Glucose-induced ERM protein activation and translocation regulates insulin secretion

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Lopez JP, Turner JR, Philipson LH. Glucose-induced ERM protein activation and translocation regulates insulin secretion. Am J Physiol Endocrinol Metab 299: E772–E785, 2010. First published August 24, 2010; doi:10.1152/ajpendo.00199.2010.—A key step in regulating insulin secretion is insulin granule trafficking to the plasma membrane. Using live-cell time-lapse confocal microscopy, we observed a dynamic association of insulin granules with filamentous actin and PIP2-enriched structures. We found that the scaffolding protein family ERM, comprising ezrin, radixin, and moesin, are expressed in β-cells and target both F-actin and PIP2. Furthermore, ERM proteins are activated via phosphorylation in a glucose- and calcium-dependent manner. This activation leads to a translocation of the ERM proteins to sites on the cell periphery enriched in insulin granules, the exocyst complex docking protein Exo70, and lipid rafts. ERM scaffolding proteins also participate in insulin granule trafficking and docking to the plasma membrane. Overexpression of a truncated dominant-negative ezrin construct that lacks the ERM F-actin binding domain leads to a reduction in insulin granules near the plasma membrane and impaired secretion. Conversely, overexpression of a constitutively active ezrin results in more granules near the cell periphery and an enhancement of insulin secretion. Diabetic mouse islets contain less active ERM, suggestive of a novel mechanism whereby impairment of insulin granule trafficking to the membrane through a complex containing F-actin, PIP2, Exo70, and ERM proteins contributes to defective insulin secretion.

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THE PATHOGENESIS OF TYPE 2 DIABETES MELLITUS is characterized by both insulin resistance and insulin deficiency. Our understanding of the molecular causes involved in defective insulin secretion from the β-cell is limited. However, it is clear that impaired insulin secretion may be due, at least in part, to a reduced number of docked granules at the plasma membrane (31, 41). In the β-cell, emerging evidence implicates the ERM proteins as a critical factor in regulated insulin secretion (37). Additionally, in β-cells, Sec6, Sec8, and Sec10 are reported to be essential in insulin granule docking and secretion (48). Although Exo70 has not been studied in insulin-secreting cells, it is required for proper GLUT4 vesicle docking to the plasma membrane and is compartmentalized to lipid rafts (18). Lipid rafts associate with SNARE proteins in PC12 cells (38), although in insulin secretion the role of lipid rafts in insulin exocytosis is controversial (30, 40, 54). However, SNARE proteins do appear to target lipid rafts in β-cells (40, 54). Therefore, PIP2, F-actin, lipid rafts, Exo70, and ERM proteins may concentrate on the plasma membrane to regulate insulin secretion.

In this study, we examined the role of ERM proteins in regulating insulin granule trafficking, docking, and exocytosis. We show that insulin granules dynamically associate with PIP2, F-actin, and ERM proteins by live-cell time lapse confocal imaging. ERM proteins are expressed in MIN6 cells and the plasma membrane in a variety of cell types (1). However, there are no studies examining the role of ERM proteins in insulin secretion.

Ezrin was the first member of the ERM family identified (6). This protein, also known as p81, cytovillin, villin 2, and AKAP78, contains an F-actin binding domain and a PIP2 binding domain (29, 49). Ezrin, radixin, and moesin are highly homologous, and only recently have unique roles of individual ERM proteins been identified (9). The F-actin binding domain and PIP2-associated domains of ERM proteins interact, resulting in inactive ERM monomers and dimers (8). This autoinhibition of ERM proteins is relieved through sequential binding of PIP2 and subsequent phosphorylation on the conserved actin binding domain of ERM proteins (10). Through these interactions, active ERM proteins regulate a whole suite of cellular processes, including morphology, signaling, and trafficking (7).

In pancreatic β-cells, the formation and regulation of PIP2, the membrane target of ERM proteins, is highly dynamic and dependent on intracellular calcium and ATP (44). Glucose stimulation of β-cells results in PIP2 cleavage by phospholipase C, forming the products inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (21), and this process of IP3 formation can oscillate simultaneously with intracellular calcium oscillations (43). PIP2 has been determined biochemically to directly associate with insulin granules via direct electrostatic associations between vesicle-associated membrane protein-2/synaptobrevin 2 (VAMP2) and PIP2 (53), although the dynamics of this interaction have not been directly assessed. F-actin and PIP2 have both been demonstrated to associate with Exo70 of the Sec6/8 exocyst complex, and Exo70 marks the sites on the membrane for vesicle docking (5, 14, 55). In β-cells, Exoc3L, a novel component of the exocyst complex, has been identified as a critical factor in regulated insulin secretion (37). Additionally, in β-cells, Sec6, Sec8, and Sec10 are reported to be essential in insulin granule docking and secretion (48). Although Exo70 has not been studied in insulin-secreting cells, it is required for proper GLUT4 vesicle docking to the plasma membrane and is compartmentalized to lipid rafts (18). Lipid rafts associate with SNARE proteins in PC12 cells (38), although in insulin secretion the role of lipid rafts in insulin exocytosis is controversial (30, 40, 54). However, SNARE proteins do appear to target lipid rafts in β-cells (40, 54). Therefore, PIP2, F-actin, lipid rafts, Exo70, and ERM proteins may concentrate on the plasma membrane to regulate insulin secretion.
pancreatic islets and are activated via phosphorylation in a glucose- and calcium-dependent manner. Following this activation, ERM proteins translocate to the cell periphery and link PIP2 to F-actin. Furthermore, ERM proteins target sites on the plasma membrane enriched in lipid rafts and Exo70 of the exocyst complex. Overexpression of a truncated dominant-negative ezrin construct impairs insulin granule trafficking and docking to the membrane and reduces glucose-stimulated secretion. Conversely, overexpression of constitutively active ezrin promotes insulin granule docking and enhances glucose- and high potassium-stimulated insulin secretion. For the first time, we have identified an assembly of molecules, including PIP2, F-actin, lipid rafts, Exo70, and ERM proteins, coordinating insulin granule trafficking to sites marked for exocytosis. ERM protein activity is also downregulated in islets from diabetic ob/ob mice, suggesting a novel mechanism of reduced ERM protein activity leading to impaired insulin secretion.

