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Review

Regulation of connexin biosynthesis, assembly, gap junction formation, and removal

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Abstract

Gap junctions (GJs) are the only known cellular structures that allow a direct transfer of signaling molecules from cell-to-cell by forming hydrophilic channels that bridge the opposing membranes of neighboring cells. The crucial role of GJ-mediated intercellular communication (GJIC) for coordination of development, tissue function, and cell homeostasis is now well documented. In addition, recent findings have fueled the novel concepts that connexins, although redundant, have unique and specific functions, that GJIC may play a significant role in unstable, transient cell–cell contacts, and that GJ hemi-channels by themselves may function in intra-/extracellular signaling. Assembly of these channels is a complicated, highly regulated process that includes biosynthesis of the connexin subunit proteins on endoplasmic reticulum membranes, oligomerization of compatible subunits into hexameric hemi-channels (connexons), delivery of the connexons to the plasma membrane, head-on docking of compatible connexons in the extracellular space at distinct locations, arrangement of channels into dynamic, spatially and temporally organized GJ channel aggregates (so-called plaques), and coordinated removal of channels into the cytoplasm followed by their degradation. Here we review the current knowledge of the processes that lead to GJ biosynthesis and degradation, draw comparisons to other membrane proteins, highlight novel findings, point out contradictory observations, and provide some provocative suggestive solutions.

Keywords: Cell–cell communication; Connexin; Gap junction; Membrane channel; Oligomeric membrane protein; Protein biosynthesis

1. Introduction

Gap junctions (GJ), tight junctions, adherens junctions, desmosomes, hemi-desmosomes, focal adhesions, chemical synapses, and immunological synapses (the specialized adhesive contacts that form between activated T cells and antigen-presenting cells) are complex multi-unit plasma membrane structures that assemble in a localized spatial and temporal organization to maintain structural tissue organization, and to provide cell-signaling functions. Central to all these structures are distinct trans-membrane proteins that cluster together to arrange into arrays, strands, or focal contacts (connexins in gap junctions, claudins in tight junctions, cadherins in adherens junctions, desmoglein and desmocollin in desmosomes, integrins in focal adhesions and hemi-desmosomes, acetylcholine receptors and other membrane channels in chemical synapses, and antigen-presenting MHC class I receptors in immunological synapses) (reviewed in Refs. [1–11]). In general, scaffolding proteins (such as ZO-1), anchor proteins (such as catenins, vinculin, α-actinin, plakoglobin, desmoplakin and talin) and signaling proteins (such as focal adhesion kinase), assemble adjacent to the trans-membrane proteins and often link the latter to cytoskeletal elements of the actin, microtubule, and intermediate filament network to build the complex, spatially ordered structures. Although gap junctions appear to be built more simply than other plasma membrane signaling structures (see below), this just might reflect our limited knowledge of proteins that bind to them. Recent approaches that used yeast-two hybrid screens, GST-pull-down assays, antibody arrays, and proteomic analysis actually identified quite a number of potential connexin binding proteins [12–18] whose functions are currently being elucidated.

Abbreviations: Cx, connexin; ER, endoplasmic reticulum; GJ, gap junction; GJIC, gap junction mediated intercellular communication; TM, trans-membrane

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In mammals, gap junction channels are composed of two hemi-channels, termed connexons, each provided by one of the two neighboring cells. Although a "gap" is left between the adjacent cell membranes, two connexons interact and dock in the extra-cellular space to form a tightly sealed, double-membrane intercellular channel [19,20]. Structural analyses have shown that each connexon is composed of 6 polytopic trans-membrane protein subunits, termed connexins (Cx) [19,21–26]. Connexins are encoded by a large gene family predicted to comprise at least 20 isoforms in humans. All represent structurally conserved non-glycosylated trans-membrane proteins 25 to 62 kDa in size that differ chiefly in the length of their C-terminal domain. Based on amino acid similarities, connexins have been classified into subgroups, with α and β being the major subgroups [27]. With so many different gap junction channel subunit isoforms expressed it is clear that the biosynthesis of gap junctions, their structural composition, and their degradation have to be regulated precisely for gap junctions to function properly. Exciting recent results from several laboratories including the authors'—especially obtained by studying gap junctions in living cells—provide new insights into these fundamental processes of gap junction biology. Here we will concentrate on providing an updated view of previously reviewed issues of gap junction biosynthesis and degradation [28–31], discuss novel aspects and findings, and draw comparisons to other membrane proteins.

1.1. Connexin polypeptide biosynthesis

The different membrane compartments of a eukaryotic cell require a sophisticated machinery to synthesize and sort membrane proteins to the appropriate targets and balance rates of delivery and removal. This machinery has its roots in simple prokaryotes, and common principles of membrane protein translocation have been characterized in all organisms [32–34]. In eukaryotes, trans-membrane proteins (like secretory proteins) are synthesized by ribosomes that are bound to the endoplasmic reticulum (ER) membrane. They encode hydrophobic domains (at their N-terminus or further downstream in the polypeptide sequence) that are recognized by a signal recognition particle (SRP). SRP binding is required for docking of the SRP/ribosome/nascent-polypeptide-chain/mRNA complex to a protein-channel in the ER membrane (Sec 61 complex, or Translocon) [35–37]. Translation of the nascent chain then proceeds until protein synthesis is complete. Surprisingly, even membrane proteins that encode large hydrophobic domains, such as connexins, initially are confined to the hydrophilic lumen of the translocon channel [38–41]. How these hydrophobic domains are stabilized in the hydrophilic channel environment is not yet clear. Once complete, secretory proteins are released into the lumen of the ER, while membrane proteins are somehow translocated out of the channel lumen into the hydrophobic ER membrane environment [42]. Many channel proteins, including connexins, have charged residues within their hydrophobic trans-membrane regions that might be shielded from the hydrophobic bilayer environment through oligomerization.

Trans-membrane channels, pumps, and receptors in general are built from membrane proteins that traverse the membrane several times (polytopic) (for a review see Ref. [43]). Examples of channel-proteins whose N-termini face the lumen of the ER include the members of the ligand gated ion channel super-family (acetylcholine, glycine, GABA, and glutamate receptor subunits). Those whose N-termini face the cytoplasm include the voltage-gated ion channels (K^+; Na^+-channel subunits), water channels (aquaporins), synaptophysin, as well as the connexins. N-glycosylation mutagenesis, site-specific antibody binding studies, and protease protection assays demonstrated that connexin polypeptides assume a final, functional trans-membrane topology of four trans-membrane spanning domains (TM1 to TM4), two extracellular loops (E1 and E2), and cytoplasmically located amino- and carboxyl termini. This trans-membrane topology is achieved during integration into the ER membrane [44,45]. Studies from several laboratories including our own [44–46] have demonstrated that connexin biosynthesis and membrane translocation, in general, conforms with the pathway that has been described for membrane proteins with cytoplasmically located N-termini (Fig. 1, step 1).

However, additional factors appear to be involved in connexin membrane insertion. The cell-free translation/microsomal membrane translocation systems in which these experiments were performed, permit 'signal peptidase' (SPase) to access cryptic cleavage sites at the end of the first trans-membrane spanning domain that probably functions as internal SA sequence in connexins [44,45]. Membrane proteins that are oriented with their N-terminus facing the cytoplasm, such as connexins, use internal trans-membrane segments for SRP-binding, targeting and anchoring of the proteins in the membrane bilayer (signal anchor, SA sequences). Internal SA sequences are structurally similar to N-terminal signal peptides (SP) of secretory and membrane proteins that are oriented with their N-terminus facing the ER-lumen. Only N-terminal SPs are cleaved from pre-proteins by the ER lumenal protease SPase during or shortly after translocation while internal SA sequences normally are left untouched (see Refs. [47–49] for reviews). Thus, cryptic cleavage that occurred very efficiently with all tested connexins in cell-free translation assays, and to some extend in over-expressing cells in culture [44], indicates that additional factors are required for successful connexin membrane integration. In intact cells, chaperones that bind to connexins in the ER might prevent SPase from accessing the cryptic sites, possibly by accurately positioning the connexin trans-membrane regions within the membrane bilayer.

