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Degradation of Connexins Through the Proteasomal, Endolysosomal and Phagolysosomal Pathways

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Abstract Connexins comprise gap junction channels, which create a direct conduit between the cytoplasms of adjacent cells and provide for intercellular communication. Therefore, the level of total cellular connexin protein can have a direct influence on the level of intercellular communication. Control of connexin protein levels can occur through different mechanisms during the connexin life cycle, such as by regulation of connexin gene expression and turnover of existing protein. The degradation of connexins has been extensively studied, revealing proteasomal, endolysosomal and more recently autophagosomal degradation mechanisms that modulate connexin turnover and, subsequently, affect intercellular communication. Here, we review the current knowledge of connexin degradation pathways.

Keywords Connexin · Degradation · Proteasome · Lysosome · Autophagy

Introduction

Gap junctions are plasma membrane channels that allow the passive diffusion of molecules less than 1,000 daltons directly between the cytoplasms of neighboring cells (Goodenough et al. 1996). The molecules include secondary messengers (cAMP), small metabolites (ATP) and ions (Ca^{2+}). The regulation of gap junctions and gap junctional intercellular communication (GJIC) is critical as the channels contribute to the normal homeostasis of cells, tissues and organs, as well as being important during development (Vinken et al. 2006; Wei et al. 2004; White and Paul 1999). Furthermore, misregulation of gap junctions and the corresponding intercellular communication can result in a number of human diseases, including heart arrhythmias (Martin and Evans 2004; Severs et al. 2008; van Veen et al. 2001), developmental diseases (Laird 2008) and cancers (Naus and Laird 2010).

The complete gap junction channel is comprised of two hexameric structures (connexons or hemichannels), one contributed by each of the neighboring cells. Connexons contain six connexin proteins that form either homomeric oligomers or, in cells expressing more than one type of connexin, heteromeric oligomers if the expressed connexins are compatible. Connexins are four-pass transmembrane proteins, containing cytoplasmic N- and C-terminal domains, two extracellular loops and one intracellular loop. There are 21 members of the human connexin protein family, expressed to different degrees depending on the tissue or cell type. Of these, connexin43 (Cx43) has been the most widely studied family member. Generally, connexins are cotranslationally inserted into the endoplasmic reticulum (ER) membrane, where they undergo proper folding and are then transported through the Golgi network. During the ER to Golgi transport, a number of connexins have been demonstrated to oligomerize into hemichannels (Das et al. 2009; Das Sarma et al. 2002; Diez et al. 1999; Koval 2006; Maza et al. 2003, 2005). The hemichannels are then transported to the plasma membrane to dock with hemichannels on the neighboring cell surface to form the

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gap junction channels. Large accretions of gap junctions may form between cells, which are generally known as gap junction plaques. Undocked hemichannels have more recently been shown to have their own activity, with important roles in cell death (Bargiotas et al. 2009; Contreras et al. 2003; Goodenough and Paul 2003; Sato et al. 2009; Stout et al. 2004) and tissue remodeling (Knight et al. 2009; Siller-Jackson et al. 2008). Hemichannels can be opened under specific conditions, such as membrane depolarization (Contreras et al. 2003), changes in ionic concentrations (Gómez-Hernández et al. 2003; Srinivas et al. 2006) and mechanical shear stress (Cherian et al. 2005; Siller-Jackson et al. 2008). All of these conditions can regulate the passage of ions and metabolites through the hemichannels (Anselmi et al. 2008; Cherian et al. 2005; Garré et al. 2010; Siller-Jackson et al. 2008).

From the plasma membrane, undocked hemichannels, intact gap junctions and even large sections of gap junction plaques are internalized and primarily degraded (Laird 2006). Remarkably, for transmembrane proteins, connexins have a short half-life of 1.5–5 h depending on the cell type (Beardslee et al. 1998; Darrow et al. 1995; Fallon and Goodenough 1981; Laird et al. 1991; Musil et al. 2000). The degradation mechanisms for connexins have been studied for years, with reports demonstrating the involvement of the proteasomal, lysosomal and autophagosomal degradation pathways (Beardslee et al. 1998; Fong et al. 2012; Girao and Pereira 2003; Guan and Ruch 1996; Hesketh et al. 2010; Kelly et al. 2007; Laing and Beyer 1995; Laing et al. 1997, 1998; Leithe et al. 2006; Leithe and Rivedal 2004a, b; Li et al. 2008; Lichtenstein et al. 2011; Musil et al. 2000; Qin et al. 2003; Su et al. 2010; Thomas et al. 2003; VanSlyke et al. 2000; VanSlyke and Musil 2002, 2005). Proper regulation of these pathways that are responsible for connexin turnover is essential as the amount of connexin protein can have a direct effect not only on the level of GJIC (Leithe et al. 2009; Leithe and Rivedal 2004a; Musil et al. 2000; VanSlyke and Musil 2003) but also on the activities of hemichannels and other functions of connexins, which are unrelated to intercellular communication (Goodenough and Paul 2003; Scemes et al. 2009; Stout et al. 2004). All of these connexin activities are critical for normal cellular and tissue functions.