MATERIALS AND METHODS

Reagents and antibodies. Latrunculin A and BODIPY-GM1 conjugated to BSA were purchased from Invitrogen (Carlsbad, CA) and both were used at 1 μM. Nifedipine was purchased from Sigma-Aldrich (St. Louis, MO) and used at 1 μM. To examine ERM proteins in islets and β-cells, we employed antibodies against ezrin (mouse monoclonal clone 3C12; Invitrogen), radixin (rabbit polyclonal; Sigma-Aldrich), moesin (mouse monoclonal clone 38/87; Sigma-Aldrich), vesicular stomatitis virus G protein (VSV-G, rabbit polyclonal; Sigma-Aldrich), and ezrin/radixin/moesin phosphorylated at Thr⁵⁶⁷, Thr⁵⁶⁴, Thr⁵⁵⁸, respectively (rabbit polyclonal; Cell Signaling, Beverly, MA). Mouse monoclonal anti-GAPDH was purchased from Fitzgerald Industries (Acton, MA) and used as a loading control.

Immunofluorescence. For immunofluorescence of phosphorylated ERM, MIN6 cells were seeded onto 35-mm glass bottom dishes (MatTek, Ashland, MA). Cells were fixed in cold 10% trichloroacetic acid (13) and permeabilized with 0.2% Triton X-100, and immunofluorescence was performed using anti-phospho-ERM (1:250; Cell Signaling) and FITC-conjugated donkey anti-rabbit Fab2’ IgG secondary antibodies (Jackson Immunoresearch, West Grove, PA). To label F-actin, cells were fixed and permeabilized with 2% paraformaldehyde and 0.2% Triton X-100 for 30 min at 4°C, blocked with 2% normal donkey serum for 20 min at room temperature, and stained with 1:50 Alexa 488-conjugated or Texas Red X-conjugated phallopain for 30 min at 4°C (Invitrogen), followed by subsequent washing.

Confocal microscopy. Two confocal imaging systems were employed in this study. One confocal imaging system is a custom-built instrument based on a Yokogawa CSU10 spinning disk confocal unit and an inverted Olympus IX70 microscope. The system is equipped with an Ar/Kr laser (Series 43; Omnichrome, Chino, CA) with excitation lines appropriate for Cherry (568 nm) and GFP-FITC (488 nm). The emitted light was filtered and recorded with a CoolSNAP HQ digital camera (Roper Scientific, Tucson, AZ). Acquisition was controlled with MetaMorph software (Molecular Devices, Sunnyvale, CA). Cells were maintained at 37°C with a TC-202 temperature controller (Harvard Apparatus, Holliston, MA).

The second confocal imaging instrument was a Leica SP5 tandem scanner spectral 2-photon confocal system (Leica Microsystems, Wetzlar, Germany) with a custom heating stage. The Leica SP5 is an instrument maintained by and located in the University of Chicago’s BSD Light Microscopy Core Facility. Confocal images were analyzed following deconvolution using the classic maximum likelihood estimation algorithm in Huygens Professional (Scientific Volume Imaging, Hilversum, Netherlands) and subsequently processed using an unsharp mask filter and/or brightness/contrast adjustments in ImageJ (NIH).

The high spatial and temporal resolution of the Leica SP5 system was critical in obtaining the images and supplemental movies in this paper. We employed a ×100 NA 1.46 oil objective with superb optical characteristics, which, following deconvolution, yields a full width at half-maximal resolution of ~170 nm for GFP fluorescence. Raw Z axis resolution is typically two times worse than lateral resolution, but the deconvolution process results in nearly isotropic detail. The red insulin granule point spread function is obviously larger than that for GFP, ~200 nm, close to the accepted physical size of an insulin granule of ~250 nm. Colocalization statistics are of course related to this resolvable limit. Actin fibers are not single filaments (~10 nm each) and are thus easily detected, but singular F-actin structures might not be resolvable. Those that are imaged would appear to be the size of the PSF of ~170 nm despite being merely 10 nm. Given these limits for particle resolution, if no GFP signal is measured in proximity to a red structure, the structures are clearly outside the range of physical interaction between these proteins. For colocalization analysis, we generated a green signal within the larger PSF of the red insulin granules. Colocalization of signals means that there is a possible interaction, since the signals co-occur within a 200-nm range. The image size was 512 × 512, resulting in a frame rate of approximately one frame per second. Other features of our confocal approach included employing a high degree of signal averaging at 128 line average, the high speed 8-kHz Leica scanner with low photobleaching during recording, and 80-nm Zstep oversampling. Together, these features of the instrument yield better than average confocal resolution and allow a cautious approach to interpretation of particle interactions.

