Cx36 Preferentially Connects β-Cells Within Pancreatic Islets

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Previous studies have provided evidence for the transcripts of Cx43 and Cx45 within pancreatic islets. As of yet, however, it has proven difficult to unambiguously demonstrate the expression of these proteins by islet cells. We have investigated whether Cx36, a new connexin species recently identified in mammalian brain and retina, may also be expressed in pancreatic islets. Using probes that permitted the original identification of Cx36 in the central nervous system, we show that a transcript for Cx36 is clearly detectable in rat pancreatic islets. Using novel and affinity-purified polyclonal antibodies, we have found that Cx36 is actually expressed in pancreatic islets. Both in situ hybridization and immunolabeling indicated that this connexin is abundant in the centrally located insulin-producing β-cells and is expressed much less, if at all, by the other endocrine cell types. This differential expression was further confirmed on fluorescence-activated cell sorter–purified preparations enriched in either β- or non–β-cells. The finding of a differential distribution of Cx36 within distinct regions of pancreatic islets creates the possibility that this connexin may provide the establishment of selective pathways of communication between the different types of endocrine cells comprising the pancreatic islet. *Diabetes* 49:727–734, 2000

Gap junctions are specialized membrane regions composed of aggregates of channels that permit a free diffusion of ions, metabolites, and other molecules <1 kDa between adjacent cells (1–3). These intercellular channels consist of dodecameric assemblies of connexin (Cx) proteins. So far, 16 different species of such proteins, each encoded by a distinct gene, have been described in mammals (4–6).

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BSA, bovine serum albumin; Cx, connexin; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; FAD, flavin adenine dinucleotide; FCS, fetal calf serum; KRB, Krebs-Ringer bicarbonate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcriptase; SSC, sodium chloride–sodium citrate.

Gap junction channels connect almost all types of vertebrate cells, including those of pancreatic islets (3,7,8). In these endocrine micro-organs, gap junctions are frequent between the insulin-producing β-cells as well as between the other islet cell types that produce glucagon, somatostatin, and pancreatic polypeptide (9,10). As of yet, islet cell gap junctions have been thought to consist mostly of Cx43 and Cx45, in view of the consistent finding of the transcripts for these two connexins within pancreatic islets (8,11) and cell fractions purified thereof (12). However, the corresponding proteins have been difficult to be positively demonstrated in a reliable manner, particularly in situ. Also, several groups studying pairs of β-cells have failed to detect intercellular electrical conductances characteristic of either Cx43 or Cx45 channels, in spite of the presence of detectable electrical coupling (8,13). These negative findings usually have been attributed to low levels of connexin expression, consistent with the small size of the gap junction plaques joining islet cells (14,15). However, no data have yet dismissed the additional possibility that islet cells, like most other types of vertebrate cells, may be linked by other connexin species. Candidates have been sought among members of the α- and β-subgroups of the connexin family (4). However, whereas several of these proteins could be easily located between the acinar (Cx26 and Cx32) and endothelial (Cx37 and Cx40) cells of the pancreas, none of them has yet been shown to be expressed within pancreatic islets (8,11,12).

Recently, another subgroup of connexins has been identified that comprises proteins with a high degree of sequence homology, which are encoded by genes markedly different from those encoding all other known connexins (5,16). Thus, in this γ-group, an intron at a conserved location interrupts the coding region, contrary to what is seen in genes for both α- and β-connexins (5,6,16). In mammals, a single γ-connexin, referred to as Cx36, has been identified so far (5,6). While the distribution of this protein remains to be fully assessed, the initial observations have suggested that its transcript is highly expressed in neuronal cells of either central nervous system or retina (5,6). In view of the many characteristics that are shared by neurons and pancreatic β-cells (17–24), we have now explored whether Cx36 may also be expressed in pancreatic islets. Using probes that permitted the original identification of Cx36 in the central nervous system as well as novel antibodies that we generated against a synthetic peptide representing a portion of the COOH-terminal tail of rat Cx36, we found that this protein is expressed in pancreatic islets at levels that allow for its easy detection between the insulin-producing β-cells but not the other endocrine islet cell types.

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RESEARCH DESIGN AND METHODS

Animals. All animals were kept under standard housing conditions with free access to water and food. Experiments were conducted according to the regulations of our institutional and state committees on animal experiments. Rats of the OFA strain were obtained from Service de Zootechnie (CMU, Geneva, Switzerland).

Tissues and cells. Adult rats weighing 250–350 g were anesthetized by inhalation of 5% Ethrane (Abbott Laboratories, Cham, Switzerland), killed by bleeding, and immediately used for pancreas, brain, and heart sampling.

Islets of Langerhans were isolated from the pancreas of adult rats by collagenase digestion and purification on a histopaque gradient (25). The isolated islets were washed twice in phosphate-buffered saline (PBS) prepared without adding Mg^{2+} and Ca^{2+} and containing 0.2 mmol/l EDTA and were then exposed for 6–7 min at 37°C to the same medium supplemented with 0.16 mg/ml trypsin (1:250) (Gibco, Grand Island, NY). Periodic aspiration was performed through a pipette tip. The incubation was stopped by the addition of 10 ml ice-cold Krebs-Ringer bicarbonate (KRB) buffer, supplemented with 0.5% bovine serum albumin (BSA), 2.8 mmol/l glucose, and 10 mmol/l HEPES, pH 7.4. The resulting suspension, which comprised mostly single cells, was centrifuged for 5 min at 130*g*. To obtain a final concentration of 3×10^6 cells/ml, the pellet was resuspended in KRB buffer containing 2.8 mmol/l glucose.

Islet cell sorting was performed with a Epic-V flow cytometer equipped with an excitation argon laser (Innova90; Coherent, Palo Alto, CA) tuned to either 488 nm (flavin adenine dinucleotide [FAD] fluorescence) or 360 nm [NAD(P)H fluorescence] at 500–600 mW output power and connected to an MDADS microcomputer (Coulter Electronics, Hialeah, FL). β-Cells were sorted from the other islet cell types according to both FAD autofluorescence (510–550 nm) and forward light scatter (25).

Anesthetized OFA rats were decapitated. Olfactory bulbs and inferior olivary complex were removed by dissection, frozen in liquid nitrogen–cooled methylbutane, and stocked at –80°C until use.

