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Molecular cloning and functional analysis of a novel Cx43 partner protein CIP150

Motofusa Akiyama, Norihiro Ishida, Takuya Ogawa, Keiichiro Yogo, Tatsuo Takeya *

Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0129, Japan

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Abstract

We identified and cloned a novel gene encoding a partner protein, CIP150, of connexin 43 (Cx43). CIP150 associates with Cx43 through its carboxyl terminal domain. Conversely, a region consisting of 16 amino acids in the juxtamembrane region (amino acids 227–242) in the carboxyl terminal tail of Cx43 was identified to be responsible for the association. A variant of Cx43 lacking this region was expressed only in a nonphosphorylated form and appeared to lose the capacity to localize to the region of cell–cell contact and dye transfer activity. When the expression of CIP150 was suppressed using small interfering RNA, the localization to the plasma membrane as well as dye transfer activity of Cx43 was significantly reduced. These results suggest that the newly identified domain is essential for the proper phosphorylation and localization of Cx43, and CIP150 is a novel partner protein targeting this domain.

Keywords: Connexin 43; Carboxyl terminal tail; Partner protein; CIP150; Subcellular localization; Dye transfer

Gap junctions are specialized plasma membrane associations containing intercellular channels that are formed by the association of two connexons, one provided by each of the communicating cells [1–3]. Each connexon is composed of hexameric connexin which is essential to form a central pore and is a product of members of a multigene family [4]. Connexins follow the general secretory pathway for membrane protein in their synthesis, assembly, recruitment to the membrane, and turnover. All through their life cycle, connexins are known to be regulated through phosphorylation by protein kinases and/or protein–protein interaction with other auxiliary proteins [5–9]. The overall functional importance of this is not fully understood, and the characterization and a functional understanding of the protein–protein interaction of connexins are thus of importance for elucidating how cell–cell interaction is regulated.

Gap junctional channel (GJ)-mediated intercellular communication has been implicated in many cellular functions [10–12]. Connexin 43 (Cx43) was the first connexin gene to be knocked out [13] and also one of the first genes to be linked to a human disease [14]; Cx43 was shown to play an essential role in heart morphogenesis and heart conduction. Cx43-mediated GJ activity is also known to play an indispensable role in both germline development and postnatal folliculogenesis [15–17]. We previously showed that FSH, a gonadotropin, stimulated the phosphorylation of Cx43 in ovarian granulosa cells and further identified four sites of phosphorylation in its CT tail [18]. Sites phosphorylated by a variety of protein kinases and regions responsible for the binding of auxiliary proteins have been identified in the carboxyl terminal (CT) tail of Cx43, which hence has been a target of extensive research

* Corresponding author. Fax: +81 743 72 5549.
E-mail address: ttakeya@bs.naist.jp (T. Takeya).

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[6,7,9,12]. Since Cx43 is widely distributed throughout vertebrate tissues, the identification of partner protein(s) in a distinct tissue would contribute to a better understanding of the functional role as well as the regulatory mechanism of Cx43. In this study, we tried to identify proteins that can associate with Cx43 by applying the yeast two-hybrid screening method using a rat ovary cDNA library as prey. As a result, we identified a novel gene encoding a 150-kDa protein, named Cx43-interacting protein of 150-kDa, CIP150, which associates with the juxtamembrane region in the CT tail of Cx43 through its carboxyl terminus. The significance of the identification of a new functional domain in Cx43 as the target of CIP150 is discussed.

Materials and methods

Yeast two-hybrid screening. The rat Cx43-CT (amino acid residues E227 to I382: [19]) was subcloned into the yeast expression vector pAS2 (CLONTECH), generating pAS2-Cx43-CT. The plasmid pAS2-Cx43-CT was introduced into yeast strain Y190, and then screening was performed using the MATCHMAKER two-hybrid system with a rat ovarian cDNA library (CLONTECH) as prey according to the manufacturer's directions. Yeast colonies that grew in medium lacking histidine were removed, and the β-galactosidase activity in each clone was assayed on filters. cDNA clones thus isolated were subjected to DNA sequence analysis and used for further characterization.

RNA extraction, reverse transcription, and PCR cloning. Total RNA was purified and used for RT-PCR under conditions described previously [20]. The region covered by KIAA1432 (GenBank Accession No. AB037853) and DKFZp434D105 (GenBank Accession No. AL136875) was divided into three and a full-length cDNA of human CIP150 was obtained by amplifying and joining each fragment. To this end, primers corresponding to the termini of the three fragments were prepared; fragment 1 (nucleotides 1–1307) forward [5′-TGAATGG AGGCCAGATAGTACC-3′] and reverse [5′-CCAAACTTGCAAC CACAG-3′]; fragment 2 (nucleotides 1226–2734) forward [5′-CCTATCTAGAGAGCAATTGGCC-3′] and reverse [5′-CAGATTC TCCAGAGCCAATGG-3′]; fragment 3 (nucleotides 2659–4030) forward [5′-GCACTAGAACAAGGCAAGTGG-3′] and reverse [5′-ACAACGTACTGAGCTGCACG-3′]. The three fragments thus obtained were joined together in pBluescript II KS^+, resulting in the full-length CIP150. Full-length human CIP150 cDNA was also obtained by PCR using placental cDNA as a template. The forward primer [5′-TGAATGGAGGCCAGATAGTACC-3′] and reverse primer [5′-GA TGGAACCTCACTGTTAGG-3′] contain the start codon of a complete DKFZp434D105 cDNA sequence and the stop codon of a partial KIAA1432 cDNA sequence, respectively.