Proteasomal Degradation

Protein degradation through the proteasomal pathway typically involves the 26S proteasome holoenzyme. This complex consists of the 20S core particle (CP) and the 19S regulatory particle (RP) (reviewed in Voges et al. 1999). The 20S CP consists of four rings stacked upon each other. Each ring contains seven individual protein subunits. The innermost two rings contain the proteolytic activity with the β-type subunits, while the α-type subunits comprise the outer two rings. The core is flanked on each side by one complete 19S RP, which is made up of the base and cap, also consisting of many protein subunits. The 19S RP subunits are classified as either ATPases (*Saccharomyces cerevisiae* Rpt proteins) or non-ATPases (*S. cerevisiae* Rpn proteins) (Glickman et al. 1998). The ATPase activity of the 19S subunit most likely is responsible for the unfolding of substrate proteins, to allow the polypeptide chain to enter the 20S CP for degradation.

The tag that marks a substrate for proteasomal degradation is often considered to be a polyubiquitin chain. Ubiquitin is a 76–amino acid protein that is highly conserved and expressed in all eukaryotes. The covalent linkage of ubiquitin to substrates occurs through a highly regulated process that is mediated by a number of enzymes (Fang and Weissman 2004). The E1 ubiquitin-activating enzyme, multiple E2 ubiquitin-conjugating enzymes and several E3 ubiquitin ligases facilitate the specific ubiquitination of cellular proteins. The E1 enzyme activates ubiquitin, enabling ubiquitin to be transferred to one of the E2 enzymes. The ubiquitin-loaded E2 then associates with an E3 ligase, which results in the covalent bonding of ubiquitin to the target protein or, alternatively, to another ubiquitin to create a polyubiquitin chain. Ubiquitination of proteins is a mechanism that provides for the selective degradation of cellular proteins. In fact, the 19S RP contains subunits that are ubiquitin-binding proteins, such as Rpn1, Rpn10 and Rpn13 (Deveraux et al. 1994; Elsasser et al. 2002; Husnjak et al. 2008). Almost all proteins known to be degraded by the 26S proteasome are ubiquitinated, with a small number of exceptions. It should be noted, however, that a large number of proteins, such as oxidized proteins, are able to be degraded without ubiquitination specifically through the 20S CP alone (Davies 2001; Ferrington et al. 2001; Grune et al. 1997; Jariel-Encontre et al. 2008; Orlowski and Wilk 2003; Shringarpure et al. 2003). Presumably, oxidation causes sufficient unfolding of the target protein to allow it to directly enter the 20S CP barrel without the unfolding activity of the ATPase activity resident in the 19S RP.

Cx43 Proteasomal Degradation

The first documented study of the involvement of proteasome degradation in connexin turnover came in 1995 by Laing and Beyer, who reported that pharmacological inhibition of proteasomal activity using ALLN resulted in increased levels and reduced turnover of Cx43 protein in Chinese hamster ovary (CHO) E36 and rat heart–derived BWEM cells, respectively. In addition, the authors used the ts20 cell line, which contains a thermolabile E1 enzyme

and where heat treatment inactivates the E1 activity, resulting in loss of the ubiquitination process. It was demonstrated that Cx43 required active ubiquitination in order to be degraded after heat treatment. Furthermore, sequential immunoprecipitation indicated that Cx43 was ubiquitinated. In a subsequent study, neonatal rat ventricular myocytes subjected to heat treatment displayed loss of Cx43, which was prevented with proteasomal inhibition using ALLN or lactacystin, further supporting a role for the proteasome in Cx43 degradation (Laing et al. 1998).

Proteasomal degradation is typically associated with ER-associated degradation (ERAD). During ERAD, misfolded or unfolded proteins are dislocated out of the ER and into the cytoplasm, where the proteins are thought to be polyubiquitinated (Smith et al. 2011). This ubiquitination then marks these proteins for proteasomal degradation. Studies have indicated that Cx43 can be degraded via ERAD. Treatment of BWEM cells with the fungal metabolite brefeldin A (BFA) to prevent transport of newly synthesized Cx43 to the plasma membrane, with a concurrent inhibition of proteasomal degradation using ALLN, resulted in increased intracellular pools of Cx43 (Laing et al. 1997). With the block in transport to the membrane, newly synthesized Cx43 would accumulate in the ER. Simultaneously blocking proteasomal degradation would then reveal the pool of Cx43 bound for ERAD. DTT treatment of CHO cells to prevent formation of the intramolecular Cx43 disulfide bonds necessary for function (in effect causing Cx43 to be misfolded) in combination with ALLN blockage of proteasomal degradation resulted in a dramatic increase of Cx43 protein levels (Musil et al. 2000). An analysis of cellular stress on Cx43 ER dislocation and degradation was conducted using multiple cell lines, where DTT treatment (an ER stressor) more than doubled the amount of cytoplasmic Cx43 present in BFA-treated cells (VanSlyke and Musil 2002). In the absence of BFA, little Cx43 was found in the cytoplasm, suggesting that DTT treatment/Cx43 protein unfolding resulted in enhanced dislocation of Cx43 from the ER. Additional proteasomal inhibition using ALLN or epoxomicin further increased the amount of cytoplasmic Cx43 that was reversed upon ALLN washout, suggesting that the ER-dislocated Cx43 was bound for proteasomal degradation and further supporting the concept that Cx43 is subject to ERAD.

