc18-1 is required for the first-phase of insulin secretion. Conversely, human islets expressing elevated levels of Munc18-1 elicited significant potentiation of only first-phase insulin release. Insulin secretory changes positively correlated with insulin granule number at the plasma membrane: Munc18-1-deficient cells lacked 35% of the normal component of pre-docked insulin secretory granules, whilst cells with elevated levels of Munc18-1 exhibited a ~20% increase in pre-docked granule number. Pre-docked Syntaxin 1-based SNARE complexes bound by Munc18-1 were detected in beta cell lysates, yet surprisingly, were reduced by elevation of Munc18-1 levels. Paradoxically, elevated Munc18-1 levels coincided with increased binding of Syntaxin 4 to VAMP2 at the plasma membrane. Accordingly, Syntaxin 4 was requisite for Munc18-1 potentiation of insulin release. Munc18c, the cognate SM isoform for Syntaxin 4, failed to bind SNARE complexes. Given that Munc18-1 does not pair with Syntaxin 4, these data suggest a novel indirect role for Munc18-1 in facilitating Syntaxin 4-mediated granule pre-docking to support first-phase insulin exocytosis.

Munc18-1/nSec1 is one of three plasma membrane-localized Sec1/Munc18 (SM) proteins expressed in mammalian cells. SM proteins are essential regulators of SNARE-protein mediated vesicle docking/fusion events, acting as high affinity binding partners for target membrane SNARE (t-SNARE) Syntaxin proteins. The other two Munc18 proteins, Munc18b and Munc18c, share greater than 50% sequence similarity with Munc18-1. Munc18-1 expression is restricted to neuronal, adrenal chromaffin, and pancreatic islet beta cells, while Munc18b and Munc18c are ubiquitously expressed (1,2). Munc18-1 and Munc18b pair similarly with plasma membrane localized Syntaxin isoforms 1-3.
but not 4, while Munc18c pairs exclusively with Syntaxin 4 (1,3). Despite sharing a similar three-dimensional crystallographic structure (4-6), it remains unknown how Munc18-1 and Munc18c are specified to differentially pair with Syntaxin 1 versus Syntaxin 4, respectively.

Recent studies show that Munc18-1 contains a cleft which binds to and chaperones Syntaxin 1 through its multiple binding modes (7,8). Munc18-1 was initially found to associate with the ‘closed’ form of Syntaxin 1, and this closed form is presumed to prevent its participation in SNARE core complexes (5,9). SNARE core complexes are formed once the v-SNARE protein present on the vesicle membrane docks with the two t-SNARE proteins present on the plasma membrane (PM) to form a bundle composed of four alpha helices, or a trans-SNARE complex (10). However, Munc18-1 was later reported to bind to the SNARE core complex, wherein Syntaxin 1 is presumed to be in its “open and accessible” conformation (11-13). How Munc18-1 can accommodate binding to Syntaxin 1’s variable conformations has recently been proposed to proceed via structural changes in Munc18-1 in tandem with Syntaxin 1 (6). Despite this plethora of compelling in vitro and liposome-based binding evidence, Munc18-1 binding to the SNARE complex in cells or cell lysates has yet to be confirmed. Moreover, cell-system based studies of Munc18-1 over-expression have shown both enhancement and inhibition of synaptic vesicle exocytosis (14-16), confounding the designation of the primary functional role for Munc18-1 in neurotransmitter release.

Islet beta cells are unique in that they both express and functionally require Syntaxin 1- and Syntaxin 4-based SNARE complexes for insulin granule exocytosis. Syntaxin IA⁻/⁻ mouse islets exhibit impaired first-phase insulin secretion with normal levels of second phase secretion (17). Syntaxin 4⁻/⁻ mouse islets show impairments in both phases of insulin release (18), although Munc18c⁻/⁻ and Munc18c RNAi-depleted mouse islets show loss of exclusively second-phase insulin release (19). That Syntaxin 4 but not Munc18c is required for first phase remains inexplicable at present, since the prevailing concept is that syntaxin functions are coordinated via their SM partner specificity. While Munc18-1, partner of Syntaxin 1A, has been implicated as a necessary factor in insulin secretion from clonal beta cell lines (20), its role and requirement in biphasic islet secretion has yet to be tested, with the islet being the definitive physiologically relevant system. Of interest from a potential therapeutic standpoint is the ability of some but not all of these four proteins to enhance exocytosis mechanisms upon their over-expression in a cellular context: Munc18-1 and Syntaxin 4 enhance (14,18,20,21), whilst Munc18c and Syntaxin IA are inhibitory (21,22). However mechanisms by which these differences in function occur in terms of SNARE complex formation in beta cells remain unexplored.

Taking advantage of conventional Munc18-1⁻/⁻ and β-cell specific Munc18-1⁻/⁻ (β-cell Munc18-1KO) in vivo model systems in the present study, we identify Munc18-1 as a required SM protein mediator of first-phase insulin release. Munc18-1 depleted islet β-cells contained 35% fewer morphologically pre-docked insulin granules under basal/unstimulated conditions, consistent with a function for Munc18-1 in acute insulin release. Furthermore, functional and mechanistic studies provide evidence to suggest that increased Munc18-1 expression in human islets can preferentially potentiate acute insulin release. β-cell protein-protein interaction studies revealed that underlying this enhancement of insulin release, Munc18-1 over-expression resulted in enhanced binding of VAMP2 to Syntaxin 4, rather than to Syntaxin 1A, as would otherwise been expected based upon Munc18-Syntaxin isoform binding specificity. However, this observation may reconcile the functional requirement for Syntaxin 4 but not Munc18c for first-phase insulin release. From a broader cell biological perspective, this may represent a new mechanism by which Munc18 proteins regulate exocytosis in the context of a complex cellular milieu that is abundant with multiple SM and SNARE protein isoforms.

**EXPERIMENTAL PROCEDURES**

**Materials** The mouse anti-Munc18-1, mouse Munc13-1 and mouse VAMP2 antibodies were obtained from Synaptic Systems (Gottingen, Germany). The rabbit polyclonal anti-Munc18c antibody was generated as described (23). The mouse Syntaxin 1A and rabbit Syntaxin 4 antibodies
were purchased from Sigma (St. Louis, MO) and Chemicon (Temecula, CA), respectively. The clathrin and SNAP-25, SNAP-23 and Doc2b antibodies were purchased from BD Bioscience (San Jose, CA), Affinity BioReagents (Golden, CO), and Abcam (Cambridge, MA), respectively. MIN6 cells were a gift from Dr. John Hutton (University of Colorado Health Sciences Center). Anti-insulin, GLUT4 and donkey anti-goat horseradish peroxidase antibodies were purchased from Santa-Cruz (Santa Cruz, CA). Goat anti-rabbit horseradish peroxidase and anti-mouse horseradish peroxidase secondary antibodies were acquired from Bio-Rad (Hercules, CA). Enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences (Pittsburgh, PA). The RIA grade bovine serum albumin, Ponceau S stain and D-glucose were obtained from Sigma (St. Louis, MO). The sensitive rat insulin, human C-peptide and human ultrasensitive RIA kits were purchased from Millipore (Billerica, MA). The peroxidase substrate was obtained from Vector Labs (Burlingame, CA).

