Human Molecular Genetics, 2009, Vol. 18, No. 3 **428–439**
doi:10.1093/hmg/ddn370
Advance Access published on November 10, 2008

Cx36 makes channels coupling human pancreatic β-cells, and correlates with insulin expression

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Previous studies have documented that the insulin-producing β-cells of laboratory rodents are coupled by gap junction channels made solely of the connexin36 (Cx36) protein, and have shown that loss of this protein desynchronizes β-cells, leading to secretory defects reminiscent of those observed in type 2 diabetes. Since human islets differ in several respects from those of laboratory rodents, we have now screened human pancreas, and islets isolated thereof, for expression of a variety of connexin genes, tested whether the cognate proteins form functional channels for islet cell exchanges, and assessed whether this expression changes with β-cell function in islets of control and type 2 diabetics. Here, we show that (i) different connexin isoforms are differentially distributed in the exocrine and endocrine parts of the human pancreas; (ii) human islets express at the transcript level different connexin isoforms; (iii) the membrane of β-cells harbors detectable levels of gap junctions made of Cx36; (iv) this protein is concentrated in lipid raft domains of the β-cell membrane where it forms gap junctions; (v) Cx36 channels allow for the preferential exchange of cationic molecules between human β-cells; (vi) the levels of Cx36 mRNA correlated with the expression of the insulin gene in the islets of both control and type 2 diabetics. The data show that Cx36 is a native protein of human pancreatic islets, which mediates the coupling of the insulin-producing β-cells, and contributes to control β-cell function by modulating gene expression.

INTRODUCTION

Gap junctions are specialized membrane domain concentrating channels that mediate direct exchanges of cytoplasmic ions and molecules between adjacent cells (1,2). Gap junction channels consist of dodecameric assemblies of transmembrane proteins called connexins (Cx), which functionally couple almost all types of vertebrate cells (1,2), including the main secretory cells of the exocrine and endocrine pancreas (3,4). In the rodent pancreas, the exocrine acinar cells are coupled by Cx26 and Cx32, whereas the insulin-secreting β-cells of pancreatic islets express Cx36, a quite different connexin isoform (3–5). Accordingly, the connexin-dependent signaling differently modulates the biosynthesis, storage and release of specific secretory products in the exocrine and endocrine pancreas (3,4).

With regard to Cx36, a large number of *in vitro* and *in vivo* studies converge to indicate an obligatory role in the proper control of insulin secretion. Thus, single β-cells, which can no more establish intercellular communication via connexin channels, show alterations in secretion and insulin gene expression, which are rapidly corrected after restoration of β-cell contacts (3–6). These alterations are mimicked by the acute exposure of either aggregated β-cells, intact islets or

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the entire pancreas to a drug, which blocks connexin channels (3–7). Further data have shown that the *in vitro* inhibition of Cx36 expression (4,8), as well as a large over-expression of this connexin, significantly perturb the secretion of insulin-producing β-cells, notably with regard to glucose-induced insulin secretion (4,8,9). These alterations are because of loss of the intercellular synchronization, which allows for the recruitment of increasing numbers of cells into both insulin biosynthesis and release (10–12), and that is normally enhanced by natural secretagogues, and particularly glucose (10,11). Accordingly, mice lacking Cx36 feature an intercellular desynchronization of glucose-induced $[Ca^{2+}]_i$ oscillations, which is associated with a loss in the normal pulsatility of insulin release, with an increased basal release of the hormone, a nil increase in insulin release in response to physiologically relevant glucose concentrations, and a delayed return of β-cells to basal secretion upon the end of such a stimulation (13–15).

Several alterations observed in Cx36 null mice, including the loss of oscillatory insulin release, increased basal secretion of the hormone and loss of response to physiologically relevant glucose concentrations, are reminiscent of the pancreatic defects reported in either prediabetic human conditions or type 2 diabetes (16–19), raising the question of whether connexin signaling may also be central to the function of human islet cells. This question cannot be answered at this time since, besides the demonstration of gap junctions in human islet cells (20), no study has yet investigated human islet cells for the expression of connexins and for the pattern of β-cell coupling that the intercellular channels made by these proteins may form. This information cannot be merely extrapolated from studies on rodents, inasmuch as growing evidence indicates significant structural and functional differences between human and rodent islets, including the expression of key membrane proteins (21–24). Most importantly, there is still no evidence of whether the expression and/or function of Cx36 is modulated with the function of human β-cells.

To address these questions, we have explored the human pancreas, and specifically its endocrine islets, for the expression of the numerous connexin genes that have been found in the human genome (1,2). We have further assessed the expression of the major endocrine Cx36 protein, and assessed the permeability of the functional channels it establishes between human islet cells. We have eventually compared whether the expression of Cx36 significantly changes with β-cell function in the islets of control and type 2 diabetics. We report that human islets and β-cells express a pattern of Cx36-dependent coupling similar to that observed in rodents, and that the expression of this connexin isoform correlates with that of the insulin gene. Data show that Cx36 is a native protein of the human β-cell membrane, likely to play a significant role in the control of insulin production and secretion.

RESULTS

Different connexins are expressed in the exocrine and endocrine human pancreas

Reverse transcriptase-polymerase chain reaction (RT–PCR) amplification of total human pancreas RNA revealed abundant transcripts for Cx32, Cx36, Cx43 and Cx45, lower levels of Cx31.9 and Cx37 mRNAs and minimal levels of Cx26 mRNA (Fig. 1). In contrast, no transcript was detected for Cx30, Cx30.3, Cx31, Cx31.1 and Cx40, although the cDNAs of these connexins were easily detectable using the same experimental conditions in the tissues (human brain, skin and heart), which were used as positive controls (Fig. 1). Using the same approach, transcripts for Cx30.3, Cx31, Cx31.1, Cx31.9, Cx36, Cx37, Cx43 and Cx45 were also amplified from human isolated islets (Fig. 1). In contrast, no transcript for Cx26, Cx30, Cx32 and Cx40 could be amplified in these samples (Fig. 1).

