Connexin 36 is expressed and associated with zonula occludens-1 protein in PC-12 cells

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Abstract. Connexin 36 (Cx36) is the predominant connexin isoform expressed in the mammalian neurons of the central nervous system (CNS). PC-12 cells, a neuronal-like cell line, are widely used for neuron functional studies. Many connexins have been shown to interact with zonula occludens-1 protein (ZO-1), a tight junction associated with protein. The present study is intended to investigate whether Cx36 is expressed in PC-12 cells and is associated with ZO-1. Cx36 transcripts were amplified and verified by RT-PCR. 2.9 kb Cx36 mRNA was detected in PC-12 cells through Northern blot hybridization. Western blotting showed a 36-kDa protein band in the homogenates of PC-12 cells. Immunofluorescence labeling revealed that Cx36 was present in cell-cell contacts of PC-12 cells and colocalized with ZO-1. The association of Cx36 and ZO-1 in PC-12 cells was also demonstrated by coimmunoprecipitation. In conclusion, PC-12 cells express Cx36 mRNA and Cx36 proteins that are associated with ZO-1. These results enhanced our understanding of the function of Cx36 in PC-12 cells.

Key words: Connexin 36 — Zonula occludens-1 protein — PC-12 cells

Introduction

Signal transmission between neurons takes place at the specialized sites of functional interaction that can be broadly distinguished as two main types: chemical and electrical synapses. Chemical synapses have been extensively studied and their molecular aspects have been elucidated in great detail. However, electrical synapses have received far less attention. The most common form of interneuronal electrical transmission is mediated by gap junctions; specialized membrane regions contain aggregates of transmembrane channels that directly connect the cytoplasm of adjacent cells. In gap junctions, intercellular channels permit the direct flow of ions between the cytoplasm of adjacent cells and allow passage of second messengers and small metabolites, leading to chemical communications and metabolic couplings (Kumar and Gilula 1996; Kam et al. 1998). Each intercellular channel is comprised of two hemichannels, or connexons, that are embedded in adjacent membranes. Each connexon contains six protein subunits known as connexins, which are arranged radically around a central pore (Beyer et al. 1990). Connexins are encoded by a multigene family, whose members are differentiated according to the species of origin and the predicted molecular mass in kDa (e.g., Cx32, Cx43) (Goodenough et al. 1996).

Gap junction proteins play a pivotal role in the regulating of cell growth, development, differentiation and synchronous activity in central nervous system (CNS) (White and Paul 1999). The importance of gap junction intercellular communication is evidenced by the specific connexin gene knock out studies where connexins mutations are found to be associated with certain diseases (Willecke et al. 2002). The connexins that are known in

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the mammalian CNS include Cx26, Cx29, Cx30, Cx32 and Cx36. Of these connexins, Cx36 is expressed only in neurons of CNS (Condorelli et al. 2000; Li et al. 2002; Connors and Long 2004).

PC-12 cell, a cell line substitute neuron, was originally cloned from rat phaeochromocytoma cells (Greene 1978; Byrd et al. 1986). However, whether PC-12 cells can express Cx36 mRNA and translate Cx36 protein has not been well studied. Emerging evidences has shown that several connexins (e.g. Cx43, Cx32, Cx45, Cx31.9) can interact with zonula occludens-1 protein (ZO-1) (Toyofuku et al. 1998; Kojima et al. 2001; Laing et al. 2001; Nielsen et al. 2002). ZO-1 is a membrane protein associated with the guanylate kinase family, and contains a number of PDZ (PSD95/D1g/ZO-1) domains (Lorenza et al. 2000). However, whether Cx36 is associated with ZO-1 is unclear. The primary purpose of this study was to investigate whether Cx36 transcripts and protein are expressed in PC-12 cells, and whether Cx36 is associated with ZO-1 in PC-12 cells.

