Doc2β Is a Novel Munc18c-interacting Partner and Positive Effector of Syntaxin 4-mediated Exocytosis*

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The widely expressed Sec/Munc18 (SM) protein Munc18c is required for SNARE-mediated insulin granule exocytosis from islet beta cells and GLUT4 vesicle exocytosis in skeletal muscle and adipocytes. Although Munc18c function is known to involve binding to the t-SNARE Syntaxin 4, a paucity of Munc18c-binding proteins has restricted elucidation of the mechanism by which it facilitates these exocytosis events. Toward this end, we have identified the double C2 domain protein Doc2β as a new binding partner for Munc18c. Unlike its granule-vesicle localization in neuronal cells, Doc2β was found principally in the plasma membrane compartment in islet beta cells and adipocytes. Moreover, co-immunoprecipitation and GST interaction assays showed Doc2β-Munc18c binding to be direct and complexes to be devoid of Syntaxin 4. Supporting the notion of Munc18c binding with Syntaxin 4 and Doc2β in mutually exclusive complexes, in vitro competition with Syntaxin 4 effectively displaced Munc18c from binding to Doc2β. The second C2 domain (C2B) of Doc2β and an N-terminal region of Munc18c were sufficient to confer complex formation. Disruption of endogenous Munc18c-Doc2β complexes by addition of the Doc2β binding domain of Munc18c (residues 173–255) was found to selectively inhibit glucose-stimulated insulin release. Moreover, increased expression of Doc2β enhanced glucose-stimulated insulin secretion by ~40%, whereas siRNA-mediated depletion of Doc2β attenuated insulin release. All changes in secretion correlated with parallel alterations in VAMP2 granule docking with Syntaxin 4. Taken together, these data support a model wherein Munc18c transiently switches from association with Syntaxin 4 to association with Doc2β at the plasma membrane to facilitate exocytosis.

Glucose homeostasis is maintained by a balance of insulin secretion and insulin action. Insulin is secreted from islet beta cells filled with mature insulin-containing granules which traffic to and fuse with the cell surface upon stimulation by elevated blood glucose. Upon detection of insulin and elevated circulating glucose levels by the skeletal muscle and adipose tissues the intracellular vesicles containing the insulin-responsive glucose transporter GLUT4 translocate to the plasma membrane and facilitate glucose uptake into the cell (1, 2). These “vesicle exocytosis” events are known to be regulated by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)2 proteins (2, 3). Vesicle exocytosis entails the specific pairing of a vesicle-associated membrane protein v-SNARE (VAMP) with a binary cognate receptor complex t-SNARE composed of SNAP-25/23 and Syntaxin proteins at the target membrane to form the SNARE core complex (3, 4). SNARE protein functions are further regulated by interaction with the Sec1/Munc18 (SM) secretory proteins, of which three plasma membrane-localized homologues exist in mammalian cells: Munc18a, Munc18b, and Munc18c (5, 6). However among these, only the Munc18c isoform can bind with and regulate the t-SNARE protein Syntaxin 4 (7, 8), and Syntaxin 4 is the singular functional Syntaxin isoform in insulin-stimulated GLUT4 vesicle exocytosis (9–11) and one of two functional Syntaxin isoforms identified in glucose-stimulated insulin exocytosis (12–15). Whereas we and others (11, 16) have shown that either depletion or overexpression of Munc18c in vivo dramatically alters glucose homeostasis via disruption of skeletal muscle GLUT4 translocation and pancreatic islet function, the mechanism by which Munc18c regulates these particular exocytotic events remains unknown.

Crystallographic and NMR studies support the concept that the Munc18 protein may keep its cognate Syntaxin in a “closed” conformation (17–19). Very recent studies further suggest that the Munc18 protein assists the transition of Syntaxin to the open state, possibly by stabilizing a labile transition half-open state of Syntaxin (20, 21). Previous studies of Munc18c-Syntaxin 4 kinetics in 3T3L1 adipocytes are consistent with this model (9, 22). In addition, we have recently demonstrated that Munc18c becomes tyrosine-phosphorylated and transiently dissociates from Syntaxin 4 upon stimulation in both islet beta cells and in 3T3L1 adipocytes (23), and this dissociation was correlated with increased SNARE docking. However, it has recently been demonstrated that in vitro, Munc18c can associate with the heterotrimeric SNARE core complex, with VAMP2

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2 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Gran, granule; GFP, green fluorescence protein; MKRBB, modified Krebs-Ringer bicarbonate buffer; RIA, radioimmunoassay; MOI, multiplicity of infection; PM, plasma membrane; GST, glutathione S-transferase; PVDF, polyvinylidene fluoride; siRNA, small interfering RNA; BSA, bovine serum albumin; VAMP, vesicle-associated membrane protein; SM, Sec/Munc18.
included (24). These data suggest that a cellular factor may be required to mediate the transient displacement of Munc18c.

There is precedence for the existence of proteins that bind and impact SM-Syntaxin complexes. For example, the yeast protein Ypt1p, a Rab GTPase, binds the Sly1p-Sed5p complex, yeast SM-Syntaxin homologues (25). In neurons, the SM protein Munc18-1 has several known binding proteins other than its cognate Syntaxin, several of which are C2 domain-containing proteins (26–28). One of these is Doc2β, which was shown to bind directly to Munc18-1 (27). Doc2β binds to a range of ligands including calcium, phospholipids, and intracellular proteins. Whereas a second isoform, Doc2α, is found primarily in brain/neuronal tissue, Doc2β is more widely expressed (27, 29–32).

