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Review

Diversity in protein–protein interactions of connexins: emerging roles

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Abstract

Gap junctions, specialised membrane structures that mediate cell-to-cell communication in almost all tissues, are composed of channel-forming integral membrane proteins termed connexins. The activity of these intercellular channels is closely regulated, particularly by intramolecular modifications as phosphorylations of proteins by protein kinases, which appear to regulate the gap junction at several levels, including assembly of channels in the plasma membrane, connexin turnover as well as directly affecting the opening and closure (''gating'') of channels. The regulation of membrane channels by protein phosphorylation/dephosphorylation processes commonly requires the formation of a multiprotein complex, where pore-forming subunits bind to auxiliary proteins (e.g. scaffolding proteins, catalytic and regulatory subunits), that play essential roles in channel localisation and activity, linking signalling enzymes, substrates and effectors into a structure frequently anchored to the cytoskeleton. The present review summarises the up-to-date progress regarding the proteins capable of interacting or at least of co-localising with connexins and their functional importance.

Keywords: Gap junction; Channel gating; Channel assembly; Intracellular trafficking

1. Introduction

Intercellular communication is of paramount importance in allowing individual cells to successfully accomplish their task in a coordinated manner in multicellular organisms and tissues. Gap junctional intercellular communication (GJIC) relies on the existence of intercellular protein channels that span the lipid bilayers of adjacent cells and allow the direct exchange of ions and small molecules between neighbouring cells. Transmembrane proteins, termed connexins, have a common topology, with four alpha-helical transmembrane domains, two extracellular loops, a cytoplasmic loop, and N- and C-termini located on the cytoplasmic membrane face. The sequences are most conserved in the transmembrane and extracellular domains, yet many of the key functional differences between connexins are determined by amino acid differences in these domains. The junctional channel is composed of two end-to-end hemichannels, each of which is a hexamer of connexin subunits forming an aqueous pore in the lipid bilayer.

GJIC was for a long time regarded as a relatively passive way of intercellular signalling through cell-to-cell tunnels before it finally appeared that the degree of intercellular coupling is finely regulated in three main aspects: (i) the number of channels present in the membrane, (ii) their functional state and (iii) their unitary permeability.

The availability of membrane proteins, including junctional proteins, at the cell surface can be regulated at different locations within the cell: (1) the amount of protein synthesised in the endoplasmic reticulum (ER) is largely controlled by gene transcription. In addition, the ER quality control system regulates the exiting of properly folded proteins from the ER. (2) In the trans-Golgi network, proteins can either be diverted directly to the lysosomes or be transported to the cell surface. (3) At the plasma membrane, the endocytic machinery can select proteins for endocytosis via clathrin-coated pits or proteins may be subject to proteolysis, resulting in shedding of the extracellular domain. (4) In endosomes, internalised proteins are either recycled back to the plasma membrane or targeted to the lysosome for degradation.

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The open probability and elementary permeability of gap junctional channels (as of a number of membrane channels) are regulated by protein phosphorylation and dephosphorylation processes and several factors (including not only intracellular pH and calcium concentrations, voltage but also different chemicals). It is now becoming clear that such regulations require the formation of multiprotein complexes (for recent reviews regarding the involvement of connexins in protein–protein interactions, see for example Refs. [1,2]). While pore-forming-subunits of many channels bind to auxiliary channel subunits, they also associate with scaffolding proteins that play essential roles in channel localisation and activity [3]. Scaffolding proteins link signalling enzymes, substrates, and potential effectors (such as channels) into a multiprotein signalling complex that may be anchored to the cytoskeleton. Besides an obvious role in targeting the channel to a particular location on the cell membrane, there are several advantages to having a membrane channel in a multiprotein complex. Indeed, the substrate selectivity of some enzymes, essential to determining specificity in several signal transduction pathways, is largely determined by their subcellular location (e.g. for protein phosphatases). There is also a large increase in the efficiency of the reaction kinetics when an enzyme is localised with its substrate and effector in a microenvironment with restricted diffusion. Moreover, the anchoring of enzyme complexes to some channels may be necessary for the extremely rapid transmission of signals required to regulate them. However, if the proteins in signalling cascades are considered spatiotemporarily organised in such a way to achieve efficiency and specificity, the physical association of components to form signalling complexes or their close proximity to facilitate random collisions remains poorly understood.

Many of the signalling pathways and regulatory systems in eukaryotic cells are controlled by proteins with multiple interaction domains that mediate specific protein–protein and protein–phospholipid interactions. Pairs of interacting partners at protein–protein (or protein–peptide) interfaces interact via modular protein domains, well demarcated and independently folded portions of proteins, typically comprising 40 to 200 amino acids. These domains are non-catalytic and bind specifically to short continuous peptide sequences in their binding partner(s) via one or more exposed, ligand-binding surfaces (‘ligand recognition pockets’). Ligands interact with their complementary domains through short and generally continuous sequence motifs. In some cases, certain amino acids of the ligands must be post-translationally modified (e.g. phosphorylated or acetylated) before recognition and binding can occur (for review, see Ref. [4]).

2. Methodological approaches

As protein–protein interactions are generally mediated by the recognition of short continuous amino acid stretches on target proteins by specific binding domains, several strategies have successfully been used to define recognition sequences for a large series of such protein domains. The immunofluorescence confocal microscopy is used to investigate co-localisation of connexins with protein partners, to examine if the similar subcellular localisation of two proteins makes possible a physical interaction between them. The two-dimensional distribution of integral membrane proteins in cellular membranes can be studied by exposing freeze-fracture replicas to antibody immunogold complexes. Main biochemical approaches consist, in co-immunoprecipitation experiments and pull-down assays, using peptides corresponding to known connexin sequences, fused to GST, in which protein partners are identified by Western blot or mass spectrometry. The two-hybrid system is the most commonly used genetic method to identify the proteins with which a particular protein associates via transcriptional activation of one or several reporter genes. Such a method is used to investigate whether two known proteins interact in vivo and in native conformation, if the genes encoding both proteins are available, or to identify previously unknown proteins that interact with a protein of interest using a specially constructed cDNA library. These same techniques, coupled with truncation and mutagenesis experiments, are used to define the region of interaction between pairs of proteins. Surface plasmon resonance-based biosensor technologies allow the determination of the affinity and kinetics of a wide variety of molecular interactions (including protein–protein) in real time, without the need for a molecular tag or label. Connexin–connexin interactions can be investigated by translational diffusion analysis, using nuclear magnetic resonance (NMR). In vitro cell-free assays are performed either by means of liposomes reconstituted with connexins extracted from plasma membrane post-translationally inserted connexins or by expression of different connexin isotypes in translation-competent lysates and ER-derived membrane vesicles (microsomes).

3. Connexin–connexin interactions

The assembly of six connexin molecules into a connexon, made of single (homomeric connexon) or multiple (heteromeric connexon) connexin types appears to be based on specific signals located within the connexin polypeptides. The end-to-end binding between two connexons occurs via non-covalent interactions between the extracellular loops of connexins, which each contains three conserved cysteine residues that form intramolecular disulfide bridges. Mutagenesis studies suggest that the docking region contains beta structures, and may resemble to some degree the beta-barrel structure of porin channels. The two hemichannels that compose a junctional channel are rotationally staggered by approximately 30° relative to each other so that the alpha helices of each connexin monomer are axially aligned with the alpha helices of two adjacent monomers in

the apposed hemichannel (for review, see Ref. [5]). The second extracellular (E2) domain [6] together with the middle cytoplasmic portion [7] were found determinant of heterotypic compatibility between connexins. The docking process of connexons may be hindered by incubation of cells with synthetic oligopeptides homologous to a specific region of the second extracellular loop of connexins, plausibly mimicking connexin–connexin binding, resulting in a decrease of the activity of junctional channels in an aortic smooth muscle cell line expressing Cx40 and Cx43 [8]. A chimera in which the third transmembrane (and proposed channel lining sequence of Cx32) was substituted by a transmembrane sequence of the cystic fibrosis transmembrane conductance regulator (CFTR), did not oligomerise in vitro and it accumulated intracellularly when expressed in COS-7 cells [9].

Connexin–connexin interactions have been suggested to be responsible for the interruption of the cell-to-cell communication elicited by some agents (including pH, insulin or $pp60^{v\text{-}Src}$, for example), via interactions between the channel pore and a discrete C-terminal domain of the connexin serving as gating particle, a "ball and chain", model [10–17] similar to the inactivation of Shaker-type K^+ channels [18]. Several lines of evidence indeed imply that the carboxyl terminal (CT) domain of connexins is a movable structure, able to behave like an independent domain. Indeed, the truncation of this domain (of Cx40 or Cx43 for example) resulted in alterations in their single channel activity, but normal functions were restored by coexpression of the CT domain, even of another connexin. It was for example the case for Cx40 truncated at amino acid 248 (Cx40tr248), where separate coexpression of either the homologous (Cx40) or the heterologous (Cx43) CT fragments restored, at least partially, channel functions [16,19,20]. These hetero-domain interactions (occurring between the CT domain of one connexin isotype and the pore-forming region of another) suggest that, despite their differences in primary sequence, the CT domains of Cx40 and of Cx43 can reach a similar conformation, allowing their interaction with a common binding domain. Such intramolecular interaction, by analogy with other biological reactions involving two separate molecules (e.g. hormone and receptor), is seen as a intramolecular noncovalent interaction of the CT domain (acting as a ligand) with an amino acid domain in or near the mouth of conductive path, (acting as a receptor). Further investigation is needed to determine the regions of the CT and of the inner pore-forming region involved in this gating.

