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Pathways and control of connexin oligomerization

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Connexins form gap junction channels that link neighboring cells into an intercellular communication network. Many cells that express multiple connexins produce heteromeric channels containing at least two connexins, which provides a means to fine tune gap junctional communication. Formation of channels by multiple connexins is controlled at two levels: by inherent structural compatibilities that enable connexins to hetero-oligomerize and by cellular mechanisms that restrict the formation of heteromers by otherwise compatible connexins. Here, I discuss roles for secretory compartments beyond the endoplasmic reticulum in connexin oligomerization and evidence that suggests that membrane microdomains help regulate connexin trafficking and assembly.

Introduction

Gap junctions interconnect cells by forming a direct link to enable the diffusion of small aqueous molecules and ions from one cell to its nearest neighbor [1]. This enables the flow of specific intercellular signals and metabolic cooperation between communicating cells in a tissue. The importance of such communication is shown by the increasing number of human diseases that are directly attributable to mutants and deficiencies of the gap junction protein, connexin [2]. Mutations range from those that subtly change gap junctional permeability [3] to mutations that cause severe changes in connexin trafficking and that completely inhibit gap junctional coupling [4–7].

Connexins are a multigene family of transmembrane proteins [8] (Figure 1). Each connexin forms a channel with unique permeability characteristics, which is reflected in the types of metabolite and signaling molecule that flow through it [9]. A complete channel is formed from two hexameric hemichannels, one in each cell, that meet at the cell surface (Figure 2). Gap junction channels are organized into higher order semicrystalline arrays, known as plaques. In addition, connexin hemichannels function as bona fide plasma membrane channels that enable the diffusion of ATP and other aqueous molecules from the cytosol to the extracellular environment [10].

Connexins span the membrane bilayer four times with the N and C termini oriented towards the cytoplasm. The four connexin transmembrane domains are largely α helical [11]. Fleishman *et al.* [12] used a best-fit algorithm to assign the transmembrane domains of connexin 32 (Cx32) mathematically to the corresponding α helices in the structural model (Figure 2). On the basis of their model, the best fit was obtained by assigning the third transmembrane domain as the predominant pore-lining helix [12], although this is controversial [13].

Different types of cell express different connexins and cells frequently express two or more connexins [14–16]. Because an individual gap junction channel is composed of 12 connexins, cells that express multiple connexins can produce mixed channels, provided that the connexins are able to hetero-oligomerize. The formation of gap junction channels by two or more connexins enables cells to produce channels that have unique permeability and gating characteristics that could not be obtained using a single connexin [17]. However, the rules that govern connexin oligomerization and compatibility are complex.

Determinants of connexin compatibility

Innate heteromeric compatibility

Figure 2 shows the different classes of channel that can be formed when cells express two or more connexins. Connexins do not ubiquitously intermix; instead, compatibility is based on their protein structure. When two connexins expressed in the same cell form a mixed channel, this is referred to as heteromeric compatibility. Examples of heteromeric channels formed by endogenously expressed connexins include Cx32–Cx26 in the liver [15,18], Cx46–Cx50 in the lens [19] and Cx43–Cx46 in the lung [20]. Heteromeric connexin compatibility has been tested using transfected cell models, although there are many combinations that have not been examined. The heteromeric compatibility groups correspond loosely to α and β connexin subfamiles [15,17]. Connexins that do not belong in either of these subgroups have been found to form heteromers with either α connexins (e.g. Cx45–Cx43) [21] or β connexins (e.g. mCx29–Cx32) [22]. To date, no connexin has been identified that forms normal heteromeric channels with both α and β subfamily connexins.

