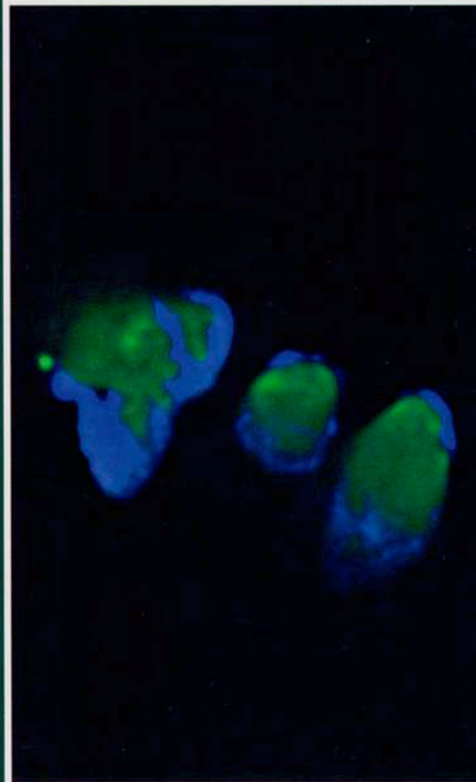
ISSN 1061-5385 CADCEF
PAGES 153–476

Volume 8
Issue 4–6

Cell Communication & Adhesion



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DISEASES LINKED TO CONNEXINS

Deafness & Skin

Human Connexin Disorders of the Skin
Gabriele Richard 401

Cx26 Affects the In Vitro Reconstruction of Human Epidermis
Ludovic Wiszniewski, Denis Salomon, and Paolo Meda 409

Connexin 26 Expression and Mutation Analysis in Epidermal Disease
Wei-Li Di, John E. A. Common, and David P. Kelsell 415

Inherited Connexin Mutations Associated with Hearing Loss
Karen B. Avraham 419

Functional Analysis of a Dominant Mutation of Human Connexin26 Associated with Nonsyndromic Deafness
R. Bruzzone, D. Gomès, F. Denoyelle, N. Duval, J. Perea, V. Veronesi, D. Weil, C. Petit, M.-M. Gabellec, P. D'Andrea, and T. W. White 425

Cancer

Aggregated DsRed-Tagged Cx43 and Over-Expressed Cx43 are Targeted to Lysosomes in Human Breast Cancer Cells
Hong Qin, Qing Shao, Daniel J. Belliveau, and Dale W. Laird 433

Determination of a Potential Role of the CCN Family of Growth Regulators in Connexin43 Transfected C6 Glioma Cells
Tamara L. McLeod, John F. Bechberger, and Christian C. G. Naus 441

Connexin 32 Fused to the Green Fluorescent Protein Retains its Ability to Control the Proliferation of Thyroid Cells
Virginie Flachon, Christine Durand, Samia Selmi-Ruby, Bernard Rousset, and Yvonne Munari-Silem 447

Cover illustration: Gap Junctions in Hawaii: Appropriate as the signature image for the recent international meeting of the gap junction field is this microscopic image of the Hawaiian islands on the surface of a cell. In fact, this high resolution deconvoluted fluorescence image taken by Matthias Falk and colleagues shows three gap junction plaques expressed between Hela cells co-transfected with Cx43-CFP (blue fluorescence) and Cx26-YFP (green fluorescence) constructs. Notably, the beaches around the islands are made predominantly of Cx43, while the green mountainous interior indicates a predominance of Cx26, demonstrating that these connexins segregate within gap junction plaques. In contrast, other co-expressed connexins like Cx32 and Cx26 show complete mixing within a plaque.

Cell Communication and Adhesion, 2001, Vol. 8, Nos. 4–6, pp. 237–242
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1061-5385/01 $12.00+.00

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Distribution and Dynamics of Gap Junction Channels Revealed in Living Cells

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To study the structural composition and dynamics of gap junctions in living cells, we tagged their subunit proteins, termed connexins, with the autofluorescent tracer green fluorescent protein (GFP) and its cyan (CFP) and yellow (YFP) color variants. Tagged connexins assembled normally and channels were functional. High-resolution fluorescence images of gap junction plaques assembled from CFP and YFP tagged connexins revealed that the mode of channel distribution is strictly dependent on the connexin isoforms. Co-distribution as well as segregation into well-separated domains was observed. Based on accompanying studies we propose that channel distribution is regulated by intrinsic, connexin isoform specific signals. High-resolution time-lapse images revealed that gap junctions, contrary to previous expectations, are dynamic assemblies of channels. Channels within clusters and clusters themselves are mobile and constantly undergo structural rearrangements. Movements are complex and allow channels to move, comparable to other plasma membrane proteins not anchored to cytoskeletal elements. Comprehensive analysis, however, demonstrated that gap junction channel movements are not driven by diffusion described to propel plasma membrane protein movement. Instead, recent studies suggest that movements of gap junction channels are indirect and predominantly propelled by plasma membrane lipid flow that results from metabolic endo- and exocytosis.

