Abstract

Gap junction proteins, connexins, are dynamic polytopic membrane proteins that exhibit unprecedented short half-lives of only a few hours. Consequently, it is well accepted that in addition to channel gating, gap junctional intercellular communication is regulated by connexin biosynthesis, transport and assembly as well as the formation and removal of gap junctions from the cell surface. At least nine members of the 20-member connexin family are known to be phosphorylated en route or during their assembly into gap junctions. For some connexins, notably Cx43, evidence exists that phosphorylation may trigger its internalization and degradation. In recent years it has become apparent that the mechanisms underlying the regulation of connexin turnover are quite complex with the identification of many connexin binding molecules, a multiplicity of protein kinases that phosphorylate connexins and the involvement of both lysosomal and proteasomal pathways in degrading connexins. This paper will review the evidence that connexin phosphorylation regulates, stimulates or triggers gap junction disassembly, internalization and degradation.

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Keywords: Connexin; Gap junction; Internalization; Degradation; Phosphorylation

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1. Introduction

Since Paul and colleagues cloned and sequenced the first connexin (Cx) Cx32 in 1986 [1], the connexin family has grown to 19 mouse and 20 human members [2]. Not surprisingly, the connexin gene family shares indisputable sequence and structural similarities that fit to a model where these polytopic integral membrane proteins pass through the membrane four times yielding two extracellular loops with the amino and carboxyl termini exposed to the cytoplasm [3–8]. Connexins share common features of having extra-cellular loop disulfide bonds thought to impose a conformation critical for the docking and functional assembly of gap junction channels [9]. While connexins collectively share many similar features, they differ considerably in the size of the cytoplasmic loop region and the length of the carboxy terminal tail [10–12]. In the case of Cx43, the carboxy terminus is extensively phosphorylated by at least five different protein kinases at 12 or more different motifs containing serine or tyrosine residues [13–15]. The vast majority of connexin phosphorylation studies have focused on Cx43 due to the fact that it is the most extensively found connexin in mammals, many antibodies and mutant cDNAs exist and, most importantly, it is highly regulated by direct phosphorylation events. Nevertheless, most other connexins are also phosphorylated lending credence to the hypothesis that connexins may share some common regulatory events associated with phosphorylation. While this is an appealing thought, Cx26 is not phosphorylated [16], highlighting the fact that there are no generic phosphorylation properties that apply to all connexins. In this review, we will focus on events that occur at the cell membrane that may regulate connexin disassembly, internalization and degradation.

2. Connexins, connexons and gap junctions

Gap junctions are specialized intercellular membrane channels composed of connexins that allow small molecules of less than a 1000 Da, including secondary messengers, to selectively pass from one cell to another [17,18]. Gap junction channels typically form between cells of the same type (homocellular gap junctions) although they have been found to form between cells of different types (heterocellular gap junctions) [19–21]. Six connexins oligomerize to form a hemichannel or connexon [11]. It has long been known that most apposing cells in mammals contain gap junctions and there exists strong evidence indicating that many, if not most, cells express more than one member of the connexin family [10,22–24]. Both homomeric connexons (composed of the same connexin) and heteromeric connexons (composed of two or more different connexins) exist in vitro and in vivo [25–30]. In homotypic gap junction channels, connexons from one cell pair precisely with identical connexons from an apposing cell [31,32]. It has been demonstrated in vitro that heterotypic gap junction channels between apposing cell membranes can form where connexons of one type dock with connexons of a different type [33–36]. Therefore, in addition to homomeric, homotypic and homocellular organization of connexins, a diverse arrangement of heteromeric, heterotypic and heterocellular gap junctions exists between apposed cells. Importantly, the connexin constituents of a gap junction channel convey selectivity in the array of small molecules that are exchanged between neighboring cells [17,18].