Insulin secretion assays. MIN6 cells stably transfected with human ezrin mutants, as indicated, were seeded at 40–60% confluence in six-well tissue culture-treated dishes. Five hours prior to the insulin secretion assay, cells were switched to Krebs-Ringer buffer with HEPES (KRBH) containing (in mM) NaCl 119, KCl 4.7, HEPES 25 (pH 7.35), MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, and d-glucose 2 supplemented with 0.5 mM/ml BSA. Cells were washed twice in this solution and subsequently assayed. Cells were maintained in KRBH with 2 mM glucose-BSA for 30 min and subsequently stimulated with KRBH with 20 mM glucose-BSA for 90 min, or 30 mM KCl-BSA for 15 min, or maintained in KRBH with 2 mM glucose-BSA at 37°C. Following treatments, the supernatant was sampled, and the cells were harvested with 1% Triton X-100. This assay was performed on each cell line in triplicates in three to six independent experiments. The samples were analyzed by an ultrasensitive mouse insulin ELISA (Merckodia, Uppsala, Sweden) following sample dilution to remain within the dynamic range of the assay. Secreted insulin was normalized to insulin content or as a fold increase in secretion or as a fold increase in secretion.

Constitutive and transient transfection. Human ezrin T567D/ VSV-G was a gift of Dr. M. Arpin (Louis Pasteur Institute) and is described in Ref. 34. Exo70-GFP was a gift of Dr. W. Guo (University of Pennsylvania) and was examined previously (55). Lifeact-GFP was generated as reported (36), using mouse-specific codons to enhance expression in MIN6 cells. GFP fused to the pleckstrin homology domain of phospholipase C₆ (GFP-PHD), human ezrin-1(309)- VSV-G, human insulin C-peptide-GFP, and human insulin C-peptide-Cherry were used as previously described (35, 39, 43). To generate mouse ERM-Cherry constructs, total RNA was isolated from C57BL6/J mouse islets, and 5 μg of this islet RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) and random hexamer primers. PCR was performed on 1 μg of this cDNA using primers specific for mouse ezrin (NCB accession no. NM_009510) and mouse radixin (NCB accession no. NM_009041). PCR was performed on a mouse moesin cDNA clone purchased from Open Biosystems (Huntsville, AL) (IMAGE ID 3711212). These PCR products were then subcloned into pcDNA3.1(+) Zeo with Cherry in

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frame at the carboxyl terminus of the cDNA using the following restriction enzymes: ezrin, EcoRI and HindIII; radixin, BamHI and XhoI; moesin, EcoRI and XhoI. Site-directed mutagenesis was performed on ezrin-Cherry, radixin-Cherry, and moesin-Cherry, yielding ezrin T567D-Cherry, radixin T564D-Cherry, and moesin T585D-Cherry by use of the Site-Directed Mutagenesis Kit II (Stratagene) per the manufacturer’s recommendations. Mouse ezrin-(1-309)-Cherry was subcloned from full-length mouse ezrin-Cherry to truncate the ezrin cDNA at amino acid position 309 and subsequently inserted into the pcDNA3.1(Zeo-Cherry) vector using ezrin cDNA at amino acid position 309 and subsequently inserted into the pcDNA3.1(Zeo-Cherry) vector using HindIII and EcoRI sites. Constructs were transfected into MIN6 using Lipofectamine 2000 (Invitrogen) per the manufacturer’s protocol. Sequences were confirmed through the Cancer Research Center DNA Sequencing and Genotyping Core Facility at The University of Chicago.

*Mouse islet isolation, cell lines, and tissue culture.* All animals were used in accordance with The University of Chicago IACUC and ACUP protocol no. 71492. Islets were isolated from pancreata of 2- to 3-mo-old C57BL/6j wild-type mice (Jackson Laboratory, Bar Harbor, ME) using collagenase P (Roche Diagnostics, Basel, Switzerland) digestion and a Ficoll gradient, as previously described (33). Islets were allowed to recover in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin and maintained in a humidified incubator at 37°C under an atmosphere of 95% air-5% CO2 and subsequently used for experiments. Similarly, islets from 10-wk-old Lepob/Lepob (ob/ob) males in the C57BL/6j background, and age-matched, sex-matched C57BL/6j islets were isolated and immediately analyzed by Western immunoblotting in three separate, independent experiments. MIN6 cells were cultured in DMEM supplemented with 15% FBS, 10 IU/ml penicillin and 10 μg/ml streptomycin and maintained in a humidified incubator at 37°C under an atmosphere of 95% air-5% CO2. All MIN6 cells were used between passages 20 and 35. Following transfection (24–48 h), cells were either used for experiments or selected with an appropriate antibiotic. Zeocin and neomycin were purchased from Invitrogen and used at concentrations of 250 and 500 μg/ml, respectively, in generation of stably transfected MIN6 cells. Stably transfected cell lines were of polyclonal origin.

Quantitative real-time polymerase chain reaction. Total RNA was extracted from MIN6 cells and freshly isolated C57BL/6j islets by use of the TRI Reagent method according to the manufacturer’s guidelines (Sigma-Aldrich). Total RNA (5 μg) was reverse transcribed using SuperScript III (Invitrogen) and random hexamers per manufacturer’s protocol. Following cDNA synthesis, 50 ng of islet and MIN6 cell DNA was analyzed for relative ERM expression in islets and MIN6 cells, respectively, in generation of stably transfected MIN6 cells. Stably transfected cell lines were of polyclonal origin.