4. Connexins–partner proteins interactions

The peptide chains contain domains that mediate protein–protein interactions and, among them, postsynaptic density-95/discs large/zona occludens-1 (PDZ) motifs are one of the most commonly found. These domains are 80–100-amino acid protein binding cassettes, identified initially in PSD-95, DLG, and ZO-1, that recognise a three-residue peptide motif in the CT of their binding partners [21]. Among these domains, PDZ motifs bind to CT ends of various proteins, especially integral membrane proteins, most of which end in valine. PDZ proteins can be classified into three principal families according to their modular organisation (for review, see Ref. [22]). The Src homology 3 (SH3) domains (approximately 50–70-amino acid-long domains) interact with proline-rich peptide motifs whereas Src homology 2 (SH2) domains associate with short amino acid sequences containing phosphotyrosine. Several domains may be present in the sequence of a given protein. Proteins of the membrane-associated guanylate kinase (MAGUK) family for example are characterised by a multidomain organisation that includes several PDZ domains, one SH3 domain, and one GUK-like domain (for review see Ref. [23]). This organisation allows MAGUK proteins to serve as linkers between integral membrane proteins and cytoskeletal networks. They can indeed cross-link multiple integral membrane proteins at the cytoplasmic surface of plasma membranes to establish specialised membrane domains. The list of MAGUK proteins, linkers between integral membrane proteins and cytoskeletal networks, is growing rapidly. Several proteins have been reported able to interact or to be co-localised with different connexins (see Table 1).

4.1. Calmodulin

Calmodulin (CaM) now appears as a constitutive or dissociable Ca^{2+}-sensing subunit of a surprising variety of ion channels found in a wide range of species from Homo to Paramecium (for recent review, see Ref. [24]). The ability of a non-specific CaM antagonist (trifluoperazine) to prevent CO_2-induced uncoupling of Xenopus embryonic cells led to suggest that CaM may participate in channel gating [25]. This hypothesis was strengthened by evidence for CaM binding to Cx32 in gel overlays [26] and that a fluorescent CaM derivative binds to Cx32 and Cx32 fragments in vitro [27]. With inhibition of CaM expression, the slow gating and CO_2-sensitive gating virtually disappeared whereas the expression of a CaM mutant (the N-terminal, NT, hand pair replaced by a copy of the CT pair) dramatically increased the chemical gating sensitivity of gap junction channels composed of Cx32 and decreased their sensitivity to transjunctional voltage [15], suggesting a CaM participation in this mechanism. A direct CaM-connexin interaction at junctional and cytoplasmic spots was demonstrated by confocal immunofluorescence in HeLa cells transfected with Cx32 and in cryosectioned mouse liver [28]. Mirror resonance assays with peptides corresponding to some parts of the CT domain of Cx43 did not detect interactions with CaM, but the authors suggested it might occur via other regions of the Cx43 CT or indirectly [17].

Table 1
Reported proteins able to interact or to co-localise with different connexins

Partner protein	Cx	Cx motif	Main approaches[a]	Cell type[b]	Refs.
Cx-Cx	Cx43, Cx46, Cx50		bt	*Xenopus oocytes*	[6]
	Cx32, Cx38, Cx43		bt	*Xenopus oocytes*	[7]
	Cx32	M3, CT tail	bt ci	COS-7 cells	[9]
	Cx40, Cx43	Second extracellular loop of Cx43	bt	A7r5 cells	[8]
	Cx32, Cx43		bt	*Xenopus oocytes*	[10]
	Cx32, Cx43		bt	*Xenopus oocytes*	[11]
	Cx43		bt	*Xenopus oocytes*	[12]
	Cx43		bt	*Xenopus oocytes*	[13]
	Cx32, Cx43		bt	*Xenopus oocytes*	[14]
	Cx32	CT tail	bt	Xenopus oocytes	[15]
	Cx32, Cx43		bt	*N2a cells*	[16]
	Cx43	CT-L2	bt nmr at		[17]
	Cx37, Cx40, Cx43, Cx50		bt	*Xenopus oocytes*	[19]
	Cx40, Cx43		bt	*N2a cells, Xenopus oocytes*	[20]
Calmodulin	Cx32	NT (1-21) ; CT (216-230)	bt		[27]
Calmodulin	Cx32	CT tail	bt	Xenopus oocytes	[15]
		M3, CT tail	bt ci	COS-7 cells	[9]
			cl	Cx32-HeLa, *cryosectioned mouse liver.*	[28]
ZO-1	Cx31.9		cl ci	HEK 293 cells	[48]
	Cx32		cl ci	Cultured rat hepatocytes	[47]
	Cx43		cl	Epithelial cells of pig thyroid	[32]
		CT 5 amino-acids	cl ci at	Cx43-HEK 293 cells	[35]
		Extreme C-terminus	cl ci dh	Cx43-COS-7 cells	[40]
		Very CT isoleucine residue	ci bt at	*COS-7*, A431, Beas2B	[76]
			cl	Porcine and equine uterine endometrium	[37]
			cl	42GPA9 Sertoli cell line	[39]
			cl ci at	ROS 17/2.8 cells	[31]
			cl	Rat adult ventricular myocytes	[33]
			cl ci em	Rat adult ventricular myocytes	[34]
			cl	Immortalised mouse hepatocytes	[46]
			cl ci	Rat cardiomyocytes	[36]
	Cx45	CT 4 amino-acids	cl ci dh	MDCK cells	[30]
		12 most CT amino-acids	cl ci at	ROS 17/2.8 cells	[31,45]
	Cx46, Cx50		cl ci	Mouse lens	[49]
			cl ci em at	Mouse lens	[50]
ZO-2	Cx32		cl	Cultured rat hepatocytes	[47]
Occludin	Cx26		at	Human intestinal cell line T84	[52]
			cl ci	CHST8 cells	[38]
			cl ci	Cultured rat hepatocytes	[47]
			cl	Immortalised mouse hepatocytes	[46]
	Cx43		cl	Rat sciatic nerves	[51]
Lin-7	Cx43	CT tail	at bt cl		[57]
Claudin-1	Cx32		cl ci	Cultured rat hepatocytes	[47]
			cl	Immortalised mouse hepatocytes	[46]
β-catenin	Cx43		cl ci	Neonatal rat cardiomyocyte in culture	[53]
			cl	Mouse neural crest cells	[54]
α-catenin	Cx26 and Cx32		cl em	Mouse liver	[55]
	Cx43		cl ci	Neonatal rat cardiomyocytes	[36]
p120	Cx43		cl	Mouse neural crest cells	[54]
E-Cadherin	Cx26 and Cx32		cl em	Mouse liver	[55]
N-Cadherin	Cx43		*cl*	Mouse neural crest cells	[54]
$pp60^{c\text{-}src}$	Cx43	Y265	ci	Rat-1 cells, COS-7, *A431*, HeLa cells	[67]
		CT tail	at	Neonatal rat cardiomyocytes, *HEK 293*	[44]
$pp60^{v\text{-}src}$	Cx43	CT-proline-rich region+ a phosphorylated tyrosine residue	at ci	*Rat-1 fibroblasts, HEK 293 cells, v-Src-transformed fibroblasts*	[66]
		CT tail	cl ci	*v-Src-transformed fibroblasts*	[64]
PKCε	Cx43		cl ci	Neonatal rat cardiomyocyte in culture	[68]
PKCα and PKCε	Cx43		cl ci	Human cardiomyocytes	[69]
PKCα	Cx43		cl	Minipig hearts	[70]

(continued on next page)

Table 1 (*continued*)

Partner protein	Cx	Cx motif	Main approaches[a]	Cell type[b]	Refs.
DMPK	Cx43		cl fs	Rat papillary muscles	[72]
			cl	Rat adult heart	[73]
MAPK	Cx43		at	NRK, FT210 cells	[74]
P38MAPK	Cx43		cl	Minipig hearts	[70]
α-or β-tubulin	Cx43	35 amino-acid domain juxta-membrane region CT tail	bt cl fs at	Rat-1 cells, *COS-7*, A431, *MDCK*, *HEK 293*, T51B, Beas2B and human fibroblasts	[75,76]
Actin	Gap junctions		cl em	Human and monkey lenses	[79]
	Cx43		cl	SW-13 cells	[77]
F-actin	Cx43		cl	Rat cultured astrocytes	[80]
Drebrin	Cx43		ci		[81]
Caveolin-1	Cx43		cl ci fs	NIH 3T3, *HEK 293T and COS-7 cells*	[82]
Clathrin	Cx43		cl	Cultured cardiomyocytes of adult Guinea pig	[84]
Ubiquitin	Cx43		ci	E36, BWEM cells	[88]
			em	Cx43 HeLa cells	[89]
			em	BICR/M1R$_k$cells	[90]
CIP 62	Cx43	CT tail	dh		[91]
AP26	Cx26		cl	P3/22, P3E1 cells	[94]
NOV	Cx43		cl	C6 glioma cells	95
YAF2-like protein	Cx26		cl dh		[97]

([a]) bt : biochemical techniques (cell-free assays, chimeras, truncated connexins, mimetic peptides, etc); cl: colocalisation; ci: co-immunoprecipitation; fs: co-fractionation or co-sedimentation; dh: double hybrid; em: electron microscopy immunolabelling: at: affinity techniques (pull-down, affinity binding assays, surface plasmon resonance); nmr: nuclear magnetic resonance.
([b]) in roman characters, cells where Cx were endogenously expressed; in italics, cells where Cx were exogenously expressed, surexpressed or mutated.

