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The Life Cycle of a Connexin: Gap Junction Formation, Removal, and Degradation

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Gap junction proteins, connexins, possess many properties that are atypical of other well-characterized integral membrane proteins. Oligomerization of connexins into hemichannels (connexons) has been shown to occur after the protein exits the endoplasmic reticulum. Once delivered to the cell surface, connexons from one cell pair with connexons from a neighboring cell, a process that is facilitated by calcium-dependent cell adhesion molecules. Channels cluster into defined plasma membrane domains to form plaques. Unexpectedly, gap junctions are not stable (half-life <5 h) and are thought to be retrieved back into the cell in the form of double membrane structures when one cell internalizes the entire gap junction through endocytosis. Evidence exists for both proteasomal and lysosomal degradation of gap junctions, and it remains possible that both mechanisms are involved in connexin degradation. In addition to opening and closing of gap junction channels (gating), the formation and removal of gap junctions play an essential role in regulating the level of intercellular communication.

KEY WORDS: Connexins; gap junctions; trafficking; assembly; degradation.

INTRODUCTION

Gap junctions were first described in the 1960's as being closely apposed plasma membranes with a distinct 2–4 nm gap (Robertson, 1963; Revel and Karnovsky, 1967). In later years these structures were described in freeze-fracture replica as being composed of packed arrays of membrane particles or plaques (Goodenough and Revel, 1970). Makowski *et al.* (1977) used data from x-ray diffraction to construct a model where a hexamer from one cell pairs with a hexamer from an adjacent cell to form a complete intercellular channel. Although this model was constructed with limited insight into the molecular constituents of the gap junction, it is still the working model of today. Innovative studies using atomic force microscopy by Hoh and others affirmed the packed array of gap junction channels in split gap junction plaques that were imaged under aqueous conditions (Hoh *et al.*, 1991a, 1993; Lal *et al.*, 1995).

One of the more surprising discoveries made by Fallon and Goodenough (1981) was that gap junctions from mouse liver have a half-life of only 5 h. This original observation was re-established several years later *in vitro* when the half-life of the protein constituents of gap junctions, connexins, was examined in pulse-chase experiments (Traub *et al.*, 1987; Laird *et al.*, 1991; Musil *et al.*, 1990a,b; Laird *et al.*, 1995). In fact, these assays revealed that connexin32 (Cx32) and connexin43 (Cx43) have half-lives as short as 1.5–3.5 h. Contrary to original expectations, gap junctions are not static long-lived structures but rather they undergo a continual process of formation and removal. It has now been established that cells can regulate their intercellular communication needs by assembling more channels or by down-regulating existing channels. This mini-review will focus on the key events in gap junction formation and removal, namely: connexin biosynthesis, trafficking, oligomerization into connexons, assembly of gap junction plaques, and finally internal-

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ization and degradation of gap junctions (see Fig. 1 for overview).

IMPORT OF CONNEXINS INTO THE ENDOPLASMIC RETICULUM AND TOPOLOGY

The evidence that exists to date suggests that connexins are co-translationally imported into the endoplasmic reticulum (ER) in cultured cell lines. Recent work by Falk and coworkers established that several connexins can be co-translationally imported into pancreatic ER-derived microsomes in a signal recognition particle-dependent manner (Falk *et al.*, 1994). However, these studies also suggest that there was some additional processing of connexins by signal peptidase although connexins have no cleavable signal sequence. Therefore, specific factors must be established in normal cells that prevent this aberrant processing of connexins (Falk *et al.*, 1994).