Materials and Methods

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from 10-day-old rat brain tissues and PC-12 cells as previously described (Chomczynski and Sacchi 1987). The RT reaction was conducted using 1×10^{-3} mg of total RNA in a solution containing 2×10^{-3} ml of 5 × RT buffer, which was comprised of 0.1 mol/l dithiothreitol, 2.5 mmol/l dNTP, 0.01% bovine serum albumin, 5×10^{-4} ml dimethylsulfoxide, 10 units of RNA guard (Pharmacia Corporation, Peapack, NJ, USA), 500 ng oligo (dT)-15 primer and 20 units of RT in a total volume of 1×10^{-2} ml. The mixture was incubated for 1 h at 37°C, and then for 10 min at 95°C. Primers chosen for the PCR were designed according to rat Cx36 sequence (GenBank accession No. NM 019281): sense primer 5'-ATGGGGGAATGGACCATC-3' and antisense primer 5'-AGTTTGATCTTCCGCCAT-3'. PCR was conducted in 2×10^{-2} ml of solution containing 2×10^{-3} ml 10 × PCR buffer, 8×10^{-4} ml of 50 mmol/l $MgCl_2$, 200 μmol/l dNTP, 100 ng sense and antisense primers, 1 unit of Taq DNA polymerase and 1×10^{-3} ml of template cDNA. PCR was performed as follows: at 95°C for 4 min, then at 95°C for 1 min, at 55°C for 1 min, and at 72°C for 1.5 min. After 32 cycles of PCR, the mixture was further incubated for 10 min at 72°C. The PCR products were separated by electrophoresis on 1% agarose gel stained with ethidium bromide and further purified using a gel purification kit. Fresh PCR products were ligated into pBluescript-SK (+) T-vector as previously described (Li et al. 1999). The plasmids were sequenced on an ABI-377 DNA sequencer using T7 sequencing primer.

Northern blot

100 ng 845bp Cx36 cDNA was labeled with 50uCi 32P-dATP using the random labeling kit (Promega). Northern blot was performed as previously described (Zhurinsky et al. 2000). Briefly, 5×10^{-2} mg total RNA from rat brain and PC-12 cells was denatured in 2.2 mol/l formaldehyde, 50 de-ionized formamide, 20 mmol/l morpholionpropanesulfonic acid (pH 7.0) at 65°C for 20 min then on ice for 5 min. The RNA samples were separated on 1.2% agarose gel containing 2.2 mol/l formaldehyde with 30 V overnight, and transferred overnight to a non-charged nylon membrane by capillary action in 20 × SSC buffer (3 mol/l sodium chloride and 300 mmol/l sodium citrate; pH 7.0). After rinsed in 6 × SSC buffer, the membrane was dried in Baker for 1 h at 80°C then UV-irradiated for 3 min. The membrane was then pre-hybridized for 24 h at 42°C in solution containing 50% formamide, 5 × SSC buffer, 5 × Denhardt´s reagent, 0.25 mg/ml Salmon sperm DNA at hybridization oven. For hybridization, 108 cpm of probe was applied in hybridization buffer containing 50% formamide, 5 × SSC buffer, 5 × Denhardt´s reagent, 0.1 g/ml dextran sulfate and incubated for 24 h at 42°C. Following hybridization, the membrane was washed three times in 2 × SSC buffer and 0.1% SDS at 65°C for 20 min. Then the membrane was wrapped in a plastic cover and placed into a film cartridge (Kodak) and exposed at –80°C for 4–7 days. Equal RNA loading was valuated by measurement of GAPDH mRNA with cDNA probe prepared to nucleotides 388–727 of GAPDH (GenBank accession No. NM 017008.1).

Cell culture and immunofluorescence

PC-12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% fetal bovine serum and 1% penicillin streptomycin.

Confluent PC-12 cells were rinsed in 10 mmol/l phosphate buffer saline (PBS, containing 0.9% saline) and fixed in ice-cold 4% paraformaldehyde, incubated overnight at 4°C with rabbit anti-Cx36 antibody diluted at 1 : 400 in Tris buffered saline (TBS) buffer (pH 7.5) containing 50 mmol/l Tris, 1.5% saline, 0.3% Triton X-100 and 4% normal donkey serum, then washed with 50 mmol/l Tris buffer (pH 7.5) for 1 h, incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit immunoglobulin G (IgG) diluted at 1 : 200 for 1 h, and finally washed for 1 h and coverslipped. For double immunofluorescence labeling of Cx36 and ZO-1 in PC-12 cells, the rabbit anti-Cx36 antibody and monoclonal anti-ZO-1 antibody were used, with FITC-conjugated donkey anti-rabbit IgG diluted at 1 : 200 labeling Cx36 and CY3-conjugated goat anti-mouse IgG diluted at 1 : 200 labeling ZO-1 (antibodies of Cx36 and ZO-1 obtained from Zymed Laboratories Incorporated, South San Francisco, CA, USA).

