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Review

Incorporation of connexins into plasma membranes and gap junctions

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Abstract

Gap junctions are polymeric assemblies of aligned pairs of interacting hexameric connexon hemichannel units facilitating direct intercellular communication. The principal process leading to assembly of gap junctions involves the cotranslational insertion of connexin (Cx) proteins into the endoplasmic reticulum, followed by their rapid oligomeric association into homo- or heteromeric connexons that are trafficked via the Golgi apparatus to the plasma membrane. Oligomerisation is a high-fidelity process that determines connexon channel stoichiometry and conductance characteristics. A large number of mutations in Cx26 and Cx32 detected in genetic diseases have emphasised the requirement for precise oligomerisation of connexins into hexameric connexons that traffic to the plasma membrane. Mutations in Cx43 are rare, and in the cardiovascular system, where it is the dominant connexin, disease changes are linked to its abundance and to gap junction remodelling. Connexins with short carboxyl tails may also be post-translationally inserted as oligomeric channels directly into plasma membranes. This mechanism of channel assembly is highly dependent on microtubule integrity and may allow cells to rapidly modulate gap junctional cross talk.

Keywords: Connexins; Trafficking pathways; Connexin chimeric proteins

1. Introduction

The coordination of cellular events in tissues and organs is mediated by intercellular communication across gap junctions, tightly packed clusters of channels directly connecting the cytoplasms of adjacent cells. Gap junctions are constructed of a multigene family of integral membrane proteins, the connexins (Cx), with over 20 connexin isoforms extending from 25 to 60 kDa found in human and mouse genomes [1]. Connexin 43 (Cx43) is the predominant connexin expressed in the mammalian heart; Cx45 and Cx40 are also present at lower levels at the nodal-crista terminalis border [2] and in the atrium [3], respectively. Gap junction channels connecting cardiac myocytes are mainly interspersed among macula and fascia adherens junctions in the intercalated disc of the cardiac myocytes plasma membrane [4]. A low-resistance intercellular pathway for the conduction of electrical impulses critical for ensuring the synchronous beating of the myocytes extends across gap junction channels and facilitates the propagation of voltage-mediated signals across the heart [5]. In non-excitable cells, gap junctions also provide a means for the exchange of regulatory molecules less than 1 kDa including ATP, NAD, IP3, cAMP and ions [6]. Gap junctions constructed of different connexins have varying permselectivities to metabolites such as ATP [7]. A high-resolution model of Cx43 gap junctions, 7A in the membrane plane and 21A in the vertical plane, has been described [8].

Connexin proteins show a strong proclivity to form connexon channels or unapposed hemichannels in which six connexins are arranged around a central aqueous pore. Cells may express more than one connexin isoform, which can result in the formation of heteromeric Cx channels and heterotypic gap junctions. Variation in connexin stoichiometry provides a basis for the selectivity of channels and a mechanism allowing cells to dynamically regulate their

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intercellular communication properties [6]. In addition to the modulation of channel composition and properties, cells actively adjust the extent of intercellular coupling via gap junctions by other mechanisms including changes in connexin expression, regulation of connexin trafficking and metabolic turnover.

This article focuses on mechanisms for the assembly of connexins into gap junctions and discusses those protein domains that regulate trafficking routes. This biogenetic process commences as connexins, emerging from ribosomes, assume the characteristic membrane topography displayed by all connexins. Connexins traverse the membrane four times with the amino and carboxyl termini accessing the cytoplasmic intracellular environment (Fig. 1). This arrangement in the lipid bilayer generates two disulphide linked loops, EL1 and EL2, that are lumenal when located inside the cell and face the external environment at the plasma membrane and after assembly of connexons into gap junctions. The loops have highly conserved amino acid sequences, thus contrasting with the highly variable amino acid sequences found in the single intracellular loop. The amino terminus is probably partially exposed to the cytoplasm and is well conserved between connexins. Thus, connexins differ from each other mainly in the amino acid sequences in the intracellular loop and the variable length carboxyl terminal tail [9].

