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The role of the Cx43 C-terminus in GJ plaque formation and internalization

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ABSTRACT

Connexin 43 (Cx43) is a major gap junction (GJ) protein found in many mammalian cell types. The C-terminal (CT) domain of Cx43 has unique characteristics in terms of amino acid (aa) sequence and its length differs from other connexins. This CT domain can be associated with protein partners to regulate GJ assembly and degradation, which results in the direct control of gap junction intercellular communication (GJIC). However, the essential roles of the CT regions involved in these mechanisms have not been fully elucidated. In this study, we aimed to investigate the specific regions of Cx43CT involved in GJ formation and internalization. Wild type $Cx43_{(382aa)}$ and 10 CT truncated mutants were stably expressed in HeLa cells as GFP or DsRed tagged proteins. First, we found that the deletion of 235–382aa from Cx43 resulted in failure to make GJ and establish GJIC. Second, the Cx43 with 242–382aa CT deletion could form functional GJs and be internalized as annular gap junctions (AGJs). However, the plaques consisting of Cx43 with CT deletions (Δ242–382aa to Δ271–382aa) were longer than the plaques consisting of Cx43 with CT deletions (Δ302–382aa). Third, co-culture experiments of cells expressing wild type $Cx43_{(382)}$ with cells expressing Cx43CT mutants revealed that the directions of GJ internalization were dependent on the length of the respective CT. Moreover, a specific region, 325–342aa residues of Cx43, played an important role in the direction of GJ internalization. These results showed the important roles of the Cx43 C-terminus in GJ expression and its turnover.

1. Introduction

Gap junction (GJ) channels connect the cytoplasms of two apposing cells by docking via a half channel termed a connexon to provide cell–cell communication. Gap junction intercellular communication (GJIC) allows small molecules of less than 1 kDa, including secondary messengers, to pass from one cell to another [1]. Connexons are composed of hexamers of four-transmembrane protein termed connexins (Cxs) [2]. Once transported to the plasma membrane, connexons (hemichannels) routinely assemble in clusters as a double-membrane spanning GJ plaques that are composed of a few to thousands of channels. The size of GJ plaques may vary from a few square nanometers to many square micrometers [3]. The communication via GJ is regulated by the dynamic processes of GJ formation and removal [4]. The removal of GJ from the cell surface involves a unique process. The entire GJ, or a part of it, is endoexocytosed into only one of the two contacting cells [5]. This internalization process generates double-membrane vesicles, termed annular gap junctions (AGJs) or connexosomes. The formation of AGJ vesicles from entire GJs resulted in a rapid reduction in the intercellular communication between two cells [6].

Connexin 43 (Cx43) is a 43 kDa GJ protein widely expressed in many mammalian cell types [7]. It has a short half-life of approximately 1–5 h. Since the turnover of Cx43 is rapid, it has been proposed that Cx43 degradation is important for the regulation of GJIC under pathophysiological conditions [8]. The 17-kDa carboxyl terminal (CT) domain of Cx43 has special features in terms of amino acid sequence and length, which differ from other Cxs [9–11]. It is reported that the Cx43CT domain is extensively phosphorylated by different protein kinases at different motifs containing serine or tyrosine residues [12]. Moreover, this CT domain has been reported to be associated with other proteins to stabilize Cx43 anchoring in plasma membranes [13–15]. Associations of Cx43CT with protein partners have also been proposed to regulate Cx43 assembly and degradation, but the overall functions remain unclear. The aim of this study was to investigate the role of the CT domain of Cx43 in the formation of GJ plaques and in internalization of GJ plaques as an AGJ vesicle. We found that three different amino acid regions in the CT domain of Cx43 played different roles as follows: (1) the region between 235 and 242aa is important for GJ plaque assembly at the plasma membrane; (2) the region between 271 and 302aa is important for sizing of the GJ plaque and AGJ; and (3) the region

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between 325 and 342aa is important for the directionality of GJ internalization. These findings suggest that the formation and internalization of GJ are dependent on the regions of the Cx43 CT domain.

2. Materials and methods

2.1. Cx43-GFP and Cx43-DsRed constructs

DNA fragments coding full-length rat Cx43 (382aa, GenBank ID: NM_012567) and CT truncated mutants [235aa(Δ236–382aa), 242aa(Δ243–382aa), 251aa(Δ252–382aa), 260aa(Δ261–382aa), 271aa(Δ272–382aa), 302aa(Δ303–382aa), 325aa(Δ326–382aa), 342aa(Δ343–382aa), 370aa(Δ371–382aa), and 378aa(Δ379–382aa)] were amplified by a polymerase chain reaction (PCR)-based technique using proofreading DNA polymerase (Pyrobest, Takara, Japan). These DNA fragments were cloned into pEGFP-N3 or a pDsRed monomer (Clonetech, USA). Then, the coding regions of fusion protein were subcloned into a retrovirus expression vector, pQCXIP (Clonetech, USA) (Fig. 1A). To make the deletion mutant Cx43(Δ325–342aa) expressing vector, the Cx43(1–324aa) and Cx43(343–382aa) coding regions were amplified, then these two fragments were ligated via a *Pst*I restriction site (Fig. 3A). All constructs were verified by analysis of restriction enzyme digests and DNA sequencing.

