Cytosolic Stress Reduces Degradation of Connexin43 Internalized from the Cell Surface and Enhances Gap Junction Formation and Function

Judy K. VanSlyke and Linda S. Musil

Division of Molecular Medicine, Oregon Health and Science University, Portland, OR 97239

Submitted May 11, 2005; Revised August 11, 2005; Accepted August 19, 2005
Monitoring Editor: Asma Nusrat

The protein constituents of gap junctions, connexins, have a rapid basal rate of degradation even after transport to the cell surface. We have used cell surface biotinylation to label gap junction-unassembled plasma membrane pools of connexin43 (Cx43) and show that their degradation is inhibited by mild hyperthermia, oxidative stress, and proteasome inhibitors. Cytosolic stress does not perturb endocytosis of biotinylated Cx43, but instead it seems to interfere with its targeting and/or transport to the lysosome, possibly by increasing the level of unfolded protein in the cytosol. This allows more Cx43 molecules to recycle to the cell surface, where they are assembled into long-lived, functional gap junctions in otherwise gap junction assembly-inefficient cells. Cytosolic stress also slowed degradation of biotinylated Cx43 in gap junction assembly-efficient normal rat kidney fibroblasts, and reduced the rate at which gap junctions disappeared from cell interfaces under conditions that blocked transport of nascent connexin molecules to the plasma membrane. These data demonstrate that degradation from the cell surface can be down-regulated by physiologically relevant forms of stress. For connexins, this may serve to enhance or preserve gap junction-mediated intercellular communication even under conditions in which protein synthesis and/or intracellular transport are compromised.

INTRODUCTION

Reducing the rate of degradation (turnover) is an efficient means to increase the level of a short half-life protein. Although a well-established mechanism to enhance the activity of several types of cytosolic proteins, relatively little is known about how the stability of plasma membrane proteins can be up-regulated. As assessed by pulse-chase analysis, connexins, members of the family of polytopic plasma membrane proteins that form gap junctions, are degraded with a protein half-life of 1.5–5 h (Fallon and Goodenough, 1981; Musil *et al.*, 1990; Beardslee *et al.*, 1998). We have previously reported that a substantial fraction of newly synthesized, topologically normal connexin molecules are rapidly turned over by endoplasmic reticulum-associated degradation (ERAD) (VanSlyke *et al.*, 2000; VanSlyke and Musil, 2002). ERAD of connexins, as well of the gap junction-unrelated major histocompatibility complex class I heavy chain, was inhibited by treatments that induce either mild hyperthermic or oxidative stress. Wild-type connexin molecules spared from ERAD in this manner could then be transported to the plasma membrane and participate in the enhanced formation of functional clusters of gap junctional channels, referred to as plaques, between adjoining cells.

Unlike most plasma membrane proteins, connexins remain metabolically unstable even after they have been trans-

ported to the cell surface as evidenced by the rapid turnover of gap junctional plaques in tissue culture cells and in vivo (Fallon and Goodenough, 1981; Musil and Goodenough, 1991; Gaietta *et al.*, 2002). The sole documented exception to this rule is gap junctions in mature lens fibers, a cell type in which essentially all energy-requiring processes are slowed. Little is known about how connexins are degraded from the plasma membrane. Previous studies have documented the formation of “annular” gap junctions, in which an intact gap junctional plaque is internalized as a double-membraned structure into the cytosol of one of the two partner cells. Annular gap junctions have been reported to be associated with lysosomes, within which they seem to be degraded (Qin *et al.*, 2003). The frequency with which annular gap junctions are formed is highly dependent on the type of cell and its physiological state and does not necessarily correspond to the rate at which connexins are turned over (Jordan *et al.*, 2001). Although this implies that a mechanism other than annular gap junction formation must exist for clearing connexins from the cell surface, the route by which such turnover takes place has not been determined. Moreover, no studies have directly addressed how connexins that have been transported to the plasma membrane, but have not been assembled into gap junctional plaques, are degraded.

Musil and Goodenough (1991) examined the transport of pulse labeled, endogenously expressed connexin43 (Cx43) to the plasma membrane by using cell surface biotinylation. Three classes of cells were studied: 1) assembly-efficient cells, which constitutively assemble Cx43 into abundant gap junctional plaques; 2) assembly-inefficient cells, which are comparatively less proficient in gap junction assembly under basal conditions; and 3) assembly-incompetent cells, defined as forming only very small and sparse gap junctions (Musil *et al.*, 2000). In all cases, the level of gap junction formation and function were closely correlated as assessed

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-05-0415>) on August 31, 2005.

Address correspondence to: Linda S. Musil (musill@ohsu.edu).

Abbreviations used: BFA, brefeldin A; CLQ, chloroquine; Cx43, connexin43; ERAD, endoplasmic reticulum-associated degradation; MesNa, sodium 2-mercaptoethanesulfonate.

by intercellular dye coupling. Although all three cell classes oligomerized comparable quantities of Cx43 into connexons (hemichannels) and then transported them to the cell surface at similar rates, only in assembly-efficient cells did the majority of cell surface-biotinylated Cx43 subsequently become incorporated into gap junctional plaques (Musil *et al.*, 2000).

In this investigation, we demonstrate that degradation of cell surface-biotinylated Cx43 is reduced by the same non-toxic stress-inducing conditions that inhibit the degradation of connexins from the endoplasmic reticulum (ER). These treatments interfere with delivery of connexins to the lysosome, allowing connexins in assembly-competent cells to recycle back to the plasma membrane and accumulate in functional gap junctional plaques. Postendocytic inhibition of plasma membrane protein turnover is a novel cellular response to mild environmental stress.

MATERIALS AND METHODS

Cell Surface Biotinylation and Treatment with Stressors or Inhibitors

Newly confluent monolayers of adherent cells, cultured as described in Musil *et al.* (2000), were cell surface biotinylated at 4°C as described in Musil and Goodenough (1991), except that the reaction was quenched after 30 min with five rinses of 100 mM glycine, pH 7.6, in phosphate-buffered saline (PBS) + Ca²⁺/Mg²⁺. Sulfo-NHS-SS-biotin (0.5 mg/ml; 1.5 ml/35-mm cell culture) was used for all experiments unless the cells were chased in dithiothreitol (DTT), in which case NHS-LC-biotin was used. Where indicated, cells were then chased in complete tissue culture medium (containing 10% fetal calf serum) at 37°C in either the absence (control) or presence of the following inhibitors: 210 μ M leupeptin, 80 μ M sodium arsenite, 10 μ M epoxomicin (Calbiochem, San Diego, CA), 6 μ g/ml brefeldin A (BFA; Epicentre Technologies, Madison, WI), 2 mM DTT (Roche Diagnostics, Indianapolis, IN), or chloroquine (CLQ; used at the minimum concentration required to neutralize the luminal pH of lysosomes as assessed by acridine orange staining, 200 μ M in S180 and normal rat kidney [NRK] cells and 500 μ M in Chinese hamster ovary [CHO] cells). Unless otherwise specified, all reagents were from Sigma-Aldrich (St. Louis, MO). Heat shock (42°C) and mock shock (37°C) treatments were conducted as described previously (VanSlyke and Musil, 2002), using a thermocouple thermometer to verify that the growing surface of the tissue culture dish was maintained at the desired temperature.