In this report we demonstrate that Doc2β is expressed in insulin-secreting beta cells as well as insulin-responsive adipocytes, in which it associates with Munc18c in a manner mutually exclusive of Munc18c’s other known binding partner Syntaxin 4. Our results delineate binding domains of each protein sufficient to confer the interaction and further show that endogenous Munc18c-Doc2β association is functionally important for glucose-stimulated insulin secretion. Loss of association was coupled to decreased SNARE docking (Syntaxin 4 association with VAMP2 granules), whereas increased association or Doc2β expression was correlated with increased SNARE docking. In vitro studies revealed that Syntaxin 4 could displace Munc18c from preformed Munc18c-Doc2β complexes in a dose-dependent fashion, altogether supportive of a model in which Munc18c switches binding partners from Syntaxin 4 to Doc2β at the plasma membrane to promote exocytosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit anti-Munc18c and anti-GLUT4 antibodies were generated as previously described (9). The rabbit polyclonal anti-Syntaxin 4 and mouse monoclonal anti-VAMP2 antibodies were obtained from Chemicon (Temecula, CA) and Synaptic System (Gottingen, Germany), respectively. The rabbit polyclonal anti-Doc2 antibody was a kind gift from Dr. Matthijs Verhage (Vrije Universiteit, Netherlands). FLAG and clathrin antibodies were obtained from Sigma and BD Transduction Labs (Franklin Lakes, NJ), respectively. Rabbit polyclonal GFP and Syntaxin 1A antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). The monoclonal anti-Myc (9E10) antibody and protein G plus agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), respectively. MIN6 cells were a gift from Dr. John Hutton (University of Colorado Health Sciences Center). Goat anti-mouse and anti-rabbit horseradish peroxidase secondary antibodies and transfectin lipid reagent were acquired from Bio-Rad. Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Radioimmunoassay (RIA) grade bovine serum albumin and d-glucose were purchased from Sigma. Enhanced chemiluminescence reagent and Hyperfilm-MP were obtained from Amer sham Biosciences. The human C-peptide and rat insulin RIA kits were purchased from Linco Research Inc (St. Charles, MO).

Doc2β siRNA oligonucleotides were purchased from Ambion (Austin, TX): siDoc2 number 2-GGCAAAUAAGCUCAAGAC-A, siDoc2 number 1-UCAUCACACGCAGAUCGc.

**Plasmids**—The pcDNA3.1-Doc2β-myc DNA construct was generated by subcloning a PCR-generated mouse Doc2β fragment into the Xhol and HindIII sites of the pcDNA3.1/myc-His(−) vector (Invitrogen). The plBlueScriptIIK (−)-Doc2β (a kind gift from Dr. Mitsu nori Fukuda) was used as the template in a GC-rich PCR reaction system (Roche Applied Science) using the following primers: forward (5′-AGACTC-GAGGCCTGCATGACCTC) and reverse (5′-AGAAAGCT-TGGTCCTGAGTAC). GST-Doc2β fusion protein constructs pGEX-2T mouse Doc2β-C2A (amino acids 123–257), pGEX-2T mouse Doc2β-C2B (amino acids 257–375), pGEX-2T mouse Doc2β-C2AB (amino acids 123–375) were gifts from Dr. Mitsu nori Fukuda. The pGEX-4T3-Doc2β plasmid was a gift from Dr. Alexander Groffen (Vrije Universiteit, The Netherlands). The pcDNA3.1-His-Doc2β construct was made by subcloning a PCR-generated mouse Doc2β fragment into the BamHI and XhoI sites of the pET-28a(+) vector (Novagen, San Diego, CA).

The Munc18c-GFP deletion constructs were generated as previously described (23). An additional deletion construct, Munc18c-(173–255)–GFP was generated by subcloning a PCR-generated fragment of the 173–255 region into the BamHI and EcoRI sites of the pEGF-P-N3 vector (Clontech), using the following primers: forward (5′-AGAGATCCATGGAGGCAA-TGGCT), reverse (5′-AGAVAATTCTCATGCTGAAAGGCTG). GST-Doc2β fusion protein constructs were made by subcloning a PCR-generated full-length Munc18c fragment, using pcDNA3.1-Munc18c DNA as template (9), engineered with a 5′ NheI site and a 3′ EcoRI site for insertion into like sites present in the multiple cloning region of the pET28a(+) vector. The pAd5CMV–FLAG–Munc18c was generated by subcloning FLAG-Munc18c fragment excised from pcDNA3.1-FLAG–Munc18c (33) using Spel for insertion into SpeI-cutter pAd5CMV vector. All constructs were verified by DNA sequencing.

**Cell Culture, Transient Transfection, and Secretion Assays**—MIN6 beta cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM with 25 mm glucose) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml 1-glutamine, and 50 µM β-mercaptoethanol as described previously (15). MIN6 beta cells at 50–60% confluence were transfected with 40 µg of plasmid DNA per 10 cm² dish using transfectin (Bio-Rad) to obtain ~30–50% transfection efficiency. After 48 h of incubation, cells were washed twice with and incubated for 2 h in freshly prepared modified Krebs-Ringer bicarbonate buffer (MKRBB: 5 mM KCl, 120 mM NaCl, 15 mM Heps pH 7.4, 24 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂, and 1 mg/ml BSA). Cells were stimulated with 20 mM glucose or 50 mM KCl for the times indicated in the figures. Cells were subsequently lysed in Nonidet P-40 lysis buffer (25 mM Tris, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 µM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 5 µg/ml pepstatin), and lysates were cleared by microcentrifugation for 10
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min at 4 °C for subsequent use in co-immunoprecipitation experiments. For measurement of human C-peptide release, MIN6 beta cells were transiently co-transfected with each plasmid plus human proinsulin cDNA (kind gift from Dr. Chris Newgard, Duke University), using transfectin with 2 μg of each DNA per 35-mm dish of cells at 50% confluence. 48 h following transfection, cells were preincubated for 2 h in MKRBB buffer and stimulated with 20 mM glucose for 1 h. MKRBB was collected for quantitation of human C-peptide released.

Transfection of siRNA oligonucleotides into MIN6 cells was achieved using Lipofectamine 2000 (Invitrogen) with 100 nM oligonucleotides to obtain ~70–80% transfection efficiency. A non-targeting RNA (scrambled siRNA, obtained from Ambion) was included as a control in parallel experiments. Transfected cells were maintained in supplemented DMEM for 48 h, starved in MKRBB and stimulated as described above, and insulin-secreted into the MKRBB quantitated by RIA. Cells were harvested in 1% Nonidet P-40 lysis buffer for detecting Doc2β depletion.

