Role of iduronate-2-sulfatase in glucose-stimulated insulin secretion by activation of exocytosis

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1Endocrinology and Diabetes Unit, Laboratory of Diabetes and Obesity, Institut d’Investigacions Biomèdiques August Pi i Sunyer-Fundació Clinic, Hospital Clinic, Barcelona; 2Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas, Spain; and 3Instituto de Bioingeniería, Universidad Miguel Hernández, Elche, Spain

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Piquer S, Casas S, Quesada I, Nadal A, Julià M, Novials A, Gomis R. Role of iduronate-2-sulfatase in glucose-stimulated insulin secretion by activation of exocytosis. Am J Physiol Endocrinol Metab 297: E793–E801, 2009. First published July 14, 2009; doi:10.1152/ajpendo.90878.2008. —Iduronate-2-sulfatase (IDS) is a lysosomal enzyme expressed in pancreatic islets responsible for the degradation of proteoglycans such as perlecan and dermatan sulfate. Previous findings of our group demonstrated the involvement of IDS in the normal pathway of lysosomal degradation of secretory peptides, suggesting a role of this enzyme in β-cell secretory functionality. The present study was undertaken to characterize the effect of IDS overexpression on insulin release. INS1E cells were transiently transfected with a construct encoding human IDS (hIDS). hIDS overexpression was associated with a gain of function detected by a reduction in heparan sulfate content. hIDS potentiated the glucose-stimulated insulin secretory response compared with controls (61%) with no changes in insulin mRNA levels or insulin peptide content. Results on quantification of the exocytic process showed a significant increase in hIDS-transfected cells compared with controls. Furthermore, ultrastructural analysis demonstrated an increase in the number of granules in the immediate vicinity of the plasma membrane in hIDS-transfected cells and a decrease in total vesicles per square micrometer. hIDS overexpression induced phosphorylation of protein kinase C (PKC) α and its newly myristoylated alanine-rich C kinase substrate, MARCKS. We conclude that IDS has a role in glucose-stimulated secretion via a mechanism that involves the activation of exocytosis through phosphorylation of PKCα and MARCKS.

exocytosis; insulin secretion; lysosome; phosphorylation; β-cell

**PANCREATIC β-CELL FAILURE** plays a critical role in the onset of diabetes. Therefore, understanding the molecular mechanisms through which β-cells respond to glucose and other secretagogues is important for the development of new therapeutic approaches to this important disease.

Our group recently discovered that the lysosomal enzyme iduronate-2-sulfatase (IDS) is expressed in pancreatic islets and that its expression is regulated by glucose (5). IDS is an enzyme involved in the degradation of glycosaminoglycans (EC 3.1.6.13). Its activity is based on the removal of the 2-sulfate group of the L-iduronate 2-sulfate units of dermatan sulfate proteoglycan 3 and heparan sulfate proteoglycan 2 (HSPG2), also known as perlecan (9). HSPGs are proteoglycans with heparin sulfate chains attached to the core protein. Some, such as glypican, are present on the cell surface, and others are present in the basement membrane and extracellular matrix like perlecan, which has been identified in islet cells (10).

Proteoglycans are important components of the extracellular matrix and cell surface. A variety of functions have been recognized for HSPGs, including cell signaling, mediation of growth factor effects, directions of neurite outgrowth, and receptor internalization for various ligands (4). Although the study of HSPGs has mainly focused on the function of heparin sulfate chains, more recently, attention has been paid to the role of the proteoglycan core protein. In the case of syndecan-4, the transmembrane domain of the protein is responsible for the assembly of syndecan-4 tetramers with phosphatidylinositol 4,5-bisphosphate (PI_{2}) and activated protein kinase Cα (PKCα) (3). Recently, α-actinin has also been shown to bind to the cytoplasmic region of syndecan-4. Studies with *Caenorhabditis elegans* have identified several signaling molecules associated with the cytoplasmic domain of perlecan, such as ILK, through which perlecan can regulate vinculin and thus influence α-actinin polymerization on actin filaments (15), a process that is related with secretory granule transport and exocytosis.