Plasmids - The pGEX-VAMP2 and pSilencer-Syn4 plasmids have been described previously (18,24). Human proinsulin cDNA was a gift from Dr. Chris Newgard (Duke University). The Munc18-1 cDNA was excised from pQE9-nSec1 (gift from Dr. Richard Scheller, currently at Genentech) using SalI and HindIII and subcloned into the 5’-XhoI and 3’-HindIII sites of pcDNA3.1-myc-his vector (Invitrogen), respectively. The pAd5CMV-Munc18-1 plasmid was generated by excision of the rat Munc18-1 cDNA from pGEX-KG-nSec1/Munc18-1 (also from Dr. Scheller) using SalI and HindIII for subclone into the 5’XhoI and 3’HindIII sites in the pAd5CMV vector (gift from The University of Iowa Gene Vector core facility); this core facility performed recombination and CsCl-particle synthesis. Control (LacZ) and Munc18c adenoviruses were generated similarly, as previously described (19).

Munc18-1 knockout mouse models – The Munc18-1+/- mice are a classic whole body gene-ablation model on the C57Bl6J strain background, generated as previously described (25). All mice for studies here were obtained by heterozygous crossing and paired littersmates used as controls. Tamoxifen (TM)-inducible beta cell specific Munc18-1+/- mice were generated and assessed in the Indiana University School of Medicine Laboratory Animal Research Center according to approved guidelines for use and care of animals. LoxP-Munc18-1 mice (26) were crossed with PdxER-cre+/+ transgenic mice (single copy; (27)). Offspring genotypes followed mendelian ratios; no gender bias was noted. Tamoxifen (Sigma) was solubilized at 20 mg/ml in corn oil and administrated to mice at 8 weeks old age by oral gavage treatment (0.05 mg/g body weight, once per day for 5 days) (27,28). Control mice were gavaged in parallel with an equivalent volume of vehicle (corn oil).

Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) – Male Munc18-1 knockout and wild-type mice (4-6 months old) were fasted overnight for 18 h. Blood was collected from the tail vein and blood glucose monitored (Hemocue, Inc.). Following sample collection of fasted blood, mice were administered glucose (2 g/kg body weight) by intraperitoneal injection and subsequent blood glucose readings taken at 30 min intervals over 120 min. For the insulin tolerance test (ITT), mice (4-6 months old) were fasted for 6 h. After fasted blood was collected, animals were injected intraperitoneally with Humulin R (0.75 units/kg body weight) and blood glucose readings were taken after 15, 30, 60 and 90 min.

Perifusion for human islets or mouse islets – Pancreatic human islets (obtained through the Integrated Islet Distribution Program, IIDP, donor information listed in Table S3) were used for perifusion in a strategy similar to that used for mouse islet perifusion, as previously described (29,30). Criteria for human donor islet acceptance: receipt within 36 hr of isolation, and of at least 80% purity and 75% viability. Upon receipt, human islets were first allowed to recover in CMRL medium for 2 h, and then were handpicked under a light microscope equipped with a green gelatin filter to discriminate residual non-islet material. Islets of non-diabetic donors were immediately transduced at MOI of 100 with Control (LacZ), Munc18-1 or Munc18c CsCl-purified adenoviral particles for 1 h at 37°C. Transduced islets were then washed twice and incubated for 48 h in medium at 37°C, 5% CO2. Fifty transduced islets were handpicked onto a column for perifusion analysis (29). Control-infected islets were run in parallel columns with experimental islets. Islets were then perifused at a
flow rate of 0.3 ml/min, and insulin secreted into eluted fractions was quantitated by the ultrasensitive human or mouse insulin RIA kits (Millipore).

**Morphometric assessment of islet cell mass**—Mouse islet morphometry was evaluated using anti-insulin immunohistochemical staining of pancreatic sections as described (31). Briefly, pancreata from wild type (Munc18-1+/+) or Munc18-1+/− 5 month old male mice were fixed with 4% paraformaldehyde, paraffin embedded, and longitudinally sectioned at 5-μm thickness and 100-μm intervals. The sectioned tissues were deparaffinized, rehydrated and blocked in 5% horse serum, and incubated overnight at 4°C with rabbit anti-insulin antibody. Immunohistochemistry was also performed using Munc18-1 and Munc18c antibodies for localization assessments, and insulin antibodies for morphometry analyses. Following PBS washes and incubation with HRP-conjugated secondary antibody, the sections were incubated in peroxidase substrates and counterstained with hematoxylin. Digital images were acquired on an Axio-Observer Z1 microscope (Zeiss) fitted with an AxioCam high resolution color camera. Percentage of insulin-stained β-cell area was calculated using Axio-Vision software. Data shown are representative of 4 sections per pancreas and 3 pancreata from each group. For pancreatic immunohistochemistry, DAB (3,3′-diaminobenzidine, brown color) substrate was used to stain for Munc18-1 and insulin. NovaRed (Vector laboratories, Burlingame, CA) was used for Munc18c staining. Hematoxylin (blue-violet) was used as counterstaining.

Islet EM analysis and insulin granule localization—Islets were isolated from 12 Munc18-1+/+ or Munc18-1+/− mice and incubated overnight in CMRL medium. Islets were then incubated 2 h in KRBB and immediately fixed in a 0.1 M cacodylate-buffered mixture of 2% glutaraldehyde and 4% paraformaldehyde for 2 h at room temperature followed by overnight incubation at 4°C and then postfixed in 1% OsO₄ for 1 h. En bloc staining in 1% aqueous uranyl acetate in Maleate buffer was performed for 1 h followed by washing with Maleate buffer. Dehydration was done in the following sequence: 25% ethanol, 50% ethanol 70% ethanol, 95% ethanol, 100% ethanol and 100% propylene Oxide. Infiltration entailed propylene oxide and resin in the order of 2:1 (1 h), 1:1 (overnight) and 100% resin (6 changes each 1 h). The Thermonox coverslips were inverted over a 1.5-ml centrifuge tube filled with resin and polymerized for 48 h at 60°C. Thin (90 nm) sections were cut using the microtome (Reichert-Jung Ultracut E). The thin sections were stained with uranyl acetate and lead citrate and viewed on the 300KV FEI Tecnai F30 (Gatan CCD digital micrograph).