Streptavidin Precipitation of Cell Surface-biotinylated Cx43

Surface-biotinylated cells were solubilized in 0.6% SDS and then boiled for 3 min as reported in VanSlyke and Musil (2000), with the addition of 15 mM glycine to the lysis buffer. The lysate from a 35-mm plate of cells was incubated with 50 μ l of a 25% slurry of streptavidin agarose beads (Pierce Chemical, Rockford, IL) on a rotator overnight at 4°C. The samples were washed three times with borate buffer (0.1 M NaCl, 20 mM sodium borate, and 0.02% sodium azide, pH 8.2) supplemented with 0.5% Triton X-100, 0.1% SDS, and 0.5% bovine serum albumin (BSA), once in the same buffer with detergents minus BSA, and finally with AP reaction buffer (150 mM NaCl, 10 mM MgCl₂, and 50 mM Tris-HCl, pH 8.0) plus 0.5% Triton X-100 and 0.1% SDS. The beads were then resuspended in 100 μ l of detergent-free AP reaction buffer containing 3 U of calf intestinal alkaline phosphatase (ALP) (no. 713023; Roche Diagnostics). After a 2- to 3-h incubation at 37°C, the beads were washed two times with borate buffer plus 15 mM EDTA, 15 mM EGTA, supplemented with 0.5% Triton X-100 and 0.1% SDS for the first wash and with 0.05% Triton X-100 and 0.1% SDS for the second.

Western Transfer and Quantitation

Dephosphorylated samples prepared as described above were resuspended in SDS-PAGE sample loading buffer containing 2% β -mercaptoethanol and boiled for 5 min to elute the precipitated proteins from the beads. The samples were then resolved on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Billerica, MA) at 65 V for 50 min. Blots were probed with primary antibodies raised against the indicated peptides in Cx43: affinity-purified rabbit 7298 (aa 252–271; Musil *et al.*, 1990) and mouse monoclonal antibodies Cx43CT-1 (aa 360–382) and Cx43NT-1 (aa 1–20) (Fred Hutchinson Cancer Research Center Hybridoma Development Facility; Seattle, WA). Immunoreactive protein bands were detected using ALP-conjugated secondary antibodies with ECF substrate (RPN 5875; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), and scans of the fluorescent reaction product were captured and quantitated on the FX Molecular Imager using the Quantity One software (Bio-Rad, Hercules, CA).

Unless indicated otherwise, all experiments were repeated a minimum of three times, and representative images are shown.

Microscopy

CHO cells grown on glass coverslips were fixed in 2% paraformaldehyde in PBS and processed for immunocytochemical detection of Cx43 as described previously (Musil *et al.*, 2000). Gap junction-mediated intercellular communication was measured using the scrape-loading/dye transfer assay exactly as in Musil *et al.* (2000).

Plasmids and Transient Transfection of CHO Cells

One day after plating, CHO cultures were transfected using FuGENE 6 (Roche Diagnostics) as specified by the manufacturer with one of the following cytomegalovirus (CMV) promoter-driven plasmids: GFP250 (Garcia-Mata *et al.*, 1999), EGFP (BD Biosciences Clontech, Palo Alto, CA), or the lacZ gene product β -galactosidase (CS2+c gal; Turner and Weintraub, 1994). Cells were analyzed 24 h after transfection.

Sodium 2-Mercaptoethanesulfonate (MesNa) Stripping of Cell Surface-biotinylated Cx43

Biotinylated cell cultures were treated with the membrane-impermeant reducing agent MesNa or mock treated as described by Schmidt *et al.* (1997), except scaled down for 35-mm culture dishes. Cultures that were to be further incubated at 37°C were rinsed four times with NT buffer (0.2% BSA, 0.15 M NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0) and once with growth medium before being returned to the tissue culture incubator in growth medium. All cells were rinsed multiple times with NT buffer and then incubated with freshly prepared 50 mM iodoacetamide in NT buffer for 10 min at 4°C before cell lysis.

RESULTS

Basal Rate of Degradation of Cell Surface-biotinylated Cx43 in S180 Cells

In S180 cells, oligomerization of newly synthesized Cx43 into connexons (hemichannels) and their subsequent transport to the plasma membrane proceed normally, but very little of this cell surface, connexon-assembled Cx43 pool then becomes incorporated into gap junctional plaques (Musil *et al.*, 1990). S180 cells were incubated at 4°C with the membrane-impermeant biotinylating reagent sulfo-NHS-SS-biotin under conditions previously shown to restrict biotin conjugation to the extracellular domain of plasma membrane proteins (Musil and Goodenough, 1991). After the biotinylating reaction was quenched with excess glycine, the cells were lysed in SDS-containing buffer either immediately or after a 1- to 6-h “chase” at 37°C in tissue culture medium. Cell extracts were precipitated with streptavidin beads to capture total biotinylated proteins, which were then subjected to SDS-PAGE and probed for endogenously expressed Cx43 by Western blotting using antibodies directed against either the cytoplasmic tail (7298 and Cx43CT-1) or amino terminal (Cx43NT-1) domains of Cx43. Samples were routinely dephosphorylated with ALP before SDS-PAGE to ensure that the epitopes recognized by these antibodies were not blocked by phosphorylation (Nagy *et al.*, 1997). A rapid decline in the level of biotinylated Cx43 during the course of the chase was detected by all three antibodies, establishing that connexin degradation, and not proteolytic clipping or epitope masking, was responsible for this loss (Figure 1A). The fast rate of turnover of largely gap junction-unassembled cell surface-biotinylated Cx43 in S180 cells is comparable with that reported for intact gap junctional plaques in assembly-efficient cell types (Fallon and Goodenough, 1981; Musil and Goodenough, 1991; Gaietta *et al.*, 2002).