Many mutations in connexins lead to channelopathies, and these have provoked studies that highlight the requirement for accuracy in the assembly of gap junction channels. Over 200 site-specific mutations scattered throughout Cx32 are associated with Charcot Marie Tooth X linked disease, a peripheral neuropathy [10]. Mutations detected in Cx26, Cx30 and Cx31.1 are associated with non-syndromal deafness [11,12] and inherited skin disorders [13]. Mutations in Cx50 and Cx46 in the lens are linked to cataract formation [14]. Although mutations in Cx43 have been reported to be associated with hearing loss [15], they are comparatively rare. The present position is that, paradoxically, no mutation in the Cx43 gene seems to affect the overall function of the heart. However, Cx43 expression is decreased and gap junctional coupling is impaired in heart diseases and such changes have been implicated in the pathogenesis of lethal ventricular arrhythmias associated with myocardial infarction, ischaemia and myocardial preconditioning [16–18].

2. Biogenesis of connexons

Connexins are synthesised mainly by membrane-bound ribosomes and are delivered from the endoplasmic reticulum to the plasma membrane as connexons in membrane vesicles that transit along the cell's secretory pathway [19]. Cell-free translation approaches showed that connexin polypeptides were co-translationally integrated into the endoplasmic reticulum [20,21]. In vitro protease protection and *N*-glycosylation assays showed that membrane integration occurred in the endoplasmic reticulum where the transmembrane topology of the connexin is achieved although no crucial chaperones have been identified [22]. Connexins do not possess a signal peptide sequence and no amino acids are glycosylated. As discussed below, Cx26 and possibly other connexins are also integrated post-translationally into membranes [23,24]. After integration into the endoplasmic reticulum, connexins form hexameric connexons possibly via dimeric and tetrameric oligomeric intermediates [21]. The precise intracellular location of oligomerisation is unclear and may differ between connexins and even cell types [25–30]. This process is generally completed by the arrival of the connexons in the Golgi apparatus [25,26]. In one study, oligomers of Cx26 are detected earlier in the secretory pathway than those of Cx32 and Cx43 [26,27], but others point to the TGN as the site where oligomerisation of Cx43 and Cx46 is completed [28–30], with Cx32 oligomerisation occurring earlier [30]. The general consensus emerging is that trafficking of connexins to the plasma membrane first requires their rapid oligomerisation into connexons [31]. In vitro approaches have shown that oligomerisation of connexins is a calmodulin-dependent step [32] since it interacts with connexins at an early stage of gap junction assembly [33] and may "plug" connexon channels during intracellular transit [34]. During intracellular transit, connexons are probably in a closed configuration in the membrane to maintain steep ionic gradients between the cell's lumenal and cytoplasmic environs. However, connexons in an open configuration have been detected at the plasma membrane [35,36]. Such hemichannels have also been shown on the basis of dye uptake and ATP release by a variety of cells [37,38] that was blocked by connexin mimetic peptides [39]. Indeed, connexon hemichannels are increasingly associated with pathophysiological conditions such as metabolic stress in astrocytes [40] and in ventricular myocytes [41].

3. Trafficking of connexons to the plasma membrane

Electron microscopy and immunocytochemical approaches have provided a static picture of the distribution of gap junctions and precursors, or degradative products in a variety of cell types. In cultured cells, especially those overexpressing transfected connexins, intracellular stores are identified immunocytochemically in the endoplasmic reticulum–Golgi interfacial regions as well as by characteristic punctate staining observed at points of cell to cell contact that corresponds to gap junction plaques [42].

4. Chimeric connexins as trafficking monitors

Connexins tagged at the carboxyl terminus with chemiluminescent aequorin [43], autoflourescent Green Fluorescent Protein (GFP) and its cyan and yellow variants, have

