Gap Junction Turnover, Intracellular Trafficking, and Phosphorylation Of Connexin43 in Brefeldin A-treated Rat Mammary Tumor Cells

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Abstract. Intercellular gap junction channels are thought to form when oligomers of connexins from one cell (connexons) register and pair with connexons from a neighboring cell en route to forming tightly packed arrays (plaques). In the current study we used the rat mammary BICR-M1R_k tumor cell line to examine the trafficking, maturation, and kinetics of connexin43 (Cx43). Cx43 was conclusively shown to reside in the Golgi apparatus in addition to sites of cell–cell apposition in these cells and in normal rat kidney cells. Brefeldin A (BFA) blocked Cx43 trafficking to the surface of the mammary cells and also prevented phosphorylation of the 42-kD form of Cx43 to 44- and 46-kD species. However, phosphorylation of Cx43 occurred in the presence of BFA while it was still a resident of the ER or Golgi apparatus yielding a 43-kD form of Cx43. Moreover, the 42- and 43-kD forms of Cx43 trapped in the ER/Golgi compartment were available for gap junction assembly upon the removal of BFA. Mammary cells treated with BFA for 6 h lost preexisting gap junction "plaques," as well as the 44- and 46-kD forms of Cx43 and functional coupling. These events were reversible 1 h after the removal of BFA and not dependent on protein synthesis. In summary, we provide strong evidence that in BICR-M1R_k tumor cells: (*a*) Cx43 is transiently phosphorylated in the ER/Golgi apparatus, (*b*) Cx43 trapped in the ER/Golgi compartment is not subject to rapid degradation and is available for the assembly of new gap junction channels upon the removal of BFA, (*c*) the rapid turnover of gap junction plaques is correlated with the loss of the 44- and 46-kD forms of Cx43.

Gap junctions are dynamic structures as evident from pulse-chase studies where the constituent of gap junctions, connexin, possesses a half-life of 1–3 h in primary cultures of neonatal cardiac myocytes (Laird et al., 1991), chick lens epithelial cells (Musil et al., 1990*a*), and embryonic mouse hepatocytes (Traub et al., 1987, 1989). In vivo turnover studies suggest that liver gap junction plaques have a half-life of 5 h (Fallon and Goodenough, 1981) or longer (Yancey et al., 1981). Whether unassembled connexins are subject to an intracellular turnover rate that is distinct from assembled gap junction plaques has not been well studied in vivo or in vitro. The life cycle of connexins involves the oligomerization of subunits into hemichannels (connexons), translocation of assembled hemichannels to the cell surface, intercellular pairing of connexons, and channel clustering to form morphologically identifiable gap junction plaques. Removal of gap junction plaques from the cell surface has been proposed to entail the internalization of the entire junctional complex as a double membrane annular junction that is targeted to lysosomes for degradation (Naus et al., 1993). Little is known about folding events, interaction with molecular chaperones or posttranslational modifications that connexins may be subjected to early in the secretory pathway. Falk et al. (1994) have suggested that an unidentified factor may be necessary to prevent aberrant processing of connexins before or during membrane insertion. The studies of Musil and Goodenough (1993) have indicated that Cx43 oligomerization into connexons occurs after the protein leaves the ER. Hence, this integral membrane protein must possess intramolecular properties or intermolecular associations in the ER that prevent oligomerization or further modifications to Cx43 may be required to induce its assembly. It is possible that maintenance of Cx43 as a monomer early in the secretory pathway may be linked to a transient posttranslational modification.

It is now well established that at least 4 connexins are phosphoproteins (Sáez et al., 1986; Traub et al., 1987, 1989; Yancey et al., 1989; Crow et al., 1990; Musil et al., 1990*a,b*; Sáez et al., 1990; Kadle et al., 1991; Laird et al., 1991; Naus et al., 1992; Goldberg and Lau, 1993; Jiang et al., 1993; Gupta et al., 1994; Laing et al., 1994; Nnamani et al., 1994). Unphosphorylated Cx43 matures to slower SDS–polyacryl-

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amide gel migrating Cx43 isoforms (Crow et al., 1990; Musil et al., 1990*b*; Laird et al., 1991) commonly designated as P_1 and P_2 (Musil et al., 1990*b*) and the presence of the P_2 Cx43 isoform has been correlated with gap junction plaque formation and communication competence (Musil et al., 1990*b*; Musil and Goodenough, 1991). Cx43 phosphorylation has also been linked to changes in gap junction channel unitary conductance (Moreno et al., 1994). Unphosphorylated and P_1/P_2 phosphorylated forms of Cx43 have been identified in many primary cell cultures (Musil et al., 1990*a*; Laird et al., 1991) and established cell lines (Crow et al., 1990; Musil et al., 1990*b*; Budunova et al., 1993). It is also known that Cx43 is phosphorylated extensively in vivo (Laird and Revel, 1990). Hence, the phosphorylation of Cx43 has been widely observed and may be linked to both gap junction assembly and gating events.

To elucidate our understanding of Cx43 maturation, trafficking, and gap junction turnover, we chose to modulate protein synthesis and trafficking in a fibroblastoid (BICR-M1R_k) cell line from a rat mammary tumor that expresses high levels of Cx43. Cx43 is known to be a major connexin in normal mammary epithelium and surrounding stromal cells (Lee et al., 1992; Wilgenbus et al., 1992). Modulation of Cx43 translocation was done by using the fungal antibiotic Brefeldin A (BFA)[1], which is known to block protein trafficking within a fused ER/Golgi compartment (Misumi et al., 1986; Lippincott-Schwartz et al., 1989, 1991). BFA was used to uncouple events leading to gap junction assembly from those related to gap junction removal. Our results provide compelling evidence that: (*a*) Cx43 is phosphorylated during its transient residency in the ER/Golgi compartment; (*b*) Cx43 that is trapped in the ER/Golgi compartment is long lived and upon the removal of BFA, available for gap junction assembly; and (*c*) rapid "plaque" turnover is accompanied by the loss of the 44- and 46-kD phosphorylated forms of Cx43.

Materials and Methods

Cell Culture and Drug Treatments

The rat BICR-M1R_k cell line was derived from a spontaneously formed Marshall rat mammary tumor. This established cell line has been characterized extensively for cell growth in culture (Rajewsky and Gruneisen, 1972) and has been the subject of several ionic and dye coupling studies (Hulser and Demsey, 1973; Hulser and Webb, 1973; Brummer et al., 1991). Normal rat kidney (NRK-52E) cells were purchased from American Type Culture Collection (1571-CRL; Rockville, Maryland). All cells were cultured in 60-mm dishes, with or without 12-mm prewashed round glass coverslips, containing DME supplemented with 10% fetal calf serum, 100 units/ml penicillin/streptomycin, and 2 mM glutamine (GIBCO BRL, Burlington, ON). The cells were maintained in 5% CO_2 at 37°C and used 2–3 d after subculturing. In some studies, BICR-M1R_k cells were treated with 1–2 μg/ml BFA (Cedarlane Labs. Ltd., Hornby, ON) for up to 10 h and/or 10 μg/ml cycloheximide (CHX) (Sigma Chem. Co., Saint Louis, MO) for up to 6 h. In recovery studies, cells were BFA-treated for 6 h, washed, and then allowed to recover for 1 h in the presence or absence of CHX. To ensure that CHX was inhibiting all protein synthesis, rat BICR-M1R_k cells were ^{35}S-trans labeled in the presence or absence of CHX for 2 h. After lysing the cells and resolving the radiolabeled proteins on SDS-PAGE in triplicate (as described below), the radioactivity associated with each sample was counted. CHX treatment of rat BICR-M1R_k cells was found to inhibit protein synthesis by 97.9%.