Although glucose metabolism is the primary initiator and regulator of most pancreatic β-cell functions, including those related to the production, storage, and exocytosis of insulin, many proteins and signaling pathways are involved in the regulation of all these processes (17). PKC is a member of a family of Ser Thr phosphotransferase, which plays an important role in many intracellular signaling events, cell growth, and differentiation. Several isoforms of PKC have been identified in pancreatic β-cells, and many have been reported to undergo translocation after stimulation by glucose. Among them, PKCα, -ε (22), and -λ (8) have been shown to have a role in glucose-induced insulin release, particularly in the last steps of secretion (11, 21). One of the newly described substrates for PKC is the myristoylated alanine-rich C kinase substrate (MARCKS), an acidic myristoylated protein that has been implicated in several cellular processes such as motility, mitosis, cytoskeleton dynamics, phagocytosis, and cell transformation (20). In intact and digitonin-permeabilized chromaffin cells, MARCKS phosphorylation is accompanied by cortical F-actin disassembly and enhancing of secretion (16).

Because IDS is expressed in pancreatic β-cell in a glucose-inducible manner, we further explored its function by overexpression of human IDS (hIDS) in INS1E cells, which resulted in an increase in glucose-induced insulin secretion. The present work is focused on examining the role of IDS in the regulation of some of the several processes that participate in insulin release. Different approaches as ultramorphological analysis, quantitative real-time PCR, monitoriza-

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tion of exocytotic responses, measurement of intracellular Ca^{2+}, and phosphorylation assays indicate that IDS plays a role in glucose-stimulated insulin secretion by a mechanism that involves the phosphorylation of PKCα and the subsequent activation of exocytosis.

**MATERIALS AND METHODS**

**Materials.** Tissue culture media and supplements were from Bio-
sera (East Sussex, UK) and Biowhitaker (Cambrex Bio Science, Verviers, Belgium). Antibodies against phospho-MARKCS (Ser152/156), phospho-PKCα/β-II (Thr638/641), and PKCα were purchased from Cell Signaling (Beverly, MA). Anti-hIDS antibody was obtained from R&D systems (Minneapolis, MN). MARCKS antibody was from Calbiochem (Nottingham, Nottinghamshire, UK). Heparan sulfate was from Seikagaku (Chuo-ku, Tokyo, Japan). Perlecan antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Myc-tag antibody was obtained from Upstate (Lake Placid, NY). Actin was purchased from Sigma (Sigma-Aldrich). Horseradish peroxidase-conjugated sec-
ondary antibody was from Amersham Biosciences (Freiberg, Ger-
many). Biochemical reagents were from Sigma, unless otherwise indicated. All other chemicals were of analytical grade.

**Human islet isolation.** Pancreases were obtained from three human cadaveric organ donors after informed consent of their families and approval of the Hospital Ethics Committee. Islets were isolated by collagenase digestion of the pancreas (SERVA Electrophoresis, Heidelberg, Germany) and separated from exocrine tissue by Biocoll density gradient (Biochrom, Berlin, Germany), as previously de-
scribed (13).

**Cell culture.** INS1E cells (passages 80–90) were cultured in a humidified atmosphere containing 5% CO_{2} in a medium composed of RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% mercaptoethanol.

**Plasmid construction.** The DNA sequence encoding the hIDS (EMBL/GenBank acc. no. NM 000202) was amplified by PCR using the Pfu enzyme (Stratagene, Amsterdam, The Netherlands), and the primers hIDS/Xbal (forward: 5′-AATCTATCTAGAACCATGCGCAG-
CACCCCAGA-3′) and hIDS/ApaI (reverse: 5′-ACTAGGGCCCCAG-
GCATCAACACTGG-3′) were designed to create Xbal and ApaI sites at 5′- and 3′-tails, respectively. The PCR-generated fragment was digested with Xbal and ApaI and subcloned directly into pBluescript II KS (+/−) vector (Stratagene). The 3′-end of hIDS was then fused in-frame with the 5′-end of a c-myc epitope tag. Correct fragment insertion was confirmed by automatic sequencing. hIDS c-myc was finally subcloned into pcDNA 3.1/Hygro (−) (Invitrogen, Paisley, UK).