Connexins pass through the membrane several times and thus are considered class III membrane proteins. The establishment of proper connexin topology occurs in the endoplasmic reticulum. A combination of protease protection assays and antibody binding studies on isolated liver and heart gap junction membranes established that Cx32 and Cx43 pass through the membrane four times forming two extracellular loops and a cytoplasmic loop with both the amino and carboxy termini exposed to the cytoplasm (Beyer *et al.*, 1987; Milks *et al.*, 1988; Hertzberg *et al.*, 1988; Beyer *et al.*, 1989; Yancey *et al.*, 1989; Laird and Revel, 1990). Although the topologies of other connexins have not been extensively studied, sequence homologies within the transmembrane domains suggest that the remaining members of the connexin family will have similar or identical organizations in the membrane.

It is well established that the lumen of the endoplasmic reticulum is the site of intramolecular disulfide bond formation. In 1991 several investigators cleaved Cx43 and Cx32 and examined the proteolytic products on both reducing and nonreducing gels (John and Revel, 1991; El Aoumari *et al.*, 1991; Rahman and Evans, 1991). The availability of antibodies to specific segments of the connexins allowed for conclusive evidence that both Cx32 and Cx43 have at least one disulfide bond linking the two extracellular loops. Sequence examination of all connexins revealed that there are three cysteine residues per extracellular loop that are positionally conserved in all connexins except Cx31 where an additional amino acid is inserted between two of the cysteines (Hoh *et al.*, 1991b). Whether there is more than one interloop disulfide bond or whether intraloop disulfide bonds exist remains to be demonstrated.

Cx43 does not oligomerize in the ER (Musil and Goodenough, 1993). This surprising finding raises the question as to what prevents connexin oligomerization within the ER where many well-characterized integral membrane proteins are known to fold and assemble (Hurtley and Helenius, 1989). In recent years, ER chaperones such as BiP (Pelham, 1989) and calnexin

Gap junction formation, removal and degradation

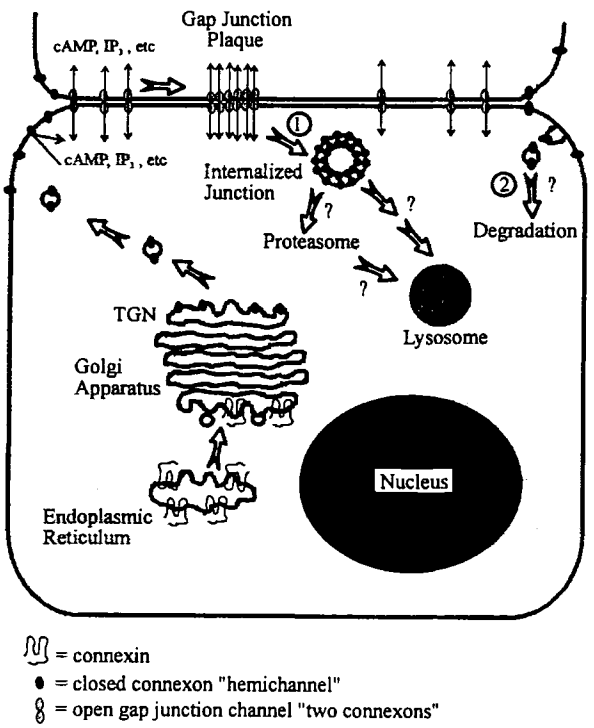

Fig. 1. Model illustrating the formation and removal of gap junctions. Connexin topology is established in the endoplasmic reticulum where the protein is observed traversing the membrane four times. Oligomerization of connexins into connexons is thought to occur in the *trans* Golgi network (TGN) and these hemichannels must remain closed until proper intercellular connexon docking occurs at the cell surface where the channels cluster into plaques and function. One mechanism of gap junction internalization is via a double-membrane system (1) to form an internalized junction called an annular junction. Alternate routes for the internalization of connexons (2) have not been ruled out. Degradation of gap junctions is complex and evidence suggests roles for both proteasomes and lysosomes. Gap-junction-like membrane fragments have been identified in lysosomes.