The ubiquitin-like (UbL) and ubiquitin-associated (UBA) domain protein family has been documented to be involved in proteasomal degradation (reviewed in Su and Lau 2009). It is generally thought that these proteins act as shuttle or adaptor proteins to transport their substrates from the ER to the proteasome. The UBA domain can interact with ubiquitin and ubiquitinated proteins. The UbL domain of these proteins interacts with subunits of the 19S RP, specifically Rpn1 and Rpn10. These two domains provide these proteins with the proper interactions necessary to function as a shuttle factor that is involved in proteasomal degradation or ERAD. The identification of the UbL-UBA protein CIP75, which is able to interact with Cx43, provides further insight into the mechanisms that regulate Cx43 proteasomal degradation (Li et al. 2008). The CIP75 UBA domain interacted with a region of the Cx43 C-terminal tail, a domain that has been found to interact with many other proteins. In human HeLa and mouse S180 cells, the interaction between CIP75 and Cx43 affected the Cx43 half-life, where increased levels of CIP75 reduced Cx43 half-life, while siRNA knock-down of CIP75 had the opposite effect. The CIP75-facilitated degradation was inhibited with MG132, which blocks proteasomal degradation. Furthermore, the CIP75 UbL domain was demonstrated to interact with the Rpn1 and Rpn10 components of the 19S RP, supporting a role for CIP75 as an adaptor between Cx43 and the proteasome. Finally, colocalization of CIP75 and Cx43 at the ER indicated that the role of CIP75 in Cx43 proteasomal degradation might involve ERAD (Li et al. 2008). Interestingly, while almost all substrates of the 26S proteasome have been found to be ubiquitinated, the ubiquitination of Cx43 was demonstrated to be nonessential for interaction with CIP75. By using a series of Cx43 point mutants with mutations of the various lysine residues that could potentially serve as ubiquitin attachment sites, CIP75 was demonstrated to still be able to interact with the mutated Cx43 (Su et al. 2010). Significantly, this is one of the unique situations where a non-ubiquitinated substrate undergoes 26S proteasomal degradation as only a limited number of proteins have been conclusively demonstrated to also undergo ubiquitin-independent degradation by the proteasome holoenzyme (Bercovich et al. 1989; Glass and Gerner 1987; Jariel-Encontre et al. 1995, 2008; Murakami et al. 1992).

Proteasomal Degradation of Membrane-Localized Cx43

Many studies using proteasomal inhibitors have reported increased Cx43 at the plasma membrane. Treatment of BWEM cells with the inhibitor lactacystin, ALLN or MG132, concurrently with BFA or monensin (to disrupt Cx43 trafficking to the plasma membrane), resulted in increased cell surface Cx43 compared to treatment with BFA/monensin alone (Laing et al. 1997). Furthermore, treatment of gap junction assembly-inefficient CHO cells or serum-starved mouse S180L cells with ALLN resulted in the upregulation of gap junction assembly and GJIC (Musil et al. 2000). Additional studies also employed proteasomal inhibitors to demonstrate that proteasomal degradation is responsible for the turnover of Cx43 from the cell surface and the corresponding reduction in

intercellular communication (Fernandes et al. 2004; Girao and Pereira 2003, 2007; Kimura and Nishida 2010; Leithe and Rivedal 2004a, b; Simeckova et al. 2009; VanSlyke and Musil 2005). The observation of the ubiquitination of Cx43 localized in the gap junction plaques at the cell surface provided additional support of a role for the proteasome in regulating Cx43 at the plasma membrane (Leithe et al. 2009; Leithe and Rivedal 2004a, b; Rutz and Hülser 2001).

A recent report, however, has provided evidence for an indirect mechanism involving proteasomal degradation that influences the Cx43 protein level and localization at the plasma membrane (Dunn et al. 2012). By utilizing a Cx43 point mutant that cannot be ubiquitinated (containing lysine to arginine mutations of all the lysine residues that would act as ubiquitin acceptor sites), the proteasome was demonstrated to regulate the levels of Akt/protein kinase B which caused the resulting stabilization of Cx43 at the cell surface. Using canine MDCK and rat NRK cells expressing the Cx43 lysine mutant, the mutant Cx43 was found to traffic to the plasma membrane and to participate in channel formation and intercellular communication. Inhibiting proteasomal degradation with MG132 elicited an increase in Cx43 at the plasma membrane in cells expressing either the wild-type Cx43 or lysine mutant, which suggested a possible indirect effect on Cx43 stabilization that resulted from the proteasomal inhibition. Furthermore, these data demonstrate that the Cx43 that is localized to the plasma membrane and creates gap junctional channels is not necessarily ubiquitinated in order for the channels to be functional.

Internalization of connexins has been linked to post-translational modifications of connexins, including phosphorylation, ubiquitination, acetylation and SUMOylation (Colussi et al. 2011; Kjenseth et al. 2012; Leithe et al. 2012; Locke et al. 2009; Shearer et al. 2008; Solan and Lampe 2005, 2007, 2009; Su and Lau 2012). Cx43 phosphorylation at the serine^{255} and serine^{262} residues by the MAP kinase ERK can occur in response to epidermal growth factor (EGF) or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) exposure, which is followed by internalization of Cx43 from the plasma membrane and subsequent lysosomal degradation (Leithe et al. 2006; Leithe and Rivedal 2004b; Sirnes et al. 2008, 2009). Akt had also previously been demonstrated to phosphorylate Cx43 (Park et al. 2007), and inhibition of Akt activity using Akt VIII treatment or expression of the dominant negative Akt-K179A mutant reduced the amount of Cx43 at the cell surface (Dunn et al. 2012). Furthermore, closer examination of Akt revealed an increase in ubiquitinated Akt and, in general, Akt kinase activity in response to MG132 treatment, blocking proteasomal degradation (Dunn et al. 2012), which has been demonstrated to increase Akt translocation to the plasma membrane and increased activity due to a phosphorylation event (Feng et al. 2004; Sarbassov et al. 2005). Furthermore, Akt-phosphorylated Cx43 was present at higher levels after MG132 treatment (Dunn et al. 2012). These results suggested that proteasomal degradation of ubiquitinated Cx43 at the cell surface is not the cause for the turnover of Cx43 gap junctions.