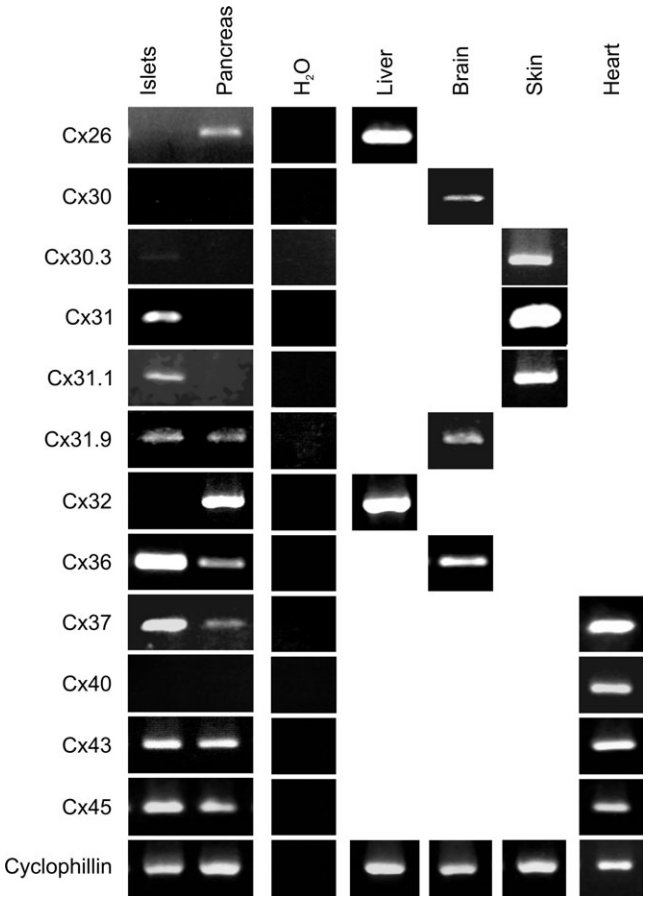

Figure 1. Different connexin transcripts are expressed in the adult human pancreas and its endocrine islets. Reverse transcriptase-polymerase chain reaction revealed the expression of Cx26, Cx31.9, Cx32, Cx36, Cx37, Cx43 and Cx45 mRNAs in intact adult human pancreas, and of Cx30.3, Cx31, Cx31.1, Cx31.9, Cx36, Cx37, Cx43 and Cx45 mRNAs in the endocrine islets, which were isolated from the gland. In all the experiments, samples of water and of human liver, brain, skin and heart served as negative and positive controls, respectively. Amplification of the cyclophillin mRNA provided an internal standard.

To determine whether the above-mentioned transcripts actually resulted in the expression of cognate proteins, we run western blots of total proteins from either intact pancreas or isolated islets with antibodies targeted to specific connexin isoforms. A 25 kDa protein corresponding to Cx26 was detected in extracts of total pancreas but not of purified islets (Fig. 2A). A 30–32 kDa protein corresponding to Cx32 was detected in total pancreas but not islet extracts (Fig. 2B), whereas a

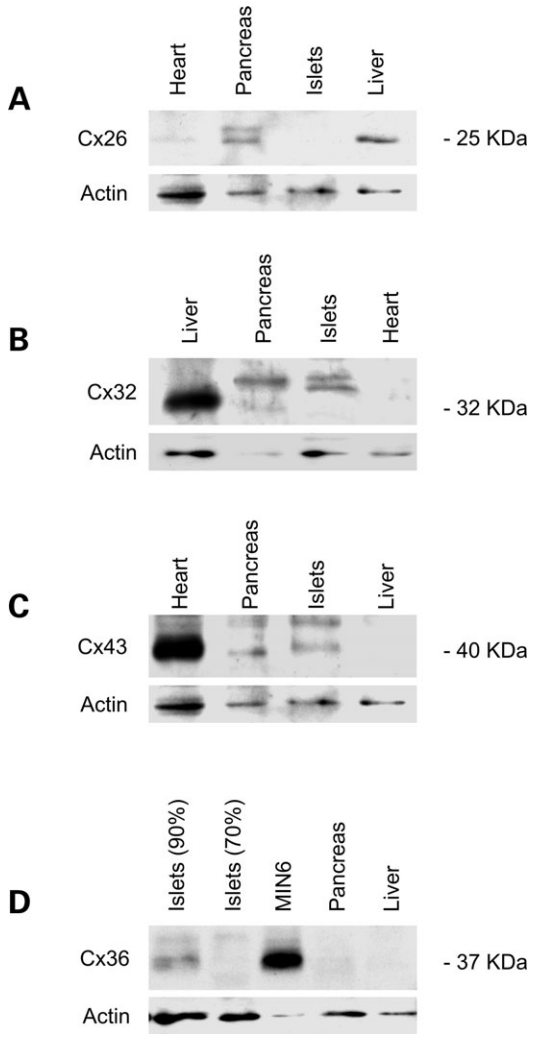

Figure 2. The major pancreatic connexins are differentially expressed in the exocrine and endocrine portions of the human gland (A–C). Western blots detected Cx26 (**A**) in extracts of total pancreas but not of purified islets. A faint 32 kDa band, corresponding to Cx32, was also detected in total pancreas, but not isolated islets (**B**). Cx43 (**C**) were found in both total pancreas and islet extracts. (**D**) In contrast, antibodies detected Cx36 in extracts of purified islets but not total pancreas. More Cx36 was detected in highly purified (90%) than in poorly purified islets (70%). Twenty micrograms of protein were loaded per lane. Immunolabeling of α-smooth muscle actin served as loading reference. Extracts of human liver and heart, as well as of the insulin-producing MIN6 cells served as positive and negative controls, depending on the connexin isoform.

38–43 KDa protein corresponding to Cx43 was detected in both organs (Fig. 2C). In contrast, antibodies to Cx36 detected a 29–30 kDa band in extracts of purified islets but not total pancreas (Fig. 2D). These data show that multiple connexins are expressed in the human pancreas, in a pattern that differs in the exocrine and endocrine portions of the gland. Thus, whereas Cx26 and Cx32 are major connexins of the exocrine region, Cx36 is the prominent connexin of endocrine islets.

Cx36 is expressed by pancreatic β-cells

To evaluate which cell type expressed Cx36 within pancreatic islets, we first dispersed islet cells, transduced them with a rat insulin promoter-green fluorescent protein (RIP-GFP) construct and FACS-purified the GFP-expressing β-cells from the GFP-negative non-β-cells. Using RT–PCR, we found that the cells of the GFP-positive fraction expressed Cx36, but no Cx26, Cx32, Cx43 and Cx45 (Fig. 3A). In contrast, the cells of the GFP-negative fraction co-expressed Cx43 and Cx36 but no Cx26, Cx32 and Cx45 (Fig. 3A). These data indicate that islet β- and non-β-cells express different connexin patterns.

Since these data also indicated that Cx36 may be the only connexin expressed by pancreatic β-cells, we immunostained the protein in sections of control human pancreas. Using peroxidase-conjugated antibodies, modest levels of the connexin were observed in most of the cells making the islets (Fig. 3B). The staining had the characteristics of specificity, inasmuch as it was not seen when the sections were incubated with a pre-immune serum and, in the presence of antibodies to Cx36, was restricted to cells containing insulin, and absent in islet cells lacking the hormone, as well as in the islet vessels (Fig. 3B). Using fluorochrome-tagged antibodies, most Cx36 was shown to be distributed in small, discrete spots, all along the membrane of the insulin-containing β-cells (Fig. 3C). These data show that, in the human pancreas, Cx36 is selectively expressed by differentiated β-cells.

β-Cells form Cx36 gap junctions in selected domains of their membrane

To ascertain the insertion of Cx36 into β-cell membranes, we immunolabelled the protein in western blots of total extracts and purified membranes of isolated pancreatic islets. Cx36 was detected in the two fractions at comparable levels (Fig. 4A). However, whereas a single band was labelled in total cell extracts, two bands were stained by the antibodies against Cx36 in the purified membrane fraction (Fig. 4A).

To specify the membrane domain containing Cx36, a cold lysate of human islets was subjected to a discontinuous sucrose density gradient centrifugation. We observed that most Cx36 was contained in fractions 4–7, which were enriched in lipid rafts as judged by the presence of the ganglioside GM1 marker (Fig. 4B). In contrast, only minimal amounts of Cx36 were found from fraction 8 (Fig. 4B) onwards, which contained marker proteins of the Golgi apparatus, lysosomes and mitochondria. Comparable observations were made concerning Cx43 (Supplementary Material, Fig. S1).

