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Aberrant Accumulation of the Diabetes Autoantigen GAD65 in Golgi Membranes in Conditions of ER Stress and Autoimmunity

ISLET STUDIES

Pancreatic islet β-cells are particularly susceptible to endoplasmic reticulum (ER) stress, which is implicated in β-cell dysfunction and loss during the pathogenesis of type 1 diabetes (T1D). The peripheral membrane protein GAD65 is an autoantigen in human T1D. GAD65 synthesizes γ-aminobutyric acid, an important autocrine and paracrine signaling molecule and a survival factor in islets. We show that ER stress in primary β-cells perturbs the palmitoylation cycle controlling GAD65 endomembrane distribution, resulting in aberrant accumulation of the palmitoylated form in *trans*-Golgi membranes. The palmitoylated form has heightened immunogenicity, exhibiting increased uptake by antigen-presenting cells and T-cell stimulation compared with the nonpalmitoylated form. Similar accumulation of GAD65 in Golgi membranes is observed in human β-cells in pancreatic sections from GAD65 autoantibody-positive individuals who have not yet progressed to clinical onset of T1D and from patients with T1D with residual β-cell mass and ongoing T-cell infiltration of islets. We propose that aberrant accumulation of immunogenic GAD65 in Golgi membranes facilitates inappropriate presentation to the immune system after release from stressed and/or damaged β-cells, triggering autoimmunity.

The γ-aminobutyric acid (GABA)–synthesizing enzyme GAD exists in two isoforms, GAD65 and GAD67, encoded by different genes (1). The smaller isoform, GAD65, is an early target of autoimmunity in 70–80% of patients who develop type 1 diabetes (T1D) (2–4). Rat and human β-cells primarily express GAD65, but no GAD65 is detected at the protein level in mouse β-cells (5,6). Our understanding of how GAD65 becomes an autoantigen in T1D is limited due to the lack of this protein in β-cells of mice, and therefore, an apparent lack of a significant role in the pathogenesis of diabetes in the highly studied NOD mouse model of T1D (7).

The GAD65 enzyme is synthesized in the cytosol as a hydrophilic cytosolic molecule that undergoes hydrophobic posttranslational modifications in the N-terminal domain to become membrane anchored (8–11). The first step of hydrophobic modifications is irreversible and results in a hydrophobic form that targets specifically to the cytosolic face of endoplasmic reticulum (ER) and *cis*-Golgi membranes, establishing an equilibrium between membrane and cytosolic pools (12). The second step of modifications, which include stabilization of membrane anchoring, followed by a reversible double palmitoylation of cysteines 30 and 45 (10) by a Golgi localized protein acyl transferase DHHC17 (also known as HIP14) (13), result in trapping of GAD65 in Golgi membranes, sorting to the *trans*-Golgi network (TGN) and targeting to an axonal vesicular pathway in route to synaptic vesicles in presynaptic clusters in

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neurons and to peripheral vesicles in β-cells (12,14,15). Palmitoylation is not required for anchoring of GAD65 to Golgi membranes but is critical for anterograde targeting of GAD65 from cis-Golgi to TGN membranes and post-Golgi peripheral vesicles (12). A depalmitoylation step by an acyl protein thioesterase can release GAD65 from peripheral vesicle membranes and/or TGN membranes, mediating retrograde trafficking back to Golgi membranes by a nonvesicular pathway. The protein can then enter a cycle of repalmitoylation and depalmitoylation (12). Palmitoylation is suggested to serve a critical function in regulating the rate of GABA synthesis in the presynaptic compartment of neurons (16,17).

Pancreatic β-cells have a well-developed, extensive, and highly active ER, reflecting their role in synthesizing and secreting large amounts of insulin. When the protein synthesis and secretion machinery becomes overloaded, due to a high physiological demand on a limited number of cells or from exogenous stressors, such as inflammation or a diet high in fatty acids, the accumulation of unfolded and improperly folded proteins transiting through the ER results in the cell experiencing a state of ER stress (18). β-Cells are highly sensitive to ER stress, which is implicated in the pathogenesis of T1D (19–22). It has been proposed that the initiation of autoimmunity against the β-cell follows an initial period of prolonged β-cell ER stress and apoptosis, induced by inflammatory cytokines secreted by early invading immune cells as well as by the β-cells themselves (19,23).

Here we report a dramatic effect of ER stress on the subcellular distribution of the T1D autoantigen GAD65 in primary rat and human β-cells and accumulation of a more highly immunogenic palmitoylated form in Golgi membranes. A similar accumulation is detected in pancreatic sections of human GAD65 autoantibody-positive individuals and patients with T1D.

RESEARCH DESIGN AND METHODS
Cell Cultures
INS-1E rat insulinoma cells (24) were cultured in RPMI 1640 with GlutaMAX, 10% FBS, 1% penicillin/streptomycin (P/S), 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, and 50 μmol/L β-mercaptoethanol. The DR4 (DRA1*0101, DRB1*0401)-positive human Epstein Barr virus–transformed B-cell line Priess (25) was cultured in Iscove's Modified Dulbecco's Medium GlutaMAX medium with 10% FBS, and 1% P/S. A DR4 (DRA1*0101, DRB1*0401) restricted mouse T-cell hybridoma cell line T33.1, recognizing the GAD65 aa 274-286 epitope (GAD65[274-286]) (26), was cultured in RPMI 1640 with GlutaMAX, 10% FBS, 1% P/S, and 0.1% β-mercaptoethanol. Primary rat hippocampal neurons were prepared from 2- to 3-day-old Sprague-Dawley rats, as previously described by Codazzi et al. (27).

Islet Culture
Rat islets isolated from P5 Sprague-Dawley rats as previously described (15) were cultured in RPMI 1640, 10% FBS,

and 1% P/S at 37°C in 5% CO_2. Human islets were cultured in Connaught Medical Research Laboratories 1066 with 2 mmol/L L-glutamine, 25 mmol/L HEPES, 10% FBS, and 1% P/S at 25°C in 5% CO_2.

Islet Single-Cell Cultures
Rat or human islets dissociated into single cells by digestion with trypsin-EDTA were cultured at 50,000 cells/well on laminin-coated Thermanox coverslips (Nunc) or 100,000 cells/well on Fluorodishes (World Precision Instruments) in minimum essential medium, 11 mmol/L glucose, 5% FBS, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 1× B-27 (Gibco), and 1% P/S at 37°C in 5% CO_2.

Human Pancreatic Sections
Human pancreatic sections were obtained from the JDRF Network for Pancreatic Organ Donors with Diabetes (nPOD) tissue bank (28,29). Sections were obtained from eight autoantibody-negative healthy donors with normal islets (Supplementary Table 1), eight individuals who were potentially prediabetic and positive for GAD65 autoantibodies (GADA[+]) (Supplementary Table 2), and eight autoantibody-positive patients with T1D, including six who were GADA[+] (Supplementary Table 3). Donors were selected to have remaining insulin-positive β-cells to enable analyses of the subcellular localization of GAD65. Furthermore, we sought to include individuals who were reported by nPOD as positive for CD3^{+} T-cell infiltration and/or insulitis (Supplementary Tables 2 and 3). Insulitis is defined by nPOD as the presence of six or more CD3^{+} T cells immediately adjacent to or within three or more islets of a defined minimum size in pancreatic sections (30).

Immunofluorescence Staining
Human pancreas sections were deparaffinized, followed by acidic-pH heat–mediated antigen retrieval. Cultures of single pancreatic islet cells were fixed with 4% paraformaldehyde. Samples were blocked and permeabilized in PBS with 0.3% Triton X-100 and 10% goat or donkey serum. Primary antibodies CHOP (1:100; sc-575, Santa Cruz Biotechnology), GAD6 mouse monoclonal antibody (mAb) against C-terminus of GAD65 (31) (1:1,000); N-GAD65 mouse mAb against the N-terminus of GAD65 (32) (1:300), giantin (1:1,000; ab24586, Abcam); insulin (1:10,000; 4011-01, Linco); insulin (1:2,000; ab14042, Abcam), and CD3 (1:30; M7254, Dako) were incubated overnight at 4°C in PBS with 0.3% Triton X-100 and 1% goat or donkey serum. Alexa Fluor conjugated secondary antibodies (Molecular Probes) were incubated at 1:200 dilution in PBS with 0.3% Triton X-100 for 30 min at room temperature.

