Research Article

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Alteration of protein localization and intracellular calcium content due to connexin26 D50A and A88V mutations

D50A ve A88V mutasyonlarına bağlı connexin26 protein lokalizasyonunun ve hücre içi kalsiyum miktarının değişimi

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Abstract

Introduction: Connexins (Cx) play essential roles in cellular homeostasis by forming gap junctions and non-junctional hemichannels. In vitro characterization of Cx26 mutations causing keratitis-ichthyosis-deafness (KID) syndrome, were shown to form leaky hemichannels. The molecular/cellular mechanisms affected by aberrant hemichannels have recently been elucidated. Here, we further wanted to characterize Cx26 KID syndrome mutations, D50A and A88V, which were shown to form aberrant hemichannels and remained unaddressed in the literature.

Methods: Neurobiotin uptake assay in HeLa and N2A cells transfected with Cx26-WT, D50A or A88V verified the presence of aberrant hemichannels and immunofluorescent staining with fluorescent microscopy determined cellular localization of Cx26. Finally, intracellular calcium content was examined by using calcium indicator, Fluo-3AM, and flow cytometer.

Results: Cx26-D50A and A88V mutations prevented the formation of gap junction plaques at cell-cell appositions and mutant proteins were observed to localize to the Golgi apparatus. Further, comparison of intracellular calcium content showed an increase in calcium amount in cells containing Cx26-D50A and A88V relative to Cx26-WT.

Conclusion: Retention of Cx26 in the Golgi apparatus and alteration in the intracellular calcium content due to KID syndrome mutations may influence various cellular processes that might contribute to development of epidermal phenotypes.

Keywords: Connexin26; Mutations; Keratitis-ichthyosis-deafness (KID) syndrome; Intracellular calcium; Golgi apparatus.

Özet

Giriş: Connexin molekülleri (Cx) kurdukları hücrelerarası kanallar ve hücrelerarasi olmayan yarık kanallar sayesinde hücre yaşamını dengelemeye önemli roller üstlenirler. İn vitro analizlerde keratitis-ichthyosis-deafness (KID) sendromuya ilişkin Cx26 mutasyonlarının düzgün kapanmayan yarı kanallar oluşmasına sebep olduklarını gösterilmiştir. Bu tip anormal çalışan yarı kanalların etkilediği moleküler hücresel mekanizmalar son zamanlarda araştırılmaya başlanmıştır. Bu çalışmada amaçımız, daha önce yarı kanallar yaptıkları gösterilen Cx26'da oluşan KID sendromu mutasyonlarından D50A ve A88V'in hücre biyolojisinde yaptığı değişiklikleri incelemektir.

Yöntemler: Cx26-WT, D50Y ve A88V ile transfecte edilen HeLa ve N2A hücrelerinde neurobiotin boya alım deneyleriyle anormal yarı kanalların varlığı tespit edilmiş ve fluoresan mikroskop ve immunobiyolasıyla Cx26'nın hücre içi lokalizasyonu belirlenmiştir. Son olarak, kalsiyum belirtici, Fluo-3AM ve akış sitometresi kullanarak hücre içi kalsiyum içeriğini belirlenmiştir.

Bulgular: Cx26-D50A ve A88V mutasyonları hücreler arasını kanalların oluşumunu engellemiş, aynı zamanda mutant proteinler hücrede Golgi aygıtındaki lokalize olmuştur.
Introduction

Connexins (Cx) are one of the building blocks of gap junctions in vertebrates. At least 21 connexin isoforms have been identified in the human genome which are expressed throughout the body in a cell/tissue type and developmental stage-specific manner [1, 2]. Gap junctions mediate various cellular processes including both the delivery of nutrients and removal of waste from avascular organs, electrical and metabolic coupling of cells, cell growth, proliferation, and differentiation by allowing the passage of molecules between adjacent cells [3–5]. In addition to the formation of intercellular channels that couple adjacent cells, connexins also function as non-junctional hemichannels that can facilitate the exchange of molecules across the plasma membrane and regulate cellular/tissue homeostasis [6]. Under physiological conditions, hemichannels are closed to prevent the leakage and uncontrolled uptake of molecules across the plasma membrane [7–9]. On the other hand, external insults, stress or mutations in connexin genes can disrupt proper sealing of hemichannels and negatively affect cellular physiology [10–15].