CHO-K1 cells were purchased from the American Type Culture collection (Manassas, VA) and cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL l-glutamine. At 80–90% confluence, cells were electroporated with 40 μg of DNA as previously described (9). After 48 h of incubation, cells were harvested in 1% Nonidet P-40 lysis buffer and lysates cleared by centrifugation at 14,000 × g for 10 min at 4 °C for subsequent use in co-immunoprecipitation experiments.

**Subcellular Fractionation**—Subcellular fractions of beta cells were isolated as described previously (34). Briefly, MIN6 beta 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 dithiothreitol, and 1 mM sodium orthovanadate containing the protease inhibitors leupeptin (10 μg/mL), aprotinin (4 μg/mL), pepstatin (2 μg/mL), and phenylmethylsulfonyl fluoride (100 μM)). Cells were disrupted by 10 strokes through a 27-gauge needle, and homogenates were centrifuged at 900 × g for 10 min. Postnuclear supernatants were centrifuged at 5,500 × g for 15 min, and the subsequent supernatant centrifuged at 25,000 × g for 20 min to obtain the secretory granule fraction in the pellet. The supernatant was further centrifuged at 100,000 × g for 1 h to obtain the cytosolic fraction. 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 × g for 1 h to obtain an interface containing the plasma membrane fraction. The interface was collected and diluted to 2 mL with homogenization buffer for centrifugation at 6,000 × g for 10 min, and the resulting pellet was collected as the plasma membrane fraction. All pellets were resuspended in 1% Nonidet P-40 lysis buffer to solubilize membrane proteins.

Subcellular fractions of 3T3L1 adipocytes were obtained using the differential centrifugation method as described previously (9). Briefly, 3T3L1 adipocytes were washed with and resuspended in HES buffer (20 mM HEPES pH 7.4, 1 mM EDTA, and 255 mM sucrose containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin, 10 μg/mL aprotinin, and 5 μg/mL leupeptin). Lysates were sheared 10 times through a 22-gauge needle and centrifuged at 19,000 × g for 20 min at 4 °C. The low speed (HDM) fraction was obtained by centrifugation of the resulting supernatant at 41,000 × g for 20 min at 4 °C. The supernatant was removed and centrifuged at 180,000 × g for 75 min at 4 °C to generate the high speed (LDM) fraction. The plasma membrane fraction (PM) was obtained by resuspending the pellet from the initial 19,000 × g centrifugation in HES buffer followed by layering onto a 1.12 M sucrose cushion for centrifugation at 100,000 × g for 60 min. The plasma membrane fraction was then removed from the cushion and centrifuged at 40,000 × g for 20 min, and that pellet then resuspended in HES buffer.

**Co-immunoprecipitation and Immunoblotting**—MIN6 beta cells were preincubated in MKRBB for 2 h followed by glucose stimulation. Cells were subsequently lysed in Nonidet P-40 lysis buffer. MIN6 beta cell-secreted detergent homogenates (2–3 mg) were combined with rabbit anti-Munc18c antibody, rabbit anti-Syntaxin4 antibody, or rabbit anti-Doc2β antibody for 2 h at 4 °C followed by a second incubation with protein G Plus-agarose for 2 h. The resultant immunoprecipitates were subjected to 10% SDS-PAGE followed by transfer to PVDF membranes for immunoblotting. Munc18c, Syntaxin 4, and Doc2β antibodies were used at 1:5000, 1:500, and 1:1000 dilutions, respectively, and secondary antibodies conjugated to horseradish peroxidase were diluted at 1:5000 for visualization by chemiluminescence. Immunoprecipitations using CHO-K1 detergent-cleared cell lysates were performed similar to that of the MIN6 cell lysates.

**Recombinant Proteins and Interaction Assays**—GST-Doc2β, GST-Doc2β-C2AB, GST-Doc2β-C2A, GST-Doc2β-C2B, and GST-Syntaxin 4 fusion proteins were expressed in *Escherichia coli* and purified by glutathione-agarose affinity chromatography as described previously (35). Recombinant Syntaxin 4 and Doc2β proteins were obtained following thrombin cleavage of GST-Syntaxin 4, GST-Doc2β, respectively. Syntaxin 1A protein was purchased from Synaptic Systems. BSA control protein was purchased from Pierce. Recombinant His-tagged Munc18c was also expressed in *E. coli* and purified by Ni-NTA nickel-chelating resin (Invitrogen) under native conditions (50 mM NaH₂PO₄, 0.5 mM NaCl, pH 8). Eluted protein was further dialyzed overnight in 50 mM Tris, pH 8, supplemented with 1 mM dithiothreitol. The interaction of GST-Doc2β with His-Munc18c was performed by incubating 2 μg of each GST-Doc2β-C2AB, GST-Doc2β-C2A, GST-Doc2β-C2B linked to Sepharose beads with 2 μg of recombinant His-Munc18c protein in Nonidet P-40 lysis buffer for 2 h at 4 °C. Following three washes with lysis buffer, proteins were eluted from the Sepharose beads and subjected to 10% SDS-PAGE followed by transfer to PVDF membrane for immunoblotting.

**Adenoviral Transduction of MIN6 Cells**—MIN6 cells at 60% confluence were transduced with pAd5CMV-Munc18c CsCl-purified particles (generated by the University of Iowa Gene Targeting Vector Core, Iowa City, IA) for 2 h at 37 °C (MOI =
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**A)** Islets

![Expression of Doc2β in islet beta cells and adipocytes. A](image1.png)

**B)** 3T3L1 adipocytes

![Expression of Doc2β in 3T3L1 adipocytes.](image2.png)

**FIGURE 1.** Expression of Doc2β in islet beta cells and adipocytes. A, lysate prepared from 100 human or mouse islets, MIN6 cells (40 µg), and plasma membrane fractions isolated from MIN6 cells (10 µg) were resolved on 10% SDS-PAGE gel, transferred to PVDF membrane, and immunoblotted with anti-Doc2β and anti-Syntaxin 1A antibodies. Purified recombinant Doc2β protein used as a control for antibody specificity and protein migration (lane 4). B, 3T3L1 adipocyte lysates and subsequent subcellular fractions (10 µg protein/lane) were resolved by 10% SDS-PAGE and immunoblotted for the presence of Doc2β, GLUT4, and Syntaxin 4 proteins. Data are representative of at least two independent sets of fractions each.

