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Kinase programs spatiotemporally regulate gap junction assembly and disassembly: effects on wound repair

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Abstract

Gap junctions are highly ordered plasma membrane domains that are constantly assembled, remodeled and turned over due to the short half-life of connexins, the integral membrane proteins that form gap junctions. Connexin 43 (Cx43), by far the most widely expressed connexin, is phosphorylated at multiple serine residues in the cytoplasmic, C-terminal region allowing for exquisite cellular control over gap junctional communication. This is evident during epidermal wounding where spatiotemporal changes in connexin expression occur as cells are instructed whether to die, proliferate or migrate to promote repair. Early gap junctional communication is required for initiation of keratinocyte migration, but accelerated Cx43 turnover is also critical for proper wound healing at later stages. These events are controlled via a "kinase program" where sequential phosphorylation of Cx43 leads to reductions in Cx43's half-life and significant depletion of gap junctions from the plasma membrane within several hours. The complex regulation of gap junction assembly and turnover affords several steps where intervention might speed wound healing.

Keywords

Gap junctions; Connexin43; wound repair; phosphorylation; src; Protein kinase C; Mitogen-activated protein kinase

1. Introduction

1.1. Gap junctions play diverse and essential roles in cells of different tissues

Vertebrate gap junctions are composed of proteins from the 21 gene connexin gene family [1–6]. These collections of intercellular channels, termed gap junction plaques, permit passage of metabolites of less than approximately 1000 Da between cells while macromolecules are excluded (although small RNAs may pass) [7, 8]. Many cell processes effectively require gap junctionalcommunication including control of cell proliferation,

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embryonic development, cell differentiation and the coordinated contraction of heart and smooth muscle [2, 3, 9–12]. Connexins have been implicated by genetic linkage analysis in at least 14 human diseases - many of which can be recapitulated in mutant connexin mouse models with common forms of hereditary deafness being the most prevalent in humans [12]. Connexins are expressed in a tissue specific manner allowing them to fulfill a variety of physiological roles. Cx43, the focus of this review, is by far the most abundantly and widely expressed gap junction protein (> 34 tissues and 46 cell types [5]). Cx43 knockout mice die within hours of birth [13]. Oculodentodigital dysplasia is caused by many different mutations in the Cx43 gene and exhibits a variety of different symptoms including small eyes, underdeveloped teeth, syndactyly and palmoplantar keratoderma – a skin disease that can be caused by frame shift mutations [14] that result in loss of the C-terminal region where Cx43 is phosphorylated. Wounding of the epidermis leads to significant changes in Cx43 expression that faciliate healing. These changes are, at least in part, a result of altered Cx43 phosphorylation.

1.2 Connexin Phosphorylation

Many connexins (e.g., Cx31, 32, 37, 40, 43, 45, 46, and 50) have multiple phosphorylation sites – with Cx43 being the most prevalent in both tissues and cultured cells. Connexins have 4 transmembrane domains with the N- and C-termini on the cytoplasmic side. Many reports, both *in vivo* and in cell culture, indicate that Cx43 has a half-life in the range of 1–3 h [5, 15–25] - much faster than typical integral membrane proteins (17–100h) [26, 27]. At least nineteen of the twenty-six serines and 2 of the 6 tyrosines in the C-terminal region of Cx43 have been identified as phosphorylation sites present in cells or tissue, and there has been some progress in the characterization of the network of kinases that phosphorylate Cx43 (Table 1). Cx43 that lacks the last ~140 residues of the C-terminal portion can form gap junctions but the resulting gap junctions have different permeability/ electrophysiological properties [28–30]. A “knock-in” mouse expressing Cx43 lacking this C-terminal region developed rigid skin with a defective epidermal layer that readily peels off. Almost all homozygote mutant mice died shortly after birth due to dehydration and Cx43 was found mis-localized throughout the stratified layers of the epidermis rather than restricted to the basal cells [31].

2. Cx43 assembly into gap junctions and turnover

2.1. Cx43 lifecycle is dynamic and complex

Cx43 is characterized by a short half-life and extensive regulation that allows the cell to exquisitely control gap junctional communication. This is evident through biochemical analyses, where Cx43 phosphorylation has been shown to regulate protein localization and behavior in a rapid and coordinated manner. Recently developed live cell imaging modalities have been particularly useful for visualizing dynamic interactions of Cx43 with the cytoskeleton and events at the gap junction. An understanding of how different subcellular pools of Cx43 are regulated and feedback on each other is critical to understanding gap junction biology. While intercellular transfer of molecules is a major function of the gap junction plaque, the sheer number of molecules that interact with Cx43 and are found at the gap junction plaque certainly argue for an important role in cell

signaling. Gap junction plaques have been suggested to provide a type of "nexus" for coordinating subcellular events [32]. In the subsequent sections of this review, we will follow Cx43 through its lifecycle with a particular emphasis on how its coordinated interactions with kinases appears to regulate and potentially provides feedback to tightly control gap junction assembly and disassembly.

2.2.1. Gap junction assembly—As an integral membrane protein, Cx43 is synthesized and traffics through the endoplasmic reticulum. It lacks a canonical membrane signal sequence and delays oligomerization into a hexameric hemi-channel or “connexon” until reaching the trans-Golgi network [33]. This delay may provide a quality control step and allows feedback control as GJ assembly can be downregulated through Endoplasmic Reticulum Associated Degradation (ERAD) during conditions of cellular stress [34, 35]. Live cell and other imaging modalities show that Cx43 can traffic to the plasma membrane via multiple mechanisms including the secretory pathway or microtubule based vesicle targeting and there is evidence that these pathways can be differentially utilized under different conditions [25, 36, 37]. Cx43 typically moves from the plasma membrane into the periphery of the plaque, thus plaques "grow" from the outside in and the oldest proteins are found in the center of the plaque where they get selectively turned over [38].

