Gap junctions are the intercellular channels localized to the plasma membrane of cell-cell contact between adjacent cells. The existence of these channels allows an intercellular exchange of low molecular weight molecules including most second messengers like cAMP, calcium, and inositol 1,4,5-triphosphate (1, 2). The building block of gap junctions is a transmembrane protein called a connexin. Six connexin molecules oligomerize into a hemichannel termed a connexon. After being transported to the plasma membrane, two hemichannels supplied by adjacent cells dock with each other to form a complete gap junction channel. The connexin family consists of at least 20 members (3), but Cx43 has attracted the most attention as it is widely expressed in many tissues and Cx43 gap junctions correlate with many physiological functions such as cardiac excitability (4). As expected, Cx43 has been shown to co-translationally insert into endoplasmic reticulum (ER)1 membranes followed by oligomerization during its transport from the ER to the Golgi apparatus (5–7). Further maturation of newly synthesized Cx43 includes phosphorylation, a process that is closely related to gap junction assembly and turnover (8). As analyzed by SDS-PAGE, Cx43 is normally reported as being represented by a faster migrating, non-phosphorylated species (Cx43-P 0) and two more slower migrating, phosphorylated species (Cx43-P 1 and Cx43-P 2) that are both sensitive to phosphoserine-specific phosphatases (8, 9). Due to the lack of insight into the phosphorylation events that lead to these two Cx43 species, in the present study we refer to these species collectively as Cx43-P. The COOH-terminal portion of Cx43 contains 21 serines, and at least 5 protein kinases have been shown to phosphorylate Cx43, suggesting that this region of molecule contains a complex array of potential regulatory sites (10).

Cx43 is a short-lived protein with a half-life of only 1–3 h in cultured cells and intact rat heart (9, 11). Therefore, an efficient degradation mechanism is indispensable and is necessary to ensure the dynamic turnover of Cx43. In general, lysosomes are involved in the degradation of integral membrane proteins and receptor-mediated internalized proteins (12). The proteasome, in addition to taking part in degrading most cytosolic and nuclear proteins (13–15), plays a role in endoplasmic reticulum localized degradation of misfolded proteins. Interestingly, both lysosomes and proteasomes have been reported to be involved in the degradation of Cx43 (16–18). Lysosomal degradation of Cx43 was evidenced by the finding that Cx43 annular profiles were associated with lysosome-like structure in C6 glioma cells (19). However, Laing and Beyer (16) showed that by treating E36 Chinese hamster ovary cells with proteasomal inhibitors and by using cells with defective ubiquitination, Cx43 accumulated accompanied by a prolonged half-life, providing the first evidence of the involvement of proteasome in Cx43 degradation (16). Later pulse-chase experiments revealed that inhibitors of both proteasomes and lysosomes caused a prolonged half-life of Cx43 to a similar extent in rat cardiac myocytes indicating the

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† Supported by a Canadian Institutes of Health Research Studentship.

‡ To whom correspondence should be addressed: Centre for Gap Junction Research, Dept. of Anatomy and Cell Biology, University of Western Ontario, London, Ontario N6A 5C1, Canada, Tel.: 519-661-2111 (ext. 86827); Fax: 519-850-2562; E-mail: dlaird@uwo.ca.

The present study was designed to determine the specific roles played by lysosomes and proteasomes in the degradation of Cx43 in both gap junctional intercellular communication-deficient MDA-MB-231 and -competent BICR-M1R k cells. In MDA-MB-231 cells, immunolocalization and brefeldin A protein transport blocking studies revealed that there was a propensity for newly synthesized Cx43 to be transported to lysosomes. On the other hand, light and electron microscopic analysis of BICR-M1R k cells showed that Cx43 gap junctions were prevalent with a subpopulation of intracellular Cx43 localized to lysosomes. In both cell types, Western blots revealed a notable increase in total cellular Cx43 in response to lysosome inhibitors. Interestingly, lactacystin inhibition of proteosomal degradation in MDA-MB-231 cells resulted in a marked increase in phosphorylated Cx43 at the expense of non-phosphorylated Cx43, and this change corresponded with an increase in “oversized” gap junction plaques. In BICR-M1R k cells, lactacystin treatment partially prevented the BFA-induced loss of gap junctions. Together, our data suggests that lysosomes play a key role in not only degrading internalized gap junction in BICR-M1R k cells but also in degrading Cx43 delivered from early secretory compartments to lysosomes in MDA-MB-231 cells. Overall proteosomal degradation regulates the stability of phosphorylated Cx43 and appears to promote the internalization of Cx43 from the cell surface.