(Ahluwalia *et al.*, 1992) have been characterized and shown to transiently interact with a variety of proteins either to promote folding or retain proteins in the ER until proper folding is achieved. Attempts to co-immunoprecipitate Cx43 and calnexin have been unsuccessful, suggesting that Cx43 does not interact with calnexin (unpublished results). No further data exists as to whether connexins interact with molecular chaperones while in the ER, but this possibility remains an exciting area of exploration.

CONNEXINS IN THE GOLGI

The classical secretory pathway involves protein trafficking to the Golgi apparatus, and in this regard Cx43 is no exception to the rule. We and others have demonstrated in primary cultures of cardiac myocytes (Puranam *et al.*, 1993), myometrial cells (Hendrix *et al.*, 1992), normal rat kidney cells, and transformed BICR-M1R_k cells (Laird *et al.*, 1995) that Cx43 colocalizes with constitutive markers of the Golgi apparatus. Subcellular fractionation of rat liver suggests that hepatocytes also have a reservoir of Cx32 in the Golgi apparatus (Rahman *et al.*, 1993). It is important to note that connexins have no suitable site for N-linked glycosylation and are not glycoproteins (Rahman *et al.*, 1993). Interestingly, inhibition of protein trafficking with monensin or brefeldin A revealed that Cx43 is modified into an alkaline phosphatase-sensitive form in primary cultures of cardiac myocytes (Puranam *et al.*, 1993) and rat mammary BICR-M1R_k tumor cells (Laird *et al.*, 1995), suggesting that the initial phosphorylation of Cx43 occurs prior to its exit from the Golgi apparatus. Crow *et al.* (1990) observed that Cx43 was modified in vole fibroblasts within 15 min, providing further evidence that a post-translational modification occurs early in the secretory pathway. Whether phosphorylation of Cx43 early in the secretory pathway occurs in all cell types is not clear, nor is it clear whether this phosphorylation of Cx43 is essential for trafficking or correct assembly. Obviously phosphorylation is not essential for all connexins as Cx26 is not a phosphoprotein.

Musil and Goodenough (1993) provided the first information on the intracellular compartment where connexin oligomerization occurs. Using inhibitors of protein trafficking, sucrose gradient fractionation, and chemical crosslinkers they concluded that Cx43 oligomerization into connexons occurred after exiting the ER, probably in the *trans* Golgi Network (TGN). These intracellular hemichannels (connexons) would be expected to be closed in order to maintain the integrity of the intracisternal space and prevent small molecular weight components of the cytosol from entering Golgi cisternae. Why connexins oligomerize in such a late compartment and not in the ER is not understood, but it may be linked to the inability of cells to prevent connexon pairing in earlier secretory compartments. Furthermore, whether intramolecular aspects of connexins prevent oligomerization or whether oligomerization is promoted in the TGN by a yet undefined TGN chaperone is unknown. The complexity in understanding connexons has recently increased since not only do homomeric connexons exist (Sosinsky, 1995) but Stauffer (1995) provided evidence that heteromeric connexons can form in insect cells that express two connexins. Furthermore, heteromeric (Cx46 and Cx50) connexons have been characterized in lens gap junction channels (Jiang and Goodenough, 1996).

CONNEXON DELIVERY TO THE PLASMA MEMBRANE, INTERCELLULAR CONNEXON DOCKING, AND PLAQUE FORMATION

It is thought that connexons are delivered from the TGN to the plasma membrane by the classical vesicular transport mechanism (Fig. 1). At least two hypotheses exist as to where connexons are inserted in the plasma membrane of nonpolarized cells; (a) they may be delivered to the plasma membrane at random and later resorted within the lipid bilayer to predefined flattened regions (i.e., formation plaques; Johnson *et al.*, 1974) for proper intercellular connexon docking and eventual plaque formation; (b) they may be delivered directly to regions of the plasma membrane where intercellular docking and plaque formation can occur within defined boundaries. In polarized cells there is no evidence that connexons are inserted into the apical surface as relocation to the basolateral domains would be impaired by the tight junctions. Interestingly, in polarized thyroid epithelial cells, connexins were shown to be sorted to distinct plasma membrane domains (Guerrier *et al.*, 1995). However, we have found in nonpolarized BICR-M1R_k cells that express endogenous Cx43 and transfected Cx32 that both connexins were sorted to the same gap junction plaques (D. Laird, S. Bond, and C. Naus, unpublished results).

