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Connexin 30.2 is expressed in mouse pancreatic beta cells

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ABSTRACT

Nowadays, connexin (Cx) 36 is considered the sole gap junction protein expressed in pancreatic beta cells. In the present research we investigated the expression of Cx30.2 mRNA and protein in mouse pancreatic islets. Cx30.2 mRNA and protein were identified in isolated islet preparations by qRT-PCR and Western blot, respectively. Immunohistochemical analysis showed that insulin-positive cells were stained for Cx30.2. Confocal images from double-labeled pancreatic sections revealed that Cx30.2 and Cx36 fluorescence co-localize at junctional membranes in islets from most pancreases. Abundant Cx30.2 tiny reactive spots were also found in cell cytoplasms. In beta cells cultured with stimulatory glucose concentrations, Cx30.2 was localized in both cytoplasms and cell membranes. In addition, Cx30.2 reactivity was localized at junctional membranes of endothelial or cluster of differentiation 31 (CD31) positive cells. Moreover, a significant reduction of Cx30.2 mRNA was found in islets preparations incubated for 24 h in 22 mM as compared with 3.3 mM glucose. Therefore, it is concluded that Cx30.2 is expressed in beta and vascular endothelial cells of mouse pancreatic islets.

1. Introduction

Gap junctions (GJ) are conglomerates of intercellular channels. In vertebrates they are mainly formed by a family of homologous transmembrane proteins (~20) termed connexins (Cxs). These channels allow the direct intercellular transfer of molecules and second messengers between adjacent cells. A connexon or hemichannel is an hexamer. Docking of two connexons forms an intercellular channel. Since cells, from most tissues, express more than one Cx subtype, intercellular channels may be homotypic (both connexons formed by the same Cx subtype), heterotypic (each connexon formed by a different Cx subtype) or heteromeric (each connexon formed by more than one Cx). Moreover, hemichannels at the cell membrane may be active [1]. Alterations in Cxs are involved in human pathology and their deficiency is critical for cell development [2,3].

Diabetes is a chronic disease that involves different metabolic disorders where subjects have in common high blood glucose levels. Its world prevalence has increased around ten times in the last 4 decades [4]. Among the different hormones that regulate glucose homeostasis, insulin is the only one that decreases glucose levels. Insulin is produced in beta cells that, in mouse, constitute most of the islet central mass (~80%). The glucose insulin release response curve from isolated perfused islets is well fitted by a sigmoidal function, with a threshold at ~6 mM followed by a linear increase that reaches it saturating point at ~22 mM [5]. Insulin secretion depends critically on electrical activity of beta cells induced by glucose. This electrical activity consists of bursts of action potentials which happen synchronously and in phase in most beta cells from a single islet. This high electrical synchrony produces cyclic changes in the interstitial $[K^+]$ and $[Ca^{2+}]$ [6] and in the intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations [7,8] in the different islet regions. The latter determines pulses of insulin release [8–10].

Electrical coupling [11] through GJ [12] intercommunicates most pancreatic beta cells [13,14]. Cx36 is localized at junctional membranes of beta cells in rodent [14–17] and human islets [18]. Isolated islets cells from Cx36-deficient mice lack synchronization in their $[Ca^{2+}]_i$ oscillations and exhibit loss of pulses of

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insulin release induced by glucose [8]. Moreover, Cx36-deficient mice are glucose intolerant [19]. These alterations have been explained to result from beta cells uncoupling since neither GJ particles [8] nor electrical or chemical coupling [20,21] were detected in islet cells from these animals.

From above, Cx36 is considered the only Cx expressed in beta cells. However, besides Cx36, the mRNA of Cx43 and Cx45 have been identified in rodent beta cells purified by cell sorting [22] and of Cx30.3, Cx31, Cx31.1, Cx31.9, Cx37, Cx43 and Cx45 in human islets by RT-PCR [18]. However the cell-specific distribution of most of these Cxs remains to be determined.

Moreover, we have previously proposed that GJ channels in mouse beta cells are heteromeric [14]. This was based on the characterization of the biophysical properties of the junctional currents recorded in dual voltage clamped freshly isolated beta cell pairs [13,14]. Intercellular channels in beta cells are weekly voltage sensitive and have a tiny main unitary conductance (γ_j, ~6 pS) [14]. In the present research, we looked for the expression of Cx30.2 because it is the other member from the Cx family, besides Cx36, whose biophysical properties are most compatible with those recorded in isolated beta cell pairs.