Cx32 Proteasomal Degradation

Similar to Cx43, Cx32 is also degraded by ERAD (Fig. 1). The degradation of Cx32 is of particular interest because of the identification of Cx32 mutations in the human peripheral neuropathy X-linked Charcot-Marie-Tooth disease (CMTX). Using rat pheochromocytoma PC12J cells stably expressing wild-type Cx32, significantly more Cx32 accumulation was detected upon proteasomal inhibition compared to blocking lysosomal degradation (VanSlyke et al. 2000). In NRK fibroblasts, Cx32 was also demonstrated to undergo ER dislocation (VanSlyke and Musil 2002), like Cx43; and accumulation of Cx32 following DTT treatment (inducing ER stress) occurred only after treatment with the proteasomal inhibitors epoxomicin and ZL_3VS (Kelly et al. 2007; VanSlyke and Musil 2002). Interestingly, a study, using the prostate cancer cell line LNCaP stably expressing exogenous wild-type Cx32, also demonstrated that Cx32 undergoes ERAD, which occurs at a faster rate after androgen depletion (Mitra et al. 2006). This degradation is rescued upon exposure to androgens, resulting in increased Cx32 trafficking to the plasma membrane and increased intercellular communication. Additional studies utilizing the Cx32 E208K mutant,

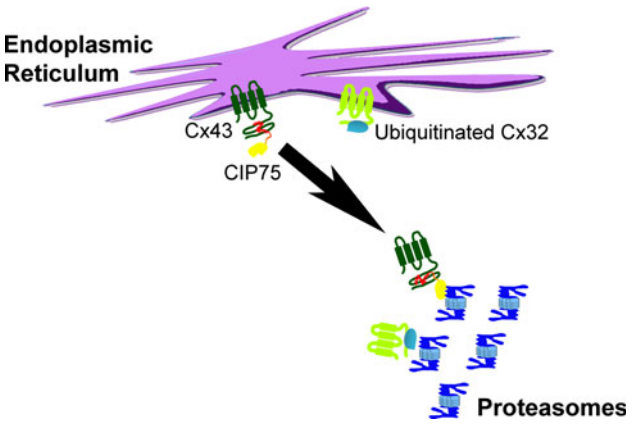

Fig. 1 Proteasomal degradation of connexins. During protein synthesis, connexins are cotranslationally inserted into the ER membrane, where the proteins undergo proper folding. Misfolded connexins can be polyubiquitinated and degraded by the proteasome through ERAD, as in the case of Cx32. Alternatively, nonubiquitinated Cx43 interacts with the accessory factor CIP75, which facilitates Cx43 ERAD by interacting with the proteins in the proteasome 19S RP

identified in CMTX patients (Fairweather et al. 1994), enabled further observation of Cx32 ERAD. This mutant exhibits an intracellular trafficking defect as it fails to form connexon hexamers and does not traffic to the plasma membrane (VanSlyke et al. 2000). Cx32 E208K localizes to the ER, and the degradation appeared to be affected by proteasomal inhibitors but not lysosomal inhibitors, indicating that turnover occurred via ERAD. The E208K mutant protein was detected in both polyubiquitinated and nonubiquitinated forms (Kelly et al. 2007). Significantly more polyubiquitinated E208K was detected than non-ubiquitinated protein, particularly in the cytoplasm. These data indicated that, as is the general case for 26S proteasomal degradation, the pool of Cx32 that undergoes ER dislocation and ERAD via the proteasome is polyubiquitinated.

Lysosomal Degradation

While early reports suggested the involvement of proteasomes in the turnover of connexins from the plasma membrane, lysosomes have been demonstrated to directly facilitate the degradation of connexins internalized from the plasma membrane. Lysosomes are cytoplasmic membrane-enclosed intracellular vesicles whose low internal pH provides the optimal environment for acidic hydrolytic enzymes to function. Chloride and proton pumps (hydrogen ion ATPases) maintain the acidic environment by pumping H^+ ions into lysosomes (Saftig and Klumperman 2009). The hydrolytic enzymes are targeted to the Golgi-derived lysosomes by the addition of a mannose-6-phosphate tag following their synthesis in the ER (González-Noriega et al. 1980; Mari et al. 2008). These enzymes include glycosidases, proteases, lipidases, acid phosphatases and sulfatases that degrade proteins and other cellular debris taken up in vacuoles that fuse with lysosomes (Bright et al. 2005; Lubke et al. 2009). Products of lysosomal digestion can be reused to synthesize new cellular components following transport to the cytosol by solute transporters in the lysosomal membrane (Jeyakumar et al. 2005; Puri et al. 1999).