Role of the Lysosome in the Turnover of Cx43 from the Surface of S180 Cells

Pulse-chase experiments have revealed that the half-life of metabolically labeled connexins is prolonged by inhibitors of lysosome-mediated degradation in both assembly-effi-

Figure 1. Turnover of cell surface-biotinylated, gap junction-unassembled Cx43 in S180 cells is blocked by lysosomal inhibitors. S180 cells were cell surface biotinylated at 4°C and chased for up to 6 h in culture medium at 37°C in the absence or presence of either 200 μ M CLQ or 210 μ M leupeptin as indicated. In C, the cultures were incubated with leupeptin for 12 h before biotinylation. After cell lysis, streptavidin-isolated biotinylated proteins were dephosphorylated before Western blot analysis with one of the three Cx43-specific antibodies indicated (A) or with AP7298 (B and C). The lanes depicting the 0-h chase sample are duplicated in B to facilitate comparison of the rate of biotinylated Cx43 turnover in control and treated cells.

cient and assembly-deficient cell types (Musil *et al.*, 2000). It has not, however, been established whether plaque-unassembled connexins are transported to the lysosome from the cell surface or from pre-plasma membrane compartments. Precedence for the latter route has been obtained in several systems (VanSlyke *et al.*, 2000; Qin *et al.*, 2003; reviewed by Berthoud *et al.*, 2004). To elucidate the role of lysosomes in the turnover of Cx43 after its transport to the plasma membrane, S180 cells were cell surface biotinylated and then chased at 37°C in either the absence or presence of the lysosomal inhibitor CLQ. As assessed with the 7298 anti-Cx43 antibody, 200 μ M CLQ very effectively blocked the turnover of biotinylated Cx43 during the course of the experiment ($87.2 \pm 2.7\%$ remaining at 6 h compared with $19.7 \pm 8.9\%$ in untreated control cells; $n = 3$) (Figure 1B). A comparable fraction of biotinylated Cx43 was recovered when blots were probed with either the amino terminal-directed NT-1 or the carboxyl tip-specific CT-1 antibodies ($92.8 \pm 7.7\%$ after 6 h with CT-1, $n = 3$; our unpublished data), confirming that the biotinylated Cx43 remained completely intact. Turnover of biotinylated Cx43 was also significantly reduced by leupeptin ($54.7 \pm 3.2\%$ remaining at 6 h; $n = 3$) (Figure 1C). The effect of leupeptin was weaker than CLQ, in keeping with its much slower rate of accumulation in lysosomes and the smaller number of proteases sensitive to it (Chu *et al.*, 1998).

Mild Hyperthermia or Oxidative Stress Reduces the Degradation of Cell Surface-biotinylated Cx43 in S180 Cells

We have previously reported that treatments that induce nonlethal hyperthermic (42°C for 30 min) or oxidative (80 μ M sodium arsenite) stress reduce the rate at which newly synthesized Cx43 is degraded by ERAD (VanSlyke and Musil, 2002). Whether the ER is the only cellular compartment in which turnover of Cx43 is sensitive to these agents was not addressed. To investigate whether heat shock affects the half-life of connexin molecules already on the cell surface at the time the stress is imposed, S180 cells were cell surface biotinylated and then immediately subjected to a 30-min

incubation at 42°C. The rate at which biotinylated Cx43 was turned over during a subsequent 37°C chase was greatly reduced relative to controls continuously kept at 37°C (Figure 2A) ($76.9 \pm 4.4\%$ remaining after a 6-h chase in heat-shocked cells, relative to $25.2 \pm 3.3\%$ in mock-shocked cells; $n = 3$). Degradation of cell surface-biotinylated Cx43 was also markedly inhibited by the addition of 80 μ M sodium arsenite to the chase medium (Figure 2B) ($54.4 \pm 6.0\%$ remaining after a 6-h chase, relative to $9.4 \pm 6.3\%$ in untreated controls in three independent experiments). Comparable levels of inhibition were observed when blots were probed with either the anti-Cx43 AP7298 antibody (Figure 2) or an antibody (CT-1) directed against the proteolytically sensitive distal carboxyl tail domain of Cx43 (percentage remaining after 6 h in heat-shocked or arsenite-treated cells: 67.5 ± 1.3 and $78.6 \pm 4.8\%$, respectively). In contrast, DTT, an agent that induces ER instead of cytosolic stress, had no significant, reproducible effect on the turnover rate of cell surface-biotinylated Cx43 ($20.1 \pm 7.9\%$ remaining after a 6-h chase in the presence of 2 mM DTT, relative to $16.3 \pm 2.7\%$ in untreated controls; $n = 3$; our unpublished data). Induction of ER stress was previously shown to be ineffective in slowing Cx43 degradation from the ER (VanSlyke and Musil, 2002).

Inhibition of the Proteasome Reduces the Degradation of Cell Surface-biotinylated Cx43 in S180 Cells

Small molecule inhibitors of the proteasome slow the rate of Cx43 ERAD (VanSlyke *et al.*, 2000; VanSlyke and Musil, 2002). To determine whether these compounds also reduce the degradation of Cx43 from the plasma membrane, S180 cells were cell surface biotinylated and then chased at 37°C in the presence of 10 μ M epoxomicin. Epoxomicin is a potent and highly specific, irreversible inhibitor of the proteasome that has no activity against the lysosome (Marques *et al.*, 2004). As shown in Figure 2C, epoxomicin significantly slowed the turnover of cell surface-biotinylated Cx43 ($36.6 \pm 0.8\%$ remaining after 6 h with epoxomicin, relative to $12.3 \pm 7.5\%$ in its absence; $n = 3$), albeit to a somewhat lesser extent than either hyperthermia or arsenite even if cells were pre-incubated with the inhibitor before biotinylation.

Figure 2. Stabilization of cell surface-biotinylated Cx43 by hyperthermia, sodium arsenite, and proteasome inhibition in S180 and CHO cells. (A and E) Immediately after cell surface biotinylation, S180 (A) or CHO (E) cells were subjected to either a mock ($\Delta 37^{\circ}\text{C}$) or mild ($\Delta 42^{\circ}\text{C}$) heat shock for 30 min and harvested after a 0- to 6-h chase in culture medium at 37°C . (B and C) Biotinylated S180 cultures were chased in the absence (control) or presence of either 80 μM sodium arsenite (B) or 10 μM epoxomicin (C). All samples were then processed as in Figure 1. Lanes containing the 0-h chase sample are duplicated. (D and F) Graphs summarizing the percentage of biotinylated Cx43 remaining after a 6-h chase in S180 (D) or CHO (F) cells under the conditions indicated (values for CHO cells in F; control, $21.8 \pm 5.8\%$; CLQ, $81.1 \pm 8.3\%$; heat shock, $75.7 \pm 8.0\%$; arsenite $74.1 \pm 3.5\%$; epoxomicin, $43.9 \pm 6.3\%$; $n \geq 3$).

Epoxomicin and other proteasome inhibitors block the degradation of misfolded or aged substrates of the proteasome, whereas arsenite and hyperthermia prevent cytosolic proteins from achieving and/or maintaining a properly folded conformation (Beckmann *et al.*, 1992; Lepock *et al.*, 1993). These treatments therefore share the ability to increase the level of unfolded protein in the cytosol and are henceforth collectively referred to as cytosolic stressors. Importantly, arsenite and hyperthermia do not inhibit the catalytic activity of the 20S proteasome under the conditions used in this study (see *Discussion*).