Image Capture, Analysis, and Quantification
Samples were imaged on a Zeiss LSM700 confocal microscope with 63×/1.40 NA Plan-Apochromat oil-immersion objective for single islet cells and 40×/1.30 NA Plan-Apochromat oil-immersion objective for pancreatic tissue sections. All images for quantification within a single experiment were captured with the same laser power and

detector gain. The ratio of GAD65 mean fluorescence intensity (MFI) in the Golgi compartment and post-Golgi vesicles compared with the rest of the cytosol was calculated with a custom ImageJ macro. Individual β-cells in a given field of view were identified and outlined by hand. For each cell, the macro automatically defined a region of interest (ROI) outlining the Golgi compartment, identified by giantin costain or by characteristic morphology and brightness thresholding of GAD65 stain, and GAD65⁺ vesicles, identified by brightness thresholding of GAD65⁺ bright puncta. A second ROI defined the remainder of the cell, excluding the Golgi, GAD65⁺ vesicles, and nucleus. GAD65 Golgi accumulation was reported as the ratio of MFI for the two ROIs.

SDS-PAGE and Western Blotting
Gel electrophoresis was performed with the NuPAGE system (Invitrogen) with transfer onto polyvinylidene fluoride membranes with the iBlot 2.0 (Life Technologies) device. Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences), incubated in primary antibody GAD1701 (a custom antibody against C-terminus of GAD67 that reacts equally well with GAD65 and GAD67 [5], 1:5,000) overnight at 4°C, and detected with secondary antibody IRDye 800CW (LI-COR Biosciences). Blots were imaged on the LI-COR Odyssey scanner.

Treatment of Cells to Induce ER Stress
Sodium palmitate (1 mmol/L) (Sigma-Aldrich) was conjugated to fatty acid–free BSA (0.17 mmol/L) (Calbiochem) in 150 mmol/L NaCl (pH 7.4) for 1 h. Stock palmitate-BSA (1 mmol/L palmitate and 0.17 mmol/L BSA) was added to culture media to achieve a final palmitate concentration of 0.1 mmol/L or 0.5 mmol/L. A stock solution of BSA (0.17 mmol/L BSA) without palmitate was added to the control (untreated) wells. Thapsigargin (Invitrogen) was used at a final concentration of 2 μmol/L. Rat interferon-γ and rat interleukin (IL)-1β (R&D Systems) were used at a final concentration of 10 units/mL for each.

Fluorescence Recovery After Photobleaching Imaging and Analysis
Fluorescence recovery after photobleaching (FRAP) experiments were performed on a Zeiss LSM700 confocal microscope with environmental stage at 512 × 512 pixel resolution, 1% laser power, and 1.94-s frame interval. GAD65-GFP fluorescence in the Golgi compartment of transfected cells, treated or not to induce ER stress, was bleached with 50% laser power. Time-stacks of GAD65-GFP fluorescence recovery in the Golgi compartment were double normalized for percentage of initial intensity and whole-cell photobleaching in ImageJ. Two-phase and single-phase association curves were plotted in GraphPad Prism software to obtain half-time of recovery.

S-Acylation Resin-Assisted Capture Palmitoylation Assay
S-Acylation resin-assisted capture (Acyl-RAC) on recombinant human GAD65 (rhGAD65; FIRS Laboratories, RSR) was performed as described by Forrester et al. (33).

Uptake of GAD65-488 by Priess Cells
rhGAD65 was depalmitoylated overnight by treatment with 200 mmol/L hydroxylamine (HA) and labeled with DyLight 488 NHS Ester (Thermo Fisher Scientific). Palmitoylated or depalmitoylated GAD65-488 was incubated at 10 μg/mL with 50,000 Priess cells/well in 96-well plates. Cells were stained with LIVE/DEAD Aqua (Molecular Probes) and analyzed by Cyan Flow Cytometer (Beckman Coulter) and FlowJo software.

Activation of GAD65-Specific T Cells
Unmodified rhGAD65 containing native palmitate modifications or HA-treated depalmitoylated GAD65 were incubated overnight with 50,000 Priess cells/well at 2 μg/mL in 96-well plates. GAD65 loaded Priess cells were then incubated for 24 h with 30,000 T33.1 T cells. IL-2 secretion was analyzed by ELISA kit (eBioscience).

Plasmids
Generation of GAD65-GFP and GAD65(C30,45A)-GFP was described previously (11,12,14). INS-1E and primary rat islet cells were transfected by Lipofectamine 2000 (Invitrogen).

Statistics
Means among three or more groups were compared by ANOVA in GraphPad Prism 6 software. If deemed significant, Tukey post hoc pairwise comparisons were performed. Means between two groups were compared using the Student t test. A 95% CI was considered significant.

Ethical Approval
Animals were used under École Polytechnique Fédérale de Lausanne (EPFL) animal regulation guidelines and a protocol approved by the Institutional Animal Care and Use Committee. Human islets were received from the University Hospital of Geneva and San Raffaele Scientific Institute, Milan, through the European Consortium for Islet Transplantation (ECIT) islets for basic research program and were approved by the University Hospital of Geneva Institutional Review Board (CER No. 05-028) and by the San Raffaele Scientific Institute of Milan Ethics Committee (IPF002-2014). Human pancreatic sections obtained from the nPOD tissue bank, University of Florida, Gainesville, FL, were harvested from cadaveric organ donors by certified organ procurement organizations partnering with nPOD in accordance with organ donation laws and regulations and were classified as "nonhuman subjects" by the University of Florida Institutional Review Board (28,29). EPFL grants permit for the use of human material as long as the provider can certify that the samples were obtained according to local laws and regulations as well as good practices in the country where they were collected.

RESULTS

### ER Stress Results in Accumulation of GAD65 in the Golgi Compartment
Primary rat and human β-cells derived from dissociated whole islets were cultured as monolayers on coverslips to

allow for high-resolution confocal microscopy. The mono-layer islet cells were fixed and immunostained for insulin, GAD65, and the Golgi marker giantin (Fig. 1). As previously reported (15), GAD65 is detected diffuse in the cytosol as well as in Golgi membranes and post-Golgi vesicles in β-cells. Insulin is detected in distinct large dense core vesicles.

To induce mild ER stress, primary islet cell monolayers were subjected to 48 h of low levels (10 units/mL) of the inflammatory cytokines IL-1β and interferon-γ or over-night treatment with the saturated fatty acid palmitate (500 μmol/L) (Fig. 2), both of which have been previously

shown to trigger ER stress in β-cells (34–36). Cells were also treated for 8 h with thapsigargin, which is a potent ER stress–inducing agent (37) (Fig. 2). We monitored ER stress by nuclear translocation of CHOP, a multifunc-tional transcription factor in the ER stress response (Fig. 2).