2.2.2. Gap junction assembly and Cx43 phosphorylation—Cx43 phosphorylation events can occur within 15 min of synthesis [16] and several kinases appear to affect the assembly of gap junctions. Cx43 migrates as multiple bands in SDS-PAGE with many cell types displaying prominent bands often labeled P0, P1 and P2 which represent sequential stages of Cx43 processing from the plasma membrane to the gap junction in untreated cells. These SDS stable conformational changes in Cx43 structure have been shown to be due to sequential phosphorylation first on S365 then some combination of residues S325/328/330. S365 phosphorylation was found to be present in the P1 and P2 phosphoisoforms and occurs during the transition from the cytoplasm to the plasma membrane [39, 40]; see Fig. 1. Furthermore, phosphorylation at S365 plays a “gatekeeper” role by preventing downregulation of gap junctional communication by subsequent Cx43 phosphorylation at S368 [39]. Conversely, prior phosphorylation at S368 due to PKC activation could decrease gap junction assembly. Activation of cAMP-dependent protein kinase (PKA) can increase Cx43 phosphorylation at S364 and S365 and stimulate trafficking to the plasma membrane resulting in enhanced gap junction assembly (see Fig. 1 and Table 1). Casein kinase 1 (CK1) phosphorylates Cx43 on S325/328/330 during the transition of Cx43 from the plasma membrane into the gap junction – staining with an antibody to these phosphorylated residues exclusively recognizes the P2 form of Cx43 in SDS-PAGE and only Cx43 present in the gap junction [41]. Phosphorylation of serine 373 by Akt allows gap junctions to grow in size (see Fig. 1) but is an event that can be linked to gap junction disassembly (discussed further below). Activation of PKC can halt assembly of new junctions through an unknown mechanism [17]. Thus, the process of gap junction assembly is regulated by a succession of kinases that constitute a “kinase program” with multiple built-in checkpoints.

2.3 Cx43 and gap junction turnover

The regulation of connexin turnover and gap junction turnover are still not well understood. This is, in part, due to the fact that while the turnover of connexin molecules is fairly consistent across cell types and conditions, the stability of individual gap junction plaques can be highly variable [42]. This is presumably due to altered kinetics of connexin assembly into and removal from a given plaque, as described in section 2.2.1. What is known is that inhibition of lysosomal degradation can prolong the half-life of Cx43 protein but not necessarily gap junctions while proteasomal inhibition increases and stabilizes Cx43 present in gap junctions in most cell types. In some situations, plaques can remain stable for many hours, presumably through equilibrium of protein moving in and out of the gap junction, while at other times a single plaque can be rapidly and wholly disassembled. As an example, Fig. 2A and the associated movie show a time lapse series of Cx43-green fluorescent protein (GFP) fusion where one gap junction breaks up and is internalized (white arrow) while another gap junction remains relatively stable (white arrowhead) throughout the 30 min movie (frames taken at 20 second intervals). Lysosomes are visualized in red (via LysoTracker) and appear to be partially co-labeling with the gap junction throughout the disassembly process in the time lapse movie. To illustrate the dynamic nature of this interaction, Fig. 2B shows a time lapse series taken at 2 second intervals showing co-labeling of lysosomes with membranes containing Cx43-GFP. The dynamic interactions observed in these types of images suggest that lysosomes may be involved in more than simply engulfing and degrading already internalized membranes containing Cx43.

We also do not understand the range of potential cellular machinery involved in connexin and gap junction turnover. There is good evidence that a gap junction can be internalized in its entirety via formation of a double membrane structure termed an annular junction or connexisome [5, 43–50] (see mode 1, Fig. 3). Annular junctions have a distinct structure that is recognizable by electron microscopy, and are increased during the process of autophagy where they co-localize with the clathrin adapter proteins Dab2 [49], Atg14 and 9 [51] and the autophagosome membrane protein LC3 [44, 52, 53]. These structures are generally accepted to be precursors to lysosomal degradation. Whether annular junction formation is the exclusive way gap junctions are turned over and the process is just upregulated during autophagy is less clear. Given that annular junctions appear to vary widely in their prevalence within different cell types and cellular treatments while the half-life of Cx43 appears to be consistently short leads us to hypothesize that annular junction formation may be a mechanism utilized specifically during enhanced turnover, such as reduced nutrient triggered autophagy and growth factor induced gap junction turnover.

Another potential mechanism would posit that gap junctions can be turned over via loss of extracellular Cx interactions (i.e., “unzippering” of the gap junction) followed by endocytosis from a single membrane (see mode 2, Fig. 3). At first glance such a mechanism seems unlikely given the stability of isolated gap junctions to relatively harsh conditions and a lack of direct evidence, but time-lapse movies of Cx43-GFP present in gap junctions make it clear that there are dynamic interactions occurring within and around gap junctional plaques as shown in the Fig. 2. It is also clear that kinases and phosphatases regulate the assembly and stability of gap junctions which is, at least, consistent with the idea of an

intracellular pathway controlling extracellular disassembly. Finally, a third mechanism for loss of gap junctions would simply result from blockage of new gap junction formation while existing gap junctions are turned over. We would predict that further studies will show that these and perhaps other mechanisms of internalization will be utilized by cells in a context specific manner.