**Transfection procedure.** Cells were plated on culture dishes that included 20-mm glass cover slips on the bottom. Plasmid hIDS c-myc pcDNA 3.1/Hygro(−) and pcDNA 3.1/Hygro(−) were transfected into INS1E cells using lipofectamine2000 (Invitrogen) following the manufacturer’s instructions. Treatments were performed after 72 h in culture. Cover slips were removed before culture harvesting.

**Immunofluorescence and transfection efficiency.** Cells were fixed and permeabilized with 4% (vol/vol) paraformaldehyde and 0.1% (wt/vol) BSA-0.1% (vol/vol) Triton X-100, respectively. Cells were then treated in blocking solution containing 1% BSA in PBS, incubated with anti-myc tag antibody (1:35) overnight at 4°C, and then with an anti-mouse Cy2 antibody (1:7,000) in blocking solution for 2 h in the dark. Cells were washed with PBS between incubations and mounted on cover slips with Mowiol (Calbiochem) before analysis. Immunostaining results were analyzed in a Zeiss LSM510 confocal laser microscope with an oil immersion ×63/numerical aperture 1.3 objective.

**Before experiments, removed cover slips from cell culture were used to estimate transfection efficiency. Transfection optimization to ensure maximum plasmid uptake was performed by immunofluoros-
cence against anti-myc tag antibody following the above-described protocol. Cover slips from cultures treated with pcDNA 3.1/Hygro(−) or hIDS c-myc pcDNA 3.1/Hygro(−) were used as negative or positive control, respectively. Transfection efficiency expressed as the percentage of green fluorescent-positive cells was 86±3% (mean±SE from 6 independent experiments).

**Transmission electron microscopy.** For cellular ultrastructural anal-
ysis, transfected INS1E cells were fixed in 2.5% glutaraldehyde (wt/vol) in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1.5 h, rinsed in 0.1 M phosphate buffer, and postfixed in 1% osmium tetroxide (wt/vol) in the same buffer for 1 h. The samples were dehydrated in an ascending series of acetone concentrations, embedded in Spurr resin, and polymerized at 60°C for 48 h. Ultrathin sections (60–90 nm) were cut using a Reichert-Jung Ultracut E ultramicrotome. They were then placed on 200 mesh copper grids and double stained with uranyl acetate (2%) and lead citrate. The grids were examined using a Jeol 1010 transmission electron microscope (Jeol-USA) operating at an accelerating voltage of 80 kV. Only INS1E cells containing liposomes on the cytoplasm were evaluated.

**Analysis of cell viability.** Cells were stained with propidium iodide (PI) (Becton-Dickinson, San Jose, CA) following the manufacturer’s instructions. Thereafter, cells were fluorescence-activated cell sorter (FACS) analyzed within 1 h on FACS Calibur (Becton-Dickinson) with Cell Quest software (Becton-Dickinson). FACS gating based on forward and side scatter of 100,000 cells was included for analysis. PI negative cells are viable cells.

**Western blot analysis.** Total lysates were subjected to SDS-PAGE on polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with selected antibodies; the immunoblots were developed using an enhanced chemiluminescence detection system (Amersham Biosciences), following the manufacturer’s instructions. Changes in protein levels were evaluated by Quantity One software (Bio-Rad Laboratories, Hercules, CA).

**Quantiﬁcation of total heparan sulfate.** Total protein lysates were used to quantify heparan sulfate content by a heparan sulfate enzyme-linked immunosorbent assay (ELISA) kit (Seikagaku).

**Insulin secretion assay.** A total of 0.9 × 10^{6} cells were seeded and immediately transfected in six-well plates. After 72 h of culture, the medium was removed, and cells were washed one time with RPMI glucose, glutamine, and FBS-free medium and preincubated in the same medium at 37°C in a 5% CO_{2}-95% air atmosphere for 2.30 h. Thereafter, the cells were washed one time with glucose-free KRB [150 mM NaCl, 6.6 mM KCl, 3.3 mM CaCl_{2}, 1.3 mM MgCl_{2}, 32 mM NaHCO_{3}, 0.1% BSA, and 25 mM HEPES (pH 7.4), equilibrated with 5% CO_{2}, preincubated in the same buffer for 30 min, and incubated for an additional 30 min with KRB containing different concentrations...
of glucose (0, 2.5, and 22.7 mM). After incubation, the supernatant was collected to measure insulin release, and INS1E cells remaining in the wells were extracted with an acid-ethanol solution to measure insulin content. Insulin was determined by radioimmunoassay (Insulin-CT; CIS bio International, Gif-Sur-Yvette Cedex, France).