**MIN6 Cell culture, transduction and transient transfection**—MIN6 beta cells were cultured as described previously (32) and transduced at MOI=50-100 for 2 hr, washed with PBS and incubated in MIN6 medium for 48 h. Cells were then preincubated for 2 h in glucose-free modified Krebs ringer bicarbonate buffer (MKRBB: 5 mM KCl, 120 mM NaCl, 15 mM Hepes pH 7.4, 24 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂ and 1 mg/ml BSA), stimulated with 35 mM KCl and supernatant collected for quantitation of insulin released by insulin RIA (Millipore). Cells were transiently co-transfected using Transfectin (BioRad) with three plasmids: pSil-Syn4 (siSyn4) or pSil-Con (siCon), pcDNA3.1-Munc18c or pcDNA3.1 empty vector, plus human proinsulin plasmid as described previously (29). After 48 hr incubation cells were preincubated in MKRBB and KCl-stimulated. Buffer containing secreted human C-peptide was collected for RIA analysis (Millipore). Detergent cell lysates were prepared from transduced or transfected cells by harvesting in 1 % Nonidet P-40 lysis buffer (1% NP-40, 25 mM HEPES pH 7.4, 10% glycerol, 50 μM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 137 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin and 10 μg/ml aprotinin) and lysates cleared by microcentrifugation for 10 min at 4°C for use in immunoprecipitation and immunoblotting.

**MIN6 subcellular fractionation**—Subcellular fractions were isolated as previously described (24). In brief, MIN6 cells at 80-90% confluence were harvested into 1 ml of homogenization buffer (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 1 mM DTT and 1 mM sodium orthovanadate) containing the protease inhibitors leupeptin (10 μg/ml), aprotinin (4 μg/ml), pepstatin (2 μg/ml) and PMSF (100 μM). Cells were disrupted by 10 strokes through a 27 gauge needle and homogenates were centrifuged at 900 x g
for 10 min. Plasma membrane fractions (PM) were obtained by mixing the postnuclear pellet with 1 ml of Buffer A (0.25 M Sucrose, 1 mM MgCl₂ and 10 mM Tris-HCl, pH 7.4) and 2 volumes of Buffer B (2 M sucrose, 1 mM MgCl₂ and 10 mM Tris-HCl, pH 7.4). The mixture was overlaid with Buffer A and centrifuged at 113,000 x g for 1 h to obtain an interface containing the plasma membrane fraction. Interface was collected and diluted to 2 ml with homogenization buffer for centrifugation at 6,000 x g for 10 min, and the resulting pellet was collected as the plasma membrane fraction. All pellets were resuspended in 1% NP-40 lysis buffer to solubilize membrane proteins.

**Calcium imaging assays** - Intracellular Ca²⁺ was measured using the ratiometric Ca²⁺ indicator Fura-2 AM, as described previously (33). Briefly, MIN6 cells transduced as described above were preincubated in MKRBB for 2 h, with Fura-2 AM (5 μM) added to the cells for an additional 25 min. Cells were washed with warmed MKRBB to remove excess Fura-2 AM and placed in fresh MKRBB containing low (2 mM) glucose and KCl (5 mM). Cells were imaged under constant perfusion (1 ml/min) for 200 seconds, followed by stimulation with 35 mM KCl to elicit calcium influx for 200 seconds. Fura-2 AM was excited at 340 and 380 nm, emission captured at 510 nm on a Zeiss Axio Observer Apochromat 100X/1.46 objective equipped with a Hamamatsu Orca-ER digital camera and analyzed using AxioVision 4.7 software (Carl Zeiss, Germany), and data expressed as the change in ratio (ΔF) over the initial ratio (F₀).

**Recombinant proteins and interaction assays** - The GST-VAMP2 protein was generated in E. coli and purified by glutathione-agarose affinity chromatography as described previously (34) for use in the Syntaxin accessibility assay. GST-VAMP2 linked to sepharose beads was combined with 2 mg detergent MIN6 cell lysate for 2 h at 4°C in NP-40 lysis buffer, followed by three stringent washes with lysis buffer, and associated proteins resolved on 10-12% SDS-PAGE followed by transfer to PVDF membrane for immunoblotting for Syntaxin 1A and Syntaxin 4. GST-Munc18-1 was generated similarly, followed by thrombin cleavage to eliminate the GST and capture the purified Munc18-1 protein (Novagen, San Diego, CA).

**RESULTS**

**Munc18-1 is required for normal islet function and maintenance of whole-body glucose homeostasis**

While Munc18-1 has been postulated to be functional in calcium-stimulated insulin release from islet beta cells, its requirement in islets for this process remains untested. To address this, we perifused islets isolated from Munc18-1⁻/⁻ mice for insulin secretory capability in response to KCl-stimulation. Insulin exocytosis evoked by KCl stimulation elicits fusion of predominantly preDocked granules during first-phase insulin release, and first-phase release defects are considered early indicators of glucose intolerance/pre-diabetes. Munc18-1⁻/⁻ mice were used as they recapitulate the level of deficiency of this protein seen in islets from human Type 2 diabetic patients (35). Munc18-1 protein is only expressed in brain and pancreatic islets, as opposed to its paralog Munc18c which is expressed ubiquitously (Fig. S1A,B). Ex vivo, insulin secretion under basal unstimulated conditions was similar amongst Munc18-1⁺/⁺ and Munc18-1⁻/⁻ islet groups. KCl stimulation (35 mM) elicited a transient 20-fold increase in insulin release from Munc18-1⁻/⁻ islets lasting ~5 min, consistent with a first-phase response (Fig. 1A), while Munc18-1⁻/⁻ showed a significantly diminished response (Fig. 1A, B). The total islet insulin content in Munc18-1⁻/⁻ islets was comparable to that from control Munc18-1⁺/⁺ mice (Fig. 1C), suggesting that the genetic ablation did not exert effects upon insulin synthesis. Munc18-1⁻/⁻ mouse islets displayed the expected
~50% deficiency in Munc18-1 protein, with no alterations in other functional Munc18 and Syntaxin isoforms of the islet (Fig. 1D). Islet cell morphometry and mass was also normal in Munc18-1<sup>−/−</sup> pancreata (Fig. 1E), suggesting that the genetic ablation did not exert effects upon islet development. Thus, these data suggest that partial ablation of Munc18-1 is sufficient to impair KCl (calcium)-stimulated insulin release.