Expression plasmids and siRNA. A cDNA fragment encoding 164 amino acids of the rat CIP150 carboxyl terminus was subcloned into pFLAG-CMV-2 (Sigma) to produce FLAG-1432-CT. Rat Cx43 was cloned into the expression vector pMH. Deletion mutants of Cx43 and Cx43-CT were produced by PCR and verified by DNA sequencing. Two kinds of *CIP150* small interfering (si)RNA expression vectors (pSUPER-si*CIP150*-1 and -2) were generated according to the manufacturer's instructions (OligoEngine) using the following targeted sequences; pSUPER-si*CIP150*-1 [5′-AACCCAGTTCAAGTGGTGG AT-3′] and pSUPER-si*CIP150*-2 [5′-AAGCAGCAATATGGTCA GCCG-3′].

Cell culture and transfection. HEK293, HeLa, COS7, and NRK cells were maintained in D-MEM supplemented with 10% fetal calf serum. T47D cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum. Human granulosa cell tumor KGN cells [21] were maintained in D-MEM/Ham's F12 supplemented with 10% fetal calf serum. Vectors were co-transfected with humanized GFP (pCMX-SAH/Y145F; a gift from Dr. K. Umesono) as an internal control of transfection efficiency. Cells used for scrape-loading dye transfer and siRNA knockdown experiments were selected by introducing a puromycin resistance gene (pCX4-puro) [22] together with the indicated expression vector, and resistant cells were used after a 24-h culture in the presence of puromycin (2 μg/ml). The endoplasmic reticulum (ER) was visualized by rhodamine-conjugated concanavalin A (ConA; Vector Laboratories) staining as described previously [23].

Antibody, Western blot analysis, and immunostaining. Western blot analysis and immunofluorescence microscopy were carried out as described previously [18]. A rabbit anti-CIP150 polyclonal antibody against the protein's Mid-region (CIP150-M) was raised using His_6-tagged polypeptides corresponding to human CIP150 amino acid residues 783–1024. Mouse anti-CIP150 polyclonal antibodies against the amino terminal (CIP150-N) and carboxyl terminal (CIP150-C) regions were prepared with GST-tagged polypeptides consisting of 109 amino acids from the amino terminus and 88 amino acids from the carboxyl terminus, respectively, as antigens. Mouse monoclonal antibodies against β-actin (AC-74) and FLAG epitope (M2), and rabbit polyclonal antibody against Cx43 were obtained from Sigma. Mouse monoclonal antibody against GFP (JL-8) was purchased from Clontech Laboratories.

Pull-down assay. GST-fused Cx43-CT variants were expressed in bacteria and purified using glutathione–Sepharose beads. Cell lysates were prepared from FLAG-1432-CT-transfected COS7 cells in lysis buffer. Binding was performed by incubating GST-fused proteins (10 μg) immobilized on the beads with cell lysates at 4 °C for 2 h, and the protein complexes were washed with the same buffer. Bound FLAG-1432-CT was detected by immunoblotting using anti-FLAG antibody.

Scrape-loading dye transfer method. Cx43-mediated channel activity was measured as described previously [18,24]. Cells cultured until they had formed a monolayer were scraped with 27-gauge needles before the transmission of Lucifer yellow (LY; 0.25%; molecular weight, 476 Da) CH and rhodamine dextran (RD; 0.75%; molecular weight, 10 kDa). The transfer of LY into neighboring cells was monitored and captured images were analyzed using NIH image software. Images of four different views per sample were scanned along lines perpendicular to the scrape and at least ten lines were scanned. The distance between the internal border of the outermost cell layer and distal edge was measured in micrometers and mean values were estimated.

Results

Isolation and molecular cloning of CIP150

Using the yeast two-hybrid screening system with a rat ovary cDNA library as prey, we searched for partners of Cx43. Since the CT tail of Cx43 was implicated in the regulation of GJ activity, we used the CT region as bait. We eventually isolated seven independent clones which gave positive signals in yeast cells. However, when these clones were expressed as FLAG-tagged proteins, only one showed interaction in mammalian cells as well as in yeast cells (Fig. 1A), and this clone was used for the subsequent study. The clone thus identified encoded a 492-bp cDNA insert and sequence analysis predicted an open-reading frame consisting of 164 amino acids which was identical to that of the human KIAA1432