2.4 Cx43 turnover and the role of ubiquitinylation

Cx43 has been reported to undergo ERAD [34, 35] but the process was found to be independent of Cx43 ubiquitinylation as another protein, CIP75, regulated its degradation by the proteasome [34]. Cx43 contains 2 putative tyrosine-based sorting signals (Yxxφ; where φ=hydrophobic) [54] including a key one involving residues 286–289 as Cx43 with a V289D mutation had a 3-fold increase in protein half-life [55]. However, this sorting signal was reported not to regulate ubiquitin-mediated Cx43 internalization [56]. Furthermore, gap junctional stability can be dramatically reduced in response to growth factors and phorbol esters [17, 57–64]. Many different reagents that can efficiently inhibit lysosomal or proteasomal protein degradation partially affect gap junction and Cx43 turnover but do not have complete effects. These and earlier results [65, 66] have led several groups to propose multiple and alternative pathways involving the proteasome and lysosome in Cx43 and gap junction degradation. There are many studies that have shown that if cells are treated with a proteasomal inhibitor and then Cx43 is immunoprecipitated and immunobloted with an antibody to ubiquitin, one sees multiple bands at higher molecular sizes implying poly-ubiquitination [65–67] or multiple instances of mono-ubiquitination [64, 68]. In addition, a recent study indicated that a deubiqutinase plays a key role in regulating gap junction turnover [67] and non-directed screens for ubiquitinated proteins have identified Cx43 [69]. However, we are not aware of reports where inhibition of the proteasome or other factors potentially involved in Cx43 degradation show significant accumulation of Cx43 at higher molecular sizes consistent with addition of ubiquitin when compared to total Cx43 levels with an antibody to Cx43, so it seems that the level of any Cx43 ubiquitination even in the presence of inhibitors is very low. One study that tried to estimate the level of ubiquitinated Cx43 found that it was less than 1% [70] and in another, the putative ubiquitinated species appeared to be even lower [64, 68]. Furthermore, conversion of all 27 of the lysines in Cx43 to arginines did not eliminate the increase in Cx43 in gap junctions in response to proteasomal inhibitors [71] nor did it affect Cx43 turnover due to ERAD [34]. One of the main issues with these inhibitors is that their effects can impact many different pathways. In fact, an indirect role for proteasomal inhibition on Cx43 has been shown where blocking the proteasomal degradation of activated Akt allows it to phosphorylate Cx43 on S373 and eliminates Cx43:ZO-1 interactions thus, resulting in larger junctions [60]. Thus, it seems that direct ubiquitination of Cx43 does not play a very significant role in proteasomal degradation of Cx43, though there may be specific conditions (e.g., autophagy) and cell types where it is important.

2.5. Regulation of Cx43 and gap junction turnover by Cx43 phosphorylation

A variety of stimuli including epidermal growth factor (EGF), TPA (12-O-Tetradecanoylphorbol acetate), src activation, wounding and extracellular ATP [57–62] cause spatiotemporal changes in Cx43 phosphorylation and loss of gap junctions. Many of

these treatments result in Cx43 phosphorylation on S368 via PKC and on S255, S279, S282 via MAPK (see Table 1). Phosphorylation at these residues has been shown to affect gap junction channel gating properties and/or is associated with decreased gap junction assembly and increased gap junction turnover. Furthermore, TPA prevents assembly of new gap junctions and reduced the half-life of Cx43 [17]. Transformation of cells by v-Src has also been shown to downregulate gap junctional communication coincident with an increase in tyrosine phosphorylation on Cx43 [16, 72]. Using LA25 cells that express temperature-sensitive v-Src showed that Y247, Y265, S255, S262, S279/282 and S368 are all phosphorylated in response to v-Src activity indicating co-activation of MAPK and PKC [73]. Interestingly, immunofluorescence studies indicated that phosphorylated Y247 appeared to be preferentially present in “larger” gap junction plaques [74]. This distinct pY247 staining could potentially "mark" a portion of the gap junction for internalization, possibly through stimulating interaction with components of the endocytic system.

Recently, we found that if cells are treated with TPA, one also sees a rapid increase in phosphorylation at S373 via Akt [60]. This was somewhat surprising as we also found that phosphorylation at S373 dramatically increased apparent gap junction size and gap junctional communication in a similar manner to proteasomal inhibitor treatment [71]. Results from Gourdie and colleagues have shown that ZO-1 interaction with the C-terminal region of Cx43 near S373 causes a reduction in gap junction size and conversely, elimination of the ZO-1 interaction leads to larger gap junctions [75, 76]. Consistent with this, we found that Akt phosphorylation of Cx43 could regulate ZO-1 interaction, providing a mechanistic explanation for changes in gap junction size during proteasomal inhibition. By mutating Akt phosphorylation sites we found that cells expressing wild type Cx43, S365/369/373A or S373D mutants showed intermediate, extensive and no co-immunoprecipitation of ZO-1 and intermediate, very limited and large gap junctions, respectively. Also extensive Cx43-ZO-1 co-localization was lost in cells expressing the S373D mutant [60].

Thus, at least 4 kinases potentially play a role in the regulation of gap junction turnover – Akt [60], PKC [17, 77, 78], MAPK [79] and Src [73]. Treatment of cells with reagents, such as TPA or EGF, provide a model system to examine spatiotemporal changes in Cx43 phosphorylation during this “activated” or “acute” turnover process. Figure 4 shows that in TPA treated cells, Cx43 phosphorylation at the Akt site is maximal at 5 minutes, shown both by immunoblot, and immunofluorescence, where colocalization of pS373 (red) and total Cx43 (green) antibodies appear as yellow. Phosphorylation at S279/282 follows, peaking at 15–30 min after treatment (Fig. 4). Phosphorylation at S368 (Fig. 4) and Y247 (not shown) show a more gradual and steady increase, perhaps due to effects on newly synthesized Cx43 or protein en route to the plasma membrane.

Thus, Cx43 is sequentially phosphorylated by Akt, MAPK, Src and PKC in response to growth factors, wounding and other stimuli which induce acute gap junction turnover. In Fig. 5, we present a model for gap junction turnover that incorporates these phosphorylation events. It seems paradoxical that an initiating signal for gap junction turnover is an Akt-mediated transient increase in gap junction size. However, formation of larger gap junctions could facilitate rapid clearance of Cx43 from the plasma membrane in 2 ways: 1) depletion