Motifs that dictate innate heteromeric compatibility have been studied most extensively using two incompatible connexins, Cx32 and Cx43. A series of truncation and point mutants that alter connexin heteromeric specificity showed that the motifs that prevent hetero-oligomerization of Cx32 and Cx43 include pairs of amino acids in the N

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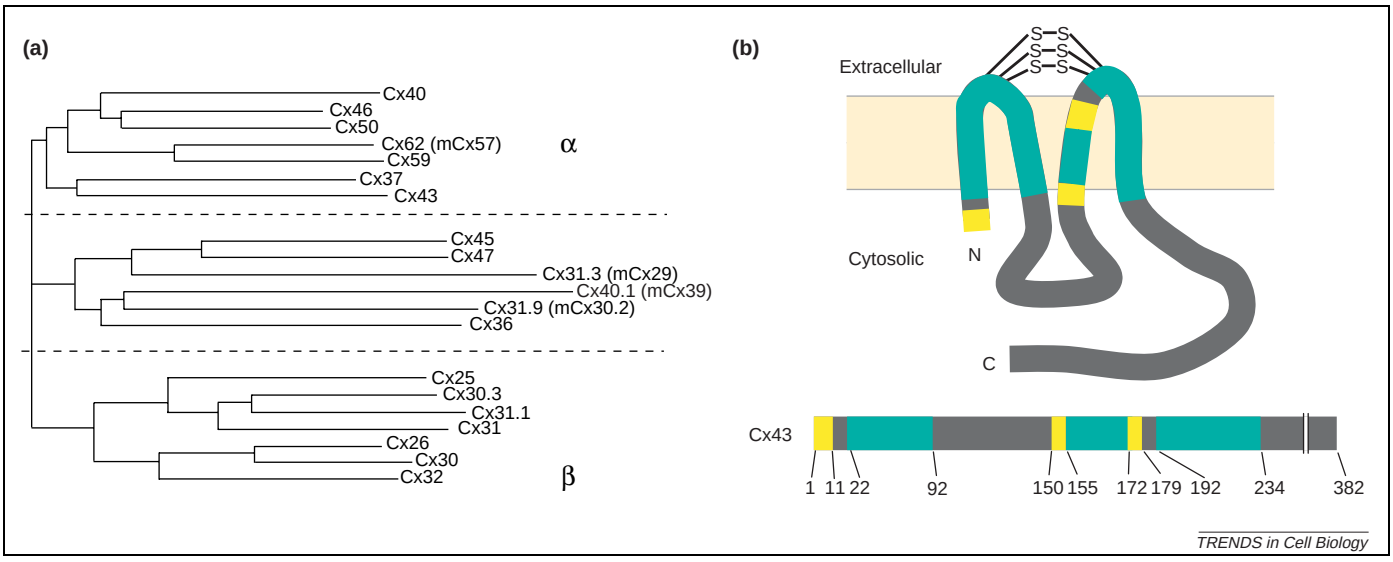

Figure 1. Connexin homology and structure. **(a)** Phylogram of 20 human connexins calculated using ClustalW and omitting C-terminal domains [85]. By protein homology, connexins form two major subgroups, α and β, with an additional group of connexins with intermediate homology [8]. Different connexins are denoted by Cx plus a number corresponding to the predicted molecular mass based on the amino acid sequence. Mouse connexin names that differ from their human orthologs are shown in parentheses*. **(b)** Line diagram corresponding to an individual connexin. The two extracellular loop domains are interconnected by three disulfide bridges. Green denotes regions with high amino acid sequence homology within the entire connexin protein family, yellow denotes regions in which amino acids are homologous within a subset of connexins and gray denotes divergent regions of connexins that vary in amino acid sequence and size. Numbers correspond to positions of Cx43 amino acids that define different classes of connexin homology domains.

terminus and at the cytosolic end of the third transmembrane domain [23].

An aberrant interaction between α and β connexins can have pathologic consequences. For example, although Cx26 and Cx43 are normally incompatible [24], some Cx26 mutants that are associated with the skin disorder palmoplantar keratoderma have a dominant-negative effect on Cx43, which is probably due to formation of an aberrant heteromer [25]. However, other Cx26 mutations that are associated with non-syndromic deafness are more specifically limited to the disruption of Cx26 and other β connexins, indicating that different mutations in the same connexin can cause different human diseases.