Endolysosomal Degradation of Cx43

Early studies identified gap junctions and, specifically, Cx43 in endosomal and lysosomal membrane compartments (Murray et al. 1981; Naus et al. 1993; Sasaki and Garant 1986). Multiple studies provided evidence that Cx43 at the cell surface undergoes internalization via the endocytic pathway. In human, rat and mouse cells, intracellular Cx43 was observed in endocytic compartments through colocalization with the endosomal markers EEA1, Rab5 and Rab7 (Boassa et al. 2010; Gilleron et al. 2008; Govindarajan et al. 2010; Leithe et al. 2006, 2009; Segretain et al. 2003). Furthermore, the delivery of connexins to lysosomes for degradation was demonstrated to be facilitated by interactions with proteins involved in endocytosis and intracellular trafficking. During endocytosis, clathrin is recruited to the membrane through interactions between proteins at the plasma membrane and clathrin adaptor proteins, such as adaptor protein complex-2 (AP-2) and Disabled-2 (Dab2). After endocytic vesicles, such as clathrin-coated pits, bud from the plasma membrane, the large GTPase dynamin protein is required for the endosome to pinch off into the cytoplasm. Cx43 colocalizes with clathrin, AP-2, Dab2 and dynamin2 at cell surface gap junction plaques (Gilleron et al. 2011; Gumpert et al. 2008; Piehl et al. 2007). Additionally, the loss of dynamin GTPase activity or the reduction of dynamin2, clathrin, AP-2 or Dab2 proteins inhibited the internalization of Cx43 as observed by a significant decrease in the number of annular gap junctions (AGJs). AGJs are cytoplasmically localized double-membrane structures containing intact gap junction channels that are derived from the membranes of both neighboring cells. These data indicate that the proteins involved in clathrin-mediated endocytosis are necessary for the internalization of gap junctions, which subsequently may be degraded by lysosomes. Additionally, the Cx43-interacting protein of 85 kDa, CIP85, is a Rab GTPase activating protein that was found to colocalize with Cx43 at the plasma membrane; and the interaction was required for Cx43 degradation by lysosomes (Lan et al. 2005).

The internalized AGJ structures and Cx43 were observed to be fused with structures resembling lysosomes by electron microscopy (Murray et al. 1981; Naus et al. 1993; Sasaki and Garant 1986), providing some of the initial evidence that internalized connexins associated with lysosomes. Laing and Beyer (1995) were the first to demonstrate that pharmacological inhibition of lysosomal function, using primaquine to treat E36 cells, resulted in a twofold accumulation in Cx43 levels and a minor prolongation of its half-life from 2.5 to 3 h. Additional studies in rat and human cells confirmed the role of lysosomes in the degradation of Cx43 internalized from the plasma membrane. These studies observed an increased accumulation of connexins in lysosomes and detected increased total levels of connexins, following treatment of cells with a wide variety of lysosomal inhibitors (Guan and Ruch 1996; Laing et al. 1997, 1998; Leithe et al. 2006; Qin et al. 2003; Simeckova et al. 2009; Thomas et al. 2003). Furthermore, biotinylated cell surface Cx43 degradation was blocked in S180 cells upon treatment with the lysosomal inhibitors chloroquine and, to a lesser extent, leupeptin, supporting the conclusion that intact gap junctions and undocked

hemichannels can be internalized and subsequently degraded by lysosomes (VanSlyke and Musil 2005). Other studies have demonstrated similar mechanisms for additional members of the connexin family. Cx32 was observed in endosome- and lysosome-containing subcellular fractions of rat liver cells (Rahman et al. 1993). Following treatment of HeLa cells with multiple lysosomal inhibitors, accumulation of Cx31 was observed (He et al. 2005). These results suggested that these connexins, and perhaps others, are internalized and can be degraded by endolysosomal mechanisms.

One area that is still unresolved is the molecular mechanism(s) responsible for the possible fusion of the double membrane of internalized AGJs with single-membrane endosome or lysosome compartments. Under physiological conditions, there is little evidence that intact gap junctions can be separated back into separate hemichannels on single membranes (Ghoshroy et al. 1995; Goodenough and Gilula 1974). However, there are electron microscopic observations of nonjunctional membrane domains within AGJs where the two membranes are not attached to each other (Fong et al. 2012; Leithe et al. 2012; Piehl et al. 2007). These membrane areas may provide AGJs an opportunity to fuse with other single-membrane vesicles. There is also evidence that the double-membrane structure of internalized AGJs becomes disrupted in coordination with the formation of single membranes and intraluminal vesicles. These multivesicular structures appeared to be able to fuse with other vesicles including lysosomes (Leithe et al. 2006, 2009).

While many reports have proposed an endocytic pathway of Cx43 internalization from the plasma membrane to the lysosome, an alternative mechanism for the delivery of Cx43 to the lysosome has been reported. One study using breast tumor cell lines found that blocking Cx43 trafficking to the plasma membrane from the Golgi with BFA did not affect the steady-state level of Cx43 that was otherwise significantly decreased by inhibiting protein synthesis with cycloheximide treatment (Qin et al. 2003). In addition, BFA treatment did not diminish the localization of Cx43 in lysosomal compartments. These results suggested that Cx43 might also be delivered directly to lysosomes from early secretory compartments (Fig. 2). A similar observation was made for the CMTX-linked Cx32 mutant R142W in PC12J cells (VanSlyke et al. 2000). As with the E208K mutant, the R142W Cx32 mutant does not traffic to the cell surface, although it can pass through the secretory pathway to reach the distal compartments of the Golgi. Inhibition of the lysosome with leupeptin increased Cx32 R142W levels in lysosomes despite the inability of the mutant to traffic through to the plasma membrane, which suggested the possibility of direct Cx32 transport from the secretory pathway to the lysosome (VanSlyke et al. 2000).