Lysosomal and Proteosomal Degradation Play Distinct Roles in the Life Cycle of Cx43 in Gap Junctional Intercellular Communication-deficient and -competent Breast Tumor Cells

Hong Qin, Qing Shao, Suleiman A. Igdoura, Moulay A. Aloufi-Jamali, and Dale W. Laird

From the Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario N6A 5C1, Canada, the §Departments of Biology and Pathology, McMaster University, Hamilton, Ontario L8S 4L8, Canada, and the ¶Department of Medicine, Pharmacology, and Therapeutics and Centre for Translational Research in Cancer, Lady Davis Institute for Medical Research, McGill University, Montreal, Quebec H3T 1E2, Canada

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co-existence of these two proteolytic pathways for Cx43 degradation (17). More recently, Musil et al. (2000) observed that inhibiting proteasome degradation of Cx43 increased gap junction assembly and intercellular dye coupling and subsequently suggested a mechanism where gap junctional intercellular communication could be regulated at the level of connexin turnover (18). Subcellular isolation of Cx43 and Cx52 suggested that a small population of ER-retained connexins was presumably made available to proteasomes by being translocated into the cytosol in the presence of proteasomal inhibitors (20). Interestingly, little data exist to show that either soluble or insoluble connexins become ubiquitinated.

Although good evidence exists that suggest that both lysosomes and proteasomes are involved in Cx43 degradation, it is difficult to reconcile why both pathways are available and needed for Cx43 degradation. It is also not clear whether both pathways degrade Cx43 derived from cell surface gap junction plaques as well as possible immature Cx43 localized to the ER or other compartments involved in the secretory pathway. Moreover, it is possible that these degradation pathways are differentially active in cells that are gap junctional intercellular communication (GJIC)-competent as opposed to cells that reflect gap junction assembly defects and are GJIC-deficient. In addition, the direct evidence that Cx43 is a substrate for proteasomal degradation is limited.

Our present data revealed that intracellularly accumulated Cx43 in GJIC-deficient MDA-MB-231 cells and internalized endogenous Cx43 in BICR-M1Rk cells were both targeted to lysosomes for degradation suggesting that lysosomes play a major role. Interestingly, a transport pathway is available to direct Cx43 from early secretory compartments to lysosomes for degradation. Active proteasomal degradation was found to reduce the phosphorylated species of Cx43 and destabilize gap junctions at the plasma membrane.

**MATERIALS AND METHODS**

**Cell Preparation**—MDA-MB-231 human breast tumor cells (ATCC) or BICR-M1Rk cells, a rat mammary tumor cell line (7), were grown to confluence in RPMI 1640 or Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cells were maintained at 37 °C with 5% CO2. Since MDA-MB-231 wild-type cells have heterogeneous low expression of endogenous Cx43 we chose to overexpress exogenous Cx43. To generate Cx43 or GFP-tagged Cx43 (Cx43-GFP) expressing MDA-MB-231 cells, cDNAs were cloned into replication-defective AP2 retroviral cDNA vector as reported previously (21). Approximately 1×106 expressing MDA-MB-231 cells, cDNAs were cloned into replication-permissive low expression of endogenous Cx43 we chose to overexpress exogenous Cx43 in GJIC-deficient MDA-MB-231 cells and internalized endogenous Cx43 in BICR-M1Rk cells were both targeted to lysosomes for degradation suggesting that lysosomes play a major role. Interestingly, a transport pathway is available to direct Cx43 from early secretory compartments to lysosomes for degradation. Active proteasomal degradation was found to reduce the phosphorylated species of Cx43 and destabilize gap junctions at the plasma membrane.

**Inhibition of Lysosomal or Proteasomal Protein Degradation**—The inhibitors of proteasomes (lactacystin) and lysosomes (chloroquine, leupeptin, and ammonia chloride) were all purchased from Sigma. Cells were treated with the inhibitors at final concentration as follows: 10 μM of lactacystin, 200 μM of chloroquine, 100 μg/ml of leupeptin, and 10 mM of NH4Cl. To block ER-Golgi translocation or protein synthesis 5 μg/ml of brefeldin A (BFA) or 20 μg/ml of cycloheximide (CHX) was employed (7, 18). For these experiments, equal amount of cells were grown in 60-mm tissue culture dishes with or without glass coverslips to 70–80% confluence. Cells treated with the various reagents for 6 h at 37 °C were then either fixed for immunofluorescent staining or lysed to collect proteins for Western blots.