Innate heterotypic compatibility

A gap junction channel formed by a head-to-head interaction between two different connexins is known as a heterotypic channel. Heterotypic channels have been identified in several settings *in vivo*, most notably in the central nervous system (CNS), in which astrocytes and oligodendrocytes are interconnected exclusively through heterotypic channels [26,27]. Although such gap junctions can contain channels with unique permeability characteristics, a more prominent role for specific heterotypic interactions is to form intercellular networks by either promoting or restricting cell interconnectivity. Disulfide bridges that interconnect the two extracellular domains are crucial for channel formation [28,29]. Two key amino acids in the second extracellular loop are the major determinants of heterotypic compatibility [15,30], although motifs in the first extracellular loop and other parts of the protein might also have a role [31]. In contrast to innate heteromeric compatibility, innate heterotypic compatibility does not correlate well with whether a given connexin is an α or β family member [15,32]. For example, Cx32 and Cx43 cannot form heterotypic channels; however, the α connexins Cx46 and Cx50 can form heterotypic channels with either Cx32 or Cx43 [30]. In addition, some connexins, including Cx31 and Cx36, appear to only form homotypic channels [32,33].

Regulated connexin compatibility

Although connexins have structural determinants that define their ability to form heteromeric channels, there are several examples showing that this ability is also dependant on the context of cell expression. For example, two compatible connexins, Cx43 and Cx46, are expressed by type I alveolar epithelial cells and form heteromeric channels [20]. However, these same connexins, when expressed by type II alveolar epithelial cells or osteoblastic cells, do not hetero-oligomerize (Figure 3) [20,34,35]. Thus, formation of heteromeric Cx43–Cx46 channels depends on cell type and is a regulated process.

Another example of regulated connexin targeting is the myoendothelial junction between vascular endothelium and smooth muscle cells. These cells express different levels of Cx37, Cx40 and Cx43, all of which can hetero-oligomerize with one another; this has been demonstrated in transfected cell models [17]. Using a co-culture model, in which the cells maintained polarity and phenotype, Isakson and Duling [36] found that Cx37 was specifically excluded from myoendothelial junctions between

* At the 2005 International Gap Junction Conference, a proposal to develop a revised nomenclature to unify connexin naming between species was approved, but has not been finalized. Changes to the connexin nomenclature will be published elsewhere and posted to the Human Genome Organization website (http://www.gene.ucl.ac.uk/nomenclature/).