The importance of connexin mediated cellular communication for human physiology is better understood with characterization of various human disorders and their associations to mutations in several connexin isoforms, such as cataract (Cx46, Cx50), hearing loss, skin disorders (Cx26, Cx30) and hereditary peripheral neuropathy (Cx32) [3, 16–19]. In vitro characterization of disease-associated connexin mutations showed that mutations affect various processes in connexin biogenesis and function including but not limited to the generation of truncated proteins, altered trafficking, prevention of gap junction channel formation and function as well as altered hemichannel activities [20]. For example, all of the characterized Cx26 mutations associated with keratitis-ichthyosis-deafness (KID) syndrome were shown to lead to the formation of aberrant hemichannels [13–15, 21]. Therefore, the formation of abnormal hemichannels due to Cx26 mutations was suggested to be the underlying cause of epidermal anomalies observed in KID syndrome patients. In this respect, mutant Cx26 hemichannels are thought to interfere with keratinocyte homeostasis by releasing signaling molecules such as Ca\(^2^+\) and ATP into the extracellular environment as a paracrine element and alter the epidermal physiology [22, 23]. However, the exact mechanisms of how aberrant hemichannels influence keratinocyte homeostasis and generate skin phenotypes of KID syndrome are not completely known. One of the components important for the epidermis is the maintenance of calcium homeostasis as abnormal calcium signaling was implicated with several human disorders [24, 25]. In that respect, here we aimed to determine alterations in the Cx26 biogenesis and the intracellular calcium content caused by KID syndrome associated Cx26-D50A and A88V mutations, which were previously shown to cause elevated membrane currents in *Xenopus* oocytes and HeLa cells [26].

Materials and methods

Site-directed mutagenesis to generate Cx26 mutant clones

Human Cx26 WT (wild-type) cDNA was used to generate Cx26-D50A and A88V missense point mutations by using site-directed mutagenesis. After cloning of PCR products into pBlueScript cloning vector, location and insertion of mutations were verified by sequencing (Macrogen Europe, The Netherlands). Then, Cx26-WT, D50A and A88V cDNAs were subcloned into pIRES2EGFP2 and pCS2+ mammalian expression vectors.

Cell culture

HeLa and neuro-2A (N2A) (ATCC, USA) were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Thermo Scientific HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% penicillin/streptomycin (GIBCO, USA) in a humidified chamber with 5% CO\(_2\) and 37\(^\circ\)C. Cells were plated in 6 well plates for transfection with Lipofectamine 2000 reagent (Invitrogen, USA) on the following day with 1 : 2 DNA to
Lipofectamine 2000 ratio following the manufacturer’s protocol. 3.2 mM CaCl₂ was added in medium to close prospective hemichannels and cells were used within 24–36 h after transfection.

**Neurobiotin dye uptake assay**

N2A cells transfected with pIRES2EGFP2 clones were used for fluorescent dye uptake assays with neurobiotin (NB, 287 Da, +1 charge, Vector Labs, USA). Twenty four hours after transfection, cells were washed with PBS and incubated with Ca²⁺ free medium for 20 min at 37°C. Cells were then incubated with 0.5 mg/mL NB for 20 min and were washed with PBS containing 3.2 mM CaCl₂ three times for 10 min. After fixation of cells with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT), cells were permeabilized with 0.1% Triton-X 100 for 10 min, blocked with 3% BSA-0.1% TritonX-100 for 15 min and then were incubated with tetra-methyl rhodamine isothiocyanate (TRIT-C) conjugated streptavidin (1:1000 dilution, Pierce, USA) for 30 min at RT in dark. Cells were then incubated with 0.5 mg/mL NB for 20 min and washed with PBS containing 3.2 mM CaCl₂ three times for 10 min. After fixation of cells with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT), cells were permeabilized with 0.1% Triton-X 100 for 15 min and then were incubated with tetra-methyl rhodamine isothiocyanate (TRIT-C) conjugated streptavidin (1:1000 dilution, Pierce, USA) for 30 min at RT in dark. Images were acquired with a fluorescence microscope (IX71, Olympus, Japan) using the same exposure times for all conditions. Image analysis for signal intensity was performed with ImageJ (NIH, USA) program. Background subtraction was applied to all images before thresholding and signal intensities of fluorescence within cells were determined.

**Immunofluorescent staining**

HeLa cells (2.5×10⁵) were cultured on glass coverslips in 6 well plates and transfected with pCS2+ clones. After washing with PBS, cells were fixed with 4% PFA for 20 min, permeabilized with 0.1% Triton-X 100 for 15 min and blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature (RT). A 1:500 dilution of a polyclonal rabbit anti-Cx26 antibody (Invitrogen, USA) was applied for 1 h at RT followed by an incubation with Alexa555-conjugated goat anti-rabbit antibody (Invitrogen, USA), 1:200 dilution of Alexa Fluor 488 conjugated Phalloidin (Invitrogen, USA) and 1 μM DAPI for 45 min at RT in dark. Antibodies and DAPI was removed by PBS wash and coverslips were dipped in distilled water, dried and mounted on glass slides. Staining was verified under fluorescence microscope (IX83, Olympus, Japan) using ×40 objective and images were taken with a CCD digital camera.