1. *Abbreviations used in this paper*: BFA, Brefeldin A; Cx43, Connexin 43; NRK, normal rat kidney; PDI, protein disulfide isomerase.

Immunofluorescent Labeling, Microinjection, and Confocal Microscopy

Control, BFA- or CHX-treated BICR-M1R_k cells grown on glass coverslips were ethanol-fixed, blocked of nonspecific binding sites, and then immunolabeled as described (Laird and Revel, 1990). In single antibody labeling experiments, BICR-M1R_k cells were labeled with 1–5 μg/ml affinity purified anti-Cx43 antibody (CT-360) in the presence or absence of the 360–382 COOH-terminal connexin peptide (20 μg/ml) used to generate the antibody. For experiments where cells were labeled for two proteins, 100-fold diluted monoclonal anti-Cx43 antibody (Ingram & Bell, Montreal, PQ) was used followed by goat anti-mouse secondary antibody conjugated to fluorescein or Texas red as in single labeling experiments. The same cells were then labeled with a 500-fold dilution of anti–MG-160 serum (resident protein of *medial*-Golgi cisternae) (Gonatas et al., 1989; Croul et al., 1990) followed by goat anti–rabbit antibody conjugated to rhodamine or fluorescein. Alternatively, the cells were labeled with a 50-fold dilution of anti-protein disulfide isomerase (PDI) (StressGen, Victoria, British Columbia) followed by anti-MG-160 or anti-Cx43 (CT-360) antibodies as described above. In some cases, cells were labeled with anti–MG-160 antibody followed by anti-Cx43 antibody directly conjugated to rhodamine according to procedures described by Puranam et al. (1993).

Untreated, 6 h BFA-treated or cells that were allowed to recover from BFA treatment for 1 h were pressure microinjected with 10 mM 6-carboxyfluorescein (pH 7.0 made in distilled H_2O; Molecular Probes, Eugene, OR) to assay for gap junction coupling. Microinjected dye was allowed to spread for 10–20 min before the cells were fixed in 3.5% formaldehyde in PBS for 5 min. Cells were subsequently viewed on a Zeiss axiophot fluorescent microscope or a Zeiss LSM 410 confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

Cells immunolabeled for Cx43 or double-labeled for Cx43 and constitutive proteins of the ER (PDI) or Golgi apparatus (MG-160) were analyzed on a Zeiss LSM 10 or 410 inverted confocal microscope. The fluorescein signal was imaged by exciting the sample with a 488-nm line from an argon or an argon/krypton laser and the resulting fluorescence was collected on a photomultiplier after passage through FT510, FT560, and BP515-540 filter sets. Likewise, the same field was excited with a helium/neon (543-nm line) laser and the Texas red or rhodamine signal was imaged on a second photomultiplier after passage through FT510, FT560 and LP590 filter sets. In addition, a z-dimension correction was made before the collection of the red images to compensate for wavelength aberration. In cases where cells were microinjected with 6-carboxyfluorescein, the fluorescent image was overlaid with a transmitted light image to denote the morphology of the cells. All images were archived on an optical disk or a Bernoulli multidisk. In some cases, the fluorescein and Texas red images were overlaid and pseudo-colored using built-in LSM software. Finally, images were printed on a Kodak XLS8300 high resolution (300 DPI) color printer.

Metabolic Labeling and Immunoprecipitation

Near confluent cell cultures were rinsed 3× in methionine-free DME media and starved for 30–45 min in the presence or absence of 1–2 μg/ml BFA. Cells were pulsed with ^{35}S-trans label (100 μCi/ml; ICN Biomedicals Inc., Mississauga, ON) for 1.5–2 h in the presence or absence of 1–2 μg/ml BFA. In some cases, cells were rinsed 3× and chased for 1–7 h in 2 mM methionine supplemented medium with or without BFA. Radiolabeled cells were rinsed 3× in cold PBS and lysed with 1.1 ml of cold RIPA cell lysis buffer supplemented with 2 mM PMSF, 2 mM sodium orthovanadate, 100 mM NaF, 5 μg/ml aprotinin, and 1–10 μM leupeptin for 5–10 min on ice. Cells were scraped and lysates were centrifuged at 40,000 *g* for 50 min.

The lysates were immunoprecipitated with 5 μl of immune serum raised against the amino terminus (AT-2) of Cx43 (Yancey et al., 1989; Laird et al., 1991) or 10–30 μg/ml of affinity-purified antibody (CT-360) specific for the carboxy terminus of Cx43 (Laird et al., 1991) overnight at 4°C with gentle agitation. Protein A–agarose beads (100 μl) (Inter Medico, ON) were added and left under gentle agitation for 1 h at 4°C. The beads were collected by gentle centrifugation and washed 3× with Wash Buffer I (0.5% Tween-20, 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1 mM EDTA) and 3× with Wash Buffer II (0.5% Tween-20, 100 mM Tris, pH 7.5, 200 mM NaCl, and 2 M urea). After a final wash with distilled water, the beads were solubilized in Laemmli sample buffer (4% SDS, 10% 2-mercaptoethanol, 0.010% bromophenol blue, and 60 mM Tris, pH 6.8) for 15 min at room temperature or boiled for 5 min. Finally, the samples were

resolved on a 10% SDS–polyacrylamide gel (Laemmli, 1970) with a bisacrylamide/acrylamide ratio of 0.4:30. Gels containing ^{35}S-labeled proteins were treated with fluorophor (En3hance; Du Pont Co., Wilmington, DE) before drying. In some cases, immunoprecipitated Cx43 was quantified by exposing to a Phosphorimager overnight (Molecular Dynamics, Sunnyvale, CA).

Alkaline Phosphatase Treatments

To examine phosphorylation events, cell lysates were dialyzed in two changes of low detergent buffer, (100 mM Tris-Cl, pH 8.0, 40 mM NaCl, 1 mM $MgCl_2$, and 0.1% SDS) overnight at 4°C. Molecular biology grade calf alkaline phosphatase (100 units; Boehringer Mannheim, Laval, PQ) was added to equal amounts of cell lysates in the presence or absence of 4 mg/ml sodium orthovanadate and digestion was for 4–8 h at 4°C with gentle agitation. Finally, alkaline phosphatase treated cell lysates and controls were immunoprecipitated as described above.

Western Immunoblotting

Untreated or drug-treated BICR-M1R_k cells grown to 70–90% confluency in 75-cm^2 flasks were rinsed several times and scraped into 10 ml of PBS. The cells were pelleted in a clinical centrifuge and sonicated in 800 μl of modified Laemmli sample buffer containing 0.062 M Tris, pH 6.8, 10% glycerol, 4% SDS, 0.01% bromophenol blue, 10% 2-mercaptoethanol, 2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM EDTA, 50 mM NaF, and 500 um $NaVO_4$ as described by Lampe (1994). Samples were resolved on a 10% polyacrylamide gel as described above. Resolved proteins were transferred to nitrocellulose paper in transfer buffer (0.19 M glycine, 0.025 M Tris, 0.1% SDS, 20% methanol) for 40 min at 75 V.