100). Transduced cells were then washed twice with phosphate-buffered saline and incubated for 48 h in complete medium at 37 °C, 5% CO2. Transduced cells were subsequently preincubated in MKRBB for 2 h and stimulated with 20 mM d-glucose for 5 min. Cleared detergent lysates were prepared as described above for the GST pull-down assay.

**Statistical Analysis**—All data are expressed as mean ± S.E. Data were evaluated for statistical significance using the Student’s t test.

**RESULTS**

**Doc2β and Munc18c Associate in MIN6 Beta Cells**—Doc2β is considered to be a ubiquitously expressed protein, enriched in brain, heart, and lung tissues (27, 31), but expression in adipocytes and islet cells has not yet been established. To address this, tissue and cultured cell lysates were used for immunoblotting to detect the presence of Doc2β protein using a previously validated antibody provided by Dr. Matthias Verhage. The Doc2β antibody recognized a single 45-kDa band in both human and mouse islet lysates, MIN6 beta cell lysate and recombinant purified full-length Doc2β protein (Fig. 1A). Moreover, of the three subcellular fractions of MIN6 beta cells examined, Doc2β was almost exclusively localized to the PM fraction (Fig. 1A, lane 4), and was nearly undetectable in granule and cytosolic fractions (data not shown). The purity of the fractions was validated by the presence of the protein Munc18c 1A exclusively in the plasma membrane fraction (Fig. 1A) and by insulin content as we have documented previously (15, 34).

In 3T3L1 adipocytes, Doc2β was also primarily localized to the PM fraction (Fig. 1B), with very little present in intracellular vesicle fractions (LDM and HDM). Subcellular fractions prepared from insulin-stimulated 3T3L1 adipocytes were validated by detection of GLUT4 protein in PM, LDM, and HDM fractions and detection of Syntaxin 4 protein principally in the PM fraction. RT-PCR was also used to confirm the presence of Doc2β mRNA in the MIN6 cells (data not shown). Thus, Doc2β and Munc18c both localize to the PM fraction in cell types where Munc18c-Syntaxin 4 complexes are known to be important for regulated exocytotic events.

To next determine whether Doc2β could bind to Munc18c, Doc2β was expressed as a GST fusion protein in E. coli and attached to beads as bait for precipitating interacting proteins. To increase the abundance of Munc18c and enhance detection of a binding event, MIN6 lysates prepared from cells transduced to express recombinant FLAG-tagged Munc18c were initially used. Both anti-FLAG and anti-Munc18c antibodies detected Munc18c in precipitates with GST-Doc2β but not GST control (Fig. 2A). Coimmunoprecipitation was used as an independent approach to confirm this interaction. MIN6 cell lysates prepared from cells transfected to express recombinant Myc-tagged Doc2β were immunoprecipitated with anti-Myc antibody or with the vector control (pcDNA3.1/myc-His) or pcDNA3-Doc2β/myc-his DNAs were used for immunoprecipitation (IP) with anti-Myc antibody. Proteins were subjected to 10% SDS-PAGE and immunoblotted with anti-Munc18c and anti-Myc antibodies. C, MIN6 lysates were used for immunoprecipitation with either anti-Munc18c antibody or rabbit IgG control. Proteins were subjected to 10% SDS-PAGE for subsequent immunoblotting with anti-Munc18c and anti-Doc2β antibodies. Ponceau S staining shows equal input of antibody (heavy chain). Data are representative of three independent experiments.

**FIGURE 2.** Doc2β interacts with Munc18c in MIN6 beta cells. A, MIN6 beta cells were transduced with pAd5CMV-FLAG-Munc18c adenovirus (MOI = 100). Lysates were subsequently prepared and combined with GST-Doc2β (linked to beads) for 2 h of incubation. Eluted proteins were subjected to 10% SDS-PAGE and immunoblotted (IB) for the presence of a single ~68-kDa band detected by both anti-FLAG and anti-Munc18c antibodies. Ponceau S staining shows loading of GST fusion proteins. B, lysates prepared from MIN6 cells transfected with either vector control (pcDNA3.1/myc-His) or pcDNA3-Doc2β/myc-his DNAs were used for immunoprecipitation (IP) with anti-Myc antibody. Proteins were subjected to 10% SDS-PAGE and immunoblotted with anti-Munc18c and anti-Myc antibodies. C, MIN6 lysates were used for immunoprecipitation with either anti-Munc18c antibody or rabbit IgG control. Proteins were subjected to 10% SDS-PAGE for subsequent immunoblotting with anti-Munc18c and anti-Doc2β antibodies. Ponceau S staining shows equal input of antibody (heavy chain). Data are representative of three independent experiments.
irrespective of whether epitope tags were placed at the N or C termini of either protein, suggesting that the interaction may occur through more internal regions of each.

The C2B Domain of Doc2β Is Sufficient to Mediate Direct Binding to Munc18c—To determine whether the association of Doc2β and Munc18c is direct or indirect, we combined bacterially expressed and purified recombinant proteins in an in vitro binding assay. A truncated form of GST-Doc2β (residues 123–375) was found to confer binding to His-tagged Munc18c (Fig. 3A), indicating that the Doc2β N-terminal Munc13-interacting domain (MID) was dispensable for interaction with Munc18c, similar to an earlier finding with Doc2β-Munc18-1 interaction (27). To further delineate the minimal binding domain of Doc2β sufficient to confer direct binding to Munc18c, each individual C2 domain was tested (Fig. 3B). Whereas the GST-C2A protein was incapable of interaction with Munc18c, the GST-C2B protein showed nearly equal affinity for Munc18c as did the full GST-C2AB protein. This binding specificity of Munc18c for the C2B domain of Doc2β is distinctly different from that of Munc18-1, which was shown to associate with the C2A domain (27). This suggests that unlike the Munc18-isofrom specific binding to cognate Syntaxins, Doc2β is not discretely paired with one particular Munc18 protein, but that its interaction with each Munc18 protein is partitioned by differential affinity of each to different C2 domains.