2.2. Cell line and cell culture conditions

Human epitheloid cervix carcinoma cells (HeLa, RIKEN BioResourse Center, Japan) and 293FT cells (Invitrogen, USA) were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C, 100% humidity and 5% CO_2/95% air. Cells were routinely subcultured by trypsinization with 0.05% Trypsin-0.02% EDTA in PBS, and the medium was changed twice a week.

2.3. Establishment of clones expressing the Cx-GFP/DsRed fusion protein

The retrovirus vector and pCL-10A1 (amphotropic vector, Imgenex Corp., USA) were co-transfected to the packaging cell line 293FT using Lipofectamine 2000® (Invitrogen, USA) as described previously [16]. Three days after the transfection, a virus containing conditioned medium was harvested. Then, HeLa cells were infected with the virus containing medium with polybrane (8 μg/ml, Millipore, USA). Three days after infection, Cx43-GFP- or Cx43-DsRed-expressing cells were cultured with 2 μg/ml of puromycin (InvivoGen, USA) containing 10% FBS DMEM for 2 weeks to establish stable clones.

2.4. Immunoblot analysis

Parental HeLa and Cx43 infected cells were harvested on ice in lysis buffer (0.02 M Tris–HCl, 0.137 M NaCl, 0.002 M EDTA, 1% Nonidet P-40 and 10% glycerol, pH 7.6) containing a protease and phosphatase inhibitor cocktail (Sigma, USA). The lysates were then ultrasonicated on ice for 20 s. Total protein concentrations were determined by a Protein Assay kit (Bio-Rad, USA). The protein samples were separated on 8% SDS–polyacrylamide gels, then transferred onto PVDF membranes, and blocked with 5% skim milk (BD transduction, USA) in TBS-T (0.05 M Tris–HCl, 0.150 M NaCl and 0.2% Tween-20, pH7.6). The membranes were incubated with anti-GFP antibody (BD Biosciences, USA) at 1:500 dilution in 5% skim milk in TBS-T at 4 °C overnight. After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG (GE Healthcare, UK) at 1:1500 dilution at 4 °C for 2 h. The blots were developed using a chemiluminescence method (ECL-plus; GE Healthcare) and were detected by LAS-1000 luminescent image analyzer (Fujifilm, Japan). The blots were reprobed with HRP-conjugated anti-β-actin rabbit polyclonal IgG (MBL, Japan) at 1:500 dilution.

2.5. Cell imagings of Cx43-GFP and -DsRed in living cells

Fluorescence images of GFP- and DsRed-tagged Cx43 were acquired by a LSM 510 META (Carl Zeiss, Germany) confocal microscope. For live cell imaging, cells were placed on a temperature-, CO_2– and humidity-controlled stage. Focus, contrast and brightness settings remained constant during the course of imaging acquisition.

2.6. Quantitative and statistical image analyses

GJ plaque length and AGJ diameter were measured using Zeiss software. Statistical analyses of GJ plaque sizes and AGJ were done in a total of 50 cell-pairs. The experiments were repeated in triplicate. GJ plaques and AGJs were categorized into three groups by size. Then, the ratio of each group was calculated and represented by percentages. Statistical analysis of AGJs internalization directions was also done by counting the number of cells containing internalizing vesicles in a total of 50 cell-pairs in triplicate experiments. The ratio of Cx43-GFP and -DsRed expressing cells containing internalizing AGJs was calculated and represented by percentage values. Data are shown as means ± SEM. Comparisons were made using an independent *t-test* and one-way ANOVA. In all analyses, a p value less than 0.05 was considered statistically significant.

3. Results

3.1. Wild type Cx43 and CT truncated mutants form different sizes of GJ plaques and AGJs

To examine the role of CT in the formation of GJ plaques and AGJs, the wild type Cx43 (382aa) and CT truncated mutants were expressed in non-endogenous connexin-expressing HeLa cells as fusion proteins (with GFP or DsRed) (Fig. 1A). The expression of Cx43-GFP fusion proteins was confirmed by immunoblotting using an antibody against GFP (Fig. 1B). These Cx43 expressing cells showed no different characteristics in terms of cell shape and size compared to parental HeLa cells (data not shown). The wild type $Cx43_{(382aa)}$-GFP and -DsRed were assembled into typical GJ plaques as green and red fluorescent lines at the junction of the infected cells, respectively (arrows in Fig. 1C). The observed GJ plaques from wild type $Cx43_{(382aa)}$-GFP showed variation in length from shorter than 1 μm to a maximum length of 15 μm. Besides GJ plaque formation, wild type $Cx43_{(382aa)}$-GFP formed internalizing AGJ vesicles that originated from a small region or the entire GJ plaque. The AGJs were observed as a circular structure in the cytoplasm. The AGJs from wild type $Cx43_{(382aa)}$-GFP showed variation in diameter from smaller than 1 μm to a maximum of 5 μm (arrow heads in Fig. 1C).

$Cx43_{(235aa)}$-GFP, the shortest CT mutant, did not show any formations of GJ plaque or AGJ (Fig. 1D). On the other hand, $Cx43_{(242aa)}$-GFP, the 7 aa longer CT mutant than $Cx43_{(235aa)}$-GFP could form GJ plaques and AGJ vesicles. This finding suggested an important role of the CT domain 235–242aa of Cx43 in GJ formation at the plasma membrane. Interestingly, GJ plaques and AGJs formed by four mutants ($Cx43_{(242aa)}$-GFP to $Cx43_{(271aa)}$-GFP) were larger than that formed by wild type $Cx43_{(382aa)}$-GFP. The