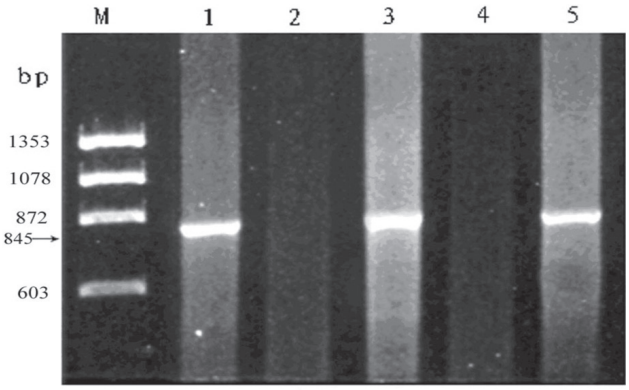

Figure 1. RT-PCR amplification of 845bp Cx36 cDNA. Lane 1: rat brain tissue; lanes 3 and 5: PC-12 cells; lanes 2 and 4: control groups without performing reverse transcription. M, PhiX-174/HaeIII marker.

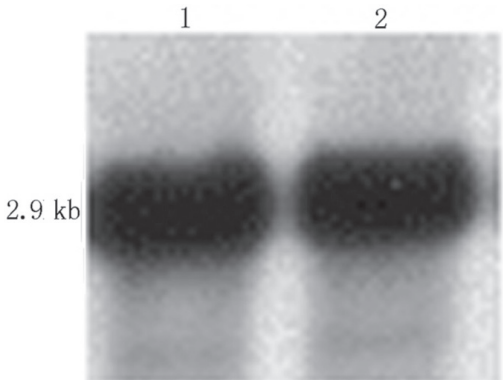

Figure 2. Northern blot detection of Cx36 mRNA expression in 10 days-old rat brain and PC-12 cells. 2.9 kb Cx36 mRNA is detected from PC-12 cells (lane 1) and rat brain (lane 2).

Western blotting

The cells were rinsed in PBS and lysed in 40 mmol/l Tris buffer (pH 7.4) containing 1 mmol/l PMSF, 1 mmol/l sodium orthovanadate, 1% NP-40, 5×10^{-3} mg/ml of leupeptin, pepstatin A, and aprotinin, respectively. The lysate was briefly centrifuged and protein concentration was determined by using a kit (Bio-Rad Laboratories, Hercules, CA, USA), and samples were electrophoresed on 12% sodium dodecylsulphate (SDS)-polyacrylamide gels and transblotted to nitrocellulose membranes (Bio-Rad) in standard Tris-glycine transfer buffer. Membranes were blocked for 1 h at room temperature in 10 mmol/l TBS (pH 8.0) with 150 mmol/l NaCl containing 5% non-fat milk powder, washed in TBS containing 0.2% Tween-20 (TBST), and incubated overnight at 4°C with rabbit anti-Cx36 antibodies diluted (1 : 500) in TBS containing 1% non-fat milk powder. Membranes were then washed in TBST for 40 min, incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1 : 2500 (Sigma), washed with TBST for 40 min and then reacted by enhanced chemiluminescence (ECL, Amersham PB).

Coimmunoprecipitation

Lysates were sonicated and centrifuged at 20,000 × *g* for 10 min at 4°C. 2 mg supernatant protein was washed for 1 h at 4°C with 2×10^{-2} ml protein A-coated agarose beads (BioTech, Santa Cruz, CA, USA) and then centrifuged at 20,000 × *g* for 10 min at 4°C. Supernatants were incubated with 2×10^{-3} mg anti-ZO-1 antibody or anti-Cx36 antibody with shaking 16 h at 4°C, followed by 1 h incubation with 2×10^{-2} ml protein A-coated agarose beads, and then centrifuged at 20,000 × *g* for 10 min at 4°C. The beads were washed vigorously five times with 1 ml washing buffer (20 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl and 0.5% NP-40) and then incubated at 60°C for 2 min in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 10% β-mercaptoethanol. Samples were processed by SDS-PAGE and the membranes were probed with anti-Cx36 or anti-ZO-1 antibodies. Control samples were taken through the immunoprecipitation procedures with exclusion of primary antibody.