Keywords Cyan fluorescent protein, gap junctions, green fluorescent protein, life cell imaging, protein dynamics, yellow fluorescent protein

INTRODUCTION AND METHODS

Gap junction (GJ)channels provide direct cell-to-cell communication. They form transmembrane channels that traverse the adjoining plasma membranes of neighboring cells, creating a cytoplasmic continuum. Channels are constructed from connexins (Cxs) that are encoded by a large gene-family predicted to comprise of at least 20 structurally homologous transmembrane protein isoforms in humans.

Received 1 September 2001; Accepted 15 September 2001.
This work was supported by grants from the NIH awarded to MMF and S-CC.
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Channels typically cluster in large arrays, termed plaques, composed of many closely packed individual channels. Gap junctions are widely distributed and many cell-types express more than one connexin isoform, allowing the possible formation of hetero-oligomeric gap junction channels, composed of more than one connexin isoform, besides homo-oligomeric channels, composed of a single connexin isoform.

To investigate the structural composition and dynamics of gap junction channel plaques in living cells we have genetically engineered three different connexin isoforms, Cx26, 32, and 43 that were tagged with enhanced green fluorescent protein (EGFP), or its spectrally shifted cyan (ECFP) and yellow (EYFP) isoforms. Our group has previously shown that excitation and emission spectra of CFP and YFP are shifted far enough apart to allow simultaneous detection of two different connexin isoforms (Falk 2000a; Falk and Lauf 2001). Comprehensive analysis has demonstrated that tagged connexins behaved similar to untagged connexins and that tagged channels were functional as monitored by dye transfer (Falk 2000a). Fusion proteins were expressed in transient, and stable transfected epitheloid HeLa cells (human cervix carcinoma), which are not contact inhibited and grow partially on top of each other. This allows gap junction observation onto their surface (en face), in addition to the more common view onto their edge (Figure 1). We combined this approach with high-resolution fluorescence deconvolution microscopy and used an over-expression system that provided the synthesis of large enough gap junction plaques suitable for light microscopic examination.

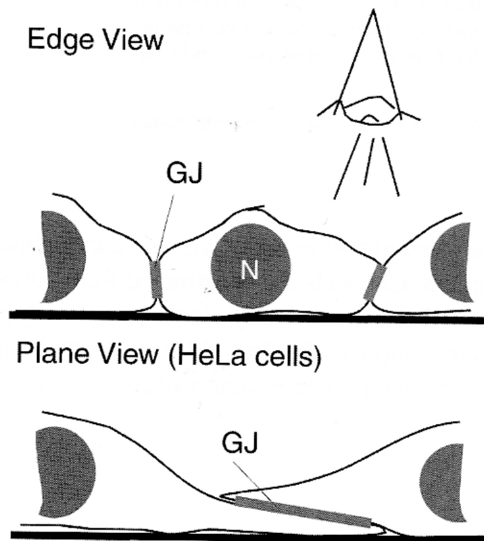

FIG. 1. Schematic representation of gap junction plaques normally visible on their edge in contact inhibited cell lines and often visible on their surface in HeLa cells that lack contact inhibition.

RESULTS AND DISCUSSION

Structural Composition of Gap Junctions Assembled from Two Different Connexin Isoforms

Gap junction plaques assembled in double-transfected cells from CFP and YFP tagged connexins were generally composed of both connexin isoforms. However, connexin isoforms were either homogenously co-distributed (Cx26 and 32), or segregated into well-separated domains (Cx26 and 43, and Cx32 and 43), indicating that channel distribution within the channel cluster is strictly dependent on the connexin isoforms (Figure 2). Further experiments indicated that segregation or mixing of different connexin isoforms is independent of tags, plaque size, or connexin ratio (Falk 2000a). A comparable study performed in vivo in which the distribution of Cx26, and 43, co-expressed in embryonic epidermis cells was investigated revealed a similar segregation into red and green domains (Risek et al. 1994). Large yellow plaque-domains also detected in this study probably represent imaging artifacts due to the insufficient spatial resolution achieved.