3. Overview of the life cycle of connexins

Gap junctions are dynamic with an in vivo half-life of ~5 h in liver hepatocytes [37] and 1.3 h in the heart [38]. Likewise, in vitro pulse-chase studies demonstrated that Cx32 and Cx43 have a t_{1/2} of 1.5–3.5 h [39–44]. While it is apparent from pulse-chase studies that most connexins examined have similar turnover rates in vitro, it is also clear that these features are not entirely generic to all connexins. Cx56 in lens cultures was found to have a subpopulation that had a long half-life of over 36 h [45]. Moreover, cells treated with brefeldin A to inhibit protein transport to the cell surface revealed that some cells, like BICR-M1R_{12} cells, will clear pre-existing gap junctions from the cell surface at a rate that equates with the predictions from pulse-chase studies [42]. However, in NRK cells it is not possible to completely clear the pre-existing gap junctions using the same brefeldin A block [42]. Collectively, these studies suggest that in some cell types or for some connexins there may exist a more stable subpopulation of connexins.

Consistent with other polytopic membrane proteins, connexins are co-translationally inserted into the endoplasmic reticulum [46] (Fig. 1). We and others have demonstrated that Cx43 follows a classical secretory pathway involving transport through the Golgi apparatus [42,47–49]. Cx43 and possibly Cx46 do not appear to oligomerize into connexons while in the endoplasmic reticulum but most likely in the trans Golgi Network (TGN) [49,50]. However, substantial evidence also exists that at least Cx32 and Cx26 can oligomerize within endoplasmic reticulum membranes [6,7,51]. To ensure the integrity of the compartments involved in connexin secretion, our model proposes that connexons would be closed while retained within the Golgi apparatus and during their transport via vesicles and/or tubular extensions (Fig. 2, pink arrows) to the cell surface where they appear as hemichannels [52] (Fig. 1). While cell surface hemichannels can be gated open [53], they are expected to primarily exist in a closed state. In co-ordination with intercellular adhesion [54,55], connexons from one cell would pair with connexons from a neighboring cell to form intercellular gap junction channels. These channels cluster to form tightly packed arrays of gap junction
channels referred to as gap junction plaques (Figs. 1 and 2, white arrows). Studies using green fluorescent protein-tagged Cx43 suggest that gap junction channels may not open until they cluster into plaques [56]. Interestingly, the tight junction-associated protein, ZO-1, has been shown to bind to Cx43, Cx45 and other members of the connexins family via a PDZ domain [57–59]. More recent evidence suggests that ZO-1 interactions with connexins may play a role in regulating the size of the gap junction plaque [60]. It remains to be determined if ZO-1 plays an inhibitory role in gap junction formation or facilitates assembly.

One mechanism of gap junction internalization is via the formation of annular junctions where an entire, or fragment of a, gap junction is removed into one of the two contacting cells [61]. This unique mechanism, while considered an endocytic pathway, is akin to phagocytosis where one cells ingests a large fragment of a contacting cell. Annular junctions are well supported by both classical electron microscopy studies [61] and by more recent studies using epitope and fluorescent protein-tagged Cx43 [62,63] (Figs. 1 and 2 blue arrows). Annular junctions are distinctive in that hundreds of gap junction channels are removed from the cell surface at once, thus dramatically and rapidly reducing gap junctional intercellular communication. An alternate pathway of gap junction removal where gap junctions disassemble into small aggregates [64] or connexons that subsequently internalize into endosomes has not been ruled-out. Also, there is little evidence that connexons once internalized...
can recycle to form new gap junction channels but this issue remains poorly studied.

Interestingly, there is evidence to support the view that gap junctions and connexins are degraded by both a lysosomal pathway and a ubiquitin-dependent proteasomal pathway [39,44,65–69] (Fig. 1). Electron microscopy studies have described annular junctions in close association or within endosomes/lysosomes [69–71] and other biochemical studies have concluded that connexins may be degraded, at least in part, within the lysosome [44,69]. However, studies using proteasome inhibitors and cell lines that lack the E1 enzyme necessary for ubiquitination have revealed that proteasomes play a critical role in both wild-type and mutant connexin degradation [39,44,65]. Moreover, since the turnover of Cx43 in the heart is rapid, it has been proposed that connexin degradation may be important for the regulation of intercellular communication under pathophysiological situations [72]. Interesting, mutagenesis of a putative tyrosine-based sorting domain (286YKLV289) encoded within Cx43 significantly stabilized Cx43 and may act as a determining motif for Cx43 internalization/degradation and, thus, overall levels of gap junctional intercellular communication [73]. Most recently, it has been documented that the binding of ubiquitin may serve as a molecular internalization signal which has been shown for the E-cadherin complex [74]. While two papers have reported that Cx43 is ubiquitinated [65,68], it remains unclear if other connexins are ubiquitinated and whether proteasomes and lysosomes work in tandem for complete connexin degradation. In the absence of the polytopic connxin being extracted from the plasma membrane into the cytosol, it is difficult to model a mechanism where the proteolytically active groups that reside on the inner most surface of the proteasome [75] would be capable of degrading a connexin that is retained within a lipid bilayer.