Results

Expression of Cx36 mRNA in PC-12 cells

Cx36 coding region was not located in the same exon and interrupted by intron (Belluardo et al. 1999), so sense primer from exon 1 and antisense primer from exon 2 were designed. Therefore, genomic DNA contamination was identified after RT-PCR. 845bp cDNA was amplified from PC-12 cells and rat brain by RT-PCR, which was not amplified in the control group without performing reverse transcription (Fig. 1). The resulting PCR products were subcloned and partially sequenced; the sequence was identical to the published Cx36 sequencing by using blast search program (www.ncbi.nlm.nih.gov). 845bp Cx36 cDNA was used as a probe for northern blot hybridization, which GADPH probe was used as a control for quantity the equal loading. 2.9 kb mRNA band was detected in PC-12 cells and 10 days-old rat brain tissue (Fig. 2).

Cx36 protein expression in PC-12 cells

After the extraction of protein from PC-12 cells, SDS-PAGE and Western blotting were carried out. A 36-kDa band was shown in the homogenates of PC-12 cells, with Cx36 transfected HeLa cells as positive control, and vector transfected HeLa

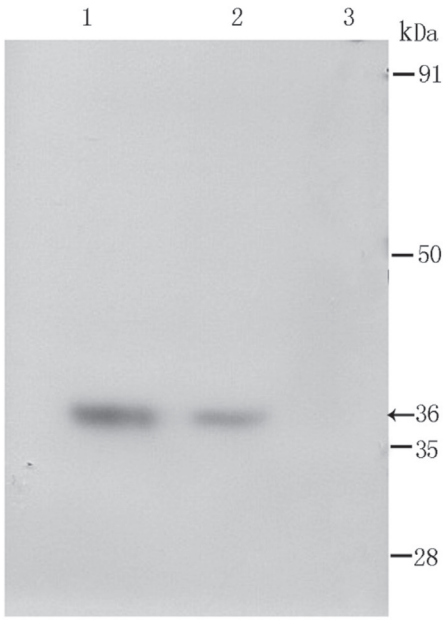

Figure 3. Western blotting of Cx36 from homogenates of PC-12 cells. 36-kDa band was showed in HeLa cells transfected with Cx36 cDNA (lane 1), and PC-12 cells (lane 2), but was no band in HeLa cells transfected with pcDNA3 vector without Cx36 cDNA (lane 3).

cells as negative control (Fig 3). The immunofluorescence staining revealed that Cx36 was lined and punctate distributing in the plasma membrane of cell-cell contacts in PC-12 cells (Fig. 4B). After the omission of the primary antibody or preadsorption of Cx36 antibody with its specific peptide, Cx36 was not detected in PC-12 cells (Fig. 4A and C).

Cx36 colocalized with ZO-1 in PC-12 cells

The colocalization of Cx36 with ZO-1 was performed by double immunofluorescence labeling of Cx36 and ZO-1 in PC-12 cells. Fig. 5A showed the appearances of lined Cx36. Fig. 5B presented that ZO-1 was also lined and punctate at the same filed. Furthermore, both of them were distributed around the periphery of cells. However, as shown in Fig. 5C, Cx36 and ZO-1 were most overlain, indicating a high degree of Cx36 and ZO-1 colocalization.

Cx36 associated with ZO-1 in PC-12 cells by coimmunoprecipitation

The lysates of PC-12 cells were immunoprecipitated with monoclonal anti-ZO-1 antibody, and omission of anti-ZO-1 antibody was used as control. The immunoprecipitation were electrophoresed on 12% SDS-polyacrylamide gels and transblotted to nitrocellulose membranes, and probed with anti-Cx36 antibody. As shown in Fig. 6, Cx36 was detected in immunoprecipitates with monoclonal anti-ZO-1 in PC-12 cells (lane 1), while there was no Cx36 detected in control sample with anti-ZO-1 omission (lane 3). Detection of Cx36 from lysates of PC-12 cells was used as positive control (lane 2). The lysates of PC-12 cells were precipitated with monoclonal anti-Cx36 antibody, and omission of anti-Cx36 antibody was used as control. Western blots of the immunoprecipitates probed with anti-ZO-1 antibody showed ZO-1 in the precipitates of PC-12 cells (lane 1). The lysates from PC-12 cells were used as positive control (lane 2). There was no ZO-1detected in the immunoprecipitation with omission of anti-Cx36 antibody (lane 3).