A7r5, an aortic smooth muscle cell line
BICR/M1R$_k$, a permanently growing cell line derived from a spontaneous rat mammary tumour
BWEM, rat cardiomyocyte-derived cells
CHST8 cells, immortalised mouse hepatocytes
COS7, monkey African green kidney cells
E36 cells, Chinese hamster ovary cells
FT210 cells, a mutant cell line derived from FM3A, a murine mammary carcinoma cell line
HEK293 cells, human embryonic kidney 293 cells
MDCK, epithelial Madin–Darby Canine Kidney cells
NIH 3T3, mouse fibroblasts
NRK, rat kidney cells
N2a, murine neuroblastoma cells
P3/22, mouse skin papilloma cell line and its derivatives P3E1 (in which E-cadherin gene is transfected)
ROS 17/2.8, osteosarcoma cell line
SW-13, human adrenal cortical tumor cells
T51B cells, rat liver epithelial cells.

4.2. Tight junction-associated proteins

4.2.1. Zonula occludens proteins

The ZO proteins are members of the MAGUK family but, in addition to the presence of the characteristic modules of this family (PDZ, SH3 and GUK domains), ZOs have a distinctive CT comprising acidic- and proline-rich regions, and splicing domains. The modular organisation of these proteins allows them to function as scaffolds, which associate to transmembrane junctional proteins, the cytoskeleton and signal transduction molecules. ZO-1 for example is a 220 kDa peripheral membrane protein, which tethers transmembrane proteins such as occludin, claudin and junctional adhesion molecule to the actin cytoskeleton. ZO-1 is both an actin-binding and cross-linking protein and interacts with a large number of proteins that are associated with the tight junctions and the adherens junctions (for review, see for example Ref. [29]).

The growing number of connexins that can associate with ZO proteins indicates that the latter may play a more general role in organising gap junctions and/or in recruiting signalling molecules that regulate intercellular communication [30]. It was proposed that ZO-1 might make a scaffold to temporarily secure the different connexins in gap junction plaques at the cell–cell boundary, different domains of ZO-1 serving as docking modules for kinases and phosphatases that interact with the different connexin polypeptides [31].

4.2.1.1. Cx43. Cx43 and ZO-1 were found co-localised in cell types as diverse as epithelial cells of the thyroid gland [32], cardiac cells [33–36], endometrial epithelial cells [37], an hepatocytic cell line [38], osteoblastic cells [31], a Sertoli

cell line [39]. The fact that the association between ZO-1 and Cx43 was surprisingly limited to low to moderate levels in intact ventricular myocardium led Barker et al. [34] to suggest that disruption of intercellular contacts between myocytes might result in a dynamic change in the pattern of association between ZO-1 and Cx43. The interaction of Cx43 with ZO-1 occurred through the extreme carboxyl terminus of Cx43 and the second PDZ domain of ZO-1 [40]. Moreover, in cardiac cells, ZO-1 would link Cx43 with α-spectrin [35].

Gap junctions in cardiomyopathic heart cells were reported to appear, in immunofluorescence microscopy, as disparate rather than as dense particles as they do in normal cardiac myocytes [41], suggesting an improper incorporation of Cx43 into the gap junctional plaque in the cardiomyopathic heart. Moreover, cardiomyopathic heart cells might reduce gap junctional communication through c-Src-mediated tyrosine phosphorylation of Cx43 [42]. The PDZ domain of ZO-1 has been proposed to participate in such protein–protein interactions [43], and in vitro binding assays using recombinant proteins synthesised from regions of Cx43 and ZO-1 showed that the CT tetrapeptide of Cx43, DLEI, has the capacity to bind to the ZO-1 PDZ2 domain [44]. According to these authors, the regulation of channel functions by c-Src might be indirect, due to a conformational hindrance, the c-Src-mediated tyrosine phosphorylation allowing a binding with a SH2 domain, inducing a structural change in the CT region of Cx43, thereby hindering the interaction between Cx43 and ZO-1. In the adult rat ventricle, ZO-1 and Cx43 co-distribute within intercalated discs, but levels of association between Cx43 and ZO-1 increased rapidly and significantly after partial or complete enzymatic dissociation of myocytes from intact ventricle, a treatment known to induce gap junction endocytosis.

4.2.1.2. Cx45. In transfected epithelial MDCK cells, Cx45 co-localises with endogenous ZO-1 at or near tight junctions and co-precipitation experiments show that Cx45 and ZO-1 directly interact [30]. Cx45 binds to the PDZ domains of ZO-1 and ZO-3, but not of ZO-2, via a short CT PDZ binding motif. Inactivating this motif in Cx45 affects its co-precipitation and co-localisation with ZO-1 [30]. ZO-1, Cx43, and Cx45 were found co-localised in osteoblastic cells, suggesting that all three proteins are normally associated in the cells; the interaction between the carboxyl terminus of Cx45 and ZO-1 is likely to occur via binding to a ZO-1 PDZ domain in the same manner as Cx43 CT [45]. The amino acid sequences of the CT of all connexins, with the exception of Cx32, end in a hydrophobic residue suggesting that they might bind to PDZ domains [21] in ZO-1. This might indicate that sets of connexins that bind to ZO-1, like Cx45 and Cx43, could be found in the same gap junctions along with ZO-1.

4.2.1.3. Other Cxs. ZO-1 proteins were co-localised with Cx32, occludin and claudin-1 at cell borders of immortalised mouse hepatocytes [46], whereas in primary cultured rat hepatocytes, Cx32 also co-localised with occludin, claudin-1, ZO-1 and also ZO-2 (on the most subapical plasma membrane) [47]. In lateral plasma membranes of epithelial cells of pig thyroid, ZO-1 was co-localised with Cx43 but not with Cx32 [32]. In human embryonic kidney HEK293 cells transfected with the Cx31.9 gene, most ZO-1 staining co-localised to membrane domains with Cx31.9 [48]. As Cx43–ZO-1 interaction involves the most CT residues of Cx43 and the second PDZ-domain of ZO-1, Nielsen et al. [49,50] examined if two connexins of the lens, Cx46 and Cx50, which possess CT residues similar to Cx43, also interact with ZO-1. Both connexins were found to co-immunoprecipitate and to co-localise with ZO-1.

4.2.2. Occludins and claudins

Occludin, a 65 kDa transmembrane phosphoprotein of the tight junction with two extracellular loops, which frequently co-localises with ZO-1 at regions of cell–cell contact, where occludin can co-localise with Cxs, for example in rat sciatic nerves with Cx43 [51] or in immortalised mouse hepatocytes (Cx32-CHST8), where Cx32-binding was demonstrated by immunoprecipitation [38]. These results indicate that connexin can be aligned side-by-side with occludin into ordered, linear fibrils when it is fused to the ZO-binding region, thus, suggesting a scaffolding function for ZO-1, ZO-2, or ZO-3. A potential link between a coiled-coil domain of occludin and Cx26 was suggested in T84 cells [52].

Claudins form a multigene family (20 members) of ≈ 22 kDa transmembrane proteins of the macromolecular tight junction complex, and claudin-1 was found co-localised with Cx32, occludin and ZO-1 at cell borders of rat hepatocytes [47] and of a hepatocytic cell line [46].

4.3. Desmosomes and adherens junctions-associated proteins

Adherens junctions and desmosomes, responsible for the mechanical coupling of cardiac myocytes, are found closely apposed to gap junction plaques at the intercalated discs, which they link to the actin cytoskeleton.

Catenins are multifunctional proteins which include α-catenin, β-catenin and plakoglobin (γ-catenin), whose activities depend on their subcellular localisation; plasma membrane-associated β-catenin acts as a component of cell-adhesive junctions, and it was found co-localised and coimmunoprecipitated with Cx43 in neonatal rat cardiomyocytes [36,53]. Catenin was suggested to interact, directly or indirectly via other components of cell junctions (such as members of the cadherin family, PDZ-domain-containing ZO-1 protein, or other cellular constituents), as part of a complex within the junctional membrane. The intracellular application of antisera against α-catenin, β-catenin (or ZO-1) in cardiomyocytes cultured in low Ca^{2+} medium inhibited the redistribution of Cx43 to the plasma membrane following Ca^{2+} switch [36]. In mouse neural crest cells, p120 catenin-related was found co-localised with Cx43 and N-

cadherin [54]. In mouse hepatocytes, both E-cadherin and α-catenin were found co-localised with Cx26 and Cx32 during gap junction reappearance [55].

Lin-7 is a PDZ-domain-containing protein able to form with Lin-2 and Lin-10 a heterotrimeric PDZ complex involved in the organisation of epithelial and neuronal junctions. Co-immunoprecipitation and in vitro binding assays revealed that this protein directly binds to the C-terminal sequence of β-catenin (for review, see for example Ref. [56]). In a preliminary report, Singh et al. [57], using tandem mass spectrometry analysis, identified mammalian Lin-7 among proteins from whole cell lysates able to interact with the C-terminal of Cx43. Deletion constructs of Cx43 and the Lin family members confirmed, as well in vitro as after expression in cells, these interactions, both biochemically and via microscopic co-localisation.