Cytosolic Stressors Up-Regulate the Assembly of Cell Surface Cx43 into Functional Gap Junctions in CHO Cells

Although it was shown that Cx43 saved from ERAD by cytosolic stress was capable of exiting the ER and becoming incorporated into gap junctional plaques in otherwise gap junction assembly-inefficient CHO cells (VanSlyke and Musil, 2002), whether other cellular pools of Cx43 could also participate in stress-induced gap junction formation was not addressed. To investigate the possible contribution of Cx43 molecules already present on the cell surface at the time the stress was imposed, we first demonstrated that each of the three cytosolic stressors as well as CLQ reduced the turnover of cell surface-biotinylated Cx43 in CHO cells to an extent comparable with that observed in S180 cells (Figure 2, E and F). We then conducted a series of immunocytochem-

ical and dye transfer studies in the presence of BFA. Control experiments demonstrated that [^{35}S]methionine-labeled Cx43 synthesized in the presence of BFA was inaccessible to cell surface biotinylation as indicated by its inability to be precipitated by streptavidin beads and that BFA has no effect on the uptake and degradation of biotinylated Cx43 from the plasma membrane (our unpublished data). These findings are in keeping with previous studies demonstrating that BFA prevents the export of newly synthesized proteins from the ER, Golgi, and *trans*-Golgi network (TGN) to the plasma membrane (Miller *et al.*, 1992; Crespo *et al.*, 2004), but it does not block endocytosis from the cell surface or subsequent degradation (Klausner *et al.*, 1992). Immunostaining of Cx43 in control CHO cells (Figure 3A, a) and in cells treated with BFA alone (b) revealed only very low levels of gap junctions, as expected for an assembly-inefficient cell type. If, however, the cells were subjected to a 30-min heat shock at 42°C before being returned to 37°C for 3.5 h, all in the continuous presence of BFA, a large number of gap junctional plaques were observed at cell-cell interfaces (Figure 3B, f). Cells incubated for a similar period with BFA in combination with either 80 μM sodium arsenite (Figure 3B, g) or 10 μM epoxomicin (h) also showed a substantial increase in gap junction number. Because BFA blocks the delivery of newly synthesized connexins to the plasma membrane, these gap junctions were assembled from connexin molecules already on the cell surface, or possibly in the TGN/endosomal sys-

Figure 3. Cytosolic stressors up-regulate the assembly of cell surface Cx43 into long-lived, functional gap junctions in CHO cells. (A) a–e, CHO cells were incubated for 4 h at 37°C in serum-containing medium in the absence (a, c, and e) or presence (b and d) of BFA. Cells in e were then incubated for an additional 7 h in the presence of BFA. All cells were fixed and immunostained with anti-Cx43 antibodies. Cells in c–e had been serum starved for 12 h before the experiment. In a'–e', cultures treated identically to those in a–e were assessed for their ability to carry out gap junction-mediated intercellular transfer of Lucifer yellow. (B) f–k, CHO cells were incubated at 37°C in serum-containing medium in the presence of BFA for either 4 h (f–i) or 7 h (j and k) with the following additions: arsenite (g), epoxomicin (h and k), or CLQ (i). Cells in f and j were heated at 42°C for 30 min and then returned to 37°C for the rest of the incubation in the continuous presence of BFA. All cells were then either fixed and immunostained with anti-Cx43 antibodies (f–k) or subjected to scrape-loading/dye transfer analysis of gap junction function (f'–k').

tem, at the time the stress was applied. In contrast, cells exposed to the lysosomal inhibitor CLQ in either the presence (Figure 3B, i) or absence (Musil *et al.*, 2000) of BFA showed no increase in junctional staining, but instead seemed in many cases to accumulate Cx43 intracellularly. To assess gap junction function, confluent monolayers of CHO cells were scraped with a 26-gauge needle in the presence of the membrane-impermeant gap junction tracer Lucifer yellow. The extent of gap junction-mediated diffusion of Lucifer yellow from the interior of the scraped cells to the cytosol of the neighboring unwounded cells remained low after exposure to CLQ (compare Figure 3A, b' with B, i') or leupeptin (our unpublished data). Intercellular transfer of Lucifer yellow was, however, greatly enhanced in cells subjected to cytosolic stress in the presence of BFA, indicating that the gap junctional channels formed in response to these conditions were active (f'–h'). The level of gap junction formation and function induced by cytosolic stress was comparable with that attained when cells were subjected to serum starvation/repletion (Musil *et al.*, 2000) (Figure 3A, c and c'). Unlike cytosolic stress, however, serum repletion did not enhance gap junctions in the presence of BFA (d and d'). Agents that induce ER instead of cytosolic stress (DTT and tunicamycin) were previously shown to have no ability to enhance gap junction formation in CHO cells (VanSlyke and Musil, 2002).

Exposing gap junction assembly-efficient cells to BFA for more than 6 h greatly reduces the number of morphologically and functionally detectable gap junctional plaques (Musil and Goodenough, 1993; Laing *et al.*, 1997; Qin *et al.*, 2003), as expected given the rapid rate of retrieval and degradation of connexins from the surface of unstressed cells. This was also the case for CHO cells in which gap junctions had been induced by serum starvation/readdition (Musil *et al.*, 2000) (Figure 3A, compare e with c). In contrast, extending the time for which heat-shocked CHO cells were exposed to BFA from 4 to 7 h had only a minimal effect on the level of immunocytochemically detectable gap junctions (Figure 3B, compare j with f). Moreover, gap junction-medi-

ated intercellular transfer of Lucifer yellow was also largely preserved (Figure 3B, compare j' with f'). Similar results were obtained with epoxomicin (Figure 3B, compare k with h; k' with h'). We conclude that gap junctions formed in response to cytosolic stress are exceptionally long lived, in keeping with the slower turnover of cell surface-biotinylated Cx43 observed under these conditions. The combination of arsenite and BFA could not be meaningfully tested because of its apparent toxicity at later time points.

Cytosolic Stress Stabilizes Cx43 on the Surface of Assembly-efficient Cells

Unlike either S180 or CHO cells, NRK fibroblasts constitutively form large gap junctional plaques. As expected (Musil and Goodenough, 1993; Laird *et al.*, 1995), gap junctional plaque staining of endogenous Cx43 was extensive in assembly-efficient NRK cells under basal conditions, but it was greatly diminished after a 9-h treatment with BFA (Figure 4A, compare a with b). If, however, the cells were exposed to 42°C for 30 min in the presence of BFA before being returned to 37°C (+ BFA) for 8.5 h, gap junctional staining was largely preserved (Figure 4A, c). Heat shock also efficiently reduced the rate of turnover of cell surface-biotinylated Cx43 (86.6 ± 9.6% remaining after a 6-h chase in heat-shocked cells, relative to 31.3 ± 8.8% in mock-shocked cells; n = 3) (Figure 4B). In contrast, treatment of NRK cells with BFA plus CLQ caused Cx43 to accumulate in intracellular vesicles likely to be part of the endosome/lysosome system (Figure 4A, d), consistent with results reported in other cell types (Laing *et al.*, 1997; Qin *et al.*, 2003). Exposure of NRK cells to epoxomicin plus BFA yielded results similar to those obtained after heat shock (Figure 4A, e). The level of gap junction function correlated with the number of immunodetectable Cx43 plaques under all conditions tested (Figure 4A, a'–e'). Comparable results were obtained in LA25 cells, a line of kidney-derived cells related to, but distinct from, NRK cells (our unpublished data).