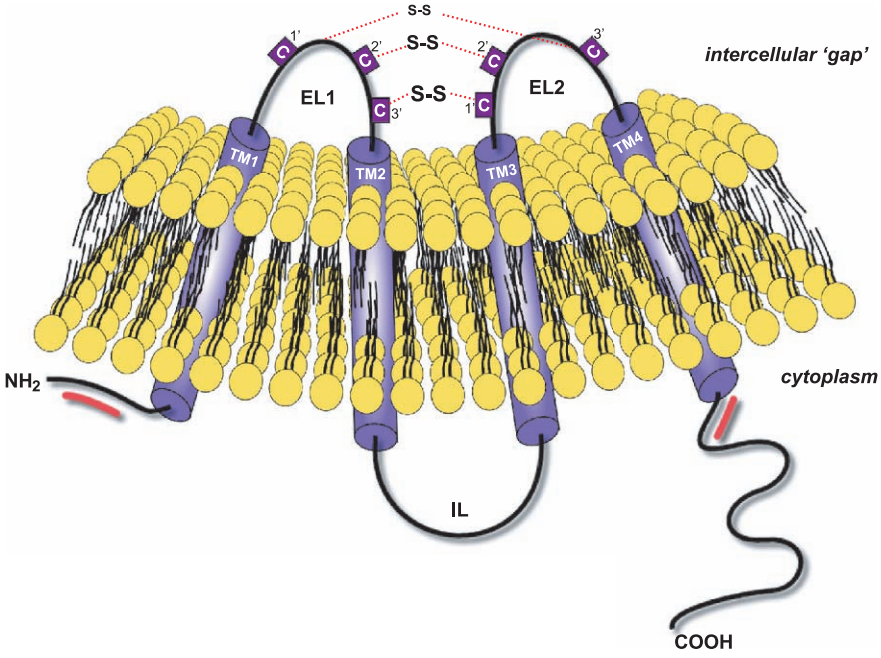

Fig. 1. Functional map of gap junction assembly determinants. Connexins span the membrane four times with two highly conserved extracellular loops (EL1 and EL2) facilitating docking and recognition of compatible connexins. The amino, intracellular loop (IL) and carboxyl termini (COOH) interface with the cytoplasm. Although most domain maps of connexin proteins have emerged through functional studies of naturally occurring mutations, especially in Cx32 and Cx26, the highly conserved nature of the proteins can permit analogies to Cx43 to be made (see Table 1 and text). The two calmodulin binding sites at the amino and carboxy termini and the six cysteine sites in the extracellular loops are indicated.