of the incoming pool of Cx43 by rapid incorporation into a gap junction, and/or 2) to reduce the energetics of annular junction formation/internalization by requiring less membrane curvature during internalization. Once Cx43 is concentrated into the gap junction, the channels are closed via MAPK and PKC phosphorylation (Fig. 5). In keratinocytes, Cx43 is subsequently phosphorylated by Src. We hypothesize that Src phosphorylation of Cx43 at Y247 initiates recruitment of the gap junction internalization machinery (Fig. 5). Inhibition of Src activity via the src kinase inhibitor PP2 blocks growth factor-induced gap junction turnover [80, 81]. Glycyrrhetinic acid-related gap junctional communication inhibitors remodel gap junctions into a looser packing arrangement [82] in a process that involves Src binding [83] and leads to disruption of Cx43-ZO-1 interaction [80]. Src can directly interact with ZO-1 and compete for binding to the C-terminal region of Cx43 [80, 84, 85]. Clearly, Src plays a role in gap junction turnover, but it is not yet clear whether Src phosphorylation of Cx43 plays a direct role. Src phosphorylation of the NMDA receptor, GluN3A [86] has been shown to trigger its endocytosis, so one possibility is that Src similarly stimulates Cx43 internalization. Src phosphorylation of Cx43 may, in fact, direct the endocytic route of internalization through annular gap junction formation (mode 1, Fig. 3) or by “unzippering” gap junctions via loss of extracellular interactions followed by endocytosis and degradation of each connexon within the same cell where it was synthesized (mode 2, Fig. 3). The formation of double membrane endocytic vesicles (i.e., annular junctions) appears to be fairly specific to gap junctions though there are a few reports of “transendocytosis” occurring in dendritic cells [87] and in response to receptor ligand complex formation during neural (Eph:Ephrin [88]) and Drosophila development (Notch:Delta [89], Hedgehog:Patched [90] and Boss:Sevenless [91]).

3. Regulation of wound repair by Cx43 expression and phosphorylation

3.1. Cx43 expression regulates wound repair

Cx43 is abundantly expressed in skin and is known to play a key regulatory role during different stages of the repair process [92–95] via its expression and phosphorylation status changes [78, 96, 97]. Proliferation continually occurs in the basal layer of the epidermis to replace dead keratinocytes and is upregulated dramatically during wounding to provide a source of cells for wound repair. Wounding of the epidermis activates changes in gap junctional communication that synchronize keratinocyte migration across the wound bed [92, 93, 95]. Both rodent [92] and human skin [93] show decreased connexin expression at the edge of a wound within a day and a return to homeostatic levels upon wound closure [92, 98]; see Fig. 6. Many (up to 9) connexins can be detected in epidermis, but Cx43 is the predominant one in vivo and in cultures of human keratinocytes [99]. Cx43 regulation may play the primary role during early stages of wound healing as modulation of Cx43 expression directly affects wound repair [78, 92, 100–106]. Specifically, immediately after wounding there is a requirement for gap junctional communication to initiate efficient migration [78] but a reduction in intercellular communication between cells at the leading edge of the wound rapidly follows. Several results implicate Cx43 as a key regulator of repair. Diabetic mice that display high levels of Cx43 expression [106] or mice with Cx43 overexpression [107] have delayed wound closure. Conversely, mice with reduced

epidermal Cx43 can show more rapid healing [104], and Cx43 antisense application accelerated keratinocyte migration and wound repair resulting in less scarring [105].

3.2. A model to explain gap junctional upregulation and turnover in response to injury

In response to wounding, we find that Cx43 is sequentially phosphorylated at specific sites by (a) Akt at 5–30 min [60, 71], (b) PKC [78] and MAPK [79] at 15–60 min (Fig. 6), and (c) Src at 30 min–24h. These events are coincident with changes in gap junction localization and function including a transient increase in gap junction size and gap junctional communication followed by gap junction internalization. We hypothesize that this sequence of events represents a wound induced spatiotemporally-regulated “kinase program” that provides a mechanism to promote increased gap junctional communication and signals for keratinocyte activation prior to rapid gap junction internalization. As described above for EGF and TPA and in Fig. 5, the first step is characterized by the increase in gap junction size and gap junctional communication observed early upon activation of Akt [60]. We suggest that this provides the robust gap junctional communication that seems to initiate the changes necessary for cells to adopt a more migratory or proliferative phenotype. Figuring out the role of this first step is potentially important since results with reagents (i.e., potential drugs) that affect Cx43 expression appear to have disparate effects. For example, treatment of wounds with Cx43 antisense results in less inflammation and faster wound healing [108]. However, treatment of diabetic wounds with ACT1 peptide, which mimics the C-terminus (residues 374–382) resulting in increased gap junction size and communication, speeds healing [109]. Part of the issue may be related to timing, as the ACT1 peptide is short-lived and may facilitate early signaling events that promote healing while allowing normal gap junction closure via MAPK and src. Connexin *downregulation* appears to be important after this early phase so reagents that reduce Cx43 might be helpful at later stages. Another explanation might be related to the target – i.e., whether the reagent is affecting Cx43 connexons (hemichannels) or gap junctions. In either case, it is clear that early events after wounding are dramatically affected by Cx43.

There is strong evidence that a reduction in Cx43 expression and gap junctional communication can be beneficial to wound healing. This evidence includes the observation of what happens to Cx43 during a healthy wound response [93, 94, 101] and how that process is disrupted by Cx43 overexpression or sped by Cx43 downregulation [78, 101, 105–107, 110]. The benefit from reduced Cx43 might be related to the promotion of proliferation and migration or a reduction in inflammatory response. The activation of MAPK and Cx43 phosphorylation at S279 and S282 observed at 15–30 min post wounding would lead to gap junction channel closure [111]. Activation of PKC leads to inhibition of new gap junction assembly [17]. Activation of Src and phosphorylation of Cx43 on Y247 from 30 min to several hours could lead to internalization of gap junctions from the plasma membrane (Fig. 5) [73]. This sequence of Cx43 phosphorylation events during wounding is consistent with what we observe during growth factor treatment. Thus, consistent with the model we propose in Fig. 5, we hypothesize that wound induced gap junction disassembly is driven by a kinase program that regulates the accumulation of Cx43 into gap junctions in preparation for disassembly and “marking” of specific plaque domains for internalization via Cx43 phosphorylation at specific residues by at least 3 kinases. Our model predicts pS373

first promotes Cx43 accumulation into larger gap junctions with increased gap junctional communication. This step also effectively "clears" the plasma membrane of connexons that may exhibit hemichannel activity. Larger junctions could promote annular junction formation through decreased membrane and energy restraints compared to that needed for smaller vesicles. Next, pS279/282 rapidly closes the gap junction and then pY247 initiates accumulation of the internalization machinery – a timeline consistent with the observed kinetics of phosphorylation at these sites during wounding and in response to EGF or TPA, as shown in the previous sections (Fig. 5).