To determine the effects of Munc18-1 haploinsufficiency upon whole-body glucose tolerance, 4-6 month old littermate Munc18-1<sup>+/−</sup> and Munc18-1<sup>−/−</sup> mice were subjected to intraperitoneal glucose tolerance tests (IPGTT). Glucose tolerance after 18 h fasting in Munc18-1<sup>−/−</sup> male mice was significantly impaired in comparison to wild type mice (Fig. 1F). Munc18-1<sup>−/−</sup> mice showed equivalent body weight and tissue/organ weights (Table S1), as well as fasting serum analytes (triglycerides, cholesterol, non-esterified fatty acids, glucose and insulin) (Table S2). Because whole body glucose intolerance could also be attributable to deficits in peripheral insulin sensitivity, causing insulin resistance, we also performed insulin tolerance tests (ITT). As expected, insulin injection resulted in a sharp ~50% decline in blood glucose within 60 min in Munc18-1<sup>−/−</sup> mice (Fig. 1G) and Munc18-1<sup>+/−</sup> mice responded similarly, consistent with the fact that we detected no Munc18-1 expression in insulin target tissues (Fig. S1A).

To discern that the phenotype and dysfunction of the islets of the Munc18-1<sup>−/−</sup> mice was attributable solely to the loss of Munc18-1 in the β-cell, and not due to loss in neuronal cells, we used tissue-specific cre-lox Munc18-1<sup>−/−</sup> mice crossed with β-cell specific and tamoxifen-inducible PdxERcre mice (27) to generate β-cell specific and tamoxifen-inducible Munc18-1<sup>−/−</sup> (β-cell Munc18-1KO) mice. Islets from the β-cell Munc18-1KO showed less than 5% residual Munc18-1 protein upon induction of the PdxERcre using tamoxifen (TM) (Fig. 2A). β-cell Munc18-1KO hypothalamic and cerebellar expression levels of Munc18-1 were normal (Fig. S2A), indicating specificity of the gene ablation to the islet beta cell. TM-induced β-cell Munc18-1KO islets showed an even greater attenuation of response to KCl when compared to the TM-induced Cre<sup>−/−</sup> control islets (Fig. 2B,C), with secretion reduced by 60% (as opposed to 20% in the Munc18-1<sup>+/−</sup>). In contrast, islets from vehicle-injected Cre<sup>−/−</sup> and β-cell Munc18-1KO (Cre<sup>−/−</sup>) mice showed the full 20-fold response to KCl (Fig. S2B,C). Moreover, β-cell Munc18-1KO mice displayed significant glucose intolerance relative to littermate TM- and Vehicle (corn oil)-injected control mice, and showed normal insulin sensitivity (Fig. 2D,E). Together, these data suggest that Munc18-1 deficiency in the beta cell exerts a substantial negative effect upon whole body glucose homeostasis in vivo, likely due to the requirement for Munc18-1 in islets to evoke acute insulin release.

**Impaired insulin granule docking in islets isolated from Munc18-1<sup>−/−</sup> mice**

To determine if the impairment in insulin release in human islets partially deficient in Munc18-1 might be related to the requirement for Munc18-1 in insulin granule docking/fusion, we next evaluated the size of the readily releasable pool (RRP) in islet beta cells from the Munc18-1<sup>−/−</sup> mice. In the islet beta cell, the quantity of insulin granules localized within ~50 nm of the plasma membrane, under resting conditions, are considered to be morphologically docked in the RRP, and to constitute that quantity of insulin released during the first-phase/acute phase of insulin secretion. Mature insulin granules located beyond this distance are considered to be located in a storage pool. To determine the size of the RRP, islets were isolated from Munc18-1<sup>−/−</sup> and Munc18-1<sup>+/−</sup> mice and cultured under low glucose conditions (2.8 mM, basal) for 2 h and subsequently fixed and processed for electron microscopic analysis. Granule location relative to the PM was tabulated; insulin granules located within 50 nm of the PM are termed ‘morphologically docked’ (37,38). As shown in Fig. 3A, granules juxtaposed to the PM were clearly visible in the Munc18-1<sup>−/−</sup> beta cells (arrow denotes PM location). In contrast, Munc18-1<sup>+/−</sup> cells contained 35% fewer granules within the 50 nm range (Fig. 3B), while the total number of granules within each field of Munc18-1<sup>−/−</sup> and Munc18-1<sup>+/−</sup> cells was similar (Fig. 3C). These data suggested that defective insulin granule docking under basal conditions, termed insulin granule pre-docking, in the Munc18-1<sup>−/−</sup> islet beta cells could represent a mechanism to explain the deficit in acute KCl-stimulated insulin release.
Human islets with increased Munc18-1 exhibit enhanced acute insulin release

Based upon our observations of Munc18-1 requirement for full KCl-stimulated insulin release, we questioned whether increased expression of Munc18-1 would enhance first-phase glucose-stimulated insulin secretion (GSIS). Human islets were obtained from normal BMI non-diabetic donors and transduced to express recombinant Munc18-1 (Fig. 4A). Transduced islets expressed ~10-fold more Munc18-1 over endogenous levels, without altering expression of the relevant Syntaxin isoforms 1A and 4. Remarkably, Munc18-1 overexpression exclusively potentiated the first phase of GSIS by ~2.5-fold, while second phase GSIS was unaffected (Fig. 4B,C). Consistent with this, KCl-stimulated insulin secretion was similarly enhanced. This is the first demonstration of Munc18-1’s potentiation of acute phase insulin release in human islets.

Elevated Munc18-1 expression enhances granule accumulation at the β-cell PM

We next sought to determine the mechanism by which increased Munc18-1 expression enhanced first-phase insulin release. Like human islets, Munc18-1-transduced MIN6 cells showed an enhanced KCl-stimulated insulin response compared to control-transduced cells within 2 min (Fig. 5A), showing that the effect is due to alterations in the beta cells of the islet and not due to other islet cell types. MIN6 beta cells are ideal for these studies given that they recapitulate the requirement for Munc18-1 in insulin release (20) and are considered to be one of the clonal lines that has retained some level of biphasic secretion (39,40). However, Fura-2 calcium imaging experiments revealed no differences in KCl-stimulated increases in cytosolic calcium concentration \([\text{Ca}^{2+}]_c\) in Munc18-1 overexpressing cells versus control-transduced cells (Fig. 5B), suggesting that the Munc18-1 overexpression does not enhance acute insulin secretion via simply promoting calcium influx/elevation of \([\text{Ca}^{2+}]_c\) per se. Based upon our observations of Munc18-1 requirement for normal insulin granule docking to support first-phase secretion, we questioned whether increased expression of Munc18-1 would increase the number of docked granules at the PM. Munc18-1-overexpressing MIN6 cells were partitioned to isolate plasma membrane enriched subcellular fractions using differential centrifugation, a method we have previously shown to mimic quantitative changes in granule accumulation at/juxtaposed to the PM (24,34). Indeed, Munc18-1 transduced cells showed ~20% more VAMP2 abundance (reporter for granules) in PM fractions compared with control cells under basal conditions (Fig. 5C). These data support the concept that Munc18-1 enhances granule accumulation/docking at the PM to enhance first-phase insulin release.