It is likely that intercellular connexon docking and/or plaque formation involves cell adhesion molecules. Musil *et al.* (1990b) demonstrated that S180 cells that biosynthesized Cx43 but failed to assemble gap junctions could be induced to make gap junctions when transfected with L-CAM. Furthermore, this same group showed that Fab' fragments from antibodies to L-CAM could be used to disassemble gap junctions (Musil *et al.*, 1990b). The following year, Jongen *et al.* (1991) demonstrated in mouse epidermal cells that the calcium-dependent regulation of gap junction intercellular communication is controlled by E-cadherin. Later monovalent antibodies to N-cadherin were found to inhibit gap junction assembly in reaggregating Novikoff cells (Meyer *et al.*, 1992). Thus, at least two members of the calcium-dependent cell adhesion family of molecules may be involved in positioning cell membranes for gap junction assembly, yet their large extensions into the extracellular space prevents them from actually being part of the gap junction plaque.

Proper intercellular connexon docking occurs when noncovalent forces seal two apposing hemichannels in such a fashion that small molecules passing through the channel do not leak into the extracellular space. Docking may occur within the domain of a formation plaque where the membranes are brought close together (Johnson *et al.*, 1974), or transient intercellular docking may occur at other cell surface locations where the apposing membranes are suitably positioned. While monovalent antibodies to the extracellular domains of Cx43 could inhibit gap junction assembly (Meyer *et al.*, 1992), synthetic peptides that constituted the majority of the two Cx43 extracellular loops were incapable of preventing gap junction assembly in cardiac myocytes, reaggregating Novikoff cells, and BICR-M1R$_k$ tumor cells (unpublished results). Dahl and colleagues, however, were able to inhibit homotypic docking of connexons in paired *Xenopus* oocytes using synthetic peptides that represented the extracellular loop regions of Cx32 (Dahl *et al.*, 1992). Thus, under certain conditions the extracellular loop regions of connexins alone encode sufficient information to inhibit proper intercellular connexon docking. Moreover, mutational analysis where extracellular loop cysteines were changed into serines resulted in the loss of connexon function, strongly suggesting that cysteine conservation is critical for tight intercellular connexon interactions (Dahl *et al.*, 1992).

Stability of gap junction channels appears to be achieved when several channels cluster to form a plaque. Plaque sizes are variable but can be over 1 μm in diameter. Musil and Goodenough (1991) first demonstrated in NRK cells that Triton X-100 resistance was associated with gap junction plaques, and this was also correlated with extensive phosphorylation of Cx43. We have seen a similar correlation of phosphorylated species of Cx43 with Triton X-100 resistance in BICR-M1R$_k$ rat mammary tumor cells (Fig. 2). Virtually all the nonphosphorylated form of Cx43 at 42 kDa was Triton X-100 soluble (Fig. 2, lane b)