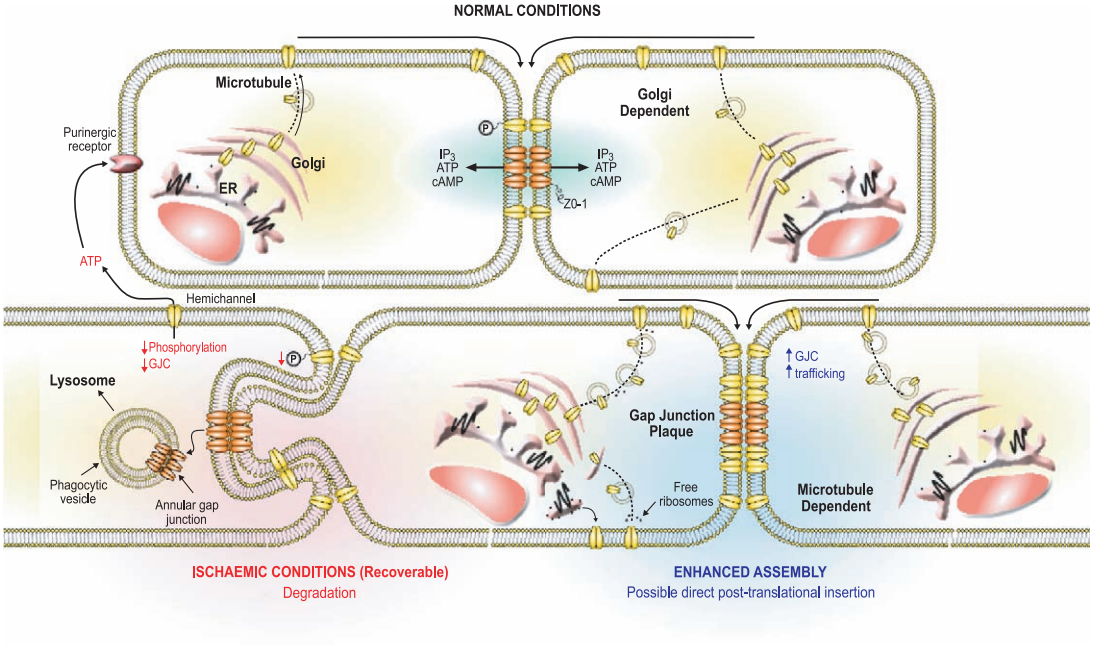

Fig. 2. The life cycle of gap junctions under different physiological conditions. Connexons are synthesised in the ER and occur as oligomers in the Golgi apparatus. Under normal conditions, connexon hemichannels are carried, in a closed configuration, by small vesicles to the plasma membrane from the Golgi with associated interactions with microtubules. Once inserted into the plasma membrane hemichannels diffuse laterally and dock with counterparts contributed by a neighbouring cell to form functional gap junction units that interact to generate plaques. Hemichannels may, under certain physiological conditions, also have a signalling role in the release of ATP leading to paracrine signalling via purinergic receptors in neighbouring cells. Enhanced assembly of gap junctions occurs, for example, following hypertrophy or treatment with cAMP. An alternative gap junction assembly pathway that can account for enhanced synthesis is highly dependent on intact microtubules. As discussed in the text, it may involve direct post-translational insertion of connexins into the plasma membrane. Under conditions of stress, accelerated removal of gap junction units occurs mainly by phagocytosis from the centre of a plaque to form annular gap junctions associated with large endocytic vesicles that are targeted to the lysosome.

been used to track the movement of connexins to gap junctions [44–46]. Short amino acid sequences incorporating tetracysteine residues [47] have also been used to investigate intracellular trafficking events leading to functional gap junction assembly [48]. High-resolution and time-lapse microscopy has permitted spatial and temporal analysis of connexins traversing the cell's secretory pathway and the kinetics of connexin turnover to be explored. Ultimately, fluorescence energy transfer (FRET) will allow heteromeric connexin interactions underpinning specific channel formation to be studied in live cells.

Protein reporter tags attached to the carboxyl terminal tail of connexins approximately double the molecular mass and can restrict the flexibility of the tail. However, they do not significantly change trafficking characteristics (and thus connexin oligomerisation events), nor docking of connexons with partners in contacting cells, nor do they alter their ability to pack into gap junction plaques. Connexin 43 when covalently linked to aequorin, GFP and tetracysteine tags, oligomerises into channels that display conduction properties similar to their wild-type counterparts in terms of dye transfer, propagation of intercellular Ca^{2+} waves and electrical coupling [43,49,50], although in some cases subtle differences in channel conductance of chimeric gap junctions have been detected [43,51]. Similar results were obtained with Cx26 and Cx32 tagged with GFP [46,52]. In contrast, fusion of Cx43 with large reporter proteins such as β-galactosidase resulted in a chimeric construct that was retained in the cytoplasm [53,54]. A red fluorescent Cx43 chimera was also non-functional in homomeric conformation but functionality was returned after co-oligomerisation with wild-type Cx43 [55]. Cytoplasmic retention of chimeric connexins was probably due to incorrect oligomerisation. Cx26 fused to aequorin, a Ca^{2+}-sensitive reporter protein, was also non-functional, probably because the short 16 amino acid carboxyl tail prevented correct folding of the reporter protein. Distancing of reporters as far as possible from juxtamembrane areas favours optimal functional outcomes [43].

Connexin-fluorescent proteins have enabled the life cycle of gap junctions to be studied in live cells in real time. The Golgi apparatus is an important transit station for the transport of Cx43, as its disruption by brefeldin A prevents delivery to the plasma membrane [26,45,56]. The cytoskeletal network also plays a variable and often critical role in connexin trafficking events [45,57,58]. Connexons reside in highly mobile vesicular carriers that move to and from the gap junction. Vesicles of <0.5 μm traffic towards the plasma membrane and larger vesicles 0.5–1.5 μm may correspond to internalised gap junctions [44,59]. Insertion of connexons into the plasma membrane occurs over large areas of the cell's surface where they diffuse laterally joining the periphery of pre-existing gap junction plaques. The dynamics of the generation and removal of gap junctions has been highlighted using tetracysteine tags attached to the carboxyl terminus of Cx43 that are successively labelled with either red or green biarsenical fluorophores thus allowing differential labelling of 'new' and 'old' connexons [47]. The picture emerging is of a dynamic assembly process as new channels are continuously added to the edge of the gap junction plaque structure and older paired connexons are removed from its centre area [47,59] (Fig. 2). After disruption of the Golgi apparatus with brefeldin A, gap junction plaque renewal continues for up to 20 min presumably due to the arrival of new connexons in transit in the plasma membrane [59]. Co-cultures of NRK cells expressing Cx43GFP and cells endogenously expressing Cx43 internalised the fluorescently tagged variant into non-Cx43GFP-expressing cells, thus showing that gap junction plaques are internalised by only one cell partner [60]. These >0.5 μm vesicles correspond to annular gap junctions labelled by immuno gold electron microscopy [61,62] (Fig. 2).