**RESULTS**

Insulin granules have a time-dependent association with F-actin. We first examined the relationship between F-actin distribution and insulin granules in live β-cells. To achieve this, we first generated Lifeact-GFP using mouse-specific codons. Lifeact-GFP has previously been used to specifically label F-actin in live cells and has not been shown to negatively affect F-actin dynamics, as is the case with actin-GFP (36). This is the first use of Lifeact-GFP in β-cells and the first visualization of F-actin and insulin granule dynamics in live β-cells. We transiently cotransfected MIN6 cells with Lifeact-GFP and human insulin C-peptide-Cherry. Using high-resolution confocal microscopy, we imaged insulin granules and Lifeact-GFP at the F-actin layer near the cell-glass interface in live MIN6 cells (n = 6; Fig. 1A). We observed insulin granules residing along and immediately adjacent to F-actin in low glucose (2 mM). We examined these interactions between insulin granu-
ules and F-actin in low glucose because high glucose results in highly dynamic changes in F-actin. Minimal colocalization between Lifeact-GFP and insulin C-peptide-Cherry was seen. This colocalization would be indicated by the presence of yellow granules, which were rarely observed. To assess the dynamic relationship between Lifeact-GFP and insulin C-peptide-Cherry, we imaged MIN6 cells cotransfected with these two constructs by time lapse live-cell confocal microscopy (Supplemental Movie 1; supplementary materials are found with the online version of this paper on the Journal website). By this method, we again noticed insulin granules traveling along and residing immediately adjacent to F-actin. This is in agreement with a previous study, which concluded that insulin granules travel along cortical actin via myosin Va (50). Very little direct colocalization between F-actin and insulin granules was seen in our studies; however, insulin granules were occasionally observed crossing over F-actin bundles by this method (n = 7).
To quantify this association between F-actin and insulin granules, we analyzed the time lapse movies of live MIN6 cells cotransfected with Lifeact-GFP and human insulin C-peptide-Cherry in low glucose (2 mM) with Imaris tracking software analysis as described in MATERIALS AND METHODS. Insulin granules were first fitted to spots in this analysis, and the subsequent displacements were tracked in relation to Lifeact-GFP dynamics (Fig. 1B). The fluorescence of Lifeact-GFP beneath the displacements of insulin C-peptide-Cherry was quantified using Imaris software (Fig. 1C). Shown are four representative graphs demonstrating changes in the Lifeact-GFP fluorescence signal beneath the granule tracks in this representative recording. An increase in fluorescence is interpreted as an increase in the association of F-actin with insulin granules, and a loss in fluorescence indicates a loss of this association. We observed and quantified granules in four categories: not associated, increasing association, decreasing association, and remaining associated with F-actin for the duration of the recordings. However, it cannot be excluded that some of these insulin granule dynamics represent random motions independent of F-actin. This is the first report of such quantified dynamic insulin granule associations with F-actin over time.

Next, we assessed the distribution of insulin granules in relation to F-actin in live cells in three dimensions (3-D). MIN6 cells cotransfected with Lifeact-GFP and human insulin C-peptide-Cherry were imaged by serial confocal optical sectioning through the z-axis. These stacks were subsequently deconvolved, processed, and displayed as maximum projections and orthogonal sections (Fig. 1D). At the bottom of the cell, we observed insulin granules residing in, above, and below the cortical F-actin layer (n = 3). The 3-D imaging observations, demonstrating an interaction of insulin granules with F-actin, support our 2-D time lapse data showing insulin granules having an active and time-dependent association with F-actin.

Insulin granules associate with PIP2, and PIP2 distribution is F-actin regulated. Similarly to the examination of dynamic insulin granule associations with F-actin, we next investigated the time-dependent association of insulin granules with PIP2. To achieve this, we cotransfected GFP-PHD (GFP fused to the pleckstrin homology domain of phospholipase Cδ3) as a probe for PIP2 with human insulin C-peptide-Cherry into MIN6 cells. These cells were subsequently observed via 2-D time lapse confocal microscopy (n = 65). We have found that insulin granules traffic along and adjacent to PIP2-enriched structures on the bottom of the cell in low (2 mM) glucose (Fig. 2A). Similar dynamics were also observed in high glucose (20 mM), where increased intracellular calcium activation of PLC would occur (data not shown). Limited colocalization between insulin granules and PIP2 was observed, which would be indicated by the presence of yellow granules. However, a subset of granules displayed a high affinity for PIP2 (Supplemental Movie 2). This supports a previous study indicating a strong electrostatic interaction between PIP2 and VAMP2 on the insulin granule (53). To quantify this dynamic association of insulin granules with PIP2, we analyzed these time lapse confocal movies using Imaris tracking software. Insulin granules were fitted to spots, and the dynamic association of these spots relative to GFP-PHD was quantified (Fig. 2, B and C). We found that a subset of insulin granules does not associate with PIP2. Another subpopulation of insulin granules becomes dynamically associated with PIP2 over time, while others dissociate from PIP2. An additional population of granules displays complex time-dependent associations, dissociations, and reassociations with PIP2. For the first time, we directly observed that insulin granules display complex dynamics with PIP2-enriched regions of the plasma membrane over time in live cells.