Cadherins are an important family of cell adhesion molecules, cell surface glycoproteins responsible for the Ca^{2+}-mediated adhesion of epithelial cells. Double staining with anti-pan-cadherin and anti-Cx43 antibodies suggested that Cx43 and cadherin were closely associated in mouse heart sections [58]. N-cadherin is frequently co-localised or closely juxtaposed with Cx43, for example in Novikoff cells [59], adult rat cardiomyocytes in culture [60], or in mouse neural crest cells [54]. In adult rat heart, only low to moderate point-by-point co-localisation of Cx43 and ZO-1 was found within intercalated discs compared with the relatively high level of co-localisation between N-cadherin and ZO-1 [34]. In mouse hepatocytes, double-immunogold electron microscopy using a polyclonal antibody for connexins (Cx26 and Cx32) and a monoclonal antibody for E-cadherin or alpha-catenin revealed co-localisation of these molecules at cell-to-cell contact sites during the reappearance of gap junction plaques [55]. A reduced level of E-cadherin expression is frequently associated with invasive tumours of epithelial origin, and E-cadherin transfection in communication-deficient mouse skin papilloma cell line P3/22 restored the function of connexins [61,62].

Cx43 and desmoplakin, a major component of the desmosomal plaques (the structures which serve as attachment sites for intermediate filaments) were observed in close proximity in rat cardiomyocytes [60] and in mouse heart [58].

4.4. Enzymes

Up to now, all enzymes found associated or co-localised with connexins are protein kinases (PK). Among them, Src, protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and casein kinase 1 (CK1) phosphorylate Cx43 at different sites of its CT tail.

4.4.1. Scr protein tyrosine kinases

Expression of $pp60^{v\text{-}Src}$ completely blocked the communication induced by Cx43 but had only a modest effect on communication through Cx32-made channels [63]. Cx43 appeared as an endogenous substrate of $pp60^{v\text{-}Src}$ in v-Src-transformed fibroblasts, and $pp60^{v\text{-}Src}$ and Cx43 were shown to be partially co-localised in regions of the plasma membrane; furthermore, $pp60^{v\text{-}Src}$ could phosphorylate co-immunoprecipitated Cx43 in an immune-complex kinase assay [64]. Cx43 and $pp60^{v\text{-}Src}$ co-immunoprecipitated from v-Src-transformed fibroblasts, indicating that the two proteins were associated and form a stable complex (see Ref. [65]).

In vitro, Cx43 bound to the SH3 domain of v-Src, but not of c-Src, and mutations in the SH3 and SH2 domains of v-Src as well as in the proline-rich region or tyrosine 265 of Cx43, reduced interactions between v-Src and Cx43 in vivo [66]. In contrast, in cardiac myocytes, constitutively active c-Src inhibits the endogenous interaction between Cx43 and ZO-1 by binding to Cx43 [44]. Indeed, c-Src (Y527F) prevents binding of Cx43 to the cytoskeletal protein ZO-1 by phosphorylating Tyr265, thereby providing a binding site for the SH2 domain of c-Src, which directly disrupts the interaction between the C-terminus of Cx43 and ZO-1. The c-Src-mediated tyrosine phosphorylation of Cx43 not only reduced gap junctional conductance but also decreased the stability of Cx43 at the cell surface, leading to conclude that the function of gap junctions is established by the interaction of Cx43 with ZO-1 and is down-regulated when that interaction is interfered by c-Src binding to a different site [44,67].

4.4.2. Protein kinase C

The C-terminal tail of Cx43 has several serine residues that could serve as PKC substrates. Co-localisation of the various PKC isoforms with their putative substrates is proposed to ensure their preferential and rapid phosphorylation on activation, and there is evidence suggesting that PKC bind to specific anchoring proteins. Two PKC isoforms, PKCα and PKCε, localise to plasma membrane sites. In non-stimulated myocytes of newborn rat, PKCε displayed a discontinuous pattern of localisation at intercellular contact sites and partial co-localisation with Cx43. Treatment with FGF-2 or phorbol 12-myristate 13-acetate (PMA) induced a more continuous pattern of PKCε distribution, and the anti-Cx43 staining appeared to overlap extensively with that of PKCε [68]; in failing or non-failing human myocardium, both PKCα and PKCε co-localised with Cx43 [69]. In immunoprecipitation experiments, PKCε but not PKCα co-precipitated with Cx43 in rat cardiomyocytes [68], whereas PKCα was also present in the coimmunoprecipitated complex with either Cx43 or PKCε antibodies in human cardiomyocytes [69]. In the last study, recombinant PKCε or αincreased PKC activity significantly above endogenous levels in the co-immunoprecipitated Cx43 complexes, but phosphorylation of purified human Cx43 by recombinant PKCα or ε resulted in only PKCε-mediated Cx43 phosphorylation. Thus, in the human heart, PKCα, PKCε and Cx43 appear to form a closely associated complex, and if only PKCε directly phosphorylates Cx43,

both PKC isoforms result in increased phosphorylation within the Cx43 co-immunoprecipitated complex. In preconditioned hearts of minipig, the co-localisation of PKCα with Cx43 at intercalated discs increased during ischemia [70].

4.4.3. Other protein kinases

Cellular co-immunoprecipitation experiments and in vitro CK1 phosphorylation reactions shown that CK1 interacted with and phosphorylated Cx43, initially on serine(s) 325, 328, or 330, and specific inhibition of CK1 activity resulted in a decrease in assembled Cx43 [71].

Among myotonic syndromes, the myotonic dystrophy (DM) is a dominantly inherited disease involving skeletal muscles, heart, endocrine organs, lens and the central nervous system. The molecular basis of this autosomal disorder has been identified as the expansion of a CTG repeat in the 3' untranslated region of a gene encoding a protein kinase, Myotonic Dystrophy Protein kinase (DMPK). The pathophysiology of the disease and the role of DMPK are still obscure. The main localisation sites of this enzyme are neuromuscular and myotendinous junctions, terminal cisternae of the sarcoplasmic reticulum in the skeletal muscle and at intercalated discs in the cardiac muscle. In the last site, DMPK was found co-localised with Cx43 [72,73].

In NRK and FT210 cell lysates, Cx43 was seen to interact and to be phosphorylated by activated MAPK (ERK1 and ERK2; Ref. [74]). In tissue sections of minipig heart, isoforms α and β of p38MAPK co-localised with Cx43 and, in preconditioned hearts, this co-localisation increased during sustained ischemia [70].

4.5. Cytoskeletal proteins

A 35-amino acid juxtamembrane region in the CT tail of connexin-43 was proposed to contain a presumptive tubulin binding motif [75]; α-tubulin and β-tubulin appeared equally bound [76]. In contrast, in SW-13 adrenal cortical tumour cells, no physical association between tubulin and gap junction protein could be established [77]. In NRK cells, the disruption of microtubules with nocodazole inhibited the recruitment of Cx43 into gap junctions whereas it had limited effect on the transport and clustering of Cx26 [78].

A co-localisation of actin filament bundles and gap junctions was observed in cortical fiber cells of human and monkey lenses [79], and of F-actin and Cx43 in rat cultured astrocytes [80]. In SW-13 adrenal cortical tumour cells, Cx43 was observed at areas of cell-to-cell contact but also within the cytoplasm as annular profiles, where it was closely associated with actin and myosin II [77]. Drebrin, one of the actin-binding proteins which regulate the structure and dynamics of the actin cytoskeleton, was found to co-immunoprecipitate with Cx43 in brain membrane fractions [81].

4.6. Proteins involved in intracellular protein trafficking

Caveolins are structural proteins acting as scaffolding proteins to cluster lipids and signalling molecules within caveolae and sometimes to regulate those proteins (including different membrane channels) that are targeted to the caveolae. They are present in specialised lipid raft domains, cholesterol-sphingolipid-rich microdomains that function as platforms for membrane trafficking and signal transduction. Cx43 was seen to co-localise, to cofractionate, and to coimmunoprecipitate with caveolin-1 [82]. In the latter study, some other connexins (Cx32, Cx36, and Cx46) also seem targeted to lipid rafts, while Cx26 and Cx50 were specifically excluded from these membrane microdomains.

In Cx43-C6 glioma cells, immunogold labelling with anti-Cx43 serum revealed that some of membrane Cx43 proteins were in proximity to clathrin-coated pits [83]. In cardiomyocytes of adult guinea pig cultured for 2 weeks at low density to prevent formation of cellular contacts, immunoreactive Cx43 decreased and became progressively localised under a nearly uniform punctate pattern throughout the sarcoplasm, costained with clathrin, without detectable co-localisation with actin or tubulin [84].

Ubiquitination is a post-translational modification in which ubiquitin chains or single ubiquitin molecules are appended to NT amino acid residue of the target proteins, giving rise to poly- or monoubiquitination, respectively. However, in some cases, as for p53, the ubiquitination does not occur in the N-terminus [85]. Polyubiquitination targets selected proteins for destruction by the proteasome via two discrete and successive steps: (1) conjugation of multiple moieties of ubiquitin to the protein, and (2) degradation of the conjugated protein by the 26S proteasome complex with the release of free and reusable ubiquitin [86]. In a few cases, it has been reported that monoubiquitination targets membrane-anchored proteins to degradation or recycling in the lysosome/vacuole [87]. In E36 Chinese hamster ovary cells, immunoprecipitation using anti-Cx43 antibody followed by re-immunoprecipitation by anti-ubiquitin antibody demonstrated polyubiquitination of Cx43 [88]. Immunogold labelling of replicated material with an anti-ubiquitin antibody revealed that gap junctional plaques contained ubiquinated proteins [89]; under control conditions, 50% of the detected Cx43 junctional plaques were ubiquitinated [90]. In these cases, the presence of ubiquitinated proteins might reflect ubiquitination of either Cx43 or another protein present in these structures.