Proteosomal and lysosomal pathways account for the degradation of Cx43 with the extent of their involvement varying between cells. Proteosomal degradation is a general mechanism for disposing of mutationally faulty or overproduced connexins, and occurs at early points in the secretory pathway [63–65].

5. Proteins associated with gap junction formation

Connexins interact with several other proteins and especially components of other intercellular junctions. This is especially apparent in the heart where assembly and turnover of gap junctions and the numerous other adhesion junctions that characterise the intercalated disc are closely coordinated.

Cx43 interacts with tubulin and demonstrates cell type specific interactions with actin. Nocodazole, a microtubule dissociating drug, reduced but did not prevent trafficking of Cx43 and Cx32 but stopped movement of Cx26 to the plasma membrane [26]. Studies using glutathione-*S*-transferase fusion protein deletion constructs of the carboxyl terminus of Cx43 showed that β-tubulin interacts directly with a juxtamembrane domain of the carboxyl tail of Cx43 [57]. In cells co-transfected with Cx43 tagged to GFP and tubulin tagged to a yellow fluorescent protein, connexin transporting vesicles were tracked along microtubules, with smaller vesicles trafficking towards the ends of the microtubules and the cell periphery [59]. Cx43 also has independent binding domains for cell junction proteins especially ZO-1, a membrane associated guanylate kinase (MAGUK) protein, proposed to link connexins to cytoskeletal actin. Interaction of Cx43 with ZO-1 involves a carboxyl terminal isoleucine and may generate a scaffold for recruiting other regulatory proteins into the gap junction. In the heart, Cx43 interacts with alpha spectrin via ZO-1, possibly a key event in localising Cx43 to myocyte-intercalated discs [66]. Such interactions are thought to constitute a general adapter mechanism for targeting connexins to specific domains in polarised cells. Similar interactions between ZO-1 and Cx46

and Cx50 occur in lens [67], and between Cx43 or Cx32 in liver-derived cell lines [68]. The stoichiometry of association of Cx43 with ZO-1 is also crucial for remodelling of cardiac gap junctions [69]. Other proteins such as catenin may also influence Cx43 trafficking and gap junction formation [70,71].

Cx43 may associate with lipid rafts where it interacts with caveolin 1 [72]. Cx32, Cx36 and Cx46 were also targeted to these specialised lipid domains and interacted with caveolin 1 but Cx26 and Cx50 were excluded. Phosphorylation of Cx43 is not involved since removal of the carboxyl tail of Cx43 had no effect on targeting to lipid rafts or its interaction with caveolin 1 [72].

PTX-sensitive G proteins have been implicated in Cx43 trafficking since inhibition of these G proteins results in a decrease in the number of hemichannels at the cell surface available for assembly into gap junctions [73]. The distribution of Cx43 involved G1α proteins and may provide a mechanism that can explain how enhanced forms of gap junction assembly and communication between cells occurs by a mechanism that complements transcriptional control.

6. Phosphorylation of Cx43

An unresolved aspect of connexin biochemistry concerns the functional role(s) of the extensive phosphorylation of Cx43. Although the carboxyl tail of connexins with its multiple phosphorylation sites is suggested to function as a regulatory element in channel gating [74], phosphorylation is unlikely to be a major requirement for trafficking since connexins with short non-phosphorylated carboxyl tails are efficiently assembled into functional gap junctions. The precise biochemical composition of non-phosphorylated Cx43 and polyphosphorylated isoforms especially in relation to the 21 serine and 2 threonine residues in the carboxyl tail remains unclear. In a trafficking context, phosphorylation commences early in the secretary pathway [75], with other phosphorylated isoforms predominantly associated with gap junctional plaques. Such conclusions are collated mainly on the basis of the insolubility of gap junction plaques and the solubility of connexins and connexons in Triton X-100 [76]. With the increasing appreciation that connexon hemichannels constructed of Cx43 are involved in the release of ATP by cells [39], it can be speculated that Cx43 phosphorylation may regulate the operation of hemichannels residing in non-junctional regions of the plasma membrane. Numerous protein kinases have been implicated indirectly in Cx43 phosphorylation, and alterations in the phosphorylation status of Cx43 correlate with a wide number of connexin-mediated processes, but the results vary especially between tissues [77]. Clarification of the level of contribution of connexin phosphorylation to gap junctional assembly and operation may require the study of a series of specific connexin phosphorylation deficient mutants as exemplified by the phosphorylation of Cx43 on serine 368 by protein kinase C and its effects on gap junctional communication [78].