Insulin granules dynamically interact with both F-actin and PIP2. Previous studies have reported that a decrease in the concentration of PIP2 available in the cell leads to F-actin depolymerization (4, 12). Conversely, alterations in F-actin affect PIP2 levels in cells. Depolymerization of F-actin by latrunculin treatment leads to increases in PIP2 (4). Additionally, treatment with latrunculin A enhances insulin secretion (45). Because of this demonstrated cross-talk between PIP2 and F-actin, we examined how treatment of β-cells with latrunculin A, an agent that binds G-actin, leading to F-actin depolymerization, affects PIP2 distribution (Fig. 2D). MIN6 cells treated with 1 μM latrunculin A for 15 min results in a dramatic redistribution of PIP2. PIP2, originally in organized puncta and structures, becomes disorganized and more diffuse following treatment with latrunculin A (Fig. 2D; n = 6).

ERM proteins are expressed in β-cells and target both F-actin and PIP2. ERM proteins are expressed in a wide variety of cell types, though which ERM is predominantly expressed is tissue dependent. We detected all three ERM proteins in isolated mouse pancreatic islets and in MIN6 cells (Fig. 3A). At the mRNA level, radixin is the ERM protein most highly expressed in pancreatic islets and MIN6 cells, as determined by quantitative RT-PCR (n = 3; Fig. 3B). Moesin, which accounts for ~20% of the ERM message in pancreatic islets, makes up less than 2% of the total ERM message in MIN6 cells. These data are also highly similar to the public database expression data for ERM proteins in mouse islets and mouse β-cell lines, with radixin being the ERM most abundantly expressed in mouse islets and β-cell lines and moesin found to be less abundant in β-cell lines than in mouse islets (17). Next, we assessed whether ERM proteins target both F-actin and PIP2. Toward this end, we generated mouse ERM constructs fused to the fluorescent protein Cherry at the ERM COOH terminus. ERM proteins fluorescently tagged on their carboxyl terminus retain cellular localization closely resembling endogenous ERM targeting (2). Radixin-Cherry was transected into MIN6 cells, followed by fixation and labeling of F-actin with Alexa 488-conjugated phalloidin. We observed a high degree of colocalization between radixin-Cherry and phalloidin-labeled F-actin (Fig. 3C). Similar colocalization was observed with ezrin-Cherry and moesin-Cherry (data not shown). This colocalization of radixin-Cherry and the actin cytoskeleton was apparent at both the midplane of the cells and at the interface between the MIN6 cells and the glass substrate (bottom plane) (Fig. 3C). Two different planes were imaged through the cells, as these two planes contain different amounts of observable F-actin. This is in agreement with studies describing ERM protein targeting to F-actin in other cell types (1, 23, 49). To assess ERM targeting to PIP2 in live β-cells, MIN6 cells were cotransfected with a construct expressing only the amino terminus of mouse ezrin (1-309), the proposed PIP2 binding domain, fused with Cherry and GFP-PHD, to label PIP2. Using confocal microscopy, we observed a high degree of colocalization between ezrin-(1-309)-Cherry and GFP-PHD (Fig. 3D). These colocalization data indicate that the amino
terminus of ezrin does target PIP2 in β-cells, supporting previous studies indicating that the amino terminus of ERM proteins targets PIP2 on the plasma membrane (3, 29) and acts in a dominant-negative manner (DN ezrin) to suppress endogenous ERM activity (39).

ERM proteins are activated via phosphorylation in β-cells in a glucose- and calcium-dependent manner leading to ERM protein translocation. We found that ERM proteins are phosphorylated in response to stimulatory glucose in pancreatic islets (Fig. 4A) and MIN6 cells (Fig. 4C) in the actin binding domain of ERM proteins following 10 min of glucose stimulation. Representative blots from three independent experiments are shown and quantified (Fig. 4, B and D). In Western blots from islets, all three ERM proteins have detectable phosphorylated bands, whereas in MIN6 cells phosphorylated ezrin and radixin are the predominant phosphorylated ERMs. In both islets and MIN6 cells, glucose leads to an increase in the abundance of phosphorylated ERM. However, the increase in phosphorylated ERM in response to glucose was greater in islets than in MIN6 cells, likely representative of the differences in physiology between primary islets compared and a cultured insulinoma cell line. ERM phosphorylation is a [Ca^{2+}]-dependent process in islets and MIN6 cells. Glucose stimulation of islets (14 mM) and MIN6 cells (20 mM) results in a marked rise in [Ca^{2+}], whereas the addition of nifedipine (1 μM) to block L-type voltage-dependent calcium channels reduces glucose-stimulated increases in [Ca^{2+}] in islets and MIN6 cells (Fig. 4, E and F). Blockade of L-type calcium channels results in a reduction in phosphorylated ERM in mouse islets and in MIN6 cells in response to a glucose challenge (10 min) (Fig. 4, A–D). Concomitant with the general increase in phosphorylated ERM in response to glucose stimulation, an increase in the abundance of phosphorylated ERM at the periphery of MIN6 cells was found by immunofluorescence. This increase in abundance of phosphorylated ERM at the periphery is blocked by the addition of nifedipine (Fig. 4G). Phosphorylated ERM was also imaged in low glucose by optical z-sectioning confocal imaging and presented as a tilting volume reconstruction movie (Supplemental Movie 3). Through this method, we observed that the appearance of active ERM is punctate along the membrane and at the interface between cells. These results indicate that glucose stimu-
The translation of islets and MIN6 cells results in an increase in \([\text{Ca}^{2+}]_i\), which in turn leads to an increase in the abundance of active ERM at the membrane. Glucose leads to translocation of ezrin and radixin to the cell periphery which is dependent upon COOH-terminal phosphorylation.