Nitrocellulose papers were blocked of nonspecific binding by incubating 2 h or overnight in PBS containing 5% powdered milk. Blots were incubated with anti-Cx43 antibody (CT-360, 1 μg/ml) for 1 h and washed 6 times in 0.15 M NaCl, 1 mM EDTA, 1 mM NaN_3, 0.05% Tween, and 20 mM Tris-acetate, pH 7.4. Finally, the blots were incubated in ^{125}I-goat anti-rabbit IgG (130,000 cpm/ml; ICN, Mississauga, Ontario) for 1-2 h, washed as before, air dried and exposed either to Amersham Hyperfilm-MP with an intensifying screen or a Phosphorimager.

Results

BICR-M1R_k Cells Express High Levels of Endogenous Connexin43 and Are Well Coupled

Rat BICR-M1R_k cells are fibroblastoid in nature and grow equally well on glass or plastic with a doubling time of ~14 h (Rajewsky and Gruneisen, 1972). The distribution pattern of the endogenous gap junction protein, connexin43 (Cx43) (Beyer et al., 1987), was determined by immunofluorescent labeling with a site-directed anti-Cx43 antibody (CT-360) (Laird and Revel, 1990) in conjunction with confocal microscopic imaging. Punctate staining was observed between apposing cell membranes (*arrowheads*) and at paranuclear locations (Fig. 1 *A*, *arrows*). In the presence of synthetic peptide used to generate the antibody (amino acid segment 360-382) no labeling was observed (Fig. 1 *B*). Microinjection of 6-carboxyfluorescein (molecular weight 376) was used to show that BICR-M1R_k cells are well coupled as dye spread extensively to 1st, 2nd, and 3rd order cells (Fig. 1 *C*).

Connexin43 in the Golgi Apparatus

The identity of the Cx43-positive intracellular paranuclear compartment was determined by double immunofluorescent labeling of BICR-M1R_k cells with anti-Cx43 antibody (CT-360) and an antibody directed against the *medial*-Golgi cisternae (MG-160) (Gonatas et al., 1989; Croul et al., 1990). Confocal images of the same field revealed that Cx43 (Fig. 2 *A*) and MG-160 (Fig. 2 *B*) were colocal-

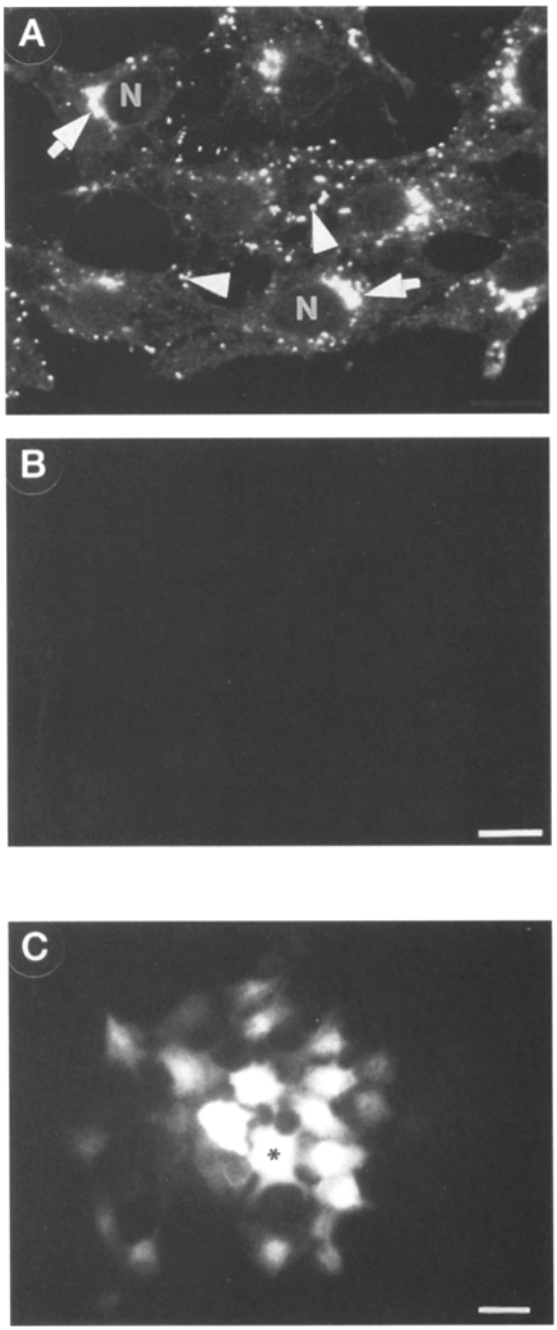


Figure 1. Immunolocalization of Cx43 and dye coupling in cultured BICR-M1R_k cells. Cultured cells were permeabilized and immunolabeled with anti-Cx43 antibody (CT-360) followed by goat anti–rabbit secondary antibody conjugated to rhodamine (*A*). Note the punctate labeling at sites of cell–cell apposition (*arrowheads*) and the paranuclear staining (*arrows*). *N*, nucleus. As a control, the CT-360 antibody and the peptide used to generate the antibody (amino acids 360–382) were coincubated with the cells and no immunostaining was observed when images were collected using similar conditions as in *A* (*B*). A BICR-M1R_k cell was microinjected with 6-carboxyfluorescein (*asterisk*) and dye was allowed to spread for 15 min before fixation (*C*). Note that dye spread to 1st, 2nd, and 3rd order cells. Bar, 10 μm.

ized to the same paranuclear region indicating an intracellular pool of Cx43 located in the Golgi apparatus (Fig. 2, *A* and *B*, *arrows*). Examination of normal rat kidney (NRK) cells double labeled for Cx43 (Fig. 2 *C*) and MG-160 (Fig. 2 *D*) revealed that these cells also contained an intracellular pool of Cx43 in the Golgi apparatus.

Cx43, MG-160 and Protein Disulfide Isomerase Have Similar Distributions in BFA-treated BICR-M1R$_k$ Cells

To determine if Cx43 and constitutive markers for ER and Golgi compartments had similar distributions in BFA-treated cells, BICR-M1R$_k$ cells were double immunolabeled for MG-160 (Fig. 3 *A*) and PDI (Fig. 3 *B*) or Cx43 (Fig. 3 *C*) and PDI (Fig. 3 *D*). The effect of BFA on the organization of the Golgi apparatus is dramatic as the paranuclear distribution of MG-160 is replaced by reticular-like staining pattern throughout the cytoplasm of the cells. Consistent with at least partially fused ER and Golgi compartments, MG-160 and PDI showed similar reticular staining patterns (Fig. 3, *A* and *B*). While Cx43 and PDI also had similar intracellular distributions, Cx43 was found more widespread throughout the BFA-treated cells that included areas not immunostained for PDI.

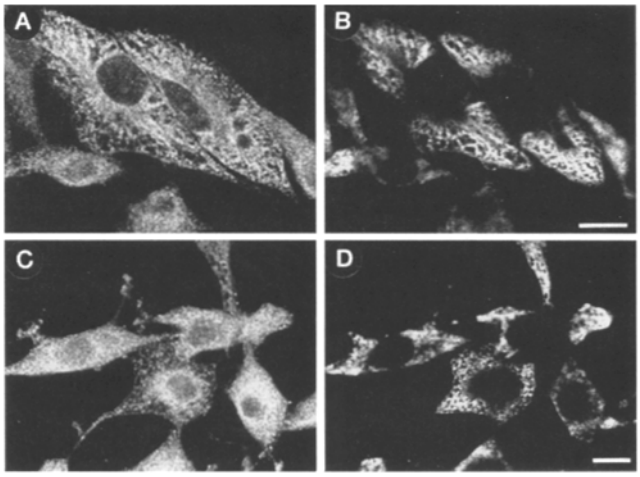


Figure 3. Double immunolabeling of BFA-treated BICR-M1R$_k$ cells for PDI and MG-160 or Cx43. BICR-M1R$_k$ cells were treated with BFA for 6 h, washed and double immunolabeled for MG-160 (*A*) and PDI (*B*) or for Cx43 (*C*) and PDI (*D*). Note that MG-160 and PDI have similar reticular staining patterns. While Cx43 and PDI appear to have intracellular areas with similar distributions, Cx43 is found more widespread in the cells. Bar, 10 μm.