7. Remodelling of gap junctions

Rapid transcriptional modulation of connexin-mediated communication occurs by acutely enhancing levels of mRNA as exemplified in the uterus. Prior to parturition, smooth muscle shows a 5–10-fold rapid increase in Cx43 and its mRNA and connexins are transported to the plasma membrane where they are assembled into gap junctions that underpin synchronised smooth muscle contraction [79]. Connexins present in cytoplasmic pools in uterine cells allow for fast track assembly possibility utilising multiple trafficking mechanisms. The short half-life of connexins provides a means for regulatory intervention, as also seen in the upregulation of Cx43 gap junctions during early stages of human coronary atherosclerosis and changes in connexin profiles observed during wound healing [80–82]. Cardiac myocytes regulate Cx43 expression over very short time periods, as shown in conditions that stimulate a hypertrophic response such as increased external load or by treatment with chemical mediators such as cAMP [83] or angiotensin II [84]. These mediators rapidly enhance Cx43 synthesis and result in increased Cx43 levels at the cell surface [85]. Indeed, treatment of cells expressing Cx43GFP with cAMP increased the regulated aggregation of gap junction plaques independently of Cx43 protein synthesis and the kinetics of vesicular trafficking events [86]. On the contrary, cardiac myocytes uncouple in acute ischaemia and there is rapid translocation of Cx43 from the cell surface into intracellular pools and Cx43 is dephosphorylated [87]. Shortly after ischaemia phosphorylated Cx43 is again detected [88]. Further analysis of the trafficking pathways and kinetics involved in the remodelling of Cx43 in the heart during hypertrophic and ischaemic conditions can provide a platform for the development of pharmacological reagents that counter cardiac arrhythmia (Dhein, this issue).

Emerging from a broad analysis of the rapid response of gap junction assembly and turnover to a range of external factors described above is the possibility that trafficking of connexons to gap junctions via the secretory pathway is supplemented by other assembly mechanisms. These may be brought into play to allow rapid enhancement of intercellular communication across gap junctions.

8. Are there multiple gap junction assembly mechanisms?

Many lines of evidence accrued from different model systems point to the existence of other independent routes of gap junction assembly that complement the primary mechanism described above. Cx26 is inserted into micro-

somal membranes post-translationally by an ATP-dependent process and Cx43 also exhibits this property but to a lesser extent [23,24]. Substitution of microsomes (that contain some plasma membrane vesicles) by liver plasma membranes resulted in a two- to threefold increase in the efficiency of connexin oligomerisation. The hemichannels inserted into liposomes were permeable to ascorbic acid and sucrose showing that connexins had integrated correctly into membranes and oligomerised [23]. These results may be explained in vivo by the involvement of free cytosolic ribosomes in the synthesis of Cx26 followed by its direct insertion and oligomerisation in plasma membranes (Fig. 2). Post-translational protein assembly mechanisms are recognised as contributing to the polarised distribution of proteins in embryonic cells, where turnover is extremely rapid and crucially dependent on microtubules [89]. Indeed, 'non-classical' pathways of protein targeting and membrane insertion may occur more widely than previously thought [90]. For example, co- and post-translational translocation mechanisms regulate transmembrane assembly of the cystic fibrosis conductance regulator [91]. Alternative biogenesis pathways have been intensively investigated in the context of organelle biogenesis in yeast where proteins synthesised on free ribosomes are inserted into peroxisome membranes in a similar post-translational manner to Cx26 [92,93]. Other mechanisms of protein trafficking assuming insertion into endoplasmic reticulum but adopting a direct route into the plasma membrane should not be disregarded, especially with connexins, proteins that are not glycosylated. Exceptions to traditional trafficking routes via the secretory pathway are being described as biochemical and proteomic analysis approaches unfold a direct route from the endoplasmic reticulum to the plasma membrane [94]. Also emphasising the presence of undiscovered minor trafficking routes is a direct connection between the endoplasmic reticulum and peroxisomes in splenic cells [95]. Such new and poorly mapped routes out of the endoplasmic reticulum and to the plasma membrane would allow rapid connexin recycling and breakdown and implicating lysosomal structures.