**Immunocytochemistry, Quantification of Cx43 Gap Junctions, and Live Confocal Microscopy**—Control or proteasome/lysosome inhibitor-treated Cx43-overexpressing MDA-MB-231 (MDA-MB-231×Cx43) or BICR-M1Rk cells grown on glass coverslips were fixed in 80% methanol, 20% PBS at 4 °C for 20 min and immunolabeled as described previously (7). Cx43 Western blot was detected using a 500-fold dilution of Cx43 polyclonal antibody (Sigma). In double labeling experiments, the MDA-MB-231×Cx43 cells labeled with a polyclonal Cx43 antibody followed by goat anti-rabbit IgG conjugated with fluorescein isothiocyanate were double labeled with a 100-fold dilution of human LAMP-1 monoclonal antibody (24) followed by goat anti-mouse secondary antibody conjugated to Texas Red. The images were captured on a Zeiss LSM 410 inverted confocal microscope as described previously (7).

Gap junction plaques in MDA-MB-231×Cx43 and BICR-M1Rk cells were quantified based on the confocal images acquired using 63× oil lens. The images were digitally enhanced to clearly show the cell-cell appositions so that only the punctate gap junction plaques on the cell-cell contacts that were a minimal size of 0.5 μm were counted. This method thus eliminated the Cx43-positive structures that may represent internalized gap junctions or transport intermediates. In untreated and drug-treated MDA-MB-231×Cx43 cells, we counted the total number of gap junction plaques on 20 cell-cell interfaces that represented four to six randomly chosen confocal images. The semi-quantification data was then statistically analyzed using the Student’s t test and represent the average value of at least three independent experiments ± S.E.

In lactacystin-treated MDA-MB-231×Cx43 cells, we found some Cx43-positive gap junction plaques that had a continuous length in the x and y dimensions of 1 μm or greater. These plaques were termed oversized as endogenous plaques of this size were rarely seen in cultured mammalian cells. In untreated and drug-treated BICR-M1Rk cells, we randomly chose a confocal image representing 10 contacting cells and counted the total number of punctate gap junction plaques along all the interfaces of these ten cells. The semiquantification data was statistically analyzed using the Student’s t test and represent the average value of at least three individual images ± S.E.

For live imaging, MDA-MB-231 cells overexpressing Cx43-GFP were cultured on dishes containing glass coverslip bottoms. LysoTracker Red (Molecular Probes, Leiden, Netherlands) was dissolved in complete RPMI 1640 medium to a final concentration of 50 nM and prewarmed at 37 °C for 10 min. Cells were then incubated with LysoTracker Red containing medium for 30 min at 37 °C. After washing cells with medium to remove excess LysoTracker red, cells were switched to optimal medium for live imaging. LysoTracker Red and Cx43-GFP images were acquired simultaneously using both 488 nm/568 nm laser lines on a Zeiss LSM 410 inverted confocal microscope.

**Electron Microscopic Studies**—BICR-M1Rk cells were fixed with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer containing 50 mM lysine at pH 7.4. Cells within blocks of agarose were washed several times with phosphate buffer, dehydrated in a graded series of methanol up to 90%, and then embedded in Lowicryl K4M as described previously (25). Ultrathin sections were cut and mounted on 200-mesh Formvar-coated nickel grids. Sections were blocked for 15 min with 10% goat serum in Tris-buffered saline and incubated for 1 h with Cx43 polyclonal antibody of a dilution of 1:20 in Tris-buffered saline. After washing, the sections were subsequently incubated for another 1 h with colloidal gold (10 nm) conjugated with goat anti-rabbit secondary antibody (Cedarlane Laboratories, Hornby, Ontario, Canada). Sections were counterstained with uranyl acetate for 2 min followed by lead citrate for 1 min. Electron micrographs were taken on a Philips 400 electron microscope.

**Western Blot Analysis**—MDA-MB-231, MDA-MB-231×Cx43, or BICR-M1Rk cells, either untreated or inhibitor-treated, were washed in phosphate-buffered saline and harvested by scraping. Cells were pelleted by centrifuge, resuspended in lysis buffer containing 200 mM EDTA and protease inhibitors, and ruptured by sonication. Protein concentration was measured using BCA protein assay reagent kit (Pierce). 10–20 μg of protein were loaded and separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes that were subsequently immunoblotted with anti-Cx43 NH-terminal antibody at a dilution of 1:5000 for MDA-MB-231×Cx43/BICR-M1Rk cells and 1:50 000 for MDA-MB-231 cells (Oncogene Science, Cambridge, MA). The membranes were re-probed with vimentin-specific monoclonal antibodies (Oncogene Science) to ensure equal loading. The relative intensity of bands along all the interfaces of these ten cells. The semiquantification data was statistically analyzed using the Student’s t test and represent the average value of at least three independent Western blots ± S.E.