4. Summary and Perspective

Gap junction biology affects several fundamental cell processes including wound repair. This review mainly discussed the short terms effects of Cx43 phosphorylation on the wound healing process but Cx43 is dynamically regulated for at least 72 h after wounding [78, 92, 93, 101] so those effects warrant further investigation. For example, wound-dependent phosphorylation at the PKC site S368 at 24 hours creates specialized communication boundaries within the basal cells of the epidermis [78]. In addition our discussion has only considered a few Cx43 interacting proteins, but CASK and CADM1 directly interact with hypo-phosphorylated Cx43 one hour post-wounding in human keratinocytes to apparently regulate activation and migration [96]. So at this point, we are left with a number of questions related to the role of Cx43 in wound healing: i) What is the role of the increased gap junctional communication early after wounding?; ii) How long is gap junctional communication necessary after the initial wounding event to maximize wound healing?; iii) Can we effectively target specific cells such as migratory or proliferative cells with connexin reagents?; iv) Can we target specific subcellular pools of Cx43 to induce the desired results?

Obviously, a more complete understanding of Cx43 biology in the epidermis would allow for more targeted and rational drug design to facilitate wound repair. Some of our understanding of the regulation of Cx43 assembly and disassembly has been affected by the use of broad specificity reagents that might be affecting the expression of many different Cx43 interacting and regulating proteins. Through the use of more targeted reagents such as phosphospecific antibodies, mutant versions of the protein and targeted knockdown of the connexin interacting protein, the field is starting to, at least, identify the important steps and players if not to fully grasp their implications. Furthermore, drugs based on that knowledge could target Cx43, the kinase important at a particular step, important interacting proteins or other key actors. Given the extensive pharmaceutical development of kinase inhibitors, we believe it will be important to test whether kinase or gap junction activators/inhibitors could be topically applied in a manner dictated by the wound status (i.e., fresh, ulcerated, diabetic, etc.) to yield better healing and reduce the need for amputations. For example, we envision speeding up the wound healing process via the use of different "band aids" embedded with distinct gap junction or kinase inhibitors/activators. One would utilize a specific bandage on a fresh wound (i.e., applied within 0–30 min of wounding and kept on for 4 hours) and then change to a second type of bandage that might be applied 4–24 hours post wounding and a third that would be good until healing is complete and might be specifically designed to reduce scarring. In this way, development of a clear spatiotemporal map of Cx43

phosphorylation and activation of its cognate kinases during pathogenic processes could yield real clinical value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Da	daltons
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
Cx43	Connexin 43
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
EGF	epidermal growth factor
TPA	12-O-Tetradecanoylphorbol acetate
ERAD	Endoplasmic Reticulum Associated Degradation
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
CK1	casein kinase 1
MAPK	mitogen-activated protein kinase
ZO-1	zonula occludens-1
GFP	green fluorescent protein

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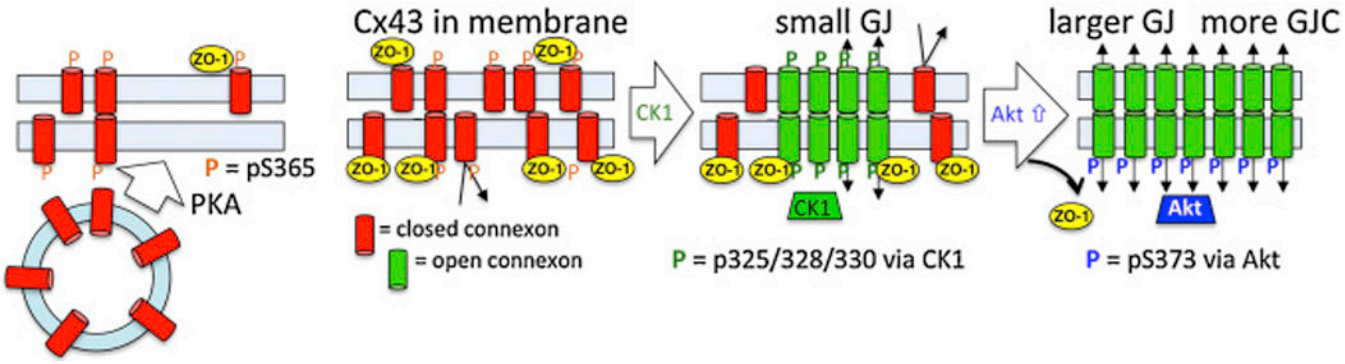

Fig. 1.

Model of the steps in gap junction assembly regulated by Cx43 phosphorylation. Our model first shows how activation of PKA causes increased trafficking of Cx43 vesicles to the plasma membrane. Cx43 in the plasma membrane then gets phosphorylated first at S365 (shown in orange) and subsequently at S325/328/330 (via CK1, shown in green). ZO-1 can associate with Cx43 and when it is present it keeps junctions small. Phosphorylation of Cx43 at S373 by Akt eliminates ZO-1 interaction and allows Cx43 accumulation into larger gap junctions with increased intercellular communication.