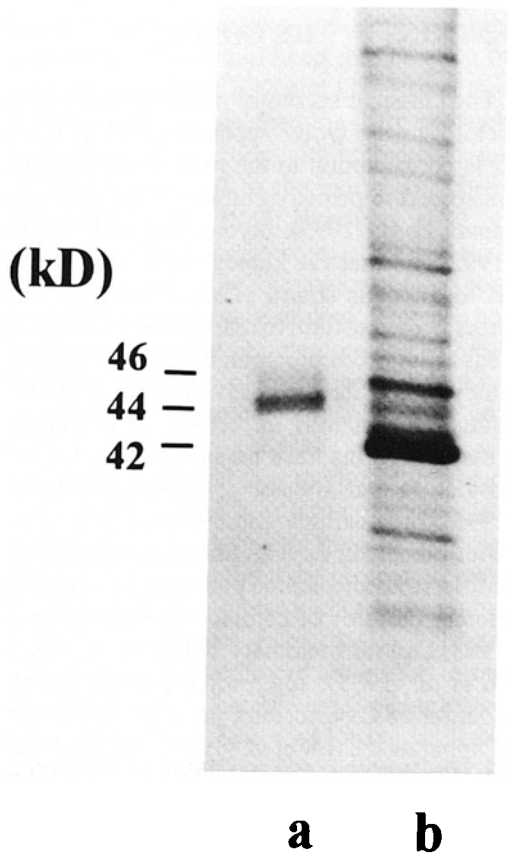

Fig. 2. Separation of Triton X-100 soluble Cx43. Rat Mammary tumor BICR-M1R$_k$ cells grown on culture dishes were labeled with ^{35}S-*trans* label for 2 h and lysed *in situ* with 1% Triton X-100. The cells were scraped from the dish, centrifuged at 40,000*g* for 50 min, and the Triton X-100 soluble fraction was separated from the Triton X-100 insoluble fraction. The Triton X-100 resistant fraction only was further treated with RIPA buffer and Cx43 was immunoprecipitated as described in Laird *et al.* (1991). The phosphorylated forms of Cx43 at 44 and 46 kDa were detected in the Triton X-100 insoluble fraction (lane a) while the unphosphorylated form of Cx43 at 42 kDa was seen only in the Triton X-100 soluble fraction (lane b). Note that several proteins co-immunoprecipitated with Cx43 in the Triton X-100 soluble fraction, but it remains unclear if this reflects specific interactions that occur in the cell.

while phosphorylated species of Cx43 between 44 and 46 kDa were observed in the Triton X-100 insoluble fraction (Fig. 2, lane a). The bulk of Cx43 phosphorylation appears to occur when Cx43 reaches the plasma membrane, although whether it is causal or coincidental with Cx43 assembly into detergent-resistant fractions or plaques remains to be conclusively demonstrated. Oh *et al.* (1993) showed that incompletely phosphorylated species of Cx43 could also assemble into gap junction plaques although these channels were not functional. Moreover, the phorbol ester, TPA, that activates protein kinase C was shown to inhibit gap junction plaque formation in reaggregating Novikoff cells (Lampe, 1994).

INTERNALIZATION OF GAP JUNCTIONS

The mechanism of how gap junctions are removed from the cell surface and/or are disassembled is not well understood. One of the first models proposed for gap junction removal from the cell surface was based on the identification of double membrane intracellular structures that were defined as annular gap junctions (Larsen *et al.*, 1979) (Fig. 1). These structures found in both normal and tumor cells were proposed to have originated from the cell surface where the entire gap junction was removed into one of the two apposing cells. The annular gap junction consists of only gap junction membranes as no other membrane components were visible by electron microscopy. However, Larsen *et al.*, (1979) reported the presence of actin associated with internalized gap junctions and described patches of clathrin-like bristles affiliated with invaginations of the junctional membrane in granulosa cells. Annular gap junctions have been described morphologically in many reports, and in later years antibodies to connexins were used to demonstrate that these structures were indeed connexin-positive (Dermietzel *et al.*, 1991; Risley *et al.*, 1992; Naus *et al.*, 1993). In immunogold labeling studies, Cx43-containing gap junctions were heavily decorated with gold particles at cardiac myocyte cell interfaces (Fig. 3, arrowheads). In addition, immunolabeled gap junction structures that may be in the process of entering the cell as well as structures that appeared to be inside the cell were observed (Fig. 3 arrows). However, the static nature of electron microscopic experiments prevents the origin of annular gap junctions from being conclusively determined.