4.7. Other partner proteins of connexins

Using a two-hybrid screen of an embryonic mouse cDNA library, Jin et al. [91,92] identified 15 cDNAs coding for protein domains that interact with the C-terminus of Cx43. One of these cDNAs encoded the PDZ domain of ZO-1, another a SH3 domain. A full-length 2.7 kb cDNA

Cx43 Interacting Protein 62 (CIP62) was isolated using as a probe the cDNA coding for the SH3 domain. CIP62 was predicted to encode a 760 amino acid protein with a molecular weight of 82 kDa. Sequence analysis of the derived peptide sequence revealed that the amino acid region 497–545 was 64.5% similar to the SH3 domain of human Grb2 proteins. The rest of the sequence was without significant homology to proteins of known functions. Predominantly localised at the plasma membrane, this protein might interact in vivo by its SH3 domain with the CT of Cx43.

Organ of Corti Proteins 1 and 2 (OCP1 and OCP2), low-molecular weight acidic proteins predominantly expressed in the vertebrate auditory system (they each represent 5% of the total proteins found in the OC) are components of OC-specific ubiquitin ligases, which presumably bind selected protein targets, positioning them for ubiquitination. Both OCP1 and OCP2 were found co-localised with Cx26 in postnatal guinea pig (see Ref. [93]).

In a preliminary report [94], a gene coding a protein which specifically binds to the cytoplasmic loop domain of Cx26, named Cx26-associated protein (AP26) and present in many tissues of mouse and various cell lines, was cloned. The co-localisation of AP26 and Cx26 and their coordinated movement lead to suggest that AP26 might be a Cx26-anchoring protein involved in Cx26 functions.

Nephroblastoma OVerexpressed (NOV) is a matricellular protein, member with connective tissue growth factor (CTGF) and Cyr61/Cef-10, of the CCN family, with negative growth-regulatory functions. High levels of NOV (CCN3) expression are observed in smooth muscle cells of the arterial vessel wall, in derivative of germ layers during development, and its expression is altered in a variety of tumours. Tumour cells often exhibit a low level of Cx and a decreased GJIC. NOV co-localised with Cx43 at the cell membrane of C6 glioma cells transfected with Cx43 [95]. There is an inverse relation between NOV expression in glioma cells and their tumorigenicity, and the establishment of functional GJIC was found associated with NOV expression [96]. These authors also suggested that the antiproliferative activity of NOV might involve a reorganisation of cellular contacts, which play a crucial role in tumorigenesis.

In a preliminary report, a yeast two-hybrid screen of Cx26-interacting proteins allowed to isolate a mouse clone homologous to the previously described human YY1-associated factor (YAF2), a protein that interacts and modulates the activity of the transcription factors YY1, Myc, and MycN. In HeLa cells co-transfected with Cx26 and YAF2, confocal microscopy revealed a co-localisation of those proteins on the plasma membrane [97].

5. Possible structural domains of connexins involved in interactions with partner proteins

Cx43 is the most ubiquitous and the best known connexin; its CT tail is not required for formation of functional channels but is critical for the regulation of GJIC. Several of its domains appear involved in interactions with partner proteins (Fig. 1). Cx43 associates with ZO-1 via its extreme CT, which interacts with the second, but not the first, PDZ domain of ZO-1 [40], and the very CT isoleucine residue of Cx43 was reported to be critical for ZO-1 binding [75].

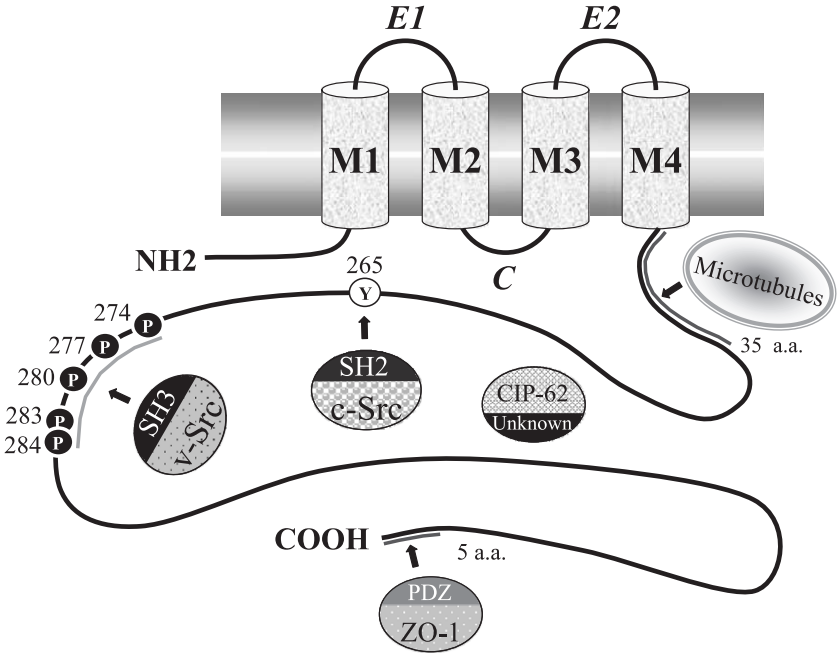

Fig. 1. Possible domains of the C-terminal tail of Cx43 involved in interactions with partner proteins. Several domains of the C-terminal tail of the protein have been found involved in protein–protein interaction with partner proteins.

Cx43 specific 35-amino acid juxtamembrane region contains a presumptive tubulin binding motif, necessary and sufficient for tubulin binding. This motif (^{234}KGVK DRVKGK^{243}, residues typically found in tubulin binding domains are underlined) is unique for Cx43 and was not found in the sequence of other connexin family members [75].

Proline-rich regions of Cx43 associate with v-Src by interacting with its SH3 domain (see Ref. [65]). Such a region (50–70 amino acids long) possesses three shallow pockets where the peptide ligands bind. Two of the pockets are 25 Å long and 10 Å wide, large enough to accommodate each of the prolines in the PxxP motif, accompanied by a hydrophobic residue (i.e. A, I, L, V, and P). Proline is indeed unique among the 20 common amino acids in having the side-chain cyclised onto the backbone nitrogen atom. This means that the conformation of proline itself is limited, with backbone angles of $\sim -65°$. It also restricts the conformation of the residue preceding the proline because of the bulk of the N-substituent and results in a strong preference for a β-sheet conformation. As a consequence, polyproline sequences tend to adopt the PP II helix, which is an extended structure with three residues per turn. This implies that the two prolines in the SH3 domain ligand core, PxxP, are on the same face of the helix and are thus well placed to interact with the protein (see Ref. [98]). Such sequence-specific ability of proline-rich regions to interact with other proteins would explain how a peptide of the same sequence as amino acids 271–287 of Cx43 (CSSPTAPLSPMSPPGYK) can prevent pH gating of Cx43-expressing oocytes [99]. According to these authors, the proline-rich region of the Cx43 CT is implicated in a binding reaction that is critical for acidification-induced channel closure. Proline-rich regions are also known capable of binding to tubulin [98]. A mutational analysis of Cx43 revealed that the hypothesised PDZ- and presumptive SH2/SH3-binding domains within the Cx43 carboxyl terminus are not required for this targeting event or for its stable interaction with caveolin-1. In this study [82], Cx43 appears to interact with two distinct caveolin-1 domains, i.e. the caveolin-scaffolding domain (residues 82–101) and the CT domain (135–178).

Phosphorylated tyrosine residues in the CT tail of Cx43 are able to bind to SH2 domains, of v-Src for example [66], and it was suggested that such interaction might induce a structural change in the CT region of Cx43, thereby hindering the interaction between Cx43 and the ZO-1 PDZ-2 domain [44]. The region surrounding S364 in the CT was suggested involved in interaction of Cx43 with intracellular trafficking proteins [100].

Cx43, Cx46 and Cx50 have similar C-terminal tails; transiently expressed Cx46 and Cx50 lacking the C-terminal residues formed structures resembling gap junctions but did not bind to the second PDZ domain of ZO-1, suggesting that, as Cx43, their interaction with ZO-1 occurs via their C-terminal residues [50]. Cx31.9 was suggested to interact with ZO-1 through a molecular mechanism similar to that described for Cx43 [48]. Cx45 was also found to interact with the PDZ domains of ZO-1 and ZO-3 via its C-terminus [30], and in vitro translated ZO-1 bound to an oligopeptide corresponding to the final 12 amino acids in Cx45 [45].

Truncation experiments of Cx32 suggested that a crucial gap-junction targeting sequence resides between amino acid residues 207 and 219 on the cytoplasmic CT tail [101]. In hepatocytes from Cx32-deficient mice transfected with Cx32, Cx26, or Cx43 cDNAs, endogenous occludin was in part localised at cell borders, co-localised with Cx32, whereas neither was detected in parental cells or in Cx26 or Cx43-transfected cells. However, this co-localisation was not observed when hepatocytes were transfected with Cx32 truncated at position 220 [46]. Several amino acid sequences in Cx32 were proposed as CaM-binding motifs, including residues 1–21 (NT) and 216–230 (CT) [27] and parts of the third transmembrane domain and of the CT-tail [9].