The different treatment conditions resulted in varying degrees of ER stress, as shown by the time frame until nuclear translocation of CHOP, indicating that the different treatment conditions affected the cells with variable strength and specificity. Although incubation with thapsigargin or palmitate induced CHOP activation at 8 and 18 h,
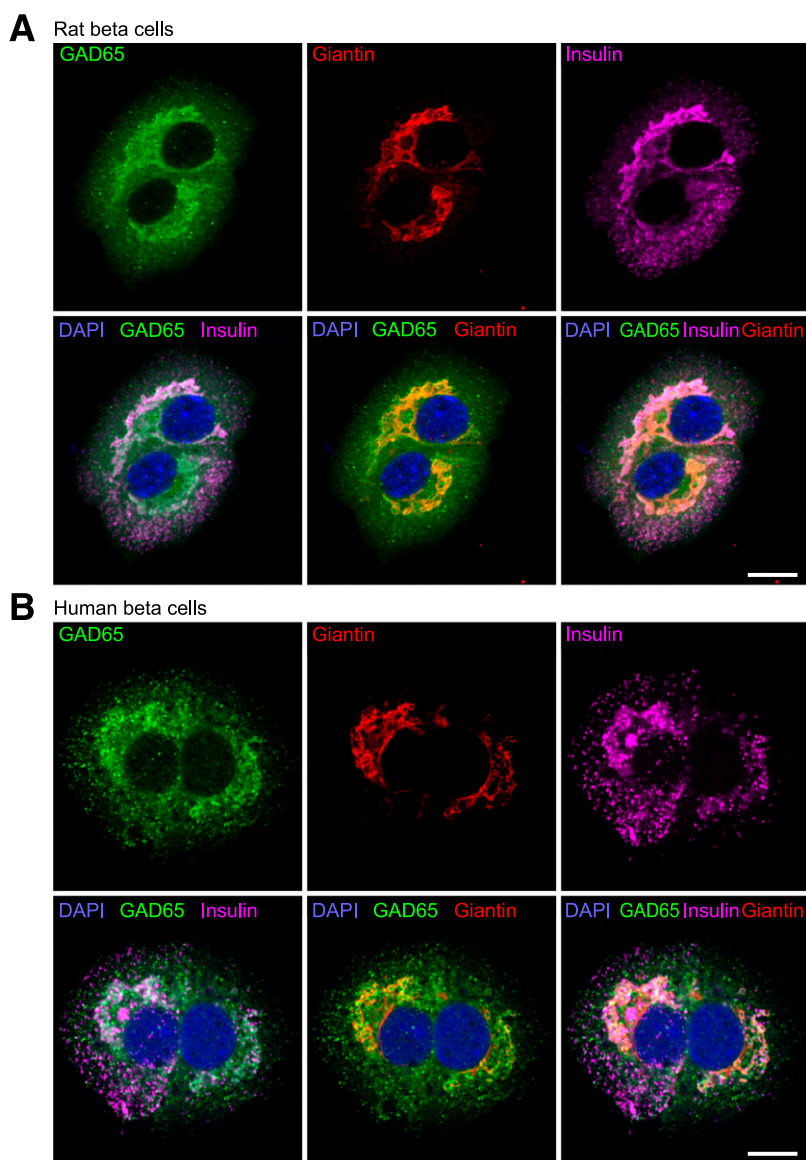
Figure 1—Confocal analyses of GAD65 localization in primary β-cells. *A*: Primary rat β-cells immunostained for GAD65 and giantin (Golgi) show a subcellular distribution of GAD65 in the cytosol and the Golgi compartment. Apart from the Golgi compartment, GAD65 is localized to vesicles, which are distinct from insulin granules. The confocal images are representative of similar analyses of seven independent isolations of rat islets. Scale bar: 10 μm. *B*: Immunostaining of primary human β-cells for GAD65, giantin, and insulin reveals localization of GAD65 in the Golgi compartment as well as in peripheral vesicles distinct from insulin-containing vesicles. The confocal images are representative of similar analyses of independent isolations of human islets from four donors. Scale bar: 10 μm.
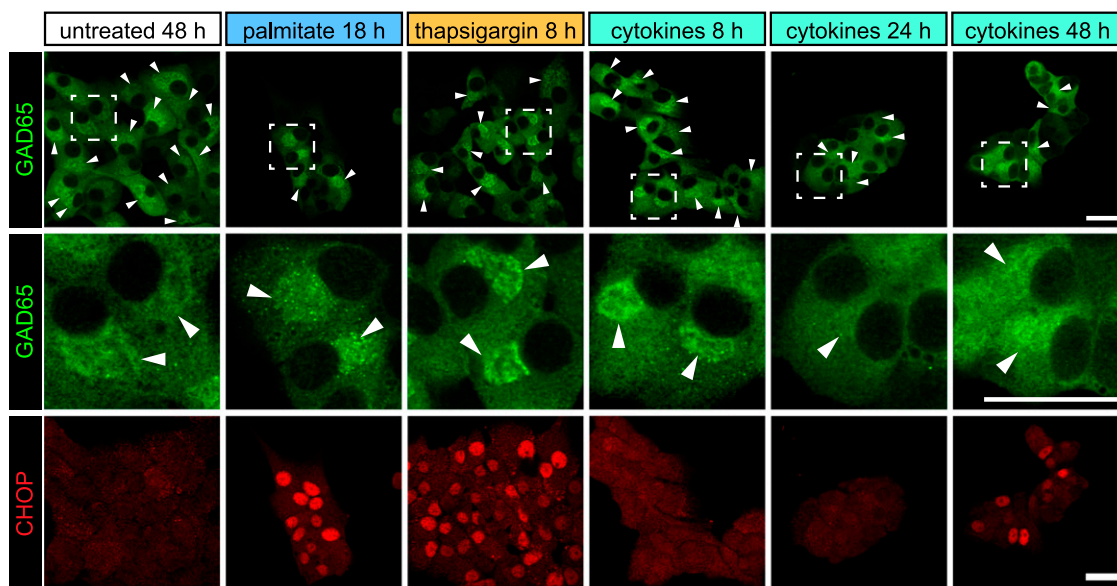
Figure 2—Activation of ER stress pathways in β-cells by thapsigargin, palmitate, or inflammatory cytokines. Primary rat β-cells were treated with palmitate (500 μmol/L, 18 h), thapsigargin (2 μmol/L, 8 h), or cytokines (10 units/mL, 8, 24, or 48 h) to induce ER stress and immunostained for GAD65 (top two panels) and CHOP (bottom panel). GAD65-positive Golgi compartments are indicated by arrowheads. For all three treatment modules, GAD65 expression in the Golgi compartment becomes noticeably brighter. The middle panels show increased magnification of framed regions in the top panels. The confocal images are representative of similar analyses of six independent isolations of rat islets. Scale bars: 10 μm.

respectively, CHOP activation was detected at 48 h (but not 24 h) of cytokine treatment (Fig. 2). Notably, all three treatment conditions resulted in a marked increase in the Golgi-localization of GAD65 (Fig. 2). Accumulation of GAD65 in Golgi membranes during palmitate-induced ER stress was also observed in primary human β-cells and in another cell type expressing GAD65, primary rat hippocampal GABA-ergic neurons (Supplementary Fig. 1).

Accumulation of GAD65 in the Golgi compartment during induction of ER stress by palmitate or thapsigargin was confirmed by costaining for the Golgi marker giantin (Fig. 3A and Supplementary Fig. 1A–C), but Golgi structures are also clearly identifiable by GAD65 localization in β-cells and neurons (12,15,38). Quantification after a 2-h incubation with palmitate or thapsigargin revealed a significant accumulation of GAD65 in the Golgi compartment (Fig. 3B and Supplementary Fig. 1D).

The time course of accumulation of GAD65 in Golgi membranes during palmitate-induced ER stress was studied further. Rat islet cell monolayers were treated with 100 or 500 μmol/L palmitate for 10 min, 2 h, and 18 h and immunostained for CHOP, GAD65, and insulin (Fig. 4 and Supplementary Fig. 2). Golgi accumulation of GAD65 occurred almost immediately upon addition of palmitate and was clearly visible at 10 min (Fig. 4A and Supplementary Fig. 2A). The strong Golgi accumulation of GAD65 persisted at 2 h and was still visible at 18 h, although the intensity began to fade coinciding with activation of CHOP (Fig. 4C and D). Quantification of

GAD65 membrane accumulation showed a statistically significant increase in the ratio of MFI for GAD65 in the Golgi compartment for cells incubated for 10 min and 2 h in 100 or 500 μmol/L palmitate (Fig. 4B). Across all conditions and times, there were no notable changes in the subcellular localization of insulin (Supplementary Fig. 2B). Taken together, the results indicate that induction of ER stress results in aberrant accumulation of GAD65 in the Golgi compartment.