**RESULTS**

Endocytosed Cx43 in GJIC-competent BICR-M1Rk and Intracellular Accumulated Cx43 in GJIC-deficient MDA-MB-231 Cells Were Both Targeted to Lysosomes—We chose two breast...
tumor cell models to study the destinations and degradation of the gap junction protein, Cx43. BICR-M1R<sub>k</sub> cells, a transformed rat breast tumor cell line, were characterized to have abundant endogenous Cx43 that assembled into punctate gap junction plaques at cell-cell appositions (Fig. 1A, arrows). As expected these cells displayed efficient intercellular transfer of Lucifer yellow, indicating that Cx43 functional gap junction channels were formed among the contacting cells (Fig. 1A, arrows) and dye microinjection studies revealed that these cells were efficiently coupled (B, C). However, overexpressed Cx43 accumulated within the cytoplasm of MDA-MB-231 cells (D), and no, or little, dye coupling was observed (E, F). Bar = 10 μm.

 Unlike MDAMB-231 cells, Cx43 did not accumulate within lysosomes of BICR-M1R<sub>k</sub> cells (see Fig. 1A), suggesting that lysosomes may either not actively participate in Cx43 degradation or Cx43 is rapidly degraded once it reaches the lysosomes. To ascertain if Cx43 enters lysosomes, BICR-M1R<sub>k</sub> cells were examined by immunogold electron microscopy for evidence of Cx43 within lysosomes. Typical gap junction profiles (Fig. 3A, arrows) were positively stained for Cx43 (Fig. 3A, small arrowheads) were observed at the cell surface. Collapsed immunogold-labeled, double-membrane Cx43 junction plaques (Fig. 3B, arrowheads) were seen within a membrane-bound and electron-dense organelle that was reminiscent of a lysosome. In still other electron dense organelles that resemble lysosomes, immunolabeled membrane debris was evident suggesting that this represented a gap junction that was in the final stages of degradation (Fig. 3C). Collectively, the electron morphological data suggested that internalized gap junctions in the form of annular junctions (26) were targeted to the endosome/lysosome pathway for degradation.

It could be possible that lack of Cx43 immunostaining at the cell surface in MDA-MB-231 cells was due to the formation of unstable gap junctions that were quickly internalized into the cells. Alternatively, newly synthesized Cx43 could be delivered to lysosomes without first going to the plasma membrane. To evaluate these possibilities we employed BFA to block Cx43 transport through the Golgi apparatus and compared the subsequent change in the steady-state levels of Cx43 between the two cell conditions. When communication-deficient MDA-MB-231vCx43 cells (Fig. 4A) were treated with BFA for 6 h (Fig. 4B) intracellular Cx43 remained prominent and was localized to lysosomes (Fig. 4D). Since the lysosomal pool of Cx43 diminished dramatically in the presence of the protein synthesis inhibitor, cycloheximide (Fig. 4, C, E, and F), this suggested
that lysosomal function was intact in MDA-MB-231vCx43 cells, and Cx43 was continually transported to lysosomes in the presence of BFA. In GJIC-competent BICR-M1Rk cells, the blocking of ER-Golgi transport eliminated the mature phosphorylated species of Cx43 (Fig. 4, E and G), consistent with our previous studies (7). As seen in GJIC-deficient cells, cycloheximide dramatically reduced the cellular levels of Cx43 in BICR-M1Rk cells consistent with the continued degradation of Cx43 during the inhibition of protein synthesis (Fig. 4, E and G).

Together, our data suggest that there exists a by-pass pathway where newly synthesized Cx43 can be targeted to lysosomes in GJIC-deficient MDA-MB-231vCx43 cells allowing for the lysosomal pool of Cx43 to be sustained in the presence of BFA.