**Measurement of intracellular calcium levels**

Intracellular calcium content in N2A cells (5×10⁵) transfected with pCS2+ clones was determined by using Fluo-3AM Ca²⁺ indicator (Invitrogen, USA) and flow cytometry [23]. After washing cells with Ca²⁺ free PBS twice for 5 min, cells were incubated with 5 μM Fluo-3AM at 37°C for 30 min. Then, cells were washed with PBS, trypsinized, pelleted and resuspended in 300 μL Hank’s Balanced Salt Solution (HBSS) [28] and analyzed with FACSCANTO (BD Biosciences, USA).

**Statistical analysis**

All results were expressed as mean (±standard deviation). Groups were compared using ANOVA followed by Student-Newman-Keuls post-hoc comparison analysis. Statistical significance was considered for p<0.05.

**Results**

**Verification of the ability of Cx26-D50A and A88V mutations to form aberrant hemichannels**

Cx26-D50A and A88V mutations were previously shown to cause elevated membrane currents in cRNA injected Xenopus oocytes and in HeLa cells, suggesting the presence of active hemichannels [26]. We wanted to confirm if these mutations were also able to facilitate the uptake of fluorescent dyes in transfected N2A cells (Figure 1). In sample images, neurobiotin fluorescent intensities were observed to increase in cells with Cx26-WT relative to the negative control, pIRES2EGFP2 transfected cells. Further, cells with Cx26-D50A and A88V had higher neurobiotin...
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signals than cells with Cx26-WT (Figure 1A). Fluorescent signal intensity analysis from at least 15 images from three different experiments suggested that the fluorescent intensity in cells transfected with Cx26-D50A and A88V were increased by 3.2 and 3.6-fold (p < 0.001) compared to pIRES2EGFP2 (Figure 1B). Further, Cx26-D50A and A88V resulted in 1.6 and 1.8-fold (p < 0.001) increase in neurobiotin uptake relative to Cx26-WT, D50Y and A88V mutant constructs (Figure 1A, white arrows), suggesting that they impaired the formation of gap junction channels between adjacent cells as also shown by Mhaske et al. [26].

Effect of Cx26-D50A and A88V mutations on the Cx26 protein biosynthesis and trafficking

To examine the effects of KID syndrome associated Cx26-D50A and A88V mutations on protein biosynthesis and trafficking, HeLa cells transiently transfected with pIRE-S2EGFP2 Cx26-WT, D50A and A88V constructs were used for immunofluorescent staining (Figure 2). Cells expressing Cx26-WT, D50A and A88V constructs were able to synthesize Cx26 proteins as observed by Cx26 signals in representative images in Figure 2 (red signals). Furthermore, Cx26-WT expressing cells had gap junction plaques at the cell-to-cell contact sites between adjacent cells (Figure 2A, white arrows). On the other hand, no gap junctional plaques were observed between neighboring cells transfected with either Cx26-D50A or A88V mutant constructs (Figure 2A, white arrows), suggesting that they impaired the formation of gap junction channels between adjacent cells as also shown by Mhaske et al. [26].

To examine whether these mutations caused defects in Cx26 protein trafficking, co-immunostaining of Cx26 and golgin-97, a Golgi apparatus marker, was performed (Figure 2B). There was no co-localization between Cx26 (red signal) and golgin-97 (green) in Cx26-WT expressing cells. In contrast, Cx26-D50A and A88V mutant proteins were detected to extensively overlap with golgin-97 in transfected cells as observed by yellow signals in transfected cells (Figure 2B). These suggested that a fraction of proteins retained in the Golgi apparatus while others formed hemichannels on the plasma membrane.

Effect of Cx26-D50A and A88V mutations on intracellular calcium signals

Calcium signals are indispensable for proper functioning of keratinocytes and abnormalities in calcium homeostasis have been shown to lead skin disorders [25]. In order to investigate whether Cx26-D50A and A88V mutations cause any change in the internal calcium levels, we determined cellular calcium content by a calcium indicator Fluo-3AM using flow cytometry (Figure 3). Comparison of Fluo-3AM signals among groups suggested that calcium amount in cells with Cx26-WT and cells transfected with the negative control, pCS2+ were similar (p = 0.12). However, cells with
Figure 2: Comparison of Cx26-WT, D50A and A88V localization and trafficking in gap junctional communication deficient HeLa cells. (A) Cx26-WT, D50A and A88V transfected HeLa cells were co-stained with phalloidin for actin (green) and Cx26 antibody (red). Blue is DAPI staining of the nucleus. White arrows point out the gap junction plaques between Cx26-WT cells and red arrows show cell-cell contact sites of cells transfected with Cx26-D50A and A88V. Cells with only green and blue signals indicate untransfected cells. Scale bar 20 μm. (B) Cx26-WT, D50A and A88V transfected cells were co-stained with Cx26 (red) and the Golgi apparatus marker, golgin-97 (green) antibodies. Blue shows DAPI staining of the nucleus. Yellow signals in Cx26-D50A and Cx26-A88V point out the co-localization of Cx26 and golgin-97. Scale bar 20 μm.