Our laboratory recently took a novel approach to addressing this issue of how gap junctions are removed from the cell surface. Live normal rat kidney cells were microinjected with affinity-purified anti-Cx43 antibodies that recognize only the carboxy termini of the molecule (Chodock *et al.*, unpublished). These antibodies targeted and specifically labeled the gap junctions in the normal rat kidney cells. After incubating the cells at 37°C for up to 6.5 h, intracellular structures that carry the microinjected antibody were found by confocal microscopy in cells that contact the microinjected cell. These results demonstrate for the first time in live cells that connexin constituents from one cell are removed into a neighboring cell. Electron microscopic analysis will be necessary to confirm if these intracellular structures have the same ultrastructural characteristics as annular gap junctions. Furthermore, whether other mechanisms of gap junction removal from the cell surface exist cannot be ruled out.

CONNEXIN DEGRADATION

The mechanism of connexin degradation has recently become an area of focus. If annular gap junctions represent a major pathway for gap junction removal, the question remains as to how these structures are degraded. To date there is no evidence that gap junctions or their connexin constituents are recycled to the cell surface. In fact, the molecular complexity of assembled gap junctions would likely prohibit channel reutilization.

Several lines of evidence suggest that lysosomes are involved, at least in part, in connexin degradation. Subcellular fractionation of rat liver revealed Cx32 in lysosomes at levels comparable to the Golgi apparatus (Rahman *et al.*, 1993). We and others have identified gap junctions and/or connexins in lysosomes or phagolysosomes (Ginzberg and Gilula, 1979; Murray *et al.*, 1981; Larsen and Tung, 1978; Larsen and Risinger, 1985; Naus *et al.*, 1993; Chodock *et al.*, unpublished). Thirdly, inhibitors of lysosomal enzymes such as leupeptin and ammonium chloride result in an accumulation of intracellular Cx43 in BICR-M1R_k cells (Chodock *et al.*, unpublished).

This issue of connexin degradation recently became more complex when Laing and Beyer (1995) demonstrated that specific inhibitors of proteasomal enzymes (*N*-acetyl-L-leucyl-L-leucinyl-norleucinal)