Inhibition of Lysosomal Degradation Resulted in the Accumulation of Total Cellular Cx43, whereas Blocking Proteasomal Degradation Preferentially Accumulated Phosphorylated Cx43—Although our data revealed that Cx43 was targeted to lysosomes in both cell lines, degradation of connexins has been reported to involve both lysosomes and proteasomes (17). To better understand the specific roles of both proteolytic pathways in the life cycle of Cx43, we employed a series of inhibitors for both lysosomes and proteasomes. As expected, non-phosphorylated Cx43 (Cx43-P0) was detected in untreated MDA-MB-231vCx43 cells, whereas the phosphorylated Cx43 species (Cx43-P) were much less apparent (Fig. 5B), consistent with the finding that little Cx43 was localized to gap junction plaques. Similarly, Cx43-P0 was more abundant than Cx43-P in the cells treated with lysosomal inhibitors (chloroquine, leupeptin, or ammonia chloride); however, inhibition of lysosomes caused a significant increase in total cellular Cx43 by 69–75% (Fig. 5, B and C) confirming the critical role of lysosomes in Cx43 degradation in MDA-MB-231vCx43 cells. Surprisingly, inhibiting proteasomal function by lactacystin increased Cx43-P at the expense of Cx43-P0 and consequently kept the total cellular Cx43 unchanged (Fig. 5, B and C). Likewise, in cells where both proteolytic pathways were simultaneously blocked, inhibition of proteasomes did not amplify the lysosomal inhibitor-induced accumulation of total cellular Cx43, but only enriched Cx43-P at the expense of Cx43-P0 (Fig. 5, B and C). Consequently, inactivating proteasomal degradation significantly increased the ratio of Cx43-P to Cx43-P0 by 1.3–2-fold, whereas no difference in this ratio was observed when lysosomal activity was inhibited (Fig. 5D). Lactacystin-dependent accumulation of Cx43-P was also observed in wild-type MDA-MB-231 cells that express limited amounts of endogenous Cx43 (Fig. 5A), which eliminated the possibility that overexpressed Cx43 may be selectively targeted to an alternate degradation pathway not indicative of native Cx43. Together, our findings strongly suggest that lysosomes are a major site for Cx43 degradation in MDA-MB-231 cells, while proteasomes may play a more indirect role in reducing Cx43-P possibly by degrading unknown proteins that allow Cx43-P to stabilize.

Proteasomal, but Not Lysosomal Inhibition, Correlated with Increased Gap Junction Plaques in MDA-MB-231 Cells—Since phosphorylation is generally associated with the assembly of Cx43 into gap junctions (8), we next examined whether lactacystin-induced accumulation of Cx43-P would correlate with an increase in the number of gap junction plaques. Unlike control cells where gap junctions were rarely detected (Fig. 6A), the incidence of gap junction plaques in cells treated with lactacystin increased by 120% (Fig. 6I). A striking feature of the plaques found in lactacystin-treated cells was their enlarged size (Fig. 6, B and B-1, double arrows) in comparison with the more “normal” appearing punctate gap junction plaques (Fig. 6, B and B-2, arrows). These oversized plaques represented 47% of the total Cx43 gap junction plaques found in lactacystin-treated MDA-MB-231vCx43 cells (Fig. 6J). In cells treated with lysosomal inhibitors, although Cx43 plaques could occasionally be seen (Fig. 6, C, E, and G, arrows), punctate and oversized gap junction plaques were not significantly different from those in control cells (Fig 6, I and J). However, the incidence of gap junction plaques did increase by 61–88% when lysosomal and proteasomal degradation were simultaneously inhibited (Fig. 6, D, F, and H, double arrows, and I), suggesting that it was proteasomal, but not lysosomal, inhibition that was responsible for the increased incidence of gap junction plaques in these cells.

Fig. 3. Cx43 was delivered to lysosomes in BICR-M1Rk cells. A typical gap junction profile (A, arrows) immunogold labeled for Cx43 (A, arrowheads) was observed at the cell surface (×75,000), while collapsed Cx43 gap junctions were found within membrane compartments reminiscent of lysosomes (B, arrowheads) (×56,000). In other electron-dense organelles resembling lysosomes, membrane debris was found to be immunolabeled for Cx43 (C, arrowheads) (×56,000).
for the increase in number of gap junction plaques in MDA-MB-231vCx43 cells. Moreover, 42–57% of the Cx43 plaques found in these cells were classified as oversized (Fig. 6J). Together with Western blot analysis, our localization studies suggest that the lactacystin-induced increase in the phosphorylated species of Cx43 is directly correlated with an increase in the number of oversized gap junction plaques.

Inhibition of Proteasomes Allows for the Persistence of Gap Junction Plaques in GJIC-competent BICR-M1Rk Cells, Even in the Presence of BFA—Degradation studies using MDA-MB-231vCx43 cells indicate that the inhibition of proteasomes allows for the maturation of Cx43-P0 and the formation of detectable gap junctions at the cell surface. We next determined whether this mechanism was also involved in BICR-M1Rk cells that have apparently a normal complement of gap junctions and extensive GJIC. Confocal microscopic images revealed that punctate Cx43 gap junction plaques were abundant at cell-cell oppositions in BICR-M1Rk cells (Fig. 7A, arrows). Cells treated with BFA for 6 h lost 90% of their gap junctions, but intracellular Cx43 immunofluorescence was abundant (Fig. 7, B and G), consistent with our previous studies (7). Cells treated with lactacystin had an unchanged complement of plaques (Fig. 7C, arrows, and G); however, in the cells treated with NH4Cl, gap junction plaques were reduced by 63% compared with control and there was an increase in intracellular organelle-compartmentalized Cx43, suggesting a critical role of lysosomes in degrading Cx43 in GJIC-competent BICR-M1Rk cells (Fig. 7, E and G). As expected, when the cells were co-incubated with NH4Cl and BFA, Cx43 accumulated in a cytoplasmic compartment, but not at the plasma membrane resulting in a loss of Cx43 plaques, which was comparable with BFA treatment (Fig. 7, E, F and H). Interestingly, simultaneous cell treatment with lactacystin and BFA significantly reduced the BFA-induced loss of Cx43 gap junctions with a population of Cx43 still remaining as gap junction plaques (Fig. 7, H), suggesting that proteasomal degradation plays some role in initiating Cx43 gap junction internalization (Fig. 7, D and H).