Discussion

Cx36 gene was first cloned by Condorelli et al. (1998). Previous studies have shown that Cx36 mRNA expression in the mammalian brain neurons, including the cerebral cortex, pineal gland, hippocampus, olfactory bulb, cerebellar cortex, spinal cord, retina and inferior olive (Condorelli et al. 2000;

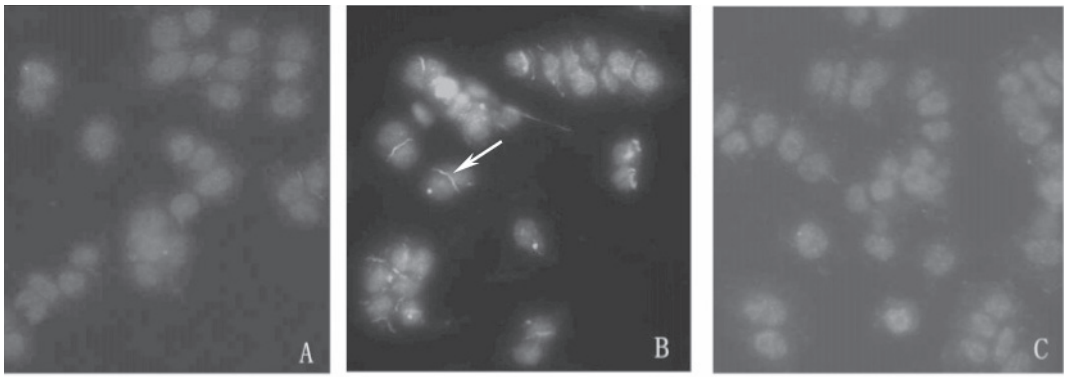

Figure 4. Immunofluorescence of Cx36 in PC-12 cells. Cx36 was lined in cell-cell contacts of PC-12 cells (B, arrow), but was not detected in Cx36 primary antiboby omission condition (A) and Cx36 antibody preabsorption condition (C). Magnification ×400.

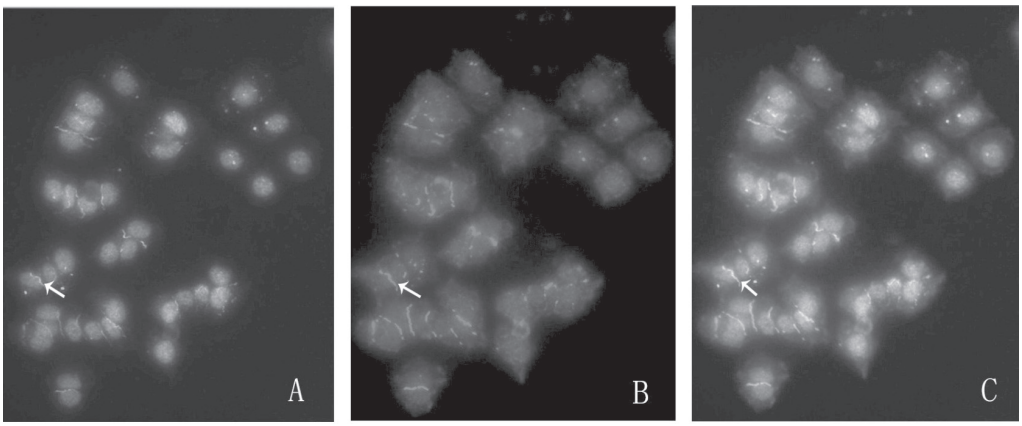

Figure 5. Double immunofluorescence labeling of Cx36 and ZO-1 in PC-12 cells showed that Cx36 colocalized with ZO-1. The overlay of Cx36 (A) and ZO-1 (B) showed that Cx36 colocalized with ZO-1 (C, arrow). Magnification ×400.