Figure 4. Effect of cytosolic stress on cell surface Cx43 in gap junction assembly-efficient and -inefficient cells. (A) Assembly-efficient NRK cells were incubated for 9 h at 37°C in either the absence (a) or presence (b–e) of BFA, with no additions (b and c), CLQ (d), or epoxomicin (e). Cells in c were heated at 42°C for 30 min and then returned to 37°C for an additional 8.5-h incubation in the continuous presence of BFA. All cells were then either fixed and immunostained with anti-Cx43 antibodies (a–e), or subjected to scrape-loading/dye transfer analysis (a'–e'). (B) NRK cells were cell surface biotinylated and subjected to either a mock ($\Delta 37^{\circ}\text{C}$) or mild ($\Delta 42^{\circ}\text{C}$) heat stress before a 0- to 6-h chase at 37°C as described in Figure 2 for S180 and CHO cells. (C) Turnover of cell surface-biotinylated Cx43 was assessed in assembly-inefficient L929 cells under control conditions or after being subjected to either mild hyperthermia or oxidative stress as described in Figure 2. Representative blots probed with anti-Cx43 AP7298 (B) or CT-1 (C) are shown, with the lanes containing the 0-h chase samples duplicated.

Cytosolic Stress Inhibits the Degradation of Cell Surface Cx43 in Cells Incapable of Up-Regulating Gap Junction Assembly

L929 fibroblasts endogenously express wild-type Cx43 that undergoes normal multisubunit oligomerization into connexons (hemichannels) before transport to the cell surface (Musil and Goodenough, 1991; Musil and Goodenough, 1993). Unlike in CHO cells, however, cytosolic stressors do not stimulate gap junction formation in L929 cells as assessed by anti-Cx43 immunocytochemistry and Lucifer yellow dye-coupling studies (our unpublished data). To determine whether cytosolic stress nonetheless slows the degradation of Cx43 from the plasma membrane, L929 cells were cell surface biotinylated and then subjected to either hyperthermic or oxidative stress as described for S180, CHO, and NRK cells. Both treatments reduced the turnover of biotinylated Cx43 to an extent comparable with that obtained in the aforementioned cell types ($83.5 \pm 12.5\%$ remaining at 6 h in heat-shocked L929 cells compared with $13.9 \pm 5.3\%$ in mock-shocked controls, and $51.8 \pm 1.2\%$ after arsenite exposure relative to $23.5 \pm 9.2\%$ in untreated controls; $n = 3$) (Figure 4C).

Effect of Cytosolic Stress on Cx43 Endocytosis and Recycling

In principle, cytosolic stress could reduce the degradation of cell surface-biotinylated Cx43 by preventing its internalization. To examine this possibility, we treated cell surface-biotinylated S180 cells with MesNa, a membrane-imper-

meant reducing agent capable of quantitatively breaking the disulfide bond that links the biotin moiety to the extracellular domain of plasma membrane proteins (Schmidt *et al.*, 1997). S180 cells were cell surface biotinylated and then incubated at either 37 or 42°C for 30 min before a 30-min chase at 37°C . As a negative control, cell surface-biotinylated S180 cells were maintained at 4°C to prevent internalization. As expected, a subsequent treatment with MesNa at 4°C removed $>90\%$ of the biotin from Cx43 in the cells continuously kept on ice (Figure 5A, lanes 1 and 2). In contrast, $\sim 30\%$ of the biotinylated Cx43 remaining after a 1-h chase at 37°C was protected from a subsequent 4°C MesNa treatment (Figure 5A, lanes 3 and 4; $29.7 \pm 11.5\%$; $n = 3$). A similar fraction of biotinylated Cx43 became MesNa-resistant in the cells exposed to hyperthermia (lanes 5 and 6; $26.8 \pm 5.5\%$; $n = 3$). Subjecting cells to the 30-min heat shock 1 h before cell surface biotinylation also failed to provide evidence for a stress-induced inhibition of endocytosis of biotinylated Cx43, despite the fact that degradation of the biotinylated connexin was still inhibited (our unpublished data). To investigate whether inhibition of lysosomal degradation affected Cx43 internalization, cell surface-biotinylated S180 cells were incubated at 37°C in the presence of CLQ for 1 h. Similar to control or heat-shocked cells, $\sim 30\%$ of the biotinylated Cx43 was resistant to a subsequent 4°C MesNa strip (Figure 5A, lanes 7 and 8; $28.9 \pm 7.7\%$; $n = 3$).

Although both heat shock and CLQ inhibit the degradation of cell surface-biotinylated Cx43 without blocking its endocytosis, only the former treatment induces Cx43 to ac-

Figure 5. Internalization and recycling of cell surface-biotinylated Cx43. (A) Internalization of cell surface-biotinylated Cx43. Duplicate plates of S180 cells were biotinylated at 4°C and either chased at 4°C for 3 h (lanes 1 and 2), subjected to mock (lanes 3 and 4) or mild (lanes 5 and 6) heat shock for 30 min followed by a 30-min incubation at 37°C, or incubated with culture medium supplemented with 200 μ M CLQ for 60 min at 37°C (lanes 7 and 8). The cells were then incubated at 4°C in either the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of MesNa to remove biotin from cell surface molecules. All samples were then processed for detection of biotinylated Cx43. (B) MesNa-resistant (i.e., internalized)-biotinylated Cx43 returns to the cell surface to become MesNa sensitive. S180 cells were biotinylated and either subjected to a 30-min mock (lanes 1 and 2) or heat (lanes 3 and 4) shock followed by a 30-min incubation at 37°C or incubated in culture medium at 37°C for 1 h (lanes 5–8). All cultures were subsequently treated with MesNa at 4°C, after which they were either maintained at 4°C (lanes 7 and 8) or incubated in culture medium at 37°C in the absence (lanes 1–4) or presence (lanes 5 and 6) of CLQ for 60 min. The cells were then incubated at 4°C and subjected to either a second MesNa treatment (even lanes) or mock treated without MesNa (odd lanes). The percentage of biotinylated Cx43 that became MesNa sensitive was calculated, and the results from three experiments were plotted. (C) Heat shock stabilizes Cx43 on the cell surface. S180 cultures were subjected to a 30-min mock or heat shock followed by incubation in culture medium at 37°C, all in the continuous presence of BFA. After a total of 2-, 4-, or 6-h incubation with BFA, the cultures were subjected to cell surface biotinylation at 4°C and then lysed. Control cultures (0 h) were untreated before biotinylation. The amount of biotinylated Cx43 recovered from BFA-treated cultures was expressed as a percentage of the biotinylated Cx43 obtained from control cultures at time 0.