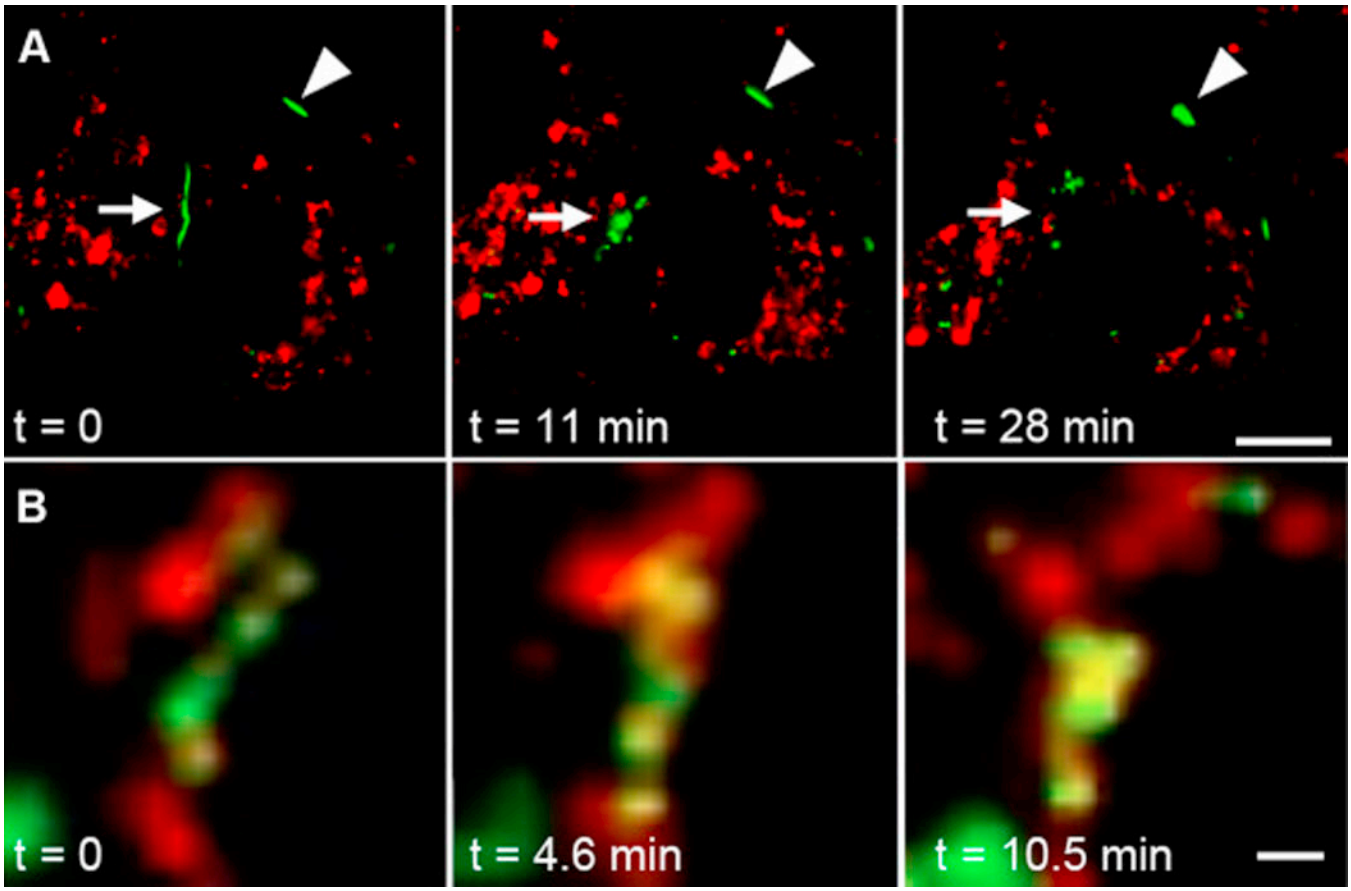

Fig. 2.

Images from time lapse series visualizing Cx43-GFP and LysoTracker Red. A) Arrow indicates a large gap junction that breaks apart and disappears, while the arrowhead points to a gap junction, in the same cell, that remains stable throughout the time course. In the associated movie, images were collected at 20 second intervals for 30 minutes. Lysosomes are seen in close proximity to the gap junctions throughout the disassembly process. Bar=10μm. B) Magnified view of interactions between Cx43-GFP and lysosomes. In the associated movie, images were collected at 2 second intervals. There appears to be a complex interplay in the membranes between Cx43-GFP and lysosomal compartments. Bar=1μm.