Evidence obtained in the Evans, and Nicholson laboratories suggests that Cx26 may insert in addition post-translationally into ER membranes [46,50,51], a feature attributed to its small size (226 amino acid residues). However, the experiments did not show unequivocally whether the poly-

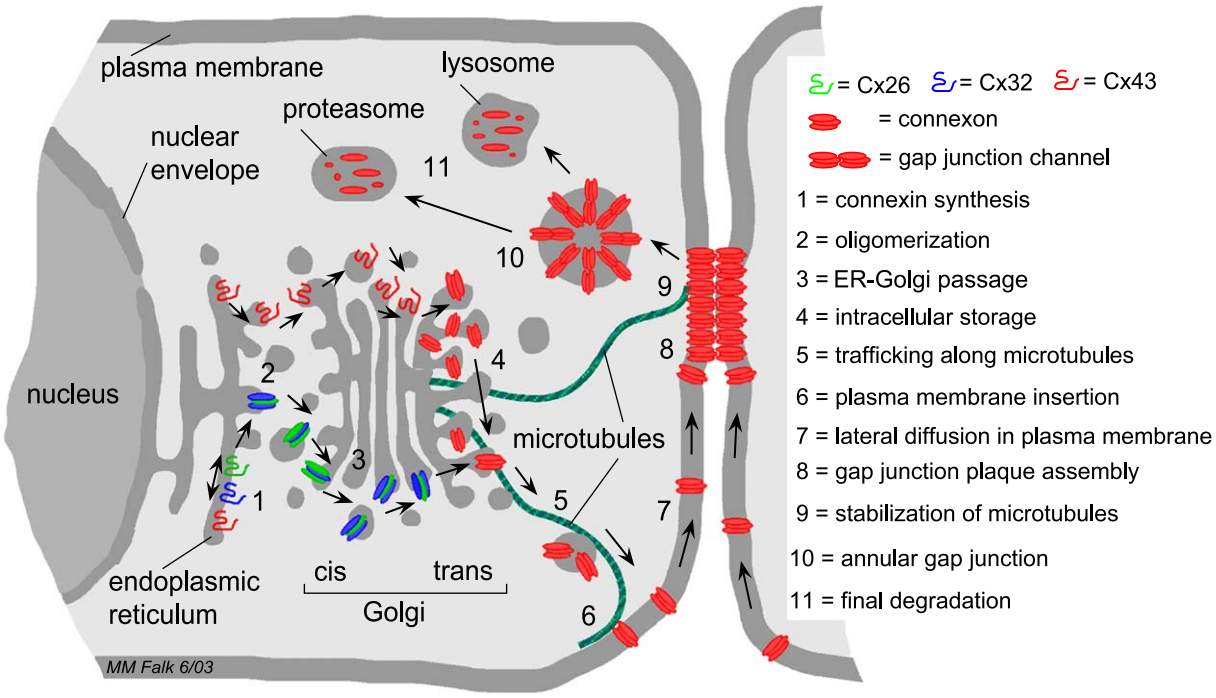


Fig. 1. Schematic representation of the steps that lead to synthesis, assembly, and degradation of gap junction membrane channels based on the current literature. Gap junction biosynthesis and degradation involves (1) synthesis of connexin polypeptides at endoplasmic reticulum membranes, (2) oligomerization into homo- and heteromeric gap junction connexons (hemi-channels), (3) passage through the Golgi stacks, (4) intracellular storage within trans Golgi membranes, (5) trafficking along microtubules, (6) insertion of connexons into the plasma membrane, (7) lateral diffusion of connexons in the plasma membrane, (8) aggregation of individual gap junction channels into plaques, (9) stabilization of peripheral microtubule plus-ends by binding to Cx43-based gap junctions, (10) internalization of the channel plaque leading to cytoplasmic annular junctions, and (11) complete degradation via lysosomal and proteasomal pathways.

peptides indeed inserted post-translationally, or simply bound to the membrane. Due to its short size, the overall hydrophobic character of Cx26 is higher than that of other connexins and thus makes this connexin isoform especially likely to bind to hydrophobic surfaces. Protease protection assays using proteases with low specificity, such as trypsin or proteinase K, might further clarify this issue.

1.2. Connexin polypeptide interactions and assembly into oligomeric connexons

Since gap junction channels like other membrane channels are oligomeric, connexin subunits must assemble before they can function. In membrane channels, four, five, or six subunits often assemble into the channel structure, with a total of 24 trans-membrane domains as a common building plan. Channels assembled from several subunits can either be homo-oligomeric (composed of identical subunits, such as certain K^+-channels), hetero-oligomeric (composed of different subunit isoforms, such as the acetylcholine receptors), or a mixture of both, as observed with gap junction channels (see below).

Assembly of oligomeric protein structures requires that first compatible subunits must recognize each other. Such subunit interactions have been investigated extensively for different ion channels. As with gap junction channels, many different ligand- and voltage-gated ion channels can oligomerize from cloned subunits into many different channel subtypes. However, the possible combinations of subunit isoforms far exceeds the actual number of different channel subtypes that are normally assembled, indicating that subunit assembly is highly regulated. Voltage-gated K^+-channels, for example, are assembled from four subunits [52]. Many different subunits can assemble into homo-oligomeric channels. In addition, certain combinations of K^+-channel proteins can co-assemble and form hetero-oligomeric channels with distinct properties, offering a possible mechanism for further diversity. The *Shaker, Shab, Shaw*, and *Shal* K^+-channels in *Drosophila* and their homologous in other species form four subfamilies, each sharing about 70% amino acid identity in the hydrophobic core region. These can co-assemble to form functional heteromeric channels, whereas members of different subfamilies, with only about 40% amino acid identity, do not. Specific structural elements have been identified that both mediate K^+-channel subunit interactions and determine the compatibility between different K^+-channel polypeptides. These elements are found in the hydrophilic amino terminal domain of K^+-channel subunits [53–56].

Acetylcholine receptors (AChRs) are pentamers that normally are assembled from four different subunits whose stoichiometry is $\alpha_2\beta\gamma\delta$. The receptor is assembled by a step-

wise pathway in which the first stage is the formation of αδ and αγ hetero-dimers. The hetero-dimers then interact with the β subunit and with each other to form the AchR (reviewed in Refs. [43,57]). Thus, during assembly, the α subunit must distinguish between the γ or δ subunits, with which it forms a dimer, and the β subunit, with which it does not. Again, specific amino acid residues in the hydrophilic amino terminal domain of the subunits were identified that regulate this subunit assembly [58–60].

Although each connexin isoform exhibits a distinct tissue distribution, many cell types express more than one connexin isoform. This makes it possible to assemble hetero-oligomeric connexons constructed from different connexin isoforms, in addition to homo-oligomeric connexons constructed from single connexin isoforms, and to considerably increase the theoretical number of different gap junction channel types [25,61–66].

When we examined the oligomerization behavior of different connexin isoforms in vitro, we found that not all connexins could participate in the formation of hetero-oligomeric connexons [25]. This observation prompted us to suggest that connexin isoform interaction is selective, restricting the possible kinds of hetero-oligomeric connexons [25]. Indeed, all hetero-oligomeric connexons reported to date are composed of two members of the same subgroup. For instance, Cx43 has been shown to hetero-oligomerize with Cx37 [61], Cx40 [62,67,68], and Cx46 [69,70] (all α-types), but not with Cx32 [25,70] (a β-type). Furthermore, Cx46 has been reported to hetero-oligomerize with Cx50 [63] (both α-types), while Cx32 can hetero-oligomerize with Cx26 [65,71] (both β-types). Although, we do not know why so many different channel types might be permitted, it appears possible that they are required to fulfill the many different specific needs of the various cell types in which they are expressed. This assumption is supported by an elegant set of experiments that used different gap junction permeable molecules to show that the specificity of gap junction channels towards size, charge, and other characteristics of permeate molecules is influenced by their connexin-subunit composition [72–76].

In addition, results obtained with ‘knock-out’ and ‘knock-in’ mice, as well as functional expression studies show that although different connexin isoforms share some common functions, at the same time they also have unique functions.

- For example, knock-in mouse lines in which the Cx43 gene was replaced with Cx32 or Cx40 coding regions rescued the lethality of Cx43-deficient mice; however, they had distinct morphological and functional defects that were different from each other, and from the defects observed in Cx43 knock-out mice [77].
- In a second example, Cx50 can take over the role of Cx46 in cataract prevention, but is required for normal ocular growth [78].
- Third, while Cx45 appears to be essential for coordinated heart muscle contraction, Cx43 appears to be required during heart development for progenitor cells to migrate out of the neural crest (reviewed in Ref. [79]).
- Fourth, mice with double knock-out of both Cx37 and Cx40 have severe vascular defects while only minor vascular abnormalities appear in single Cx37, or Cx40 knock-outs [80].
- Fifth, a recent study by Deans et al. [81] has shown that all neuronal pathways from rods to ON-type ganglion cells require Cx36, and thus this connexin is essential for night vision.
- Sixth, lentivirus-mediated expression of different connexin isoforms in pancreatic β-cells showed that adequate levels of coupling via Cx36 channels is required for the secretion of physiological amounts of insulin [82].

1.3. Signals that regulate connexin recognition and oligomerization

A puzzling question emerges from the findings described above: how is selective compatibility between different connexin isoforms achieved? Several mechanisms are possible. (1) Different connexin isoforms may be synthesized in different regions of the ER, and thus, will not get into physical contact to allow their hetero-oligomerization. (2) Specific chaperones may bind to particular connexins and prevent their interaction. (3) In analogy to ion channels, specific signals encoded in the connexin polypeptides may regulate connexin interactions, and allow hetero-oligomerization of only compatible isoforms.