The N Terminus of Munc18c Is Required for Binding to Doc2β—To determine the minimal binding domain of Munc18c required for its interaction with Doc2β, a deletion series of Munc18c-GFP was used as previously described (23). Munc18c-GFP mutants were co-electroporated with Doc2β-myc into CHOK1 cells, because CHOK1 cells transfected very efficiently (Fig. 4A). Lysates prepared from transfected cells were used in anti-Myctagged (Doc2β) immunoprecipitation reactions to determine which region of Munc18c conferred the highest level of interaction (Fig. 4B, lanes 1–6). Although both N-terminal regions containing residues 1–172 or 173–255 of Munc18c could be co-precipitated by Doc2β-myc, the 173–255 region showed consistently higher binding affinity to recombination Myc-tagged Doc2β (Fig. 4B, lanes 8 and 9); in three of five experiments no binding of the 1–172 fragment to GST-Doc2β was observed, whereas in the remaining two experiments a low level of binding was observed. This was not caused by discrepancies in protein expression or batch of cells or DNA used for electroporation. Thus it appears there could be two binding sites, but the affinity of the 1–172 fragment for GST-Doc2β is reproducibly lesser than that of the 173–255 region. The specificity of this binding was validated by lack of binding of GFP alone to Doc2β-myc (Fig. 4B, lane 12). In addition, full-length Munc18c-GFP bound less well than did the 173–255 region (Fig. 4B, lane 7), suggesting that the smaller isolated region may adopt a more accessible conformation. Interestingly, this 173–255 region is known to contain a pivotal tyrosine at position 219 that we have recently demonstrated to be required to mediate Munc18c dissociation from Syntaxin 4 (23). Thus the region required to displace Munc18c from Syntaxin 4 is also the region of Munc18c sufficient to accept binding to Doc2β, consistent with a switch mechanism of Munc18c binding to either in a mutually exclusive manner.

Syntaxin 4 Is Excluded from the Munc18c-Doc2β Complex—To test the notion that the Munc18c-Doc2β complex is mutually exclusive of Munc18c-Syntaxin 4 complex, GST-Doc2β linked to beads was incubated with MIN6 cell lysates and eluted proteins were immunoblotted with anti-Munc18c and anti-Syntaxin 4 antibodies. As shown in Fig. 5A, endogenous Munc18c but not Syntaxin 4 was precipitated from MIN6 cell lysates.

The inability of Syntaxin 4 to coprecipitate with GST-Doc2β and Munc18c may have been caused by issues of protein abundance or additional cellular factors, because the assay was conducted using MIN6 cell lysates. Therefore, to test the notion that Munc18c forms mutually exclusive complexes with Doc2β and Syntaxin 4, we performed in vitro competition assays (Fig. 5B). GST-Doc2β coupled to beads was initially preincubated with His-tagged Munc18c protein for 2 h at 4 °C. GST-Doc2β-Munc18c complexes were then pelleted by centrifugation and unbound excess Munc18c washed away. The presence of unbound Munc18c demonstrated that Munc18c protein was
experiments were repeated using the truncated GST-Doc2
specific protein interference in the assay. Furthermore, the above
by Syntaxin 4 is not simply the result of increased and nonspe-
cific binding of recombinant Syntaxin 4 in large abundance
in the PM fraction (Fig. 6A, lane 1). Trace amounts of Doc2β were also
exclusively from the PM fraction, even though Doc2β and Munc18c were
both present in the cytosolic fraction (Fig. 6A, lane 3). Co-immunoprecipita-
tions were performed from these subcellular fractions using anti-
Munc18c-EGFP or anti-Doc2β antibodies. Munc18c co-immunoprecipitated
both Syntaxin 4 and Doc2β exclusively from the PM fraction, even
though Doc2β and Munc18c were both also present in the cytosolic
fraction (Fig. 6B, lanes 1 and 3). Reciprocal immunoprecipitation of
Doc2β similarly resulted in co-precipitation of Munc18c from the PM
fraction but failed to precipitate Syntaxin 4 (Fig. 6B, lane 4). No net
changes in abundance of complexes formed between Munc18c, Syntaxin 4, or Doc2β in response to glucose stimu-
ation were observed (data not shown). These data might suggest that complexes are highly transient and/or that
there is no net switch of Munc18c to Doc2β at any one partic-
ular point in time during exocytosis.

Endogenous Munc18c-Doc2β Complexes Are Essential for Insulin Exocytosis—To evaluate the requirement for the endogenous Doc2β-Munc18c complexes in exocytosis we
used the minimal Munc18c region (173–255) as a competitive
inhibitor in a human C-peptide reporter assay. This small
region of Munc18c fails to bind Syntaxin 4 (data not
shown). MIN6 cells were co-transfected with Munc18c-
(173–255) or vector control together with human proinsulin
cDNA. Human C-peptide (derived from human proinsulin)
is synthesized and packaged in an identical fashion to mouse
C-peptide and serves as a reporter of
exocytosis from granules, but is immunologically
distinct from mouse C-peptide and insulin in granules, but is immunologically
specific binding of recombinant His-Doc2β protein (Fig. 5C). Thus, under in vitro
conditions, we have established a switching binding mode of
Munc18c to Syntaxin 4 and to Doc2β. Though the molar ratio
threshold for Syntaxin 4 to completely compete off Munc18c is high
in vitro, it is possible that there might be cellular signals required to
trigger the switch of binding partner in vivo.

Munc18c-Doc2β Complexes Exist Only at the Plasma Membrane—
We have shown previously that the dissociation of Munc18c from
Syntaxin 4 is only observed from the PM compartment of pancreatic beta
cells or 3T3L1 adipocytes although displaced Munc18c was never seen to
move into either cytosolic or granule compartments (23). Subcel-
lar fractions prepared from MIN6 cells contained Doc2β, Munc18c,
and Syntaxin 4 in large abundance in the PM fraction (Fig. 6A, lane 1).
Not limiting for the formation of complex (Fig. 5B, lanes 1–4). Increasing amounts of soluble recombinant Syntaxin 4 protein
were subsequently added for an additional 2 h. A 3-fold molar
excess of Syntaxin 4 resulted in dissociation of more than 60%
of the His-Munc18c from GST-Doc2β (Fig. 5B, lane 4). Consis-
tent with data from the GST-Doc2β interaction studies using
cell lysate above, a negligible amount of recombinant Syntaxin 4
protein was seen to precipitate with either GST-Doc2β or GST
alone, with greater than 99% of protein left unbound. Control
experiments conducted using Syntaxin 1A, a non-Munc18c-
binding protein, in place of Syntaxin 4 as the competitor failed
to show disruption of the interaction between GST-Doc2β and
His-Munc18c (Fig. 5B, lane 7), indicating that the competition
by Syntaxin 4 is not simply the result of increased and nonspe-
cific protein interference in the assay. Furthermore, the above
experiments were repeated using the truncated GST-Doc2β-
C2AB protein and gave identical results. The reciprocal exper-
iment gave similar results: GST-Syntaxin 4 failed to precipitate
recombinant His-Doc2β protein (Fig. 5C). Thus, under in vitro
conditions, we have established a switching binding mode of
Munc18c to Syntaxin 4 and to Doc2β.

