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Dynamin 2 interacts with connexin 26 to regulate its degradation and function in gap junction formation

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ABSTRACT

Connexin 26 (Cx26), a protein involved in gap junctional intercellular communication, has an essential function during organ and tissue development. Its deregulation, in part due to inherent mutations, is associated with pathological conditions including congenital deafness. Regulation of Cx26 protein level is critical for its function but the molecular mechanisms involved are partially understood. This study identifies dynamin 2 (Dyn2) as a Cx26 interactor in yeast and mammalian cells. Deletion studies revealed that Cx26–Dyn2 interaction involves the C-terminus of Cx26 and the GTPase effector domain of Dyn2, which is of particular importance for the regulation of the endocytic pathway. Dyn2 inhibition using siRNA or dynasore resulted in reduced Cx26 degradation at the plasma membrane and this was associated with change in gap junctional intercellular communication (GJIC). Furthermore, we demonstrate that Dyn2 regulates Cx26 endocytosis and ubiquitination. These results establish Dyn2 as a Cx26 partner in the regulation of GJIC.

1. Introduction

Connexin 26 (Cx26), encoded by the *GJB2* gene, is a member of the gap junction protein family responsible for gap junctional intercellular communication (GJIC). GJIC is established by the intercellular pairing of hemichannels or connexons via homomeric or heteromeric arrangements of multiple connexins, including Cx26. The resulting gap junction channels mediate the transfer of small signaling molecules, e.g., secondary messengers such as cAMP and Ca^{++} (Loewenstein, 1981).

Cx26 is essential for development and has a wide tissue distribution that includes the cochlea, mammary gland, liver and skin (Gabriel et al., 1998). In humans, Cx26 mutations have been associated with rare autosomal non-syndromic sensorineural hearing loss, which account for a significant proportion of genetic human deafness (Denoyelle et al., 1997; Kelsell et al., 1997), as well as autosomal disorders of epidermal keratinization, e.g., palmoplantar keratodermas (Maestrini et al., 1999). Collectively, these phenotypes highlight the multifunctional role of Cx26 in physiological contexts. In cancer, Cx26 expression is deregulated in several carcinoma tissue types (Kanczuga-Koda et al., 2005; Tate et al., 2006). However, the significance of Cx26 expression levels in cancer remains debated. While some studies implicated Cx26 as a potential tumor suppressor (Mesnil et al., 1995), conditional inactivation of Cx26 in mouse tissues such as liver did not result in cancer development (Bry et al., 2004).

Cx26 has been reported to co-translationally and post-translationally insert into the endoplasmic reticulum where it oligomerizes into connexons (Zhang et al., 1996). Connexons are subsequently transported to the cell surface where they mediate the formation of gap junction channels. At present, there is no firm evidence that Cx26 is phosphorylated, which rules out a possible direct involvement of kinase-mediated regulatory mechanisms during its trafficking and assembly into gap junctions. However, members of the connexin family can engage in multiple protein–protein interactions, including heterodimerization among distinct Cx members (Giepmans, 2004). In this context, only an

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ubiquitin ligase was reported to interact with Cx26 in the cochlea; this interaction is thought to position Cx26 for attack by an E2 ubiquitin-conjugating enzyme (Henzl et al., 2004).

Cx26 is organized into 4 transmembrane helices (M1–M4), two extracellular loops (E1 and E2), with the N- and C-termini facing the cytoplasm. Transmembrane domains are believed to have a role in the formation of the gap junction pore, voltage gating and closure of gap junction channels (Unwin, 1989). The two extracellular loops, which possess highly conserved cysteine residues that allow intramolecular disulphide bond formation, have been implicated in the docking of intercellular hemichannels (Unger et al., 1999). The N-terminal of Cx26 located at the cytoplasmic side of the channel pore is thought to be essential for the regulation of voltage gating and has been suggested to plug the gap junction channel pore (Maeda et al., 2009; Purnick et al., 2000). The function of the C-terminus portion, however, has not been established. Here we report that the GTPase dynamin 2 (Dyn2), a protein implicated in endocytosis of cell surface molecules, interacts with the C-terminus of Cx26. This interaction was identified using the yeast two-hybrid system and confirmed in mammalian cells. The functional implication of Dyn2–Cx26 interaction was further analyzed in relation to Cx26 degradation and gap junction formation.