2. Materials and methods

2.1. Animals

CD1. mice were maintained and handled in accordance with the national and international Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction. Animals were anaesthetized and sacrificed by decapitation before tissue dissection.

2.2. Western blot

About 500 islets were isolated from 12 pancreases and homogenized by sonication. Proteins were extracted using buffer (RIPA #20–188, Millipore Corporation, Billerica, MA, USA) with protease inhibitors (P8340, Sigma, St. Louis, MO, USA). Gels were loaded with 50 μg of total protein. The electrophoretic run and transfer protein protocols were performed as previously described [23]. The membranes were incubated overnight at 4 °C with rabbit anti-Cx30.2 (Invitrogen, CA, USA; 1:100). After rinsed, they were incubated for 1 h at room temperature with a horseradish peroxidase (HRP) goat secondary antibody (1:5000). Blots were revealed with chemiluminescence (SuperSignal West Pico, Pierce, Milwaukee, WI, USA).

2.3. Immunohistochemistry

(IHC). Pancreases (10 mice) were fixed in 4% buffered paraformaldehyde for 24 h. After antigens were retrieval with citrate buffer and endogenous peroxidase was blocked, serial sections were incubated with anti-Cx30.2 (1:50), anti-Cx36 (Invitrogen, CA, USA; 1:50) or guinea pig anti-insulin (Invitrogen, CA, USA; 1:2000) for 18 h at 4 °C. After rinsed, sections were incubated for 30 min at 37 °C with an HRP secondary antibody (1:500), and immunoreactivity was revealed by incubation in 3,3′-diaminobenzidine tetrahydrochloride (Zymed, CA. USA). As negative controls, sections were incubated only with a secondary antibody.

2.4. Immunofluorescence

Pancreatic cryosections were co-incubated overnight at 4 °C with anti-Cx30.2 (1:100) and monoclonal anti-Cx36 (1:100), or anti-cluster of differentiation 31 (CD31, BD Pharmingen, San Diego, USA; 1:500). Isolated cultured beta cells were also co-incubated with anti-Cx30.2 and anti-insulin (1:100). After rinsed, sections and cells were revealed with their corresponding secondary antibodies. Images were acquired using a confocal laser-scanning microscope (Olympus model FV1000, Japan). For each experimental condition, at least seven different pancreases were analyzed. As negative controls, sections were incubated only with the corresponding secondary antibody.

2.5. Islets and beta cells cultures

Islets of Langerhans were dissociated from minced pancreases using collagenase 3 mg/ml in Krebs ringer solution with 3% albumin, as previously described [13]. Collected islets were incubated overnight in a DMEM medium (GIBCO, Grand Island, NY, USA) with 11 mM D-glucose. The next day, islets were incubated for 24 h in the DMEM containing 3.3 or 22 mM glucose. Beta cells were dispersed from batches of 150 islets following a protocol previously described [13] and cultured in RPMI with 11 or 22 mM glucose overnight before processed for immunofluorescence studies. Islets and cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. All culture media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% of fetal bovine serum (FBS).

2.6. RNA Isolation

Total RNA was isolated from five pools of isolated islets (~450), collected from 12 pancreases for each culture condition (3.3 or 22 mM glucose), using the RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The quality and purity of RNA was confirmed as previously described [23]. Traces of DNA were removed by digestion with RNase-free DNase I (Invitrogen, CA, USA) following the manufacture's protocol.