FIGURE 4. The N terminus of Munc18c is sufficient for mediating Munc18c-Doc2β interaction. A, four
fragments traversing the length of Munc18c were linked at the C terminus to EGFP. B, DNA constructs were
co-electroporated into CHO-K1 cells with Doc2β-myc, and 48 h later detergent lysates were prepared for immu-
noprecipitation with anti-Myc antibody. Proteins were subjected to 12% SDS-PAGE and initially immunoblotted with anti-GFP antibody to detect binding of Munc18c fragments. Equal precipitation of Doc2β-myc was confirmed by anti-Myc immunoblotting. Lysate proteins (60 μg/lane) show expression of each Munc18c-EGFP fragment (left panel). Data are representative of five inde-
pendent experiments.
C-peptide secretion in response to glucose (Fig. 7A, bars 1 and 2). In contrast, cells transfected to express the 173–255 fragment showed abolished glucose-stimulated secretion (Fig. 7A, bars 2 and 3), and was without effect upon basal secretion (1.1 ± 0.1-fold of control, n = 3). However, KCl-stimulated human C-peptide release was not affected by the presence of the 173–255 region (Fig. 7A, bars 4 and 5), suggesting that the Doc2β-Munc18c is required only for glucose-stimulated exocytosis, and that expression of this region did not have a global dampening effect upon exocytosis events in general.

To gain mechanistic data underlying this alteration in function we assessed SNARE complex assembly (Syntaxin-VAMP association) in MIN6 lysates prepared from cells transfected with the 173–255 region or vector control. As we have shown previously (23), glucose stimulation for 5 min increased the co-immunoprecipitation of VAMP2-granules with Syntaxin 4 in lysates prepared from vector-treated cells (Fig. 7B). However, MIN6 cells overexpressing Munc18c-(173–255) abolished glucose-stimulated Syntaxin 4-VAMP2 association (Fig. 7C), but was without significant effect upon unstimulated levels of Syntaxin 4-VAMP2 association. SNARE complex assembly in unstimulated cells has been reported to represent predocked granules used for the initial burst of insulin release that can be stimulated by either glucose or KCl (38). Taken together, these data suggest that endogenous Munc18c-Doc2β interaction is functionally important for regulating glucose-stimulated VAMP2-granule docking with Syntaxin 4, but not necessary for predocking granules.

In a second approach to determine the requirement for Doc2β we utilized siRNA-mediated depletion. Two different commercially available siRNA oligonucleotides were transiently transfected into MIN6 cells (designated as siDoc2β) using the reagent Lipofectamine 2000 to obtain greater than 80% of cells transfected, as determined using fluorescently labeled oligonucleotides. Depletion by one of the two oligonucleotides, designated siDoc number 1, resulted in ~40% reduction in Doc2β protein (Fig. 8A). This depletion corresponded to ~40% reduction of glucose-induced insulin release relative to secretion in siControl-transfected cells (Fig. 8B). Transfection with the second siRNA, siDoc number 2, resulted in no signif-

**FIGURE 5.** Syntaxin 4 is excluded from the Munc18c-Doc2β complex and competes with Doc2β for Munc18c binding. A, MIN6 cells lysates were and combined with GST-Doc2β linked to beads for 2 h of incubation. Eluted proteins were subjected to 10% SDS-PAGE and immunoblotted with anti-Munc18c and Syntaxin 4 antibodies. Ponceau S staining showed equal input of GST fusion proteins. B, GST-Doc2β linked to Sepharose beads (0.5 μg/7 ml) was preincubated with His-Munc18c (0.5 μg/7 ml) and complexes pelleted for subsequent addition of soluble Syntaxin 4 (6–18 nM). Both bound and unbound proteins were subjected to 10% SDS-PAGE and immunoblotted with anti-Munc18c and anti-Syntaxin 4 antibodies (Syntaxin 4 failed to specifically bind GST-Doc2β or GST). Quantitation of Munc18c remaining bound in the presence of Syntaxin 4 is shown below Munc18c bands in lanes 2–4 (n = 5, p < 0.01 at the 18 nM level). Syntaxin 1A (18 nM) was substituted for Syntaxin 4-VAMP2 association. SNARE complex assembly in unstimulated cells has been reported to represent predocked granules used for the initial burst of insulin release that can be stimulated by either glucose or KCl (38). Taken together, these data suggest that endogenous Munc18c-Doc2β interaction is functionally important for regulating glucose-stimulated VAMP2-granule docking with Syntaxin 4, but not necessary for predocking granules.

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siDoc2β-treated cells failed to show any glucose-stimulated increase in SNARE complex assembly (quantified in Fig. 8D), compared with appropriate 2-fold increase in glucose-stimulated VAMP2 granule association with Syntaxin 4 in siControl-treated cells. Similar to results obtained using the 173–255 region in this assay, siDoc2β treatment had no significant effect upon unstimulated SNARE complex assembly nor Syntaxin 4-Munc18c association. Specific depletion of Doc2β was confirmed by immunoblotting of lysates using anti-Doc2β and -clathrin antibodies. Taken together these data indicate that reduction of Doc2β levels directly impact the ability of Munc18c to associate with Syntaxin 4, and that Docβ is required for Syntaxin 4-mediated exocytosis of glucose-stimulated granules.