Cells of the RIN (26) and INS-1 (27) lines were cultured in RPMI 1640 medium containing 11.1 mmol/l glucose and supplemented with 10% fetal calf serum (FCS) (heat-inactivated for INS-1), 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 µmol/l 2β-mercaptoethanol, 110 U/ml penicillin, and 110 µg/ml streptomycin. Cells of the βTC-3 line (28) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose and supplemented with 10% fetal calf serum, 110 U/ml penicillin, and 110 µg/ml streptomycin. Cells of the AR42J cell line (29) were cultured in DMEM containing 5.5 mmol/l glucose and supplemented with 10% FCS, 1 mmol/l sodium pyruvate, 110 U/ml penicillin, and 110 µg/ml streptomycin. All lines were kept at 37°C in a humidified incubator gassed with air and CO_2 to maintain a medium pH of 7.4, fed at 3-day intervals, and passed by trypsinization once a week.

RNA extraction. Rat tissues were homogenized in 2.5 mmol/l Tris-HCl, pH 7.4, containing 2 mol/l β-mercaptoethanol and 4 mol/l guanidium thiocyanate. After the addition of solid CsCl (0.4 mg/ml), the homogenate was layered on 2 ml 5.7 mol/l CsCl–0.1 mol/l EDTA (pH 7.4) and centrifuged at 150*g* and 20°C for 20 h. Pelleted RNA was resuspended in 300 µl 10 mmol/l Tris-HCl pH 8.1, supplemented with 5 mmol/l EDTA and 0.1% SDS, extracted twice with phenol-chloroform, precipitated in ethanol, and resuspended in water. Samples of total cellular RNA were similarly extracted from insulin-producing cells and AR42J cells.

Reverse transcriptase–polymerase chain reaction amplification. Aliquots (1 µg each) of total RNA were transcribed using 200 U superscript II Moloney murine leukemia virus reverse transcriptase (RT) in 50 mmol/l Tris-HCl buffer (pH 8.3) supplemented with 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, 0.5 mmol/l 4 dNTP, and 61.5 ng/µl of Random Hexamer (all reagents from Gibco, Basel, Switzerland). Samples were incubated at 42°C for 50 min. RNAse H (0.5 U) was added, and the incubation continued at 37°C for 20 min. Amplification of the resulting cDNA (20-µl samples) was performed in a final volume of 60 µl by adding 6 µl of amplification buffer containing 100 mmol/l Tris-HCl (pH 8.8), supplemented with 500 mmol/l KCl, 15 mmol/l $MgCl_2$, 1% Triton X-100, 1.2 µl 10 mmol/l 4 dNTP (Gibco), 2 U Dynazyme (Finnzymes, Espoo, Finland), and 0.167 µmol/l of both sense 5′-CA CAGCGATGGGGGAATGGA-3′ and antisense 5′-TGCCCTTTCACACATAGG CA-3′ primers. The sense primer includes the putative ATG start codon, whereas the antisense primer includes the stop codon TGA. Therefore, the entire coding sequence was included in the amplification product.

After a 5-min start at 94°C, amplification was carried out in a polymerase chain reaction (PCR) Thermal Cycler 9600 (Perkin-Elmer Cetus, Kusnacht, Switzerland) for 30 cycles, each comprising 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C. Aliquots (10 µl each) of the amplified DNA fragments were separated on a 2% agarose gel and stained with 0.5 mg/ml ethidium bromide.

Northern blot

Northern hybridization of RNA from rat tissues. Total RNA from rat tissues (8–20 µg total RNA/lane) were size-fractionated on 1% agarose gels containing 8% formaldehyde (Fluka, Buchs, Switzerland) and 1× MOPS buffer (Fluka, Switzerland) and transferred overnight onto nylon membranes (Gene Screen membranes; Du Pont de Nemours, NEN Division, Dreieich, Germany) by capillary transfer in the presence of 10× sodium chloride–sodium citrate (SSC). Membranes were ultraviolet cross-linked and vacuum-baked for 2 h at 80°C. After prehybridization, total mRNA levels were determined by hybridization with random primed cDNA probes (Boehringer Mannheim, Mannheim, Germany) specific for Cx36, which were labeled with [^{32}P]dCTP (Amersham, Zürich, Switzerland). Hybridizations were performed overnight at 42°C in the presence of 5× sodium chloride–sodium phosphate–EDTA buffer, 50% formamide, 5× Denhardt's solution, 5% SDS, 100 µg/ml purified salmon sperm DNA (Sigma, St. Louis, MO), and 100 µg/ml PolyU (Boehringer Mannheim). Blots were washed 3 times for 10 min at 42°C in 2× SSC and 1% SDS, and 3 times for 20 min in 2× SSC containing 0.1% SDS. Exposure times of all membranes to X-ray film (X-Omat AR; Kodak, Dübendorf, Switzerland) were chosen to optimize the signals under conditions preventing saturation. As a probe for Cx36, we used a 976-bp Cx36 cDNA subcloned in vector pCR-Script SK(+) (5). To normalize signal levels, the same filters were rehybridized with probes for the ubiquitously expressed gene GAPDH. We used a 1.1-kb (*Hind*III-*Eco*RI) fragment of GADH cDNA (30).