Palmitoylation Is Required for GAD65 Accumulation in the Golgi Compartment During ER Stress

The distribution of GAD65 between ER/*cis*-Golgi and TGN/vesicular membranes is controlled by its palmitoylation–depalmitoylation–repalmitoylation cycle. Although palmitoylation of cysteines 30 and 45 is not required for firm anchoring of GAD65 to Golgi membranes, it is critical for its anterograde trafficking from *cis*-Golgi to TGN membranes and targeting to post-Golgi vesicles (12). We assessed whether a palmitoylation-deficient mutant of GAD65 was capable of accumulating in the Golgi compartment in β-cells undergoing ER stress. Primary rat β-cells were transfected with wild-type (WT) GAD65-GFP or palmitoylation-deficient GAD65(C30,45A)-GFP, and treated with palmitate or thapsigargin for 1 h to induce ER stress. Cells were imaged live by confocal microscopy, and the images were measured for MFI of GAD65-GFP in the Golgi compartment (Fig. 5). ER stress induced by palmitate or thapsigargin significantly increased the fraction of WT GAD65-GFP in Golgi and vesicle membranes, but palmitoylation-deficient GAD65(C30,45)-GFP was unaffected (Fig. 5).
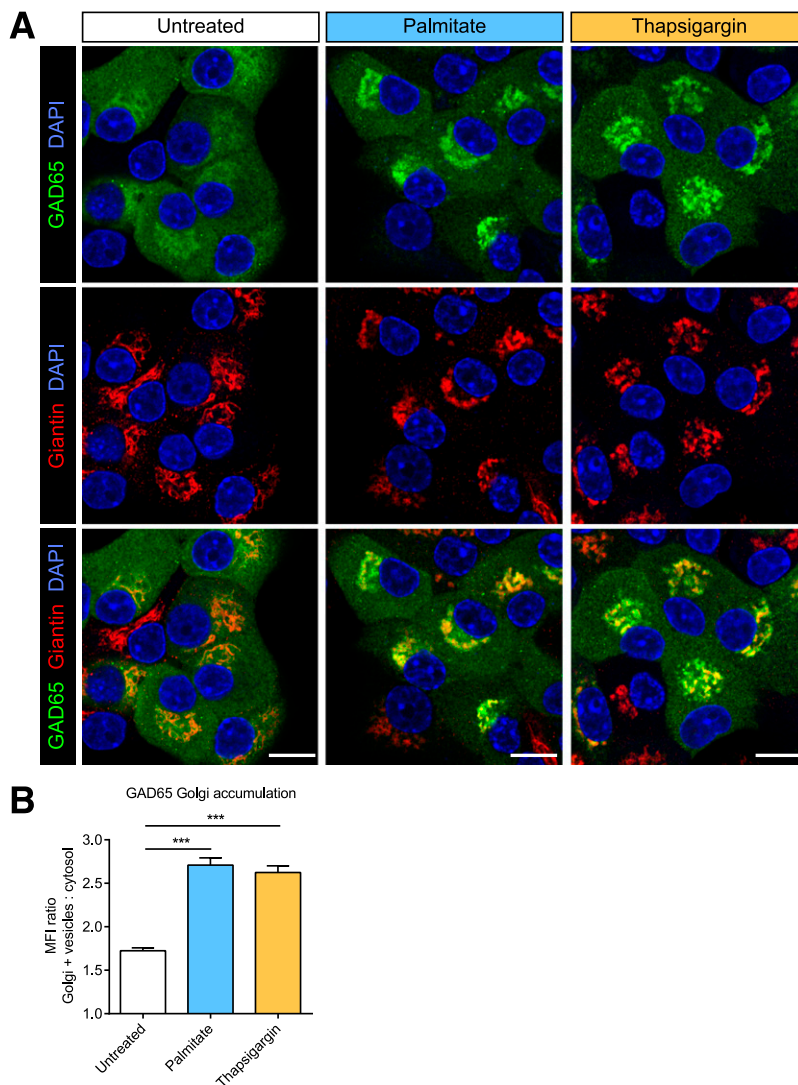
Figure 3—Treatment of β-cells with palmitate or thapsigargin promotes Golgi accumulation of GAD65. *A*: Primary rat β-cells were treated with palmitate (500 μmol/L, 2 h) or thapsigargin (2 μmol/L, 2 h) to induce ER stress and immunostained for GAD65 and giantin. Scale bars: 10 μm. *B*: Image quantification of GAD65 accumulation in the Golgi compartment reported as the ratio of MFI for GAD65 in the Golgi compartment and GAD65-positive vesicles to MFI for GAD65 in the rest of the cell, excluding the nucleus. Results are presented as mean ± SEM (*n* = 39–43 β-cells from eight image fields analyzed per condition). Cells positive for giantin and negative for GAD65 represent non–β-islet cells. Data were analyzed using one-way ANOVA, followed by the Tukey multiple comparisons test. ***P < 0.001. Analyses of accumulation of GAD65 in Golgi membranes in rat β-cells, human β-cells, and rat hippocampal neurons incubated with palmitate for 2 h are shown in Supplementary Fig. 1.

Thus, Golgi accumulation of GAD65 in response to ER stress is restricted to palmitoylation-competent GAD65.

Recovery of WT but Not Palmitoylation-Deficient GAD65 in the Golgi Compartment After Photobleaching Is Inhibited During ER Stress

We next assessed the effect of ER stress on the kinetics of replenishment of WT GAD65-GFP as well as the palmitoylation-deficient mutant GAD65(C30,45A)-GFP into Golgi membranes after irreversible photobleaching. We previously reported that the FRAP of WT GAD65-GFP in Golgi membranes involves two pools of the protein, a rapid pool and a slow pool (12). The rapid Golgi replenishment pool represents the nonpalmitoylated form of GAD65, which has undergone the first step of hydrophobic modifications resulting in weak on/off membrane association. The second and slower replenishment pool represents palmitoylation-competent GAD65, which, after anterograde vesicular trafficking to the TGN and periphery, can undergo depalmitoylation and nonvesicular retrograde trafficking back to Golgi membranes. INS-1E cells (Fig. 6A–D) and primary rat islet cells (Supplementary Figs. 3 and 4) were subjected to irreversible photobleaching of the Golgi compartment. Recovery of GAD65-GFP in Golgi membranes was recorded for untreated cells and for cells pretreated with palmitate (Fig. 6 and Supplementary Fig. 3) or thapsigargin (Supplementary Fig. 4)
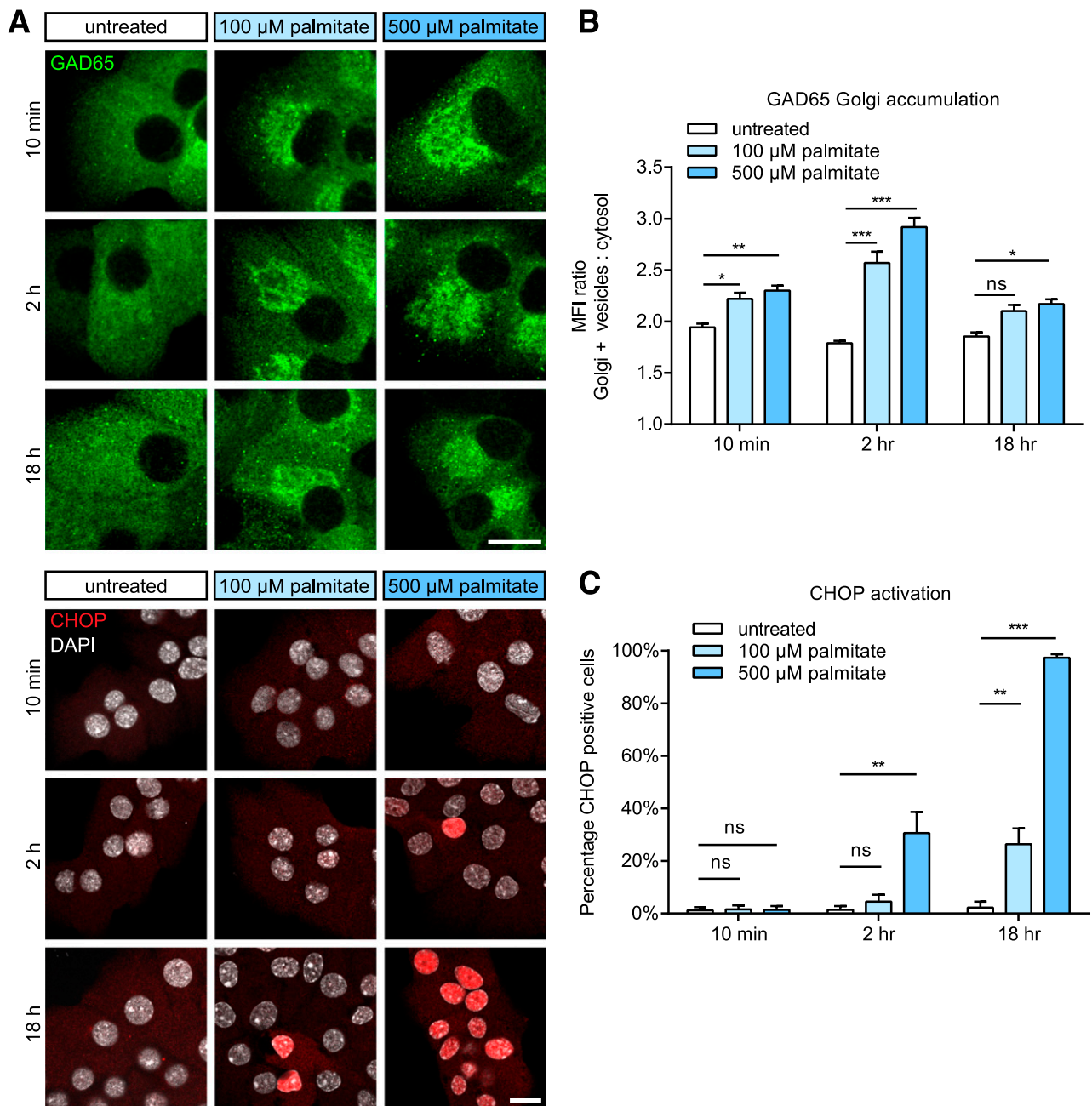
Figure 4—Time course of Golgi accumulation of GAD65 upon palmitate treatment. *A*: Confocal analyses of monolayer cultures of primary rat β-cells treated or not with 100 μmol/L or 500 μmol/L palmitate for 10 min, 2 h, and 18 h, respectively. Fixed cells were immunostained for GAD65 and CHOP. Scale bar: 10 μm. *B*: Image quantification of GAD65 accumulation in the Golgi compartment reported as the ratio of MFI for GAD65 in the Golgi and GAD65-positive vesicles to MFI for GAD65 in the rest of the cell, excluding the nucleus. Results are presented as mean ± SEM (*n* = 28–45 cells from 4–5 image fields analyzed per condition). Data were analyzed using two-way ANOVA, followed by the Tukey multiple comparisons test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. *C*: Image quantification of CHOP-positive nuclei as a percentage of total nuclei. Results are presented as mean ± SEM (*n* = 4–10 image fields analyzed per condition). Data were analyzed using two-way ANOVA, followed by the Tukey multiple comparisons test. **$P < 0.01$ and ***$P < 0.001$; ns, not significant. Corresponding lower magnification images of primary rat β-cells stained for GAD65 and insulin are shown in Supplementary Fig. 2. hr, hour.