Based on the lack of an increase in the number of gap junction plaques in BICR-M1Rk cells in the presence of lactacystin and due to the fact that plaques are often correlated with increased levels of phosphorylated Cx43, we tested the hypothesis that proteasomal inhibitors would not preferentially increase the amount of Cx43-P in GJIC-competent BICR-M1Rk cells. Cells were treated with inhibitors of proteasomes or lysosomes in combination with BFA and subjected to Western blots to both the carboxyl and amino-terminal ends of Cx43. Consistent with our previous report that BFA blocked the maturation of Cx43 to the most mature phosphorylated species, our data showed that BFA dramatically reduced Cx43-P, while accumulating immature Cx43 in BICR-M1Rk cells (E, G). CHX greatly reduced Cx43 content in both cell types (E–G). The overexposed insert in C revealed the presence of a low level of Cx43 in CHX-treated MDA-MB-231vCx43 cells. Statistical comparisons were made in reference to non-treated cells (asterisks). Bar = 10 μm.
in combination with NH₄Cl (Fig. 8A), leading to a significant increase in Cx43-P/Cx43-P₀ ratio (Fig. 8C). This latter finding would again suggest that proteosomal activity plays some roles in initiating gap junction internalization. Moreover, unlike MDA-MB-231vCx43 cells, lactacystin-treated BICR-M1Rk cells increased both Cx43-P₀ and Cx43-P (Fig. 8, A and B) keeping the Cx43-P/Cx43-P₀ ratio relatively unchanged (Fig. 8C). Together, our data strongly suggest that proteasomes play an important role in the internalization and destabilization of gap junctions.

DISCUSSION

One of the most striking features of connexins is their rapid turnover. In cultured cardiac myocytes and intact rodent hearts, the half-life of Cx43 was found to be 1–3 h (9, 11). Such a dynamic turnover, therefore, requires an efficient degradation mechanism to prevent the intracellular accumulation of Cx43. As transmembrane proteins, connexins were originally assumed, and later reported, to be degraded in lysosome after endocytosis (17–19, 27). This concept was challenged by the finding that proteasome inhibitors prolonged the turnover of Cx43 in several cell types, suggesting that proteasomes play a role in connexin degradation (16–18, 28). Since proteasomes are normally involved in degrading cytosolic or misfolded proteins, it is difficult to understand the role of proteasomal degradation in the life cycle of polytopic connexins that oligomerize into hemichannels and subsequently aggregate into plaques consisting of hundreds of tightly packed channels. Recent studies by VanSlyke and Musil (20) revealed a population of Cx43 in the cytosol that appeared to have dislocated from ER membranes in the presence of a proteasomal degradation inhibitors (20). Although it was not clear whether this cytosolic population of Cx43 was ubiquitinated, it would seem probable that proteasomal degradation would be the fate of this soluble pool of Cx43.

While considerable information exists on the degradation of connexins in GJIC-competent cell models, where connexins are efficiently assembled into gap junctions, less is known about the degradation of connexins in GJIC-deficient cells, where wild-type or mutant connexins aberrantly accumulate within cells (23, 24, 29, 30). An abnormal GJIC phenotype is predominantly observed in malignant cells that may continue to express connexins, yet little is known about the mechanisms involved in aberrant connexin processing and assembly in tumor cells (23). GJIC defects in tumor cells that express connexin...
ins could be due to the instability of connexins at the plasma membrane or defects in the secretory pathway involved in delivering connexins to the cell surface for gap junction assembly.