2.7. Real-time quantitative retrotranscription

PCR (qRT-PCR). Reverse transcription of total RNA was performed as previously reported [23]. The expression of Cx30.2 and Cx36 genes was explored in islets incubated in 3.3 or 22 mM glucose with qRT-PCR using TaqMan probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. TaqMan gene expression assays were used (Cx30.2, Mm00731344_s1, Cx36 Mm00439121_m1 and GAPDH, Mm99999915_g1; Applied Biosystems). The cDNA from five different pools were explored in duplicates in a final volume of 20 μl, including 100 ng of cDNA template, 10 μl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl of 20X TaqMan Gene Expression Assay, and 7 μl of RNase-free water. The cycling program was run in a 7500 real time PCR system (Applied Biosystems, Foster City, USA), which was set as follows: an initial PCR activation step at 50 °C for 2 min followed by 95 °C for 10 min, then 45 cycles of melting at 95 °C for 30s and annealing/extension at 62 °C for 1 min. Measurement of gene expression was based on relative standard curves constructed from a 10-fold serially diluted pool of islets cDNAs ranging from 500 to 0.05 ng. Curves for GAPDH gene were tested in three different experiments ran in duplicate. The correlation coefficient (r) average was higher than 0.98. The expression of target genes was calculated based on the GAPDH standard curve and normalized in each experiment to the intensity of the internal reference (GADPH) using a previously described method [29]. The normalized intensity values were measured in ng/μl. The statistical significance of the differences between the groups was calculated with the t-test.

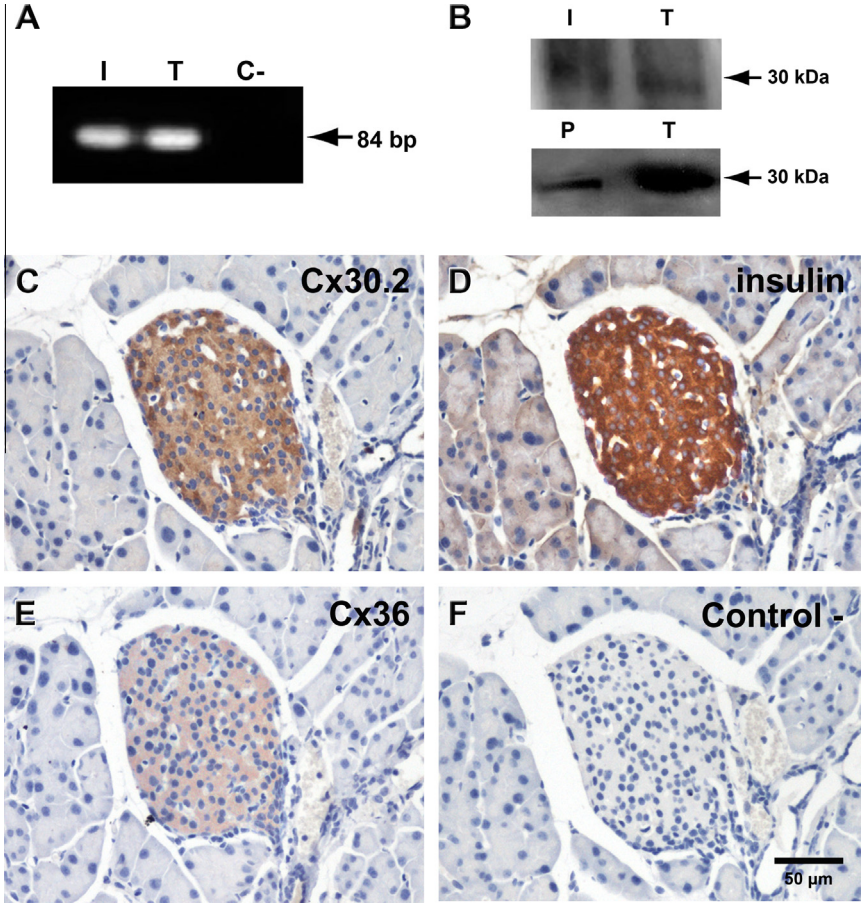

Fig. 1. Cx30.2 mRNA and protein are expressed in pancreatic islets. RT-PCR analysis of cDNA obtained from three batches of isolated islets. (A) A 84 bp band, as expected for Cx30.2, was detected in an electrophoresis gel loaded with reactions from islets (*I*) and testis (*T*), but not in reactions in which cDNA was substituted by H_2O. (B) Western blot, where a band of 30 kDa is detected in total homogenate from total pancreas (*P*), islets (*I*) and testis (*T*). (C-F). Digital images of 3 µm thick pancreatic serial sections revealed with immunoperoxidase, show that insulin positive cells (D) express Cx30.2 (C) and Cx36 (E). No reactivity was found with the secondary antibody (F).