Liver tissue expresses Cx26 and Cx32, and in guinea pig liver, these connexins are expressed in equal abundance as shown by analysis of plasma membrane fractions and gap junctions [27]. Surprisingly, in intracellular membranes, Cx26 expression was low compared to that of Cx32. It was concluded that the low Cx26 levels detected in Golgi vesicles reflected its incorporation into heteromeric connexons detected in liver [96]. The independence of Cx26 and Cx32 trafficking routes to gap junctions was also shown in hepatocytes from Cx32 knockout mice [97].

The use of tagged connexins has also shown differences in trafficking between Cx26 and connexins 32 and 43. In summary, these show that Cx32 and 43 are trafficked via the secretory pathway whereas Cx26 continued to be inserted into gap junctions after disruption of the Golgi apparatus or exposing cells to 15 °C [26]. Substitution of the carboxyl tail of Cx26 by the tail of Cx43 fused to aequorin did not change the Golgi bypassing mechanism for insertion into gap junctions [26]. These studies were corroborated by using connexins fused to GFP variants, where it was shown that Cx26GFP was inserted into gap junctions after disruption of the Golgi apparatus but this was prevented after disassembly of microtubules, although insertion of Cx32 and Cx43 continued but at lower efficiency [45,52] (Fig. 2).

A microtubule-dependent mechanism for direct insertion of connexins into gap junctions would generate homomeric connexons in the plasma membrane and could operate during cell division when the Golgi is dispersed and non-functional. This could explain the rapid synthesis of Cx26 relative to other connexins in brain [98], liver [99], during wound healing in keratinocytes [100] and lactating breast tissue [101]. In a cardiovascular context, the enhanced synthesis of gap junctions may involve the switching on of these alternative mechanisms leading to additional Cx43 gap junctions. Indeed, stimulation of cells with forskolin or IBMX, resulting in elevation of cAMP levels, caused increased intercellular communication and incorporation of Cx43 from intracellular stores into gap junction plaques at the plasma membrane [102,103]. Disruption of the microtubular network with nocodazole blocked this additional incorporation of Cx43 into gap junctions suggesting that microtubules facilitate gap junction growth [103]. Clearly, these examples show that Cx43 levels at the plasma membrane can be modulated by fast track assembly pathways that operate independently of the Golgi and that require an intact microtubular network [59,103] (Fig. 2).

9. Connexin domains implicated in the assembly of gap junctions

Genetic mutations are rarely detected in Cx43 and most connexin functional domain maps are associated with Cx32 and Cx26. However, the similar topography in the membrane and high amino acid sequence homology allows analogies to be made. Many naturally occurring mutations in connexins display functional and assembly defects and often show dominant negative characteristics [104,105].

A generalised connexin is shown in Fig. 1, and functions associated with the major domains are shown in Table 1. A range of properties has been assigned to the amino terminal domain. A calmodulin binding site identified in Cx32 may be one of the oligomerisation determinants [32,33]. This region of the protein is important in membrane insertion and/or trafficking, since several diseases exist in which mutations at amino acid position 12 result in intracellular accumulation of the protein [106]. Some mutant proteins oligomerised but are incorrectly folded and accumulate in intracellular stores corresponding mainly to the Golgi apparatus region [106,107]. Amino acids 12 and 13 in Cx43 may contribute to the oligomerisation incompatibilities of