4. Connexin phosphorylation

This manuscript will not attempt to review the literature on connexin phosphorylation as several excellent reviews on this topic have recently been published [13,15,76]. Instead we will focus on only the phosphorylation events where evidence is presented that such events may regulate, or are correlated, with gap junction turnover. The role of connexin phosphorylation may differ considerably among cell types, stages of the cell cycle, immortalization or transformation status, three-dimensional environment, growth factor milieu and extracellular matrix interactions. Without question, the vast majority of the literature on this topic focuses on Cx43 where all the reported phosphorylation sites are localized to the 17-kDa carboxyl terminal domain that extends freely into the cytoplasm. To begin to understand the role of phosphorylation in regulating Cx43, it is notable that this connexin is phosphorylated by at least protein kinase C, casein kinase 1, mitogen-activated protein kinase, Src kinase and p34cdc2 at in excess of 12 sites [13,15,76] while Cx26 is not phosphorylated at all [16]. Between these two extremes most connexins are thought to be phosphorylated to some extent and an extensive dissection of the kinases involved and the sites phosphorylated is beginning to unfold. The principal question in this review is how a phosphorylation event might drive mechanisms leading to gap junction internalization and degradation.
One of the best examples of phosphorylation being correlated with, or triggering, Cx43 internalization is when cells exit the \( G_2 \) phase and entering mitosis. Goodall and Maro [77] first showed that there was a loss of gap junction coupling during mitosis in the developing mouse embryo. This original finding was confirmed in Rat1 and human endothelial cells and further explored in parallel by three laboratories that collectively showed that Cx43 was phosphorylated by p34\(^{cd2} \) protein kinase to a unique phospho-isoform [78–80]. Kanemitsu et al. [79] and Lampe et al. [80] used an activated temperature-sensitive mutant of p34 cdc2 protein kinase to conclusively show that Cx43 became extensively phosphorylated and, furthermore, phospho amino acid analysis of tryptic peptides revealed that this phosphorylation event occurred at S255 and S262. Consistently, a direct or indirect Cx43 serine-based phosphorylation event at the onset of mitosis was supported in endothelial cells. Xie et al. [78] went on to show that sister cells could reestablish gap junction communication in the presence of inhibitors of protein synthesis suggesting that there was a pool of intracellular Cx43 available to reestablish gap junction coupling. In vitro studies by Kanemitsu et al. [79] using purified p34\(^{cd2} \) protein kinase and synthetic peptides further confirmed the Cx43 S255 phosphorylation site. p34\(^{cd2} \) protein kinase-dependent phosphorylation of Cx43 correlated with cells rounding and gap junction internalization into large structures which resemble internalized annular junctions [78,80] (Fig. 3). The fate of these internalized gap junctions is not entirely clear but protein degradation inhibitor studies would suggest that they are degraded [80] (Fig. 3).

Cell cycle-regulated Cx43 phosphorylation was further explored by Lampe et al. [81] and Solan et al. [82] when they showed that PKC phosphorylation of Cx43 at S368 was prevalent in cells at the S/G2 transition. However, this phosphorylation event was more readily correlated with p34\(^{cd2} \) kinase-dependent phosphorylation of Cx43 is correlated with gap junction internalization at the onset of mitosis.