3. Results

To address whether the Cx30.2 gene is expressed in pancreatic islets, the mRNA expression was explored by RT-PCR and the protein by Western blot. Fig. 1A shows an electrophoretic gel where a PCR product of the expected size (84 bp) for Cx30.2 mRNA is detected in lanes loaded with reactions from mouse islets and testis but not in reactions where H_2O substituted cDNA. These last ones were used as positive and negative controls, respectively. Cx30.2 mRNA was also identified in rat, hamster and guinea pig pancreases (not shown). Fig. 1B shows an image of a Western blot where a band of 30 kDa is identified in lanes loaded with homogenates from total pancreas (lower panel), isolated islets (upper panel) and testis. The latter demonstrates that the anti-Cx30.2 antibody used recognized this Cx.

To identify the cell specific distribution of Cx30.2, IHC experiments were performed in 3 µm thick serial sections of pancreases. Figure 1 shows that cells positively stained with anti-Cx30.2 (C) were also labeled with anti-insulin (D) and anti-Cx36 (E) which is another beta cell marker. In contrast exocrine acinar cells were not labeled. In addition islets from sections incubated only with secondary antibody were not stained (F). Thus, these findings demonstrate that Cx30.2 protein is localized in beta cells.

The subcellular distribution of Cx30.2 in beta cells was investigated by immunofluorescent (IF) studies. Fig. 2A–L shows confocal images from 1 µm thick pancreatic sections from three mice, double-labeled with anti-Cx30.2 and anti-Cx36. Tiny reactive spots of Cx30.2 were found in beta cells cytoplasms (Fig. 2A, E, J). In addition, in some islets, Cx30.2 reactive spots were also found at cell membrane appositions (Fig. 2A), as indicated by their spatial distribution close to Cx36-IF labeling (Fig. 2B). This spatial relation is better illustrated in merge image (Fig. 2C and insert) where Cx30.2 (green) is found adjacent to Cx36 (red). This region is also shown at a lower amplification (Fig. 2D, asterisk)

In other islets (5 of 7), Cx30.2 -reactive spots at cells membranes (Fig. 2E) were found to have a noteworthy similar spatial distribution to Cx36 protein (Fig. 2F). This is better illustrated in the merge image (Fig. 2G) where Cx30.2 (green) is shown to co-localize with Cx36 (red) at junctional membranes (yellow, arrowheads). This pattern is also shown at a lower (Fig. 2H) and higher amplification (Fig. 2I). Co-localization of Cx30.2 and Cx36 at junctional membranes is also shown in beta cells from another islet (Fig. 2J–L).

Distribution of Cx30.2 was also analyzed in beta cell cultures. Fig. 2M–T shows confocal images of insulin positive cells (red) from two different cell cultures incubated with 22 mM glucose. Cx30.2 reactivity (green) was localized in the whole beta cell volume, as shown in different optical sections (Fig. 2M–P). Furthermore, in some beta cells Cx30.2-IF reactivity was higher at the cell membrane than in the cytoplasm (Fig. 2Q–T).

High glucose concentrations increase blood flow in the islet where endothelial and beta cells are intimately associated [24].