**Doc2β Functionally Enhances Insulin Exocytosis and SNARE-mediated Vesicle Docking**—To further investigate the relationship of the Doc2β role in exocytosis with its role as a Munc18c-binding protein, we tested the ability of Doc2β overexpression to relieve the inhibition upon insulin secretion caused by increased Munc18c expression. We have shown previously that overexpression of Syntaxin 4 can act as a molecular sponge to bind excess Munc18c and resume normal exocytosis, and that non-Munc18c-binding proteins, or a non-PM localized form of Syntaxin 4 fail to rescue exocytosis (23, 39). As shown in Fig. 9A, Munc18c overexpression reduced glucose-stimulated human C-peptide release by ~50% (119% of basal versus 141% attained in vector-expressing cells, \( p < 0.05 \)). Co-expression of Doc2β with Munc18c fully counteracted this inhibition (Fig. 9A, bars 6 versus 4), consistent with the Doc2β role as a PM-localized Munc18c-binding factor. Coordinate expression of Munc18c-myc dramatically decreased VAMP2 association with Syntaxin 4, under both unstimulated and stimulated conditions (Fig. 9B, lanes 3 and 4). Of note, the recombinant Munc18c-myc protein is not recognized by the Munc18c antibody (antibody raised against the far C terminus of Munc18c protein), such that the Munc18c immunoblot in Fig. 9B represents only endogenous Munc18c binding with Syn4. Expression of Myc-tagged forms of Munc18c and Doc2β in MIN6 lysates was confirmed by anti-Myc immunoblotting. Consistent with the secretion results, the addition of Doc2β-myc protein in Munc18c-myc overexpressing cells restored the level of VAMP2 co-precipitated by anti-Syntaxin 4 antibody in unstimulated lysates to the control levels (Fig. 9B, compare lanes 1, 3, and 5). However, there was no difference between unstimulated and glucose-stimulated VAMP2 association with Syntaxin 4 in cells overexpressing both Doc2β-myc and Munc18c-myc (Fig. 9B, lanes 5 and 6).

Remarkably, Doc2β overexpression alone caused release of ~40% more human C-peptide compared with vector-transfected cells (Fig. 9A, bars 2 versus 8), and without significantly increasing basal secretion. Consistent with this, Doc2β overexpression in MIN6 cells reduced association of Syntaxin 4 with endogenous Munc18c (Fig. 9B, lanes 7 and 8) under both unstimulated as well as glucose-stimulated conditions (by 23±8% and 41±4% under unstimulated and stimulated conditions, respectively, compared with vector-expressing cells). By contrast, Doc2β overexpression resulted in significantly increased Syntaxin 4-VAMP2 co-precipitation from both unstimulated
and glucose-stimulated lysates (Fig. 9B, quantified in Fig. 9C). These data suggest that the stimulatory effect of Doc2β overexpression in glucose-stimulated insulin secretion correlated with its ability to increase the docking of granules at Syntaxin 4 sites.

**DISCUSSION**

In this report we have identified Doc2β as a new binding partner for the exocytotic protein Munc18c and demonstrated an essential functional role for this complex in insulin granule exocytosis. Doc2β was found localized to the plasma membrane compartment in both cell types known to utilize Munc18c for exocytosis, islet beta cells and 3T3L1 adipocytes, and bound to Munc18c exclusively in this cellular compartment. The N-terminal residues 173–255 of Munc18c bound directly to the Doc2β second C2 domain, and expression of the 173–255 region of Munc18c or siRNA-mediated depletion of Doc2β significantly impaired glucose-stimulated insulin exocytosis. Syntaxin 4 failed to bind Munc18c-Doc2β complexes, and in vitro binding studies revealed that Syntaxin 4 could competitively inhibit Munc18c binding to Doc2β. Furthermore, increased expression of Doc2β was found to enhance glucose-stimulated insulin exocytosis, resultant from increased VAMP2-granule docking at Syntaxin 4 sites, and decreased Munc18c occupancy of Syntaxin 4 sites. Taken together, these data support a model whereby Munc18c switches between binding partners Syntaxin 4 and Doc2β at the plasma membrane, such that Munc18c remains in close proximity to the SNARE fusion apparatus to exert its function in regulated exocytosis.

SM proteins are proposed to function in catalysis of vesicle fusion, fusion being the final and irreversible step of vesicle trafficking. However SM proteins are also thought to add another layer of specificity of vesicle docking in tandem with Rab-like proteins. In yeast the Sly1p-Sed5p complex, analogous to SM-Munc18 complexes, affinity is reduced by the addition of the Rab-like protein Ypt1p to promote SNARE pairing and fusion (40, 41). Similarly, the Munc18-1-Syntaxin 1A complex crystal structure shows a putative Rab-interacting site in the loop connecting domain 2 (composed of residues 135–245 and 480–592) with domain 3b of Munc18-1 that would serve as a hinge mechanism for releasing the constrictive hold on Syntaxin 1A (18). Consistent with this, we have previously shown that mutation within domain 2 reduces Munc18c affinity for Syntaxin 4 in 3T3L1 adipocytes (33). However, neither candidate Rab proteins Rab4 found on GLUT4 vesicles nor Rab3A in beta cells have been confirmed to carry out this disruptive function.

Alternatively, the calcium-phospholipid-binding Doc2β protein has also been localized to vesicles in neuronal cell types and shown to bind to Munc18-1 via its first C2 domain (C2A) (27, 42), making it a suitable candidate as a Munc18c-Syntaxin 4 displacement factor. However, two key findings presented here suggest a novel role for Doc2β function in endocrine cell exocytosis: 1) Munc18c does not bind the C2A domain of Doc2β, but rather binds to the C2B domain instead; 2) Doc2β
**Munc18c-Doc2β Complexes Function in Exocytosis**

exocytosis in response to Ca$^{2+}$ stimulation. Neither did we observe Doc2β translocation in stimulated 3T3L1 adipocytes (data not shown). Given the persistent plasma membrane localization of Munc18c in both MIN6 beta cells and 3T3L1 adipocytes, it seemed likely that any factor binding to displaced Munc18c would also be localized to the plasma membrane. The Doc2β plasma membrane localization in both beta cells and adipocytes fits such a prediction well. Thus, the mechanisms underlying alterations of Munc18c-Syntaxin 4 complex conformation likely differ significantly from those of yeast and neuronal cells.