There is evidence that Cx43 internalized from the plasma membrane not only may be destined for lysosomal degradation but can be recycled back to the plasma membrane. First, dye coupling was observed to resume independently of de novo protein synthesis following Cx43 internalization and cell uncoupling that occurred during cytokinesis (Xie et al. 1997). Second, recycling of cell surface Cx43 was also demonstrated in experiments where S180 cells were first biotinylated, then treated with sodium 2-mercaptoethanesulfonate (MesNa) to strip the biotin label from the protein that had not been internalized. After a period of recovery, a subset of Cx43 was found to still be sensitive to MesNa, while control cells that were not allowed to resume vesicle-mediated recycling did not. These data suggested the possibility that intracellularly localized Cx43 was able to recycle back to the plasma membrane, where it became vulnerable to MesNa treatment (VanSlyke and Musil 2005). Third, using the tetracysteine/biarsenical labeling system with fluorescent FlAsH and ReAsH ligands to bind to internal tetracysteine tags in Cx43, the return of Cx43 to the plasma membrane after mitosis was demonstrated to result from Cx43 that was labeled prior to the onset of mitosis, instead of newly synthesized Cx43. This existing pool of Cx43 was responsible for reestablishing gap junctional communication following mitosis (Boassa et al. 2010). Finally, Cx43 in AGJs and vesicles which pinched off from AGJs were shown to colocalize with Rab11, a small GTPase involved in the recycling pathway (Gilleron et al. 2011). Taken together, these data indicate that not all internalized connexins are destined for degradation by lysosomes. Despite these reports, there is a general consensus that connexins can be internalized and degraded through the endolysosomal pathway (Berthoud et al. 2000; Leithe et al. 2012; Salameh 2006; Segretain and Falk 2004).

The molecular mechanisms that dictate the fate of connexin proteins are not clearly understood. Connexin posttranslational modifications are known to affect connexin function and localization. Of the various modifications, ubiquitination has been proposed to function as part of the internalization and, possibly, the subsequent intracellular trafficking mechanism of cell surface connexins. The ubiquitination of connexins has been discussed in depth by three recent reviews (Kjenseth et al. 2010; Leithe et al. 2012; Su and Lau 2012). These reviews cover the initial reports linking ubiquitination to connexin degradation (Laing and Beyer 1995; Laing et al. 1997), as well as later studies which revealed that connexin phosphorylation by MAP kinases resulted in multiple monoubiquitination events (Leithe and Rivedal 2004a, b). The monoubiquitination resulted in the targeting of Cx43 for internalization and degradation (Leithe and Rivedal 2004a, b), which differs from polyubiquitination associated with proteasomal

