Chapter 9
Interaction Partners of Connexin Proteins

For the functioning of proteins, they need be placed at a proper location in the cell. Connexins, being the membrane proteins, are to be delivered to the membrane for the assembly and establishment of gap junction communication. However, connexins are known to be multifunctional proteins, and the mere formation of gap junction communication does not explain all the potential functions influenced by the connexins. In addition to the gap junction communication, connexins are part of various protein complexes, which control various physiological processes of the cell. Thus, connexins not only help in intercellular communication but also mediate intracellular communication by interacting with many other proteins. The number and diversity in connexin-associating partners has increased steadily over the past years, implicating the importance of these interactions for the proper functioning of gap junctions. Below are discussed the various interaction partners of connexins and the role of these interactions in regulating various cell functions.

9.1 Cytoskeleton Proteins

For the transport and proper docking of connexins to the membrane, they need to interact with various cytoskeletal proteins. This interaction is also important for the turnover of connexin proteins. Various cytoskeleton interaction partners of connexins include microtubules, actin, α-spectrin, debrin, etc.

Microtubules are dynamic structural polymers consisting of tubulin proteins. Cx43, being one of the major connexins, is known to interact with microtubules. The C-terminal domain of Cx43 has been found essential for the interaction. The amino acids in the C-terminal domain, which are critical for this interaction, have been mapped from 234 to 243 with the sequence of KGVKDRVKGL. Cx43 binds directly to α- and β-tubulin and that microtubules at the cell periphery co-localize with Cx43-based gap junctions. Based on multiple alignment studies, similar kinds of domain have been mapped in the Cx41 and Cx46. The interaction of connexin proteins with

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microtubules is essential in allowing direct transport of newly synthesized connexin hemichannels to the plasma membrane. Microtubules also interact with other junctional molecules such as cadherins, which allows increased specificity in targeting the connexins to the adherens junction, and this directs the delivery of newly synthesized connexins to areas of pre-existing adherens junctions.

Actin is the major cytoskeletal protein, and studies have revealed that Cx43 and actin co-localize in various cell types. Moreover, membrane cytoskeletal protein α-spectrin has been shown to co-immunoprecipitate with the gap junction. Another cytoskeletal protein, which has been found to interact with Cx43, is Drebin. Drebin is known to be involved in mediating cell polarity, and interestingly, studies have shown that Cx43 mediates cell migration. Whether the interaction of Cx43 with debrin is essential for mediating cell polarity and cell migration needs to be ascertained. In general, the interaction of connexin with the cytoskeletal proteins plays an important role in the life cycle of connexins by stabilization of gap junction proteins at the membrane and their internalization.

9.2 Junction Proteins

The gap junction proteins have been usually mapped in the region of plasma membrane where other junctional proteins exist. Thus, there exists a close association between the connexins and other junctional proteins. Both direct and indirect interactions of connexins with other junctional proteins have been demonstrated. The interaction between connexins and other junctional proteins is regarded to be essential for connexin assembly, trafficking, turnover, and channel gating. Hence, these interaction partners of connexin are critical for regulating the connexin life cycle, including membrane insertion, localization, and gap junctional plaque formation. At the plasma membrane, various junction proteins are known to interact with connexins, and these include Zonula occludens-1 (ZO-1), zonula occluden-2 (ZO-2), Occludin, Cadherins, and Catenins.

Zonula occludens-1 (ZO-1) and zonula occluden-2 are tight junction membrane-associated guanylate kinase proteins and are involved in the organization and trafficking of gap junctions. ZO-1 and ZO-2 interacts with the C-terminal domain of Cx43, and this interaction is mediated by the second PDZ domain of ZO-1. Similarly, studies have indicated that Cx45 interacts with ZO-1, and this interaction is mediated in a similar manner as that of Cx43. ZO-1 being a scaffolding protein can bind many other proteins, thus, increasing the potential of interaction partners of Cx43 with other proteins. Interestingly, the association between Cx43 and ZO-1 increases during certain conditions. For example, cardiac Cx43 gap junction remodelling occurs during cardiac development and in certain pathophysiological conditions. Moreover, the preferential interaction of Cx43 either with ZO-1 or with ZO-2 is determined by the stage of cell cycle. Connexin 43 interacts preferentially with ZO-1 during G0 stage, whereas the interaction with ZO-2 is preferred during the

other stages of cell cycle. Based on the homology studies, the PDZ domain has been found to be present in other connexins, thus raising the potential of other connexins to interact with ZO-1/2.