![Diagram](image-url)

**Fig. 3.** p34\(^{cd2} \) kinase-dependent phosphorylation of Cx43 correlates with gap junction internalization at the onset of mitosis. 1: In this model we propose that soluble p34\(^{cd2} \) protein kinase is directly recruited to plasma membrane at the beginning of M-phase where it phosphorylates Cx43 to a novel isoform. However, it should be noted that an indirect p34\(^{cd2} \) kinase-dependent phosphorylation of Cx43 might also exist. 2: Upon phosphorylation, Cx43 channels are predicted to close. 3a: Gap junctions (GJ) subsequently internalize as annular junctions or 3b: by undetermined pathways prior to 4: being degraded by either lysosomes or proteasomes.
decreased ability of Cx43 to be assembled into gap junctions as opposed to being a possible internalization or degradation trigger [82].

6. Phosphorylation of Cx43 in steady-state cells

One of the difficulties in examining the phosphorylation status of internalized gap junctions, commonly referred to as annular junctions, has been the inability to trap and isolate pure populations of annular junctions for biochemical and phosphorylation analysis. Moreover, the likelihood of a second mechanism for internalizing connexins remains quite high. In one study, a specific monoclonal antibody that only recognized unphosphorylated Cx43 was used to show that gap junction plaques contained both unphosphorylated and phosphorylated Cx43 while annular junctions were not labelled, indirectly suggesting annular junctions contain primarily phosphorylated species of Cx43 [83]. The vast majority of connexin phosphorylation studies have focused on treatments or conditions that either up- or down-regulate the phosphorylation status of the connexin population. In the case of Cx43, this approach is often combined with a biochemical dissection of the phosphorylation species as revealed by variations in the migratory behaviour of Cx43 on SDS-polyacrylamide gel electrophoresis [84–86]. While this remains a useful index of Cx43 phosphorylation changes, Cx43 phosphorylation events do not always correlate with changes in the mobility of Cx43 isoforms in polyacrylamide gels and, furthermore, this approach provides little information on protein kinases, degree of phosphorylation or the residues involved [15,82]. The limitations of such approaches have reduced conclusive assignment of phosphorylation events to gap junction assembly and/or removal from the cell surface. For example, as appears to be the case when Cx43 is phosphorylated by protein kinase C [82], if assembly alone is inhibited, the loss of gap junctions and accompanying gap junctional intercellular communication could be at a steady state level and not directly triggered by the phosphorylation event. Early biochemical pulse-chase studies revealed that the major phospho-isoforms of Cx43 had essentially the same half-life as unphosphorylated Cx43, suggesting that global Cx43 phosphorylation events did not drastically alter the life expectancy of Cx43 [41]. Refinements of these early experiments and additional studies on other members of the connexin family are beginning to show that this issue is more complex than first appreciated.

One of the best-studied reagents to enhance PKC activity and subsequent Cx43 phosphorylation is the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA). PKC-induced phosphorylation of Cx43 in WB-F344 rat liver cells has been shown to be correlated with gap junction internalization [87]. Interestingly, Klotz et al. [88] found that menadione induced Cx43 phosphorylation and reduced gap junction communication like TPA, but gap junctions were not stimulated to internalize. Lampe [89] found that TPA had a significant affect on Cx43 assembly in Novikoff cells and caused the Cx43 half-life to decrease from 3.1 to 2 h. Later the same authors showed that TPA activation of protein kinase C resulted in Cx43 phosphorylation at S368 [81]. Comparatively, TPA also decreased the half-life of Cx56 [90]. Consequently, collective evidence would argue for protein kinase C-based Cx43 phosphorylation and subsequent inhibition of gap junctional intercellular communication being related to both changes in gap junction assembly and degradation.