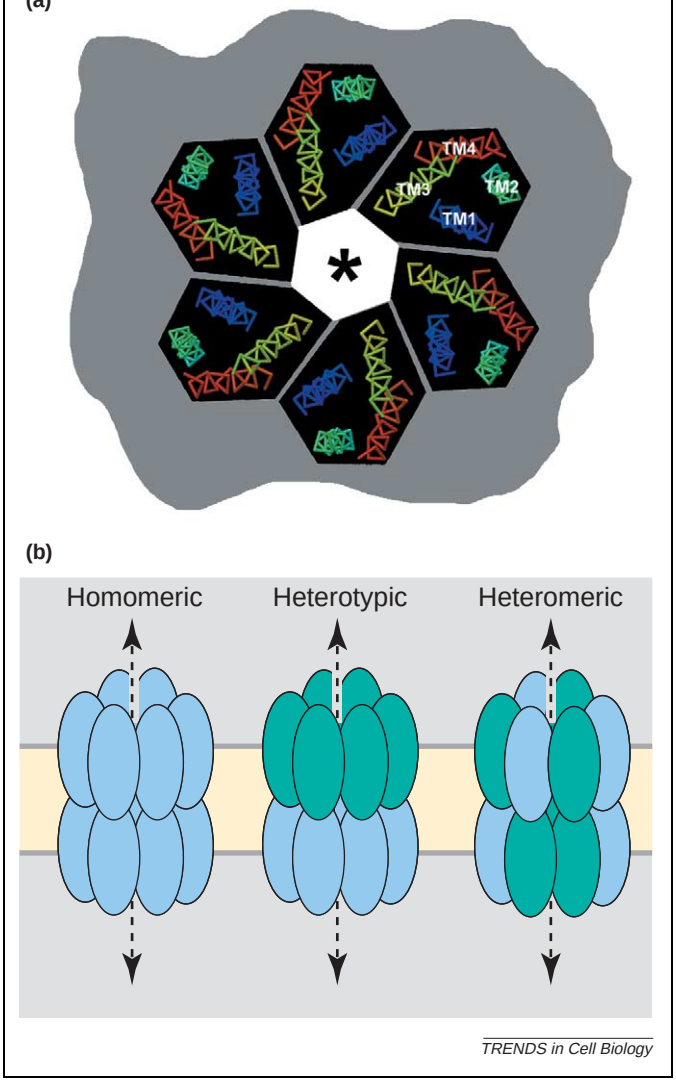

Figure 2. Gap junction channels. **(a)** *En face* view of a connexin hemichannel, showing only the transmembrane helical domains as viewed from the outside of the cell looking into the permeability pore (*). The image was generated with RasMol using coordinates from [12]. In this model, transmembrane (TM) domains correspond to TM1 (blue), TM2 (aqua), TM3 (yellow) and TM4 (red). **(b)** Types of gap junction channel. Ovals represent individual connexins and the dashed line indicates the path of the aqueous portion of the channel. Shown from left to right are a homomeric gap junction channel composed of a single type of connexin, a heterotypic channel composed of two different connexins expressed by two adjacent cells and a heteromeric channel composed of two different connexins co-expressed in the same cell.

endothelial cells and smooth muscle cells. Instead, Cx37 was incorporated into junctions interconnecting either two adjacent endothelial cells or two adjacent smooth muscle cells. This suggests that the vasculature regulates connexin hetero-oligomerization and uses polarized delivery of connexins to different plasma membrane domains to produce three distinct classes of cell–cell interface that influence intercellular signaling pathways. Polarized connexin targeting also has a role in regulating heteromer formation in the CNS [22,26,27] and oocyte–granulosa cell interfaces [37,38]. The mechanisms to control connexin hetero-oligomerization and trafficking are not well defined at present. In particular, connexin trafficking in cultured polarized cell models must be studied to define roles for

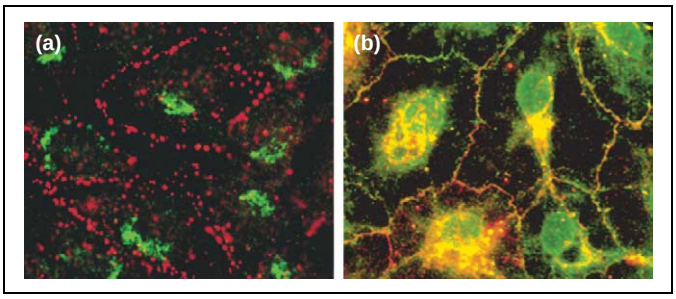

Figure 3. Cellular control of connexin oligomerization. Immunofluorescence images of **(a)** osteoblastic cells and **(b)** type I alveolar epithelial cells are shown, both of which express endogenous Cx43 (red) and Cx46 (green). (a) Osteoblastic cells prevent Cx43 and Cx46 from intermixing. Only Cx43 is targeted by these cells to assemble into gap junctions at the plasma membrane (red). However, Cx46 is retained by osteoblastic cells in the TGN (green perinuclear fluorescence). (b) By contrast, type I alveolar epithelial cells show co-localization of Cx43 (red) and Cx46 (green) to produce yellow fluorescence. Formation of Cx43–Cx46 heteromers was demonstrated by co-immunopurification. Adapted, with permission, from [20]. Scale bar = 10 μm.

differential connexin targeting to apical and basolateral secretory pathways involved in regulating the formation of heteromeric and heterotypic gap junction channels.

Connexin oligomerization after the endoplasmic reticulum

Connexins do not follow the classical pathway for transmembrane protein oligomerization when forming hexamers. Similar to most channel-forming proteins [39], connexins oligomerize into a multimeric complex before delivery to the plasma membrane. However, Cx43 oligomerizes into hexamers after exit from the endoplasmic reticulum (ER) in a secretory compartment distal to the medial Golgi, which is probably the trans Golgi network (TGN) [35,40] (Figure 4). This is consistent with the notion of a quality-control system in the Golgi apparatus [41,42] that complements ER quality-control pathways [43]. However, identifying specific components of the quality-control apparatus that regulates connexin oligomerization has been difficult.