Northern hybridization of RNA from cell lines. Total cellular RNA from cell lines was denaturated with glyoxal, electrophoresed in a 1% agarose gel (5–10 µg total cellular RNA/lane) and transferred overnight onto nylon membranes (Hybond N; Amersham International, Amersham, U.K.). Membranes were ultraviolet cross-linked, stained with methylene blue, and prehybridized for 4 h at 65°C in a 50% Pipes-buffered (pH 6.8) solution of formamide supplemented with 2 mmol/l EDTA, 0.1% SDS, and 100 µg/ml salmon sperm DNA. Filters were then hybridized 15 h at 65°C with 10^5 cpm/cm^2 ^{32}P-labeled probe, washed twice at 65°C in 3× SSC and 2× Denhardt's solution, and then washed 3 times at 70°C in 0.2× SSC, 0.2% SDS, and 0.1% sodium pyrophosphate. Filters were exposed to film (X-OMAT; Eastman Kodak, Rochester, NY) between intensifying screens at –80°C. As a probe for Cx36, we used a 256-bp Cx36 cDNA subcloned in vector pCR-Script SK(+) (5). The plasmid was linearized with *Bam*HI for the synthesis of the antisense riboprobe. The template was incubated with a mixture of transcription buffer (40 mmol/l Tris-HCl [pH 7.5], 20 mmol/l NaCl, 6 mmol/l $MgCl_2$, 2 mmol/l spermidine); 0.1 mg/ml BSA; 5 nmol ATP, CTP, and GTP and 125 pmol [α-^{32}P]UTP (Amersham International); 0.75 U/µl RNAse inhibitor; and 1.7 U/µl of T3 RNA polymerase (Promega, Madison, WI) at 37°C for 80 min. The cDNA template was digested by adding 20 ng/ml RQ1- DNAse at 37°C for 20 min. After phenol extractions, the transcripts were purified using Sephadex G50 columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Ribonuclease protection assay. Total RNA was extracted as described previously (31). A 256-bp Cx36 cDNA, subcloned in plasmid pCR-Script SK(+), was used as template for riboprobe synthesis. The plasmid was linearized by digestion with *Eco*RI and transcribed in the presence of [α-^{32}P]UTP (800 Ci/mmol) using T3 RNA polymerase (MAXIscript Kit; Ambion, Austin, TX). RNAse protection assays were performed with the RPA II Kit (Ambion) according to the manufacturer's instructions. Hybridization to yeast tRNA was used as a negative control. Protected cRNA fragments were separated on 4% polyacrylamide gels under denaturing conditions, and the gels were exposed to X-ray film at –70°C with an intensifying screen.

In situ hybridization. Anesthetized rats were perfused by intracardiac injection of 4% paraformaldehyde in PBS (pH 7.4) for 8 min. Pancreatic tissue was rapidly removed and postfixed in 4% paraformaldehyde in PBS for 1 h and then kept overnight at 4°C in 10% sucrose in PBS. Samples were frozen in isopentane cooled in liquid nitrogen and stored at –70°C until use. Cryostat sections of 10-µm thickness were thawed onto 3-aminopropyl ethoxysilane–coated slides, fixed in 4% paraformaldehyde for 15 min, and rinsed once in PBS and twice in distilled water. Tissue was deproteinated in 0.2 mol/l HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1 mol/l ethanolamine for 20 min, and dehydrated with increasing concentrations of ethanol. Slides were incubated for 16 h in a humidified chamber at 52°C with 8×10^5 cpm of probe in a 70-µl hybridization cocktail (50% formamide, 20 mmol/l Tris-HCl [pH 7.6], 1 mmol/l EDTA [pH 8.0], 0.3 mol/l NaCl, 0.1 mol/l dithiothreitol, 0.5 µg/ml yeast tRNA, 0.1 µg/ml poly-A-RNA, 1× Denhardt's solution, and 10% dextran sulfate). Slides were washed twice for 15 min in 1× SSC at 62°C and then for 30 min in formamide:SSC (1:1) at 62°C. After an additional washing in 1× SSC at 62°C, single-stranded RNA was digested by RNAse treatment (10 µg/ml) for 30 min at 37°C in 0.5 mol/l NaCl, 20 mmol/l Tris-HCl (pH 7.5), and 2 mmol/l EDTA. Tissue was washed twice for 30 min with 1× SSC at 62°C before dehydration in ethanol and air-drying. Slides were dipped in NTB-2 photoemulsion diluted 1:1 in water (Eastman Kodak), exposed at 4°C for 4 weeks, developed with D19 (Eastman Kodak), fixed with Al-4 (Agfa Gevaert, Kista, Sweden), and counterstained with cresyl violet.

A 593-bp Cx36 cDNA, subcloned in vector pCR-Script SK(+) (5), was used as template. The plasmid was linearized by digestion with *Sac*I for the synthesis

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of the antisense riboprobe or with *Eco*RI for the sense riboprobe. Each template was incubated with a mixture of transcription buffer (40 mmol/l Tris-HCl [pH 7.5], 6 mmol/l $MgCl_2$, and 2 mmol/l spermidine); 12.5 nmol ATP, CTP, and GTP; 500 pmol UTP and 125 pmol [α-^{35}S]UTP (Dupont–NEN, Boston, MA); 1 U/µl RNAse inhibitor; and 1 U/µl of appropriate RNA polymerase (T7 RNA polymerase for the antisense probe and T3 polymerase for the sense one) at 37°C for 60 min. The cDNA template was digested by adding 20 ng/ml DNAse I at 37°C for 30 min. The transcripts were purified using Nensorb columns (Dupont–NEN) and analyzed by formaldehyde gel electrophoresis under denaturing conditions. Controls included the use of single-strand sense ^{35}S-labeled riboprobes. Each control probe resulted in a signal equivalent to background.

In some experiments, sections were washed in 0.1 mol/l PBS after the last washing of the in situ hybridization procedure and incubated for 10 min in 1.5% normal goat serum and 0.3% Triton X-100 in PBS. The sections were incubated at room temperature with a primary antibody against either insulin (monoclonal antibodies diluted 1:200 in PBS with 1.5% blocking serum), glucagon (diluted 1:100), or somatostatin (diluted 1:100); washed 3 times for 5 min in PBS; and incubated 10 min with anti-mouse or anti-rabbit IgG, whichever was appropriate (diluted 1:200 in PBS with 1.5% blocking serum). After 3 5-minute washings in PBS, the sections were processed with a Vectastain kit (Vector, Burlingame, CA) for 15 min. Sections were rinsed, and the peroxidase reaction was developed with 0.05% 3,3-diaminobenzidine-4 HCl and 0.003% hydrogen peroxide. After washing in water, the sections were dehydrated in an increasing alcohol series, coated with the NTB-2 emulsion, and processed in the in situ hybridization, as outlined above.