To determine the kinetics of translocation of radixin-Cherry, we transfected radixin-Cherry into MIN6 cells and stimulated those cells with 20 mM glucose. In response to high glucose, radixin-Cherry translocates to the cell periphery (Fig. 5A) in a similar manner as ezrin-Cherry (data not shown). This translocation of radixin peaked between 4 and 10 min following glucose stimulation and likely represents translocation of phosphorylated radixin. Next, we transiently transfected MIN6 cells with ezrin-Cherry and subsequently treated with 20 mM glucose for 10 or 60 min or maintained in low glucose (2 mM). Following glucose treatment, ezrin-Cherry-transfected MIN6 cells were fixed and stained with Alexa 488-conjugated phalloidin to label F-actin. Ezrin-Cherry displayed a predominantly cytoplasmic distribution in low glucose (2 mM) but, following 10 min of high glucose stimulation, a significant fraction of ezrin-Cherry translocated to the cell periphery (Fig. 5B).

This plasma membrane-associated ezrin-Cherry displayed a high degree of colocalization with F-actin. However, by 60 min in high glucose, ezrin-Cherry no longer displayed extensive colocalization with F-actin and predominantly localized to the cytoplasm. To confirm that this translocation of ezrin and radixin was a result of COOH-terminal phosphorylation, we transfected MIN6 cells with ezrin T567D-Cherry, a constitutively active mutant of ezrin (CA ezrin). Mouse ezrin T567D-Cherry failed to translocate in MIN6 in response to glucose stimulation and remained associated with the actin cytoskeleton (Fig. 5C). These data suggest that glucose stimulation leads to phosphorylation on the COOH terminus of ERM proteins, resulting in translocation to the periphery where ERM proteins interact with F-actin.

Fig. 3. ERM (ezrin, radixin, and moesin) proteins are expressed in pancreatic islets and MIN6 cells and target F-actin and PIP2. A: ERM proteins were detected in mouse islets and MIN6 cells by SDS-PAGE immunoblotting with antibodies against ezrin, radixin, and moesin, resulting in bands corresponding to the expected size of ~80 kDa. B: ERM mRNA expression relative to ezrin in pancreatic islets and MIN6 cells as determined by qRT-PCR (n = 3). C: expression of radixin-Cherry in MIN6 cells with Alexa 488-conjugated phalloidin labeling of F-actin. Confocal images are shown through the mid-plane and bottom of the cell at the cell-glass interface. D: colocalization of mouse amino-terminal PIP2 binding domain of ezrin [ezrin-(1-309)] fused to Cherry and GFP-PH-D in MIN6 cells. Representative images shown. Scale bar, 5 μm.

Fig. 4. ERM proteins are phosphorylated in islets and MIN6 cells in response to glucose stimulation in a calcium-dependent manner. Islets (A) and MIN6 cells (C) contain significantly more phosphorylated ezrin (top), radixin (middle), and moesin (bottom) (Thr567, Thr564, Thr558 respectively) relative to total ezrin following 10 min of high glucose stimulation [islets 14 mM glucose, MIN6 cells 20 mM glucose (n = 3)]. Representative blots shown. Bands correspond to expected molecular mass of these proteins at ~80 kDa. Inhibition of L-type calcium channels with 1 μM nifedipine reduces phosphorylated ERM (n = 3) in the presence of high glucose in islets and MIN6 cells. Quantitation of blots is presented in B for islets and D for MIN6 cells. For B and D, *P < 0.05 comparing bar 1 with bar 2; #P < 0.05 comparing bar 2 with bar 3. Representative calcium traces in pancreatic islets (E) and MIN6 cells (F) stimulated with high glucose for 10 min (red squares), with high glucose and 1 μM nifedipine (blue circles), or maintained in low glucose (2 mM; green triangles). G: MIN6 cells were treated with high glucose (20 mM), high glucose with nifedipine (1 μM), or maintained in low glucose (2 mM); subsequently immunofluorescence was performed for detection of phosphorylated ERM. Representative images are shown. Scale bar, 10 μm.
Insulin granules, similar to the F-actin and PIP2 distribution (A), but resided immediately adjacent to C-peptide-GFP (Fig. 6C) tagged with Cherry also did not colocalize with insulin granules (Fig. 6D). Mouse ezrin-(1-309)-Cherry (mouse ezrin T567D-Cherry and radixin T564D-Cherry shown) resided immediately adjacent to, but did not colocalize with human ezrin-Cherry and visualized their distribution in live cells by confocal microscopy. We observed that Exo70-GFP shares similar and overlapping localizations with ezrin-Cherry in live insulin-secreting cells (Fig. 6D). Within the limits of our confocal methods (see MATERIALS AND METHODS for details), this suggests that these two proteins are in relatively close proximity in the plasma membrane.

**Insulin granules reside immediately adjacent to Exo70-targeted sites, sites enriched in F-actin, PIP2, and ERM proteins.** As a result of our finding that Exo70 targets similar sites in the cell as ERM proteins, we examined whether Exo70 targets PIP2 on the membrane, similar to ERM proteins. Therefore, we cotransfected ezrin-(1-309)-Cherry with Exo70-GFP. The PIP2 binding domain of ezrin also targeted sites in the cell as ERM proteins, F-actin, and PIP2. Because Exo70 shared similar targeting as ERM proteins, we investigated whether Exo70 would have a similar association with a subset of granules. We coexpressed Exo70 and insulin C-peptide-Cherry and found that a subset of insulin granules did associate with Exo70 (Fig. 7C), and these associations may be indicative of granules docking with the membrane.