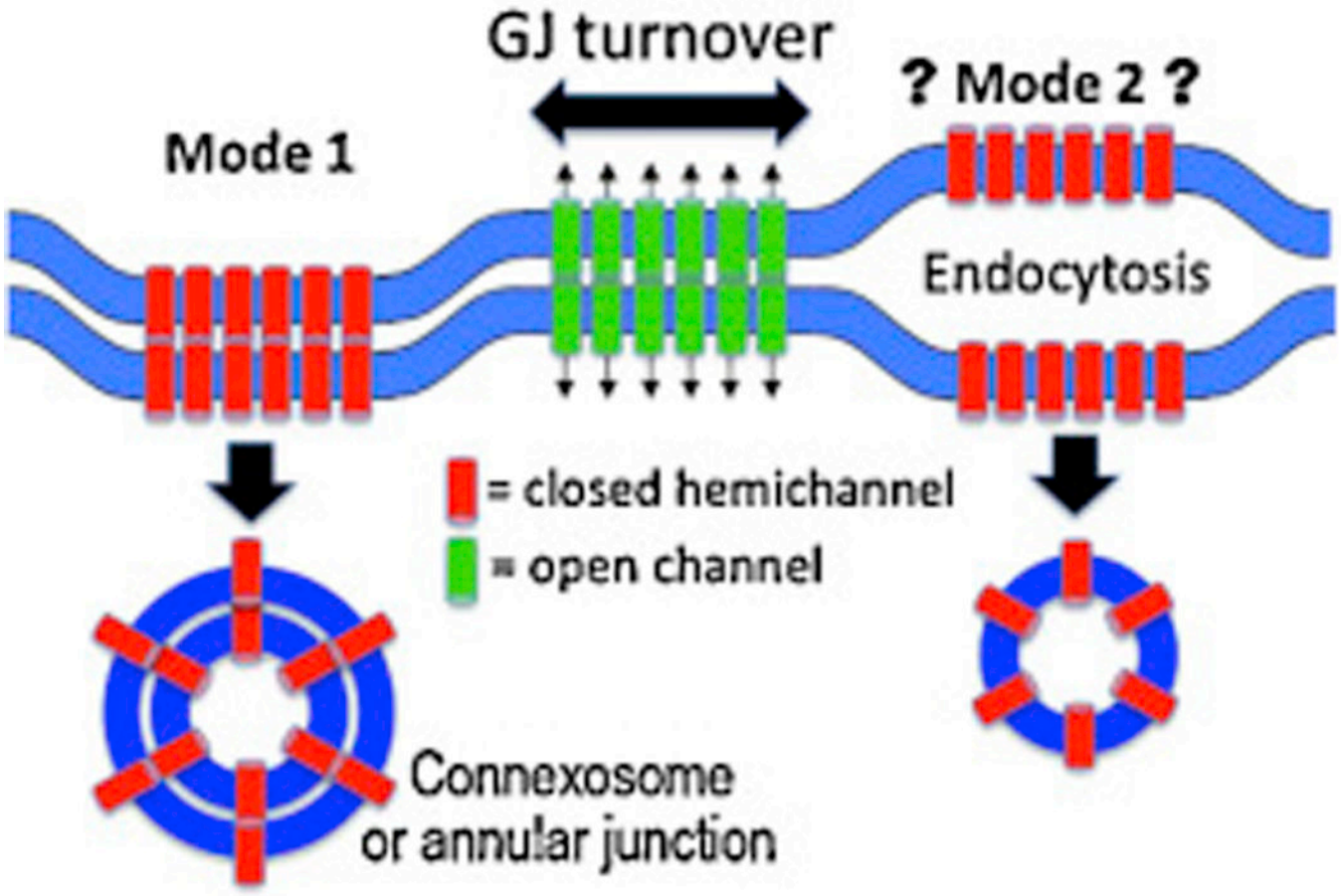

Fig. 3.

Cartoon of two potential modes of gap junction (GJ) turnover. Mode 1 proceeds through formation of a double membrane structure that will become an annular junction. Mode 2 involves opening of the gap between the two membranes by changes in phosphorylation or ubiquitinylation that destabilize the junction followed by conventional endocytosis.

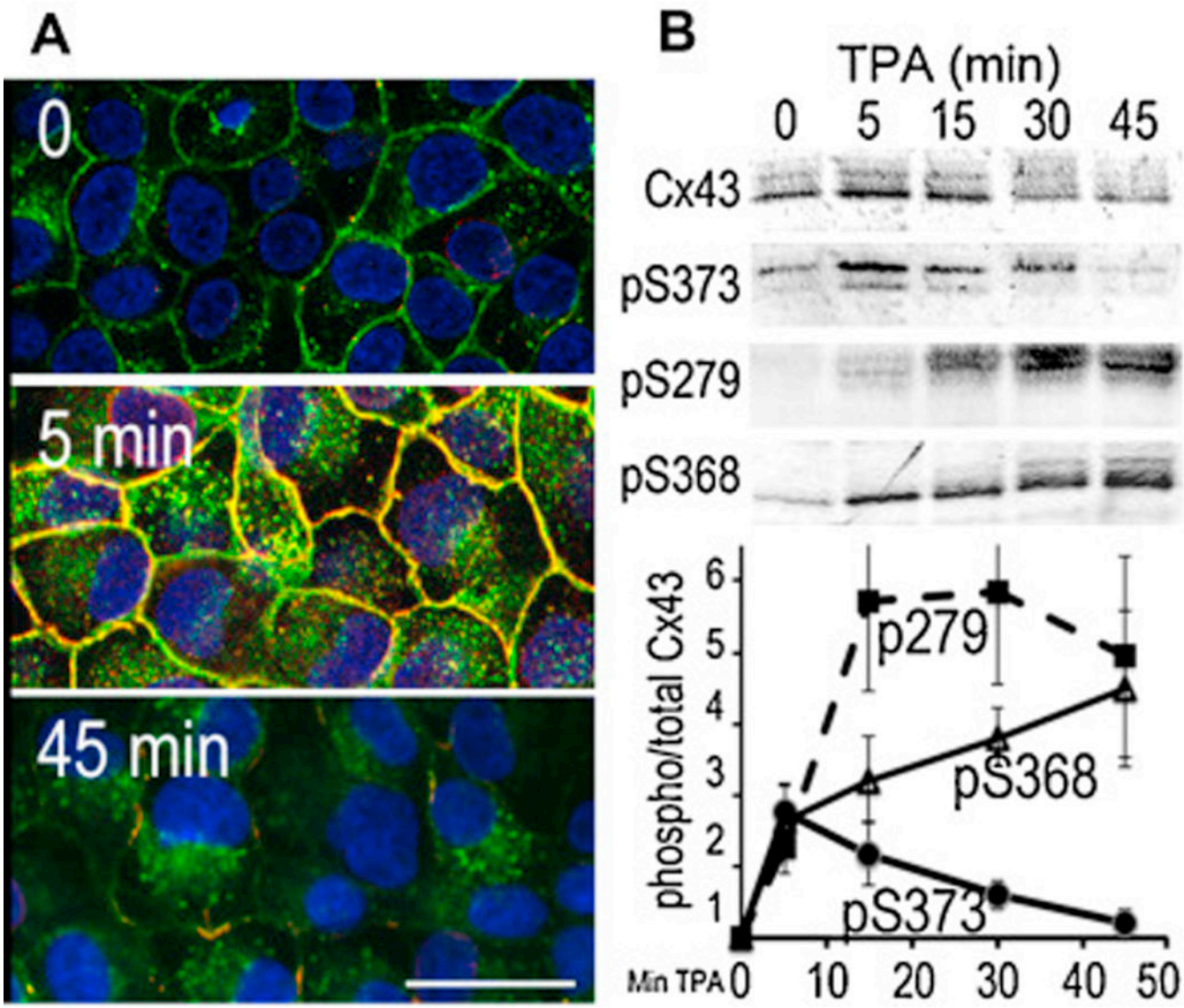

Fig. 4.