Immunolabeling of Cx36. A peptide corresponding to amino acids 289–303 (AKRKSVYEIRNKDLP) of rat Cx36 was conjugated to keyhole limpet hemacyanin and used to immunize rabbits (Eurogentec; Parc scientifique du Sart Tilman, Herstal, Belgium). Site-directed antibodies were affinity-purified by passing the crude serum diluted in PBS through a HiTrap affinity column (HiTrap NHS-activated sepharose; Amersham Pharmacia Biotech AB), which was coupled with the Cx36 COOH-terminal tail peptide. The column was washed sequentially with PBS, 0.5% PBS-Tween20, and PBS. Antibody solutions were eluted with 100 mmol/l glycine pH 2.5 in a solution of 1.5 mol/l Tris pH 8.8 to neutralize the antibody solution. The specificity of the purified fractions was assessed by labeling sections of rat hypothalamus, hippocampus, and olfactory bulbs known to express Cx36 (5).

Immediately after sampling, tissues were frozen in liquid nitrogen–cooled methylbutane, embedded in Tissu-Tek, and sectioned in a Cryocut 3000 cryostat (Leica AG, Glattbrugg, Switzerland). Sections (5 µm thick) were exposed for 3 min to –20°C acetone, air-dried, rinsed in PBS containing 0.5% BSA, and incubated for 2 h at room temperature with the rabbit polyclonal Cx36 antibody diluted 1:100. After a second hour's exposure at room temperature to a goat anti-rabbit IgG antibody labeled with fluorescein and diluted 1:500, sections were stained, coverslipped, and photographed. A similar protocol was followed to immunostain insulin-producing cell lines using the rabbit polyclonal Cx36 antibody diluted 1:200. In these experiments, controls included use of wild-type (negative control) and Cx36-transfected HeLa cells (positive control; a gift from Dr. K. Willecke).

RESULTS

To screen for the expression of a Cx36 transcript in rat pancreas, the first analysis was carried out by RT-PCR total RNA, using primers expected to amplify the entire coding sequence of Cx36 (5). These primers amplified a single band of 980 bp in olfactory bulbs and the inferior olivary complex (*lanes OB* and *IO* in Fig. 1, upper panel), the neuronal tissues used as the positive control, but not in heart (*lane H* in Fig. 1, upper panel), the tissue used as the negative control. Using these primers, no transcript for Cx36 was detectable in RNA extracted from total pancreas (*lane P* in Fig. 1, upper panel), even though transcripts for both Cx43 and Cx45 were easily detectable in the similar RNA samples (Fig. 1, middle and lower panels).

To assess whether Cx36 mRNA may have escaped detection because of a restricted expression in selected pancreatic cell types, we examined the expression of Cx36 transcript in isolated pancreatic islets. We found that these micro-organs (*lane I* in Fig. 1, upper panel) expressed a transcript of the same size (980 bp) as that of the Cx36 found in olfactory

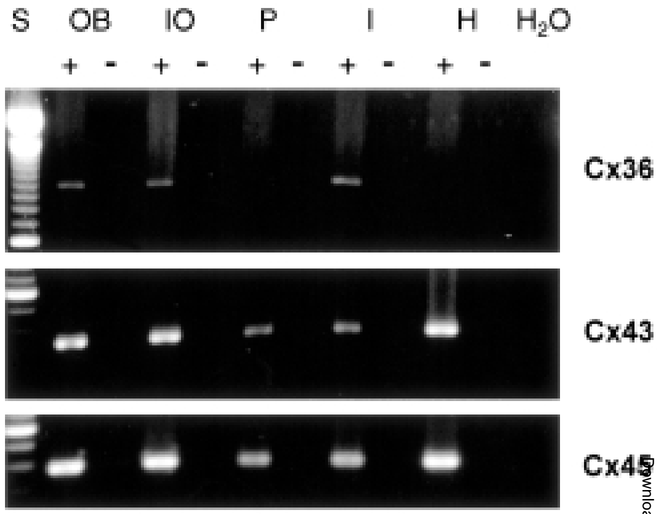

FIG. 1. A Cx36 transcript is found in selected rat tissues. RT-PCR amplification of total RNA showed that a Cx36 transcript was expressed by pancreatic islets (I) as well as by olfactory bulbs (OB) and inferior olivary complex (IO), two tissues used as positive controls. By contrast, this transcript was not detected in RNA extracted from total pancreas (P) and heart (H), a tissue used as the negative control. Middle and lower panels: in the very same RNA samples, Cx43 and Cx45 transcripts were detected in all the tissues tested. In all cases, negative controls were also provided by not reverse-transcribing the samples before PCR amplification (– lanes) and by submitting a sample of water (H_2O) to amplification. Left lane shows size standards (S). All samples were submitted to 30 amplification cycles.

bulbs and the inferior olivary complex. To assess the actual expression of this transcript, RNA from isolated rat islets was further analyzed by Northern blotting. A mRNA of ~2.9 kb was detected in the total brain extract (*lane B* of Fig. 2*A*) that served as the positive control as well as in rat pancreatic islets (*lane I* of Fig. 2*A*). In contrast, no transcript was detected by the same probe in RNA extracted from heart (*lane H* of Fig. 2*A*). RNAse protection assay confirmed the presence of a Cx36 transcript in pancreatic islets (*lane I* of Fig. 2*B*), which was not detectable in intact pancreas (*lane P* of Fig. 2*B*) and which corresponded in size to that observed in the hypothalamus, olfactory bulb, and retina (*lanes Hy*, *OB*, and *R* of Fig. 2*B*, respectively). In situ hybridization of pancreas sections showed that the distribution of Cx36 mRNA was restricted to endocrine islets, with the surrounding exocrine pancreas showing only a background level of labeling (Fig. 2*C*, left panel). The combination of in situ hybridization and immunoperoxidase labeling for glucagon, which was used to map the outermost layer of endocrine islet cells, showed that the autoradiographic grains reflecting the presence of Cx36 mRNA were predominantly distributed over the cells forming the bulk of the islet center (Fig. 2*C*, right panel), which is known to comprise mostly insulin-containing cells (32).