Altered SM and SNARE complex formations in Munc18-1 over-expressing β-cells

We next questioned whether Munc18-1 overexpression would trigger increased Munc18-1 binding to Syntaxin 1A-based SNARE complexes, which might explain Munc18-1’s functional enhancement of first-phase insulin release. While Munc18-1 has been shown \textit{in vitro} and in yeast to bind to the SNARE complex as a means to facilitate vesicle fusion (13,41,42), the ability to precipitate such complexes has yet to be demonstrated using mammalian cell lysates. GST-VAMP2-bound beads were successfully used to capture binding of Munc18-1. Since Munc18-1 does not bind directly to GST-VAMP2, it is assumed that Munc18-1 interacted through GST-VAMP2 associated with Syntaxin 1A-SNAP25 dimers; GST-VAMP2 may have also competitively replaced VAMP2 present in pre-formed SNARE complexes (SNARE complexes are predicted to exist under these detergent conditions) (Fig. 6A). Unexpectedly however, Munc18-1 overexpressing cells showed a 50% loss in Syntaxin 1A coprecipitation by GST-VAMP2 (Fig. 6A). Similarly, anti-VAMP2 co-immunoprecipitation of Syntaxin 1A was reduced by ~40% in Munc18-1 overexpressing lysates versus control lysates (Fig. 6B). SNAP-25 coprecipitation with VAMP2 was similar regardless of Munc18-1 expression levels, suggesting the effect of Munc18-1 overexpression is specific to Syntaxin 1A. Anti-Syntaxin 1A coimmunoprecipitation fully recapitulated these findings (Fig. 6C), suggesting against the concept that decreased Syntaxin 1A-VAMP2 association is an artifact of approach. Moreover, the increased immunoprecipitation of Munc18-1 from Munc18-1-Ad lysates was not paralleled by an increase in Syntaxin 1A association; instead, ~45% reduction in Syntaxin 1A/Munc18-1 ratio was observed (Fig. 6D). The flow-through (eluate) from IP reactions showed plenty of Syntaxin 1A protein remaining, with control and Munc18-1
expressing eluates containing similar quantities (data not shown). This would suggest against the notion of Syntaxin 1A being limiting for interaction. Thus, Munc18-1’s functional enhancement of first-phase insulin release appears not related to an increase in Munc18-1 binding with Syntaxin 1A-based SNARE complexes.

Intriguingly, Munc18-1 overexpression correlated with a 150% increase in the amount of Syntaxin 4 association with GST-VAMP2 in pulldown studies (Fig. 7A), as well as in VAMP2 immunoprecipitation studies (Fig. 7B). This difference could not be accounted for by an interaction between Munc18-1 and Syntaxin 4 (Fig. 7C), consistent with prior reports that Munc18c is the only SM protein known to pair with Syntaxin 4 in cells (3). To determine whether this effect was due to an artifact of over-expressing Munc18-1 for 40 h prior to assessment, possibly altering unforeseen protein expression patterns, we instead opted to titrate recombinant Munc18-1 protein directly into GST-VAMP2 pulldown reactions. Exogenous acute addition of Munc18-1 to pulldown reactions, reaching ~2.5-fold over endogenous Munc18-1, resulted in a ~30% increase in Syntaxin 4 binding to GST-VAMP2, in parallel with a decrease in Syntaxin 1A-VAMP2 association (Fig. 7D).

Munc18-1 is found localized both at the PM as well as in the cytosolic compartment. To focus our investigations of Munc18-1 binding to the PM, where SNARE complexes are formed, subcellular PM fractions were prepared from control- and Munc18-1-transduced cells and used in anti-Munc18-1 coimmunoprecipitation reactions. When normalized for the quantity of Munc18-1 immunoprecipitated from Munc18-1 overexpressing cell PM fractions versus control fractions, ~20% less Syntaxin 1A and Doc2b proteins were coprecipitated (Fig. 7E). A more severe 45% decrease in Munc18-1 association with Munc13-1 (factor involved in the priming step of exocytosis) was detected in Munc18-1 overexpressing cells. Attempts to assess association with other Munc18-1-Syntaxin 1A binding factors Granuphilin and Rab3A failed due to antibody unsuitability (data not shown). These data argue against the concept that the increased cellular content of Munc18-1 binds to more Doc2b or Munc13-1 to account for the increase in exocytosis.

These results might suggest that Munc18-1 over-expression exerts an indirect effect to yield the increase in Syntaxin 4 interaction with VAMP2. This may account for, at least in part, the increased number of pre-docked granules and the functional elevation of first-phase insulin release from islets. We tested this experimentally by evaluating the ability of Munc18-1 to potentiate insulin release in cells depleted of Syntaxin 4 using RNAi. RNAi-mediated Syntaxin 4 depletion by 50% significantly attenuates GSIS, similar to the level of impairment exhibited by Syntaxin 4-/- knockout mouse islets (18). MIN6 cells were co-transfected with shRNA to target Syntaxin 4 as used previously or a non-targeting control (siCon) (18), plus Munc18-1 plasmid DNA, all with human proinsulin cDNA to serve as a reporter of secretion selectively from transfecable cells. Human proinsulin is packaged and processed to human C-peptide and insulin in secretory granules in a manner similar to that of the mouse proinsulin present in the MIN6 cells, but the human C-peptide is immunologically distinct from that of the mouse C-peptide. Fig. 8A demonstrated effectiveness of the co-transfection, with Munc18-1 over-expression at ~2.5-3-fold over endogenous levels, and Syntaxin 4 depleted to ~40-50% of control (siCon), as detected by immunoblotting. KCl-stimulated human C-peptide secretion (stimulation index =stimulated/basal) from the siSyn4-transfected cells showed the expected attenuation (46 ± 8% of siCon, n=3; p<0.01). Secretion from the siCon-transfected cells was enhanced by over-expression of Munc18-1 (Fig. 8B), consistent with results using adenoviral expression of Munc18-1. However, Munc18-1 overexpression failed to potentiate or rescue the impaired KCl-stimulated secretion from cells deficient in Syntaxin 4.

DISCUSSION

In this study we document the detrimental effect of Munc18-1 depletion, using conventional (Munc18-1+/−) and beta cell specific (β−cell Munc18-1KO) knockout mouse models, upon whole body glucose homeostasis in vivo and acute insulin release from pancreatic islets. Commensurate with this, increased expression of Munc18-1 enhanced
first-phase insulin release from human islets. While Munc18-1 deficient islet beta cells showed fewer docked granules at the PM, Munc18-1 over-expressing cells showed increased granule docking. Unexpectedly however, Munc18-1 over-expression promoted an increase in Syntaxin 4-based SNARE complexes, rather than Syntaxin 1-based complexes as otherwise expected. Moreover, knockdown of Syntaxin 4 blunted the ability of Munc18-1 overexpression to enhance KCl-stimulated secretion. Since Munc18-1 does not directly bind to Syntaxin 4, these data suggest that Munc18-1 indirectly promotes Syntaxin 4-based granule docking and exocytosis to support the first phase of insulin secretion. This may represent an additional and new mechanism by which SM proteins regulate exocytosis in the context of a complex cellular milieu that is abundant with multiple SM and SNARE protein isoforms.