To assess whether the Cx36 inserted into β-cell membranes properly packed into gap junction plaques, freshly isolated human islets were processed for freeze-fracture electron microscopy. Analysis of the membrane of islet cells containing numerous secretory granules, revealed the presence of typical trans-membrane connexin aggregates (Fig. 4C), whose spotted distribution was consistent with that of the Cx36 immunofluorescence labeling. In most cases, these plaques were tightly intermingled with the fibrils of focal tight junctions (Fig. 4C). These data show that Cx36 is inserted into selected domains of the β-cell membrane, presumably lipid rafts, where it forms gap junction plaques.

Human β-cells are coupled by Cx36 channels

To assess whether the Cx36 expressed at the membrane of β-cells formed functional cell-to-cell channels, individual

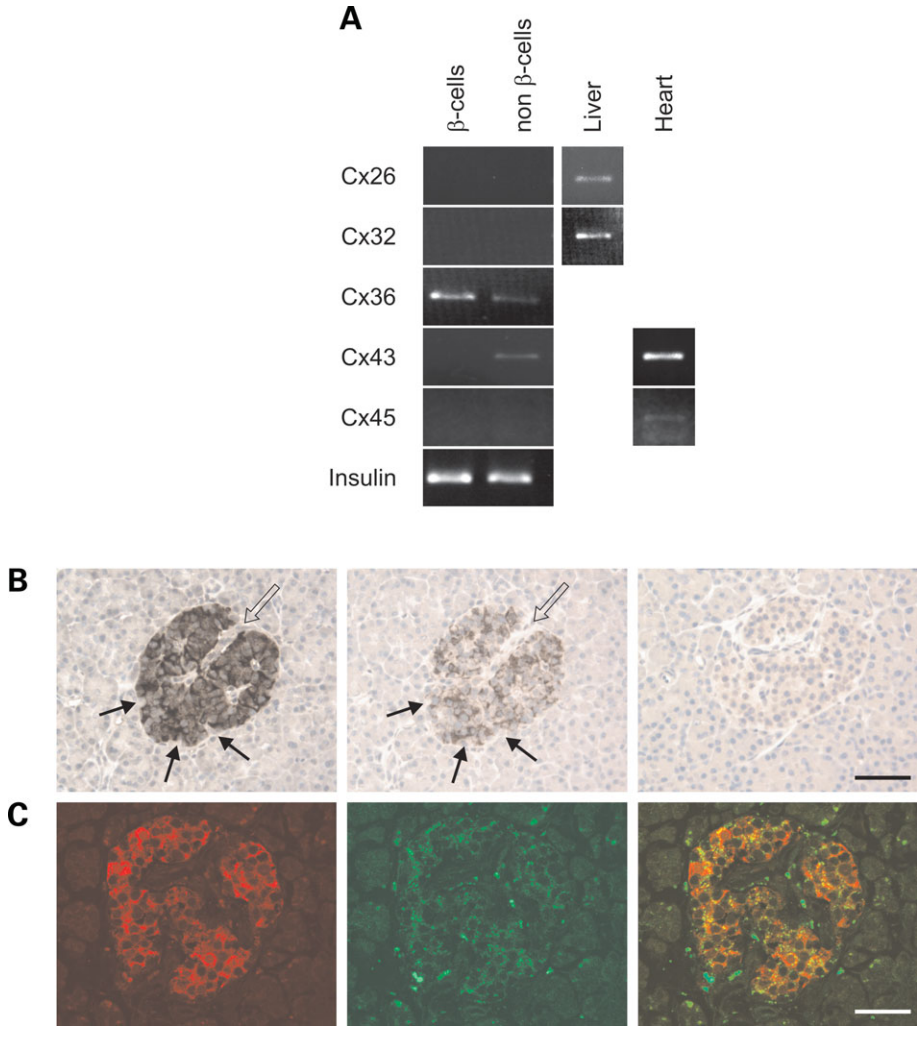

Figure 3. Cx36 is expressed by pancreatic β-cells. (**A**) Reverse transcriptase-polymerase chain reaction (RT–PCR) amplification of total RNA showed expression of Cx36 in FACS-purified and green fluorescence protein (GFP)-expressing human β-cells, and of both Cx36 and Cx43 in the GFP-negative non-β-cells. Neither fraction expressed Cx26, Cx32 and Cx45. Because of the high sensitivity of the non-quantitative RT–PCR approach, some insulin transcript was detected in both cell fractions, owing to the unavoidable contamination of the non-β-cell fraction by a limited number of small size β-cells. (**B**) Immunoperoxidase staining of a section of a control human pancreas showed that several cells containing abundant insulin (left panel) also expressed significantly lower levels of Cx36, whereas islet cells lacking detectable immunolabeling for insulin (solid arrows) as well as cells of islet vessels (open arrow) lacked Cx36 (middle panel). No islet cell was stained after incubation with non-immune IgGs (right panel). Bar, 50 μm. (**C**) Immunofluorescence showed that most Cx36 was distributed as small, discrete spots (green; middle panel) all along the membrane of the insulin-containing β-cells (red; left panel). The right panel is the merge of the left and middle panels showing the location of Cx36 in β-cells (yellow). Bar, 30 μm.

cells of human islets freshly isolated from normoglycemic, multi-organ cadaveric donors were microinjected with either Lucifer Yellow (LY) or Ethidium Bromide (EB), two tracers that do not permeate the membrane of living cells. In approximately 76% of the cases, this microinjection resulted in the diffusion of the tracer from the injected cell into some of its neighbors, whether LY or EB was used (Fig. 5A). On the average, however, LY revealed a smaller ($P < 0.001$) intercellular diffusion than EB, corresponding to an estimated number of 1.4 ± 0.2 ($n = 25$) and 5.8 ± 0.9 ($n = 25$) cells, respectively (Fig. 5B), as expected for junctional channels made of Cx36. Analogous observations were made in the islets of the single type 2 diabetic donor that we could study immediately after isolation (data not shown). The data document that Cx36 forms functional channels between adjacent islet cells.