Results from our laboratory suggest that hetero-oligomerization might be based on intrinsic signals that are specific to the connexin isoform itself. These results are based on co-immunoprecipitation of full-length and progressively truncated Cx43, Cx32, and Cx26 polypeptides [25,83]. Results suggest that a principal *“assembly” signal* that allows connexin subunits to recognize each other might be located in the C-terminal portion (preferentially third trans-membrane spanning domain TM3) of the connexin polypeptides, while a *“selectivity” signal* regulating subunit compatibility might be located in the amino terminal portion (NH_2-terminal, first trans-membrane, and/or first extracellular domain). These signals might be manifested in specific stretches of amino acid residues that differ among connexin isoforms; resulting in a different surface structure of protein/protein interfaces, and thus preventing the interaction of isoforms with differently folded motifs (=incompatible connexins), but enabling interaction of connexins with similarly folded motifs (=compatible connexins).

The structure of such protein/protein interfaces has been described in several oligomeric proteins. Protein surfaces involved in subunit contacts differ from the rest of the subunit surface; they are enriched in hydrophobic side chains, yet contain a number of charged groups, especially from arginine along with leucine which is the most abun-

dant residue at interfaces ([52,84], reviewed in Refs. [85–88]). Thus, the connexin trans-membrane (TM) spanning domains are most likely involved in such signals, since they are hydrophobic and contact the trans-membrane domains of the neighboring subunit within the connexon [19]. Interestingly, TM1 of connexins also bears a charged arginine residue in its center that plays an important role in trans-membrane positioning of TM1 [45], and thus makes this domain and its flanking domains (the N-terminus and the first extracellular loop) the prime candidates to harbor such selectivity signals. The hypothesis is supported by the identification of a number of amino acid residues located within this region that seem to play an important role in connexin subunit compatibility. Exchange of these residues results in dominant, and trans-dominant inhibitory effects of the variants on co-expressed connexins that might be based on a direct interaction and oligomerization of non-functional mutant connexin subunits with wild-type connexins [89–91] (see below).

Recently, we began to align and compare connexin sequences using the CLUSTAL W algorithm of the OMIGA sequence analysis package (Oxford Molecular Group, Inc., Oxford, UK). We characterized four amino acid positions, 11 and 12 in the N-terminal domain, and 152 and 153 in TM3 (counting corresponds to the Cx43 sequence), where the physico-chemical characteristics between α and β connexins differ, while physico-chemical characteristics within the subclasses are conserved [92]. Substitution of each of these residues in Cx43 (an α-type) with the corresponding residues of Cx32 (a β-type) resulted in the assembly of all variants into gap junction channels and plaques at cell–cell appositions, however, only the fourth variant was functional as indicated by lucifer yellow dye transfer assays. The other three variants exerted a moderate to severe dose-dependent, dominant negative (on the same connexin isoform) effect on co-expressed wild-type Cx43 channel activity. Moreover, a significant dose-dependent, trans-dominant (on a different connexin isoform) inhibition on a β-connexin, Cx32, was observed when either one of the N-terminal variants, but not the TM3 variants, was co-expressed with this connexin isoform. Assembly analyses indicated that dominant and trans-dominant inhibitory effects appear to be based on the oligomerization of wild-type and variant connexins into mixed connexons that renders the resulting gap junction channels non-functional [92]. However, additional amino acid residues located downstream in the first extracellular domain (E42, W44, D66, R75) have been characterized in Cx26 disease variants that also have a dominant inhibitory effect on co-expressed wild-type connexins [89–91], and trans-membrane domain 3 has been proposed to be involved in the hetero-oligomerization of Cx32 [93]. Thus, different signals, different structural motifs, or probably more likely, compound structural motifs, that involve different segments of the connexin polypeptides might regulate recognition and co-oligomerization of different connexin isoforms.

Interestingly, the sites we identified in the N-termini correlate with a number of point mutations in disease-linked β-connexins (Cx26, Cx30, Cx31) that appear to be responsible for their disease phenotype [94–96]. Based on the trans-dominant inhibitory effect of the Cx43 P1 and P2 amino acid exchange variants and the trans-dominant Cx26 variants described above, it is tempting to speculate whether an aberrant hetero-oligomerization between co-expressed mutant and wild-type α and β-connexins might lead to an altered activity of these errant channels and thus might lead to certain disease phenotypes in tissues in which incompatible connexin isoforms are co-expressed.

1.4. Intracellular location of connexon assembly

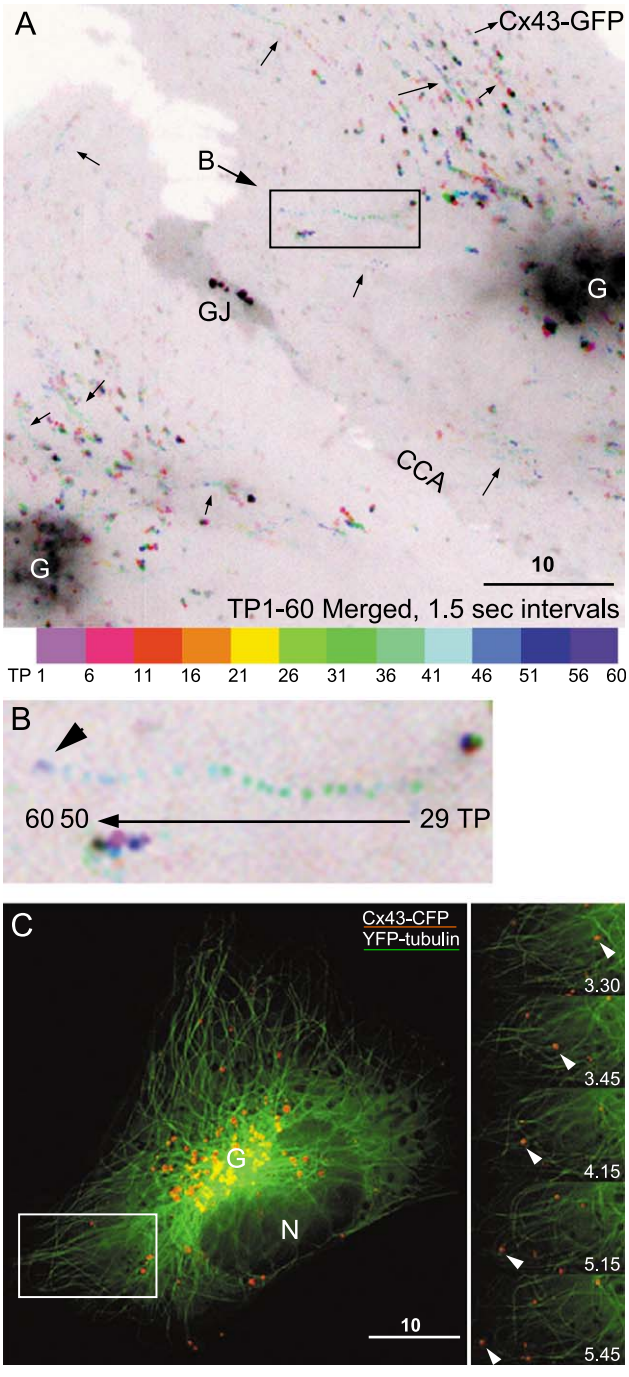
Most known oligomeric membrane proteins, including voltage- and ligand-gated ion channels, whose structure is comparable to connexons, assemble in the ER (reviewed in Ref. [43]). Moreover, for many oligomeric membrane proteins, assembly in the ER is a necessary prerequisite for further trafficking and delivery to the plasma membrane [97,98].

The intracellular location where connexins assemble has been debated. Using an integrated biochemical and biophysical analysis, we and others have observed functional assembly of gap junction connexons composed of Cx43 or Cx32 in microsomes after translation/membrane translocation in a cell-free translation system [25,50] (Fig. 1, step 2) suggesting that connexin oligomerization occurs within ER membranes. However, using cultured cells, Musil and Goodenough [22] obtained evidence for assembly of connexons after exit from the ER, probably in the trans-Golgi network (TGN), while Diez et al. [99] obtained evidence for assembly in the ER-Golgi-intermediate compartment (ERGIC) (reviewed in Ref. [100]). Recently, using genetically engineered connexins that encode ER-retention signals, Koval's [101] laboratory obtained evidence that the place of connexon assembly might be connexin isoform-specific with Cx32 assembling in the ER/ERGIC and Cx43 assembling in the TGN.