Several independent lines of evidence, such as coimmunoprecipitation (Falk et al. 1997), a trapping approach using transport-deficient DsRed-tagged connexins (Lauf et al. 2001) to capture other interacting connexin isoforms (P Lopez and MM Falk unpublished results), and fluorescence resonance energy transfer (FRET) between closely apposed Cx-CFP and Cx-YFP chromophores (MM Falk

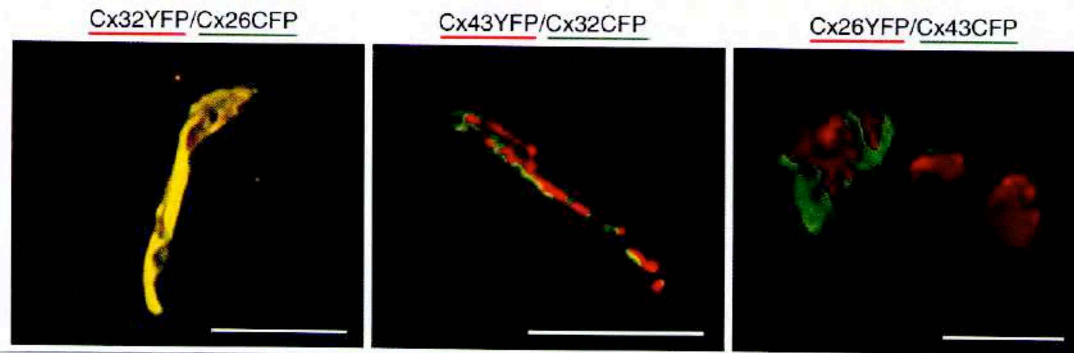

FIG. 2. Mixing and segregation of connexins. Cx32 and 26 were found to mix homogenously in gap junction plaques after coexpression of the two connexin isoforms (yellow), while Cx43 and 32, and Cx43 and 26 were found to permanently segregate into well separated domains (green and red). Connexins were tagged with CFP and YFP, respectively, and imaged in living cells. Bar = 5 μm. (See Color Plate IV).

unpublished results) indicate that the yellow plaques – consisting of evenly distributed Cx26 and 32—are composed of hetero-oligomeric channels that contain both connexin isoforms. These results are in agreement with studies by other investigators in which hetero-oligomerization between Cx26 and 32, Cx37 and 43, Cx40 and 43, and Cx46 and 50 have been proposed (Stauffer 1995; Brink et al. 1997; He et al. 1999; Jiang and Goodenough 1996).

Based on complementary studies in our laboratory in which the interaction of co-expressed full length and shortened connexins progressively truncated from their C- and N-termini (Falk 2000b), or wild type and amino acid exchange variants (V Lagrée, K Brunschwig, MM Falk, and NB Gilula unpublished results), respectively, were analyzed, we propose that hetero-oligomerization is regulated by intrinsic, connexin isoform specific signals. Recently, we have identified specific amino acid residues in the N-terminal portion of the connexin sequences that are homologues in Cx26, and 32 (and in other β connexins), but different in Cx43 (and in other α connexins) that may result in an altered surface structure of protein-protein interfaces in these connexins, and thus may prevent the interaction of Cx43 and 32, and Cx43 and 26, but enable Cx26 and 32 to interact (V Lagrée, K Brunschwig, MM Falk, and NB Gilula unpublished results). Future experiments using additional connexin isoforms may reveal whether such proposed structural differences between connexin isoforms have a functional relevance in specific homo- and heteromeric gap junction channel types composed of these connexin isoforms.

Dynamic Mobility of Gap Junction Channels and Gap Junction Plaques

Imaging gap junctions by time-lapse microscopy revealed that gap junctions, contrary to previous expectations, are mobile, and dynamic arrangements of channels which constantly undergo structural changes in the plasma membranes. Movements are complex and include (1) lateral movement of plaques in the membrane plane that can result in plaque fusion (Figure 3A) and plaque splitting (Figure 3B), (2) directional flow of channels and plaques in which channels and plaques predominantly move into one direction (note how the green domains consisting of Cx26 fuse and move from the top left to the bottom right of the plaque imaged in Figure 3C), and (3) distorted movements in which channels and channel clusters move laterally, however, without any clear direction (Figure 3D).