The expression of Cx36 parallels that of the insulin gene

To evaluate whether the expression of Cx36 changes with β-cell function, total RNA was obtained from islets freshly isolated from multi-organ cadaveric donors that were either normoglycemic and without a history of overt diabetes ($n = 10$) or that were known to be type 2 diabetics ($n = 11$). Clinical information on these individuals is given in the Materials and Methods section. Quantitative PCR (qPCR) revealed that, relative to the levels of cyclophilin mRNA, the levels of Cx36 mRNA varied widely in both the control and the diabetic samples (Fig. 6), which we obtained from co-authors in Pisa and Philadelphia, preventing a reliable assessment of any difference between the healthy and the diseased group of patients. Strikingly, however, the expression of the Cx36

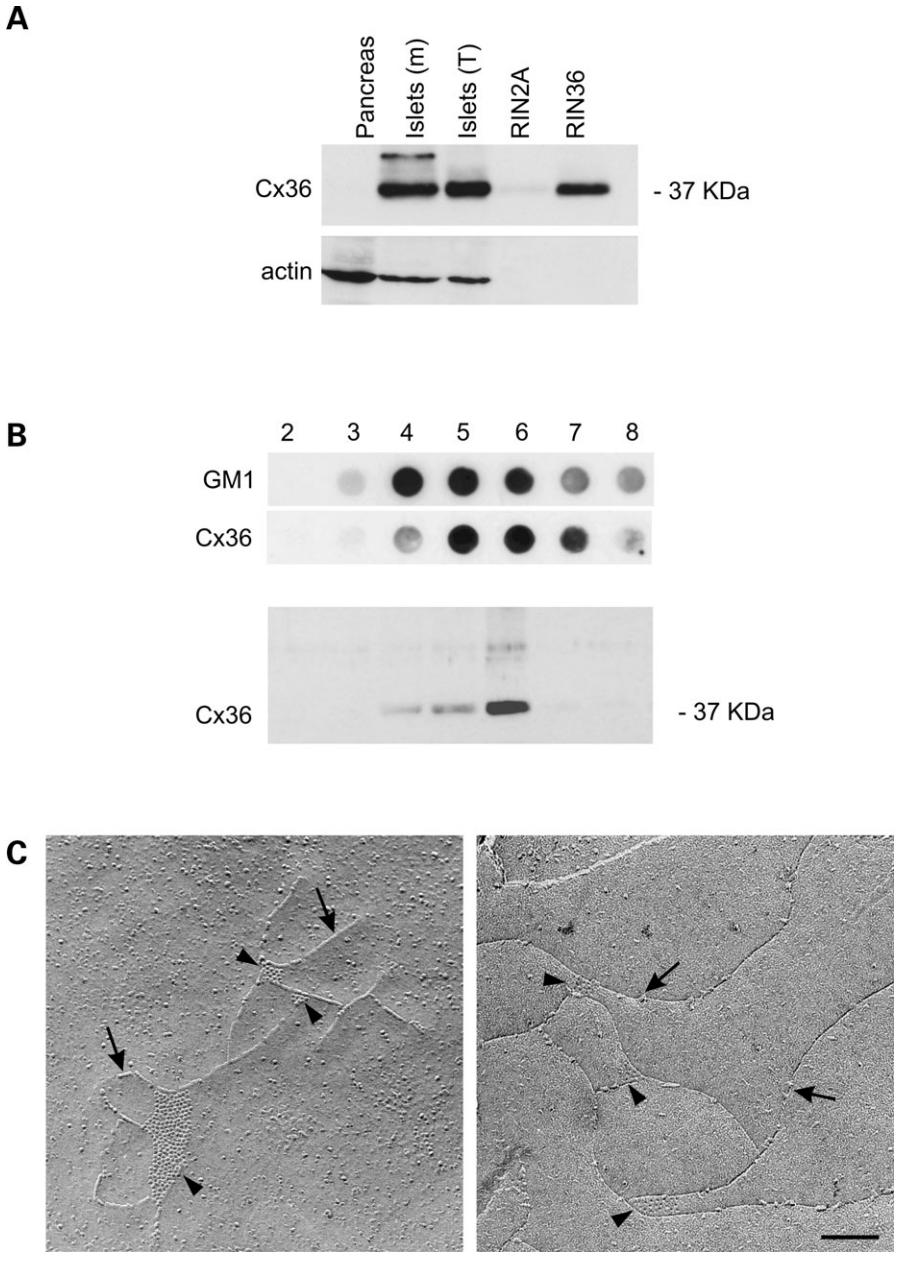

Figure 4. β-Cells form Cx36 gap junctions in lipid rafts of their membrane. (**A**) Western blots detected Cx36 in extracts of both purified islet cell membrane (m) and total isolated islets (T), but not in extracts of total pancreas. Forty micrograms of protein were loaded per lane. Immunolabeling of α-smooth muscle actin served as loading reference. Extracts of wild type (RIN2A) and Cx36-transfected RIN2A insulin-producing cells (RIN36) served as negative and positive controls, respectively. (**B**) Discontinuous sucrose density gradient centrifugation of a Triton X-100 lysate of human islets showed that most Cx36 (middle panel) was recovered in fractions of 4–7, which were enriched in lipid rafts as judged by the presence of the GM1 ganglioside (top panel). The bottom panel shows the western blot with an antibody against Cx36. Fraction 2 = 35% sucrose; fraction 8 = 5% sucrose. (**C**) Freeze-fracture electron microscopy showed that β-cell membranes contained typical gap junction plaques (arrow heads) made of connexin oligomers, that in most cases were found tightly associated to tight junctions (arrows). The presence of connexin particles on the P fracture face of one membrane (left panel) and of corresponding pits on the E fracture face of the adjacent membrane (right panel) demonstrates the trans-membrane disposition of the connexin channels. Bar, 120 nm.

gene was found to be significantly correlated ($r^2 = 0.834$, $P < 0.001$, $n = 21$) with that of the insulin gene, in both the control ($r^2 = 0.745$, $P < 0.01$, $n = 10$) and the diabetic group ($r^2 = 0.871$, $P < 0.001$, $n = 11$) (Fig. 6). The data show that Cx36 is expressed by both normo- and hyperglycemic individuals, in a pattern that varies with β-cell function, and is interdependent on the expression of insulin.

DISCUSSION

We have investigated the pattern of connexins expressed in the human pancreas, with a special focus on the endocrine portion of the gland. We show that Cx36 mRNA, and much lower levels of several other connexin transcripts, are selectively expressed in the endocrine pancreatic islets, whereas Cx26 and Cx32 mRNAs are expressed in the exocrine pancreatic

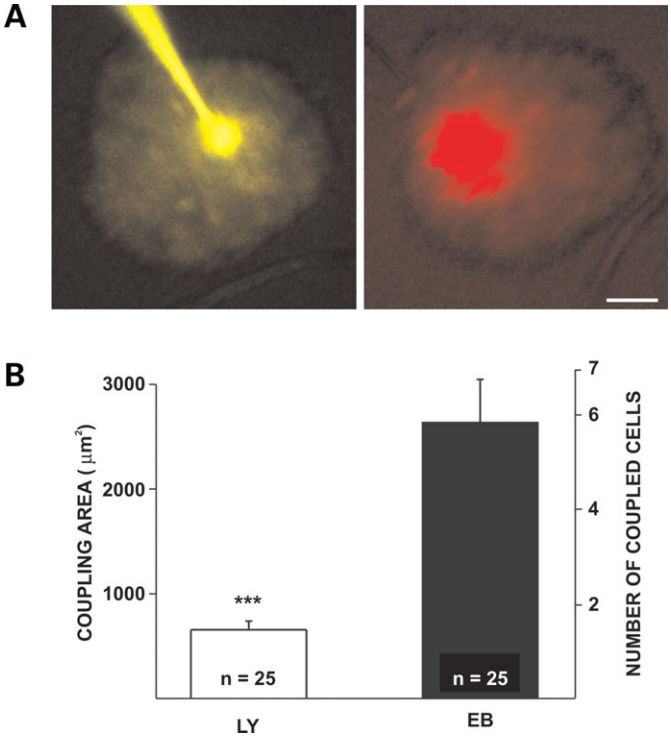

Figure 5. Human β-cells are coupled by Cx36 channels. (**A**) Microinjection of Lucifer Yellow into individual cells of freshly isolated human islets resulted in the diffusion of the tracer from the injected cell into some of its neighbors (yellow; left panel). A much larger intercellular diffusion was observed when another β-cell of the very same islet was microinjected with ethidium bromide (red; right panel) Bar, 25 μm. (**B**) Quantitative analysis showed that the average extent of β-cell coupling revealed by the small and positively charged ethidium bromide (solid bar) was three- to four-fold larger than that revealed by the larger size and negatively charged Lucifer Yellow (open bar). $^{***}P < 0.001$ versus ethidium bromide diffusion. Values within columns are number of microinjections.