1.5. Trafficking to the cell surface

Trafficking to the cell surface is normally accommodated by the budding of membrane vesicles from the ER that contain the polypeptides as cargo, and fusion of the vesicles with subsequent intracellular membrane compartments, e.g. the Golgi stacks, the TGN, and finally the plasma membrane (PM). The membrane vesicles shuttle back to their membrane compartment from which they originated [102]. Subcellular fractionation, immunoprecipitation, and immuno-colocalization with antibodies directed against subcellular compartment marker proteins were performed in various tissues [44,103,104], as well as tissue culture cell lines expressing endogenous [105,106], or recombinant connexins [107–110]. These studies repeatedly detected

connexin polypeptides in the ER, and in Golgi membranes, indicating that the connexins are transported by successive vesicle budding and fusion from the ER through the Golgi stacks, following the general intracellular transport route referred to as the secretory pathway [102,111] (Fig. 1, steps 1–4). Furthermore, no gap junction channel assembly or gap junction plaque formation was observed in cells that were treated with drugs known to interfere with the secretory pathway, such as brefeldin A (BFA), or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), or that were kept at non-permissive temperature [22,103,106].



To investigate how connexins are trafficked to the plasma membrane and gap junctions (Fig. 1, steps 5–7), Lauf et al. [110] have studied delivery of connexons assembled from GFP-tagged Cx43 in transfected living HeLa cells. Multicolor time-lapse microscopy revealed that connexons were delivered in vesicular carriers that traveled along microtubules from the Golgi to the plasma membrane. Most of the post-Golgi cargo containers were round and had a diameter of no more than 200 nm. Occasionally, elongated cargo containers were observed to exit the Golgi. Cx-containing transport containers traveled by saltatory, predominantly directional motion along curvilinear tracks that followed microtubules extending to the plasma membrane. Movements were fast, averaging 0.5±0.22 μm/s (min=0.2 μm/s, max=1.05 μm/s, *n*=80) (Fig. 2). Post-Golgi trafficking along microtubules and similar constitutive secretory vesicle characteristics were described recently for monomeric membrane proteins with only one transmembrane segment [112,113].

Martin et al. [114] reported that Cx26 and other connexins can traffic in a microtubule independent, not yet defined pathway to the plasma membrane. It is known that membranes of the rough ER can be located very close to the plasma membrane [115], and we have seen rough ER membranes reaching gap junctions in Cx43-GFP transfected HeLa cells by immunofluorescence staining, and by ultrastructural analysis (Fig. 3). Under these conditions, connexons assembled in the rER might be able to transfer directly into the plasma membrane. Furthermore, Golgi to plasma membrane secretion has been observed even in cells in which microtubules were depolymerized, providing evidence that diffusion-based secretion can occur with reasonable efficiency over short distances (reviewed in Refs. [116,117]). Thus, these observations might explain why connexins can be delivered to the plasma membrane, even under conditions were microtubules and Golgi membranes have been disrupted. Intact actin filaments have also been observed to be involved in the plasma membrane delivery, especially of Cx26. [118].

Fig. 2. Transport of gap junction hemi-channels (connexons) to the plasma membrane. (A) HeLa cells transfected with Cx43-GFP were imaged in the early phase of gap junction assembly by rapid time-lapse microscopy. Many vesicular, and occasionally tubular transport containers containing Cx43-GFP exited the Golgi (G) and were transported in all directions, predominantly distant from gap junctions (GJ) and cell–cell appositions (CCA), into the periphery of the cells. Trails (depicted with arrows) and directional movement of constitutive cargo vesicles was visualized by inverting black and white, color-coding and merging the images of the time-lapse recording. (B) Track of a single constitutive vesicle traveling 11 μm in 21 s from time point (TP) 29–50. Preceding fusion, the vesicle becomes tethered (marked with an arrowhead) and only moves locally between TP 50–60. (C) To visualize that trafficking occurs along microtubules HeLa cells were transfected with Cx43-CFP (red) and YFP-tubulin (green). Vesicular constitutive carriers, as well as larger degradative vesicular structures associated closely with, and moved along microtubules away from the Golgi (G). A slower migrating Cx43-GFP carrier (marked with arrowhead) traveling up to 0.58 μm/s is tracked. N=nucleus. Bars=μm.

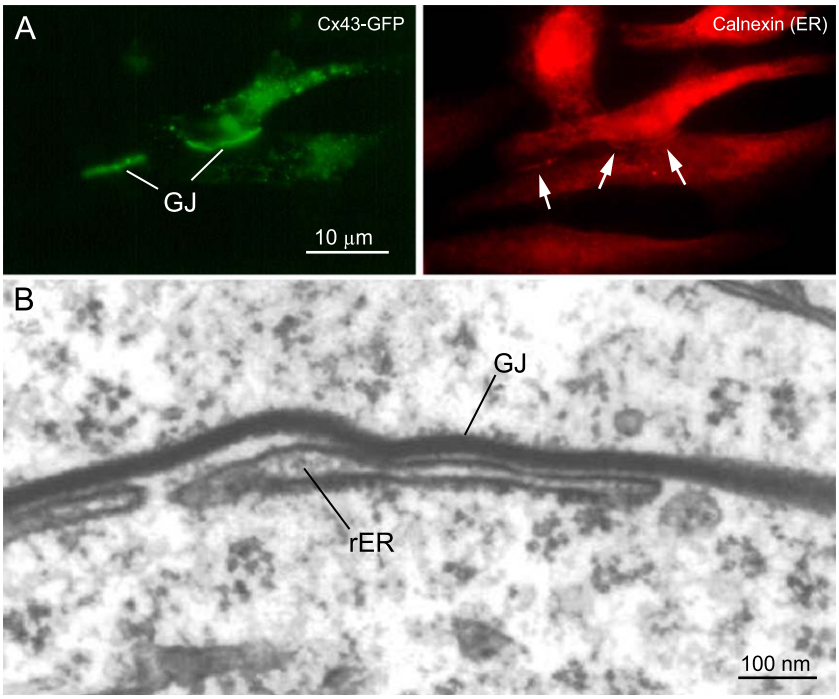


Fig. 3. Rough endoplasmic reticulum (rER) membranes can localize at gap junctions. (A) Immunofluorescence analysis of HeLa cells transfected with Cx43-GFP. rER was visualized with staining for the rER-resident marker protein calnexin (red). Calnexin staining at gap junctions (GJ) is highlighted with arrows. (B) Ultrastructural analysis of Cx43-GFP transfected HeLa cells. Note the close association of rER and gap junctions.

Paulson et al. [119] found that trafficking of Cx43 from intracellular storage sites followed by an enhanced assembly of gap junctions can be triggered by treatment of cells with cyclic AMP-elevating reagents or low density lipoprotein (LDL).

1.6. Insertion of connexons into the plasma membrane

Lauf et al. [110] found that routing and insertion of Cx43 connexons in non-polarized HeLa cells was distributed over the entire non-junctional plasma membrane surface (Fig. 1, step 6 and Fig. 2). This is consistent with (I) the delivery of a number of other membrane proteins including aquaporin-1 [120], vesicular stomatitis virus G (VSVG) protein [112,121,122], and glycosyl-phosphatidylinositol (GPI)-anchored proteins [113], (II) freeze-fracture electron microscopic studies that provided evidence for the insertion of gap junction intramembrane particles into the plasmalemma by fusion of particle-bearing cytoplasmic vesicle couriers [123], and (III) the characterization of functional gap junction hemi-channels in the plasma membrane (see below). Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss In Photo-bleaching (FLIP) experiments indicated that plasma membrane connexons can move laterally in the plane of the membrane, consistent with the movement of proteins diffusing in cellular membranes [124–127], and thus, can reach the outer margins of gap junction plaques (Fig. 1, step 7). The fast FRAP and FLIP kinetics, as well as the homogeneity of the recovered fluorescence indicate that connexons are distributed as single particles, or small groups, but not as large aggregates, however, exact diffusion constants for connexons moving laterally in the plasma membrane have not been measured yet.

Delivery of connexons via the non-junctional plasma membrane instead of routing them directly to gap junctions might be unexpected, especially since distal ends of microtubules can anchor directly at Cx43-based gap junctions [14] (Fig. 4). A 35-amino acid juxta-membrane region, which contains a presumptive tubulin binding motif that is necessary and sufficient for microtubule binding was characterized in the C-terminal tail of Cx43. The role of this interaction is not yet clear, but increasing evidence indicates that microtubule dynamics in the plasma membrane periphery is reduced in cells with well established cell–cell contacts when compared to microtubule dynamics in migrating cells [128]. A yet-unidentified microtubule "plus end" capping protein has been proposed to reside in the plasma membrane [128]. If Cx43 were this capping protein it could, in addition to its role as a channel-forming protein, function as a microtubule-anchoring protein [14] (Fig. 1, step 9).