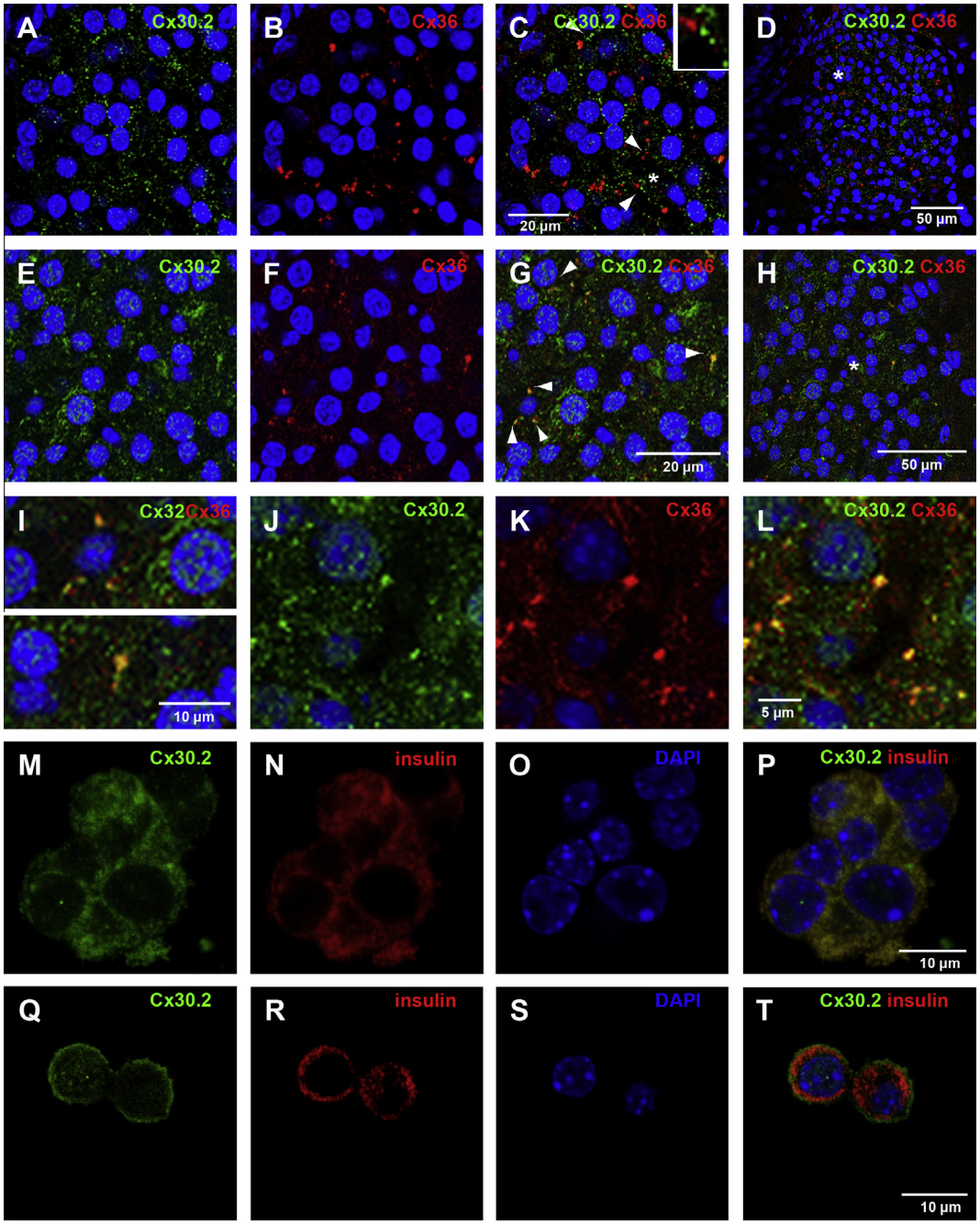

Fig. 2. Cx30.2 is expressed in beta cell cytoplasm, junctional and non junctional membranes. (A-L) Confocal images of pancreatic sections, 1 µm thick, double-labeled, with anti-Cx30.2 (green) and anti-Cx36 (red), where nuclei are stained with DAPI (blue). Abundant tiny Cx30.2-IF spots are found at pancreatic beta cell cytoplasm and nonjunctional membranes from three islets (A, E, J). Moreover, in some islets, Cx30.2 (A, green) and Cx36 (B, red) reactive spots alternate at junctional membrane areas (C arrowheads, insert, and D, asterisk). In other islets, Cx30.2 reactivity (E) is distributed at the same junctional membranes as Cx36 (F). In these areas, as shown at different amplifications, both Cxs co-localize (G, H and I, yellow). Co-localization of both Cxs is also shown for a third islet (J-L). (M-T) Images, of 1 µm thick confocal sections, of a beta cell clump cultured at 22 mM glucose, double-labeled with anti-Cx30.2 (green) and anti-insulin antibodies (red). Cx30.2 is localized in beta cell cytoplasm (M-P). In some beta cells a higher density of Cx30.2 is found at the cell membranes (Q-T).

Therefore, we investigated whether Cx30.2 is expressed in blood vessels of pancreatic islets. For this, pancreatic sections were co-incubated with anti-Cx30.2 and anti-CD31, which is a marker of the endothelial cells membranes [25]. Confocal images shown in Fig. 3A illustrate that cells positive for CD31 (red) and Cx30.2 (green) are localized in blood vessels distributed both at the periphery (asterisk) and at the center (arrowheads) of pancreatic islets. At a high magnification, Cx30.2 is shown to localize in endothelial cells closely associated with CD31 at junctional membranes (Fig. 3B and C).