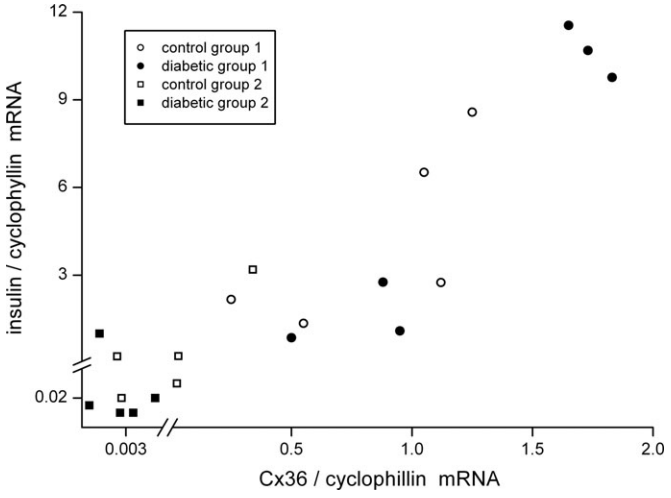

Figure 6. The expression of Cx36 in human β-cells parallels that of the insulin gene. Quantitative PCR amplification of total RNA extracted from isolated islets of normoglycemic (open symbols) and type 2 diabetics (solid symbols) revealed a quantitatively variable expression of Cx36 in all samples. The expression levels of the connexin were significantly correlated ($r^2 = 0.834$, $P < 0.001$, $n = 21$) with those of the insulin gene. Cx36 and insulin mRNAs were expressed relative to the levels of cyclophillin.

acini. Accordingly, we document a differential distribution of the cognate proteins in the two regions of the gland. These data show that the most abundant connexins of the human pancreas are expressed in a pattern comparable to that previously reported in laboratory rodents, supporting the view that different forms of connexin channels and signaling are required for the normal function of the endocrine and exocrine pancreas (3–5,24,25–29). Specifically, they show that, in contrast to a previous report (30) that contrasted with a number of observations in rodents (3–5,24,25–27), Cx26 is not detected in human pancreatic islets.

With regard to Cx36, which was the main focus of this study, our data show that it is the prominent connexin of human insulin-producing β-cells, as seen in rodents (3–5,8,13,15,25,28). Here, we provide evidence that Cx36 is concentrated at gap junction plaques closely linked to tight junctions, within Triton-insoluble and sphingolipid-enriched domains of the β-cell membrane. These data are consistent with the previous observations in other systems, documenting that both connexins and tight junction proteins are targeted to lipid rafts (31–33). The reason(s) for this selective localization remain(s) to be fully understood. Several proteins which play important roles in insulin secretion, including $K_v2.1$, calmodulin, SNARE proteins, kinases and phosphatases, are localized in lipid rafts (34,35). The present finding that Cx36 is also concentrated in a similar environment, suggests that the mechanism whereby this connexin plays a significant role in the regulation of β-cell function (8,13–15), may involve interactions with the proteins (4,5,20,36,37) and/or lipids that are segregated in these selected membrane domains (38). Whether the control of Cx36 channels is affected by their lipid environment, as is the case for other connexins (38), remains to be established. Consistent with this idea, β-cells can be rapidly and reversibly uncoupled by lipophilic drugs (7,29), whose uncoupling efficiency parallels their lipid partitioning (39). The different membrane environment of Cx36 in different cell types may also account for the observation that the channels made by Cx36 between rodent β-cells are alike, though not identical to those which the same protein makes between neurons (40), notably with regard to the sensitivity to quinine-induced blockade, or HeLa cells, notably with regard to ethidium bromide permeability (41).

Our study provides the first functional evidence that the Cx36 expressed at the membrane of human β-cells forms bona fide junctional channels, coupling most, though not all adjacent islet cells, as indicated by the rapid cell-to-cell transfer of different tracers that cannot permeate the cell membrane (42,43). Within the second-to-minute time-course of our microinjection experiments, we have observed that this coupling is limited to small communication territories that represent but a fraction of individual human islets. This pattern cannot be solely attributed to the patchy arrangement of β-cell clusters and/or to the high proportion of non-β-cells within the human islet (21), inasmuch as it was also observed after microinjection of rodent islets and monolayer cultures of transformed insulin-producing cells (4,7,9,13,14). As in these cases, the positively charged ethidium bromide revealed a significantly larger coupling of human β-cells (typically, six to seven cells were shown to be coupled using this tracer,

which is consistent with the size of the patchy clusters of different islet cell types within the human islets) than the negatively charged LY. In spite of a molecular weight close to that of ethidium bromide, this tracer revealed coupling between only one and two islet cells in both rodents (4,7,9,13,14) and human islets (this study), presumably because of the cationic permselectivity of Cx36 channels (9,41,42), the small macroscopic conductance of gap junctions at β-cell interfaces (15,43–46), and the hydrated size of the LY molecule, which approximates the functional bore of individual connexin channels (42). Because of these conditions, the limited coupling of β-cells, which is sufficient and obligatory to account for the physiologically relevant intercellular synchronization and propagation of electrical currents and Ca^{2+} waves (8,13,15,45–47), may escape detection when tested with negatively charged tracers at the periphery of rodent islets (48), where adjacent β-cells are rare, after culture of clusters of islet cells (48) or isolated islets (46) that could rapidly decrease the levels of the short-lived connexins (8,49) or at a sub-physiological temperature (46), which would decrease the motion of the tracers in the cytosol. At any rate, the finding that ethidium bromide revealed a significantly larger coupling of human β-cells than LY, rules out a significant functional contribution of other types of β-to-β-cell channels made by a different connexin isoform, that typically are much more permeable to negatively charged tracers than the channels made by Cx36 (4,9,28,42). In turn, this consideration raises questions about the reported expression of Cx26 in human pancreatic islets (30), which we could not confirm in our study, and that should have provided an extensive coupling when assessed with LY (27,42,50,51). It also rules out the potential participation to β-cell coupling of several other connexin isoforms, including Cx31.1, Cx37 and Cx31.9, which have not been investigated in the rodent pancreas, and whose transcripts we have found expressed, at levels significantly lower than those of Cx36, in human islets. By analogy with a number of other cell systems in which the cellular distribution of the corresponding proteins has been determined, it is plausible that these connexins are expressed by the cells of the vessels and connective tissue of the human islets (25,52). Strikingly, we could not detect in the pancreas and isolated islets we studied a transcript for Cx40, a connexin which couples many types of endothelial cells in several, but not all regions of the vascular system (52). Given that other bona fide 'vascular' connexins (e.g. Cx37, Cx43 and Cx45) were easily identified by the same methods in the very same samples, the simplest explanation is that islet vessels may not express Cx40 at detectable levels. These data now call for future investigations on the actual protein expression and possible function of these minoritary connexins, which may be relevant to the integrated functioning of pancreatic islets. Still, our molecular biology, biochemical, morphological and functional findings converge to show that the functional exchanges of cytosolic molecules between human β-cells are solely due to Cx36, the protein which was actually shown to be obligatory for the electrical and dye-coupling of rodent β-cells (4,8,13–15).