Quantitative real-time PCR. Total RNA was extracted and purified by the TRIzol reagent method (Invitrogen) following standard protocols. cDNAs were synthesized using a First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche, Manheim, Germany) following the manufacturer’s instructions. The primers INS1 (forward: 5'-TCTTCAGACCTGGACTGGA-3', reverse: 5'-GCTGGTGCACGCATGATCC-3'), INS2 (forward: 5'-TGACCTTCAGACCTGGACTGGA-3', reverse: 5'-ATGCTGGTGCACGCATGATCC-3'), and Taqman probe (CCCTGCAAGCCTGGATTTGT) were selected with Primer Express software (Applied Biosystems, Foster City, CA). 18S rRNA (Applied Biosystems) was used to normalize insulin expression. Amplifications were carried out using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Results were expressed as means ± SE of five to eight independent experiments.

Monitorization of exocytic response. Exocytotic response was monitored at the single-cell level in a dynamic manner using the styryl dye FM1-43 (18). Cells were incubated with 2 μM of FM1-43 for 10 min. This probe was maintained continuously throughout the experiment in a medium containing (in mM) 115 NaCl, 10 NaHCO3, 5 KCl, 1.1 MgCl2, 1.2 NaH2PO4, 2.5 CaCl2, 25 HEPES, and 5 d-glucose, pH = 7.4. All experiments were carried out at 37°C. For imaging FM1-43 fluorescence, cells were excited at 488 nm, and emission was obtained with a 560-nm long-pass filter. Fluorescence was monitored using a 10× oil immersion objective (numerical aperture 1.3) of a Zeiss LSM 510 confocal microscope. Temporal series were filtered with a spatial low-pass filter and processed using the digital image software of the confocal microscope. The changes in fluorescence over time of each individual cell were expressed in percentage as ΔF/F0, where ΔF is the difference between the fluorescence with the

![Fig. 2. hIDS overexpression in INS1E cells.](http://ajpendo.physiology.org/)

A: immunofluorescences against c-myc and confocal microscopy of hIDS c-myc-expressing cells. No staining was found in INS1E cells transfected with the empty vector [pcDNA 3.1/ Hygro(-)] alone, whereas robust staining was detected in INS1E cells transfected with hIDS c-myc (left). Immunoblot analyses using c-myc antibody were carried out to reconfirm successful transfections (right). B: morphological analysis using electron microscopy of hIDS INS1E transfected cells with respect to controls. Magnified images (bottom) confirmed no differences in organelle morphology between hIDS- and pcDNA-transfected cells (bar = 0.5 μm). Orange and yellow arrowheads indicate mitochondria and Golgi, respectively. C: analysis of cell viability was performed on INS1E cells transfected with hIDS or pcDNA. Cells were stained with propidium iodide and analyzed by fluorescence-activated cell sorter analysis. Negative cells were considered viable (n = 4; not significant). D-F: analysis of perlecan and heparan sulfate content. Protein extracts from hIDS- and pcDNA-transfected cell lysates were analyzed by Western blot with heparan sulfate antibody to detect the 470-kDa (D) and the 15- to 20-kDa (E) fractions. Bottom: representative blots including actin (loading control) (Images are representative of 3 independent experiments). F: quantification of total cellular heparan sulfate content in hIDS (filled bars) - and pcDNA (open bars) - transfected cells (n = 5–6; *P < 0.05).
stimulus and the background signal (fluorescence without stimulus, $F_0$). Each individual fluorescence record represented the averaged changes of fluorescence along the whole membrane of a single cell. The area under the curve of the different FM1-43 records was analyzed during the response to the stimulus using commercial software (Sigmaplot; Jandel Scientific).