Although there is a high degree of sequence homology between family members within the syntaxin and SM protein families, there are notable differences that have been proposed to exert conformational changes that significantly alter how the complexes form and function. For example, comparison of Syntaxin 4 with Syntaxin 1A suggests there are differences in Syntaxin 4 contacts between the Hab and H3 domains that will alter its presentation to the variable hairpin region in domain 3a of its SM partner (Munc18c) (18). In addition, very recent reports have shown that the Munc18-1 isoform binds to the far N terminus of Syntaxin 1A to allow Syntaxin 1A to adopt a transitional conformation (20, 21). A KDR motif present in the far N terminus of Syntaxin 1A mediates its interaction with Munc18-1 (46). However, we cannot necessarily expect Syntaxin 4 to bind and function as does Syntaxin 1A, since Syntaxin 4 lacks the KDR motif.

Our *in vitro* competition studies demonstrated that Syntaxin 4 displaced Munc18c from binding to Doc2β (IC$_{50}$ ~ 16 nm) suggesting that in the cell Doc2β might function either as an acceptor of displaced Munc18c, or play a more active role by inducing the disruption of Munc18c-Syntaxin complexes. However, no significant increase in abundance of Munc18c-Doc2β complexes following glucose stimulation in MIN6 beta cells was detected, analogous to Munc18c-Syntaxin 4 complexes (23). There are several possibilities for this: 1) Munc18c is not fully displaced from Syntaxin 4 in vivo; 2) the switching is highly transient and significant net changes in complex abundances are not reached at any given time point. In the case of the first possibility, it has been shown *in vitro* that Munc18c may remain attached to Syntaxin 4 during SNARE complex assembly (24); however, this has yet to be demonstrated *in vivo* in these endocrine cell types. Alternatively, SNARE complex interactions *in vitro* are known to occur more promiscuously than *in vivo* (3). This may be related to the absence of post-translational modifications, such as stimulus-induced phosphorylation of Munc18c, occurring only *in vivo*, that displace it from Syntaxin 4 (23). Even in cells the phosphorylation event was highly transient, requiring phosphatase inhibitor to trap phospho-Munc18c to detect its displacement from Syntaxin 4 (23). Thus, this second possibility is particularly intriguing because the region of Munc18c found sufficient to confer Doc2β binding contains the regulatory Tyr$^{219}$ residue.

Another possible trigger of Munc18c-Doc2β association could be elevated [Ca$^{2+}$]. Granule priming is known to require elevated [Ca$^{2+}$], in islet beta cells, and if Doc2β is involved in glucose-induced priming of granules then our data showing loss of insulin release from Doc2β-depleted cells would support
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this. Upon binding to calcium, the affinity of Doc2β for binding to phospholipids is significantly increased (47), and the C2A but not C2B domain of Doc2β mediates this in vitro (29). Interestingly, Doc2β also associated with the Munc18-1 isoform through this C2A domain in neurons, in a calcium-independent fashion (27). However our data clearly show that Doc2β binds to Munc18c through the C2B domain, unlike its interaction with Munc18-1. Given this distinction it may be that Munc18c-Doc2β binding in beta cells is calcium-dependent.

Our observation that both disrupting endogenous Munc18c-Doc2β complex and depletion of endogenous Doc2β led to reduced Syntaxin 4-VAMP2 association specific to glucose stimuli suggests that Munc18c-Doc2β interaction is dispensable for pre-docking of granules but is critically important for docking of mobilized insulin granules. This interpretation stems from several studies showing a correlation between the co-immunoprecipitation of VAMP2 with Syntaxin 4 from unstimulated beta cell lysates with the abundance of pre-docked granules, those granules released in response to [Ca\(^{2+}\)]\(_i\), elevated by either glucose or KCl in the first phase of insulin release (23, 38, 49). In contrast, overexpression of Doc2β enhanced both basal and glucose-stimulated VAMP2 association with Syntaxin 4. The mechanism behind this could lie in the ability of Doc2β to compete Munc18c away from Syntaxin 4 in the absence of stimulus, but could also be mediated by its interaction with other molecules such as Munc13-1, which has been demonstrated to influence both first phase and second phase insulin secretion in studies using islet cells isolated from Munc13-1 heterozygous knockout mice (50).

Doc2β was found to function as a Munc18c-binding protein in a rescue assay, whereby the inhibition of exocytosis caused by overexpression of Munc18c can be relieved and normal exocytosis restored by co-expression of the Munc18c-binding protein. We have previously shown that co-expression of Syntaxin 4 rescues insulin secretion as well as VAMP2 granule docking (23). However, while Doc2β overexpression also rescued secretion and pre-docked VAMP2 granules at Syntaxin 4 sites, it failed to fully restore increased docking of glucose-mobilized granules. One possible explanation could be that protein stoichiometry of Munc18c:Doc2β in islet cells is crucial for insulin granule docking and exocytosis, and alterations of this stoichiometry by overexpression of both proteins did not necessarily reconstitute the proper Munc18c:Doc2β complex molar ratio. This was not too surprising because protein stoichiometry of SNARE accessory proteins has been shown to be important for maintaining normal granule exocytosis events (48, 51). Future islet perfusion studies will be required to discern whether Munc18c, Doc2β, and the Munc18c-Doc2β complex are required for first phase or second phase insulin secretion.

In summary, we have identified Doc2β as a new binding partner for Munc18c in endocrine cells and have demonstrated a functional requirement for the Doc2β-Munc18c complex exocytosis and SNARE core complex formation. We have further revealed that Doc2β can compete with the Munc18c cognate SNARE protein, Syntaxin 4. Doc2β has the unique ability to compete with Syntaxin 4 for Munc18c binding, and may represent the missing factor in the mechanism by which Munc18c facilitates the transition of Syntaxin 4 from a monomeric closed conformation into its fusion competent form. Because Munc18c, Doc2β and Syntaxin 4 are ubiquitously expressed, the mechanism identified here may be conserved throughout numerous secretory events, particularly in endocrine cells.

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