To address what might drive the movement of gap junction channels and channel clusters we determined the speed with which plaque edges, channel domains, and channel-free areas, visible as

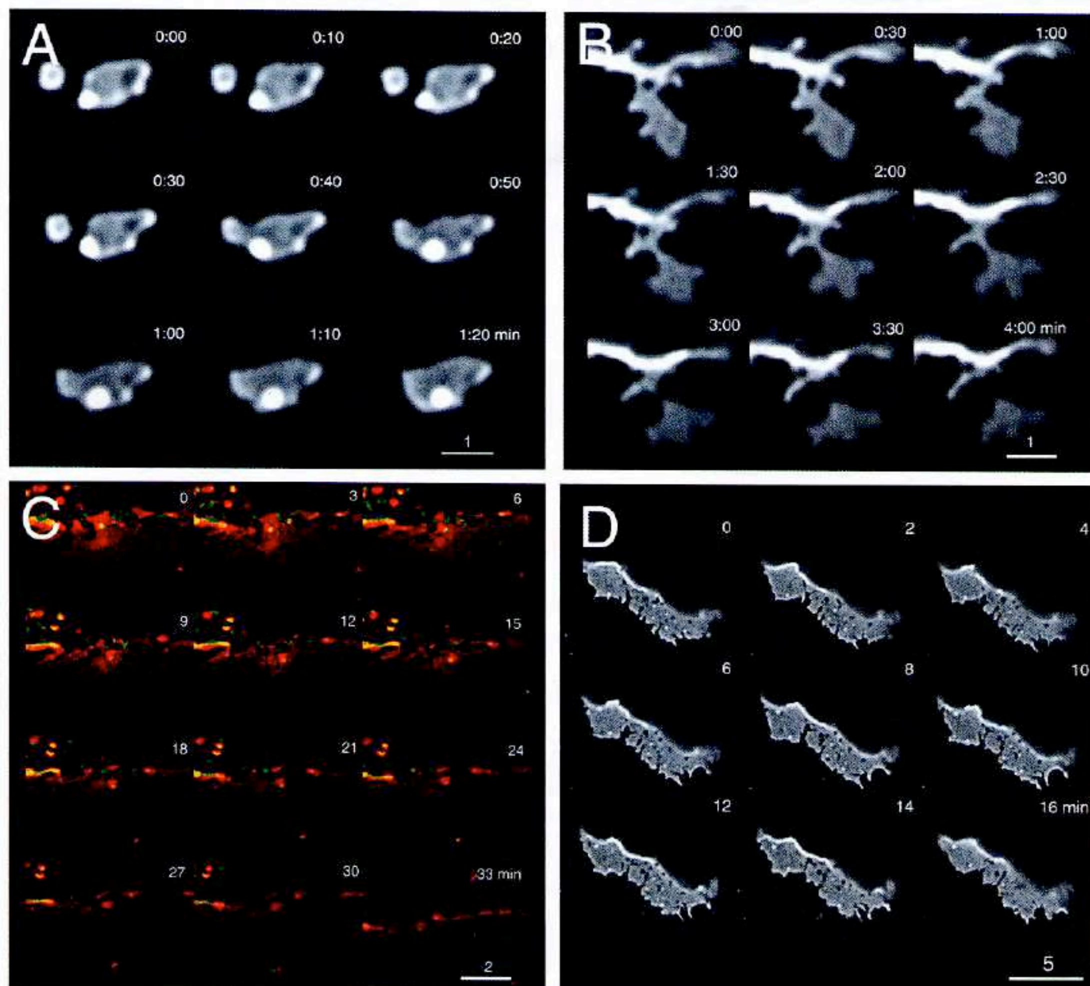

FIG. 3. Gap junction channels are assembled dynamically within gap junction plaques. Cx43-GFP (A, B, D), and Cx43-CFP (green) and Cx26-YFP (red) (C) were expressed in transfected HeLa cells and gap junction plaques were imaged by time-lapse microscopy. This approach revealed that gap junction channels are mobile which can result in plaque fusion (A), plaque splitting (B), directional (C), and distorted channel movements (D) in the plane of the membranes. Gap junction movements appear to be driven predominantly by the flow of plasma membrane lipids resulting from metabolic endo- and exocytosis. Note how in (C) domains consisting of Cx43-CFP fuse and split, but never mix with the Cx26-YFP containing domains. Also note how channel-free domains, appearing as dark areas within the fluorescent plaques (C, D) can be generated by invagination. They fuse, split, and move throughout the plaque and finally can be expelled at the plaque-edge. Bars = μm. (See Color Plate V).

dark "holes" in the fluorescent channel cluster (see below), move. We found that movements are app. 0.2–0.5 μ/min (n = 8). This corresponds to a diffusion constant D (two-dimensional diffusion) = $s^2/4t$ (s, distance; t, time) of $3-7 \times 10^{-12} cm^2/sec$. This is much slower than the diffusional speed of lipids in plasma membranes ($D = 10^{-7}-10^{-8}$ cm^2/sec), or the diffusional speed of plasma membrane proteins not anchored to cytoskeletal elements ($D \sim 10^{-9}$ cm^2/sec). These results, and results obtained by fluorescence recovery after photobleaching (FRAP) experiments (U Lauf and MM Falk

unpublished results) in which fluorescence of areas of gap junction plaques was permanently bleached, indicate that diffusion does not play an important role as driving force of gap junction channel movements.