**Fig. 3.** Influence of hIDS on insulin secretion. A: hIDS (filled bars)- and pcDNA (open bars)-transfected cells were used to assess insulin secretion. Insulin release was expressed as the ratio of the percentage of cellular insulin content ($n = 8–10$; $\dagger P < 0.005$ vs. pcDNA 2.5 mM glucose, $* P < 0.05$ vs. pcDNA 22.7 mM glucose). B and C: effect of hIDS on insulin expression. B: insulin content was measured by ELISA and expressed with respect to total protein in hIDS (filled bars)- and pcDNA (open bars)-transfected cells cultured at 11.1 mM glucose ($n = 11$). C: analysis of insulin (INS1 and INS2 mRNA expression by quantitative real-time RT-PCR in hIDS (filled bars)- and pcDNA (open bars)-transfected cells cultured at 11.1 mM glucose ($n = 5–8$). mRNA levels are normalized to 18S rRNA expression.

**Fig. 4.** Exocytotic responses in hIDS-transfected cells at the single-cell level measured by FM1-43. A: transmitted light image of a group of INS1E hIDS cells (top) and the corresponding FM1-43 fluorescence images immediately before application of 22 mM glucose (middle) and 15 min later (bottom). Arrows indicate several areas with a higher fluorescence increase. B: temporal course of FM1-43 fluorescence from single cells after stimulation with 22 mM glucose. Two representative records from a control (open circles) and a hIDS-transfected cell (filled circles) are shown. Each trace represents in percentage the increase in fluorescence ($\Delta F$) with respect to the basal fluorescence ($F_0$) of the averaged changes in the cell membrane of one cell. Analysis of both the FM1-43 fluorescence increase at the end of the experiment (C) and the area under the curve over the whole record (D) in hIDS (filled bars)-transfected cells with respect to control (open bars). Data in panel C and D are shown as means ± SE ($n = 30$ cells for each condition from at least 3 experiments; $* P < 0.001$).
room temperature. After loading, cells were washed with the extracellular solution used during experiments (in mM) 115 NaCl, 10 NaHCO3, 5 KCl, 1.1 MgCl2, 1.2 NaH2PO4, 2.5 CaCl2, 25 HEPES, and 5 d-glucose, pH = 7.4). Fura 2-loaded cells were imaged by using an inverted epifluorescence microscope (Axiovert 200; Zeiss, Jena, Germany). A ratio image was acquired approximately every 3 s with an ORCA-100 CCD camera (Hamamatsu Photonics Iberica, Barcelona, Spain) by using a Lambda-10-CS dual filter wheel (Sutter Instrument), equipped with 340- and 380-nm bandpass filters, and a range of neutral density filters (Omega Optical, Stanmore, UK). Data were acquired by using the Aquacosmos version 2.0 from Hamamatsu. Fluorescence records are expressed as the ratio of fluorescence at 340 and 380 nm. The area under the curve in each Ca2+ record was analyzed using commercial software (SigmaPlot; Jandel Scientific) after obtaining the increment of fluorescence between the fluorescence levels obtained during the stimulus and the basal fluorescence in the absence of stimulus (1). The area under the traces was calculated during 8 min from the initial fluorescence increase in response to the stimuli (1). Data obtained with this analysis shows the integrated response of the Ca2+ response during the stimulation and is an indicator of the global Ca2+ increase in the cells (1).

**Quantification of insulin vesicles.** Electron microscopy images were analyzed using Leica QUPS software (Leica Imaging Systems, Cambridge, UK). The number of vesicles per square micrometer and the percent vesicles in membrane with respect to the total were quantified by a researcher blind to the status. Fifty cells were selected randomly and included in the analysis.

**Phosphorylation assays.** Cells were deprived of glucose, glutamine, and FBS for 2.30 h, preincubated in glucose-free KRB for 30 min, and incubated with 22.7 mM glucose for different times. Western blot were performed as previously described, and protein bands were quantified using Image Gauge V4.0 (Fujifilm; Fuji Photo Film). Results were expressed as means ± SE of three independent experiments.

**Statistical analysis.** Results were expressed as means ± SE from at least three different experiments performed independently. Statistical analysis was performed by the paired two-tailed Student’s t-test and a P value of <0.05 was considered statistically significant.