To assess the actual expression of the Cx36 protein, we developed affinity-purified antibodies against residues 289–303 of the COOH-terminal tail of rat Cx36. These antibodies, labeled in a selective and punctate manner, Cx36-transfected HeLa cells and the neuronal processes comprising glomeruli of olfactory bulbs (Fig. 3*A* and *B*) and neuron bodies on either hippocampus sections (Fig. 3*C*) or cultures of hypothalamic explants (not shown). In contrast, these antibodies did not

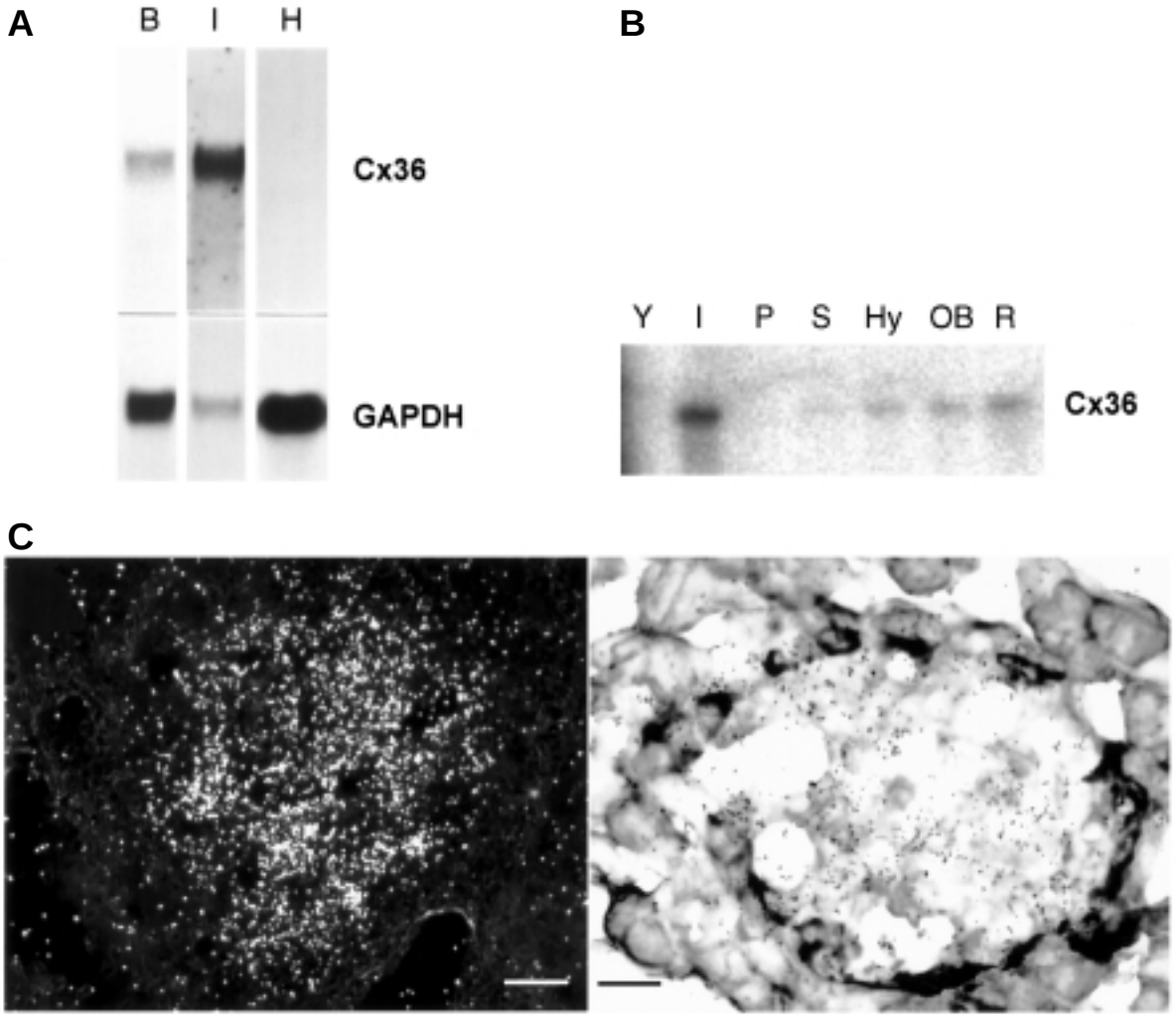

FIG. 2. A Cx36 mRNA is expressed in pancreatic islets. *A*: Top: Northern blot analysis of Cx36 mRNA in rat brain (B), islets (I), and heart (H) revealed that this transcript is detectable in the whole brain and highly expressed in rat islets. No Cx36 transcript was detectable in the heart. Lanes were loaded with 15 µg total RNA (brain), 8 µg (islets), and 20 µg (heart), as indicated by the level of the GAPDH transcript detected in each lane. *B*: Detection of Cx36 mRNA by RNAse protection assay. Analysis of total RNA (5 µg) of yeast (Y), pancreatic islets (I), total pancreas (P), striatum (S), hypothalamus (Hy), olfactory bulbs (OB), and retina (R) suggested that the levels of the Cx36 transcript may be higher in pancreatic islets (I) than in the control nervous system regions that were used as positive controls. *C*: Left panel: Dark-field microautoradiograph of a pancreatic section hybridized in situ with a probe for Cx36 shows an intense labeling, reflecting the abundance of Cx36 mRNA over a pancreatic islet. Right panel: The combination of in situ hybridization and immunolabeling for glucagon shows that autoradiographic grains, reflecting the distribution of Cx36 mRNA, were abundant over the insulin-containing β-cells that form the center of pancreatic islets. In contrast, the grain density was not above background levels over the glucagon-containing α-cells that form the islet periphery. Note the sparse background labeling of the surrounding exocrine pancreas. Scale bar equals 100 µm in both panels.

label the astrocytes that were present in the same cultures, nor did they label wild-type HeLa cells, which are known not to express connexins at detectable levels (not shown). The same antibodies resulted in a sizable punctate immunostaining of membranes in pancreatic islets but not in the surrounding exocrine acini (Fig. 3*D*). Dual immunofluorescence labeling, associating antibodies to Cx36 with antibodies to insulin, showed that most Cx36 was distributed over insulin-containing β-cells (Fig. 3*E*).