6. Physiological importance of protein–protein interactions

6.1. Intracellular trafficking of connexins

Steps in the synthesis, assembly, targeting to the membrane and turnover of gap junction channels appear to follow the general secretory pathway for membrane proteins. All through life cycle, connexins are associated with different protein partners, including other connexins molecules, in homo or hetero-oligomers, as summarised in Fig. 2.

6.1.1. Hemichannel assembly

The formation of hexameric connexon hemichannels involves dimeric and tetrameric connexin intermediates. Removing the CaM-binding site located at the carboxyl tail of Cx32 or cell exposure to either a CaM-binding synthetic peptide or W7 (a naphthalene sulfonamide CaM antagonist) limited the formation of connexons and caused their intracellular accumulation [9]. Kausalya et al. [30] suggested that their binding to ZO proteins could determine which connexin types co-assemble into a particular connexon.

6.1.2. Targeting connexons to the membrane

GJIC is impaired in most tumours; however, connexin genes are rarely mutated in tumours, whereas aberrant localisation of connexins is frequently observed. Abnormalities in connexin partner proteins, responsible for the intracellular traffic control of connexins might then cause aberrant localisation of connexins. For channel assembly, partner proteins might function as cytoplasmic scaffolds in organising membrane proteins into specialised membrane domains. It was for example possible to combine the cytoplasmic tail of occludin to transmembrane portions of Cx32 to create chimeras, and such chimeras were able to

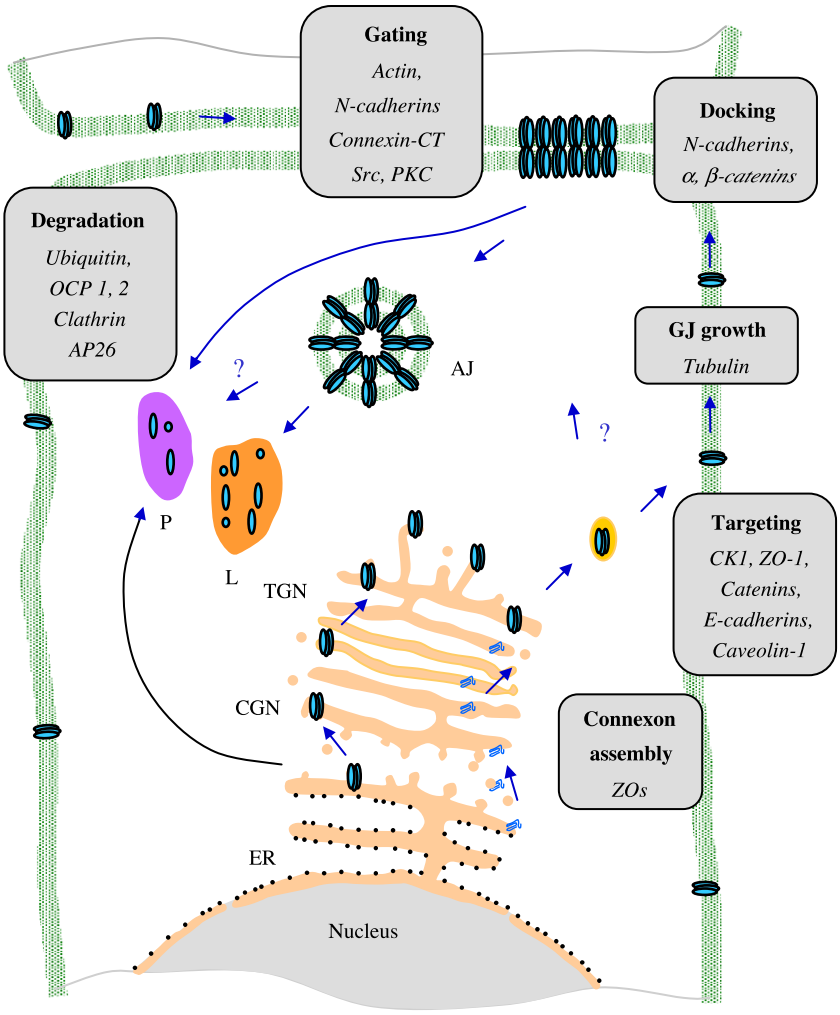

Fig. 2. Main steps of the life cycle of a connexin involving interactions with partner proteins. ER, endoplasmic reticulum; CGN, cis-Golgi network; TGN, trans-Golgi network; L, lysosomes; P, proteasomes; AJ, annular junction. Evidence has been reported for oligomerisation within the membranes of either the ER or the Golgi apparatus.

localise with ZO-1-containing cell contacts, suggesting an important role for cytoplasmic proteins in the targeting of these chimeras to the appropriate membrane subdomain [102]. ZO-1 was suggested to provide a docking that temporarily secures the different connexins in gap junction plaques at the cell–cell boundary [31,35,40]. The over-expression of the NT domain of ZO-1, which lacked the ability to localise at cell–cell interfaces, disrupted the transport of connexin-43-FLAG to the target site [35]. However, the fact that a truncated Cx43 lacking the CT phosphorylation sites and the ZO-1-binding site can nonetheless form channels [103,104] showed that the interaction with ZO-1 is not essential for the formation of functional channels of Cx43. Indeed, Cx43 lacking the ZO-1 binding domain remained able to form channels (although the level of the incorporation into the cell surface and junctional conductance was reduced by about 30–40% compared to wild-type Cx43 [44]). In KO fibroblasts transfected with rat Cx43 cDNA truncated at amino acid 257 (M257Cx43), immunolabel of M257Cx43/KO cells shown that significant levels of truncated Cx43 resided within the cell cytoplasm, with very little localising to interfaces [100]. In rat cardiomyocytes, the binding of catenins to ZO-1 was suggested to form a catenin–ZO-1–Cx43 complex, required for Cx43 transport to the plasma membrane during the assembly of gap junctions [36].

The disruption of microtubules with nocodazole was found to have varied consequences. It indeed exists several possibilities for newly assembled connexons to be recruited in GJ plaques to GJs. Connexon-containing transport vesicles may fuse with the plasma membrane within the plaques or at the outer margin of plaques, or transport vesicles may fuse with nonjunctional parts of the membrane, and connexons may then drift to the GJ plaques where they can dock into complete junctional channels. In transfected HeLa cells, the disruption of microtubules with nocodazole had little effect on the assembly of Cx43-GFP gap junctions, but perturbed assembly of Cx26-GFP gap junctions [105],

whereas in NRK cells, it inhibited the recruitment of Cx43 into gap junctions whereas it had limited effect on the transport and clustering of Cx26 [78]. In Cx43-HeLa cells, the plasma membrane pool initially enabled connexon accrual to the clusters but further accrual was inhibited, suggesting that insertion of connexons occurred predominantly into the nonjunctional areas of membrane, followed by lateral movement to reach the margins of channel clusters [106]. In Cx43-MDCK cells, nocodazole was also without effect on initiation but inhibited the growth of GJ [107].

Other different interactions with partner proteins appear involved in connexon targeting; for example the removal of the CaM-binding site located at the carboxyl tail of Cx32 resulted in an intracellular accumulation of intermediate connexin oligomers, which failed to target to gap junctions [9]. CK1 phosphorylation of Cx43 was suggested to "tag" the Cx43 connexon for assembly, and, without this marker, assembly of Cx43 gap junction structures was reduced [71]. E-cadherin junction formation at high calcium was suggested to induce the formation of actin cables, which directly or indirectly transport connexins from the cytoplasm to the cell–cell contact membranes via the Golgi apparatus [62]. Forced expression of Cx43 and Cx32 into an invasive human cell line PC-3 and in several other androgen-independent PCA cell lines resulted in intracellular connexin accumulation, but the transient expression of α-catenin facilitated the trafficking of both connexins to the cell surface and induced gap junction assembly [108]. The disruption of actin microfilaments by cytochalasin B had no significant effect on the initiation of GJ assembly in Novikoff hepatoma cells [109], whereas it inhibited the recruitment and assembly of connexins (Cx26-YFP and Cx43-GFP) into gap junctions in NRK cells, suggesting that the recruitment of these connexins to the cell surface or their lateral clustering into gap junctions plaques is, at least in part, dependent on the presence of intact actin microfilaments [78]. AP26, which was seen to move to the cytoplasmic membrane with Cx26 but not with Cx43, might be a Cx26-anchoring protein [94].

6.1.3. Movements of connexons within the membrane; growth of junctional plaques

Connexin-specific interactions, for example the stable interaction of Cx43 with caveolin-1, might allow partition into lipid raft domains [82]. In Novikoff hepatoma cells, the disruption of microtubules had no significant effect on the initiation of GJ assembly, but microtubules appeared required for GJ growth [109]. Cardiomyocytes of adult guinea pig kept in isolation maintained their ability to reconnect with each other for up to at least 2 weeks, with redistributed Cx43 co-localised with clathrin in vesicular-shaped structures [84]. In HEK293T cells, recombinant coexpression of Cx26 with caveolin-1 recruits Cx26 to lipid rafts, where it co-localised with caveolin-1. This trafficking event appeared to be unique to Cx26, since the other connexins investigated in the study did not require caveolin-1 for targeting to lipid rafts [82]. As recently suggested [57], PDZ domain-containing protein complexes could play a pivotal role in localising their target membrane proteins to specialised membrane domains.