**RESULTS**

**IDS expression on human islets.** Previous results reported by our group demonstrated *Ids* expression in mouse pancreatic islets (5). To assess *IDS* gene expression in human islets, we performed in situ hybridization analysis. IDS mRNA was detectable in islet cells [Supplemental Fig. 1 (Supplemental data for this article may be found on the American Journal of Physiology: Endocrinology and Metabolism website.)]. IDS expression in human islets was studied by Western blot analysis of human pancreatic islet extracts and exocrine tissue samples. As shown in Fig. 1, IDS expression is mostly restrictedly observed in human pancreatic islets.

**Characterization of hIDS-overexpressing cells.** The presence of hIDS on INS1E-transfected cells was evaluated in each experimental run by confocal microscopy (transfection efficiency 86 ± 3%) and confirmed by Western blot analysis using a mouse monoclonal antibody against the c-myc tag (Fig. 2A) and by quantitative real-time RT-PCR (Supplemental Fig. 2). Transfected cells (containing liposomes on the cytoplasm) showed normal ultrastructural cell morphology (Fig. 2B). Moreover, cellular viability analysis revealed no differences between hIDS c-myc- and pcDNA-expressing cells (Fig. 2C). At this point, we performed different approaches to analyze the functionality of the transfected enzyme. The content of heparan sulfate chains on hIDS c-myc- and pcDNA-transfected cells was evaluated by Western blot analysis. hIDS overexpression resulted in a decrease of heparan sulfate expression (Fig. 2D); this was confirmed by the quantification of heparan sulfate using ELISA (Fig. 2E). Taken together, these data suggested that the hIDS-transfected enzyme is fully functional. To corroborate these results, we performed a Western blot using the heparan sulfate antibody to focus on the 470-kDa protein band, the predicted molecular weight for the perlecan molecule with a reduced number of heparan sulfate chains. The mature perlecan protein with heparan sulfate chains has a predicted molecular mass of 800 kDa. We observed an increase in the 470-kDa perlecan fraction in hIDS-overexpressing cells with respect to controls (Fig. 2F). Perlecan expression was confirmed by PCR (Supplemental Fig. 3).

**hIDS overexpression leads to an enhancement of glucose-stimulated insulin secretion.** All of these data prompted us to investigate whether these changes could affect β-cell functionality. Static insulin secretion assays showed a significant increase of ~61% in 22.7 mM glucose-induced insulin secretion in hIDS-transfected cells compared with controls, whereas basal insulin secretion was not affected (Fig. 3A). To investigate the mechanism by which insulin secretion was modified, we studied insulin expression in INS1E with overexpression of hIDS compared with controls. No changes in insulin 1 and 2 mRNA levels were found (INS1: 3.76 vs. 3.54, INS2: 4.32 vs. 4.01, arbitrary
units; not significant) (Fig. 3B), and insulin peptide content was unaffected (784.47 vs. 846.39 ng INS/mg total protein; not significant) (Fig. 3C). These results indicated that the increase in insulin secretion was not due to an effect of hIDS on insulin expression.

Detection of higher exocytotic response at the single-cell level in hIDS-transfected cells. To examine whether exocytosis is enhanced in individual hIDS-transfected cells, we monitored this process at the single-cell level by confocal microscopy and measurements of FM1-43 fluorescence (18). This cell-impermeable styryl dye is a nonfluorescent probe in aqueous solution that emits intense fluorescence upon partition into the plasma membrane. Thus the incorporation of secretory granules to the plasma membrane during secretion increases fluorescence (18). The temporal course of FM1-43 fluorescence from single cells after stimulation with 22 mM glucose showed a significant increase in fluorescence compared with basal values (ΔF/F₀) in hIDS-transfected cells from 10 min onward with respect to controls (Fig. 4). Quantification of both the average fluorescence increases at the end of the record and the area under the curve (Fig. 4, C and D) also indicated a higher exocytotic response in hIDS-expressing cells compared with controls. Given that Ca²⁺ signals play an important function in the stimulation of exocytosis, we measured free intracellular Ca²⁺ concentrations in fura 2-loaded INS1E cells (Fig. 5). Although a very small difference in basal Ca²⁺ values was found in hIDS-transfected cells (Fig. 5C), this did not influence basal insulin secretion (Fig. 3A) (19). Glucose stimulation did not produce significant differences in the Ca²⁺ values between either group (Fig. 5D), and the addition of tolbutamide to completely block ATP-dependent K⁺ (K_ATP) channels induced similar Ca²⁺ responses. Given that the secretion response to both stimuli is increased in hIDS-transfected cells compared with controls.