7. EGF-induced Cx43 phosphorylation and gap junction internalization

Several studies have reported that epidermal growth factor (EGF) induces Cx43 phosphorylation to novel phospho-isoforms [68,91–93]. In some cases this has been correlated with a decrease in gap junctional intercellular communication and a loss of gap junctions from the cell surface [68,91–93]. Upon reviewing the kinetics of these events, it is reasonable to postulate that EGF-induced phosphorylation triggers the internalization of Cx43. Leithe and Rivedal [68] explored this further in IAR20 rat liver epithelial cells where the EGF-induced phosphorylation and internalization of Cx43 were inhibited by a well-described PD98059 inhibitor of MEK1, suggesting a direct role for the mitogen-activated protein kinase pathway. The EGF-induced phosphorylation of Cx43 was found to be linked to ERK5 and not ERK1/2 [97]. Importantly, EGF-stimulation of IAR20 cells resulted in the increased ubiquitination of Cx43 which may act as a signal of Cx43 internalization [68]. Monoubiquitination of integral plasma membrane proteins is widely becoming a well-understood signal for regulated internalization [98]. Leithe and Rivedal [68] have gone on to provide evidence to show that EGF-induced gap junction internalization was inhibited by hypertonic sucrose, suggesting a role for clathrin in the internalization process. While electron micrographs have occasionally revealed clathrin to be associated with internalized gap junctions [99], little additional evidence exists for the role of clathrin in the internalization process. If clathrin does indeed play a mechanistic role in the internalization role of gap junctions, this could argue for a connexin internalization pathway that may be independent of annular gap junctions.

8. Evidence for the role of phosphorylation in the stability and internalization of connexins expressed in the lens

Compared to Cx43, there is a scarcity of studies that have examined the role of phosphorylation in regulating the life cycle of other connexins. Interestingly, the lens connexin, Cx56, was found to have a rapid half-life like other
connexins, but a second population of Cx56 required 2–3 days for turnover [45]. Likewise, lens Cx46 and Cx45.6 may have subpopulations with prolonged half-lives [100,101]. All three of these lens connexins are known to be phosphorylated. Casein kinase II and protein kinase C were shown to phosphorylate Cx45.6 [102] and mutagenesis studies revealed that when S363 was mutated to alanine in Cx45.6, the resulting mutant had a prolonged half-life in cultured lens cells [102]. Collectively Yin et al. [102] provided evidence that S363 was a casein kinase II phosphorylation site responsible for destabilizing the connexin leading to its degradation. Conversely, mutations in serine residues near the carboxy tail of Cx45 resulted in a destabilization of Cx45 and a more rapid degradation of the mutant connexin [103]. Thus, it remains unpredictable how the stability of a connexin may respond upon its phosphorylation. This issue will require extensive study.

9. Phosphorylation as a prerequisite to degradation

In general, connexin phosphorylation is not a generic prerequisite for connexins to be targeted for degradation as evidenced by the fact that Cx26 is not phosphorylated but still appears to have a short half-life [104] similar to Cx32 and Cx43 [40,41]. Additional evidence for this conclusion stems from studies which show that (i) Cx32–disease linked mutants that are retained in the endoplasmic reticulum or Golgi apparatus are rapidly degraded presumably in an unphosphorylated state [39]; (ii) P0, P1 and P2 species of Cx43 have similar turnover kinetics [41,105]; and (iii) subpopulations of Cx43 and Cx32 extracted from the endoplasmic reticulum membrane prior to any, or little, phosphorylation are degraded rapidly, likely via proteasomes [67]. While major changes in the degradation of connexins are not linked to their phosphorylation status, the kinetics of degradation for some connexin species and mutants are not identical, leading to the possibility that the phosphorylation status of connexins could play a part in fine-tuning their rates of degradation.

Overall, the evidence that direct phosphorylation of connexins sensitizes connexins for degradation is mixed. To fully understand connexin degradation, it is necessary to revisit the role of both lysosomes and proteasomes. The vast majority of studies linking Cx43 to proteasomal degradation are based on the use of specific proteasomal inhibitors which include lactacystin, MG138, ALLN and ALLM [39,65–69,106]. While these studies clearly show that Cx43 degradation requires functional proteasomes, they do not directly address whether connexins are the substrates that enter the proteasome nor do they mechanistically reveal how internalized gap junctions and connexins are extracted from the membrane to enter the proteasome. To date, only Cx43 and Cx32 have been examined for their potential role as substrates for proteasomal degradation. Two reports suggest that Cx43 is indeed a substrate for ubiquitination [65,68] with the more recent study being performed on cells stimulated by EGF [68]. No direct evidence currently exists that any other connexin is ubiquitinated prior to internalization or degradation. While ubiquination is not an absolute requirement for proteasomal degradation, it is the predominant means by which proteins are targeted to proteasomes. Increasing evidence suggests that monoubiquitin is linked to triggering integral membrane protein internalization while polyubiquitination is associated with targeting of proteins to proteasomes [98]. If the EGF-induced ubiquitination of Cx43 is indeed multiple monoubiquitination events, this may act as trigger for EGF-induced gap junction internalization as reported by Leithe and Rivedal [68]. In the case where a population of connexins is extracted from the endoplasmic reticulum membrane, these now soluble, misfolded molecules could easily become substrates for proteasomes even in the absence of ubiquitination. Intriguingly, cross-talk between Cx43 and ubiquitin ligases has been reported as Cx43 expression was shown to downregulate the levels of an S-phase kinase associated protein required for the ubiquitination of p27 [107,108]. However, it is unknown if a mechanism exists where connexin expression regulates ubiquitin ligases involved in its own ubiquitination.

