A Tetracysteine-tag and HeLa Cell System for the Dynamic Analysis of the Localization and Gating Properties of a Specific Connexin Isoform

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ABSTRACT

Cell-cell communication in animals is predominantly conducted via gap junction (GJ) plaques comprising 20 connexin (Cx) isoforms. However, the GJ plaques are formed at only limited part of cell-cell interface and such an irregular localization is a current problem. To solve this problem, we developed an experimental platform to analyze the role of each Cx isoform in the regulation of GJ plaque localization and in the intercellular molecular movement. A tetracysteine-tagged Cx expressed in HeLa cell deficient in Cx was found feasible for viable imaging of GJ plaques. Femtoinjection enabled the quantitative analysis of intercellular dye diffusion.

1. Introduction

In a biological system, the cell-cell communication is essential to maintain the homeostasis of the system under stress conditions as well as during drastic transitions of differentiation. Gap junctions (GJs) are thought to be facilitating the intercellular communication by forming channels for ions, small molecules, and second messengers to pass through to the neighbor cells. The GJ comprises various isoforms of connexin (Cx), a functional protein. Actually the GJ molecular movement occurs via GJ plaque, a cluster of a number of GJ.

The maturation process from the Cx synthesis to the GJ plaques is speculated as follows. Cx proteins are synthesized in endoplasmic reticulum and transported to Golgi apparatus. During this transportation, a Cx is folded into 3-dimensional structure and then oligomerized into a hexameric hemichannel called a connexon. A monomer of Cx has four transmembrane domains, two extracellular loops, a cytoplasmic loop, N-terminus, and C-terminus. Three intramolecular disulfide bonds stabilize the extracellular loops. However, there is no covalent intermolecular bond between Cxs. Connexons in Golgi apparatus are packaged into vesicles and transported to the cell membrane. An individual connexon from one cell associates with a corresponding connexon on a neighboring cell to form a GJ channel. Usually multiple channels aggregate in the cell membrane to form GJ plaques.

To date a large number of studies have been reported about the relevance of Cx to various growth stages, metabolic conditions, tissues and organs, and diseases. Special attention has been paid to the potential role as tumor suppressors. Those studies have been concentrated on the specific roles of several isoforms such as Cx31, Cx43, and Cx45 because they are more ubiquitously expressed than other isoforms. However the specificity of their respective roles is still unclear. Moreover it should be necessary to consider the simultaneous actions of multiple isoforms rather than the specific action of a particular single isoform alone.

Our interest exists in the role of Cxs in undifferentiated states of mouse embryonic stem (ES) cells and in the initial several days of their differentiation, because a drastic change from the symmetric cell divisions in undifferentiated state to the asymmetric cell division should be the most fundamental event underlying every other differentiation step. Previously we analyzed the dynamic expression of 20 Cx isoforms and found that the GJ plaques appeared at only limited part of cell-cell interface.

The open-close properties of GJ were well analyzed formerly. The GJ comprising Cx43, for example, was analyzed by whole cell voltage clamp method. The open state, however, did not imply the single state of complete opening. Rather, there were multiple open states with different opening rates. The transition manners from one state to another were different depending upon chemical conditions in the cytosol and/or membrane potential conditions.

Once GJ plaque is formed between the neighbor cell of interest, we can understand the molecular movement via GJ plaque. However, we cannot understand why GJ plaques were not formed between every neighbor cell, though there seemed to be no difference between those neighbor cells. In order to solve this problem, we intended to develop an experimental platform to analyze the role of each Cx isoform in the regulation of GJ plaque localization and in the intercellular molecular movement.

A fluorescent probe such as GFP is useful for the visualization of a target protein in living cells. However the molecular size of GFP is as large as ca. 25 kD and therefore might disturb the intrinsic Cx function. Therefore a smaller tag might be better to reduce this influence.

Then we adopted a small motif of Cys-Cys-X-X-Cys-Cys as a visualization tag and introduced it into the C-terminal of Cx. An As-complex, FlAsH, can bind to this tetra-Cys (TC) tag to form a fluorescent conjugate. Here we have selected Cx43 as a demonstrative isoform because of its most ubiquitous expression behavior. The functional analysis needs the quantitative introduction of a diffusion marker dye into only a target single-cell. Such an experiment could be successfully conducted by means of a femtoinjection system.

2. Experimental

2.1 Preparation of pCMV-Cx43-TC

RNA was extracted from mouse ES cells and its cDNA was prepared by reverse transcription. The TC tag was designed as
AEAAAREACCRECCARA by referring to the sequence reported by Griffin et al.\textsuperscript{24} A PCR primer set for Cx43 were designed as follows. The forward primer comprised a sequence targeting Cx43 and an Agel recognition adaptor at the 5′ terminal: 5′-GGTCA-CCGGTATGGGGTACGAGGCGCCTTG-3′. The reverse primer comprised a sequence targeting Cx43 and TC tag, and an EcoRI recognition adaptor at the 5′ terminal: 5′-GCCGAATTCCTTATGGGCGGCAGCCTCCCTGCGGAGGCCCTGGCAATCTCCAGGTCATCCAGGCCGAG-3′. The cDNA prepared from mouse ES cells was analyzed by PCR with this primer set. The PCR product was treated with Agel and EcoRI and then ligated with pDsRed monomer C1 vector (Clontech) that was also treated with Agel and EcoRI beforehand.

2.2 Cell culture and transfection
HeLa cells were cultured in GMEM supplemented with 10% FBS for 24 h until a confluent condition. Then pCMV-Cx43-TC was transfected by treating with lipofectamine 2000 for 24 h. The cells were transferred to a selection medium containing G418.