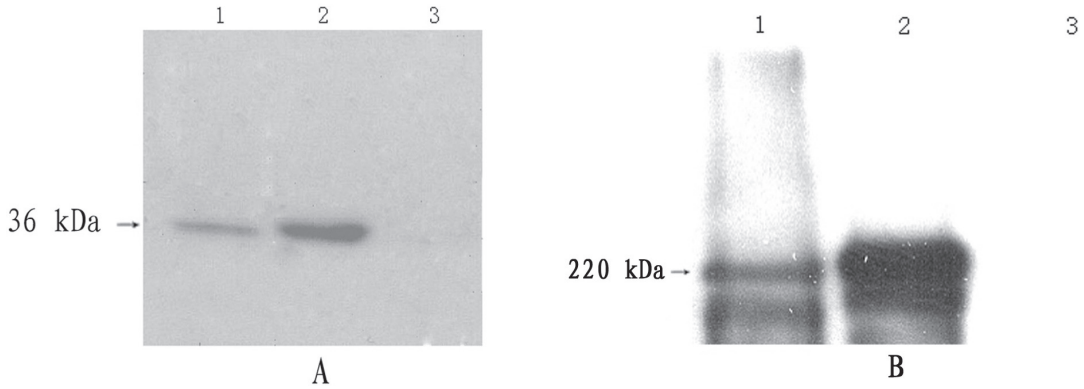

Figure 6. A. Coimmunoprecipitation probed anti-Cx36 antibody showed that Cx36 was detected in PC-12 cells (lane 1), PC-12 cells were used as positive control (lane 2), while there was no Cx36 band in the immunoprecipitation with omission of anti-ZO-1 antibody (lane 3). **B.** Coimmunoprecipitation probed with anti-ZO-1 antibody showed that ZO-1 was detected in PC-12 cells (lane 1), PC-12 cells were used as positive control (lane 2), while there was no ZO-1 band in the immunoprecipitation with omission of anti-Cx36 antibody (lane 3).

Rash et al. 2001; Kosaka et al. 2005). In addition, Cx36 and its mRNA are also found in the β cells of pancreatic islets and various insulin-producing cells including MIN3, INS-1, INS-ES cells and βTC-3 cell line, playing some roles in insulin secretion (Serre-Beinier et al. 2000; Calabrese et al. 2003; Le Gurun et al. 2003). Single cell RT-PCR techniques reveal that Cx36 mRNA is also expressed in chromaffin cells in adrenal slices (Martin et al. 2001). In chromaffin cells, Cx36 plays very important role in ensuing calcium signal and releasing catecholamine. *In vitro*, Li et al. (2004a) transfected HeLa cells with full length Cx36. They observed that immunofluoresence labeling for Cx36 was distributed around the periphery of cells.

Recently, electrical physiological studies using Cx36 knock out mice have indicated that Cx36 forms electrical synapses in retina, thalamic reticular nucleus, neocortex, and hippocampus, which plays pivotal role for night vision, synchronous activity in mammalian neuron networks (Demb and Pugh 2002; Landisman et al. 2002).

PC-12 cells are originally cloned from rat phaeochromocytoma cell. PC-12 cells and chromaffin cells of adrenal medulla have similar properties, such as catecholamine secretion and Ca^{2+} sensitivity. PC-12 cells are one of the established neuronal-like cells (Byrd et al. 1986), which are widely used for studying neurite differentiation by nerve growth factor and catecholamine secretion.

In this study, Cx36 mRNA and protein expression and the relationship between Cx36 and ZO-1 in PC-12 cells were investigated using RT-PCR, Northern blot, Western blotting, immunofluoresence and coimmunoprecipitation. We have found that Cx36 mRNA and protein were expressed in PC-12 cells, which is different from the study by Srinivas

et al. (1999) who found that Cx36 was expressed in Cx36 transfected PC-12 cells.

There are several types of connexins interacting with ZO-1. Cx43, Cx32, Cx45, Cx31.9 are associated with ZO-1 (Toyofuku et al. 1998; Kojima et al. 2001; Laing et al. 2001; Nielsen et al. 2002). In transfected HeLa cells with full length Cx36, immunofluoresence labeling for both Cx36 and ZO-1 are distributed around the periphery of cells, whereas nearly all Cx36-positive puncta are colocalized with ZO-1 (Li et al. 2004b).