This difficulty is partly due to different systems giving apparently conflicting results. For example, several connexins, including Cx26, Cx32 and Cx43, oligomerize when translated *in vitro* in the presence of microsomes [18,44,45]. Although this has been cited as evidence that connexin oligomerization naturally occurs in the ER, another possibility is that microsomes lack components of the quality-control apparatus that are present in the intact ER and that inhibit connexin oligomerization. Consistent with this, addition of liver Golgi membranes to the *in vitro* translation microsome mix enhances Cx32 oligomerization and promotes the formation of Cx26–Cx32 heteromers [18]. Also, the ER pool of Cx32 in guinea pig liver is monomeric, although the ER pool of Cx26 does show partial oligomerization [46]. Factors provided by Golgi membranes that promote connexin oligomerization remain to be identified.

Cells do not have an unlimited capacity for quality control of protein folding and assembly. This is a particular problem when analyzing the assembly of connexins transfected into cells because overexpression could drive premature connexin oligomerization.

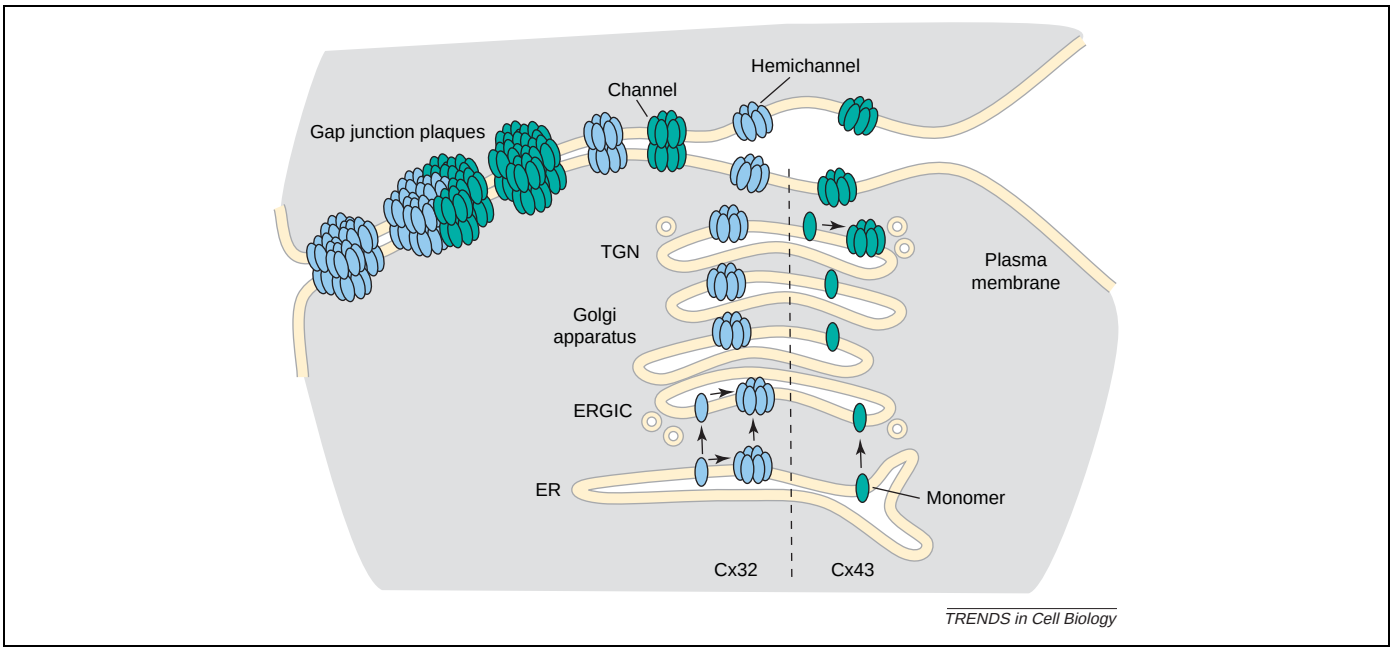

Figure 4. Connexin oligomerization pathways. Connexins are co-translationally inserted into the ER membrane. Depending on the connexin subtype, oligomerization can occur either in the ERGIC (Cx32, blue) or the TGN (Cx43, green). The potential for connexin oligomerization in the ER, driven by high levels of connexin expression, is also shown for Cx32. Hemichannels are subsequently transported to the plasma membrane, where they can function as channels or pair with hemichannels on adjacent cells to form complete intercellular channels. Channels at the plasma membrane further assemble into semi-crystalline arrays known as gap junction plaques, which can contain from tens to thousands of channels. Homogenous plaques are composed of either a single connexin or heteromeric connexins (not shown). Heterogeneous plaques contain regions enriched for different connexins.

For example, overexpression initiates the formation of intracellular plaque-like structures in the ER of transfected BHK cells suggesting aberrant connexin assembly [47]. Overexpressed newly synthesized connexins, including Cx26, Cx32 and Cx43, oligomerized in the ER, whereas endogenous Cx32 and Cx43 expressed at much lower levels did not (J.K. VanSlyke and L.S. Musil, personal communication); however, transfected HeLa cells have been used to show that Cx26 oligomerizes following exit from the ER [24]. Cx43 overexpression induces premature oligomerization [48] and it has been suggested that overexpression might underlie some of the conflicting results obtained using transfected cell models to study connexin