In the present study we used human MDA-MB-231 breast tumor cells and compared Cx43 degradation in this GJIC-deficient cell line with that in a GJIC-competent cell line, BICR-M1Rk rat breast tumor cells. Our data strongly suggest that lysosomes degrade Cx43 in GJIC-competent BICR-M1Rk cells, while proteasomes likely play a role in regulating the internalization of gap junctions. Consistently, lysosomes degrade Cx43 in GJIC-deficient MDA-MB-231, but interestingly, a by-pass mechanism appears to exist where newly synthesized Cx43 can be targeted to lysosomes for degradation. Proteasomes, on the other hand, participate by regulating the maturation or stability of Cx43 gap junctions. Importantly, our studies are consistent with proteasomes regulating the life cycle and turnover of Cx43 without Cx43 necessarily being a substrate for direct proteasomal degradation.

In GJIC-competent BICR-M1Rk cells, Cx43 gap junction plaques were present at cell-cell interface with a very limited intracellular pool of Cx43, apart from Golgi-localized Cx43. Two key lines of evidence suggest that Cx43 is degraded within lysosomes of these cells. First, electron microscopy revealed Cx43-positive, collapsed double-membrane structures and Cx43 immunolabeled membrane debris localized to membrane organelles consistent with lysosomes. We suggest that lysosomes are the final destination for annular junctions that were previously shown to be internalized gap junctions (26). Second, quantifiable Western blots revealed that inhibitors of lysosomal degradation dramatically increased total cellular Cx43. This is support further by a recent study by Thomas et al. (31) where lysosomes were found to be involved in Cx43 degradation in SKHep1, and these authors further suggested that a tyrosine-based motif on the COOH-terminal domain regulated lysosomal degradation of Cx43 (31). In addition, localization and Western blot evidence in the presence of lysosomal inhibitors in GJIC-deficient MDA-MB-231 cells revealed that Cx43 was also targeted to lysosomes for degradation.

Although the evidence for lysosomal degradation of Cx43 in GJIC-deficient MDA-MB-231 cells was conclusive, the question still remains as to how Cx43 was delivered to lysosomes. One possibility could be that transiently assembled gap junction in MDA-MB-231 cells might be unstable and internalized quickly for lysosomal degradation. Western blots performed after blocking protein secretion with BFA revealed no change in the steady-state level of Cx43, while inhibition of protein synthesis for the same period of time caused a major decrease in total

**Fig. 6. Treatment of MDA-MB-231vCx43 cells with proteasomal, but not lysosomal, inhibitors increased Cx43 gap junctions.** Untreated and cells treated with protease and/or lysosome inhibitors were immunolabeled for Cx43 (A–H). The results of semiquantification of confocal images revealed that lactacystin significantly increased both total number of Cx43 gap junction plaques found along 20 interfaces (I) and the percentage of oversized plaques (J) that were indicated by double arrows in confocal images (B, D, F, H). The inset in B-1 represents a typical oversized gap junction, while the inset in B-2, from a separate image, represents a more normal gap junction profile. Statistical comparisons were made in reference to non-treated cells (asterisks). Bar = 10 μm.
Cx43. Consequently, our data support a novel model where newly synthesized Cx43 is targeted to lysosomes without first being transported to the plasma membrane and then to lysosomes. This finding may in part explain the defect in some tumor cell situations where connexins continue to be synthesized but few gap junctions are assembled (23, 32). In addition, we previously reported that misfolded DsRed-tagged Cx43 also accumulated within lysosomes in HBL-100 cells, whereas wild-type Cx43 was readily assembled into gap junctions (24). Likewise, Cx32 mutants linked to Charcot-Marie-Tooth disease displayed altered trafficking propensities resulting in a variety of phenotypes where the mutants have an intracellular organelle localization pattern (29, 30). These latter cell models suggest that connexins with trafficking defects are unlikely to be properly assembled into gap junctions, reflecting a quality control mechanism that exists to degrade misfolded or mutant connexins.

While it is clear that lysosomes play an important role in degrading Cx43 in both cell types used in this study, a key question remained as to the role of proteasomes. Although proteasomes are mainly involved in the degradation of cytosolic, nuclear, and ER-retained misfolded or aggregated proteins, integral membrane proteins have been reported to go through proteasomal inhibitor-sensitive degradation (14, 15). The evidence linking proteasomes with connexin degradation relies primarily on the observation that proteasomal inhibitors prolonged the turnover of connexins (16, 18, 29). Consistently, we found that proteasome inhibition caused an accumulation of intracellular Cx43, whereas inhibitors of lysosomes partially prevented BFA-induced loss of gap junctions in GJIC-competent BICR-M1Rk cells. Punctate gap junction plaques were observed at cell-cell interfaces in untreated BICR-M1Rk cells (A, arrows) but scarcely detected in cells treated with BFA (B, G). Lactacystin inhibition of proteasomes did not result in the loss of gap junctions (C, arrows, and G). Moreover, gap junction plaques were readily detected in the cells simultaneously treated with BFA and lactacystin (D, arrows, and H). Treating the cells with lysosomal inhibitor NH4Cl significantly increased intracellular Cx43 staining (E), and gap junction plaques were dramatically reduced (E, arrow, and G). Gap junctions were sparse in cells treated with NH4Cl combined with BFA (F, H). Statistical comparisons in G were in reference to the “no inhibitor” treatment and in H to the “BFA” treatment (asterisks).