Next, we investigated the nature of the channel-free areas, present within the plaques. We observed that channel free-areas could arise from invaginations at the edge of plaques, that gap junction channels can flow around and pass by these areas, and that they can split apart and fuse (see Figure 3C, D). These channel-free areas were also seen commonly in electron microscopic images of gap junction freeze-fracture replicas (see e.g. Friend and Gilula 1972). These electron microscopic investigations have shown that these areas do not contain gap junction channels, or any other protein that would produce a distinct structure in such freeze-fracture replicas. We further found that the channel-free areas contain lipid (Figure 4A), and that actin filaments end in some of these areas (Figure 4B). This suggests that cell-adhesion sites or other actin filament capping proteins may occupy these areas as suggested by the work of Fujimoto et al. (1997) and movements may be driven by cytoskeletal dynamics. However, recent experiments implemented with drugs that disrupt the cytoskeletal architecture, such as nocodazole (microtubule depolimerization), cytochalasin D (f-actin fiber depolimerization), or BDM (myosin-II inhibitor) suggest that movement of gap junction channels in non-motile cells with well established cell-cell contacts is not resulting from cytoskeletal dynamics, but is predominantly propelled by "membrane flow." Membrane flow describes the directional flow of lipids from the plasma membrane through the endoplasmic reticulum (ER) and Golgi back to the plasma membrane that results from the metabolic activity of cells. When endo- and exocytosis occur in an overall oriented, polarized fashion, it will result in a rotational net-flow of plasma membrane lipids around the cell body that then can drive directional lateral streaming (shown in Figure 3C), as well as disordered lateral movements of gap junction channel clusters (shown in Figure 3D). The slow movements of gap junction channels described above correlate very well with the slow movements resulting from metabolically driven membrane flow (Sheetz 1993; Bretscher 1996; Steinman et al. 1983 and references cited therein). Currently we are carrying out experiments to inhibit endo- and exocytosis to test this hypothesis.

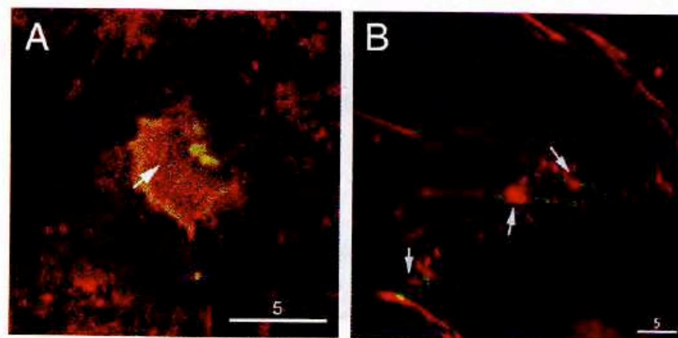

FIG. 4. Characterization of channel-free areas of gap junction plaques. (A) Channel-free areas (marked with an arrow) contain lipid as evident from plasma membrane staining with DiI (red). The gap junction plaque assembled from Cx43-GFP (green) appears yellow in the merged image. (B) F-actin staining with Rh-phalloidin (red) shows that some actin fibers end in channel-free areas (arrows), suggesting, that capping proteins of cell-adhesion sites might occupy some channel-free domains. Bars = μm. (See Color Plate VI).

CONCLUDING REMARKS

Tagging connexins with autofluorescent tracer proteins and high resolution fluorescence microscopy has allowed us to study gap junctions in living cells and to elucidate several new aspects of gap junction structure, assembly, and degradation. Entire gap junctions were imaged and reconstructed as they appear in the adjoining membranes of neighboring cells (Falk 2000a). Furthermore, a complete and permanent segregation or homogenous mixing of channels within gap junction plaques according to their characteristics was observed (Falk 2000a,b; Falk and Lauf 2001; Shen et al. 2001). Finally, time lapse recordings have demonstrated that gap junctions are mobile and very dynamic structures that constantly undergo spatial rearrangements. Channels and plaque movement appears to be indirect and predominantly result from metabolic plasma membrane lipid flow. In future experiments, it has to be established how precisely gap junctions are synthesized and degraded. Thus, high resolution fluorescence

microscopy, fluorescence tagging, time-lapse imaging, and volume rendering promise to be indispensable tools for investigating the biosynthesis, dynamics, structure and function of gap junctions.

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