trafficking and oligomerization [7,49]. Taken together, these results suggest that care should be taken in producing and interpreting these models. Conversely, it is plausible that there might be physiological circumstances in which high levels of connexin expression might regulate hexamer formation by inducing early connexin oligomerization in the ER, although, to date, this has not been directly demonstrated in a system examining endogenously expressed connexins.

Although unusual, transmembrane protein oligomerization in a post-ER secretory compartment is not unique to connexins. For example, targeting of coronavirus coat proteins [50] and Golgi resident enzymes [51] correlates with oligomerization in the Golgi apparatus. Remodeling and assembly of ion channels, such as ENaC and Na-K ATPases, can occur in late secretory compartments and at the plasma membrane [52–54]. Another class of proteins that oligomerize in the Golgi apparatus are tetraspanins, such as CD9, CD63, CD81 and CD151 [55]. However, tetraspanins also hetero-oligomerize with several other classes of transmembrane protein, such as integrins, in the ER [56,57] (Box 1).

Di-lysine-tagged connexins as probes to define sites of oligomerization

Most studies to characterize early events in connexin oligomerization require membrane trafficking inhibitors, such as brefeldin A. Although these are useful tools for identifying early events in protein assembly, they can also alter the composition of the secretory pathway [58] and can induce stress responses that are due to the accumulation of unfolded proteins in the ER [59]. Given these potential pitfalls, connexin chimeras containing a di-lysine-based C-terminal ER retention–retrieval sequence, HKKSL, were used to study early events in connexin oligomerization [60,61]. Consistent with Cx43 oligomerization in the Golgi apparatus or TGN, ER-localized Cx43–HKKSL is monomeric. In contrast to Cx43, ER-localized Cx32–HKKSL oligomerizes. Importantly, this difference was not due to protein overexpression [61].

Given that Cx32–HKKSL that was localized to the ER was oligomerized, does Cx32 normally oligomerize in the ER? This is possible; however, although the HKKSL motif is a strong ER retention signal, it also acts as a retrieval signal for proteins that have escaped from the ER [62]. Access and retrieval of proteins containing di-lysine motifs from the ER–Golgi intermediate compartment (ERGIC) and, to a lesser extent, the *cis* Golgi stack form a retrograde recycling pathway, which contributes to quality control in the secretory pathway [63,64]. By contrast, ER retrieval of di-lysine-containing proteins from late Golgi compartments and the TGN is relatively rare [65]. Consistent with this, a small fraction of

Box 1. A tetraspanin–connexin connection?