Fig. 6. Electron micrographs of hIDS-transfected cells. Analysis of transmission electron micrographs of hIDS INS1E-transfected cells compared with controls shows an increased number of vesicles at the membrane surface (A-D). Electron microscopy images of pcDNA (A)- and hIDS (B-D)-transfected cells. D: magnified image of the selected area in C showing an increased number of vesicles in close proximity to the plasma membrane. E: bar graph of the quantitative analysis showing the increased number of vesicles at the membrane with respect to the total in hIDS-transfected cells (n = 50; *P < 0.01). F: bar graph showing a decrease in the total number of vesicles present/μm² in hIDS-transfected cells (n = 50; *P = 0.024).
(Fig. 3 and Supplemental Fig. 4), these findings suggest that the effect of hIDS on insulin secretion does not result from increased intracellular Ca\(^{2+}\) signaling because of changes in the K\(_{\text{ATP}}\)-dependent stimulus-secretion coupling.

Ultrastructural evidences of higher exocytotic response in hIDS-transfected cells. Ultramorphological analysis suggested that a higher number of granules in the immediate vicinity of the plasma membrane could contribute to the increased capacity of hIDS-transfected cells (Fig. 6, A–D). Quantitation of vesicles demonstrated an increase in this value in hIDS-transfected cells (68.66 vs. 59.97%, \(P < 0.01\)) and a decrease in total vesicles per square micrometer (0.82 vs. 0.62, \(P < 0.05\)) compared with control cells (Fig. 6, E and F). Measurement of vesicle size showed no differences (data not shown). Thus the enhanced secretory capacity can be attributed, at least in part, to an increased availability of granules close to the membrane. This finding is important since these secretory granules may form the ready releasable pool (11). Although the majority of secretory granules require multiple steps to gain release competence, this pool close to the plasma membrane is immediately available for exocytosis upon stimulation without further modification. Thus the ready releasable pool may account for the first phase of insulin release, which determines the magnitude of the initial secretory response (11).

Involvement of PKC on hIDS-induced insulin exocytosis. Because the remodeling of the actin filament network has been associated with changes in exocytosis, we also studied the possible effect of hIDS overexpression in those processes. MARCKS, a substrate of PKC, has been identified as a regulator of actin cytoskeleton dynamics (20). PKC phosphorylation as well as changes in total PKC expression levels were studied by Western blot analysis, which showed no differences between hIDS-transfected and control cells (Supplemental Fig. 5, A and B). On the other hand, it is known that PKC\(\alpha\) can bind to the COOH-terminal intracellular domain of proteoglycans (3). Although no changes were observed in total PKC\(\alpha\) expression levels (Supplemental Fig. 5D), a significant increase in PKC\(\alpha/\beta{II}\) phosphorylation was detected in hIDS-transfected cells with respect to controls (Fig. 7A). Interestingly, enhanced PKC\(\alpha/\beta{II}\) phosphorylation was already identified in the non-glucose-stimulated condition and was significantly sustained for at least 5 min after the 22.7 mM glucose stimuli (Fig. 7A). Moreover, a Western-blot analysis against phospho-MARCKS revealed an increased phosphorylation of this PKC\(\alpha/\beta{II}\) substrate in hIDS-transfected cells compared with controls, which was evident at 10 min after glucose stimulation (Fig. 7B).

**DISCUSSION**

This study focuses on the effect of hIDS overexpression on glucose-induced insulin secretion. We show that the enhancement observed on insulin release is due to an activation of the exocytotic process. We also demonstrated an increase in PKC\(\alpha\) and MARCKS phosphorylation, suggesting a possible role of these molecules in the enhanced secretory capacity detected.

The lysosomal enzyme IDS has been detected in several tissues, including the pancreas (6, 12). Previous findings by our group demonstrated the presence of IDS in mouse pancreatic \(\alpha\)- and \(\beta\)-islet cells (5), which we have now detected for the first time in human pancreatic islets. Moreover, antisense inhibition of IDS in \(\beta\)-cells resulted in degradation of insulin granules that are not directed to the secretory pool, suggesting an essential role of this enzyme in the maintenance of the pancreatic \(\beta\)-cell function (5).