In this study, immunostaining analysis demonstrated ZO-1 expression in PC-12 cells. This result is consistent with the previous study from which PC-12 cells are shown to express endogenous ZO-1 (Sunshine et al. 2000). Forthermore, the double immunolabelling and immunoprecipitation techniques in this study indicated that Cx36 was colocalized and associated with ZO-1 in PC-12 cells. Hunter et al. (2005) demonstrated that ZO-1 alters the size and organization of Cx43 gap junction by influencing channel accretion. Therefore, it is suggested that ZO-1 might play the same role in the regulation of Cx36 channel as it exerts in Cx43 gap junction channels.

In summary, the present study demonstrated that PC-12 cells express Cx36 mRNA and protein and that Cx36 is associated with ZO-1 in PC-12 cells. This study also proves that PC-12 cells can be used as a cell model system for studying Cx36 function and ZO-1.

References

Belluardo N., Trovato-Salinaro A., Mudo G., Hurd Y. L., Condorelli D. F. (1999): Structure, chromosomal localization, and brain expression of human Cx36 gene. J. Neurosci. Res. **57**, 740–752

Beyer E. C., Paul D. L., Goodenough D. A. (1990): Connexin family of gap junction proteins. J. Membr. Biol. **116**, 187–194

Byrd J. C., Hadjiconstantinou M., Cavalla D. (1986): Epinephrine synthesis in the PC12 pheochromocytoma cell line. Eur. J. Pharmacol. **127**, 139–142

Calabrese A., Zhang M., Serre-Beinier V., Caton D., Mas C., Satin L. S., Meda P. (2003): Connexin 36 controls synchronization of Ca^{2+} oscillations and insulin secretion in MIN6 cells. Diabetes **52**, 417–424

Chomczynski P., Sacchi N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156–159

Condorelli D. F., Parenti R., Spinella F., Trovato Salinaro A., Belluardo N., Cardile V., Cicirata F. (1998): Cloning of a new gap junction gene (Cx36) highly expressed in mammalian brain neurons. Eur. J. Neurosci. **10**, 1202–1208

Condorelli D. F., Belluardo N., Trovato-Salinaro A., Mudo G. (2000): Expression of Cx36 in mammalian neurons. Brain Res. Rev. **32**, 72–85

Connors B. W., Long M. A. (2004): Electrical synapses in the mammalian brain. Annu. Rev. Neurosci. **27**, 393–418

Demb J. B., Pugh E. N. Jr. (2002): Connexin36 forms synapses essential for night vision. Neuron **36**, 551–553

González-Mariscal L., Betanzos A., Avila-Flores A. (2000): MAGUK proteins: structure and role in the tight junction. Semin. Cell Dev. Biol. **11**, 315–324

Goodenough D. A., Goliger J. A., Paul D. L. (1996): Connexins, connexons and intercellular communication. Annu. Rev. Biochem. **65**, 475–502

Greene L. A. (1978): Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. J. Cell Biol. **78**, 747–755

Hunter A. W., Barker R. J., Zhu C., Gourdie R. G. (2005): Zonula occludens-1 alters connexin43 gap junction size and organization by influencing channel accretion. Mol. Biol. Cell. **16**, 5686–5698

Kam Y., Kim D. Y., Koo S. K., Joe C. O. (1998): Transfer of second messengers through gap junction connexin 43 channels reconstituted in liposomes. Biochim. Biophys. Acta. **1372**, 384–388

Kojima T., Kokai Y., Chiba H., Yamamoto M., Mochizuki Y., Sawada N. (2001): Cx32 but not Cx26 is associated with tight junctions in primary cultures of rat hepatocytes. Exp. Cell Res. **263**, 193–201

Kosaka T., Deans M. R., Paul D. L., Kosaka K. (2005): Neuronal gap junctions in the mouse main olfactory bulb morphological analyses on transgenic mice. Neuroscience **134**, 757–769

Kumar N. M., Gilula N. B. (1996): The gap junction communication channel. Cell **84**, 381–388

Landisman C. E., Long M. A., Beierlein M., Deans M. R., Paul D. L., Connors B. W. (2002): Electrical synapses in the thalamic reticular nucleus. J. Neurosci. **22**, 1002–1009