Cx26-D50A and A88V had 1.6-fold increase in the intracellular calcium amount with respect to Cx26-WT cells (p = 0.04) (Figure 3). These changes in calcium content suggested that Cx26-D50A and A88V mutations interfered with calcium mechanisms in these cells.

Discussion

KID syndrome is a rare congenital disorder associated with dominant Cx26 mutations. The molecular mechanisms leading to KID syndrome skin phenotypes is not exactly known and in vitro characterization of Cx26 mutations has helped to understand the effect of mutations on cell homeostasis and hence the generation of skin anomalies in affected individuals. Here, we characterized
the properties of KID syndrome associated Cx26-D50A and A88V mutations, which were shown to form aberrant hemichannels by electrophysiological analysis [26]. D50A and A88V mutations resulted in an increase in the uptake of neurobiotin into cells from the extracellular environment, implicating the ability of mutant hemichannels to transfer larger molecules than small ions into cells. Moreover, these mutations prevented the formation of gap junction channels at the cell-to-cell junctions and a fraction of mutant proteins were observed to be retained in the Golgi apparatus compared to Cx26-WT. Finally, mutations caused an elevation in the intracellular calcium levels in cells containing D50A and A88V compared to cells with Cx26-WT. These results confirmed the activities of leaky hemichannels due to Cx26-D50A and A88V mutations and may also implicate the involvement of altered protein trafficking and elevated intracellular calcium content due to aberrant hemichannels in KID syndrome.

Cx26 mutations cause both non-syndromic and syndromic deafness. In vitro characterization of mutations leading to non-syndromic deafness suggested these mutations are loss-of-function mutations and they only affect the cochlear function but have no effect on other Cx26 expressing tissues such as the skin and the liver [15, 29–31]. On the other hand, Cx26 mutations associated with syndromic deafness were shown to result in the acquisition of novel functions which influence the homeostasis of both the inner ear and the skin and are considered as gain of function mutations [14, 32, 33]. For example, Cx26 mutations linked to KID syndrome were shown to form aberrant hemichannels and activities of abnormal hemichannels are suggested to be the main mechanisms underlying skin pathologies [22, 26, 33, 34]. The molecular mechanisms related to this phenotype might be the uncontrolled release of signaling molecules including ATP into the extracellular environment through aberrant Cx26 hemichannels and therefore the disruption of calcium signaling. Changes in calcium homeostasis can affect different cellular processes including proliferation, differentiation and survival that can contribute to the development of epidermal abnormalities [25, 35]. Here, we showed that expression of Cx26-D50A and A88V mutations in mammalian cells resulted in an increase in the intracellular calcium content which may suggest the contribution of altered calcium homeostasis that might contribute to the development of epidermal phenotypes.

Cx26-D50A and A88V mutations prevented the formation of gap junction plaques at cell-to-cell communication sites. The observation of aberrant hemichannel activities suggest the transport of connexons to the plasma membrane where they formed non-junctional hemichannels. However, these hemichannels were not able to dock with each other to complete the formation of gap junction channels on the plasma membrane. Furthermore, a fraction of Cx26-D50A and A88V mutant proteins were observed to accumulate in the Golgi apparatus, suggesting defects in Cx26 trafficking. Cx26 proteins were shown to be transported to the plasma membrane either passing through the Golgi apparatus or by bypassing the Golgi apparatus depending on the cell type, suggesting different membrane transport pathways for Cx26 [36, 37]. Further, Cx26-D66H mutation associated with Vohwinkel syndrome and R184Q mutation linked to dominant hearing loss were also shown to be retained in the Golgi apparatus [38, 39]. These mutations might impair the folding of the proteins, resulting in their accumulation in the Golgi apparatus that may play role in the generation of stress responses to influence cellular processes [40]. Thus, both the leaky hemichannel activity and retaining of mutant proteins in the Golgi apparatus can alter cellular physiology.

In conclusion, characterization of Cx26 mutations provide an invaluable tool in the elucidation of molecular mechanisms underlying the KID syndrome. It will help us to expand our understanding of the disorders, the role of Cx26 in epidermal homeostasis and also our ability to develop new therapeutic approaches for patients.

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