The conservation of Cx36 distribution and coupling organization in rodent and human islets, in spite of several major structural and functional differences, including the number and arrangement of β-cells, their Ca^{2+} handling and their equipment in ion channels (21–24) supports the view that Cx36 plays an indispensable role in β-cell function. In rodent islets, Cx36 is essential to synchronize across small groups of cells the oscillatory changes in Ca^{2+} and currents that are induced by glucose stimulation (8,15), and which, in turn, control the normal pulsatility of insulin release from individual islets (13). Human β-cells also display Ca^{2+} oscillations under glucose stimulation, although the intercellular synchronization of these events may be more limited than in rodents, presumably because β- and α-cells are intermingled within human islets (21). Whether Cx36 plays any role in the Ca^{2+} and current handling of human β-cells in such an environment awaits the possibility to modulate the levels of the protein within human islets. If technically feasible, this approach is presently complicated by the difficulty to obtain large numbers of human islets for research purposes (53). Thus, to test whether Cx36 plays a significant role in the function of human β-cells, we have analyzed by qPCR the levels of Cx36 and insulin transcripts in total RNA extracted from islets of either control individuals or type 2 diabetic patients. In spite of a large variability in expression, we have found that the levels of these two transcripts were highly and directly correlated in both the normoglycemic and the hyperglycemic individuals. This finding shows a parallel, hitherto unsuspected control of the Cx36 and the insulin genes, that is presumably accounted for by the regulation of the cognate promoters using a similar set of transcription factors. It also adds the insulin gene to the growing list of genes whose expression is coordinately controlled with that of Cx36 (54), pointing that control of gene expression is a relevant mechanism whereby connexins may affect cell function (50,54). This is the first evidence that this also applies for Cx36 in humans. Given the primordial importance of insulin expression for the identity and proper function of pancreatic β-cells, our data also provide the first evidence that Cx36 is one of the factors that are relevant for the functional control of human β-cells (55).

Previous experimental studies in rodents, have documented that loss of this connexin results in alterations of insulin secretion that are reminiscent of the main characteristics of prediabetic states and type 2 diabetes (8,13,15). Strikingly, the human *GJD2* gene that codes for Cx36 (http://www.genenames.org/genefamily/gj.php) is located on chromosome 15q14 (56), a region of the genome which has also been linked to type 2 diabetes in various populations (57–60). Thus, if the effects observed in rodents were to be extended to humans, type 2 diabetic patients would be anticipated to express lower levels of the Cx36 protein and/or decreased β-cell coupling than normoglycemic individuals (61). We found a large variability in the levels of the Cx36 transcript in both the control and the diabetic individuals that, within the limits of our small sample, could not be attributed to a specific difference in clinical phenotype, and prevented an unambiguous conclusion to be drawn with regard to the amounts of Cx36. On the other hand, our experiments show that at least some coupling is retained between the β-cells of type 2 diabetics, in agreement with the findings made in cell and animal models exposed to high glucose and/or fatty acids (62–64), suggesting that the diabetes-related Cx36 defect, if any, is likely to be quantitative rather than qualitative.

In summary, our data document that the connexin pattern of the human pancreas is similar to that previously described in rodents, notably with regard to the prominent expression of Cx36 in the insulin-producing β-cells. We show here that this protein concentrates at gap junction plaques within specific domains of the islet cell membranes, and forms junctional channels that are permeated by various molecules, still favor the β-to-β-cell exchange of cationic species. We eventually document that the expression of the Cx36 and the insulin genes are correlated. Thus, Cx36 may significantly contribute to modulate the function of human β-cells.

MATERIALS AND METHODS

Tissues, islets and sorted β-cells

Samples of control human liver, heart, skin, brain and pancreas were obtained from surgical specimens taken for diagnosis purposes at the Department of Pathology, University Hospital of Geneva, with the informed consent of the patient, and according to the rules of our institutional ethics committee for research on human beings.

Human islets from cadaveric multi-organ donors were isolated at the Cell Isolation and Transplantation Center of our institution. Briefly, pancreases harvested from multi-organ cadaveric donors were processed using a previously described modification (65) of the automated method (66). Aliquots of 20,000 islets were incubated in 30 ml CMRL 1066 medium (Sigma, St Louis, MO, USA) containing 5.6 mM glucose supplemented with 10% fetal calf serum (FCS), 25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (hereafter, referred to as complete CMRL). In most experiments, the islets were cultured 1–3 days before collection and analysis. For dye-coupling experiments, a few isolated islets were studied immediately after isolation. Human islets from 11 type 2 diabetic and 10 normoglycemic cadaveric donors without a history of the disease, to be analyzed by real-time qPCR, were isolated from the co-authors in Pisa (67) and Philadelphia. The control (six males and four females) and diabetic individuals (eight males and three females) had overlapping ages (40–72 and 33–79 years in the control and diabetic groups, respectively) and body mass index values (18–35 and 24–50 in the control and diabetic groups, respectively; NS) in the two groups. At the time of pancreas harvesting, diabetic and non-diabetic donors had blood glucose levels of 275 ± 13 mg/dl ($n = 11$) and 164 ± 9 mg/dl ($n = 10$, $P < 0.001$), respectively.

To purify β-cells, the isolated islets were dissociated by incubation at room temperature in 2 ml of enzyme-free 'dissociation buffer' (Sigma), under frequent pipetting. The reaction was stopped with the addition of F10 medium (Gibco) containing 5% (v/v) FCS, 1% (w/v) bovine serum albumin (BSA) and 7.5 mM glucose (F10/FCS/BSA). Aliquots of 2×10^6 primary islet cells were infected with 2×10^8 pfu recombinant and replication-defective adenovirus expressing GFP under control of the rat insulin I promoter (Ad-RIP-GFP) for 2 h at 37°C, washed two times in F10/FCS/BSA and cultured in this medium for 48 h (68). At this time, the infected cells were dispersed with 0.025% trypsin and 0.3 mM EDTA, washed and resuspended in Krebs-Ringer bicarbonate buffer (KRBB) containing 10 mM Hepes, 8.3 mM glucose and 0.5% BSA (KRBB–BSA). GFP-positive β-cells were sorted from non-fluorescent islet cells using a FACStar-Plus machine (Becton-Dickinson), equipped with an argon laser beam tuned to 488 nm (68). Non-infected cells served as controls.