**Overexpression of ezrin mutants results in altered distribution of insulin granules but not F-actin or PIP2.** The exocyst complex has previously been implicated in the regulation of insulin granule docking to the plasma membrane (48). Since Exo70 of the exocyst complex shares similar cellular distribution as ERM proteins, we tested whether overexpression of ezrin mutants alters the docking of insulin granules to the plasma membrane. To this aim, we cotransfected insulin C-peptide-GFP with either DN ezrin or CA ezrin in MIN6 cells.
Subsequently, cells expressing both insulin C-peptide-GFP and ezrin-(1-309)-Cherry or ezrin T567D-Cherry were visualized by optical z-sections by using 3-D confocal imaging on live MIN6 cells. Image stacks were subsequently deconvolved, and insulin granules and ezrin mutants were 3-D rendered in Imaris as spots and surfaces, respectively (see MATERIALS AND METHODS) (Fig. 8, A–D). Next, rendered granules and surfaces were mapped relative to each other. Expression of ezrin mutants resulted in an altered distribution of insulin granules (Fig. 8E).

The percentage of granules mapped to less than 0.5 μm distance away from the surface marked by the ezrin mutant was nearly threefold higher in CA ezrin-expressing cells than in cells expressing DN ezrin (Fig. 8F). Similarly, the mean distance of insulin granules from the surface was threefold higher in cells expressing DN ezrin than in CA ezrin cells (Fig. 8G; n = 4 two-tailed t-test, P < 0.05). Similar qualitative observations were seen in MIN6 cells stably expressing ezrin-(1-309)-VSV-G and ezrin-(1-309)-VSV-G (data not shown). Because overexpression of ezrin mutants resulted in altered insulin granule distribution, we examined whether this altered granule distribution was a result of disrupted F-actin or PIP2 distributions. To this end, ezrin T567D-Cherry or ezrin-(1-309)-Cherry was cotransfected with Lifeact-GFP or GFP-PH. We found that overexpression of mutant ezrin constructs did not significantly alter F-actin distribution (Fig. 8, H and I) or PIP2 distribution (Fig. 8, J and K).

Ezrin activity regulates glucose- and potassium-stimulated insulin secretion, and activities of ezrin and radixin are downregulated in diabetic islets. To determine whether modulation of ERM proteins regulates β-cell secretory function, we assessed insulin secretion in MIN6 cells stably expressing human ezrin-(1-309)-VSV-G (DN ezrin) and ezrin-T567D-VSV-G (CA ezrin). Ezrin-T567D acts as a constitutively active ezrin mutant, because this mutant mimics ezrin in its phosphorylated and active conformation. Ezrin-(1-309), on the other hand, acts as a dominant-negative, because it maintains plasma membrane-associated targeting while not interacting with F-actin. In MIN6 cells stably expressing DN ezrin, glucose stimulated insulin secretion following high glucose stimulation (90 min) was reduced by greater than 50% compared with control cells (Fig. 9A; one-way ANOVA and post hoc Tukey’s test, P < 0.05). While DN ezrin diminished glucose-stimulated insulin secretion, CA ezrin expression modestly augmented secretion relative to control cells, albeit not to significance. In the CA ezrin cell line, following glucose stimu-
lation, insulin secretion was over twofold greater than in DN ezrin-expressing cells (Fig. 9B; one-way ANOVA and post hoc Tukey’s test, \( P < 0.05 \)). So, although neither CA ezrin nor DN ezrin was significantly different from control cells, there was a significant difference in insulin secretion between CA ezrin- and DN ezrin-expressing cells. To determine whether the effect of ERM proteins on insulin secretion is dependent on glucose metabolism, we assessed insulin secretion in ezrin mutants with a depolarizing stimulation of isotonic high potassium (30 mM) for 15 min. Insulin secretion was increased nearly twofold in response to high K\(^+\) treatment in CA ezrin stable cells compared with DN ezrin (Fig. 9C; one-way ANOVA and post hoc Tukey’s test, \( P < 0.05 \)). Maintenance of the expression levels of ezrin mutants in MIN6 cells was verified by sequential Western immunoblotting for VSV-G, the tag found on mutant ERM proteins (data not shown).

Since the diabetic state is often associated with insufficient insulin secretion, we assessed whether the \( ob/ob \) diabetic mouse model also displays a downregulation of active ERM in islets. These \( ob/ob \) mice had body masses over twice those of control mice (\( ob/ob \) 52.2 ± 2.2 vs. control 25.0 ± 1.3 g), and ad libitum blood glucose of nearly three times that of control mice (\( ob/ob \) 355.2 ± 58.1 vs. control 129.4 ± 13.0 mg/dl). Freshly isolated islets from frankly diabetic \( ob/ob \) mice contained significantly less phosphorylated ezrin and radixin than control islets, as determined by Western immunoblotting and quantified by densitometry (Fig. 9, D and E). However, the abundance of phosphorylated moesin did not significantly
differ in the obese diabetic islets compared with control islets (Fig. 9, D and F).