To further study the distribution of Cx36, cells were dispersed from pancreatic islets and separated by fluorescence-activated sorting in two cell populations, hereafter referred to as "β-cells" and "non–β-cells," respectively. Using total RNA extracted from the two populations and amplified by RT-PCR, we found the transcript for Cx36 in both β- and non–β-cells (Fig. 4, upper panel). However, this transcript was more abundant in the β-cell population, which comprised almost exclusively insulin-containing cells, than in the non–β-cell population, which comprised a mixture of all islet cell types. In the same samples of total RNA, the reverse was observed for both Cx43 and Cx45. Thus, the levels of the transcripts for these two connexins were higher in the non–β-cell population than in the β-cell population (Fig. 4, middle and lower panels). Using antibodies, we found that Cx36 was abundant along the membranes of most β-cells that had reaggregated in clusters but was not detected along those that had remained single after a 1- to 3-day culture period (Fig. 5*A* and *B*). In contrast, the same antibodies labeled only rare cells in cultures made of the mixed non–β-cell population (Fig. 5*C* and *D*).

RT-PCR analysis further revealed that the 980-bp Cx36 transcript found in islets (*lane I* in Fig. 6*A*) was also ampli-

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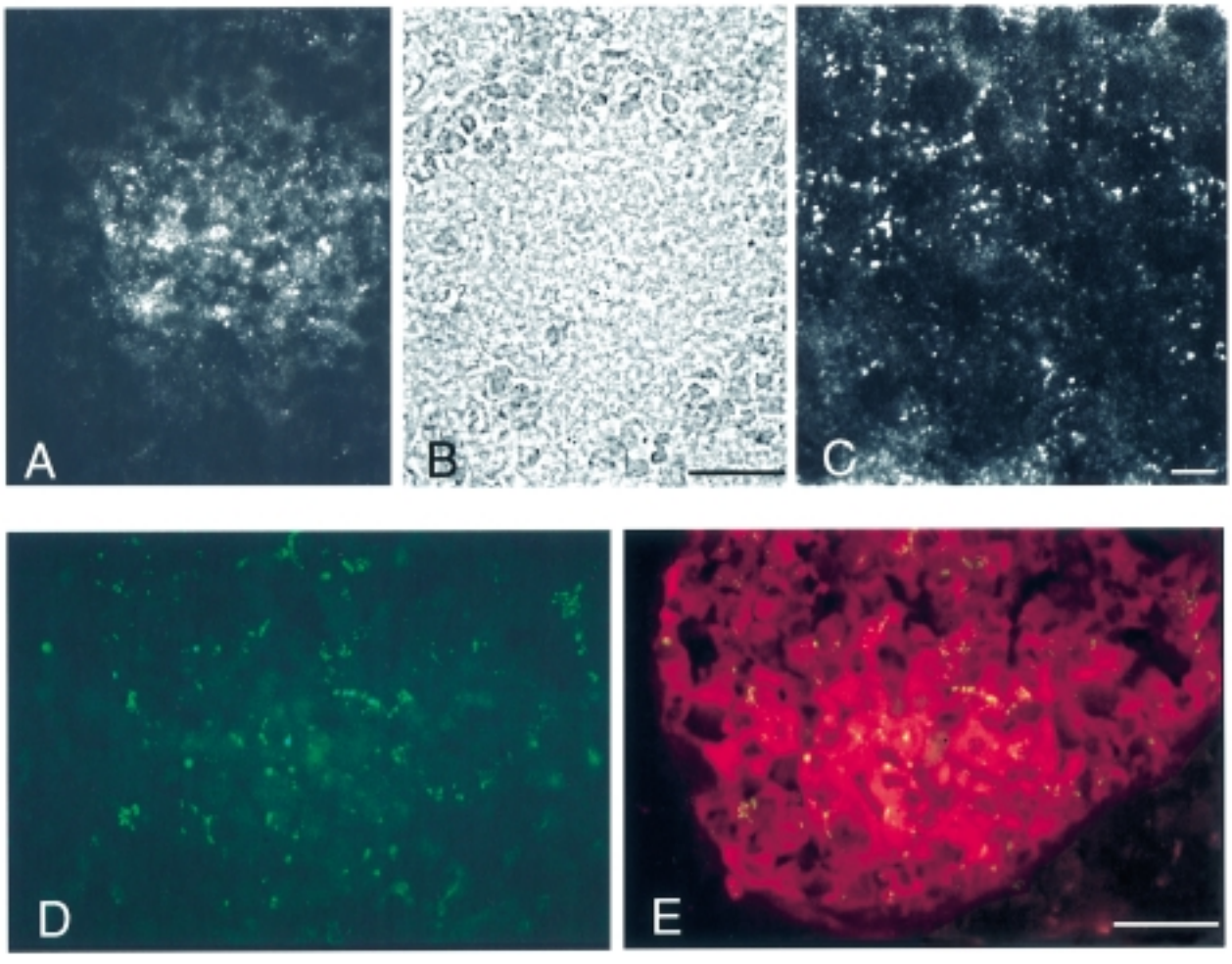

FIG. 3. Cx36 protein is expressed in pancreatic islets. *A–C*: An antibody directed against a sequence of the COOH-terminus of Cx36 immunodetected this protein in the neural processes comprising the glomeruli of olfactory bulbs (*A*). *B* is the phase contrast view of the field shown in *A*. The protein was also detected between cell bodies of neurons forming the CA3 pyramidal layer of hippocampus (*C*). *D* and *E*: The same antibodies resulted in a punctate staining of pancreatic islets (*D*) but not of the surrounding exocrine acini. Dual immunostaining for insulin (as revealed by a rhodamine fluorochrome) and Cx36 (as revealed by a fluorescein fluorochrome) revealed that the punctated staining was distributed over the insulin-containing β-cells that form the bulk of pancreatic islets (*E*). Scale bar equals 40 μm in *A–E*.

fied in several lines of insulin-producing cells, including βTC-3, INS-1, and RIN-2A (Fig. 6*A*). Northern blotting (Fig. 6*B*) further showed that this transcript was expressed at high levels in βTC-3 cells and much less in the RIN-2A cell line (*lane RIN* in Fig. 6*B*). INS-1 cells showed intermediate levels of expression (Fig. 6*B*).