The role of lysosomes in degrading connexins and gap junctions remains prominent. Connexins and gap junction fragments have been directly localized to lysosomes providing a convincing argument that lysosomes play a critical role in connexin degradation [69,70,109]. While lysosomal inhibitors, such as NH4Cl, chloroquine and leupeptin, tend not to cause as much of an increase in the half-life of Cx43 as proteasomal inhibitors in some cell types [44,66], these lysosomal inhibitors tend to be less specific and efficient. Properties of Cx43 degradation also appear to vary among cell types. MDA-MB-231 breast tumor cells appear to direct the bulk of their connexin content to lysosomes, yet proteasomal inhibitors enhance the accumulation of phosphorylated Cx43 species and increase overall gap junction plaque numbers at the cell surface [69]. In CHO cells, lysosomal inhibitors have modest effects on the half-life of Cx43 while proteasomal inhibitors are more potent at extending the life of connexins [66].

The understanding of connexin degradation pathways has additional complexity as it appears that subpopulations of Cx43 and Cx32 are translocated from the endoplasmic reticulum, possibly ubiquitinated, and assigned to a degradation fate in proteasomes [67]. Likewise, in MDA-MB-231 cells, Cx43 appears to escape the normal secretory pathway and prematurely enter lysosomes for degradation [69]. In both cases phosphorylation appears to play little or no role. In a recent study, the Cx26 disease-linked D66H mutant was found to reside in the TGN and had a delayed rate of degradation that was accompanied by changes in intracellular distribution when cells were treated with lysosomal inhib-
itors but not proteasomal inhibitors [104]. Clearly, since this is an unphosphorylated connexin, direct phosphorylation has no role in its assignment to lysosomes or proteasomes. Similarly, endoplasmic reticulum or Golgi-retained Cx32 mutants are subjected to similar turnover pathways. Similarly, endoplasmic reticulum or Golgi-ylation has no role in its assignment to lysosomes or ulation, requiring extensive connexin-specific studies. It is

10. Future perspectives

The mechanistic need for the regulation of connexins and gap junctions to be dynamic, with short half-lives, remains puzzling and virtually unprecedented for a family of polytopic membrane proteins. Consequently, while gating events regulate gap junction channel activity, the formation and removal of gap junctions will most often dictate the extent of gap junction cell–cell communication within tissues. Evidence for phosphorylation regulating Cx43 life cycle events is substantial. It is likely, however, that there are few generic connexin phosphorylation properties common to two or more members of the connexin family. In essence, early results with lens connexins would suggest that each connexin isoform may differently be regulated by phosphorylation, requiring extensive connexin-specific studies. It is intriguing to speculate that phosphorylation events can trigger gap junction internalization and the evidence that this is true for Cx43 has become substantial while not yet unequivocal. It is reasonable to propose that the internalization of gap junctions composed of other connexin family members may also be regulated by select and specific phosphorylation events.

11. Summary

Without doubt, selective and specific phosphorylation of Cx43 plays several roles in regulating the life cycle and function of Cx43. In the cases of p34^cdk2^ protein kinase and EGF-induced MAPK activation, these events have been shown to be tightly associated with Cx43 internalization and eventual degradation. It is furthermore intriguing that phosphorylation may regulate the ubiquination of Cx43. Finally, convincing data exist that functional proteasomes and lysosomes are necessary to successfully degrade select populations of Cx43 in a cell cycle- and a cell type-dependent manner.

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