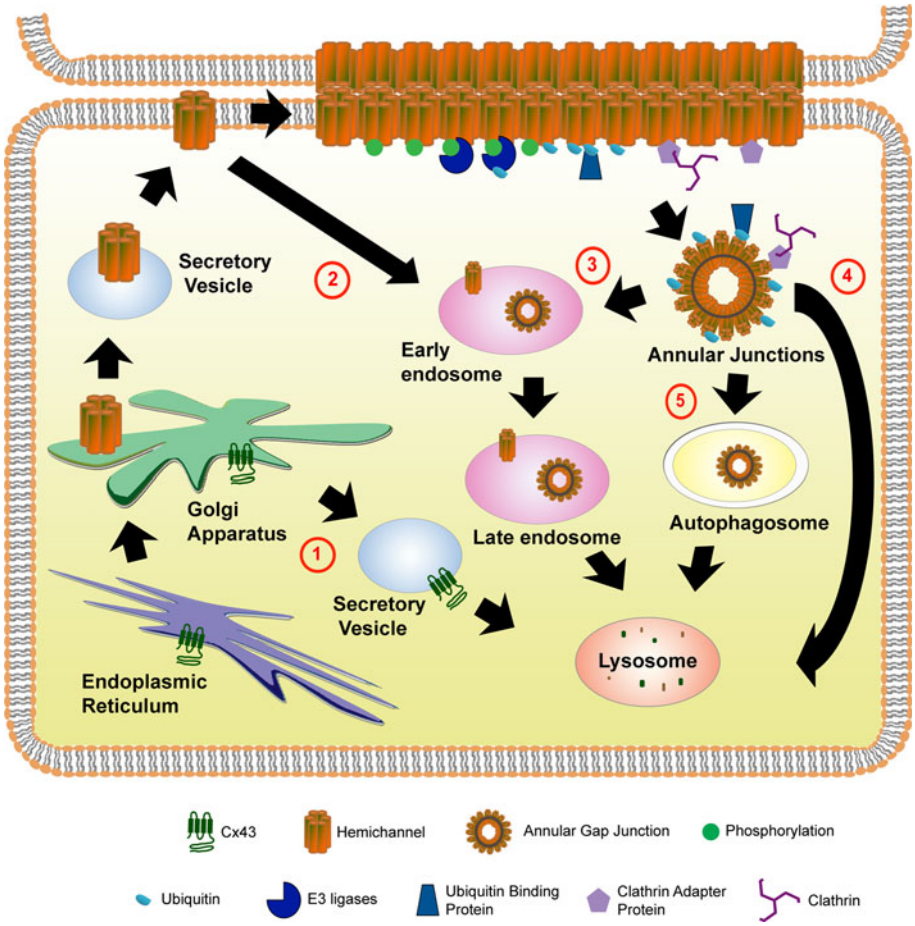

Fig. 2 Lysosomal degradation of connexins. Following synthesis in the ER, some new connexins may be transported directly to lysosomes via early secretory vesicles (*1*). Most connexins oligomerize in the Golgi to form hemichannels that are trafficked to the plasma membrane. Evidence suggests that nonjunctional hemichannels can be internalized and degraded via the endolysosomal pathway (*2*). Hemichannels that dock with hemichannels from adjacent cells to form gap junction channels will be incorporated into gap junction plaques. Phosphorylation and ubiquitination of connexins mediate gap junction internalization, which can involve the formation of AGJs. AGJs are internalized by a clathrin-dependent process and may fuse directly with early endosomes that will mature into late endosomes and fuse with lysosomes (*3*). Some observations suggest that AGJs can fuse directly with lysosomes (*4*). The direct fusion of AGJs with other vesicular membranes (*3–4*) could involve small areas of the outer AGJ membrane not occupied by gap junctions or the transformation of AGJs into single-membrane, multivesicular structures. Alternatively, phagophores may engulf AGJs into autophagosomes (*5*), which are able to fuse with lysosomes, resulting in degradation of their contents

degradation. Also, Cx43 has been found to interact with the ubiquitin-binding proteins Hrs (hepatocyte growth-factor regulated tyrosine kinase substrate), TSG101 (tumor susceptibility gene 101) and Eps15 (epidermal growth factor substrate 15), which mediate the ESCRT (endosomal sorting complex required for transport)–dependent endolysosomal trafficking of proteins (Auth et al. 2009; Girao et al. 2009; Leithe et al. 2009). Interestingly, a possible recycling of Cx43 back to the plasma membrane was observed when Hrs and TSG101 levels were reduced in siRNA-treated cells that were also treated with TPA to induce Cx43 internalization concurrently with a block in protein synthesis (Leithe et al. 2009). It has been proposed that Cx43 ubiquitination and perhaps the interactions with proteins of the ESCRT complexes dictate the trafficking of internalized Cx43, i.e., to the lysosome for degradation versus being recycled back to the cell surface.

Phagolysosomal Degradation of Cx43

Autophagy has long been recognized as a part of the connexin life cycle as a result of observations of gap junctions in autophagic structures by electron microscopy (Mazet et al. 1985; Sasaki and Garant 1986; Severs et al. 1989). Macroautophagy involves the degradation of cytoplasmic complex protein structures, where autophagic double-membrane compartments assemble de novo around protein complexes and engulf them along with surrounding cytoplasm. The engulfed proteins are subsequently degraded by acidic hydrolases following fusion of the outer

membrane of a completed phagophore with a lysosome (Yang and Klionsky 2009). AGJs, which are composed almost entirely of a double membrane fused by gap junctions, would be considered a complex protein structure that could be subject to macroautophagy. Despite previous observations of gap junctions in autophagosomes, only recently has autophagy received attention as an alternate means of delivering complex structures containing internalized connexins to lysosomes in studies that specifically investigated the role of autophagy degradation in the connexin life cycle (Fong et al. 2012; Hesketh et al. 2010; Lichtenstein et al. 2011). The most recent of these studies suggested that autophagy may be the most common pathway for degradation of internalized gap junctions (Fong et al. 2012).

Autophagy of Cx43 was observed in diseased cardiac cells (Hesketh et al. 2010). Cx43 localizes to the intercalated disc region of the plasma membrane in cardiomyocytes under normal conditions, where intact gap junction channels propagate the electrical signal required for the heart rhythm. Reduced electrical signaling in the failing heart has previously been linked to the relocalization of Cx43 from intercalated discs, which are located in the plasma membranes at the ends of cardiomyocytes, to lateral cell membranes (Beardslee et al. 2000; Peters et al. 1993; Severs et al. 2008; Smith et al. 1991). In failing canine heart myocardium, multilamellar membrane structures containing Cx43 were observed near the lateral membranes through electron microscopy. These Cx43-positive structures also colocalized with the autophagosome marker light chain 3 (LC3) (Hesketh et al. 2010). Another study demonstrated that internalized Cx43 and Cx50 colocalized with cup- or ring-shaped LC3-containing structures, which resembled autophagosomes and colocalized with p62 (Lichtenstein et al. 2011), which has been suggested to be a receptor for substrates in autophagic degradation (Komatsu and Ichimura 2010). Treatment of NRK and HeLa cells with the lysosomal inhibitor chloroquine during starvation, a condition known to induce autophagy, resulted in increased levels of Cx43 and Cx50, respectively, in autophagosomes (Lichtenstein et al. 2011). Most recently, AGJs have been demonstrated to be engulfed by autophagosomes prior to lysosomal degradation in HeLa cells (Fong et al. 2012). AGJ vesicles colocalized with the LC3 and p62 autophagic markers as well as the lysosomal markers LAMP-1 and LysoTracker. Electron microscopic studies identified the formation of phagophores around AGJ vesicles, which were subsequently degraded by lysosomal mechanisms. When autophagic degradation was inhibited or reduced, by treatment with either pharmacological agents or RNAi against requisite autophagic proteins, accumulation of total Cx43 protein in cells was observed (Fong et al. 2012). Thus, it appears that connexins that are internalized in AGJ vesicles can be targeted for degradation by autophagy. While there is significant evidence that both endolysosomal and phagolysosomal pathways participate in connexin degradation (Fig. 2), it is unclear whether these pathways are in fact part of the same overall mechanism that dictates the turnover of connexins from the cell surface or whether these pathways diverge at some point during the trafficking of connexins from the plasma membrane.