Cx43 Is Initially Phosphorylated in the Fused ER/Golgi Compartment

The identity of the protein bands immunoprecipitated with anti-Cx43 (CT-360) antibody (Fig. 4, lane *a*) from BICR-M1R$_k$ cells was established by digesting control cell lysates with alkaline phosphatase. As expected, the protein bands between 44 and 46 kD were sensitive to alkaline phosphatase indicating that they represent phosphorylated isoforms of Cx43 (Fig. 4, lane *b*). When cells were metabolically labeled with ^{35}S-trans label in the presence of BFA a doublet at 42–43 kD was immunoprecipitated while slower migrating phosphorylated Cx43 isoforms were absent (Fig. 4, lane *c*). Quantitation of these Cx43 protein bands revealed that they were present in equal amounts. When cell lysates from BFA-treated cells were digested with alkaline phosphatase, a protein band at 42 kD remained while there was a complete loss of the protein band at 43 kD (Fig. 4, lane *e*), a process inhibited by sodium orthovanadate (Fig. 4, lane *d*). The anti-Cx43 antibody directed against the amino terminus of Cx43 also immunoprecipitated a Cx43 doublet at 42–43 kD (Fig. 4, lane *f*) and the 43-kD form was found to be sensitive to alkaline phosphatase (Fig. 4, lane *g*).

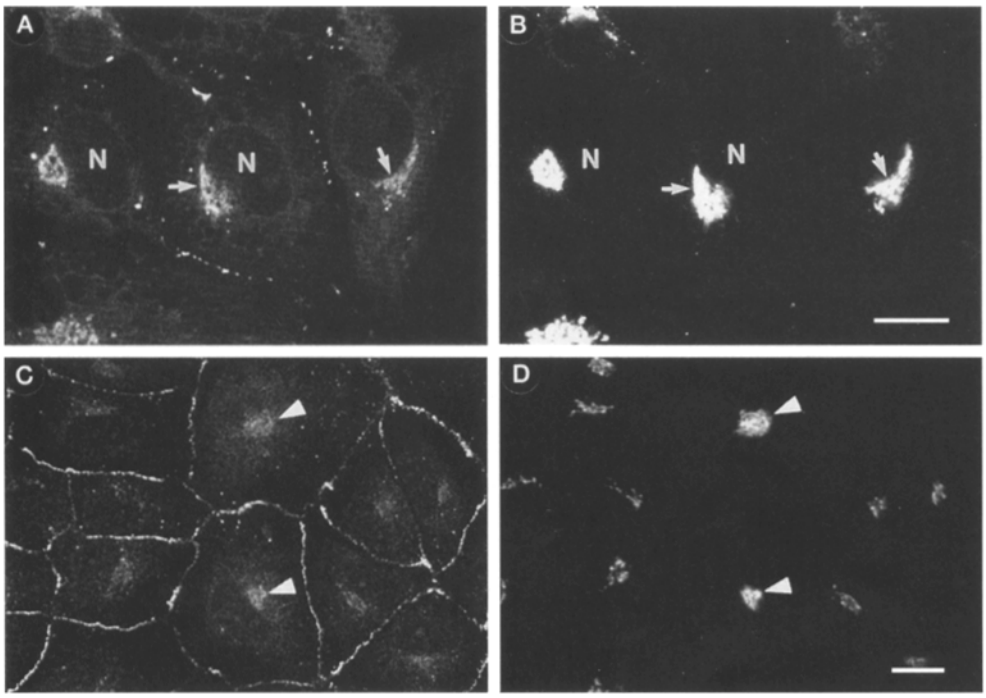


Figure 2. Confocal images of BICR-M1R$_k$ and NRK cells double immunofluorescently labeled for Cx43 and MG-160. Permeabilized BICR-M1R$_k$ cells were labeled with anti-Cx43 (CT-360) directly conjugated to rhodamine (*A*). The same cells were indirectly labeled for MG-160 (*B*). Permeabilized NRK cells were indirectly double immunolabeled for Cx43 (*C*) and MG-160 (*D*). The arrows and arrowheads denote locations where Cx43 and MG-160 are colocalized. *N*, nucleus. Bar, 10 μm.

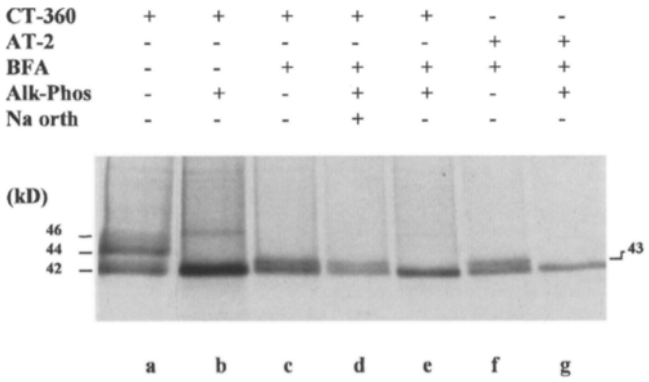


Figure 4. Inhibition of extensive Cx43 phosphorylation in the presence of BFA and the identification of an ER–Golgi phosphorylation event. Control (lanes *a* and *b*) and BFA-treated (lanes *c–g*) BICR-M1R$_k$ cells were ^{35}S-trans labeled before immunoprecipitation with anti-Cx43 site-directed antibody (AT-2 or CT-360). Unnormalized immunoprecipitates were resolved on a SDS–polyacrylamide gel and exposed for fluorography or for quantitation on a phosphorimager. In control cells, Cx43 isoforms between 42 and 46 kD were observed (lane *a*). In the presence of BFA, equally intense 42- and 43-kD forms of Cx43 were observed and the protein bands above 43 kD were not apparent (lanes *c* and *f*). When control (lane *b*) or BFA-treated (lanes *e* and *g*) cells were lysed and digested with alkaline phosphatase before immunoprecipitation, only the 42-kD unphosphorylated form of Cx43 remained. In BFA-treated cells, alkaline phosphatase was incapable of eliminating the 43-kD protein band in the presence of excess sodium orthovanadate (lane *d*). The sharp protein band at ~47 kD in lane *b* (also Fig. 5 *B*) was deemed not likely to represent Cx43 as it was not consistently immunoprecipitated and often seen when immunoprecipitations were done with preimmune serum (Laird et al., 1991).

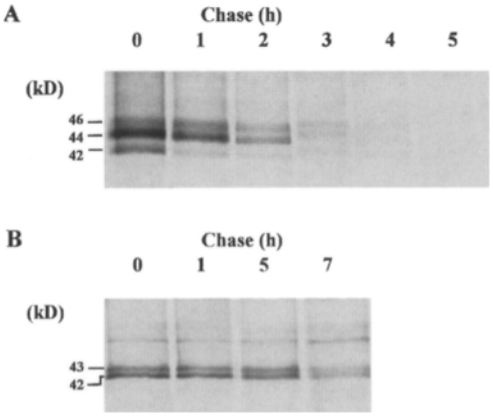


Figure 5. Turnover of Cx43 in untreated and BFA-treated cells. Control (*A*) or BFA-treated (*B*) BICR-M1R$_k$ cells were ^{35}S-trans labeled before being chased in the presence or absence of BFA for 0–7 h. Cx43 was immunoprecipitated from lysates containing an equal number of cells using an affinity purified anti-Cx43 antibody (CT-360) and exposed for fluorography or for quantitation on a phosphorimager. In control cells (*A*), the unphosphorylated form of Cx43 at 42 kD and the 44- and 46-kD phosphorylated isoforms were observed to have half lives of <2 h. In the presence of BFA (*B*), the doublet of Cx43 at 42–43 kD was seen throughout the 7-h chase period.