6.1.4. Docking of hemichannels

Gap junctions form with desmosomes, adherens junctions and tight junctions specialised complexes joining individual epithelial and endothelial cells. In mouse hepatocytes, both E-cadherin and α-catenin were found co-localised with Cx26 and Cx32 during gap junction reappearance; the cell-to-cell contact sites made by cadherin–catenin complexes as well as tight junctional strands may then act as foci for gap junction formation [55,60]. An antibody specific for N-cadherin inhibited gap junction formation in embryonic chick lens cells [110]. The assembly of the adherens junction proteins (particularly N-cadherin, α-catenin and β-catenin) was observed at cell contacts at an earlier time than Cx43 junctions [60,111] and seems to be a prerequisite for subsequent GJ formation [60]. However, in corneal epithelial cells, the presence of E-cadherin was not a prerequisite for the assembly of Cx43 gap junctions [112].

6.1.5. Degradation of connexins

The turnover of cellular proteins is a highly organised process that involves spatially and temporally regulated degradation by the ubiquitin/proteasome system. In this pathway, the covalent ligation of ubiquitin to the substrate is a signal for recognition by the 26S proteasome. Recent studies indicate that targeting of substrates of the ubiquitin pathway to the proteasome is usually accomplished by the ligation of a polyubiquitin chain assembled through K48-G76 isopeptide bonds, rather than by ligation of monoubiquitin. Sorting of transmembrane proteins to endosomes and lysosomes is mediated by signals present within the cytosolic domains of the proteins. Most signals consist of short, linear sequences of amino acid residues. These signals are recognised with characteristic fine specificity by the components of protein coats, including clathrin, adaptor protein (AP) complexes, and Disabled-2 (Dab2, which as other cytoplasmic adaptor proteins, has no catalytic domains but possesses protein binding domains and may thus participate in signal transduction pathways or regulate protein traffic inside cells). As monoubiquitination seems involved in endosome sorting and lysosome targeting, it could be of interest to examine if connexins wear such post-translational modifications. Ubiquitin-mediated proteasomal proteolysis was reported to be the major mechanism of degradation of connexin43 in E36 Chinese hamster ovary cells and rat cardiomyocyte-derived BWEM cells [88], whereas in other cell types, both the proteasome and the lysosome ways seem involved [113]. In SW-13 adrenal cortical tumour cells, the disruption of actin microfilaments by cytochalasin B exposure resulted in a decrease in the average number and

an increase in the average size of annular gap junctions compared to control populations suggested that cytoskeletal elements containing actin and myosin II were involved in the turnover of annular gap junction [77]. In contrast, it had very limited consequences on the structure or permeability of the developing junctions in Novikoff hepatoma cells [109].

In Cx43-Skep 1cells, a tyrosine-based sorting signal (YKLV) in the C-terminus of Cx43 was proposed to control Cx43 turnover by affecting internalisation and targeting of the protein for degradation in the endocytic/lysosomal compartment [114].

The fact that both the lindane-induced interruption junctional communicative between Sertoli cells [39] and the disruption of intercellular contacts between myocytes, a treatment inducing gap junction endocytosis [34], were accompanied by an increased in direct interaction between Cx43 and ZO-1 led to suggest a role for ZO-1 in the turnover of Cx43 during or after gap junctional endocytosis [34]. A Cx43 mutant that no longer binds ZO-1 exhibited an increased turnover rate [44]. A redistribution of Cx43 from cell–cell interfaces to cytoplasmic structures was for example observed after junctional uncoupling occurring during 18 beta-glycyrrhetinic acid (18 β-GA) exposure [115–117] or acute myocardial ischemia [118].

6.2. Cellular selectivity of gap junction formation

GJIC specificity allows the establishment of communication compartments, groups of cells joined to each other by gap junctions but more rarely to cells of adjacent compartments. Heterocellular coupling is observed both in vivo and in mixed cultures (see for example Ref. [119]), but its molecular basis is not elucidated. It might be based on the differential expression of connexins, but also of molecules required for GJ formation, particularly of cell adhesion molecules. Several of them were found involved, in heterocellular populations of cell types expressing Cx43, in the control of specificity of gap junction formation. In mixed cultures of rat fibroblasts and epithelial cells, exogenous E-cadherin expression resulted in a 10-fold increase in heterologous communication, whereas exogenous P-cadherin expression had no effect [120]. In contrast, mutual expression of E-cadherin was insufficient to stimulate GJ formation between bovine epithelial cells and fibroblasts [119]. In mouse L and rat Morris hepatoma cells, exogenous cadherin induced strong cell–cell adhesion in both cell types, but had opposite effects of communication [121]. In human skin, normal melanocytes established GJIC with keratinocytes, whereas melanoma cells did not; instead, melanoma cells communicated among themselves and with fibroblasts. This switch in communication partners was seen to coincide with a shift from E-cadherin to N-cadherin expression during melanoma development, and forced expression of E-cadherin in melanoma cells restored gap junctional compatibility with keratinocytes [122].

6.3. Modulation of junctional channel activity

Reversible and dynamic protein phosphorylations are considered as a pivot mechanism of the regulation of essentially all cellular functions, including GJIC, but the precise mechanisms remain unclear. The anchoring of involved enzymes at specific subcellular sites is critical for their functioning. The fact that junctional channel activity is well preserved during intracellular cell dialysis (in conventional whole cell mode of the patch-clamp technique (see Ref. [123]) and even after membrane excision (when the membrane of one of the cells is mechanically ruptured, whereas the second cell is investigated [124]) show that enzymes important in the regulation of the channel permeability are firmly anchored in the channel vicinity. In the scaffolding model proposed by Laing et al. [31], the different domains of ZO-1 serve as docking modules for enzymes (e.g. protein kinases and phosphatases), localising them in the vicinity of junctional channels.

Moreover, the modulation of GJIC by protein phosphorylation/dephosphorylation events is probably more complex than a direct connexin phosphorylation and increasing evidence indicates that protein partners are involved. This view is supported by a lack of correlation between the degree of junctional coupling and the extent of Cx43 [99,125–129] or Cx45 phosphorylation [130]. Although Cx26 cannot be phosphorylated [131], exposure of Cx26-SKHepl transfected cells to PMA reduced Lucifer yellow dye transfer and junctional conductance; moreover, it also markedly shifted the unitary conductance distribution [132]. In cultured astrocytes, hypoxia markedly reduced the degree of GJIC and Cx43 phosphorylation, but junctional uncoupling occurred prior to Cx43 dephosphorylation [133]. Conversely, various treatments induced changes in the connexin phosphorylation status without alteration of GJIC degree [134,135]. Taken together, these results suggest that disruption of GJIC is not solely mediated by modification in the connexin phosphorylation status but requires the participation of additional regulatory components.

In transfected cell pairs, even in plaques large enough to mediate coupling, only a small fraction of channels would be open at the same time [136]. These authors suggested that proteins localised in areas of cell–cell contact, might promote connexon aggregation or clustering and proposed that the latter may be necessary for channel opening. Channel gathering was also found essential for the activity of some other channels, Kv1.4 potassium channels for example, of which channel clustering appears to be obligatory for suppressing internalisation and requests the presence of a MAGUK protein [137].

The interactions among connexin isotypes with distinct regulatory mechanisms could influence channel gating properties and lead to the construction of a spectrum of channels. For example, the introduction of Cx45 into a rat osteosarcoma cell line ROS-17/2.8, which endogenous expresses Cx43, altered intercellular coupling, a most likely

result of direct interaction between Cx43 and Cx45 [138]. Hetero-domain interactions (occurring between the CT domain of one connexin isotype and the pore-forming region of another) occur, that could provide a structural basis for the charge and size selectivity of the channels, with probable functional consequences, allowing a great versatility of the functional modulation of gap junction channels (see Ref. [139]).

In murine skin papilloma cells, the degree of GJIC was significantly increased when cells were transferred from low-calcium to high-calcium medium, with concomitant depletion of G-actin stocks and rapid increase of F-actin bundles, probably due to actin polymerisation [62]. Both micro-injection of anti-actin antibodies or cytochalasin D treatment decreased GJIC between rat cultured astrocytes, suggesting that the morphological integrity of microfilaments is fundamental for GJIC [140]. Cell-to-cell dye coupling was virtually eliminated in N-cadherin-deficient cells of mouse neural crest, suggesting that N-cadherin might modulate the gating of Cx43 junctional channels [54]. The implication of sites on the CT domain of several connexins (e.g. Cx43) in their regulation by different kinases raises the question of the degree to which phosphorylation and "chemically" (pH, insulin, ...) -induced gating mechanisms may share common elements. A possible interaction of p120 and β-catenin with the cytoplasmic tail of Cx43 might be involved in conformational changes of the latter, perturbing directly or indirectly the channel activity [54]. In the "chain and ball" or "particle-receptor" model of gating process, a part of the CT domain of the connexin is able, either directly by physically blocking the pore or indirectly by allosteric mechanisms, to obstruct the intercellular pore in response to different stimuli, including reduced cytoplasmic pH [10,11], insulin and insulin-like growth factor [12], $pp60^{v\text{-}src}$ [13], transjunctional voltage dependence [14,16,19,20] or CaM ("cork gating", Ref. [15]).