Knowledge of the mechanisms of pancreatic \(\beta\)-cell stimulus-secretion coupling in response to glucose has been accumulating over several decades. After glucose stimulus, closure of the K\(_{\text{ATP}}\) channel is induced, and depolarization of the \(\beta\)-cell causes Ca\(^{2+}\) entry via voltage-dependent Ca\(^{2+}\) channels, which triggers the exocytosis of a small pool of granules that is responsible for the first phase of insulin release. It is accepted that the second phase of insulin release is the result of the K\(_{\text{ATP}}\) channel-independent pathways, and several hypotheses have been put forward to explain the mechanisms involved (19). Here we showed an increase in insulin secretion in response to a 22.7 mM glucose stimulus in INS1E cells overexpressing hIDS compared with controls, whereas no
effect was observed at basal or low glucose concentrations. The effect on insulin secretion could not be explained by an increase in insulin synthesis, since mRNA levels and insulin content were equivalent in hIDS and control transfected cells. Moreover, no significant differences were found in the intracellular Ca\(^{2+}\) responses in hIDS-overexpressing cells compared with controls in response to glucose and tolbutamide, despite the increased secretory response to these stimuli. It has been reported that increases in intracellular Ca\(^{2+}\) concentrations are required for initiation of exocytosis, but the real modulator of the process is the balance between protein phosphorylation cascades (2).

To analyze the role of IDS in glucose-stimulated insulin release, we explored the possibility in individual cells that hIDS could affect exocytotic processes. Interestingly, we found a higher exocytotic response in hIDS-expressing cells than in controls. In agreement with these results, a larger number of granules were found in the immediate vicinity of the plasma membrane in hIDS overexpressing cells compared with controls. This finding is significant given that the size of the ready releasable vesicle pool and part of the docked secretory granules, which are in close proximity to the plasma membrane, determine the magnitude of the initial secretory response (11). Interestingly, previous studies, although using different methodologies, and different strategies to deregulate IDS expression found IDS implication on the vesicular secretory pathway (5).

The present data suggest that overexpression of hIDS increases insulin release by stimulating the late stages of exocytosis processes with no effects on more proximal events such as glucose metabolism or intracellular Ca\(^{2+}\) levels. In addition to a higher number of granules close to the plasma membrane, overexpression of hIDS is likely influencing other steps involved in the exocytotic response, as explained in the next paragraph.

This study identified targets of IDS in INS1E β-cells such as perlecan and glypican 4. Those proteoglycans are important functional proteins that link extracellular matrix and plasma membrane with the cytoskeleton (4). The mechanism by which IDS stimulates exocytosis remains unknown. We found that overexpression of IDS increased the phosphorylation state of PKCo/BII, a kinase that has already been associated with glucose-induced insulin release (22), particularly at the late stages of secretion (2). PKCo has a binding and regulatory site through PIP\(_2\) within the intracellular domain of transmembrane proteoglycans (3). In agreement with these results, we also observed enhanced MARCKS phosphorylation, one of the recently described substrates for PKC (7), thus reinforcing the relevance of these findings. This result is notable since it has been reported that MARCKS phosphorylation is accompanied by a cortical F-actin disassembly (16) that has been shown to enhance insulin secretion (14). Moreover, it has been shown that perlecan can interact with vinculin regulating α-actinin polymerization on actin filaments (15). Thus our results indicate that the phosphorylation level of these enzymes is enhanced in hIDS-transfected cells. Because these proteins play an important role in the remodeling of the actin filament network, which affects the transport and access of secretory granules to the plasma membrane, their increased activity may also influence granule trafficking in these cells.

In conclusion, our studies demonstrate that overexpression of hIDS enhances glucose-induced insulin secretion in INS1E cells by activation of the exocytotic process. We attribute this burst of exocytosis to a mechanism that involves phosphorylation of PKCo and MARCKS. Because insulin secretion deficiencies are critical in the onset of diabetes, the identification of new molecules involved in the mechanism of glucose-stimulated insulin release, such as IDS, is an essential step in the search for new drug targets for diabetes treatment.

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