**DISCUSSION**

Insulin granules efficiently traffic along microtubules to F-actin and then to plasma membrane docking sites and eventual secretion. These insulin dynamics are impaired in type 2 diabetes, resulting in a relative deficiency of insulin secretion. To further our understanding of these late steps in insulin secretion, we have examined a role for ERM protein scaffolds in the regulation of insulin secretion. In response to glucose, ERM proteins are activated and translocate to the membrane where they link PIP2 with cortical F-actin in \( \text{H9252} \)-cells. These sites targeted by ERM proteins appear to be specialized domains on the plasma membrane not only comprising F-actin and PIP2 but also enriched in lipid rafts and Exo70. We have found that diabetes downregulates the activity of ERM proteins and that altered activity of ERM proteins directly causes aberrant insulin trafficking and secretion. Therefore, our results suggest that we have discovered a domain on the plasma membrane that is coordinated by the activity of ERM proteins and is critical for proper insulin secretion.

Reorganization of F-actin has long been known to be a central factor in glucose-stimulated insulin secretion (15–16, 42). These dynamics of F-actin are a complex result of glucose stimulation through the activation of gelsolin (46), Rac1 (52), Cdc42 (26–28, 51–52), and many other GTPases, actin-binding proteins, and scaffolding proteins. We have found that Exo70 of the exocyst complex targets F-actin in \( \text{H9252} \)-cells, further highlighting the central role of F-actin in insulin secretion. F-actin is considered to have an inhibitory role for regulated insulin secretion (28). However, depolymerization of F-actin with latrunculin A has the confounding effect of increasing cellular PIP2 levels (4). Additionally, we have shown that addition of latrunculin A leads to a redistribution of PIP2 to cover a larger area of the overall cell surface. This, in addition to an increase in PIP2 levels, may have the net effect of increasing the number of docking sites for insulin secretion. This is further supported by our direct observations of insulin granules associating with regions of the inner domain of the
ERM proteins regulate insulin secretion

Fig. 9. Insulin secretion is modulated by ERM activity. MIN6 cells stably transfected with human ezrin-(1-309)-VSV-G and human ezrin-T567D-VSV-G were assessed for insulin-secretory function by mouse ultrasensitive ELISA. MIN6 cells stably expressing truncated ezrin-(1-309)-VSV-G and ezrin-T567D-VSV-G were stimulated for 90 min with high glucose (20 mM) or maintained in low glucose (2 mM) and compared with vector control stable transfectants. MIN6 cells stably transfected with human ezrin-(1-309)-VSV-G and human ezrin-T567D-VSV-G were assessed for insulin-secretory function by mouse ultrasensitive ELISA. MIN6 cells stably expressing truncated ezrin-(1-309)-VSV-G and ezrin-T567D-VSV-G were assessed for insulin-secretory function by mouse ultrasensitive ELISA. MIN6 cells stably transfected with human ezrin-(1-309)-VSV-G and human ezrin-T567D-VSV-G were assessed for insulin-secretory function by mouse ultrasensitive ELISA.

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plasma membrane enriched in PIP2. Although these interactions were previously assessed biochemically, this is the first study to visualize the dynamic interactions between insulin granules and PIP2 directly with time lapse confocal micro-

copy in intact cells. This is also the only use and examination of Lifeact in β-cells to date. Fluorescently tagged Lifeact will now allow for more physiological studies of the role of F-actin in glucose-stimulated insulin secretion in β-cells. The use of Lifeact has a distinct advantage over all previous studies of F-actin in insulin secretion in that Lifeact allows the visualization of F-actin in live cells rather than in phalloidin-labeled fixed cells.

We have shown that Exo70 of the exocyst complex targets to plasma membrane domains enriched in F-actin, PIP2, and ERM proteins. This is the first study of Exo70 in β-cells. Exo70, in addition to Sec3, has been implicated in coordinating the membrane targeting for the remaining members of the exocyst complex that travel on the secretory granule (5). The exocyst complex comprises Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (55). Our findings that Exo70 associates with PIP2 and F-actin in β-cells support previous observations of Exo70 targeting in other cell types (14, 55). The exocyst complex has previously been shown to compartmentalize within lipid rafts and control GLUT4 vesicle tethering in adipocytes (18). In support of this concept in β-cells, we imaged homologous distributions of Exo70 and ERM proteins and found that ERM proteins target lipid rafts. Therefore, for the first time we have visualized targeting of the exocyst complex to highly specialized domains on the plasma membrane in β-cells.

In diabetic rat islets, overall PIP2 levels are reduced, and PIP2 dynamics in response to stimuli are diminished (25). These previously reported observations, in addition to our observed reduction in ERM protein activity in diabetic ob/ob islets, may assist us in furthering our understanding of the molecular causes of insulin insufficiency. Diabetes results in glycosylation of numerous proteins. Impairment of ERM protein activity by glycosylation has been described (11, 24). Therefore, impairment of ERM proteins may contribute directly to insulin secretion defects in diabetes.

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The mechanism by which ERM proteins exert control over insulin secretion is most likely at a critical docking step. An impairment of insulin granule docking has previously been shown in β-cells from diabetic Goto-Kakizaki rats (31). We have found that ERM protein activity directly modifies insulin granule docking. This concept is also supported by a previous study implicating the Sec6/8 complex in insulin granule docking (48). Further studies are needed to elucidate the kinase/phosphatase regulation of ERM protein activity in β-cells and the cause of ERM protein activity downregulation in diabetes. Additional investigations are also necessary to understand the molecular mechanism by which ERM proteins modulate insulin granule dynamics and the role of Exo70 in insulin granule docking. This work describes a novel mechanism whereby ERM protein activity regulates insulin granule trafficking and docking to specialized domains on the plasma membrane and advances our understanding of how altered β-cell function leads to insulin secretion defects in diabetes.
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DISCLOSURES

No conflicts of interest are reported by the authors.

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