6.4. Expression, localisation and activity of other proteins

Besides their role of pore-forming subunits, some connexins now appear to mediate nonjunctional effects. Some of their mutations may for example impair cellular cytoskeletal organisation; Cx32 formation and/or Cx32-mediated GJIC was suggested involved in the formation of functional tight junctions and actin organisation [46]. In the latter study, stable Cx32 transfection resulted in an increase in circumferential actin filaments without a change of actin protein. In cultured astrocytes, GJIC inhibition during the formation of a monolayer resulted in discordance of actin stress fibers between neighbouring cells [141], whereas actin organisation remained unchanged in Cx43 knockout mice [142]. Forced expression of connexins (Cx43 or Cx32) was found accompanied by a profound reorganisation of the actin and myosin cytoskeleton in C6 glioma cells [143].

In hepatocytes from Cx32-deficient mice, the expression of tight junction proteins (occludin, claudin-1 and ZO-1) was significantly higher after Cx32 transfection, whereas Cx26 or Cx43 transfection never induced occludin expression [46]. Conversely, in this study, treatment with a GJIC blocker (18 β-GA) resulted in decreases of occludin and claudin-1 at cell borders in Cx32 transfectants, suggesting that Cx32 formation and/or Cx32-mediated GJIC may participate in the formation of functional tight junctions and actin organisation. In Cx43-deficient cells of mouse neural crest, the subcellular distribution of p120 catenin was altered [54]. Intact microtubules seem dispensable for the regulation of Cx43 gap-junctional communication, and it was suggested that Cx43 might anchor microtubule distal ends to gap junctions and influence microtubule properties [75]. Connexins (particularly Cx43) hemichannels appear to operate in nonjunctional regions of the plasma membrane, interacting directly with intracellular signalling cascades (particularly via activation of the Src/ERKs signal transduction pathway [144]). Connexins may thus provide intercellular signalling but also sites where intracellular signalling is transduced.

7. Conclusions and perspectives

Junctional channels, as numerous other membrane channels, are associated with a variety of molecules to achieve their physiological functions. In addition to coupling to auxiliary subunits that modulate the biophysical properties of the pore-forming subunit, the channels have been shown to interact with scaffolding proteins and cytoskeleton, and such interactions are essential for the channel regulation and targeting. There is increasing evidence that gap junctions are, as tight junctions, macromolecular assemblies consisting of integral membrane proteins and other proteins that bind to them. The characterisation of protein–protein interactions of connexins and of their functional importance is a recently emerging field. In the case of Cx43, the most abundantly expressed gap junctional channel-forming protein, able of binding with different proteins, gap junctions may also form a macromolecular structure, tentatively represented in Fig. 3. As emphasised by Duffy et al. [145], it is interesting to note that connexin and claudin multigene families share analogous structural and physical properties. They both exist as four transmembrane domain (tetraspan) proteins with intracellular amino and carboxyl termini, and both bind PDZ domains (either the first, for Cx43 [40], or the second, for claudin-1 [146]) of the MAGUK protein ZO-1 within the cell. Connexins form hexamers surrounding a hydrophilic pore allowing ions and small molecules to pass from cell to cell, whereas claudins would form hexamers that may create paracellular pores for ion and molecular diffusion in the spaces between cells (for review, see Ref. [147]).

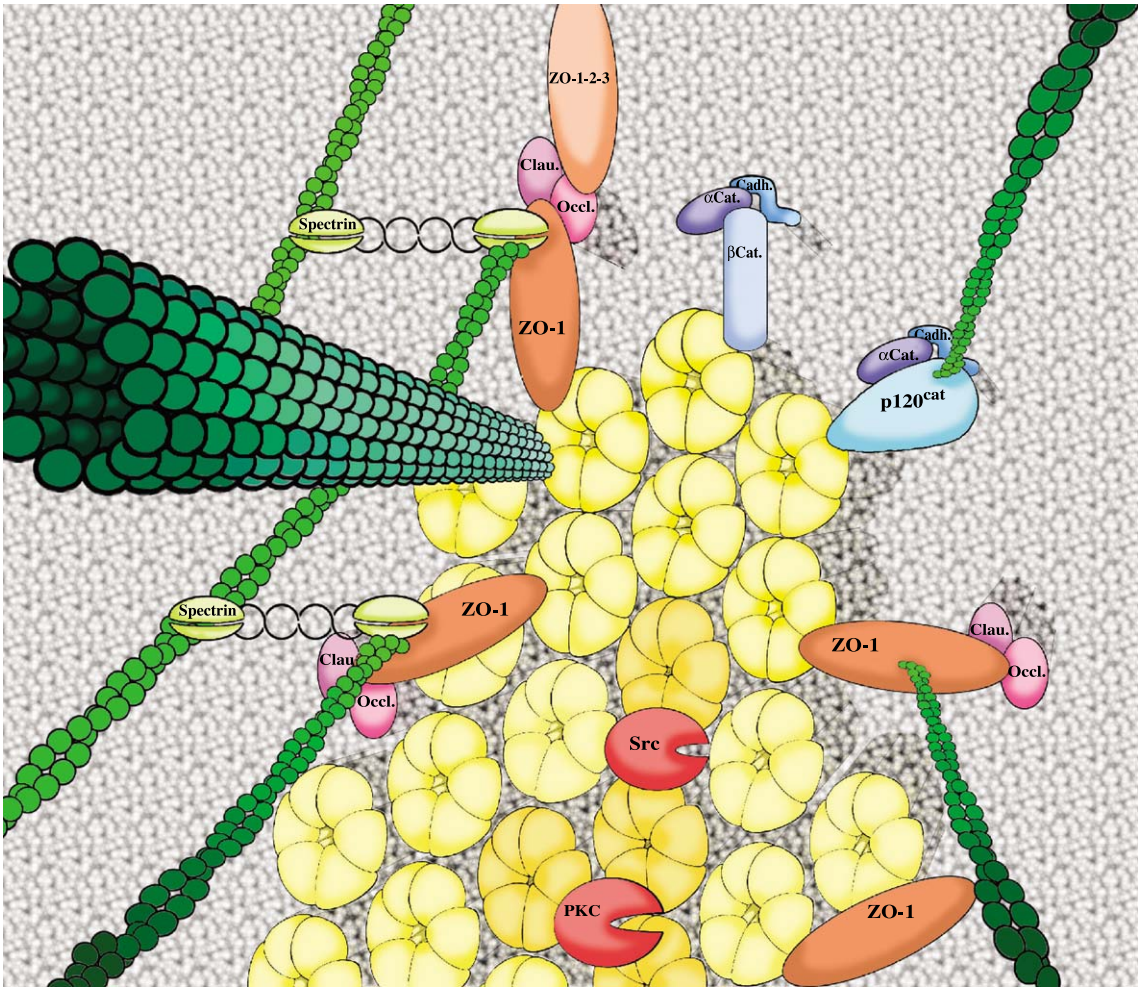

Fig. 3. Round about gap junctions. Artistic view of some of the cellular proteins found associated with Cx43-made channels or in their close surrounding.

For different membrane channels, the available evidence suggests that the association of scaffolding proteins with channels is part of a general mechanism that not only directs channel localisation within a cell but is also essential for regulation of channel function (for review, see for example Refs. [148,149]). Anchoring of membrane channels at specific subcellular sites is critical for their function, but the mechanisms underlying channel localisation and clustering are largely unknown. In the case of gap junctional channels, partner proteins appears to regulate the gap junction functions at several levels, including assembly of the junctions in the plasma membrane, connexin turnover as well as channel gating.

It is however essential to keep in mind that the identification of connexin partners (or of partners of other membrane proteins, as membrane receptors, transporters, and ion channels, of these proteins which span the plasma membrane multiple times and fold into a special structure involving non-linear peptide loops) request rigorous biochemical evidences via multiple approaches. Co-localisation observations in immunofluorescence microscopy need additional approaches (including for example in vitro binding assays, coimmunoprecipitation) to substantiate the interactions between connexins and the identified proteins. On the other hand, the screening of protein–protein interaction frequently result in identification of interactions which, although reproducible and specific, are not easily assessed. This is mainly due to the fact that proteins are misexpressed in a compartment from which they are typically excluded and therefore may be exposed to a repertoire of proteins that are not accessible in their natural situations. In addition, the fusion of a protein or a protein fragment to an effector protein (e.g. in two-hybrid or protein recruitment approaches) may result in misfolding of the protein and exposure of protein surfaces that are typically not accessible for association with other proteins.

In conclusion, the importance of protein–protein interaction in the regulation of connexin trafficking, assembly and disassembly, as well as the dynamic modulation of the opening state of junctional channels are progressively gaining support. Communication through gap junctions is sensitive to a variety of stimuli, including changes in the level of intracellular Ca^{2+}, pH, transjunctional applied voltage and phosphorylation/dephosphorylation processes. In any

case, identification of the cytoskeletal and signalling proteins that interact with connexins is only a first step in a long endeavour. The major challenges of the future are to quantify the stoichiometry of these protein interactions, to picture their geometry by microscopic and structural biological approaches, to reveal the dynamic regulation of gap junctional complexes and, perhaps most importantly, to understand the functional significance of these protein interactions in cell-to-cell communication.

Note added in proof

A receptor protein tyrosine phosphatase μ (RPTPμ) was recently found to interact via its first catalytic domain with Cx43 in diverse cell systems. RPTPμ (or a closely related PTP) interaction with the regulatory CT-Cx43 might then prevent Src-mediated closure of Cx43 gap junctional channels [150].

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