accumulate in gap junctional plaques in assembly-competent cells. One explanation for this finding would be that internalized cell surface-biotinylated Cx43 is recycled back to the plasma membrane in heat-shocked cells, but it remains in intracellular compartments in the presence of CLQ. This possibility was confirmed using a MesNa-based recycling assay (Figure 5B). S180 cells were biotinylated at 4°C as usual and then either subjected to a 30-min heat stress at 42°C followed by a 30-min incubation at 37°C, or maintained at 37°C. The cells were then treated with MesNa on ice to strip biotin from Cx43 molecules remaining on the cell surface. After MesNa washout, the still viable cells were warmed to 37°C for an additional hour to allow biotinylated Cx43 molecules to be returned to the cell surface. A second MesNa treatment at 4°C revealed that ~50–60% of the formerly MesNa-resistant biotinylated Cx43 had become MesNa sensitive (i.e., unable to be precipitated by streptavidin agarose beads) in both the heat-shocked and mock-shocked cells, indicative of its recycling to the plasma membrane (Figure 5B, lanes 1–4). Control cells kept at 4°C after the first MesNa strip to abolish all vesicle-mediated recycling retained virtually all of their biotinylated Cx43 in a MesNa-resistant (i.e., intracellular) state ($6.0 \pm 5.8\%$ MesNa sensitive; Figure 5B, lanes 7 and 8). To examine the effect of CLQ on Cx43 recycling, biotinylated cells were first incubated for 1 h at 37°C without CLQ before MesNa stripping

at 4°C. Only ~20% of the remaining biotinylated Cx43 became MesNa sensitive during a subsequent 1-h, 37°C incubation in the presence of CLQ (Figure 5B, lanes 5 and 6). Comparable results were obtained when the experiment was repeated with concanamycin A, a lysosomal inhibitor that (like CLQ) neutralizes the luminal pH of the endolysosomal system (15.3% MesNa sensitive; our unpublished data). Thus, Cx43, in common with certain other types of cell surface membrane proteins (Mellman *et al.*, 1986), recycles back to the cell surface in a pH-sensitive process.

By protecting Cx43 from degradation but still allowing its recycling to the cell surface, cytosolic stress could cause Cx43 to accumulate on the plasma membrane. The percentage of biotinylated Cx43 assessable to MesNa (and thus on the cell surface) in heat-shocked cells remained at 50–60% when measured after 3 or 6 h of chase (our unpublished data). Taking into account that heat shock more than triples the amount of biotinylated Cx43 recovered at 6 h relative to cells maintained at 37°C (Figure 2, D and F), it would be expected that at least three times as much biotinylated Cx43 accumulates on the plasma membrane in heated cells than in untreated controls during this period. This was confirmed by the experiment shown in Figure 5C. Unlabeled S180 cells were incubated for 30 min at 42 or 37°C and then returned to 37°C for up to 6 h. All incubations were conducted in the presence of BFA to prevent Cx43 pools that had not yet

traversed the secretory pathway from reaching the cell surface. The amount of Cx43 remaining on the plasma membrane was then assessed by cell surface biotinylation at 4°C. In keeping with the turnover kinetics established in Figure 1, Cx43 was rapidly cleared from the surface of cells continuously maintained at 37°C in the presence of BFA. Significantly more Cx43 was present on the surface of heat-shocked cells at each time point examined, reaching a maximum of 4.4-fold at 6 h. We conclude that cytosolic stress increases the number of Cx43 molecules that recycle back to the cell surface in an intact state. This expanded pool of stable, plasma membrane-localized Cx43 participates in the enhanced formation of gap junctional plaques observed after exposure of assembly-competent cells to cytosolic stress (see *Discussion*).

Potential Role of Unfolded Cytosolic Protein in Stress-induced Stabilization of Cell Surface Cx43

Although hyperthermia, arsenite, and proteasome inhibitors each have pleiotropic effects on cellular processes, one of the notable activities they share is that they increase the level of unfolded protein in the cytosol (Beckmann *et al.*, 1992; Lepock *et al.*, 1993). In contrast, agents (DTT and tunicamycin) that cause unfolded protein to accumulate exclusively within the secretory pathway do not stabilize Cx43 from degradation (VanSlyke and Musil, 2002). If the ability of cytosolic stressors to elevate the amount of unfolded protein in the cytosol is causally related to their effect on cell surface Cx43 turnover, then it would be expected that 1) subjecting cells to hyperthermia under conditions in which unfolded proteins do not accumulate in the cytosol would have no effect on the turnover of cell surface Cx43, and 2) increasing the level of unfolded protein in the cytosol of cells not exposed to hyperthermia should mimic the effect of stress by stabilizing Cx43 on the plasma membrane.

To test the first prediction, we took advantage of the phenomenon of acquired thermotolerance (Figure 6A). Exposing cells to a brief hyperthermic treatment is well-known to block a more severe heat shock applied several hours later from elevating the level of unfolded protein in the cytosol, apparently by increasing the expression of heat shock-inducible protein chaperones (Mizzen and Welch, 1988; Lepock *et al.*, 1990). S180 cells were subjected to either a 15-min thermal preconditioning treatment at 42°C or mock preconditioned at 37°C. The cells were then incubated for 16 h at 37°C, after which they were cell surface biotinylated and then immediately exposed to a 30-min, 42°C heat shock. As expected, the percentage of biotinylated Cx43 remaining after a 6-h chase in the cells subjected to the mock preconditioning regime before the 30-min, 42°C treatment (Figure 6A, lane 4) was much higher than in control cells maintained at 37°C throughout the course of the experiment (lane 2). In contrast, the same 30-min heat shock did not stabilize biotinylated Cx43 in cells previously made thermotolerant by a 15-min, 42°C pretreatment (lane 8); the amount of biotinylated Cx43 recovered after a 6-h chase from the doubly heated cells was 1.18 ± 0.72 -fold of that recovered from control cells continuously maintained at 37°C ($n = 3$). We have previously shown that such thermal preconditioning also abolishes the ability of a subsequent 30-min, 42°C treatment to up-regulate gap junction formation and function in CHO cells (VanSlyke and Musil, 2002).

We next transiently transfected CHO cells with a cDNA construct encoding GFP250, a folding-incompetent, cytosolic chimeric protein previously shown to accumulate in non-toxic, perinuclear aggregates referred to as aggresomes (Garcia-Mata *et al.*, 1999). Anti-Cx43 immunostaining revealed

Figure 6. Stabilization of cell surface Cx43 correlates with accumulation of unfolded proteins in the cytosol. (A) S180 cells were thermally preconditioned by a 15-min incubation at 42°C (lanes 5–8) or mock preconditioned at 37°C (lanes 1–4) before being returned to 37°C for 16 h. The cells were then cell surface biotinylated at 4°C and either lysed immediately (lanes 1, 3, 5, and 7) or subjected to a 30-min incubation at either 37 or 42°C and lysed after a 5.5-h chase at 37°C (lanes 2, 4, 6, and 8). Anti-Cx43 antibody AP7298 was used for immunodetection. Percentage of biotinylated Cx43 remaining after a 6-h chase under the conditions indicated: lane 2, 22.4%; lane 4, 87.0%; lane 6, 14.8%; lane 8, 20.0%. (B) CHO cells were transiently transfected with plasmids encoding either GFP250 or β -galactosidase as indicated. One day later, cells were incubated for 8 h in either the absence (a–d) or presence (e) of BFA. All cells were then fixed and immunostained for Cx43. a'–e' are the same fields as in a–e, respectively, but visualized with either fluorescein optics (a', b', and e') or after immunostaining for β -galactosidase (c' and d'). e + e', enlarged view of GFP250-expressing cells in e', overlaid with the corresponding region of e to show cell-cell interface staining of Cx43.