BFA Delays the Turnover of Cx43

The turnover of Cx43 was evaluated in BICR-M1R$_k$ cells that were pulsed with ^{35}S-trans label and chased in excess methionine (Fig. 5 *A*). Quantitation of radiolabeled Cx43 revealed that nascent Cx43 (42 kD) and its phosphorylated isoforms (44 and 46 kD) had an average half-life of ~1–1.5 h. However, when pulse-chase studies were performed in the presence of BFA (Fig. 5 *B*) little degradation of the 42–43-kD doublet was evident for 5 h suggesting that Cx43 trapped within the fused ER–Golgi compartment is stored and not subjected to degradation.

Fate of Preexisting Gap Junctions in BFA and Cycloheximide-treated Cells

To examine the spatial distribution of Cx43 upon BFA and cycloheximide treatment, control and drug-treated cells were double immunofluorescently labeled for Cx43 and MG-160 before analysis by confocal microscopy. In control cells, gap junction plaques were frequently observed between neighboring cells (Fig. 6 *A*) and the Golgi exhibited a paranuclear distribution (Fig. 6 *B*). When cells were treated with BFA for 6 h, gap junction plaques were not identifiable at the cell surface (Fig. 6 *D*) and Cx43 was found distributed in a reticular-like fashion similar to MG-160 (Fig. 6 *E*). The rapid clearing of gap junction plaques from the surface of these cells is consistent with gap junction plaques having a half-life of ~1.5 h. Unlike BFA-treated cells, BICR-M1R$_k$ cells treated with CHX for 6 h had observable gap junctions between many neighboring cells (Fig. 6 *G*) and the Golgi remained stacked near the nucleus (Fig. 6 *H*). The retention of a finite number of gap junction plaques in the presence of CHX suggests that an intracellular pool of Cx43 resides within the ER/Golgi apparatus that is able to regenerate a population of gap junctions throughout the 6-h time period or CHX is inhibiting the synthesis or action of factors that are necessary for the removal of gap junction plaques. Combined treatments of BFA and CHX resulted in a notable loss of gap junctions from the cell surface (Fig. 6 *J*) and the disruption of the Golgi apparatus (Fig. 6 *K*). Thus, all the cellular components or factors necessary for internalization of gap junctions are available and protein synthesis is not required.

Kinetics of Gap Junction Assembly and the Return of Intercellular Communication Upon the Removal of BFA

When BFA-treated (6 h) BICR-M1R$_k$ cells were microinjected with 6-carboxyfluorescein and incubated for 15 min, no dye transfer to neighboring cells was observed (Fig. 7 *A*). However, when BFA-treated (6 h) cells were allowed to recover for 1 h in the absence of BFA, dye was observed to transfer to first and second order cells (Fig. 7 *B*) indicating that functional gap junction channels had reformed. In multiple dye microinjections into BFA-treated cells, transfer was either completely blocked or only observed in a limited number of 1st order cells. To determine if BFA-treated (6 h) BICR-M1R$_k$ cells could assemble gap junction plaques and reassemble the Golgi apparatus upon the removal of BFA, cells were washed and incubated in BFA-free medium for 1 h before being labeled for Cx43 and MG-160. Our results show that the cells recovered from the BFA treatment as the Golgi apparatus