Cx mRNA

Total RNA was extracted from tissues as previously described (5), and reverse-transcribed for 50 min at 42°C, using 200 U superscript II Moloney murine leukemia virus reverse transcriptase in 50 mM Tris–HCl buffer (pH 8.3), supplemented with 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM 4dNTP and 61.5 ng/μl Random Hexamer (all reagents from Gibco, BRL). Total RNA from β- and non-β-cells was isolated using the QIAGEN RNeasy Mini kit (Qiagen, Santa Clara, CA, USA), denatured at 65°C for 5 min and reverse-transcribed for 60 min at 37°C using a SensiscriptTM reverse transcriptase (Qiagen), primed with 61.5 ng/μl Random Hexamers (Gibco BRL).

All cDNA samples were incubated for 20 min at 37°C in the presence of 0.5 U RNAseH. Amplification of the resulting cDNAs was performed in a final volume of 50 μl by adding 5 μl amplification buffer containing 100 mM Tris–HCl (pH 8.8), supplemented with 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100, 0.6 μl 10 mM 4dNTP (Gibco BRL), 1 U Dynazyme (Finnzymes) and 0.167 μM of the following sense and antisense primers:

Cx isoform	Sense primer	Antisense primer
Cx26	TCCCCATCTCCCACATCCGGC	AAGATGACCCGGAAGAAGAT
Cx30	GGGAAGGTGTGGATCACAGT	AAGCAGCATGCAAATCACAG
Cx30.3	CCTGTACGACAACCTGAGCA	GATACCCACCTGCATCCACT
Cx31	TATACGTGGTGGCTGCAGAG	AGGCTGAACAGGTAGGTCCA
Cx31.1	CACAAGGACTTCGACTGCAA	GCTTGGAGATGAAGCAGTCC
Cx31.9	GCCTTCCCGGTCTCCCACTAC	GAGCAGCTTCTGCGCCTCTTC
Cx32	GGGAGGTGTGAATGAGGCAG	GGTGGATTGGAGCGGCGCTGG
Cx36	AACGCCGCTACTCTACAGTCTTCC	GATGCCTTCCTGCCTTCTGAGCTT
Cx37	GGCTGGACCATGGAGCCCGT	AGGGGGCAGGGGGCATCCCA
Cx40	GGGCGATTGGAGCTTCCTGGG	TTAAAGTCGGGGGGTGGTGT
Cx43	GCAACATGGGTGACGGAGCG	GCCAGGTACAAGAGTGTGGGT
Cx45	CTCCCATGTACGCTTCTGG	CGGGTGGACTTGGAAGCCA
Cyclophilin	GGTCAACCCCACCGTGTTCT	TGCCATCCAGCCACTCAGTCT

After a 5 min start at 94°C, the amplification was carried out in a PCR T3 machine (Biometra) for 30 cycles, each comprising 1 min at 94°C, 1 min at either 52°C (for Cx30, Cx30.3, Cx31 and Cx31.1), 55°C (for Cx32, Cx36, Cx43 and Cx45), 58°C (for Cx26 and cyclophilin) or 60°C (for Cx31.9, Cx37 and Cx40), and 1 min at 72°C. A final cycle comprised of 5 min at 72°C. Aliquots (10 μl each) of the amplified DNA fragment were separated on a 2% agarose gel and stained with 0.5 mg/ml ethidium bromide (5). Samples of human heart, liver, skin and brain served as positive and negative controls for the various connexins.

For qPCR analysis of Cx36 transcripts in the islets of control and type 2 diabetic individuals, total RNA extracted from human islets was purified using the Qiagen RNeasy mini kit and 0.5 μg were reverse-transcribed into cDNA. The following sense and antisense primers were designed using the Primer Express Software (Applera Europe, Rotkreuz, Switzerland): for Cx36, 5′-AAGGCATCTCCCGCTTC TACA-3′ and 5′-GCCAACCAGGAACCCAATTT-3′, for cyclophillin, 5′-TACGGGTCCTGGCATCTTGT-3′ and 5′-CCATTTGTGTTGGGTCCAGC-3′. The qPCR was performed in an ABI 7000 Sequence Detection System (Applera Europe) and PCR products were quantified using the SYBR Green Core Reagent kit (69). Two independent amplifications of each sample were performed and each transcript evaluated in duplicates. Mean values were normalized to the mean value of the reference mRNA cyclophilin (69). Authenticity of each amplicon was verified by DNA sequencing.

Cx proteins

Total protein extracts were prepared by homogenization of human tissues and isolated islets in a 0.1 M Tris–HCl buffer, pH 7.4, supplemented with 5 mM EDTA, 5% SDS, 1 μg/ml pepstatin A, 1 μg/ml antipain, 1 mM benzamidine, 200 Kin/ml aprotinin, 2 mM phenylmethanesulphonylfluoride (PMSF) and 1 mM DFP, followed by sonication (5). After a 10 min centrifugation of the sonicate at 16,000*g* and 4°C, the supernatant was collected and stored at −20°C.

Protein content of all the samples was measured by the DC protein assay kit (Bio-Rad Laboratories). Samples of total proteins (20–40 μg protein/lane) were separated by electrophoresis in either 12% (Cx26 and Cx32) or 10% (Cx36 and Cx43) polyacrylamide gels. Electrophoresed samples were transferred onto PVDF membranes (Immobilon^{TM}-P, Millipore) in the presence of 0.01% SDS and 20% methanol, using a constant current of 400 mA for 2 h. The membranes were saturated for 30 min at room temperature in a 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl, 0.1% Tween 20 (TBS-Tween) and 5% dry milk, and then incubated overnight at 4°C with one of the following antibodies: for Cx26, mouse monoclonal antibody clone CX-12H10 (Zymed Laboratories, San Francisco, CA, USA) diluted 1:500; for Cx43, mouse monoclonal antibody clone CX-1B1 or rabbit polyclonal antibody 71–0700 (Zymed Laboratories) diluted 1:500 and 1:100, respectively; for Cx32, affinity-purified antiserum 26KAP (courtesy of K. Willecke) diluted 1:1000. For Cx36 immunoblots, the electrophoresed samples were transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences) in the presence of 0.04% SDS and 20% methanol, using a constant current of 250 mA for 1 h. After the initial experiments with PVDF membranes, we noticed that nitrocellulose membranes resulted in higher signals and lower background for most connexin immunolabelings. We therefore opted for nitrocellulose membranes for all the remaining experiments. The membranes were rapidly washed in 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl, 0.4% Tween 20 (TBS-Tween) and 5% dry milk and then incubated with one of the following antibodies: for Cx36, rabbit polyclonal (5), diluted 1:1000 and used overnight at 4°C; for smooth muscle actin, rabbit polyclonal antibody A2066 (Sigma Chemical Co) diluted 1:1000 and used 2 h at room temperature; for α1-anti-trypsin rabbit polyclonal antibody A0012 (Dako), diluted 1:2000 and used for 2 h at room temperature. After repeated rinsing in TBS-Tween, the immunoblots were incubated for 60 min at room temperature with a goat anti-rabbit or anti-mouse Ig_s, whichever appropriate, conjugated to horseradish peroxidase (Bio-Rad Laboratories) and diluted 1:6000. Membranes were then washed and developed by enhanced chemiluminescence, according to manufacturer's instructions (Amersham Pharmacia Biotech). In all blots, the amount of loaded protein was controlled by probing the very same filters with the polyclonal antibody mentioned above. All blots included, as a positive control, extracts from RIN-2A cells transfected for Cx36 (9).