that a subset of GFP250-expressing CHO cells had large gap junction plaques at their cell–cell interfaces that were absent from their untransfected neighbors (Figure 6B, a, a', b, and b'). Plaques were most commonly detected between pairs of cells in which each partner contained a prominent GFP250 aggresome. Probably because this constituted $<5\%$ of the total cell population, the turnover rate of cell surface-biotinylated Cx43 in GFP250-transfected cultures was not reproducibly slower than that in mock-transfected controls (our unpublished data). As an alternative means to demonstrate that GFP250 expression decreases the degradation of Cx43 from the cell surface, GFP250 transfectants were treated with BFA for 8 h to allow unstabilized connexins to be cleared from the cell surface and to prevent their replacement with intracellular pools of Cx43. BFA had little effect on the

Table 1. Summary of the effect of cytosolic stress on Cx43 degradation from the cell surface and gap junction assembly in the cell types examined

	S180	CHO	L929	NRK
Biotinylated Cx43 degradation				
Basal (% remaining at 6 h)	17.5 ± 8.2	20.8 ± 5.0	19.4 ± 7.2	31.0 ± 6.8
Decreased by cytosolic stress?	Yes	Yes	Yes	Yes
Level of gap junctional plaques				
Basal conditions	+ / -	+ / -	-	+++
After cytosolic stress	+	+++	-	+++

Numbers are the percentage of cell surface-biotinylated Cx43 remaining ($\pm$ SD) in the indicated cell type after a 6-h chase under control conditions.

number or size of gap junctional plaques between GFP250-expressing cells (Figure 6B, e and e'), suggesting that the degradation of plasma membrane Cx43 had been inhibited in a manner that mimics exposure of CHO and NRK cells to cytosolic stress. Cells transfected with CMV-based plasmids that drive high level synthesis of soluble, folding-competent cytosolic proteins such as β -galactosidase (Figure 6B, c, c', d, and d') and EGFP (our unpublished data) showed no significant increase in gap junctional plaques.

DISCUSSION

Our results establish inhibition of connexin turnover from the cell surface as a previously unrecognized cellular response to mild hyperthermic and oxidative stress. These findings also provide the first demonstration that the delivery of connexins from the plasma membrane to the lysosome is a down-regulatable process that can serve as a control point to promote gap junction formation and function (summarized in Table 1). Recycling of connexins back to the cell surface is a new mechanism to control the amount of gap junction protein on the plasma membrane.

Role of the Lysosome and Proteasome in the Turnover of Cx43 from the Cell Surface

The lysosome (Qin *et al.*, 2003) and/or the proteasome (Laing and Beyer, 1995; Laing *et al.*, 1997; Segretain and Falk, 2004) have been proposed to carry out the degradation of connexins internalized from the plasma membrane. Using cell surface biotinylation to directly label and monitor the fate of plasma membrane pools of Cx43, we have found that the lysosomal inhibitor CLQ and the proteasomal inhibitor epoxomicin each markedly and reproducibly reduced the rate of degradation of biotinylated Cx43 in the cell types tested. Chloroquine has no ability to decrease the proteolytic activity of the proteasome, and epoxomicin is ineffective against lysosomal hydrolases (Gelman *et al.*, 2002; Marques *et al.*, 2004). In light of this specificity, one possible explanation for our results would be that cell surface Cx43 is degraded by two independent yet parallel processes, one inhibited by CLQ but not epoxomicin, and the other sensitive to epoxomicin only. The latter pathway is, however, unlikely to exist given that ~87% (and on occasion >95%) of the total pool of cell surface-biotinylated Cx43 remained after a 6-h chase in the presence of CLQ in S180 cells, with comparable results obtained in CHO cells (Figure 2, D and F). Several observations support the alternative concept that cell surface Cx43 is degraded within the (CLQ-sensitive) lysosome and that epoxomicin inhibits this process by interfering with the targeting and/or transport of Cx43 from the cell surface to

this organelle. First, incubation of CHO and NRK cells with epoxomicin results in the accumulation of plasma membrane pools of Cx43 on the cell surface in gap junctional plaques, whereas after CLQ exposure Cx43 redistributes to the cell interior in vesicles likely to be part of the endosome/lysosome system. Second, cell surface-biotinylated Cx43 spared from turnover by CLQ is in a full-length state (Figure 1B), ruling out a previously proposed model in which proteasome-mediated clipping of Cx43 serves as a prerequisite for further destruction within the lysosome (Laing *et al.*, 1997). Third, although ultrastructural studies have found evidence of degradation of gap junctions within lysosomes (Qin *et al.*, 2003), to our knowledge colocalization of gap junctions with the proteasome has not been reported. Last, sensitivity to both lysosomal and proteasomal inhibitors after transport to the cell surface is not unique to Cx43 and has been described for several other nonconnexin plasma membrane proteins now thought to be direct substrates of the lysosome but not of the proteasome (Longva *et al.*, 2002).

How Does Oxidative or Hyperthermic Stress Reduce the Turnover of Cell Surface-biotinylated Cx43?

There is no evidence that sodium arsenite or heat shock inhibits the function of lysosomal proteases under the experimental conditions used. Indeed, if these stressors acted as a bona fide lysosomal inhibitor such as CLQ or leupeptin, Cx43 would be expected to accumulate in their presence intracellularly instead of in gap junctional plaques as we have observed in CHO and NRK cells (Figures 3 and 4). It is also highly unlikely that arsenite and heat shock are exerting their effect on cell surface Cx43 by blocking the proteolytic activity of the proteasome because 1) neither treatment prevents the degradation of peptide or cytosolic protein substrates of the proteasome, including dislocated Cx43 (VanSlyke and Musil, 2002); and 2) in both S180 and CHO cells, hyperthermia and arsenite are better inhibitors of the turnover of cell surface-biotinylated Cx43 than epoxomicin, one of the most potent and specific blockers of the proteasome known (Figure 2, D and F). The finding that hyperthermia, oxidative stress, and CLQ each individually protect >50% of cell surface-biotinylated Cx43 from degradation indicates that these three treatments cannot be exerting their effect on connexin turnover via separate, nonoverlapping pathways. Our results are most consistent with the concept that oxidative and hyperthermic stress inhibit the turnover of cell surface Cx43 by interfering with the same process as epoxomicin, namely, the targeting and/or transport of Cx43 from the plasma membrane to the lysosome. One possibility is that the postendocytic delivery of connexins to the lysosome is facilitated by a cellular component that has a high-