Cx36 mRNA has been found to decrease in rat islets maintained under glucose stimulation for 24 h [26]. To explore whether Cx30.2 gene is also modulated by glucose its mRNA was quantified by qRT-PCR after islets were incubated for 24 h in low (3 mM) and

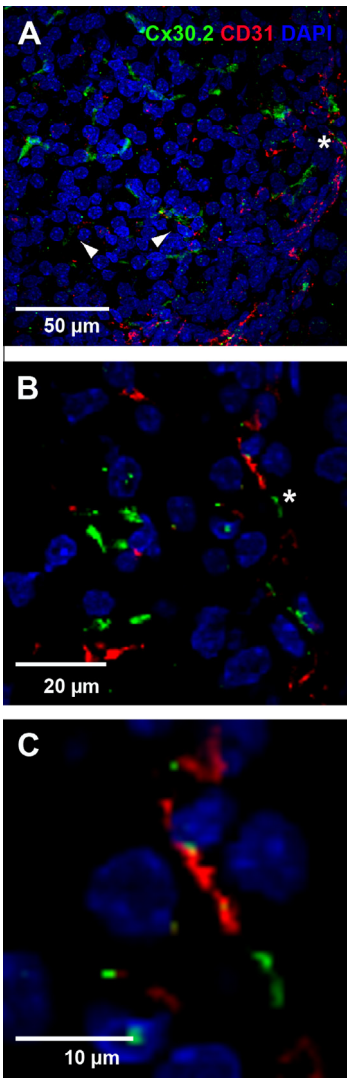

Fig. 3. Cx30.2 is expressed at junctional membranes in islet vascular endothelial cells. Confocal images of pancreatic sections of 1 µm thickness, co-incubated with anti-Cx30.2 (green) and anti-CD31 (red), an endothelial cell marker. Cx30.2 is expressed in central (arrowheads) and peripheral (asterisk) blood vessels (A), localized at junctional membranes (asterisk) of endothelial cells (B-C).

high (22 mM) glucose. A significant decrease in Cx30.2 of a 45% (Fig. 4A) and in Cx36 of a 31% (Fig. 4B) mRNAs was found in islets maintained under high glucose concentration ($P < 0.05$).

4. Discussion

This research demonstrates that Cx30.2 is expressed in pancreatic islet beta cells. This conclusion is based on the detection of Cx30.2 mRNA by qRT-PCR in rodent islet cells preparations. It is also supported by the detection of 30 kDa-band protein in islets homogenates. In addition, Cx30.2 protein was shown clearly localized in insulin positive cells by IHC. Moreover, IF experiments revealed that Cx30.2 co-localizes with Cx36 protein, which is well known to be expressed at beta cells junctional membranes. Furthermore, IF analysis in cell cultures confirmed that insulin positive cells express Cx30.2.

Here, it is demonstrated by IHC studies that both Cx30.2 and Cx36 are localized to beta cells. Moreover, by confocal images and IF studies we documented that Cx30.2 and Cx36 co-localize at junctional membranes of beta cells from most islets. This result indicates that in beta cells both connexins co-exist at the same junctional plaques.

Co-localization of Cx30.2 and Cx36 at junctional plaques is consistent with the hypothesis that GJ channels in beta cells are heteromeric [14]. Although similar, the biophysical properties of junctional conductance (g_j) recorded in dual voltage clamped beta cell pairs were not identical to those of Cx36 channels as recorded in cell expression systems. Specifically, beta cell g_j was not affected with a concentration of quinine 1 mM, that is 28 times higher [14] than the concentration found (EC_{50} 5 µM) to close the Cx36 GJ channels in cell expression systems [27]. Furthermore, the relationship between the normalized steady-state junctional conductance (G_{ss}) and the transjunctional voltage applied between dual voltage clamped isolated beta cell pairs was heterogeneous. In 55% of the cell pairs G_{ss} was voltage insensitive, while in the remaining 45% a symmetrical weak voltage sensitivity was found. In the latter group of beta cell pairs, the half transjunctional voltage dependence ($V_o = \pm 85$) was 10 mV higher [14] than in Cx36 channels ($V_o = \pm 75$) [28,29]. Further studies are required to prove this hypothesis. So far, both Cx30.2 [30] and Cx36 [28,29] intercellular channels are known to be weakly voltage sensitive, poorly anion selective and have a tiny unitary conductance ($\gamma_j \sim 10$ pS). Then, differences in biophysical properties of beta cell intercellular channels with respect to those of Cx36 [13,14] may well be explained by the co-expression of Cx36 and Cx30.2.