Laing J. G., Manley-Markowski R. N., Koval M., Civitelli R., Steinberg T. H. (2001): Connexin45 interacts with zonula occludens-1 and connexin43 in osteoblastic cells. J. Biol. Chem. **276**, 23051–23055

Le Gurun S., Martin D., Formenton A., Maechler P., Caille D., Waeber G., Meda P., Haefliger J. A. (2003): Connexin-36 contributes to control function of insulin-producing cells. J. Biol. Chem. **278**, 37690–37697

Li X. B., Zhao X. S., Tian D. Z., Zhu Y. C., Yao T. (1999): A new method for PCR products cloning – T-A clone technique. Prog. Biochem. Biophys. **26**, 187–189

Li X. B., Lynn B. D., Olson C., Meier C., Davidson K. G., Yasumura T., Rash J. E., Nagy J. I. (2002): Connexin29 expression, immunocytochemistry and freeze-fracture replica immunogold labelling (FRIL) in sciatic nerve. Eur. J. Neurosci. **16**, 795–806

Li X., Olson C., Lu S., Kamasawa N., Yasumura T., Rash J. E., Nagy J. I. (2004a): Neuronal connexin36 association with zonula occludens-1 protein (ZO-1) in mouse brain and interaction with the first PDZ domain of ZO-1. Eur. J. Neurosci. **19**, 2132–2146

Li X., Olson C., Lu S., Nagy J. (2004b): Association of connexin36 with zonula occludens-1 in HeLa cells, βTC-3 cells,

pancreas, and adrenal gland. Histochem. Cell Biol. **122**, 485–498

Martin A. O., Mathieu M.-N., Chevillard C., Guérineau N. C. (2001): Gap junctions mediate electrical signaling and ensuing cytosolic Ca^{2+} increases between chromaffin cells in adrenal slices: a role in catecholamine release. J. Neurosci. **21**, 5397–5405

Nielsen P. A., Beahm D. L., Giepmans B. N., Baruch A., Hall J. E., Kumar N. M. (2002): Molecular cloning, functional expression, and tissue distribution of a novel human gap junction-forming protein, Connexin-31.9. Interaction with zona occludens protein-1. J. Biol. Chem. **277**, 38272–3828

Rash J. E., Yasumura T., Davidson K. G., Furman C. S., Dudek F. E., Nagy J. I. (2001): Identification of cells expressing Cx43, Cx30, Cx26, Cx32 and Cx36 in gap junctions of rat brain and spinal cord. Cell Commun. Adhes. **8**, 315–320

Serre-Beinier V., Le Gurun S., Belluardo N., Trovato-Salinaro A., Charollais A., Haefliger J. A., Condorelli D. F., Meda P. (2000): Cx36 preferentially connects beta-cells within pancreatic islets. Diabetes **49**, 727–734

Srinivas M., Rozental R., Kojima T., Dermietzel R., Mehler M., Condorelli D. F., Kessler J. A., Spray D. C. (1999): Functional properties of channels formed by the neuronal gap junction protein connexin36. J. Neurosci. **19**, 9848–9855

Sunshine C., Francis S., Kirk K. L. (2000): Rab3B regulates ZO-1 targeting and actin organization in PC12 neuroendocrine cells. Exp. Cell Res. **257**, 1–10

Toyofuku T., Yabuki M., Otsu K., Kuzuya T., Hori M., Tada M. (1998): Direct association of the gap junction protein Cx43 with ZO-1 in cardiac myocytes. J. Biol. Chem. **273**, 12725–12731

White T. W., Paul D. L. (1999): Genetic diseases and gene knockouts reveal diverse connexin functions. Annu. Rev. Physiol. **61**, 283–310

Willecke K., Eiberger J., Degen J., Eckardt D., Romualdi A., Guldenagel M., Deutsch U., Sohl G. (2002): Structural and functional diversity of connexin genes in the mouse and human genome. Biol. Chem. **383**, 725–737

Zhurinsky J., Shtutman M., Ben-Ze'ev A. (2000): Differential mechanisms of LEF/TCF family-dependent transcriptional activation by β-catenin and plakoglobin. Mol. Cell. Biol. **20**, 4238–4252

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