for 1 h to induce early-stage ER stress. Analysis of the data were performed using nonlinear regression, assuming one or multiple pools of replenishing protein. Induction of ER stress significantly impaired the Golgi replenishment kinetics of WT GAD65-GFP in INS-1E cells treated with palmitate (Fig. 6*C* and *D*) and in primary islet

cells treated with palmitate (Supplementary Fig. 3) or thapsigargin (Supplementary Fig. 4). In contrast, the Golgi replenishment kinetics of palmitoylation-deficient GAD65(C30,45A)-GFP was similar for untreated and treated cells (Fig. 6*C* and *D* and Supplementary Figs. 3 and 4). Calculations of half-time of recovery of the rapid
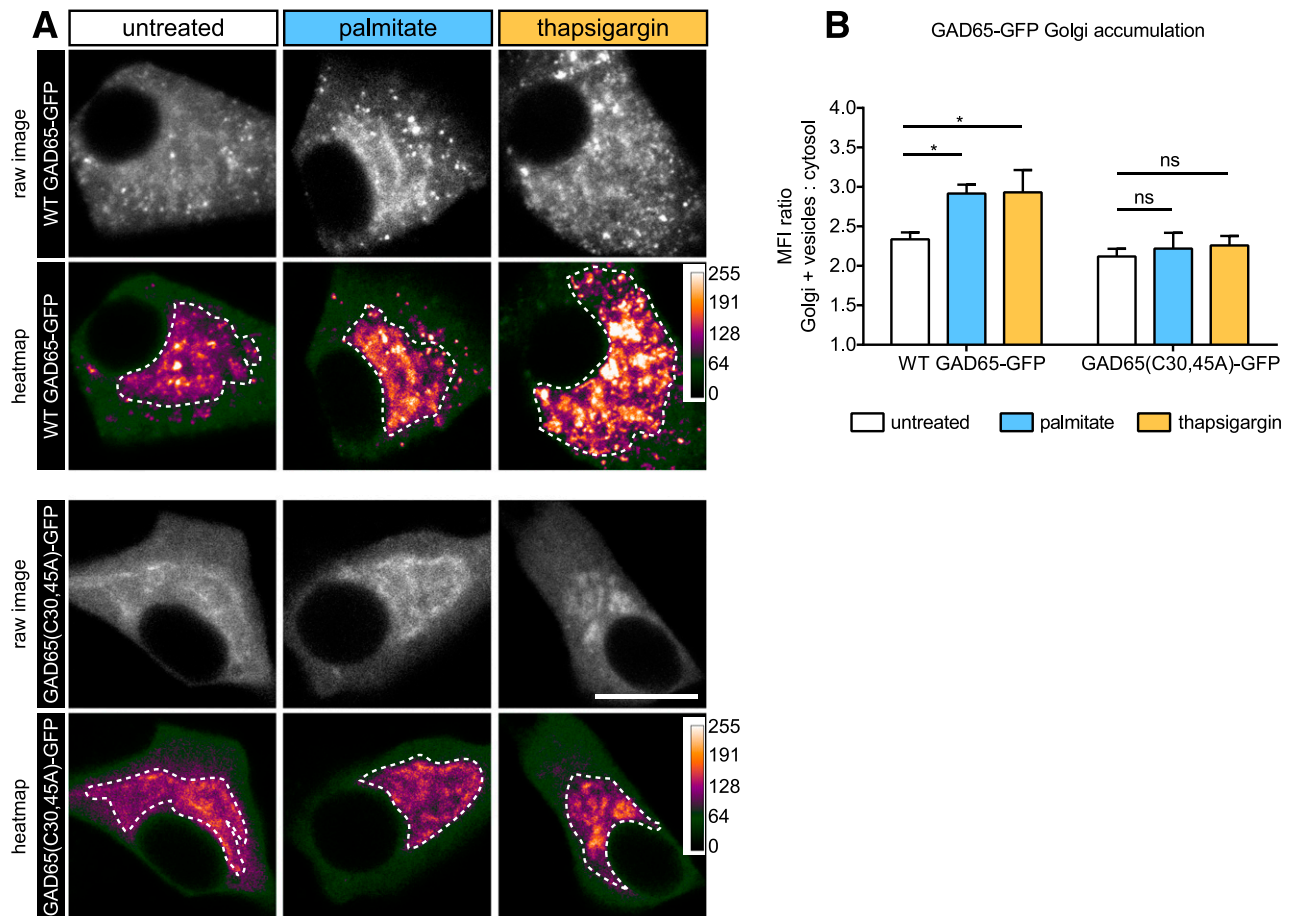
Figure 5—Palmitoylation is required for Golgi accumulation of GAD65. *A*: Primary rat β-cells were transfected with WT GAD65-GFP or the palmitoylation-deficient mutant GAD65(C30,45A)-GFP and imaged live after a 1-h treatment with palmitate (500 μmol/L) or thapsigargin (2 μmol/L) to induce ER stress. Images are also displayed with a heatmap to highlight the increase in Golgi and vesicle brightness. Golgi regions are indicated with a dashed outline. Scale bar: 10 μm. *B*: Image quantification of GAD65 accumulation in the Golgi compartment expressed as the ratio of MFI for GAD65 in the Golgi and GAD65-positive vesicles to MFI for GAD65 in the rest of the cell, excluding the nucleus. Results are presented as mean ± SEM (*n* = 6–10 cells analyzed per condition). Data were analyzed using one-way ANOVA, followed by the Tukey multiple comparisons test. *$P < 0.05$; ns, not significant.

and slow pools of WT GAD65-GFP revealed that although the half-time of recovery of the rapid pool replenishing the Golgi was minimally or not affected, the half-time of recovery of the slow pool was increased three- to fourfold (Fig. 6*C* and *D* and Supplementary Figs. 3 and 4). The results of these experiments indicate that ER stress, whether induced by palmitate or thapsigargin, inhibits and perturbs the palmitoylation cycle of WT GAD65-GFP.