Tetraspanins have been called molecular facilitators because of their ability to promote the assembly and trafficking of several classes of transmembrane proteins [86,87]. Although tetraspanins and connexins both span the bilayer four times and have similar protein orientation in the bilayer, they are structurally distinct and not homologous [88].

Intermolecular interactions involving tetraspanins are complex, because tetraspanins form homologous complexes with other tetraspanins and form heterologous complexes with other transmembrane proteins (e.g. integrins or class II major histocompatibility complex proteins). Tetraspanin–protein complexes form in the ER and the Golgi apparatus. In addition to direct interactions with other proteins, tetraspanins facilitate the formation of large-scale heterogeneous membrane lipid–protein complexes. The ability of tetraspanins to form large membrane microdomains is enhanced by palmitoylation, a post-translational modification that occurs in the Golgi apparatus.

One parallel between connexins and tetraspanins is that they partition into cholesterol- and sphingolipid-enriched membrane microdomains. Given this, and the prominent role of post-ER compartments in connexin and tetraspanin oligomerization, it is tempting to speculate that the formation of higher order complexes by these protein families might share common features. Also, given that tetraspanins function as molecular facilitators in the Golgi apparatus to regulate trafficking of other classes of junction and cell adhesion proteins, it is plausible that tetraspanins might also help regulate connexin oligomerization and/or trafficking. Further work will be required to determine whether this is the case.

Cx43–HKKSL escapes from the ER retrieval pathway and forms functional gap junction channels [60]. Thus, an alternative explanation to Cx32–HKKSL oligomerization in the ER is that Cx32–HKKSL oligomerizes in a compartment such as the ERGIC, where they are efficiently retrieved by the di-lysine tag. Given this and the arguments presented here that support connexin oligomerization after exit from the ER, I propose a model that favors the ERGIC as a major intracellular compartment in which Cx32 oligomerizes (Figure 4).

Identifying the precise subcompartments involved in connexin oligomerization requires further testing and is likely to be difficult, given the complex interrelationships and architecture of the ER, ERGIC, Golgi stacks and TGN [66]. Nonetheless, the notion that different connexins can oligomerize in different intracellular compartments provides a potential mechanism for regulating whether connexins form heteromeric hemichannels, as prior oligomerization into homomers could preclude subsequent formation of heteromers. This type of control is more important for inherently compatible connexins, such as Cx37 and Cx43 or Cx43 and Cx46, as opposed to inherently incompatible connexins, such as Cx32 and Cx43. Identification of the sites of oligomerization for other connexins is required to confirm if this is the case.

Structural determinants that regulate connexin oligomerization

The third transmembrane domain and second extracellular loop are necessary and sufficient to prevent premature Cx43 oligomerization [61]. Charged amino acid residues at both membrane-interface regions of the third transmembrane domain are crucial elements of this Cx43 motif, whereas Cx32 and other β connexins have bulky hydrophobic groups at comparable positions. Intriguingly, one of the key amino acids required to regulate the site of Cx43 oligomerization, Arg153, is also crucial for the innate heteromeric incompatibility of Cx43 and Cx32 described earlier [23]. This provides a potential link that connects the relative stability of Cx43 as a monomer and the innate compatibility of Cx43 to hetero-oligomerize with other connexins.

Importantly, mutations in amino acid residues that are localized to membrane interfaces interfere with connexin trafficking and cause connexins to accumulate in the ER and Golgi apparatus [4,6,67]. Disease-related connexin mutations can interfere with oligomerization. For example, a mutant form of Cx50 that is associated with human cataract, P88S, forms cytoplasmic accumulations in the ER with partial plaque-like characteristics [68]. In addition, two mutant Cx32 proteins that are associated with Charcot-Marie-Tooth disease and do not traffic to the plasma membrane remain monomeric and are subsequently processed by the ER-associated degradation (ERAD) quality-control pathway [67].