DISCUSSION

Connexins, gap junctions, and coupling are obligatory features of secretory cells, including those that form pancreatic islets (33–35). Cx43 has been the first gap junction protein identified in these micro-organs (8,11). Recently, however, we have shown that gap junctional plaques persist in the β-cell membranes of homozygous transgenic mice that completely lack Cx43 (36,37). When revisiting the connexin pattern of normal β-cells, we found that a transcript for Cx45 is also expressed within pancreatic islets, both by primary insulin-producing β-cells and other islet cell types (36). As of yet, however, it has proven difficult to unambiguously demonstrate the expression of Cx43 and Cx45 by islet cells using specific antibodies. Also, attempts at identifying electrical conductance characteristics of either Cx43 or Cx45 channels have failed (8,13). These negative findings prompted us to explore the expression of Cx36, a protein recently identified in mammalian brain and retina (5,6).

Using RT-PCR amplification, Northern blot analysis, and an RNAse protection assay, we now show that a transcript for Cx36 is also expressed in rat pancreatic islets. Using the same set of primers, this transcript was not detectable in total pancreas (6), reflecting a restricted expression in the gland similar to the situation in the central nervous system (5,6). In situ hybridization confirmed that the Cx36 transcript was abundant within pancreatic islets but was not expressed to a detectable level in the surrounding exocrine acini.

Using novel antibodies, we found that Cx36 is actually expressed in pancreatic islets, where it labels, in a punctate way, the membranes of hormone-producing cells. Thus, this protein can no more be considered only as a connexin of the central nervous system (5,6). Interestingly, both in situ hybridization and immunolabeling indicated that this connexin was abundant in the centrally located insulin-producing β-cells and was much less, if at all, expressed by the other endocrine cell types that form the periphery of pancreatic islets. This differential expression was further confirmed on fluorescence-activated

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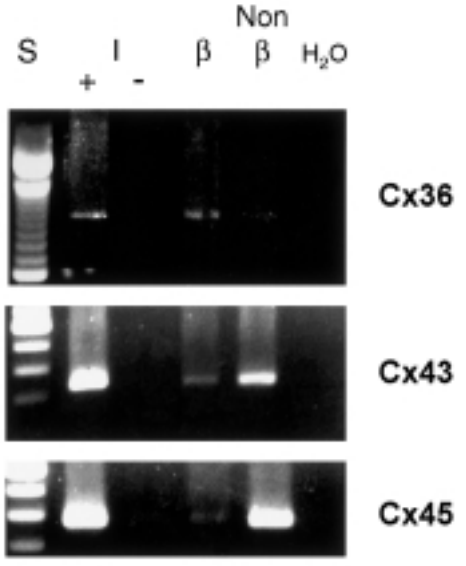

FIG. 4. Cx36 transcript is differentially distributed in pancreatic β- and non–β-cells. RT-PCR amplification of RNA from FACS-purified preparations of islet cells detected a Cx36 transcript in β- and non–β-cell fractions. However, this transcript was less abundant in preparations comprising a mixture of all islet cell types (non β) than in purified preparations of β-cells (β). In the very same RNA samples, Cx43 and Cx45 transcripts were also detected in both β-cells and non–β-cells. Positive controls were provided by amplification of Cx36 product in pancreatic islets (I). Negative controls were provided by not reverse-transcribing the samples before PCR amplification (– lanes) and by submitting a sample of water (H_2O) to amplification. The left lane shows size standards (S). All samples were submitted to 30 amplification cycles.

cell sorter (FACS)-purified preparations enriched in either β- or non–β-cells, in which both immunolabeling and RT-PCR amplification showed that Cx36 is predominantly expressed by insulin-producing β-cells. The ratio of the transcript coding for Cx36 to that coding for either Cx43 or Cx45 was high in β-cells and low in the non–β-cell population, which contains most of the glucagon-, somatostatin-, and pancreatic polypeptide–producing cells and a few insulin-producing cells. Together, the data suggest that Cx36 is preferentially, if not solely, expressed by insulin-producing β-cells.

Different connexins allow for the formation of intercellular channels with distinct conductances, permeabilities, and regulation characteristics (38–40). Cx36 provides for unique features of junctional channel in terms of both unitary conductance and sensitivity to transjunctional voltage (41). Thus, its differential expression in distinct islet cell types may provide the basis for selectivity in their communication. Such a selectivity is required for proper islet functioning, since, under most conditions, some islet cell types (e.g., β- and δ-cells) are activated or inhibited in parallel, whereas others (e.g., β- and α-cells) function in an antagonistic way (42–44). If the same ions and molecules were exchanged at the same rate throughout all the connexin channels, it would be difficult to conceive how distinct islet cell types could retain a specific secretory activity. The restricted expression of Cx36 provides β-cells with a pathway for intercellular exchange of signals that may not reach, at least at the same rate, neighboring cells producing other hormones.

Cx36 is the first connexin species unambiguously detected between insulin-producing cells. When compared with the