To the best of our knowledge, the islet beta cell is the only cell type to differentially utilize distinct Syntaxin-Munc18 protein isoform pairs to elicit an exocytosis event. Other exocytosis events consisting of multiple phases, such as those involved in neurotransmitter release, show repeat utilization of the same isoform pairings (43). Islet beta cells are known to utilize three forms of Munc18, Munc18-1 and Munc18b, but for the same rapid calcium-stimulated phase of insulin release (44); the third form, Munc18c, is required only for second phase insulin release (19). Beta cells also make use of two Syntaxin isoforms, 1 and 4, with Syntaxin 1 function exclusive to first phase and Syntaxin 4 in both phases. Although it is not clear how, each syntaxin is involved in beta cell exocytosis on the same plasma membrane; in synapses, Syntaxin 1 and 4 exhibit differential localization and function in presynaptic versus postsynaptic densities, respectively (45). In epithelial cells, Syntaxin isoforms 3 and 4 are reported to be differentially confined to mutually exclusive submicron-sized clusters of the plasma membrane possibly via differential association with microtubules versus actin cables (46). These isoforms are also differentially functional with distinct aquaporin channel isoforms in MDCK cells (47), suggesting the selective pairing is essential to the specificity of channel function. However, Syntaxin 4 has the capacity to directly interact with F-actin in vitro, and dissociation of the Syntaxin 4-actin complex in beta cells does alter exocytosis (34). Thus, it is possible that insulin granules might carry markers for docking at Syntaxin 1 versus Syntaxin 4 sites. Since Munc18-1 and Munc18c exist in the cytosol as well as at the membrane, future studies will be required to determine whether their differential association with granules trafficking to the plasma membrane correlates to the granule’s destination at a particular syntaxin docking site.

The enhancement of first phase insulin release with over-expression of Munc18-1 in first phase secretion presented an opportune cell type to test the various molecular models regarding Munc18-1 interaction with SNARE core complex. Indeed, Munc18-1 was shown to co-precipitate with GST-VAMP2/SNAP-25/Syntaxin 1A complexes from cell lysate. Paradoxically, over-expression of Munc18-1 did not result in increased abundance of Syntaxin 1-based SNARE complexes, as determined by Syntaxin 1 binding to GST-VAMP2 as well as SNARE co-immunoprecipitations. A third approach, comparing abundance of high molecular weight SNARE complexes formed under basal conditions in beta cell lysates using the classic SNARE complex boiled-unboiled/SDS buffer paradigm (48-50), showed no effect of Munc18-1 over-expression upon Syntaxin 1-based SNARE complex abundance (data not shown). Further perplexing was our finding that endogenous Munc18c neither coprecipitates with endogenous VAMP2 (data not shown) nor binds to exogenous GST-VAMP2 (51) in the manner observed with Munc18-1 (Fig. 7A). Hence, while Munc18-1 and Munc18c are structurally similar, their binding characteristics with SNARE proteins, at least in the context of a cellular milieu expressing both Munc18 isoforms, may differ. One possibility to be tested is that the differential serine/threonine and tyrosine phosphorylations of the Munc18 isoforms that occur in response to stimuli in beta cells might account for their differential functions in insulin secretion (44,52).

Munc18-1 over-expression enhanced formation of Syntaxin 4-based SNARE complexes, which is unprecedented given that Munc18-1 does not pair with Syntaxin 4. However, Syntaxin 4 does contribute to first phase insulin release, and the mechanism for this has remained enigmatic. That Munc18-1 utilizes Syntaxin 4 for promotion of
insulin release was supported by our data from Syntaxin 4-depleted Munc18-1 overexpressing cells, wherein the potentiating effect of Munc18-1 was abrogated (Fig. 8). Future studies will be required to clarify the issue of whether reduced Munc18-1 expression affects the equilibrium of VAMP2 interactions with Syntaxin 1A and Syntaxin 4.

Moreover, Munc18c, the only known SM partner for Syntaxin 4, does not participate in first phase insulin release (19), suggesting that Syntaxin 4’s participation in first-phase insulin exocytosis proceeds in a manner exclusive of its SM partner. Alternatively, our data also showed that increased levels of Munc18-1 corresponded to its decreased association with Munc13-1 and Syntaxin 1A. Both Munc18-1 and Munc13-1 interact with the N-terminus of Syntaxin 1A in a 1:1 stoichiometry and can compete for binding to Syntaxin 1A (55,56). Munc13-1 is required for both phases of insulin release (57) and its activation is proposed to convert Syntaxin 1 to the open state to prime granules (58). Since neither Munc18-1 nor Munc13-1 has been found to associate with Syntaxin 4, any ‘shifting’ of these factors towards Syntaxin 4 as an explanation falls short of accounting for the enhancing effect of Munc18-1 over-expression upon increased Syntaxin 4-based SNARE complex formation. Recently Munc13-4 (Munc13D) was shown to associate with Syntaxin 4 (59), and as such remains to be investigated as in this mechanism in beta cells.

We also show here that Munc18-1 can bind to the double C2 domain protein Doc2b in beta cells; Doc2b is calcium-activated and known to bind Munc18-1 and Munc13-1 in neuronal cells, as well as Munc18c in β-cell, fat and muscle cell types (60-62). However Doc2b-Munc18-1 binding was not enhanced in Munc18-1 overexpressing cells; rather, Doc2b-Munc18-1 binding was reduced. Our understanding of the configuration of Doc2b binding to Munc18-1 in the presence of Munc13-1 is hindered by the paucity of data describing Doc2b stoichiometry with these factors. Toward this, Doc2b-Munc18-1 binding stoichiometry experiments are currently underway.

In conclusion, the data presented here support a key role for Munc18-1 in first phase exocytotic processes relevant to the maintenance of whole body glucose homeostasis. We show for the first time that Munc18-1 engages in association with SNARE complexes in beta cells. Importantly, we show that Munc18-1 over-expression in human islets enhances first phase insulin release, the phase most commonly associated with early onset of islet dysfunction in the course of diabetes disease progression. Mechanistically, Syntaxin 4 but not Syntaxin 1 participation in SNARE complexes was fostered by Munc18-1 overexpression. This challenges existing models regarding the mechanism by which Munc18-1 facilitates vesicle fusion and exocytosis, and further suggests that non-pairing SM-Syntaxin isoforms have potential for functional cross-talk, perhaps through common binding partners such as Doc2b. From a cell biological perspective, this type of mechanism expands the repertoire of SM and SNARE isoforms capable of contributing to exocytosis.