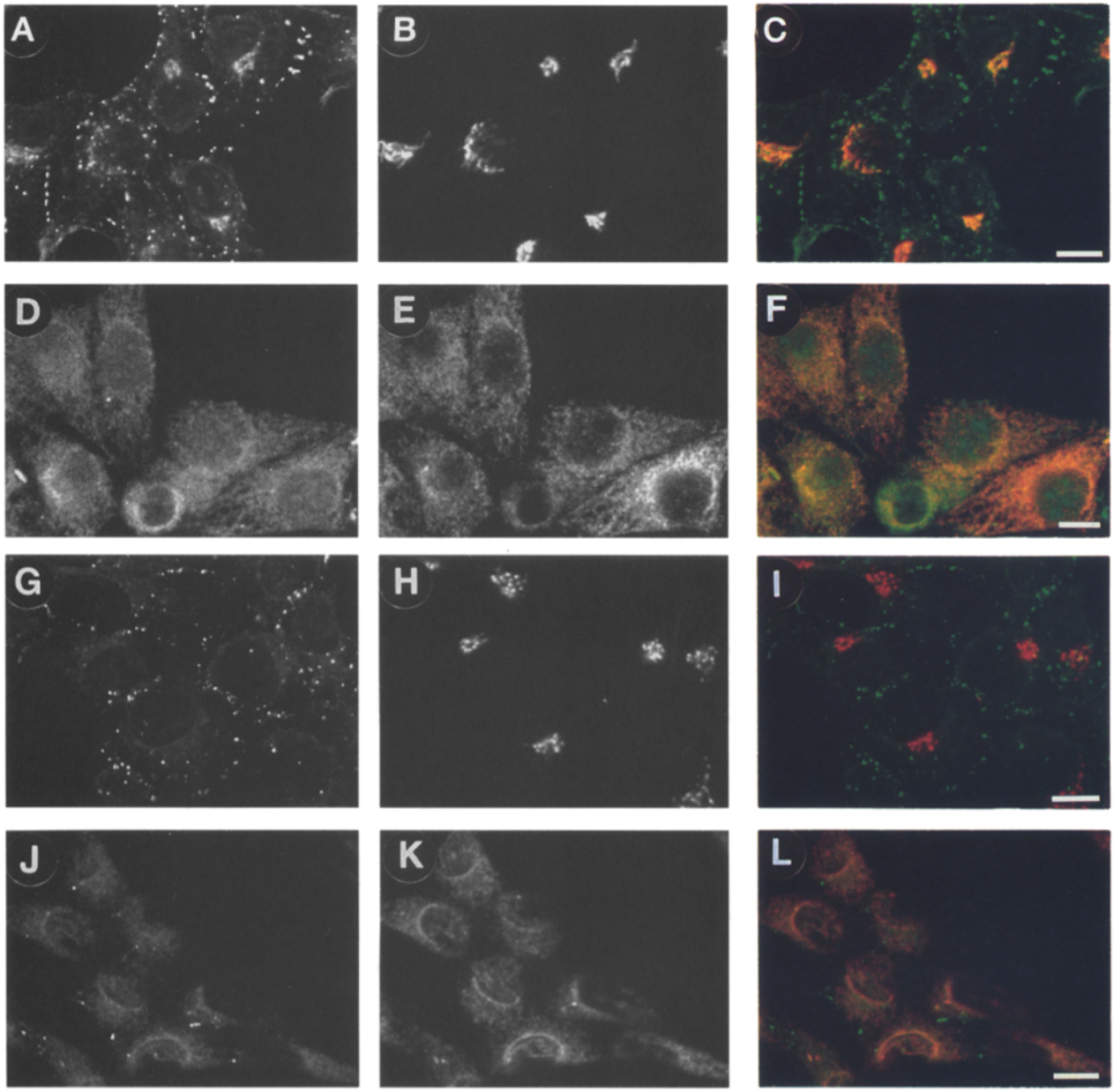


Figure 6. Immunolocalization of Cx43 and MG-160 in control and drug modulated cells. Control (*A–C*), or cells treated with BFA for 6 h (*D–F*), CHX for 6 h (*G–I*), or BFA + CHX for 6 h (*J–L*) were double labeled for Cx43 and a constituent of the Golgi apparatus, MG-160. The same fields of immunolabeled cells were imaged on a confocal microscope for Cx43 (*A, D, G,* and *J*) and MG-160 (*B, E, H,* and *K*) and the images were overlaid and pseudocolored to demonstrate the spatial distribution of both labeling patterns (*C, F, I,* and *L*). The punctate intercellular and paranuclear Cx43 staining seen in control cells (*A–C*) was eliminated when the cells were treated with BFA (*D–F*). However, in CHX treated cells, the Golgi remained organized around the nucleus and gap junction plaques were often found at locations of cell–cell contact (*G–I*). BFA/CHX-treated cells lost most of their gap junction plaques and the Golgi became disrupted (*J–L*). Bar, 10 μm.

reassembled (Fig. 8 *B*) and numerous gap junction plaques were found between apposed cells (Fig. 8 *A*). The assembly of gap junctions (Fig. 8 *D*) or the reassembly of the Golgi apparatus (Fig. 8 *E*) was not inhibited by CHX over the 1-h BFA-recovery time period.

Fate of Total Cellular Cx43 and Its Phosphorylated Isoforms in BFA and CHX-treated Cells

Western blots and phosphorimager analysis were performed to examine the biochemical changes to Cx43 associated with cell–cell uncoupling and loss of gap junction plaques that was observed in BFA-treated BICR-M1R_k cells. Western blot analysis revealed a 42–43-kD doublet of Cx43 and the extensively phosphorylated 44- and 46-kD forms of Cx43 in untreated cells (Fig. 9, lane *a*). Phosphorimager analysis revealed that the 42–43-kD forms of Cx43 constituted ~60% of the total Cx43 in these cells. When the cells were treated with BFA for 6 h (Fig. 9, lane *b*), the 44- and 46-kD forms of Cx43 were lost and there was an

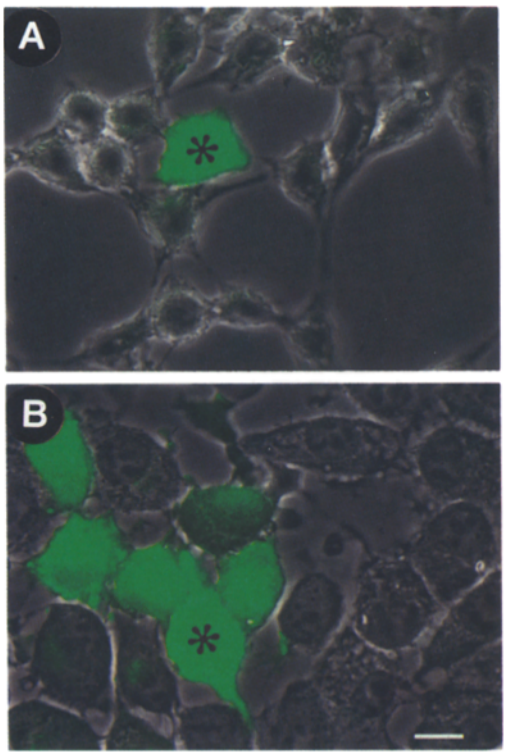


Figure 7. Reversible inhibition of dye coupling in BFA-treated cells. BICR-M1R_k cells treated with BFA for 6 h (*A*) or treated with BFA for 6 h and recovered for 1 h (*B*) were microinjected with 6-carboxyfluorescein (*asterisk*) and dye was allowed to spread for 15 min. After fixation, confocal microscopy was used to overlay the fluorescent image (*green*) with the transmitted light image (*gray*) to illustrate the spreading of the dye to neighboring cells. Bar, 10 μm.

accumulation of the Cx43 doublet at 42 and 43 kD (Fig. 9 *A*, *inset*). As expected, when the cells were treated with BFA for only 2 h, there was an increase in the 42–43-kD forms of Cx43 and a reduction in Cx43 at 44 and 46 kD (Fig. 9, lane *g*). Cx43 was greatly reduced in BICR-M1R_k cells when protein synthesis was inhibited with CHX for 6 h (Fig. 9, lane *c*), however, all forms of Cx43 were clearly identifiable. Moreover, cells treated with BFA and CHX for 6 h lost the 44- and 46-kD forms of Cx43 while nascent Cx43 at 42 kD and immature Cx43 at 43 kD were detectable (Fig. 9, lane *d*).

Recovery of Cx43 Phosphorylation Is Independent of Protein Synthesis

In recovery studies, after a 6-h BFA treatment Cx43 matured to the 44- and 46-kD phosphorylated forms after a recovery period of only 1 h (Fig. 9, lane *e*). A similar recovery of the more mature forms of Cx43 at 44 and 46 kD was seen even in the presence of CHX (Fig. 9, lane *f*).

Discussion

In this study we provide compelling evidence that Cx43 is a transient resident of the Golgi apparatus and is initially phosphorylated in this compartment or earlier in the ER. In addition, we have used BFA to separate the process of gap junction assembly from the events that lead to gap junction removal and demonstrated that both processes are rapid and independent of protein synthesis. Moreover, the loss of gap junction plaques at the cell surface in the presence of BFA is correlated with the loss of the extensively phosphorylated 44- and 46-kD forms of Cx43 and functional uncoupling of the cells.

The established rat mammary tumor cell line BICR-M1R_k was chosen to examine Cx43 trafficking and turnover for the following reasons: (*a*) these cells are well coupled via gap junctions; (*b*) they express high levels of endogenous Cx43 with no immunofluorescently detectable connexin32 or connexin26 (results not shown); (*c*) mammary epithelium in vivo contains Cx43 (Wilgenbus et al., 1992); (*d*) growing cells have identifiable pools of intracellular Cx43 in paranuclear locations suggesting that the secretory and/or degradative pathways are rich in Cx43; (*e*) similar to most in vitro studies (Musil et al., 1990*a,b*; Laird et al., 1991) Cx43 has a half-life of ~1.5 h in this cell line indicating that gap junction formation and removal is an active process. Double immunofluorescent labeling for Cx43 and a constituent of the Golgi apparatus was used for the first time to show that a steady-state pool of this nonglycosylated integral membrane protein resides within the Golgi apparatus in vitro. The Golgi protein, MG-160, and Cx43 were colocalized not only in rat mammary tumor cells but also in NRK cells suggesting that both tumor and normal rat cells have varying levels of Cx43 in the Golgi apparatus. Primary cultures of cardiomyocytes appear to have a low level of Cx43 in the Golgi apparatus as Cx43 could only be clearly identified in this compartment when protein trafficking was blocked with monensin (Laird et al., 1993; Puranam et al., 1993). However, in one in vivo study, Hendrix et al. (1992) localized Cx43 to the Golgi apparatus in the myometrium of a 21 day pregnant rat. While other reports have shown that Cx43 may have a perinuclear distribution (Berthoud et al., 1992; Naus et al., 1992), this compartment was not previously defined. Based on our results and the recent findings that rat liver Golgi membrane fractions contain immunoreactive Cx32 (Rahman et al., 1993) and Golgi membranes from transfected BHK cells contain Cx43 and Cx32 (Falk et al., 1994), it is reasonable to suggest that other members of the connexin family may also transiently reside within the Golgi apparatus.