2. Materials and methods

2.1. Yeast-two-hybrid assay and cDNA library screening

The human Cx26 expression vector was obtained from Dr. Naus (University of British Columbia, Vancouver, Canada). Cx26 nucleotide sequences G1 (1–57 bps), G2 (297–393 bps) and G3 (648–678 bps) were PCR amplified, purified, and cloned into the yeast two-hybrid plasmid pBTM116 in frame to LexA (1–202). Dynamin 2 constructs were PCR amplified from cDNA derived from a human osteosarcoma cDNA library (kindly provided by Dr. Karaplis, Lady Davis Institute, Montreal, Canada) and subcloned into pACT2 in frame to GAL4-TA. Dyn2 fragments comprising the GTPase domain (aa 6–245), middle domain (aa 246–515), PH domain (aa 516–623), GED domain (aa 644–735), and PRD domain (aa 736–864) were generated. All cDNA clones and subcloning were confirmed by sequencing at Guelph Molecular Supercentre (University of Guelph, Guelph, Canada) using a dye primer cycle sequencing kit (Perkin-Elmer, Norwalk, CT) on a DNA sequencer (Perkin-Elmer ABI 377). Sequence identity was confirmed using the DNASTAR SeqMan program (DNASTAR, Madison, WI). For the yeast two-hybrid assay, the pLexA-connexin plasmids were transformed into yeast strain L40 [MATα trp1 leu2 his3 URA3::(lexAop) 8-lacZ LYS2::(lexAop) 4-HIS3 lys2 ura3 ade2 gal80 gal4] prior to library transformation, and then assayed for LacZ reporter activity by using the liquid culture β-galactosidase assay in presence of O-nitrophenyl β-D-galactopyranoside (ONPG) as substrate as recommended by Clontech's Matchmaker protocol. A human osteosarcoma cDNA library was amplified and yeast transformation was done as previously described (Schiestl and Gietz, 1989). Briefly, growth on histidine-deficient media containing 25 mM 3-amino-1,2,4-triazole determined qualitatively by vigor of colony formation. Where two proteins are assayed for interaction, the selection media is also deficient for tryptophan, leucine and histidine. Where a protein is assayed for activation of the HIS3 reporter gene by itself, the media is deficient for either tryptophan or leucine and histidine.

2.2. Cell lines and cell culture

Cells used in this study include the BL6 cell line derived from the B16 mouse melanoma cell line (kindly provided by Dr. Linda D. Williams, Dept. of Cancer Biology, MD Anderson Cancer Center, TX) (Ito et al., 2000, 2004); human HeLa cancer cell line (ATCC, #CCL-2), HEK 293T human embryonic kidney cells (ATCC, #CRL-3216) and normal rat kidney (NRK) cells (ATCC, #CRL-1895). All these cells were maintained in a complete Dulbecco's modification Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. In the case of BL6, medium was also supplemented with 1 mM sodium pyruvate and 1% non-essential amino acids. Cells were maintained in culture at 37 °C in an atmosphere of 5% CO_2.

2.3. Generation of cells expressing stable Dyn2 shRNA

For both human and mouse cells, the 19-nucleotide sequences used to target human and mouse Dyn2 mRNA were described earlier (Gomez et al., 2005). These sequences were synthesized by Dharmacon Inc. and consist of 5′-GGACCAGGCAGAAAACGAG-3′ for human cells and 5′-GGACCAGGCAGAGAATGAG-3′ for mouse cells. In both cases scrambled sequences were used as negative control. The mouse and human target sequences were cloned as inverted repeats into pSuper-retro puromycin vector according to the manufacturer's instructions (Oligoengine). Control retroviral vector pSuper-retro puromycin alone (PSR) or expressing Dyn2 shRNA (PSR/Dyn2), or Dyn2 scrambled shRNA (PSR/NC) were transfected into Phoenix cells as we described previously (Benlimame et al., 2005). 48 h after transfection, supernatants containing retroviral particles were collected, filtered through a 0.45 μm filter and used to infect BL6 cells twice, 24 h apart, in the presence of 8 μg/ml of polybrene (Sigma–Aldrich). Polyclonal populations were selected for resistance to 1 μg/ml puromycin for a 2-week period to generate stable shRNA expressing cells and their matched controls (PSR and PSR/NC).

2.4. Immunoprecipitation and western blot analysis

Cells at 70% confluence were transfected with plasmids encoding FLAG or FLAG–Cx26 fusion proteins (the full length Cx26 coding sequence was cloned in frame into pFLAG-CMV-2 expression vector from Invitrogen) using FuGENE6 Transfection Reagent according to the manufacturer's recommendations (Roche Diagnostics) except for HEK 293T where calcium phosphate method was used. When indicated, plasmids encoding GFP-tagged Cx26 or Dyn2 were used for transfection. GFP-tagged Dyn2 construct was kindly provided by Dr. Mark A McNiven, Mayo Clinic College of Medicine, Minnesota. To investigate protein stability, cells were incubated for various times in the absence and presence of 20 μg/ml cycloheximide. Forty-eight hours after transfection, cells were washed twice with cold PBS, harvested by scraping, and lysed directly by incubation with a lysis buffer (1% Triton X-100, 10 mM Tris–HCl with pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5 mM phenylmethylsulfonylfluoride, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, and 0.01 mg/ml aprotinin, 5 mM sodium orthovanadate) for 15 min at 4 °C. Cell lysates were centrifuged at 14,000 × *g* at 4 °C for 15 min to remove insoluble debris. Supernatants were transferred to other Eppendorf tubes and kept at −80 °C until use. Aliquots containing 50 μg proteins (based on Bradford assay) were resolved on acrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked in 5% milk for 1 h and probed overnight at 4 °C with a rabbit polyclonal antibody against Dyn2 (H-300; Santa Cruz Biotechnology Inc.) used at a dilution of 1:200; a mouse monoclonal antibody against Cx26 (Zymed Laboratories Inc.) used at a dilution of 1:500, or a monoclonal antibodies recognizing either FLAG (Sigma–Aldrich) or GFP (Roche Diagnostics) proteins. Immune complexes were detected by horseradish peroxidase (HRP) conjugated to appropriate secondary antibodies (1:3000 dilution) using the enhanced