Regulation of Connexin Levels by Degradation—Relevance in Human Disease

There have been a number of reports suggesting that Cx43 degradation may play an important role in human diseases. A recent study analyzed the effect of Cx43 on cancer cell death when proteasomal degradation is inhibited (Huang et al. 2010). The rationale of studying proteasomal degradation in cancer arose from the use of the proteasomal inhibitor bortezomib as treatment for patients with multiple myeloma, among other cancers. Blocking degradation of Cx43 by the proteasome with MG132 also resulted in increased apoptosis of murine hepatoma Hepa-1c1c7 cells. Gap junction inhibitors flufenamic acid, 18-α glycyrrhetinic acid and carbenoxolone reduced the cell death induced by MG132. Using a gap junction–deficient cell line, porcine kidney epithelial LLC-PK1 cells, expression of wild-type Cx43 sensitized the cells to the apoptotic effects of MG132 that was blocked with treatment of gap junction inhibitors. Notably, expression of the Cx43 Δ130–137 deletion mutant, which does not create functional gap junctions, had the same effect as wild-type Cx43, suggesting that the apoptosis induced by MG132 was independent of the ability of Cx43 to establish GJIC. Induction of ER stress with treatment using tunicamycin and thapsigargin demonstrated a Cx43-dependent increase in cell death in response to stress (Huang et al. 2010). These observations indicated that Cx43 may have a critical role in the induction of cell death and may be an important component of successful cancer therapy.

Similarly, lysosomal degradation of connexins has been implicated as a potentially important mechanism in human cancer. The lysosome contributes to the downregulation of gap junctions at the plasma membrane in human tumors originating from human and mouse breast cancers, as well as in rat keratinocytes, rat C6 glioma cells and mouse testicular Leydig cells (Langlois et al. 2010; Naus et al. 1993; Qin et al. 2002, 2003; Segretain et al. 2003). Studies in breast tumor cells have linked decreased expression of Cx26 and Cx43 with increased tumor growth in mice and demonstrated that the connexins were localized preferentially to lysosomes in communication-deficient

MDA-MB-231 human breast tumor cells (Qin et al. 2002). Furthermore, delivery of Cx43 to lysosomes in MDA-MB-231 cells contributed to the loss of Cx43 from the cell surface, which was previously linked to tumor cell growth and the upregulation of genes involved in tumor growth and metastasis (Qin et al. 2003). Cx43 was also shown to function as a tumor-suppressor in rat endothelial keratinocyte cells (Langlois et al. 2010). Increased endolysosomal degradation of Cx43 in rat endothelial keratinocytes, following treatment with TPA or EGF, decreased Cx43 at the plasma membrane. This reduced the interaction of Cx43 with caveolin-1 and increased cell transformation (Langlois et al. 2010). Cx43 also exhibited anticancer activity in C6 glioma cells that express low endogenous levels of Cx43, where the overexpression of Cx43 resulted in reduced proliferation and tumorigenesis (Naus et al. 1993). This was linked to an increase in Cx43 and gap junction plaques observed at the surface of Cx43-overexpressing cells compared to untransfected cells, where distinctive gap junctional aggregates were not observed. In transfected as well as untransfected glioma cells, AGJs and Cx43 were both observed in lysosomes, indicating their involvement in Cx43 degradation (Naus et al. 1993).

Cx43 autophagy may also play an important role in heart disease and trauma. An elevation in autophagosomes containing Cx43 has been reported in failing canine myocardium (Hesketh et al. 2010), which suggests a potential mechanism for how Cx43 is removed from the intercalated disc region of cardiomyocytes, an observation commonly made in human heart disease.

Concluding Remarks

Aberrant connexin degradation clearly contributes to the impaired regulation of connexin functions that is associated with human diseases. These observations underscore the importance of fully elucidating the specific mechanisms of proteasomal, endolysosomal and phagolysosomal degradation pathways in connexin turnover. Recent studies demonstrating the involvement of autophagy in the degradation of internalized Cx43 reveal the need to reexamine the previous studies of connexin lysosomal degradation for the possible role of autophagy. Earlier reports proposed that lysosomal degradation occurred as part of the endosomal trafficking of Cx43. However, since lysosomal degradation is the end point for both endolysosomal and autophagic degradation, it is important to clarify whether these two pathways are separate and distinct processes or whether they are part of the same mechanism. In addition, further study of the factors that facilitate connexin proteasomal degradation is required to gain a better understanding of how the proteasome affects connexin-dependent processes as cellular and ER stresses, which influence proteasomal degradation, can have dramatic effects on connexin levels and trafficking. It has become evident that connexin turnover is controlled by a diverse array of physiological stimuli and posttranslational modifications. Understanding the molecular mechanisms by which these factors target connexins toward the different degradation pathways and influence their functioning may evoke novel approaches to restore connexin function in cells resulting from the aberrant degradation of connexins that can contribute to various pathological conditions.

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