During its life cycle Cx43 is subject to posttranslational modifications. At present, several forms of Cx43 are known to exist that are the result of phosphorylation of the nascent protein. The most well characterized of these is the unphosphorylated form and two phosphorylated species (Musil et al., 1990*a,b*; Laird et al., 1991), commonly referred to as P_1 and P_2 (Musil et al., 1990*a*). In addition, a novel elevated phosphorylated form of Cx43 has been identified in epidermal growth factor-treated T51B rat liver epithelial cells (Lau et al., 1992). In all cases the phosphorylated species of Cx43 are retarded in SDS–polyacrylamide gels. Nevertheless, it remains unresolved how many phosphate groups are added to Cx43 to generate these phosphorylated isoforms.

In our study we used BFA to impair protein trafficking within the ER/Golgi to identify intermediate processing of Cx43 that proceeds the maturation of the protein to the

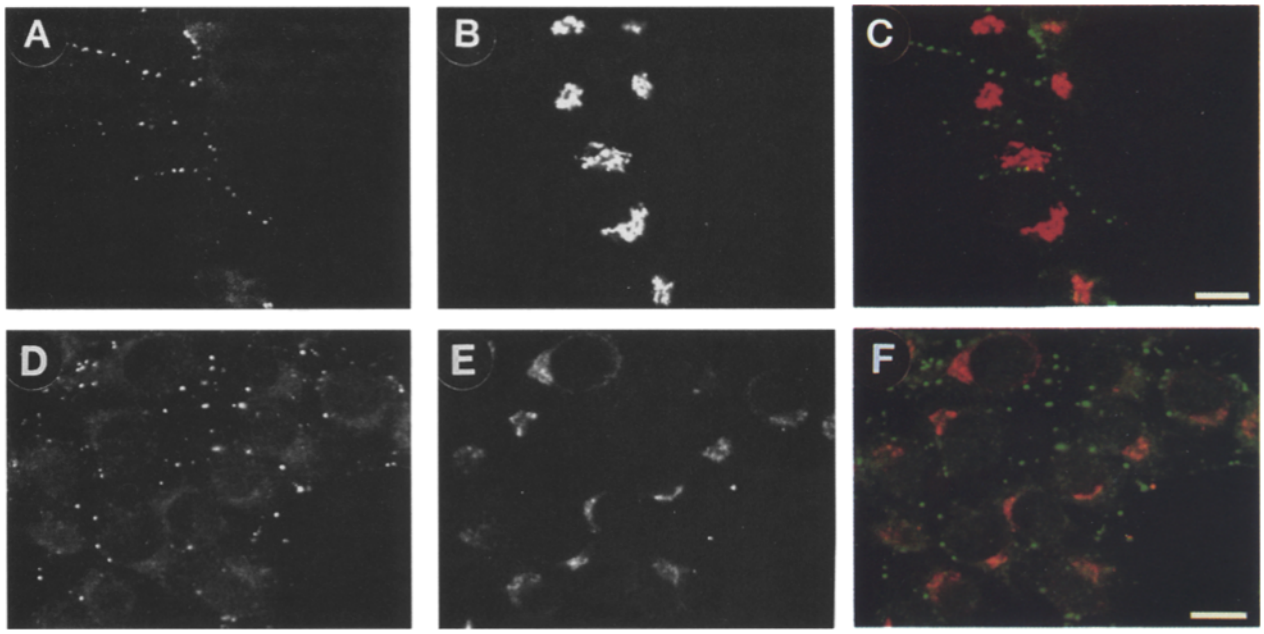
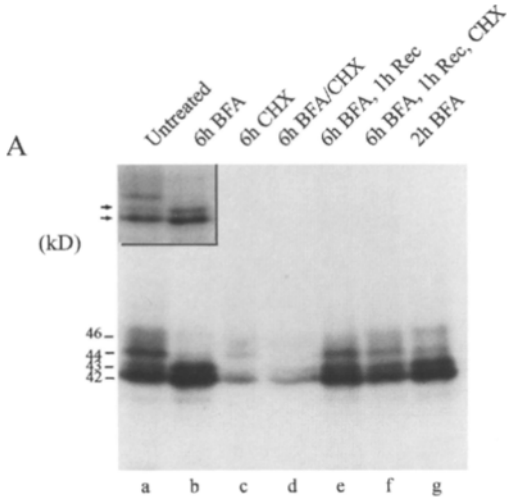


Figure 8. Assembly of cell surface gap junction plaques is independent of protein synthesis. Cells were treated with BFA for 6 h and allowed to recover in the absence (*A–C*) or presence (*D–F*) of cycloheximide for 1 h. Permeabilized cells were immunolabeled for Cx43 (*A* and *D*) and MG-160 (*B* and *E*) and optical slices of double-labeled cells were overlaid (*C* and *F*). Note that gap junction plaques assembled within 1 h and this process was not inhibited by CHX. Bar, 10 μm.

more extensively phosphorylated and well-characterized Cx43 isoforms (i.e., P_1 and P_2). In the presence of BFA, high resolution gels revealed a 42–43-kD ^{35}S-trans–labeled doublet while the more extensively phosphorylated forms of Cx43 were eliminated. Recovery from BFA treatment was coordinated with the reappearance of the more highly phosphorylated forms of Cx43 similar to that observed by Musil and Goodenough (1993) in NRK cells. In fact, the 43-kD isoform of Cx43 was found to be specifically sensitive to alkaline phosphatase strongly suggesting that Cx43 proceeds through a transient phosphorylation state within the ER/Golgi complex. Our data suggests that once Cx43 is initially phosphorylated it is either rapidly subjected to more extensive phosphorylation or a substrate for phosphatase activity. The retention of both 42- and 43-kD forms of Cx43 in the ER/Golgi suggests that neither form of the protein is recognized as being misfolded and subjected to ER degradation. We provide compelling evidence by immunoprecipitating Cx43 with antibodies to the amino and carboxy terminal ends of the molecule that the 42–43-kD Cx43 doublet represents the full length protein. Furthermore, the use of two specific site-directed anti-Cx43 antibodies rules out the likelihood that our results are due to the immunoprecipitation of a second member of the connexin family.

Upon examination of total Cx43 in Western blots of untreated BICR-M1R_k cells, a doublet at 42–43 kD is present but the 43-kD form of the protein is often masked by the intensity of the nascent form. Crow et al. (1990) first presented data suggesting that a transient posttranslational modification of Cx43 occurs within 15 min of its biosynthesis in NIH 3T3 fibroblasts. In a previous study we used monensin to trap an alkaline-phosphatase sensitive intermediate form of Cx43 in primary cultures of cardiac myocytes (Laird et al., 1993). Both cardiomyocytes and BICR-M1R_k cells, in particular, expressed high levels of endogenous Cx43 enabling ER/Golgi posttranslationally modified forms of Cx43 to be identified particularly with the aid of protein translocation inhibitors. Phosphorylation of proteins early in the secretory pathway is not an uncommon event. It is known that caseins are phosphorylated in the Golgi apparatus and that the phosphorylation of alpha casein can occur in the presence of BFA (Turner et al., 1993). Likewise, BFA was used to show that the phosphorylation of chromogranin B and secretogranin II occurs in the *trans*-Golgi (Rosa et al., 1992). Moreover, Davidson et al. (1992) showed that protein dephosphorylation is essential for vesicular stomatitis virus G protein transport from the ER to the Golgi. The role of an early Cx43 phosphorylation event in the ER or Golgi apparatus is unknown. It is possible that early phosphorylation of Cx43 is necessary to facilitate later connexin oligomerization that has been proposed to occur in the *trans*-Golgi network (Musil and Goodenough, 1993). It is also possible that Cx43 is phosphorylated early in the secretory pathway to prevent it from misfolding or being targeted for degradation before its oligomerization within the Golgi apparatus and trafficking to the cell surface. Future site-directed mutagenesis studies may determine the amino acid residue(s) that is phosphorylated and its functional role.