Table 1
Functional domains of connexins

Domain	Function	Reference
Amino terminus	Calmodulin binding site	[32,33]
	Membrane insertion and targeting domain	[105]
	Oligomerisation compatibility	[107]
TM1	Regulation of trafficking pathways	[45]
	Post-translational insertion	[45]
	Site-specific mutations cause trafficking and assembly problems	[103,104]
	Partial pore lining	
	Membrane anchoring	
Extracellular loops	Facilitate docking and recognition of compatible connexins	[112,113]
	Pore extension	[8]* [110]
	Site-specific mutations cause trafficking and assembly problems	[103]
TM2	Membrane anchoring	[110]
	Pore lining	
Intracellular loop	Chemical (pH) gating	[8]*
	Voltage gate	
TM3	Pore lining	[8]* [110]
	Oligomerisation domain	[32,54]
	Oligomerisation compatibility	[107]
	Site-specific mutations cause trafficking and assembly problems	[103]
	Membrane anchoring	
TM4	Membrane anchoring	
Carboxyl tail	Gap junction targeting domain (juxtamembrane sequence) and essential role in oligomerisation	[64,109]
	Calmodulin binding domain	[33]
	Protein/protein interactions, e.g., ZO-1, tubulin	[57,66]
	Channel regulation, e.g., fast junctional gating of Cx43	[6]*
	Phosphorylation	[77]*
	Not required for trafficking but involved in the formation of heteromers	[26]

Functional properties of the different domains of connexins are outlined and referenced. The majority of this information is accrued from studies of Cx32 and Cx26; however, as discussed in the text, analogies with Cx43 can be made.

* Indicates review articles.

alpha and beta categories of connexins [108]. The N terminal region is also associated with the entrance to the channel adopting a helical conformation, with residues 12–15 forming an open turn with mutations at the G12 position affecting the flexibility of this locus [109].

All the transmembrane domains may be regarded as membrane anchoring. Deleting the first transmembrane region prevented overall membrane insertion [110] and a mutation Cx32I28L adopted the post-translational and microtubular dependent trafficking pathways associated with Cx26 [45]. This region may determine how connexins are targeted to gap junctions and the extent of post-translational insertion properties observed. Interestingly, Cx43 possesses a leucine at position 28, which may partly explain its ability to follow a rapid microtubular dependent pathway to gap junctions under enhanced assembly conditions as discussed above [103]. The third transmembrane region is a major contributor to the channel wall especially the extracellular aspect, with the first transmembrane region contributing to the intracellular side of the channel [111]. The voltage gate is also located in the vicinity of the first transmembrane region [112] and the same region is also reported to be a crucial regulator of connexin oligomerisation [32,54].

The extracellular loops are highly conserved in connexins and are linked by disulphide bonds [113,114]. These are crucial for the docking of hemichannels and a number of genetic mutations with functionality and trafficking disorders have emphasised the importance of these loops [115,116].

The intracellular loop and the carboxyl tail constitute the most non-homologous regions of the connexin family suggesting that many of the functional differences between connexins reside here. Sequential truncation of the carboxyl tail of Cx32 had little effect on assembly of gap junctions [117]. Furthermore, exchanging the carboxyl tail of Cx26 for that of Cx43 did not alter the different trafficking pathways or the kinetics observed for Cx26, reinforcing the conclusion that at least the bulk of the carboxyl tail is not involved in trafficking [118]. Nevertheless, other studies have suggested that the carboxyl tail is required for the formation of heteromeric connexons [21] and that it contributes to differences in gating characteristics in heteromeric channels [118]. The carboxyl tail of Cx32 also contains a second calmodulin binding domain and a short juxtamembrane region within this domain incorporates a crucial gap junction targeting motif [110]. The carboxyl tail of Cx43, is modified post-translationally by phosphorylation [77] and interacts with other associated proteins such as microtubules and ZO-1 [57,69]. Undoubtedly, the carboxyl tail is an important determinant of gap junction operation [119] with extensive evidence that it interacts with the intracellular loop via a ‘ball and chain’ mechanism allowing chemical gating of the channel [120].

It is apparent that no clearcut single domains or motifs regulate assembly and trafficking of connexins to gap junctions. Indeed, extremely minor amino acid sequence changes anywhere in connexins may have critical consequences on the generation and functioning of these dynamic intercellular communication channels. The challenge now is to use current knowledge of the mechanisms of assembly of connexins into functional gap junctions to help develop therapeutic tools to overcome connexin-mediated communicationopathies [121,122].

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