1.7. Function of hemi-channels in the plasma membrane

Once delivered to the plasma membrane connexons can function in intra-/extracellular signaling. Indeed, functional gap junction hemi-channels have been known for some years [129–132]. But a number of recent reports now

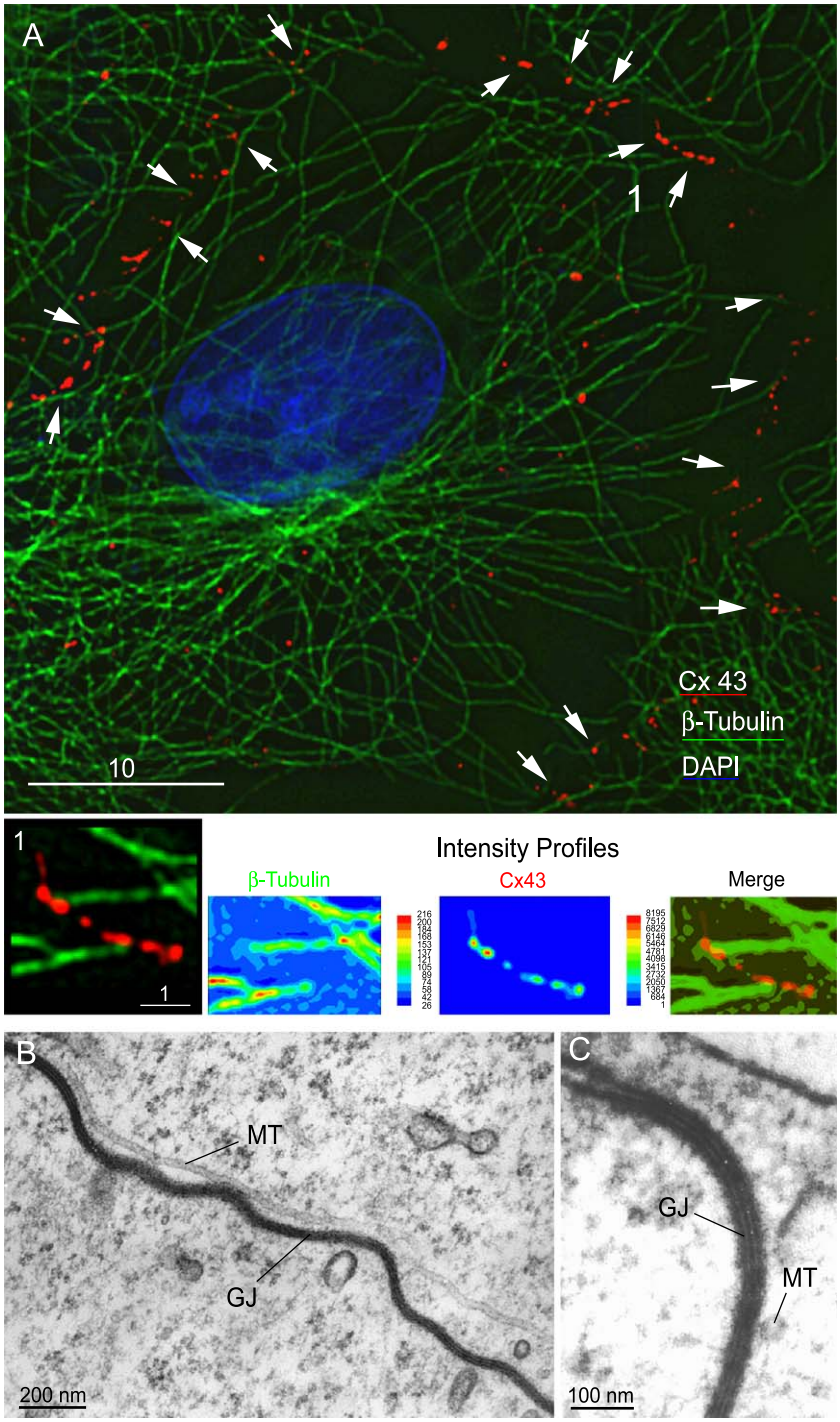


Fig. 4. Cx43 and microtubule plus-ends co-localize at cell–cell contacts. (A) Sub-confluent rat liver epithelial T51B cells were stained for endogenous Cx43 (red), tubulin (green), and chromatin (blue). Microtubules extend to and terminate at the plasma membrane in regions where gap junctions are present (marked with arrows). Fluorescence-intensity profiles of β-tubulin, and Cx43 staining are shown for one region (1). Note the yellow areas in the Merge indicating direct interaction at microtubule/gap junction contact points. Bars=μm. (B/C) Ultrastructural analysis of HeLa cells expressing Cx43-GFP. Microtubules (MT) reaching gap junctions (GJ) (longitudinal in B), and in cross-section (in C) are clearly visible.

describe that they function as independent entities between intra- and extracellular milieus. Suggested functions are diverse and include isosmotic cell volume regulation [133], inhibition of the activity of Ca^{2+}-channels and subsequent glutamate release at synapses located between horizontal cells and cones in the outer retina [134], promotion of or rescue from apoptosis [135,136], regulation of glutamate and aspartate release in astrocytes [137,138], and differentiation of teratocarcinoma progenitor cells into neuronal and nonneuronal cells ([139], reviewed in Refs. [140–

142]). Recently, gating and regulation of Cx43 hemi-channels was examined. Gating properties resembled those of the corresponding Cx43 cell–cell channels, however, open probabilities appeared to be very low under physiological conditions [137]. Gap junction hemi-channel activity was inhibited by well known gap junction channel blockers, such as carbenoxolone, octanol, heptanol, 18-α-glycyrrhetinic acid, multivalent cations, and the non-specific chloride channel inhibitor flufenamic acid, but not by the chloride channel-specific inhibitor 4,4′ -diisothiocyanatostilbene-2,2′ -disulfonic acid [133–139]. Thus, delivery of connexons to the non-junctional plasma membrane might provide a simple, two-step mechanism that would allow connexons to function first in intra-/extracellular signaling, and then in direct cell–cell communication. However, it has not yet been shown whether the same hemi-channels delivered to the plasma membrane can be used for both functions. Inhibition of both structures by identical inhibitors, however, might provide some evidence for this assumption. In many electron microscopic images of gap junction freeze-fracture replicas that were obtained from tissues, intermembraneous particles, which could be interpreted as individual connexons, can be seen dispersed around gap junction plaques [143,144]. They may reflect the pool of dispersed connexons in the plasma membrane and the accretion of channels to the plaque that Lauf et al. [110], and Gaietta et al. [145] have observed in living cells.

1.8. Docking of connexons into double-membrane spanning gap junction channels

Docking of connexons delivered to the plasma membrane is enabled, or at least facilitated by calcium-dependent cell-adhesion molecules, probably by bringing the membranes close enough together to initiate connexon interaction. This is indicated by thin-section electron microscopic images of gap junctions that show the plasma membranes within the plaque to be spaced much closer together than outside the plaques, leaving only a characteristic 30-nm-wide gap. In S180 cells, which can biosynthesize Cx43 but fails to assemble gap junctions, gap junction formation could be induced by transfecting the cells with L-CAM [146]. Fab' fragments derived from antibodies directed against L-CAM could be used to disassemble gap junctions [146]. Jongen et al. [147] reported calcium-dependent regulation of gap junction mediated intercellular communication (GJIC) by E-cadherin, and monovalent antibodies to N-cadherin were found to inhibit gap junction assembly in re-aggregating Novikoff cells [148]. Fujimoto et al. [149] suggested that adherens junctions (cadherin–catenin complex-mediated cell-to-cell contact sites) act as foci for gap junction formation. Recently, Wu et al. [150] reported evidence for the formation of a catenin/ZO-1/Cx43 complex in rat cardiomyocytes and showed that binding of catenins to the membrane-associated guanylate kinase protein, zonula occludens-1 (ZO-1), is required for Cx43 transport to the plasma membrane during the assembly of gap junctions. Other roles of ZO-1 in gap junction biosynthesis and degradation are suggested as well [151,152] (discussed below).

Structural investigations by cryo-electron microscopy revealed that the extracellular loops of apposed connexins interdigitate in the extracellular space to form the tight seal between the two connexons. This results in a 30° staggered arrangement of the two connexons [19,20]. The most notable feature of the extracellular loops is that each contains three strictly conserved cysteine residues that are essential for normal folding and channel function. Shifting four of the six conserved cysteines of Cx32, whether individually or in combination, suggested that three intramolecular disulfide bridges connect the first cysteine in E1 with the third cysteine in E2, and the third cysteine in E1 with the first cysteine in E2, consistent with a model in which the extracellular loops fold as anti-parallel β sheets [153].

As an additional complication in gap junction channel architecture, besides homo-typic channels, hetero-typic channels composed of two different connexon types can be assembled between adjacent cells that express different connexin isoforms [154]. Signals that regulate hetero-typic docking of different connexons have been identified mainly in the extracellular loops ([155,156] reviewed in Ref. [157]).

1.9. Aggregation of gap junction channels into plaques

Ultrastructural analyses revealed that gap junction channels aggregate to create characteristic two-dimensional arrays of channels, termed plaques, that are structurally distinct from other clustered arrays of particles also present in the plasma membrane [158–160]. Typically a single plaque may contain from less than a dozen to many thousand individual channels and it can extend from less than a hundred nanometers to several micrometers in diameter [158,161].