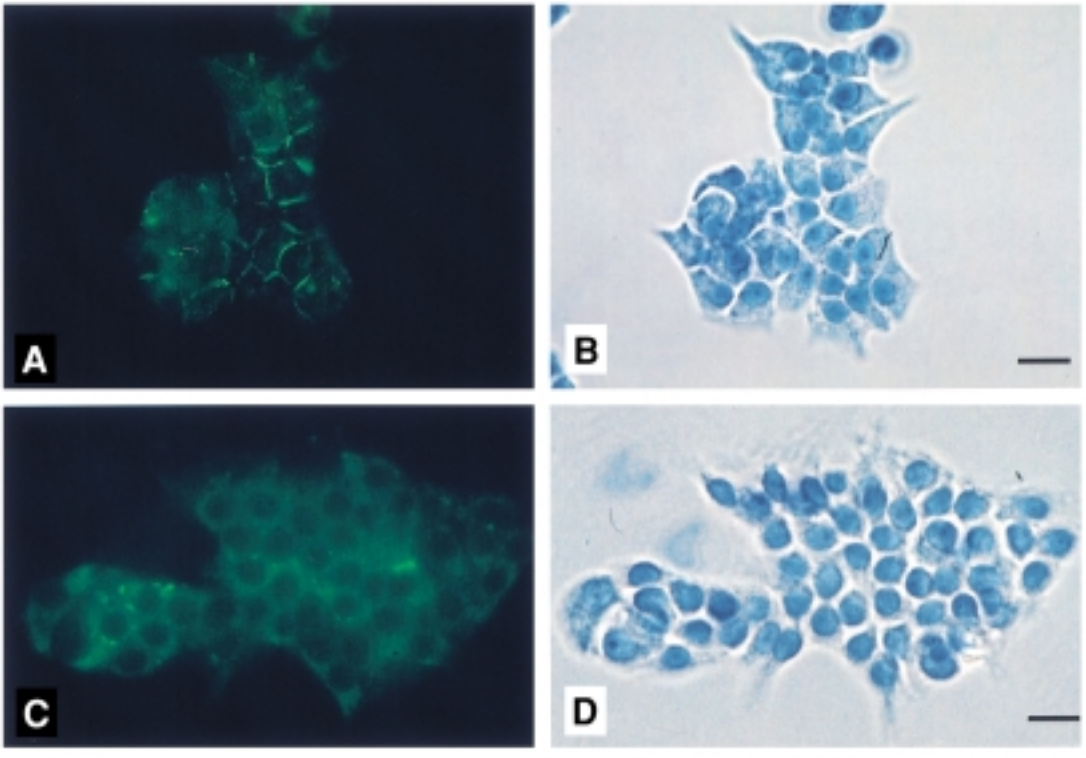

FIG. 5. Cx36 protein is differentially expressed in β- and non–β-cells. *A*: Immunolabeling of FACS-purified preparations of islet cells resulted in an abundant fluorescent labeling of most β-cell membranes. *B*: The phase contrast view of the field shown in *A*. In contrast, the same antibodies barely labeled the fraction comprising mostly non–β-cells (*C*). *D*: The phase contrast view of the field shown in *C*. Scale bar equals 10 μm in *A*–*D*.

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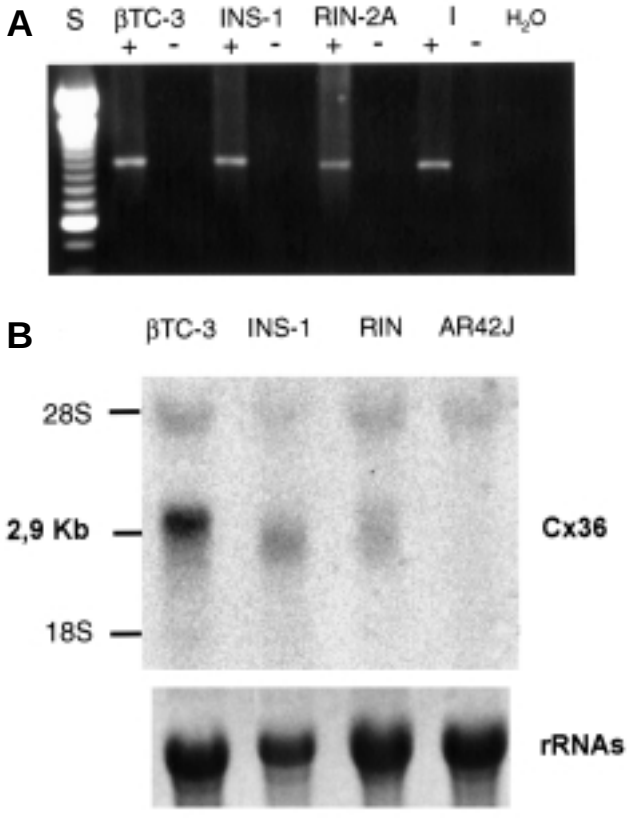

FIG. 6. Expression of Cx36 is retained in insulin-producing cell lines. *A*: RT-PCR amplification of RNA from the insulin-producing cell lines βTC-3, INS-1, and RIN-2A showed that Cx36 was expressed in all cases. Positive controls were provided by amplification of a Cx36 product in pancreatic islets (I). Negative controls were provided by omitting the reverse transcription of samples before PCR amplification (– lanes) and by submitting a sample of water (H_2O) to amplification. The left lane shows size standards (S). All samples were submitted to 30 amplification cycles. *B*: Northern blot analysis revealed an mRNA coding for Cx36 in samples of total RNA (10 μg) from all the insulin-producing lines tested. In contrast, no such transcript was detected in AR42J cells—a cell line derived from pancreatic acinar cells. The methylene blue staining of ribosomal RNA indicated a comparable loading of all RNA samples.

channels made by other connexins, homotypic channels made of Cx36 have a small single-channel conductance (~14 pS) and are weakly dependent on transjunctional voltage (41). The former characteristic presumably explains why these channels have so far escaped detection in pairs of β-cells monitored under dual patch-clamp whole-cell recording conditions (8,13). The latter characteristic, which is shared by other members of the γ group of connexins (42,45,46), suggests that the electrical coupling of β-cells may not be disrupted during either the hyperpolarization or depolarization phases that these cells undergo cyclically (47–49). Previous studies have indicated that connexin-dependent coupling may be required for the glucose-induced recruitment and synchronization of β-cells, which, in the absence of junctional communication, show marked differences in the ability to synthesize and release insulin (8,12,14,33,50). The abundance of Cx36 between β-cells and the finding that this protein provides channels that could ensure the electrical synchronization of β-cells during both resting and stimulated function are consistent with a central role of Cx36 in the regulation of insulin secretion. A central role of Cx36 in glucose-induced insulin secretion is further suggested by the observation that level Cx36 expression correlates with the glucose responsiveness of different insulin-producing lines (26–28), i.e., is high in βTC-3 cells, much lower in INS-1 cells, and minimal in RIN-2A cells.

The reason Cx36 has been selected as a prominent connexin by both insulin-producing cells and neurons remains to be understood. Contrary to many other types of endocrine cells, pancreatic β-cells do not derive from ectoderm as neurons (17,51,52) and still share with them many characteristics, including the expression of a number of "neuron-specific" molecules such as GAD (20), GLUT2 (18,21), and islet brain-1 (22). β-Cells also resemble neurons because they are electrically excitable, respond to hormonal stimuli and glucose by depolarization (23), and display neurite-like processes in culture (24). These similarities suggest that some developmental, morphogenetic, and/or secretory features common to β-cells and neurons may obligatorily require the participation of Cx36 channels.

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