Confocal images showed that in some pancreatic islets Cx30.2 IF-spots were also found adjacent to Cx36 ones. This result suggests that homomeric plaques formed only with Cx30.2 also exist in islet beta cells. It also suggests that groups of Cx30.2 inter-communicated beta cells may be involved in the Cx36-independent component of circulating blood insulin oscillations found in Cx36 deficient mice [19]. Nonetheless, since in these mice no GJ particles [8] or coupling [8,20,21] have been detected in islet beta cells that exhibited an uncoordinated $[Ca^{2+}]_i$ activity [8,21], it is possible that

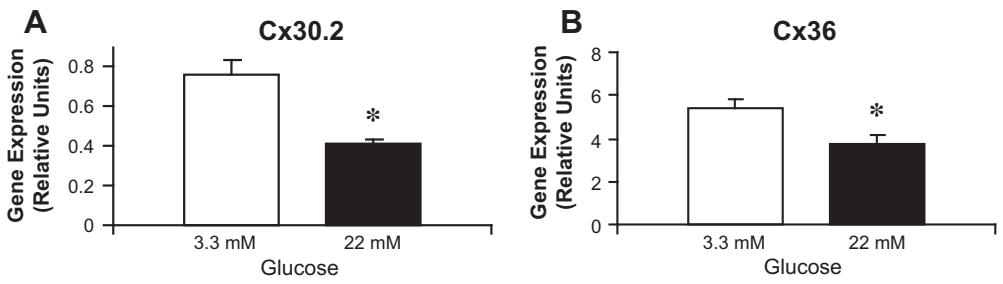

Fig. 4. High glucose concentration downregulates Cx30.2 mRNA levels in isolated islets. Quantitative qRT-PCR analysis of Cx30.2 mRNA in islets after a 24 h incubation at low (3.3 mM) and high (22 mM) glucose concentrations. Transcript levels were normalized with respect to the constitutive gene GAPDH. A 45% decrease in the Cx30.2 (A) mRNA was found under high glucose concentration whereas Cx36 mRNA, was reduced in 31% (B). Data are means ± S.E.M. ($P < 0.05$), $n = 5$ pools for each experimental condition.

Cx30.2 distribution at the junctional plaques may depend in part on Cx36 expression.

Cx30.2 was also localized in the whole volume of beta cells, as shown here by images from islet and cultured cells. This raises the possibility that Cx30.2 is also expressed at non junctional cell membranes. This is supported by the higher Cx30.2-IF reactivity found in cell membranes compared with the one found in cytoplasm in some cultured beta cells (Fig. 2Q–T). It is also supported by the abundant tiny Cx30.2-IF spots at the non junctional membrane areas. Some Cxs form hemichannels, including Cx30.2 [30]. Hemichannels may be active under in vivo conditions as previously reviewed [1]. Therefore, it is possible that Cx30.2 forms hemichannels in native pancreatic beta cells. Further studies are required to prove this hypothesis.

Here we also provide for the first time evidence that Cx30.2 is localized at GJ of endothelial cells of islet blood vessels. Cx30.2 has been described at junctional membranes of blood vessels of testis [31] and retina [32]. Incubation under high glucose concentration (30 mM) downregulates Cx30.2 protein in rat retinal endothelial cells [32]. Therefore, the relative decrease of Cx30.2 mRNA in islet preparations identified here by qRT-PCR may result from its reduction in beta and/or in vascular endothelial islet cells. Further studies are required to discern between these possibilities.

Overall, results obtained in this research demonstrate for the first time that Cx30.2 is expressed at junctional membranes of endothelial and beta cells of mouse pancreatic islets. Beyond these findings, our results demonstrate that there is an abundant expression of Cx30.2 in beta cells besides Cx36. This finding constitutes a tipping point for the understanding of how do GJ proteins regulate beta cell function.

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