Connexin partitioning into membrane microdomains

Increasing evidence suggests that cholesterol- and sphingolipid-enriched membrane microdomains, or lipid 'rafts' [69], might have roles in connexin trafficking and assembly. Consistent with a potential role for microdomains in regulating connexin assembly, treatment of cells with extracellular cholesterol enhances gap junction formation and intercellular communication [70]. Several connexins partition into biochemically isolated microdomains [71–75] and contain binding motifs for a microdomain-associated protein, caveolin-1 [73]. Whether connexins associate with microdomains enriched for caveolin-1 depends on the preparation technique. For example, hemichannels containing Cx32 are found in Triton X-100-insoluble microdomain fractions, whereas homomeric Cx26 hemichannels are not [73]. However, both Cx32- and Cx26-containing hemichannels are found in Brij-58-insoluble microdomain fractions [73]. Whether this reflects the *in vivo* partitioning of Cx26, Cx32 and Cx26–Cx32 heteromers into different classes of membrane microdomain remains to be determined. It also demonstrates the complexity of using different fractionation techniques to study the formation of membrane microdomains.

As caveolin-1 has been co-localized to gap junction plaques [72], it has been suggested that the plaques themselves are located in microdomains. However, accumulating evidence suggests that connexins are present in membrane microdomains before incorporation into gap junctions [71,73]. For example, connexin-containing microdomain fractions are enriched in glycosphingolipids, whereas the glycosphingolipid content of gap junction plaques is low [73]. Also, in studies of ZO-1, a connexin-binding scaffold protein [76,77], Laing *et al.* [71] showed that cells expressing a dominant-negative ZO-1 construct had an increase in microdomain-associated

Cx43, which localized to the region of the Golgi apparatus. By contrast, overexpressing wild-type functional ZO-1 enhanced Cx43 transport to the plasma membrane and enhanced gap junction plaque formation. Although this suggests that functional ZO-1 might enhance the transition of Cx43 from microdomains to gap junction plaques, ZO-1 binding is not an absolute requirement for their formation, because connexins that do not bind to ZO-1 can form plaques [73,77,78]. In fact, ZO-1 might also serve to limit plaque size [77]. In general, studies that focus strictly on binary connexin–ZO-1 interactions should be interpreted with caution. For example, the effect of mutant or wild-type ZO-1 on connexins might be indirect because these proteins might interfere with or promote the binding of other proteins to the connexin C terminus.

Although connexins can associate with membrane microdomains, further studies are needed to relate this to their oligomeric state. However, given that membrane lipids and microdomains are continually remodeled throughout the secretory pathway [79], this could be an intriguing potential modulator of connexin oligomerization. In addition, microdomains could also have a role in polarized delivery of connexins to distinct plasma membrane domains, comparable to roles for microdomains in the polarized trafficking of other classes of transmembrane protein [80].

Conclusion and perspectives

The context of connexin expression is an important factor in determining the characteristics of intercellular communication. Cells control whether compatible connexins oligomerize into heteromeric gap junction channels. Oligomerization of different connexins in different intracellular compartments is likely to have an important role in regulating heteromer formation. This implies that cell-specific organelle remodeling might help determine which types of gap junction channel are produced. Cell culture models that preserve cell phenotype and polarity will be useful to study how cells contribute to regulating connexin oligomerization.

The formation of gap junction channels is balanced by other control points that regulate plasma membrane gap junction content, including connexin phosphorylation, internalization and turnover [16,81–83]. In addition, identifying a mechanistic basis for regulated connexin oligomerization will require more detailed study of other less well-characterized connexins. Whether the mechanisms that control Cx43 and Cx32 are representative of other α and β connexins, or connexins with intermediate homology, remains to be determined. In addition, little is known about the trafficking and assembly of a newly discovered class of gap junction-forming proteins, known as mammalian pannexins, which are distinct from connexins and instead are homologous to invertebrate gap junction proteins [84].

Although significant progress has been made in identifying structural connexin motifs and organelles involved in connexin oligomerization, little is known about chaperones and other protein cofactors that are required to regulate this process. Further understanding of the molecular basis for regulation of gap junction channel formation will require the identification of components of the connexin quality-control apparatus.

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