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Abbreviations used: SNARE, SNAP [soluble NSF (N-ethylmaleimide sensitive factor) attachment protein] receptor; SM, Sec1/Munc18c protein; ITT, insulin tolerance test; IPGTT, intraperitoneal glucose tolerance test; NEFA, non-esterified fatty acids; PM, plasma membrane; AUC, area under the curve.

FIGURE LEGENDS

Figure 1. Munc18-1+/− mice show impaired glucose tolerance corresponding to impaired KCl-stimulated insulin release from the islets. A) Islets from Munc18-1+/− and Munc18-1−/− mice were perifused with 2.8 mM glucose followed by stimulation with 35 mM KCl, and B) quantitation of the area under the curve (AUC) for insulin secretion from three independent islet batches isolated from Munc18-1+/− (black bars) and Munc18-1−/− (grey bars), normalized to baseline; *P<0.05 versus Munc18-1+/−. C) Islet insulin content from Munc18-1+/− and Munc18-1−/− isolated mouse islets from B). D) Islets isolated from Munc18-1+/− and Munc18-1−/− mice in part B) were lysed for immunoblot detection of multiple Munc18 and Syntaxin proteins. E) Islet beta cell area from pancreatic sections from Munc18-1+/− and Munc18-1−/− mice (bar=50 μm), as determined by quantitation of insulin-stained pancreas sections from 3 pairs of mice. F) IPGTT of Munc18-1+/− and Munc18-1−/− mice was performed by intraperitoneal injection of D-glucose (2 g/kg body weight) into 11 pairs of male mice (age 4–6 months) fasted for 18 h; *P<0.05 versus Munc18-1+/−. G) Insulin tolerance testing (ITT) of Munc18-1+/− and Munc18-1−/− was performed by intraperitoneal injection of insulin (0.75 units/kg body weight) into male mice (age 4–6 months) fasted for 6 h. Blood glucose levels were normalized to basal=100% for each animal for calculation of the mean percent ± SE; *P<0.05 versus Munc18-1+/−.

Figure 2. Impaired glucose tolerance and islet defects in β-cell specific Munc18-1 knockout mice. A) Islet protein expression from TM-induced β-cell specific Cre+/−-Munc18-1 knockout (β-cell Munc18-1KO) mice, performed as described in Fig. 1 above. B) Islets from TM-induced Cre+/− and Cre−/− mice were perfused with 2.8 mM glucose followed by stimulation with 35 mM KCl, and C) area under the curve (AUC) for insulin secretion from islets quantified, normalized to baseline for each; *P<0.05 versus TM-Cre−/−. D) IPGTT and E) ITT of TM- or vehicle-induced β-cell specific Cre−/− and Cre+/−Munc18-1 (β-cell Munc18-1KO) mice (5 pair of vehicle-injected, 6 pair of TM-induced male mice), performed as described in Fig. 1; *P<0.05 versus TM-induced Cre−/−-Munc18-1. TM, tamoxifen.

Figure 3. Munc18-1+/− islet β-cells display altered insulin granule distribution. A) Transmission electron microscopy of Munc18-1+/− and Munc18-1−/− islets. Islets were incubated overnight and then placed in glucose-free MKRBB for 2 h and left unstimulated for fixation for EM as described in Experimental Procedures. Arrows denote the plasma membrane; N denotes location of the nucleus. Sections are equivalent in size, bar = 500 nm. B) Distribution of insulin granules in Munc18-1+/− and Munc18-1−/− cells. Criteria for inclusion required clear demarcation of the plasma membrane and presence of nucleus in each field tabulated. Insulin granule distance from the plasma membrane was tabulated for Munc18-1+/− and Munc18-1−/− electron micrographs from 10 sections/each of 12 mice, with a total of 2,235 Munc18-1+/− and 2,132 Munc18-1−/− insulin granule distances measured and grouped into categories based upon distance from the PM; *P<0.05 vs. Munc18-1−/−. C) Total granule number per field/section in Munc18-1−/− (black bar) and Munc18-1+/− cells (grey bar) under each condition was quantified.

Figure 4. Human islets over-expressing Munc18-1 show enhanced first-phase insulin release. Isolated human islets transduced with control (LacZ) or Munc18-1 adenovirus (MOI=100) for 40 h were: A) solubilized for immunodetection of Munc18-1 over-expression as well as syntaxin proteins, B) perfused with 2.8 mM glucose followed by stimulation with 20 mM glucose, returned to 2.8 mM glucose for 20 min, and then stimulated with 35 mM KCl. C) Quantitation of the area under the curve (AUC) for first (11-17 min) and second (18-45 min) phases as well as KCl-induced insulin secretion from human islets perfused in B), normalized to baseline; *P<0.05 versus Control-Ad. Data represent 3 independent sets of donor islets (donor profiles listed in Table S3).
**Figure 5.** Increased accumulation of VAMP2-bound insulin granules at the PM of Munc18-1 over-expressing MIN6 cells correlates with enhanced insulin secretion. MIN6 cells transduced to express Munc18-1 or control (LacZ) were subjected to 2 h incubation in glucose-free MKRBB for subsequent analyses: **A)** KCl stimulation (35 mM, 2 min) and quantitation of insulin release; **B)** assessment of KCl-stimulated calcium influx using Fura-2 imaging; **C)** assessment of VAMP2 abundance in plasma membrane (PM) fractions by immunoblotting (inset) and optical density scanning quantitation. Data in each panel represent 3-4 independent experiments; *P<0.05 versus Con-Ad.

**Figure 6.** Munc18-1 over-expression does not increase formation of Syntaxin 1A-based SNARE complexes. MIN6 cells transduced to express Munc18-1-Ad or control (LacZ, Con-Ad) were subjected to 2 h incubation in glucose-free MKRBB (basal condition) for preparation of cleared detergent cell lysates for use in binding assays. **A)** GST-VAMP2 pulldown reactions entailed incubation of recombinant GST-VAMP2 protein linked to glutathione-sepharose beads with detergent-solubilized MIN6 cell lysates, and coprecipitated proteins were detected by immunoblot (IB). Ponceau S staining of the GST-VAMP2 protein was used to gauge the equivalence of precipitation between reactions. Immunoprecipitation reactions with detergent-solubilized MIN6 cell lysates were performed using antibodies against the following: **B)** VAMP2, **C)** Syntaxin 1A, or **D)** Munc18-1. Co-precipitated proteins were resolved on 10-12% SDS-PAGE for detection by immunoblot. Panel B lysate lanes show the lack of effect of Munc18-1-Ad upon SNARE protein expression levels. Data are representative of at least three independent experiments for each data panel. Each set of experiments was quantified by optical density scanning, with bar graphs showing ratios of protein associations to the right of the corresponding set of immunoblots; *P<0.05 versus Con-Ad. Ad: adenovirus; Con, control-Ad transduced cells.