2.3 Imaging of Cx43-TC in living cells
The expression of Cx43-TC in HeLa cells was detected by staining with FlAsH (Toronto Research Chemicals Inc.) according to the procedure developed by Hoffmann et al.\textsuperscript{25} and Crivat et al.\textsuperscript{26} with slight modification. Briefly, a 500 nM FlAsH solution was added to the confluent culture of HeLa cells for incubation under a 5% CO\textsubscript{2} condition for 1 h at 37°C. Then the FlAsH solution was removed and the cells were rinsed with PBS for two times. The cells were observed with a fluorescent microscope.

2.4 Femtoinjection and fluorescence analysis
The molecular size cut off by GJs composed of Cx43 is estimated as 1000–1500. Therefore we have selected Lucifer yellow (LY, MW: 443 Da) (Sigma-Aldrich) and Dextran Texas Red (D-TR, MW: 70 kDa) (Invitrogen) as a permeable and a non-permeable dyes, respectively. A 0.9 g cm\textsuperscript{-3} LY solution or a 0.8 mol cm\textsuperscript{-3} D-TR solution was filled in a glass capillary. By means of a single-cell manipulation supporting robot,\textsuperscript{21–23} the capillary was injected into a target HeLa single-cell that expressed Cx43-TC and the dye solution was introduced into the cell under a condition of 0.7 kg cm\textsuperscript{-2} for 10 ms. Then the cells were observed with a fluorescent microscope.

3. Results and Discussion

3.1 Localization of Cx43-TC in HeLa cells
HeLa cells were transfected with pCMV-Cx43-TC and stained with FlAsH. Non-specific staining of FlAsH with cysteine-rich proteins was washed with a BAL wash buffer solution (Invitrogen, Molecular Probes). The washing conditions were studied by varying BAL concentration (50–650 µM), washing time span (10–15 min), and number of washings (1–5 times). The optimum washing condition for obtaining a clear image with a sufficient S/N level was found to be a combination of 100 µM, 10 min, and 3 times.

After washing under the optimum condition, fluorescent images supposedly specific to Cx43-TC appeared at various sites in the cell (Fig. 1). The fluorescent lines such as one encircled by a red line near cell membrane were thought to be GJ plaques that were clusters of a number of GJs. There were smaller fluorescent spots near cell membrane such as one encircled by a white line. Those seemed to be small GJ plaques of connexons. The connexon is a hexamer of Cx but still not functioned as a GJ. Fluorescent spots were observed also in cytosol such as one encircled by a yellow line. They might be Cx43-TC cluster on the way of transportation towards cell membrane or stored in Golgi apparatus.

3.2 Intercellular diffusion of dyes through the cell membrane of control HeLa cells
LY and D-TR were femtoinjected into single-cells of control HeLa single-cells, respectively (Fig. 2). LY and D-TR remained only in the injected cells and no diffusion to the outside through cell membrane was observed. It is known that there is no intercellular GJ communication in HeLa cells. Therefore even a small dye molecule such as LY could not move to the neighbor cell though those cells were in contact with each other.

Then the same dyes were femtoinjected into the transfected cells in which fluorescent lines appeared at the cell-cell contact location (Fig. 3). D-TR remained within the injected cells in the same way as

![Figure 1](image1.png)

**Figure 1.** (Color online) Expression of Cx43-TC in HeLa cells. A red circle, a plaque located at the interface of 2 cells; a white circle, a smaller plaque of connexon; a yellow circle, a Cx43-TC cluster in the cytosol. Scale bar, 30 µm.

![Figure 2](image2.png)

**Figure 2.** (Color online) Localization of diffusion marker dyes in control HeLa cells. (a) D-TR, (b) LY.

![Figure 3](image3.png)

**Figure 3.** (Color online) Three cases (a, b, c) of diffusion properties of transfected HeLa cells. (a1, b1, c1) merge of Cx-TC and D-TR; (a2, b2, c2) merge of Cx-TC and LY; (a3, b3, c3) merge of Cx-TC, D-TR, and bright field image. Scale bar, 30 µm.
the control cells. In contrast, LY diffused to the neighbor cell. It should be noted that dye diffusion occurred only at the site just near the fluorescent line, a GJ plaque.

For the case of Fig. 3a, the fluorescent intensity profiles for D-TR and LY were analyzed along the broken lines (Fig. 4). From the profiles recorded at definite time span, the dye diffusion rates not only through GJs between 2 cells but also within the dye injected cells may be estimated quantitatively.

There are occasions that one target HeLa cell is in contact with multiple neighbor cells. In such a case, GJ plaques are not necessarily formed at every intercellular contact plane as observed in Fig. 1. It is an unsolved problem how the target cell can decide a proper location position for GJ plaque formation. The target cell cannot detect the intracellular factors such as pH, K⁺ concentration, and the presence of second messengers in every contacting cell. Therefore the target cell might determine the proper site from extracellular factors such as cell surface electric potential and cadherin-like molecules of every contacting cell. This should be one of the future subjects associated with gap junctional communication.

4. Conclusion

A TC-tag and HeLa cell system was found to be feasible for the dynamic functional analysis of GJ plaques comprising specific single Cx isoform. The location of intercellular dye diffusion coincided the expression site of GJ plaque that was visualized as fluorescent line of Cx43-TC. The present system may be further developed to the experimental platform for the independent analysis of the involvement of every single isoform.

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References

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