Johnson and coworkers [162,163] have used freeze-fracture electron microscopic and electro-physiological methods to study the beginning steps of gap junction plaque formation (see above for factors enabling connexon-docking). They used Novikoff hepatoma cells that were dissociated with EDTA and allowed to re-aggregate for 5–180 min in the presence of calcium. Their results suggest that the formation of gap junctions appears in stages with the following order: first, flattened membrane regions with loosely clustered groupings of 9- to 11-nm intramembranous particles (probably undocked connexons) were seen that were termed "formation plaques." Second, a reduction of the extracellular space between matched formation plaques in adjacent cell membranes occurs, probably resulting from the docking of opposed connexons. Third, the 9- to 11-nm particles aggregate into tightly adherent arrays which are indistinguishable from small gap junctions; and fourth, growth of the small gap junctions, probably by addition of individual particles and fusion of small aggregates.

Two studies have recently investigated the turnover of channels in established gap junction plaques. By permanently photo-bleaching the GFP-fluorescence of square areas of Cx43-GFP gap junctions, or by tagging Cx43 with tetracysteine motif-encoding peptides and successive labeling with biarsenic fluorophores of different colors (FlAsH and ReAsH), Lauf et al. [110] and Gaietta et al. [145] recently demonstrated that newly synthesized channels are added along the outer margins of gap junction plaques (reviewed in Ref. [164]) (Fig. 1, step 8). In the study by Lauf et al. [110] newly added channels were recognizable by their GFP fluorescence, which formed fluorescent lines outlining the older, bleached plaque areas (Fig. 5). Fluorescence re-appeared homogeneously throughout the entire length of the bleached areas as a solid, fine line that steadily increased in width, indicating that connexons were added as single particles, or in very small groups, consistent with the homogeneous distribution of connexons in the plasma membrane, and electron microscopic observations by Johnson et al. [162,163] (described above). In the Gaietta et al. [145] study, older channels in the center of the plaques were recognizable by their green FlAsH label, while newer channels, recognizable by their red ReAsH label, formed a ring around the central, green-labeled channels.

The size of the investigated plaques remained relatively constant over the observation periods, indicating that gap junction plaques had reached a steady-state in which removal of older, photo-bleached channels in the center, and accrual of newly synthesized channels to the plaque margins was in balance [110]. Successive replacement of channels was directly visible with FlAsH-labeled gap junctions that were re-labeled after increasing time periods with ReAsH. In these plaques the size of the central green channel areas decreased in correlation with increased ReAsH incubation periods [145]. In the Lauf et al. [110] experiments, the recovered stripe of channels reached a width of up to 0.5 μm within 1 h, indicating that up to 50 layers of channels were added, if channels have the predicted center-to-center spacing of ~10 nm [19]. These kinetics mean that a 1 μm^2 gap junction, which consists of about 10,000 channels could be assembled within 2 h. This is somewhat faster than the replenishment observed by Gaietta et al. [145] who found that a 0.5–1.5 μm wide border was added within 4 h. The difference in channel accrual in the two studies is probably due to a higher protein synthesis rate in the Lauf et al. study that also resulted in a larger plaque size.

To investigate whether channel accrual depended on delivery of connexons to the plasma membrane, Lauf et al. [110] inhibited secretion of newly synthesized connexons by disrupting microtubules with nocodazole, or by treating the cells with BFA prior to photo-bleaching. BFA disrupts the Golgi apparatus by vesiculating the Golgi membranes that then rapidly re-distribute into the ER [165,166]. Under both conditions, plaques initially recovered fluorescence along their margins within 10–20 min, but further recovery was inhibited. This suggests that a pool of newly synthesized connexons is present in plasma membranes that can accrue to the edge of plaques, but further connexon accrual requires delivery of newly synthesized connexons once the plasma membrane connexon pool becomes depleted. These results are in agreement with studies performed by Johnson et al. [167], which demonstrated that initial gap junction plaque assembly can occur in the presence of drugs that

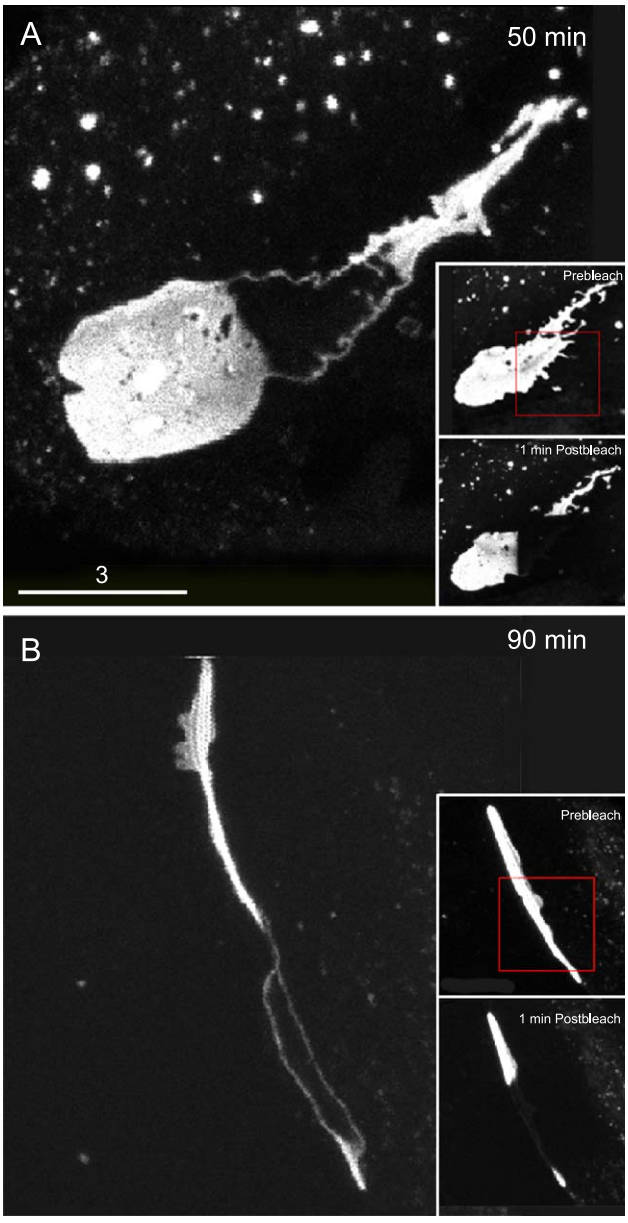


Fig. 5. Newly synthesized gap junction channels assemble along the outer edge of gap junctions. Fluorescence of selected areas (boxed) of gap junctions assembled from Cx43-GFP in transfected HeLa cells was permanently photo-bleached and recruitment of newly synthesized channels was observed over time (viewed on the plane in A, and on the edge in B). Newly accrued channels are visible by their unbleached fluorescence forming a continuous line along the outer margin of the bleached channel plaque in the images taken 50 and 90 min post-bleaching. Images of the gap junctions directly before, and 1 min after photo-bleaching are shown in the inserts. Bar=μm.

disrupt actin filaments (cytochalasin B), or microtubules (colchicine, nocodazole), respectively; however, further Cx43 channel accrual requires intact microtubules.

1.10. Why do gap junction channels aggregate?

Although, our knowledge of the organization of aggregated membrane proteins has increased over the past years, our knowledge of the signals and mechanisms that mediate their aggregation is still rather poor. Two principal mechanisms that are likely to occur in nature are feasible. In one, the proteins have a high intrinsic affinity for each other and thus stay together. In the other, the trans-membrane proteins are initially spread over the membrane and in response to specific signals, the proteins are brought and held together by extrinsic forces. Clustering of integrins, for example, and recruitment of associated proteins seems to be initiated upon ligand binding only [168], and neuromuscular AChRs cluster upon interaction with the AchR-associated protein rapsin, a process that appears to be regulated by agrin, a key nerve-derived synaptogenic factor that is activated by the small GTPases Rac and Rho [169,170].

Recordings of gap junctions tracked over time in living cells showed that gap junction plaques are not assembled in a rigid, para-crystalline arrangement, rather they show a dynamic arrangement permitting lateral movements in the plane of the membrane that can result in plaque fusion and splitting, and dynamic rearrangement of channels within the plaques [164,171,172] (Fig. 6). When gap junction plaques moved closer together by drifting laterally in the plane of the membrane, plaques were observed to suddenly fuse over their entire length (Fig. 6A), while splitting of plaques occurred more slowly and included the formation of a channel "bridge" that connected the plaque domains. The width of the connecting bridge progressively decreased as the domains moved further apart, and finally split (Fig. 6B), supporting the hypothesis that gap junction channels might have some intrinsic affinity for themselves that cohesively aggregates them, and that energy is required for their separation. From a statistical analysis of the positions of gap junction channels in plaques seen on freeze-fracture micrographs, Braun et al. [173], concluded that aggregates are maintained by the minimization of the repulsive force between apposed membranes that holds the membranes at a distance of only about 30 nm within the plaque, and thus traps the gap junction channels within this area.