Uptake and Processing of GAD65 by Antigen-Presenting B Lymphocytes Is Enhanced by Palmitoylation

Given that modification of peptides and proteins by palmitoylation may enhance their immunogenicity (39), we assessed whether the palmitoylation state of GAD65 has an effect on its uptake by antigen-presenting cells (APCs) and/or activation of T cells in an APC/T-cell coculture assay. rhGAD65 produced in yeast was depalmitoylated by thiol-acyl cleavage with HA, and depalmitoylation was confirmed biochemically by the Acyl-RAC assay (33) (Fig. 7*A*).

Analyses of uptake of DyLight-488–labeled palmitoylated and nonpalmitoylated GAD65 by the HLA-DR4 (DRB1*0401)–positive human B-cell line Priess revealed a 2.5-fold increase in uptake of palmitoylated GAD65 compared with the depalmitoylated GAD65 (Fig. 7*B*). Furthermore, Priess cells were loaded with palmitoylated or depalmitoylated GAD65 and used as APCs to stimulate the GAD65-specific HLA-DR4 (DRB1*0401)–restricted murine T-cell hybridoma line T33.1 that recognizes the GAD65[274-286] epitope. In this APC/T-cell coculture assay, presentation of palmitoylated GAD65 induced a threefold higher secretion of IL-2 by T33.1 cells compared with the depalmitoylated protein (Fig. 7*C*). Taken together, the results indicate that palmitoylation facilitates uptake and processing of GAD65 by APCs, resulting in increased antigen-specific stimulation of T cells. Thus the palmitoylated form of GAD65 that accumulates in Golgi during ER stress has higher immunogenicity, consistent with a possible role of ER stress in activating autoreactive T cells in T1D.
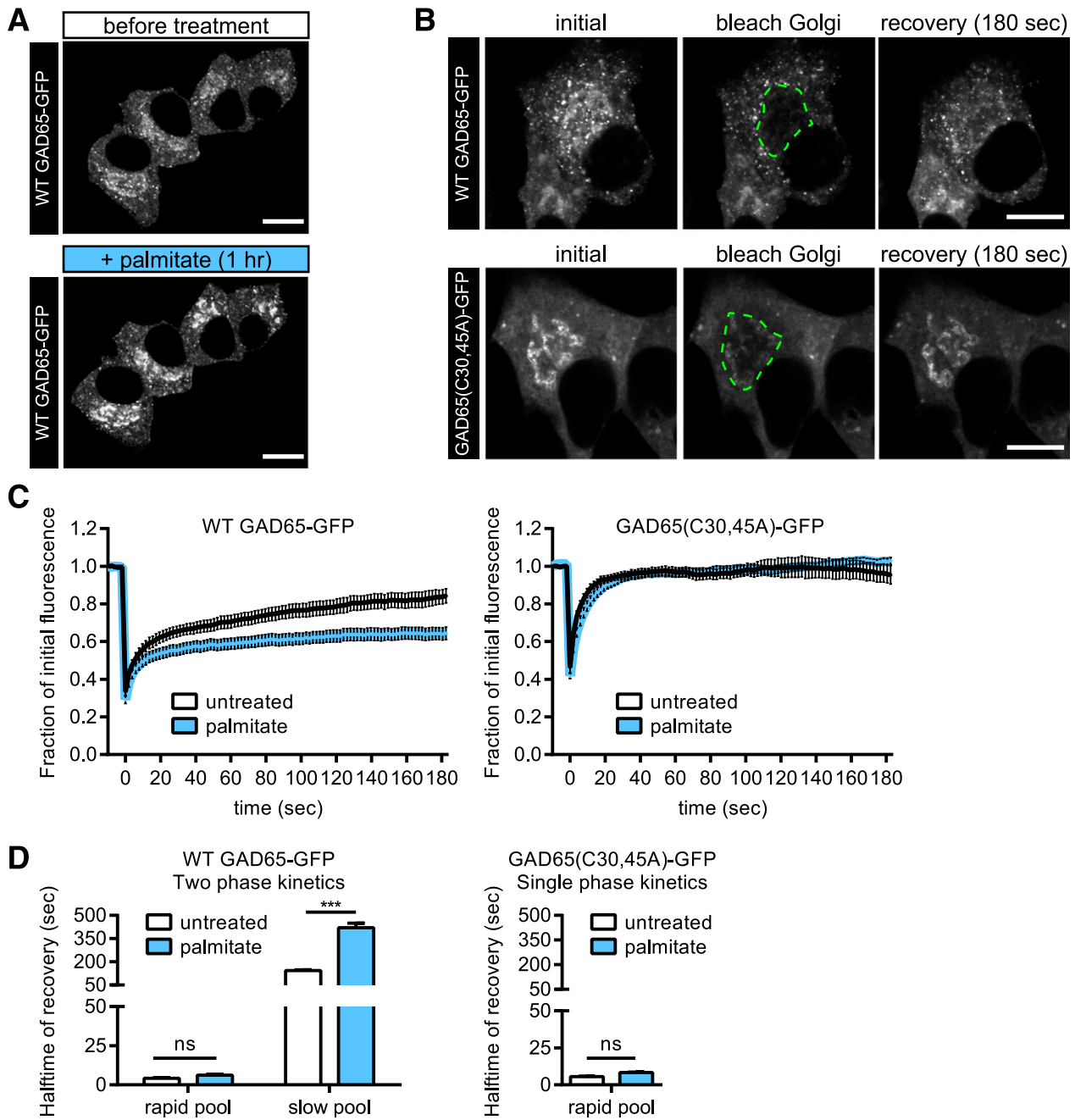
Figure 6—FRAP in the Golgi of WT but not palmitoylation-deficient GAD65 is inhibited during ER stress. *A*: Live cell images of INS-1E cells transfected with GAD65-GFP before and after treatment with palmitate (500 μmol/L) for 1 h show accumulation of GAD65-GFP in the Golgi. Scale bar: 10 μm. *B*: Representative images from FRAP analyses of INS-1E cells expressing WT GAD65-GFP or palmitoylation-deficient GAD65(C30,45A)-GFP. The entire Golgi compartment was photobleached, and recovery of fluorescence was followed by live cell imaging. Scale bar: 10 μm. *C*: Kinetics of fluorescence recovery after bleaching the entire Golgi-associated pool of WT GAD65-GFP or GAD65(C30,45A) in INS-1E cells pretreated or not with palmitate for 1 h. Results from a representative experiment are presented as mean ± SEM (*n* = 10 cells per condition). *D*: Calculated halftimes of Golgi fluorescence recovery in INS-1E cells after photobleaching. WT GAD65-GFP exhibits two-phase recovery kinetics corresponding to a rapid as well as a slow recovery pool, while palmitoylation-deficient GAD65(C30,45A)-GFP exhibits single-phase recovery kinetics corresponding to a rapid recovery pool. Results are presented as the mean halftime of recovery ± SEM (*n* = 3 independent experiments, 10 cells per experiment). Data were analyzed by Student *t* test comparing the halftime parameter between the association curve fits of the combined data set from three independent experiments. ***P < 0.001; ns, not significant. FRAP analyses of GAD65 Golgi recovery in primary rat β-cells treated with palmitate or thapsigargin are shown in Supplementary Fig. 3 and Supplementary Fig. 4, respectively. hr, hour; sec, second.
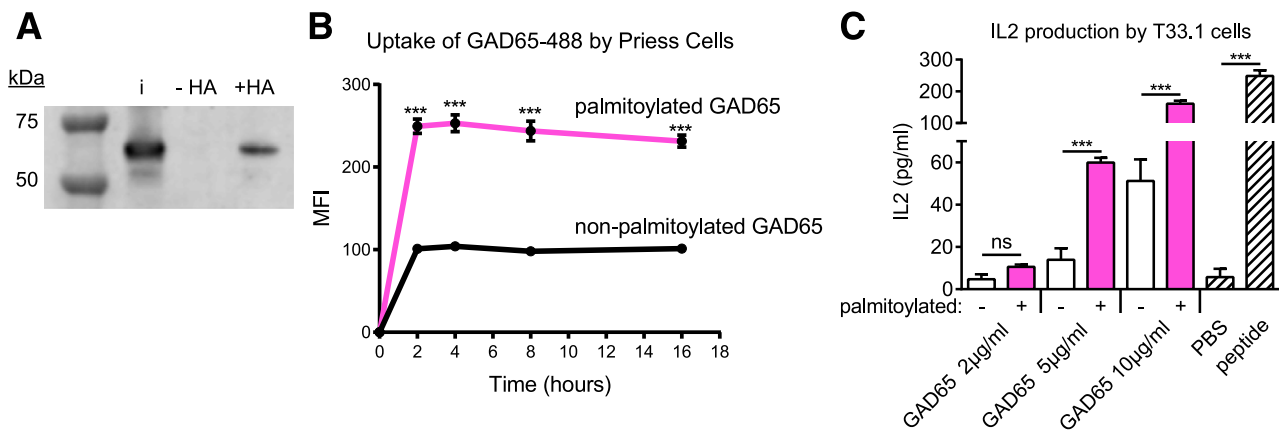
Figure 7—Palmitoylation confers increased immunogenicity upon GAD65. *A*: Recombinant human GAD65 produced in yeast was shown to be palmitoylated by the Acyl-RAC assay. Free thiols in recombinant GAD65 were capped with methyl methanethiosulfonate, followed by cleavage of palmitate-thiol modifications with HA, pulldown with a thiol-reactive pyridyl-disulfide agarose resin, and detection by Western blot. Lanes: i, input, positive control of unmodified recombinant GAD65; –HA, negative control without HA, resulting in no palmitate cleavage and no protein pulldown; +HA, HA added with palmitate groups cleaved and protein recovered by thiol-reactive pulldown. *B*: Time course of uptake of equimolar amounts of DyLight-488–labeled palmitoylated or depalmitoylated (HA cleaved) recombinant GAD65 by MHC-class II DR4–positive human Priess B-cell APCs measured by flow cytometry. Results are presented as mean ± SEM (*n* = 3 experimental replicates). Data were analyzed by two-way ANOVA, followed by the Tukey multiple comparisons test. ***P < 0.001. *C*: Priess cells were loaded with equimolar amounts of palmitoylated or depalmitoylated GAD65 (HA cleaved) and tested for stimulation of the DR4 restricted GAD65-specific T33.1 T-cell hybridoma cells by IL-2 secretion measured by ELISA. Peptide, GAD65[274-286] epitope. Results are presented as mean ± SEM (*n* = 3 experimental replicates). Data were analyzed by two-way ANOVA, followed by the Tukey multiple comparisons test. ***P < 0.001; ns, not significant.