Cx in islet cell membranes

To prepare protein extracts of membranes, human tissues and isolated islets were homogenized by sonication in 0.1 M Tris–HCl buffer, pH 7.4, supplemented with 20 mM EDTA, 1 μg/ml pepstatin A, 1 μg/ml antipain, 1 mM benzamidine, 200 Kin/ml aprotinin, 2 mM PMSF and 1 mM DFP. After a 10 min centrifugation of the sonicate at 900*g* and 4°C, the supernatant was collected and centrifuged 60 min at 100,000*g* and 4°C (70). Pelleted material was resuspended (for storage at −20°C) in 50 mM Tris–HCl buffer, pH 7.4, supplemented with 120 mM NaCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, the abovementioned concentrations of protease and phosphatase inhibitors (70), and containing 1% Triton X-100. The addition of this non-ionic detergent is known to promote the enrichment of both lipid rafts (71,72) and gap junction plaques (73).

Raft fractions of islet membranes, identified by enrichment of the GM1 ganglioside, were prepared by sucrose density gradient centrifugation, as previously described (71). To this end, aliquots of 20,000 human islet equivalents were collected by centrifugation, rinsed in PBS and lysed for 30 min on ice, in 100 μl TNE/Triton X-100 buffer (25 mM Tris–HCl, pH 7.4, containing 5 mM EDTA, 150 mM NaCl and 1% TX-100), supplemented with protease inhibitors (Roche). The samples were mixed with 162 μl 73% sucrose in Tris-KCl-$MgCl_2$ (TKM), to reach a final 40% (w/v) sucrose concentration and transferred to a 2.2 ml ultracentrifuge tube. A discontinuous sucrose density gradient was made by layering 1.2 ml 35% sucrose, and then 0.7 ml 5% sucrose (all solutions in TKM buffer). The gradients were centrifuged for 18–21 h at 258,800*g* (55,000 rpm in a S55S Sorval rotor) and 4°C. Ten 200 μl fractions were collected from the bottom of the tubes. The GM1 distribution was assessed in dot blots of 6 μl samples of each fraction, using CTxB-HRP. The distribution

of Cx36 and α1-anti-trypsin (74) were analyzed in each fraction by immunoblotting with the specific antibodies mentioned above. To this end, trichloroacetic acid was added to each fraction to a final concentration of 9%, the samples were incubated 30 min on ice, and centrifuged 10 min at 12,000*g* and 4°C. The resulting pellets were washed with 1 ml −20°C acetone and air-dried. Pelleted material was resuspended in 10 mM Tris–HCl buffer, pH 7.4, supplemented with 0.5% deoxycholate and 0.5% Nonidet P40 (NP40), and stored at −20°C.

For *in situ* immunolabeling of β-cells, fresh surgical samples of two human adult pancreas (from a 20- and a 56-year-old individual) were embedded in OCT (Sigma), frozen and stored at −80°C. Cryosections of 8 μm were cut and used immediately for staining. Sections were allowed to reach room temperature, dried for 1 h, fixed for 5 min with 2% PFA in PBS, permeabilized for 10 min at room temperature with 0.1% Triton-X100, and blocked for 10 min with 50 mM Glycin in PBS. The sections were incubated for 1 h in PBS (pH = 7.4) containing 1% BSA, 2% donkey serum and 1% cold water fish gelatin, and then 2 h with one of the following antibodies: sheep anti-human (The Binding Site) and rabbit anti-Cx-36 (C7855-14; US Biological). After rinsing, the sections were reacted for 1 h at room temperature with either Lissamine–Rhodamine-labeled donkey $F(ab)_2$ against rabbit immunoglobulins (Igs) or fluorescein isothiocyanate-labeled donkey $F(ab)_2$ against sheep Igs (both antibodies preadsorbed on multiple species serum proteins; Jackson ImmunoResearch), or with sera from the same species conjugated to peroxidase (75). Stained sections were mounted and viewed on a FV-1000 confocal microscope (Olympus). Controls included exposure of the islet sections to only the secondary appropriate antibody. None of these incubations resulted in a sizable, specific staining of the sections.

For ultrastructural assessment of gap junction plaques, isolated islets were fixed for 1 h in a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer, infiltrated with 30% glycerol, frozen in vacuum-solidified liquid nitrogen, and processed for freeze-fracture using a BAF060 Freeze Etching System (BAL-TEC, Liechtenstein), as described (76). Replicas were screened, under a Philips CM10 electron microscope.

Islet cell coupling

Human islets freshly isolated at the Cell Isolation and Transplantation Center of the University of Geneva were attached to Sylgard®- and poly-L-lysine-coated dishes and transferred onto the heated (37°C) stage of a Universal microscope (Zeiss). Individual cells were microinjected for 5 min using glass electrode pulled to a resistance of 50–60 MΩ, as assessed in 150 mM LiCl, and whose tip was filled with either 4% (w/v) LY CH or 1% (w/v) ethidium bromide (both tracers from Sigma) dissolved in 150 mM LiCl buffered to pH 7.2 with 10 mM HEPES (7,9,13,14). After back-filling of the electrode shaft with 3 M HEPES-buffered LiCl, the electrodes were used to penetrate individual cells located deep in the islet core. Following successful penetration, the tracers were iontophoretically injected for 10 min, by applying square pulses of 0.5 nA amplitude, 900 ms duration and 0.5 Hz frequency of negative (LY) or positive polarity (ethidium bromide). At the end of each experiment, the dark-field and epifluorescence views of the injected field were immediately recorded before and/or after pulling out the electrode, using an AxioCam digital camera (Zeiss, Jena, Germany) and settings for FITC or rhodamine fluorescence, whichever appropriate (9,41). Cell-to-cell coupling was defined as the tracer diffusion from the injected cell to at least one of its immediately adjacent neighbor. Uncoupling was defined as the persistence of the tracer in only the microinjected cell. The incidence of coupling was determined by scoring the number of microinjections that resulted in the cell-to-cell transfer of the tracers, as determined by inspection of the islets immediately at the end of each microinjection (9,41). The extent of coupling was determined by measuring the area of cells stained by the tracers, using a digitalizing tablet and the LeicaQWin software (Leica, Wetzlar, Germany). The number of coupled cells was estimated by dividing this area by the calculated average surface of a single β-cell profile (approximately 450 μm^2).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

ACKNOWLEDGEMENTS

The authors thank Mrs N. Dupont for excellent technical assistance.

Conflict of Interest statement. None declared.

FUNDING

The Swiss National Science Foundation (310000-122430 to P.Me), the Juvenile Diabetes Research Foundation (1-2005-1084 to V.C., 1-2007-158 to P.Me), the National Institute of Health (DK55183 to V.C.), the European Union (FP6-Integrated Project EuroDia LSHM-CT-2006-518153 to P.Ma; FP-7 BETAIMAGE 222980 to P.Me), Novo Nordisk (to P.Me) and The Larry L. Hillblom Foundation (to V.C.). Image analysis was performed at The National Center for Microscopy and Imaging Research (NIH grant RR4050 to M. Ellisman). Fresh human islets were provided by the Cell Isolation and Transplantation Center of the Geneva School of Medicine, thanks to the ECIT 'islet for research' distribution program sponsored by the Juvenile Diabetes Research Foundation (to T.B.).

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