Interestingly, proteasome activity regulates the prevalence of the phosphorylated species of Cx43 in GJIC-deficient MDA-MB-231vCx43 cells. Our data using these cells revealed that proteasome inhibitors alone did not alter the steady-state levels of total Cx43 and neither did it amplify lysosome inhibitor-induced accumulation of intracellular Cx43. Immunolocalization and Western blot analysis clearly showed that Cx43 became more phosphorylated, and more gap junction plaques were apparent when proteasomes were inactivated strongly, suggesting that proteasome inactivation promoted the maturation or stability of Cx43. These results are consistent with Musil et al. (18) where the inhibition of proteasomes rescued gap junction and cell-cell communication in gap junction assembly-deficient Chinese hamster ovary cells, suggesting that the formation and function of gap junctions could be regulated at the level of connexin turnover (18).

Together, our data revealed that proteasome activity can
transiently regulate the life cycle of Cx43 at potentially different intracellular locations. We propose that the bulk of Cx43 may in fact not be a substrate for proteasomal degradation, but rather its turnover is regulated by proteasomal degradation of other undefined molecules. Evidence for this position is based on several findings. First, the configuration of connexins, connexons, and gap junctions prevents their incorporation into the catalytic core of a proteasome unless physically removed from the membrane, disassembled, and unfolded. Second, the identification of collapsed Cx43 gap junctions in compartmentalized organelles is inconsistent with proteasomal degradation. Third, since the amino or carboxyl-terminal ends of Cx43 are chiefly not cleaved under steady-state or proteolytic inhibitor treated scenarios, our data are inconsistent with models where proteasomes cleave a significant population of Cx43 prior to delivery to lysosomes for final degradation. Finally, limited evidence exists that a substantial population of Cx43 is either ubiquitinated or polyubiquitinated (16), and thus Cx43 may need to be targeted to proteasomes via a rare ubiquitin-independent mechanism, which, to our knowledge, has not been reported for any integral membrane protein. While it is possible that ubiquitination of Cx43 blocks anti-Cx43 antibody binding sites, this argument is weakened when multiple antibodies to distinctly different Cx43 motifs are used. Our data clearly revealed that sensitive anti-Cx43 antibodies only detect non-phosphorylated and phosphorylated Cx43 species with no evidence of polyubiquitination.

In a recent paper, a population of Cx43 was found be extracted into the cytosol, leaving open the possibility that this soluble fraction of Cx43 might in fact get ubiquitinated and directed to proteasomes for degradation (20). Although some proteins can be degraded by proteasomes without ubiquitination, ubiquitin-independent proteasomal degradation has only been experimentally reported for intracellular soluble proteins such as ornithine decarboxylase, CIP-1 and calmodulin (36–40). Consequently, we propose an indirect role of proteasomes in affecting the turnover of connexins. Nevertheless, we cannot exclude the possibility that different degradation pathway preferences for Cx43 exist among various cell types. Musil and colleagues (20) have clearly established a role for proteasomes in the degradation of membrane extracted connexins, and it is likely that connexins expressed in excess together with quality control mechanisms may require their immediate degradation. It is also intriguing that Cx43 has subtle but distinct localization characteristics in cell lines and cells in situ with differential steady-state levels in organelle and membrane compartments, including lysosomes (comparing...
the two cell lines used in this study), the Golgi apparatus (7, 41) and annular junctions (26). It is unlikely that these differences in steady-state compartment pools of Cx43 highlight fundamental differences in Cx43 routing but rather quantitative kinetic variability in Cx43 transport and organelle function. In summary, our study revealed that lysosomes and proteasomes play distinctly different roles in the life cycle of Cx43, and it remains to be elucidated as to the substrates degraded by proteasomes that regulate gap junctions and GJIC.

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Lysosomal and Proteasomal Degradation Play Distinct Roles in the Life Cycle of Cx43 in Gap Junctional Intercellular Communication-deficient and -competent Breast Tumor Cells

Hong Qin, Qing Shao, Suleiman A. Igdoura, Moulay A. Alaoui-Jamali and Dale W. Laird

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