## GAD65 Accumulates in the Golgi Compartment in Human β-Cells During Progression of T1D Autoimmunity

We addressed the question of whether the experimental accumulation of GAD65 in the Golgi compartment induced by treatment with ER stressors in vitro is of relevance for human diabetes. Human pancreatic sections provided by the nPOD tissue bank (28,29) representing eight healthy donors (Supplementary Table 1), eight individuals with potential prediabetes and GADA+ (Supplementary Table 2), and eight patients with T1D with residual β-cell mass (Supplementary Table 3) were immunostained for GAD65, giantin, insulin, glucagon, and the pan–T-cell marker CD3 (Fig. 8A–D). In five of eight GADA+ individuals and seven of eight patients with T1D, the GAD65 signal in β-cell Golgi membranes compared with cytosol was higher than one SD above the mean for healthy control individuals where GAD65 immunostaining was mostly uniform (Fig. 8E and Supplementary Tables 1–3, last column). Staining of serial sections for the pan–T-cell marker, CD3 (Fig. 8A), confirmed peri- and intraislet infiltrating T cells in islets from three of eight GADA+ individuals and in six of eight patients with T1D (Fig. 8A and results not shown). Examination of the patient information (Supplementary Tables 1–3) for individual donors provided by nPOD revealed that the highest Golgi accumulation observed in the GADA+ group with prediabetes was from a single autoantibody-positive (GADA+) 2.2-year-old child expressing the HLA-class II T1D susceptibility haplotype DR4, DQ8 (Supplementary Table 2) showing infiltrates of CD3+ T cells in and around islets (Fig. 8A, nPOD 6090) but not meeting the nPOD

insulitis criteria (Supplementary Table 2). This child may represent a case of a young, genetically susceptible individual in the early stages of autoimmunity associated with development of T1D. The lowest Golgi accumulation in the GADA+ group was observed in a 31-year-old, single-autoantibody–positive (GADA+) individual with normal islet morphology and no insulitis (Supplementary Table 2, nPOD 6181). The highest Golgi accumulation observed in the group with T1D was observed in a double-autoantibody–positive (GADA+ and microinsulin autoantibody assay+) 11-year-old individual expressing the T1D HLA-class II susceptibility haplotypes DR3, DQ2 and DR4, DQ8 and with ongoing insulitis (Supplementary Table 3) (30) as confirmed by our CD3 immunostaining (Fig. 8A, nPOD 6265). Taken together, the results suggest that aberrant accumulation of GAD65 in Golgi membranes may correlate with active islet autoimmunity in human T1D.

DISCUSSION
Accumulation of GAD65 in Golgi Membranes in β-Cells Experiencing ER Stress and/or Active Autoimmunity
In this study, we show that treatment of islet cell cultures by three separate regimens known to induce ER stress in β-cells results in accumulation of GAD65 in Golgi membranes. Importantly, analysis of human pancreatic tissue sections revealed significant accumulation of GAD65 in β-cell Golgi membranes in GADA+ individuals with potential prediabetes and in patients with T1D with residual β-cell mass. Thus, aberrant accumulation of GAD65 in Golgi membranes is observed in β-cells in culture
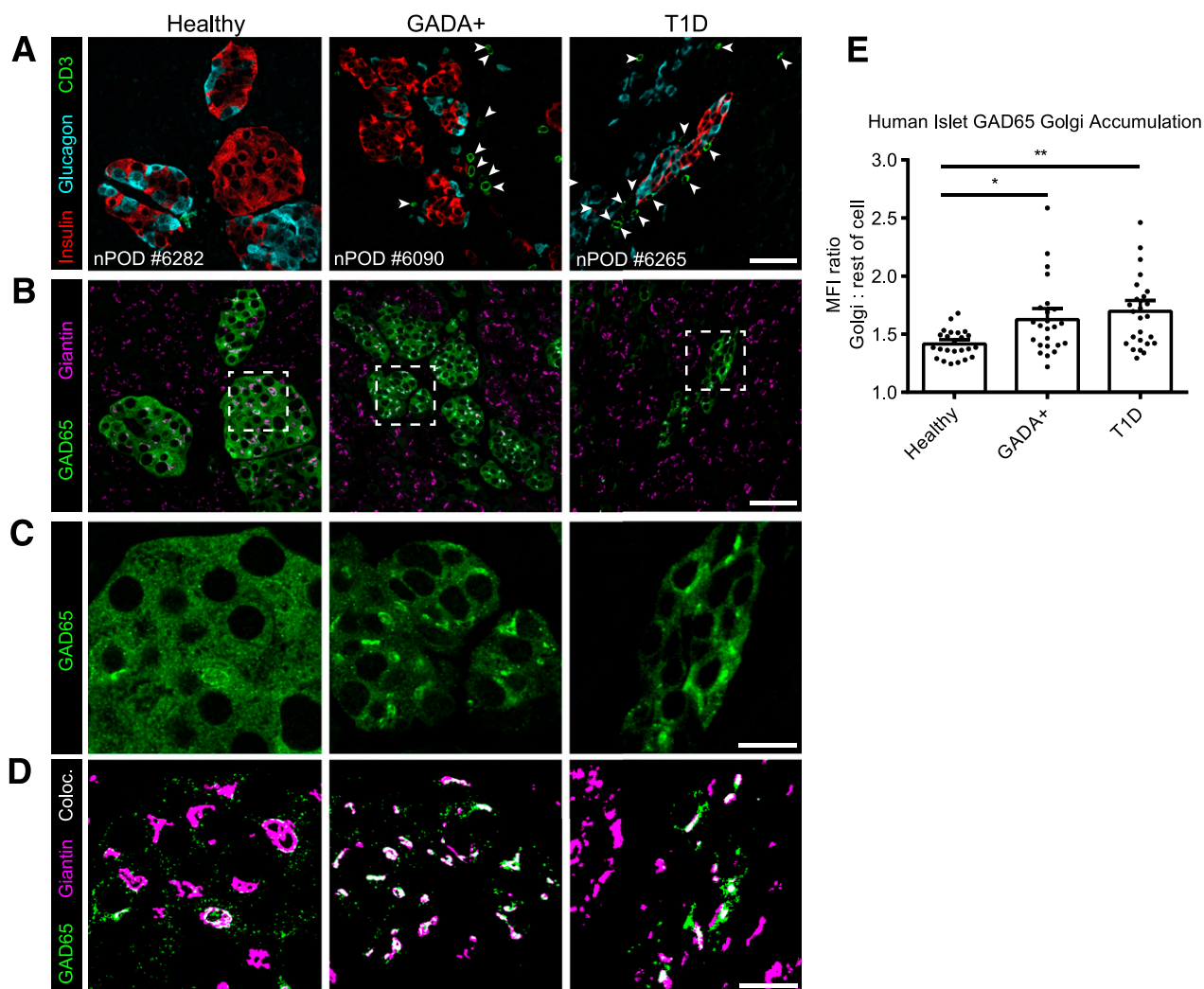
Figure 8—Confocal analyses of islets in paraffin sections of human pancreas from healthy donors, donors who were GAD65 autoantibody-positive but without diabetes, or donors with T1D with residual β-cell mass. *A*: Immunostaining of human pancreatic sections for insulin, glucagon, or CD3 from the indicated nPOD case numbers. The arrowheads indicate CD3+ T cells. Scale bar: 50 μm. *B*: The same human islet from a serial section, immunostained for GAD65 and giantin. Scale bar: 50 μm. *C*: Increased magnification of GAD65 staining in the framed region shown in *B*. Scale bar: 10 μm. *D*: Thresholded binary images of the same region in *C* show the colocalization (Coloc.) between giantin (Golgi) and the bright GAD65 signal. Scale bar: 10 μm. *E*: Image quantification of GAD65 accumulation in the Golgi compartment in three representative islets from eight individuals per donor category, reported as the ratio of MFI for GAD65 in the Golgi of β-cells to MFI for GAD65 in the rest of the cell, excluding the nucleus. Results are presented as scatter dot plots overlaying the mean ± SEM (n = 24 islets from eight donors). Data were analyzed by one-way ANOVA, followed by the Tukey multiple comparisons test. *P < 0.05, **P < 0.01. The average GAD65 Golgi accumulation calculated per individual and demographic information for healthy, GADA+, and donors with T1D are listed in Supplementary Tables 1–3.