While the life cycle of Cx43 includes a transient residency in the Golgi apparatus, this protein is generally short-lived in BICR-M1R_k cells as pulse-chase experiments show that it has a half-life of ~1.5 h. Compared to the over 30 glycosylated and nonglycosylated plasma membrane proteins analyzed in cultured rat liver hepatocytes ($t_{1/2}$ 17–100 h) (Chu and Doyle, 1985) and the 35–40 proteins studied from plasma membranes of 3T3 fibroblasts and H4-II-E-



B

44-46 kD	71	< 1	19	< 1	26	20	24
42-43 kD	107	172	11	18	102	64	144
Lane	a	b	c	d	e	f	g

Figure 9. Western blot and phosphorimager analysis of total Cx43 in BFA, CHX and recovered BICR-M1R_k cells. (*A*) Untreated (lane *a*), 6 h BFA (lane *b*), 6 h CHX (lane *c*), 6 h BFA+CHX (lane *d*), 6 h BFA + 1 h recovery (lane *e*), 6 h BFA + 1 h recovery in CHX (lane *f*), or 2 h BFA (lane *g*) BICR-M1R_k cells were lysed in cocktail buffer and subjected to SDS-PAGE. After transfer to nitrocellulose, the blots were immunolabeled for Cx43 and exposed to hyperfilm. The insert is a Phosphorimage of Cx43 from lanes *a* and *b* to clearly illustrate that there are protein bands at 42 and 43 kD (*arrows*) in both untreated and BFA-treated cells. (*B*) The gel in *A* was exposed to a Phosphorimager and the Cx43 signal from each treatment was collected and arbitrary volume counts were divided into two groups (42–43 kD and 44–46 kD) and recorded. These experiments were repeated and equivalent results were found.

C3 hepatoma cells ($t_{1/2}$ > 75 h) (Hare and Taylor, 1991), both Cx32 and Cx43 have short half-lives of 1–3 h (Traub et al., 1987, 1989; Laird et al., 1991; Musil et al., 1990*a,b*). Thus, gap junction formation and removal is believed to be an active process and possibly a means by which cells regulate intercellular communication in response to physiological stimuli. To determine if fully assembled, Cx43-containing, gap junction "plaques" have a turnover rate that equals the rate observed in pulse–chase studies where newly synthesized connexins are followed, the secretory pathway was blocked with BFA and the fate of "preexisting plaques" at the cell surface was followed. BFA treatment of BICR-M1R_k cells for 6 h resulted in the accumulation of intracellular Cx43, the elimination of preexisting gap junction plaques from the cell surface and the functional uncoupling of cells. The accumulated intracellular store of Cx43 had a disperse reticular-like pattern very similar to that seen when cells were labeled for MG-160 or the ER protein, PDI. Colocalization of constituent proteins of the ER and Golgi suggests that the ER and Golgi membranes are fused after the BFA treatment and that this compartment is rich in the 42–43-kD species of Cx43. Over the course of a 6-h BFA treatment we ascertain from our pulse chase data that Cx43 will pass through four half-lives ($t_{1/2}$ ~1.5 h) that is predicted to leave ~6% of the gap junction plaques on the surface. The observation that gap junction plaques are immunodetectably cleared from the cell surface and the cells become uncoupled strongly suggests that fully assembled "gap junction plaques" have a half-life of ~1.5 h in these cells comparable to the calculated half-life of Cx43 monomer. Moreover, a 6-h BFA treatment resulted in the loss of the phosphorylated forms of Cx43 at 44 and 46 kD. Thus, in the presence of BFA, these results are consistent with the loss of Cx43 from the cell surface. While the possibility that the 42- and 43-kD forms of Cx43 seen in Western blots represent plasma membrane forms of the protein cannot be absolutely ruled out, based on the turnover rate of Cx43, this remains unlikely. Moreover, BFA has not been reported to stabilize proteins within the plasma membrane.

The presence of Cx43 in the Golgi apparatus suggested that BICR-M1R_k cells may be able to meet their constant and continual need to assemble new gap junction plaques by accessing this intracellular store of protein. To evaluate the size and availability of this intracellular repository of Cx43, BICR-M1R_k cells were examined under conditions where protein synthesis was inhibited by CHX. Somewhat unexpectedly, when BICR-M1R_k cells were treated with CHX for 6 h, a population of plaques was observed by immunofluorescence, yet quantitatively there was over an 80% reduction in total Cx43 suggesting that immunofluorescent evaluations of Cx43 are strictly qualitative. Two possibilities exist for the partial retention of gap junctions after a 6 h CHX treatment; (*a*) CHX may be inhibiting the internalization of gap junction plaques; or (*b*) a sufficiently large pool of Cx43 may reside within the ER/Golgi complex that is able to continue to regenerate a population of plaques throughout the 6 h time course. Extended treatment of BICR-M1R_k cells with CHX (>6 h) results in a progressive reduction in gap junction plaques that also correlates with altered cell morphology and inevitable cell death (results not shown). It is unlikely that the CHX effect can be explained by the blockage of gap junction internalization as cells treated with BFA and CHX for 6 h lost the majority of their gap junction plaques. Thus, we conclude that the ER/Golgi intracellular pool of Cx43 is unavailable to assemble new gap junctions in BFA-treated cells but is available for replenishing gap junctions in CHX-treated BICR-M1R_k cells throughout the 6-h time course. Likewise, gap junction removal is not dependent on the synthesis of proteins which may be necessary to facilitate or govern the internalization process. We show here that BFA-treated cells can indeed assemble gap junctions upon the removal of BFA independent of protein synthesis. Immunostaining of BFA-treated BICR-M1R_k cells strongly suggests that Cx43 accumulates in the diffused ER/Golgi membrane compartments during the 6-h treatment and upon the removal of the drug there is an abundance of Cx43 available to traffick to the cell surface and restore gap junction plaques and functional intercellular communication. The assembly of gap junction plaques and the restoration of functional coupling is further correlated with the phosphorylation of Cx43 to the 44- and 46-kD forms. Gap junction assembly in the preimplantation embryo (McLachlin et al., 1986) and reaggregating Novikoff

cells (Epstein et al., 1977) has been shown to occur in the absence of protein synthesis. Thus, our and other studies are consistent with cells maintaining an intracellular store of connexins that are readily available for gap junction assembly. Moreover, De Sousa et al. (1993) used inhibitors of protein trafficking to show that the regulated step in gap junction plaque formation during compaction was after Cx43 translocation.

In summary, we provide evidence that Cx43 is initially phosphorylated in the ER or as it transiently resides in the Golgi apparatus. Blockage of the secretory pathway with BFA has provided a means of separating the kinetic and compartmental events that lead to gap junction formation from those related to gap junction plaque loss and functional uncoupling. Finally, the turnover of both connexins and fully assembled gap junction plaques is rapid and the loss of intercellular communication and gap junction plaques is correlated with the loss of the more highly phosphorylated forms of Cx43.

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