(A) Immunofluorescence staining with antibodies to total Cx43 (green) and phosphoS373 Cx43 (red) 0, 5 and 45 min after 50nM TPA treatment. Nuclei were labeled with DAPI (blue). Bar=25μm. Note the very rapid accumulation of Cx43 into large gap junctions upon Akt activation and phosphorylation at S373. (B) Immunoblot analysis of total Cx43, phosphoS373 (pS373), phosphoS279/282 (pS279) and phospho368 (pS368) levels at 0, 5, 15, 30 and 45 min after TPA treatment

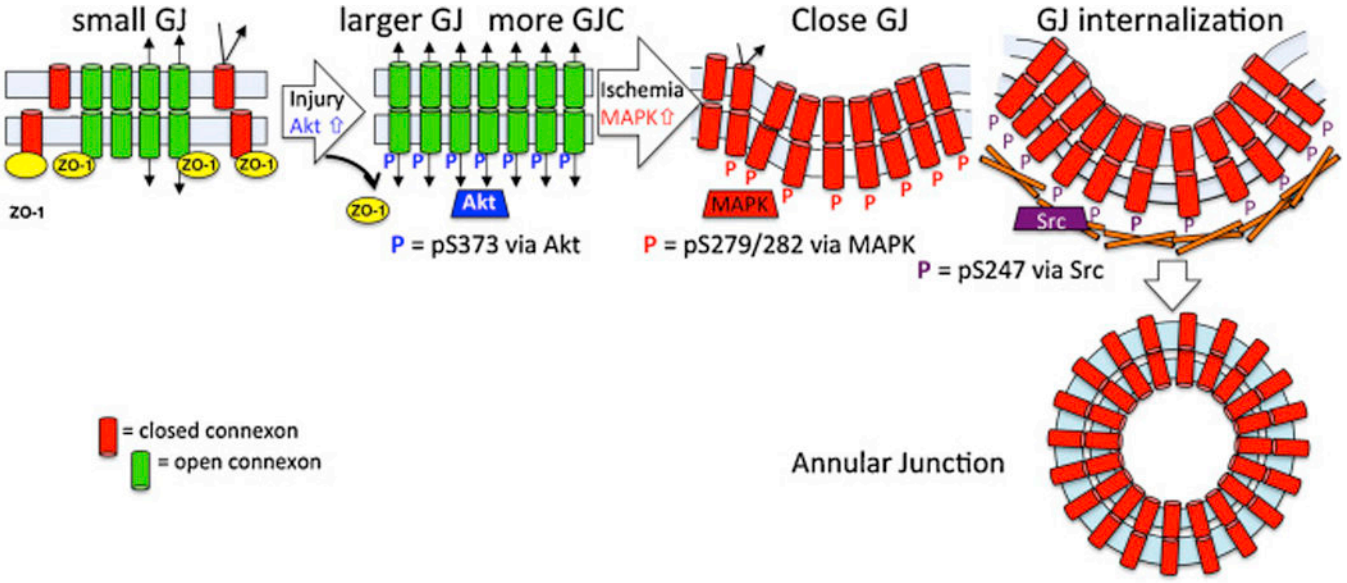

Fig. 5.

Model of the steps in gap junction turnover regulated by Cx43 phosphorylation. Our model predicts that phosphoS373 (via Akt, shown in blue) first promotes Cx43 accumulation into larger GJs with increased gap junctional communication. Larger junctions could promote annular junction formation through decreased membrane and energy restraints compared to that needed for smaller vesicles. Next, phosphoS279/282 (via MAPK, shown in red) closes the gap junction and then phosphoY247 (via Src, shown in purple) initiates recruitment of the internalization machinery – a timeline consistent with the observed kinetics of phosphorylation at these sites in response to EGF, TPA or wounding.

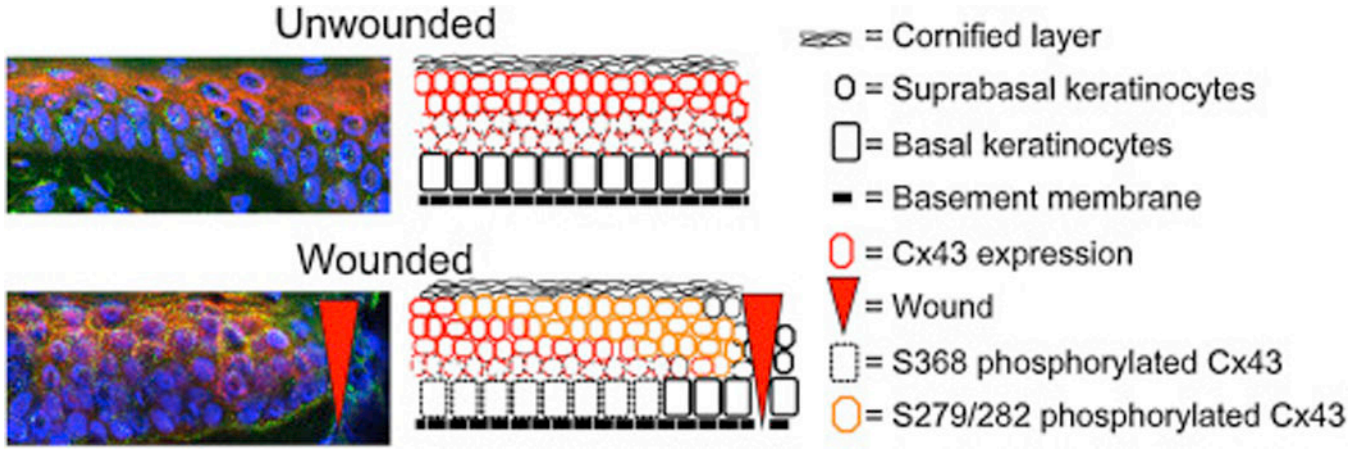

Fig. 6.

Immunofluorescence of human skin stained with antibodies to total Cx43 (green) and phospho279/282 Cx43 (red). Nuclei were labeled with DAPI (blue). Note the high level of phospho279/282 in suprabasal cells adjacent to the wound edge (noted by red arrowhead). An accompanying cartoon illustrates the changes in phospho279/282 and total Cx43 in basal and suprabasal keratinocytes.

TABLE 1

Cx43 residues phosphorylated, the kinases involved and their consequences.

Assembly residues	Kinases	References
S325/328/330	CK1, ??	[41]
S364	??	[112, 113]
S365	??	[39, 114]
S373	Akt	[60, 114–116]
Gating residues		
S255	MAPK	[117]
S262	$p34^{cdc2}$, MAPK	[118–120]
S279/282	MAPK	[117]
S306	??	[121, 122]
S368	PKC	[116, 121, 123]
Dissassembly		
Y247	Src	[124]
S368	PKC	[116, 121, 123]
Unknown		
Y265	Src	[124]
S296	??	[121]
S297	??	[121]
Y313	??	[125, 126]
S369	Akt,??	[113–115]
S372	??	[114–116]