Occludin is a transmembrane protein and has been found to co-localize with gap junction plaques. Freeze-fracture and co-immunoprecipitation studies have concluded that the Cx32 is a potential interaction partner of occludin. Similarly, Cx26 has been reported to interact with occludin. Interaction of connexins with occludin is regarded essential for the proper assembly of connexin at the membrane.

Adherens are another class of junctional proteins, which form close associations with the gap junction proteins. Many functions of gap junctions, like cell proliferation and cell growth, are thought to be mediated by forming functional associations with adherens. Moreover, adherens are regarded to modulate the assembly of connexins at the cell membrane. Thus, based on these functional links, it is presumed that adherens form important interaction partners of connexins. One such important interaction partner of Cx43 is β-catenin. β-catenin is a multifunctional protein, having both membrane and nuclear localization properties. β-catenin has both cell adhesion characteristics and transcriptional properties. Cx43 interacts with β-catenin and co-localizes mainly at the cell membrane. Both β-catenin and Cx43 play a role in cell growth, and the interaction of β-catenin with Cx43 is regarded as one of the mechanisms used by Cx43 in regulating cell growth. Besides Cx43, other connexins, like Cx26 and Cx32, have been shown to interact with the α-catenin. Similarly, it is established that the Cx30, Cx32, and Cx43 associate with both α-catenin and β-catenin in mammary epithelial cells. Studies have shown that this interaction is important for sequestering β-catenin away from the nucleus in differentiation permissive conditions. Besides the role of connexin–catenin interaction in regulating cell growth and differentiation, studies suggest that the interaction of α-catenin with connexins is critical for the assembly and trafficking of the gap junction proteins to the membrane.

Additionally, co-immunoprecipitation and co-localization studies have shown that N-cadherins are another type of adherens proteins that interact with Cx43. This association is regarded to be critical for the gating of Cx43 channel, as mice lacking N-cadherins show decreased cell coupling.

9.3 Enzymes

As mentioned previously, connexins are the target of various post-translational modifications, and among these, phosphorylation is the most prominent one. Most of the connexins are phosphoproteins and thus are the target of various kinases and phosphatases. The dynamics of phosphorylation and dephosphorylation regulates various aspects of connexin functions, like gating, interaction with other proteins, assembly and degradation, cell growth and differentiation, etc. For carrying out phosphorylation and dephosphorylation, it is imperative that

connexins interact with various kinases and phosphatases. Various kinases are known to target connexins in general and alter their function. These kinases include tyrosine kinases and serine/threonine kinases.

The tyrosine kinases are the enzymes that phosphorylated the tyrosine residues in the target proteins. Various signalling molecules activate these kinases, and the majority of these are the growth factor signalling molecules. The binding of the signalling molecules to the receptor tyrosine kinases (RTKs) activates their intrinsic tyrosine kinase activity, which in turn results in the phosphorylation of the tyrosine residues present on the cytosolic face of these receptors. This trans-phosphorylation event of the receptor subunits and/or the associated proteins is known as trans-autophosphorylation. The intracellular signalling proteins that bind to phosphotyrosine residues on activated receptor tyrosine usually share two highly conserved non-catalytic domains called SH2 and SH3 (SH stands for Src homology regions 2 and 3 because they were first found in the Src protein, which has a role in cancer). It has been demonstrated that Cx43 is the target of various tyrosine kinases and one such known kinase is v-Src. In the *Xenopus* oocytes, mutation of the putative v-Src phosphorylation site in the Cx43 results in lack of gap junction closure by v-Src. It has been shown that the phosphorylated Tyr265 in Cx43 forms a docking site for the SH2 domain of v-Src and the SH3 domain of v-Src can bind to a proline-rich stretch in Cx43. In addition, Tyr247 can be phosphorylated by v-Src. The phosphorylation of Cx43 Tyr247 leads to the channel closure. Moreover, phosphorylated Cx43 binds to c-Src via the SH2 domain, competing with ZO-1 binding to the connexin protein.