1.11. Structural composition of gap junction channel plaques

Localization of different connexin isoforms within ultrastructurally defined gap junction plaques has been reported [174–177]. When we co-expressed different combinations of the connexin isoforms Cx26, Cx32, and Cx43 in cells in culture, we found that the co-expressed connexins gathered within the same plaque. Furthermore in same cases, connexin isoforms co-distributed homogenously (Cx26 with Cx32, both β-types), while in others they segregated into well-separated domains (Cx26 with Cx43, and Cx32 with Cx43, one α and one β type) [109] (Fig. 7). Observing GJ plaques in living cells over time showed that segregation was stable, although the channels themselves were dynamically arranged and could move throughout the plaques [171] (Fig. 6C).

How is such a segregation of channels within a GJ plaque possible, and what could be the underlying mechanisms? One can postulate that connexins that are segregated within a plaque are assembled into homomeric channels, and connexins that mix within a plaque are assembled into heteromeric channels. If this hypothesis is correct, only two types of signals would be required to regulate channel assembly and plaque composition: (1) a signal that regulates subunit compatibility and allows limited hetero-oligomerization (as discussed above), and (2) a signal that keeps different homomeric channels of a plaque segregated (additional signals that regulate docking of compatible con-

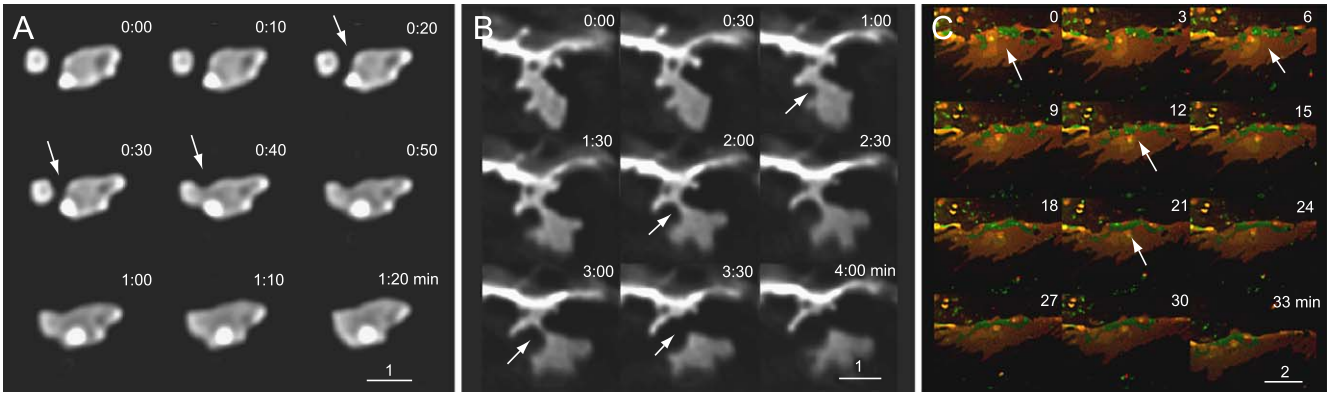
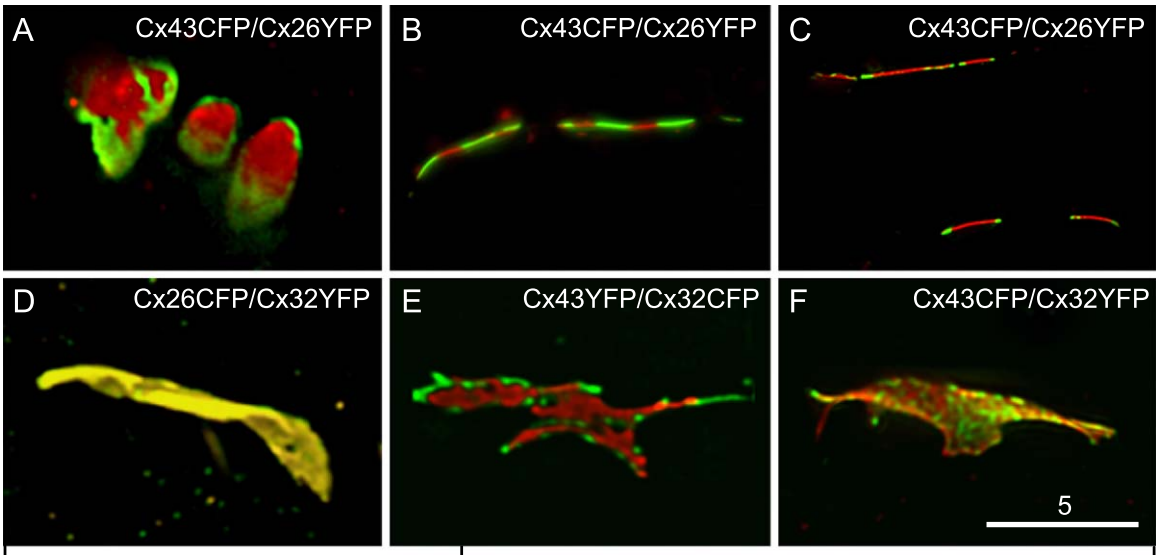


Fig. 6. Gap junctions and gap junction channels are arranged dynamically. Time-lapse microscopy of gap junctions assembled from Cx43-GFP (A, B), and Cx43-CFP (green) and Cx26-YFP (red) (C) in transfected HeLa cells revealed that gap junction channels are arranged mobile. Plaque fusion (in A), plaque splitting (in B), and lateral flow of channels in the plane of the membranes (in C) were observed that lead to dynamic gap junction re-arrangements. Note how in (C) domains consisting of Cx43-CFP fuse and split, but never mix with the Cx26-YFP containing domains. Bars=μm.

nexons into heterotypic gap junction channels have been described above). Mixing and segregation requires that the affinity among similar channels is greater than the affinity among dissimilar channels. Thus, mixing and segregation could either be based on structural motifs encoded by the connexin amino acid sequences (as discussed for connexin oligomerization compatibility), different affinities for surrounding lipids, or even involvement of connexin-binding proteins (see below).

The proteins of gap junction channels are surrounded by lipids, and early evidence suggested that cholesterol, common in eukaryotic membranes, is enriched in gap junction plaques (reviewed in Ref. [178]). A few recent reports describe a specific association of various connexins (Cx43, Cx32, Cx36, and Cx46) with lipid rafts, specialized membrane domains enriched in cholesterol, sphingolipids, and signaling proteins [179], and co-localization with the lipid raft marker-protein caveolin-1 [180,181]. Cx43-association with lipid rafts might be regulated by protein kinase Cγ that stimulates interaction after activation with the phorbol ester TPA, or insulin-like growth factor (IGF)-1 [182]. Although, the reports differ with respect to whether the associated connexin is phosphorylated or non-phosphorylated or aggregated into plaques, it appears that different connexins have different preferences for certain lipid environments and that these preferences could be involved in channel aggregation, mixing, and segregation. Furthermore, it will be interesting to find out whether "formation



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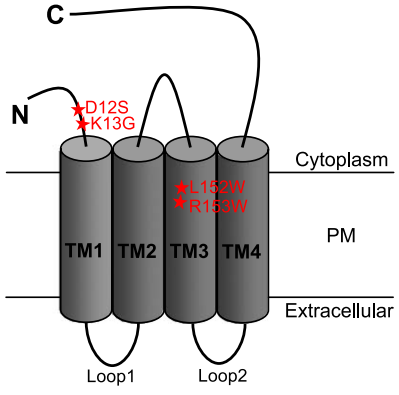

Table1. Amino acid properties of α and β connexins on discriminatory positions

Location	αConnexin	βConnexin
P1 (N-terminal tail; aa 12 in Cx43)	Negatively charged D,E	Small, non-charged S, G
P2 (N-terminal tail; aa 13 in Cx43)	Polar K, N, Q, E	Small, non-charged G
P3 (cytoplasmic site of TM3; aa 152 in Cx43)	Non aromatic L, M	Aromatic W
P4 (cytoplasmic site of TM3; aa 153 in Cx43)	Non aromatic R, G, N	Aromatic W

Fig. 7. Structural composition of gap junctions assembled from two different connexins and characterization of distinct amino acid residues discriminating α and β connexins. (A–F) Connexins were co-expressed in transfected HeLa cells. Gap junctions viewed on the plane surface (in A, D–F), and on the edge (in B, C) are shown. Co-expressed connexins assembled together within the same plaques. Co-expression of an α (Cx43), and a β-type (Cx26, Cx32) resulted in connexin segregation (A–C, E, F), while co-expression of two β-types (Cx32 and Cx26) resulted in homogenous mixing (D). Bar=5 μm. (G) Trans-membrane topology of connexins, location, and mutagenesis of identified discriminatory amino acid residues D12S (P1), and K13G (P2) in the N-terminal domain, and L152W (P3), and R153W (P4) in TM3.

plaques'' characterized earlier by Johnson et al. [162,163] (discussed above) and connexin/lipid raft associations are related structures.