undergoing experimentally induced ER stress as well as in β-cells of individuals experiencing active β-cell autoimmunity.

GAD65 in Pancreatic β-Cells Undergoes a Palmitoylation–Depalmitoylation–Repalmitoylation Cycle That Is Perturbed During ER Stress Resulting in Accumulation of the Protein in Golgi Membranes

We have shown earlier that palmitoylation of GAD65 results in anterograde trafficking from *cis*-Golgi to TGN membranes (12). The evidence presented in this study shows that only WT GAD65, but not palmitoylation-deficient GAD65(C30,45A), accumulates in Golgi membranes during ER stress. This increase of GAD65 in Golgi

membranes is consistent with the accumulated protein representing the palmitoylated form of GAD65 in TGN membranes, suggesting that the palmitoylation cycle of GAD65 significantly slows down during induction of ER stress. This retardation in transport likely affects the control of GAD65 distribution between peripheral vesicle membranes, where most of the GAD65 enzymatic functional activity is believed to take place, and Golgi membranes, which may serve as a sorting station for this protein. We therefore propose that perturbation in the regulation of GAD65 membrane distribution by ER stress may have negative consequences for GABA synthesis

and secretion. GABA serves as an important signaling molecule and survival/growth factor in islets of Langerhans (40).

The mechanisms by which cytokines, palmitate, and thapsigargin induce ER stress in β-cells may differ (34). One commonality between the three different treatments is that each is implicated in dysregulation of Ca^{2+} homeostasis (41). Inflammatory cytokines and thapsigargin have both been shown to induce β-cell ER stress through downregulation or inhibition of the sarcoplasmic/ER Ca^{2+} ATPase, resulting in elevation of cytosolic Ca^{2+} concentrations and depletion of ER Ca^{2+} stores (42). Palmitate is reported to induce ER stress through lipotoxic signaling pathways mediating a block in ER uptake of Ca^{2+} and a sustained depletion of ER Ca^{2+} stores (35,43). The idea that dysregulation of ER/cytosolic Ca^{2+} concentrations is involved in Golgi accumulation of GAD65 is supported by the fact that a similar effect could be achieved by strongly depolarizing cells by abruptly elevating extracellular glucose or KCl concentration (E.A.P., C.C., and S.B., unpublished results). Although how a change in Ca^{2+} homeostasis could affect membrane trafficking of GAD65 is currently unknown, it is of note that earlier studies have suggested modulation of the affinity of GAD65 to liposome membranes by Ca^{2+} ion concentration (44). Interestingly, there is evidence to suggest that palmitate treatment of insulinoma cells alters the lipid composition of endomembranes and specifically disrupts lipid raft microdomains (45), a dynamic hub for palmitoylated proteins (46). It is of note that distribution of H-Ras into lipid rafts is regulated by its palmitoylation–depalmitoylation cycle (47), which shares similarities with the acylation cycle of GAD65 (16). Thus, it is possible that alterations in lipid raft composition are part of the mechanism involved in perturbation of GAD65 trafficking and accumulation in Golgi membranes.

Accumulation of Palmitoylated GAD65 in Golgi Membranes May Have Implications for Initiation of Autoimmunity

Parallel to the important role of GAD65 as the highly regulated and the only GABA-synthesizing enzyme in human β-cells, it can assume a detrimental role as a major target of autoimmunity associated with pancreatic human β-cell destruction and development of T1D in genetically susceptible individuals (2). The autoantigenicity of GAD65 is in stark contrast to the highly homologous GAD67 isoform, which is not an independent autoantigen. GAD67 primarily differs from GAD65 in the N-terminal domain (48) that mediates hydrophobic posttranslational modifications, membrane anchoring, palmitoylation, and trafficking of GAD65 (12), suggesting that this region is integral to the susceptibility of GAD65 to become a pathogenic autoantigen. GAD67 does not undergo hydrophobic modifications (49) and can only be targeted to membranes by piggybacking onto other proteins (15,38).

Our data show that compared with depalmitoylated GAD65, palmitoylated GAD65 induces a significantly stronger effector T-cell response by the T-cell hybridoma T33.1, which recognizes GAD65[274-286] in the context of DR4, a T1D MHC-class II susceptibility haplotype. Palmitoylation of peptide epitopes has been shown to enhance immunogenicity of autoimmune epitopes in experimental autoimmune encephalomyelitis (50) and in synthetic peptide vaccines (51). Palmitoylation of a protein increases its avidity for binding to membranes. The uptake of palmitoylated GAD65 by the DR4-positive human B-cell line Priess was significantly enhanced compared with nonpalmitoylated GAD65. We posit that the increase in uptake, conferred by palmitoylation, may involve enhanced binding to the surface of Priess cells resulting in increased uptake by endocytosis and enhanced targeting to late endosomes for proteolytic processing and presentation to T cells in the context of MHC-class II antigens (reviewed by Blum et al. [52]). Palmitoylation of GAD65 may also affect antigen unfolding and proteolysis and alter the hierarchy of peptides displayed to CD4^{+} T cells. The T33.1 hybridoma reporter cell line is clonally restricted to a single MHC-class II binding epitope, GAD65[274-286], which is distant from the palmitoylated cysteine residues in GAD65, aa 30 and 45. Therefore, the possibility of palmitoylation increasing the MHC-class II binding affinity of this epitope can be excluded in our experimental system.

Rather, we suggest that increased stimulation of T33.1 cells reflects a quantitative increase in the levels of the GAD65[274-286] epitope available for binding to DR4 in late endosomes and elevated expression on the surface of Priess cells. If palmitoylated GAD65, which has accumulated in TGN during ER stress, is released by distressed or dying β-cells and encountered by APCs, its heightened immunogenicity compared with nonpalmitoylated GAD65 may stimulate autoimmunity in genetically predisposed individuals. An important agenda for future studies will be to elucidate the mechanisms by which palmitoylated, immunogenic GAD65 is released from β-cells undergoing ER stress, as well as the determinants of that stress, which the results presented here suggest could be key factors in the mechanism that results in autoimmunity to GAD65 associated with development of T1D.

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