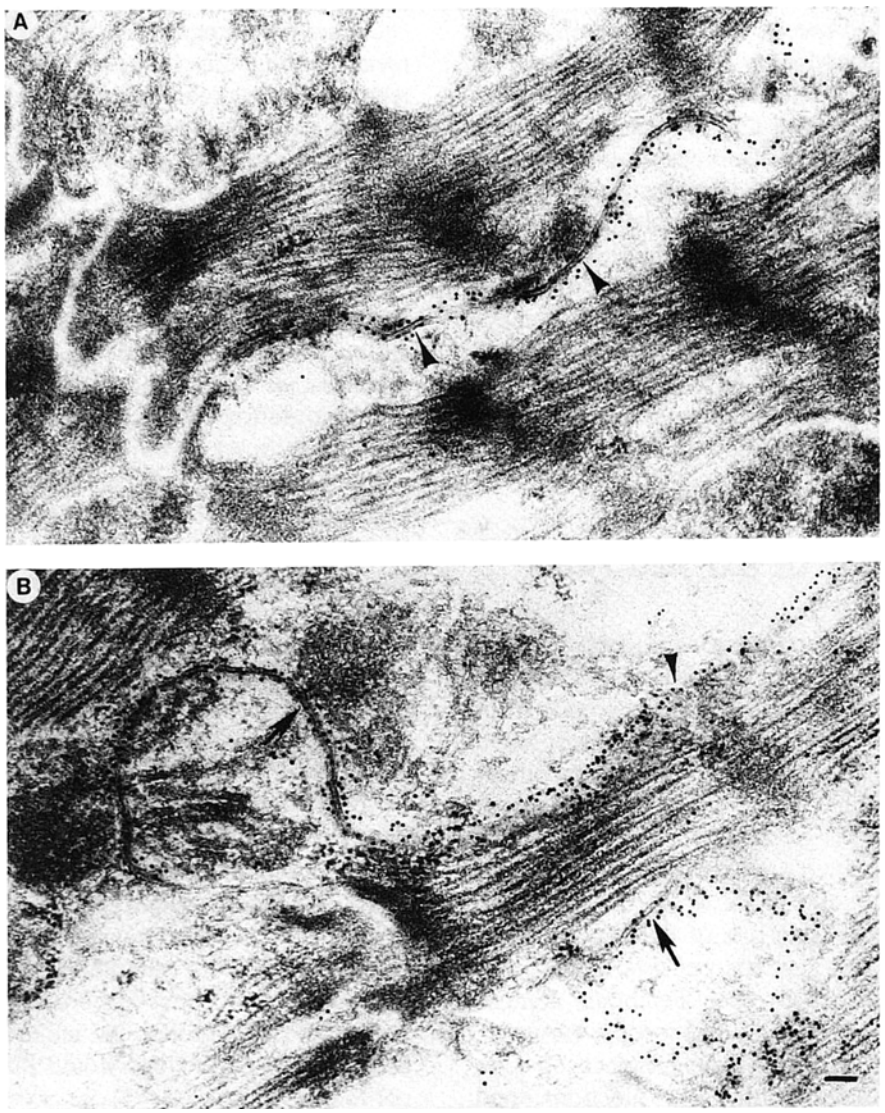

Fig. 3. Electron micrographs of anti-Cx43 immunogold labeled gap junctions in adult rat cardiac myocytes (A, B). Pieces of rat heart were fixed in 0.1 M sodium cacodylate buffer containing 0.2% glutaraldehyde and 4.0% paraformaldehyde overnight at 4°C and embedded in Lowicryl. Ultrathin sections were immunolabeled with 100-fold diluted anti-Cx43 antibody (CT-360; Laird and Revel, 1990) followed by goat anti-rabbit secondary antibody conjugated to 9 nm gold particles. Note that immunogold labeled gap junctions are not only seen at the cell surface (arrowheads), but also in membranes that appear to be in the process of entering the cell or have already entered the cell. Bar = 0.1 μm.

prolonged the half-life of Cx43 in BWEM cells. Moreover, these authors used E36 Chinese hamster ovary cells and a temperature-sensitive mutant of these cells that had a defect in the ubiquitin-activating protein, E1, to provide evidence that Cx43 can be degraded in a ubiquitin-dependent fashion and that proteasomal proteolysis may play a significant role in Cx43 degradation (Laing and Beyer, 1995). Earlier Elvira *et al.* (1993) demonstrated *in vitro* that the neutral proteases, calpains, could cleave Cx32 but not Cx26.

Several questions related to the mechanism of how connexins are degraded remain unanswered. Is it possible that proteasomes are able to attack annular junctions where the integral membrane protein is enclosed in a double-membrane system? How would the proteasome deal with the lipid bilayers associated

with annular gap junctions? Do proteasomes degrade connexins at the cell surface or within the cell? It is interesting to note that the annular gap junctions in Cx43-transfected C6 glioma cells presented in a report by Naus *et al.* (1993) are preferentially immunogold labeled for Cx43 on the inside as opposed to the outside. It is possible to speculate from this data that proteasomes were able to access and degrade the cytoplasmic domains of Cx43 that were exposed to the outside (cytoplasmic face) of the annular gap junction. An intriguing possibility would be that the proteosome conditions the annular gap junction for eventual fusion with a lysosome. This hybrid form of protein degradation would be consistent with the somewhat conflicting data that have been amassed. Alternatively, the degradation of connexins may be via both lysosomal and proteasomal pathways (Fig. 1) and possible cell type differences may exist. Clearly the internalization and degradation of connexins are much more complicated than originally thought and further experimentation is required in these areas.

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