The involvement of serine/threonine kinases in gap junction regulation has also been well documented. For example, PKC acts on Ser368 of Cx43, resulting in inhibition of gap junctional intercellular communication (GJIC). Interestingly, PKCε, which is implicated in inhibition of GJIC downstream of the fibroblast growth factor-2, is also known to co-immunoprecipitate and co-localize with Cx43 in cardiomyocytes. However, an upregulation of GJIC occurs by PKCα concomitant with Cx43 phosphorylation, and as such, maximal Cx43 phosphorylation and GJIC are observed during mammary gland differentiation and lactation. Moreover, Cx26, 32, and 43 are regulated by MAP kinases during differentiation of rat mammary epithelial cells. Interestingly, MAPK interact with Cx43 and mediate its phosphorylation; however, the effect of this phosphorylation on gap junction gating and function remains controversial. The seemingly contradictory effects of PKCs on Cx43 and GJIC likely reflect the complexity of channel regulation, where it may regulate and alter the permeability of gap junctions and hemichannels.

The phosphorylation of the connexins by various kinases demands that these events need to be regulated by dephosphorylating the target connexins. This action is mediated by the specific phosphatases. To perform such kind of reactions, the phosphatases need to interact with various connexins. In fact, numbers of interaction partners of connexins are the serine/threonine and tyrosine phosphatases. The role of serine/threonine phosphatases is to limit gap junctional conductance. These effects of phosphatases have been observed in cardiac myocytes upon conditions of hypoxia or metabolic starvation and also upon stimulation of T51B rat liver

epithelial cells with EGF. Similarly, phosphatases have been shown to function upon treatments of cells with known gap junction inhibitors such as 18-β-glycyrrhetinic acid and 2,3-butanedione monoxime. On the other hand, tyrosine phosphatases have a role in enhancing GJIC, as inhibition of the activity of these enzymes significantly decreased GJIC in various cell types.

9.4 Other Proteins

Besides the associating proteins discussed so far, other interacting connexin partners are known to interact with connexins either directly or indirectly. These include plasma membrane ion channels, membrane transport proteins, receptors, aquaporin-0, and acetylcholine receptors. Various connexins have also been shown to interact with calmodulin, which has been implicated in control of connexin channel gating. Furthermore, the calcium-/calmodulin-dependant kinase II (CaMKII) interacts with and phosphorylates Cx36 and mediates channel gating. CaMKII has also been shown to mediate enhanced gap junctional coupling, mostly through Cx43, in response to high extracellular K^+ in mouse spinal cord astrocytes. Thus, this interaction of connexins with CaMKII may have a general regulatory role in neuronal signal transmission, with a role in electrical coupling in addition to the defined role of CaMKII in chemical synaptic transmission. Other interactions include cholesterol, which was shown to interact with gap junction channels in different ratios during fibre cell differentiation in embryonic chicken lens. Moreover, Cx43 is known to translocate to mitochondria and interacts there with the heat shock protein 90 (Hsp90) and translocase of the outer mitochondrial membrane (TOM).

Furthermore, we have recently shown that Cx43 interacts with an adenosine triphosphate-sensitive K^+ channel (Kir6.1). Adenosine triphosphate-sensitive K^+channels are the protein channels responsive to changes in ATP/ADP ratio and provide a means to couple movement of potassium ions, to relate membrane potential with cellular energy status. Both Cx43 and Kir6.1 are known for their role in cell protection during stress conditions. Moreover, studies have shown that Cx43 and Kir6.1 are part of the same signalling pathway that imparts protection during ischaemia preconditioning. Interestingly, it was shown that phosphorylation at Serine 262 of Cx43 was indispensible for the interaction with Kir6.1. Studies have shown that the status of Cx43 phosphorylation is regulated in response to various physiological functions and has been attributed to alter interaction of Cx43 with other proteins. Moreover, phosphorylation of Cx43 has been shown to be indispensable for various protective treatments such as ischaemic preconditioning.