1.12. Gap junction removal and degradation

The half-life of connexins, in general ranges from about 1 to 5 h both in vivo [183,184], and in vitro [106,146,185–189], a surprisingly short period for a structural protein that forms a channel that can be opened and closed by gating. Gap junction degradation is further complicated by the observation that gap junction channels, once formed, cannot be separated again into hemi-channels under physiological conditions [190,191]. In early electron microscopic studies, vesicular double-membrane gap junction-like structures termed annular junctions or gap junction profiles were identified in the cytoplasm of cultured cells and in tissues [192–194] (Fig. 8). These annular junctions were later shown by immuno-gold labeling of connexins to consist of densely packed gap junction channels [195–197]. It has been suggested that annular junctions might be early degradation products of internalized gap junctions that are generated by the invagination, restriction, pinching off, and transport of both junctional membranes into the cytoplasm of one of the apposed cells for further degradation [192] (Fig. 1, step 10). Clathrin and actin filaments might be involved in this process [192], and thus gap junction internalization may use a comparable machinery to that of classical endocytosis. Performing time-lapse microscopy of GFP-tagged connexin43 (Cx43-GFP) and micro-injection of Cx43-specific antibodies, Jordan et al. [198] provided direct evidence that fragments of GJ plaques are internalized as annular junctions into one of the two contacting cells.

However, internalization of larger portions or entire gap junctions for degradation does not conform with recent observations of Lauf et al. [110] and Gaietta et al. [145] on gap junction channel turnover. They demonstrate that newly synthesized channels are accrued along the outer edge of existing plaques, while older channels are simultaneously released from the plaque center (discussed above).

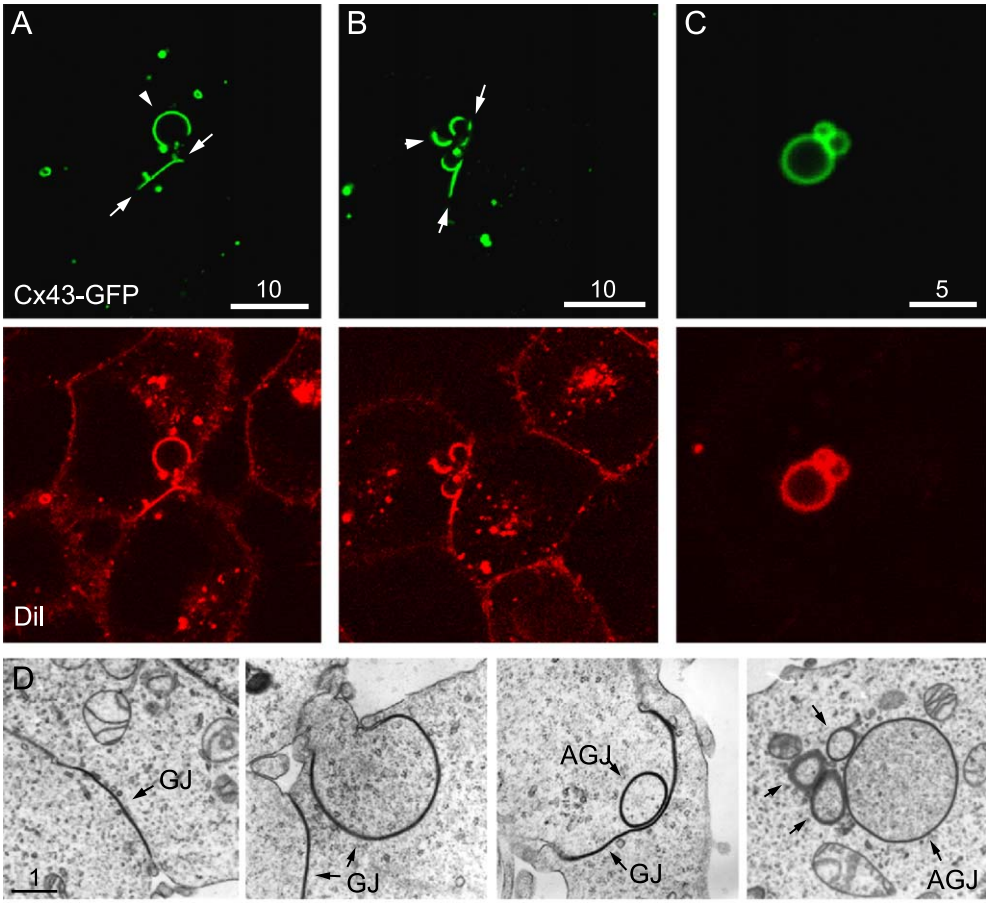

Fig. 8. Removal of gap junctions from the plasma membrane and their transition into cytoplasmic, early degradation products, termed ''annular junctions''. Gap junctions can be removed from the plasma membrane by the invagination of the entire, or large portions of the double-membrane channel plaque into the cytoplasm of one of the two adjacent cells, restriction, and pinching. This process generates the vesicular, intracellular annular junctions. (A–C) Confocal sectioning reveals the vesicular structure of the annular junctions (labeled with arrowheads). Portions of gap junctions remaining in the plasma membranes are labeled with arrows. Staining membranes with a lipophilic dye, DiI, reveals the lipid content of gap and annular junctions. (D) Ultrastructural analysis reveals the double-membrane structure of gap junctions (GJ) and annular junctions (AGJ). Bars=μm.

Therefore, an additional mechanism appears to exist that continuously removes channels from the plaques. However, such an additional GJ channel degradation mechanism has not yet been characterized.

Once internalized, further gap junction degradation appears to involve both lysosomal and proteasomal degradation pathways [198–203] (reviewed in Refs. [29,204]) (Fig. 1, step 11). Recent reports suggest that GJIC might be regulated via stabilization or removal of gap junctions from the plasma membrane, but exact mechanisms have not yet been elucidated. Qin et al. [205] obtained evidence that both pathways play distinct roles in the life cycle of Cx43, with secretory forms of Cx43 and internalized gap junctions being degraded by lysosomes, while active proteasomal degradation was found to destabilize phosphorylated gap junctions at the plasma membrane. Musil et al. [187] could demonstrate that reducing connexin degradation with inhibitors of the proteasome was associated with a striking increase in gap junction assembly at the plasma membrane, providing evidence that GJIC can be up-regulated at the level of connexin turnover. Phosphorylation of connexin 43 by several different kinases (protein kinase C (PKC), extracellular signal-regulated kinases (ERK), casein kinase 1 (CK1)) have been described to stimulate gap junction removal from the plasma membrane [180,206–208]. Girao and Pereira [209] showed that phosphorylation of Cx43 stimulates proteasome-dependent degradation of this protein in lens epithelial cells. Thalmann et al. [210] propose that connexin 26 homeostasis in the cochlea is regulated by the balance between the Wingless/Wnt signaling pathway that activates connexin expression and OCP1, an organ-of-Corti-specific ubiquitin ligase which promotes its degradation. Thomas et al. [211] recently characterized a tyrosine-based sorting signal in the C-terminal domain of Cx43 that appears to be a prime determinant of Cx43 stability, and consequently GJIC, by targeting Cx43 for degradation in the endocytic/lysosomal compartment. Finally, an increased association of ZO-1 with Cx43 after remodeling of myocyte intercellular contacts has been described that suggests a possible role of ZO-1 in gap junction turnover during cardiac development and disease processes [151,152].

1.13. Concluding remarks

It is clear that gap junction biosynthesis and degradation is a complex and highly regulated process. From biosynthesis of the connexin subunit proteins on ER membranes to oligomerization of compatible subunits into hexameric connexons (hemi-channels), delivery of the connexons to the plasma membrane, head-on docking of compatible connexons in the extracellular space at distinct locations, arrangement of channels into dynamic spatially and temporally organized gap junction channel plaques, and coordinated removal of channels into the cytoplasm followed by their intracellular degradation, all steps are interdependent and subject to regulation. Although, the function of existing gap junction channels can be controlled by opening and closing of the channels, regulated delivery, assembly, and removal appears to be a second independent mechanism to control GJIC. A number of secondary proteins will undoubtedly be found that aid multiple steps of this process. Recent approaches that used yeast-two hybrid screens, GST-pull-down assays, antibody arrays, and proteomic analysis have identified quite a number of potential connexin binding proteins [12–18] ranging from chaperones, to scaffolding proteins, kinases, phosphatases, other membrane channels, membrane receptors, cell signaling molecules, and structural proteins. The challenge of the coming years will be to identify those proteins that play a primary role in regulating gap junction channel biosynthesis and degradation, to describe the steps of this process, and to understand why such a continuous synthesis and removal of gap junction channels at cell–cell appositions is evolutionarily and physiologically desirable.

Acknowledgements

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