**Figure 7.** Syntaxin 4-based SNARE assembly is increased in Munc18-1 over-expressing β-cells. MIN6 cells transduced to express Munc18-1 or control (LacZ) were treated as described in Fig. 6 above. **A)** Recombinant GST-VAMP2 protein linked to glutathione-sepharose beads was incubated with MIN6 cell lysates and coprecipitated proteins detected by immunoblot (IB). Ponceau S staining of the GST-VAMP2 protein was used to gauge the equivalence of precipitation between reactions. Immunoprecipitation reactions with detergent-solubilized MIN6 cell lysates were performed using antibodies against **B)** VAMP2, or **C)** Munc18-1. **D)** GST-VAMP2 pulldown reactions using non-transduced MIN6 detergent lysates were supplemented with recombinantly expressed and purified Munc18-1 protein (0 or 250 ng per reaction), and coprecipitated proteins detected by immunoblot. **E)** PM subcellular fractions prepared from Munc18-1 or control-transduced cells were used in anti-Munc18-1 immunoprecipitation reactions, and co-precipitated proteins detected by immunoblot. Data are representative of at least three independent experiments for each data panel. Each set of experiments was quantified by optical density scanning, with bar graphs showing ratios of protein associations to the right of the corresponding set of immunoblots; *P<0.05 versus Con-Ad. Ad: adenovirus; Con, control-Ad transduced cells.

**Figure 8.** Knockdown of Syntaxin 4 abrogates the effect of Munc18-1 over-expression upon secretion from MIN6 β-cells. MIN6 cells were co-transfected to express either RNAi targeting Syntaxin 4 (siSyn4) or a non-targeting control (siCon), Munc18-1 or control (pcDNA3.1 vector), all with human proinsulin (reporter of secretion selectively in transfectable cells). Following 48 hr incubation, cells were subsequently incubated 2 h in glucose-free MKRBB (basal condition) and stimulated with KCl (50 mM, 20 min) for: **A)** preparation of cleared detergent cell lysates for detection of Syntaxin 4 knockdown and Munc18-1 over-expression levels, and **B)** for quantitation of human C-peptide release, reporting insulin release. No significant differences in basal secretion were detected; data are presented as the average stimulation index (stimulated/basal) in four independent experiments; *p<0.05 versus Con/siCon; **p<0.05 versus Munc18-1/siCon.
Figure 1

A) Insulin release (ng/ml) over time with 35 mM KCl.

B) Insulin release AUC comparison between Munc18-1+/+ and Munc18-1+/-.

C) Insulin content (ng/ml) comparison between Munc18-1+/+ and Munc18-1+/-.

D) Western blot analysis of islets from Munc18-1+/+ and Munc18-1+/- mice, showing IB: Munc18-1, Munc18c, Syntaxin 1A, Syntaxin 4, and GAPDH.

E) Imaging of insulin content in islets from Munc18-1+/+ and Munc18-1+/- mice, with percent islet area comparison.

F) IPGTT (Insulin Potentiation of Glucose Tolerance Test) showing blood glucose levels (mg/dl) over time after glucose injection.

G) ITT (Insulin Tolerance Test) showing blood glucose levels (% baseline) over time after insulin injection.
Figure 2

A) Islets (+ TM)

Cre: /-       +/- (β-cell Munc18-1KO)

IB: Munc18-1
IB: Munc18c
IB: Syntaxin 1
IB: Syntaxin 4
IB: Clathrin

B) Insulin release (ng/ml)

35 mM KCl

Cre +/-, TM
Cre +/-, TM (β-cell Munc18-1KO)

C) Insulin release AUC

TM: Cre/-
Cre/+ (β-cell Munc18-1KO)

D) IPGTT

Cre +/-, Veh
Cre +/-, Veh
Cre +/-, TM
Cre +/-, TM (β-cell Munc18-1KO)

E) ITT

Cre +/-, Veh
Cre +/-, Veh
Cre +/-, TM
Cre +/-, TM (β-cell Munc18-1KO)
Figure 3A) Munc18-1\(^{+/+}\)/Munc18-1\(^{+-}\)

B) 

- **Munc18-1\(^{+/+}\)**
- **Munc18-1\(^{+-}\)**

C) 

- **Total granules/section**
  - **< 50nm**
  - **50-200nm**

* *
Figure 4

A) Human Islets

| Ad:       | Control | Munc18-1 |
|-----------|---------|----------|
| IB:       | Clathrin| Munc18-1 |
| IB:       | Syntaxin 1A | Syntaxin 4 |

B) 

- 20 mM Glucose
- 35 mM KCl

Insulin release (µU/ml)

- Control-Ad
- Munc18-1-Ad

C) 

Insulin release AUC (min*ng/ml)

- Control-Ad
- Munc18-1-Ad

Glucose

- 1st phase
- 2nd phase
- KCl
Figure 5

A) 35 mM KCl, 2 min

Stimulation Index

Control-Ad  Munc18-1-Ad

B) 35 mM KCl

35 mM KCl

\( \Delta F/F_0 \)

Seconds

Control-Ad  Munc18-1-Ad

C) Ad: Con  Munc18-1

IB: Munc18-1

IB: Syntaxin 1A

IB: VAMP2

VAMP2 location at PM

Control-Ad  Munc18-1-Ad
### Figure 6

#### A) GST-VAMP2

| Ad: Con | Munc18-1 |
|---------|----------|
| IB: Munc18-1 |  |
| IB: Syntaxin 1A |  |
| IB: SNAP-25 |  |
| Ponceau (GST-VAMP2) |  |

#### B) Lysate

| Ad: Con | Munc18-1 |
|---------|----------|
| Con | Munc18-1 |
| IB: Syntaxin 1A |  |
| IB: SNAP25 |  |
| IB: VAMP2 |  |

#### C) IP: Syntaxin 1A

| Ad: Con | Munc18-1 |
|---------|----------|
| IB: Syntaxin 1A |  |
| IB: VAMP2 |  |
| IB: VAMP2 (in lysate) |  |

#### D) IP: Munc18-1

| Ad: Con | Munc18-1 |
|---------|----------|
| IB: Munc18-1 |  |
| IB: Syntaxin 1A |  |
Figure 8

A) 

|        | Con | Munc18-1 |
|--------|-----|----------|
| siCon  |     |          |
| siSyn4 |     |          |

IB: Munc18-1
IB: Syntaxin 4
IB: Clathrin

B) Human C-peptide release

Stimulation Index

- Con siCon
- Munc18-1 siCon
- siSyn4
Munc18-1 regulates first-phase insulin release by promoting granule docking to multiple syntaxin isoforms
Eunjin Oh, Michael A. Kalwat, Min-Jung Kim, Matthijs Verhage and Debbie C. Thurmond

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