affinity for un- or misfolded cytosolic proteins. According to this view, the levels of this component are sufficient to promote the efficient trafficking of connexins to the lysosome under basal conditions. After exposure of cells to hyperthermia, oxidative stress, or proteasome inhibitors, however, un- or misfolded proteins accumulate in the cytosol (Beckmann *et al.*, 1992; Lepock *et al.*, 1993), saturating the binding capacity of this component and thereby making it unavailable to mediate the transport of connexins to the lysosome. Consequently, connexin turnover is decreased. Candidates for such a cellular component include ubiquitin, HSC/HSP70, and p97, each of which associates tightly with unfolded cytosolic proteins and has been reported to be involved in protein uptake from the cell surface. This hypothesis would be in keeping with our finding that exogenous overexpression of a misfolded cytosolic protein in otherwise unperturbed cells mimics the effect of stress on cell surface Cx43 stability (Figure 6B). Future experiments will test this model as well as the possibility that other cellular responses to stress may also play a role in protecting Cx43 from degradation. Treatments that induce hyperthermic or oxidative stress have been reported by others to rapidly increase the total cellular level of Cx43 protein in tissue culture cells, although whether this was a result of increased Cx43 translation or decreased degradation at the ER and/or plasma membrane was not determined (Azzam *et al.*, 2003).

Relationship between Cx43 Cell Surface Stability, Gap Junction Assembly, and Gap Junction Degradation

In addition to reducing the rate of degradation of cell surface-biotinylated Cx43, cytosolic stressors enhanced the de novo assembly of gap junctional plaques in CHO cells even under conditions that blocked the transport of nascent connexin molecules to the plasma membrane (Figure 3). Because gap junction assembly is a cooperative process (Valiunas *et al.*, 1997), it is likely that cytosolic stress facilitates gap junction formation in such cells at least in part by slowing the turnover, and thereby raising the number, of Cx43 molecules on the cell surface. This seems to be accomplished not by blocking endocytosis but by inhibiting the delivery of internalized Cx43 to the lysosome. Thus, more Cx43 survives in an intact state to recycle back to the plasma membrane, without a requirement for stress to increase the rate of recycling. In assembly-competent C6-14 glioma cells, stable expression of a Cx43-encoding plasmid resulted in a less than threefold increase in the amount of cell surface-biotinylatable Cx43 (our unpublished data). This modest rise in plasma membrane Cx43 was nonetheless sufficient to induce both gap junction formation and function (Charles *et al.*, 1992). Hyperthermia triples the levels of Cx43 detectable on the surface of S180 cells by 4 h (Figure 5C), an amount that could account for the increase in gap junctions observed in assembly-competent cells.

The endocytosis and recycling studies were conducted in S180 cells, a line that forms only very few and small gap junctions under any of the conditions tested. This property minimized the potential for artifacts associated with restricted access of the biotinylating reagent and/or MesNa to connexins sequestered within large gap junctional plaques. An important issue that cannot be directly addressed using our methodology is the fate of Cx43 molecules already incorporated into plaques at the time the stress was imposed. Two possibilities can be envisioned. In the first, plaques continue to be rapidly degraded at the basal rate, but they are replaced by de novo assembly of stress-stabilized, previously nonjunctional pools of cell surface Cx43. Alternatively, stress may reduce the turnover of mature gap junctional

plaques by an as yet unknown mechanism. Because cell surface-biotinylated Cx43 remains competent to be assembled into gap junctional plaques (Musil and Goodenough, 1991), it is very likely that at least a fraction of biotinylated Cx43 in stressed CHO or NRK cells becomes incorporated into gap junctions during the postbiotinylation chase period. Such connexins may become preferentially incorporated into the periphery of plaques, where they might have a different rate and/or mechanism of turnover than Cx43 molecules located within the center of gap junctions (Gaietta *et al.*, 2002). Addressing such issues is likely to require a technique that can assess the relative ages of plaque-assembled and -unassembled pools of Cx43 over time.

Potential Physiological Relevance of Cytosolic Stress-induced Stabilization of Connexins on the Plasma Membrane

Increasing or maintaining the number of gap junctional channels on the surface of stressed cells could conceivably serve as a rapid means to promote the gap junction-mediated acquisition of cytoprotective substances from surrounding, less affected cells, and/or enable the intercellular dissipation of toxic metabolites. This could be especially important under conditions in which the synthesis or transport of connexins to the plasma membrane might be reduced. Such a scenario would be in keeping with reports that gap junctional transfer of glutathione can restore the function of oxidatively stressed cultured cardiac myocytes (Nakamura *et al.*, 1994). In addition to antioxidants, gap junctions serve as intercellular conduits for many other substances that can either elevate or reduce cell survival depending on the physiological context. It is intriguing that a seemingly unrelated variety of treatments including facial nerve transection (Rohmann *et al.*, 1994), cold stress (Saitongdee *et al.*, 2000), and carbon tetrachloride exposure (James *et al.*, 1986) that cause cytosolic stress also up-regulate gap junction formation in whole animals and may therefore reduce connexin turnover from the plasma membrane in vivo. We have shown that sodium arsenite and hyperthermia increase the stability of cell surface-biotinylated Cx43 even in cells in which gap junction formation is blocked at the hemichannel stage (e.g., L929 cells). Under conditions permissive for hemichannel opening, the increased number of connexons on the surface of stressed cells could significantly affect functions attributed to hemichannel activity such as ATP and NAD release, antiapoptotic signal transduction, and ephaptic neuronal communication (Goodenough and Paul, 2003).

ACKNOWLEDGMENTS

We thank E. Sztul and R. Garcia-Mata for the GFP-250 plasmid. This work was supported by Grants R01 NS40740-01 and R01 EY014622 to L.S.M.

REFERENCES

- Azzam, E. I., de Toledo, S. M., and Little, J. B. (2003). Expression of CONNEXIN43 is highly sensitive to ionizing radiation and other environmental stresses. *Cancer Res.* 63, 7128–7135.
- Beardslee, M. A., Laing, J. G., Beyer, E. C., and Saffitz, J. E. (1998). Rapid turnover of connexin43 in the adult rat heart. *Circ. Res.* 83, 629–635.
- Beckmann, R. P., Lovett, M., and Welch, W. J. (1992). Examining the function and regulation of hsp 70 in cells subjected to metabolic stress. *J. Cell Biol.* 117, 1137–1150.
- Berthoud, V. M., Minogue, P. J., Laing, J. G., and Beyer, E. C. (2004). Pathways for degradation of connexins and gap junctions. *Cardiovasc. Res.* 62, 256–267.

- Charles, A. C., Naus, C. C., Zhu, D., Kidder, G. M., Dirksen, E. R., and Sanderson, M. J. (1992). Intercellular calcium signaling via gap junctions in glioma cells. *J. Cell Biol.* 118, 195–201.
- Chu, T., Tran, T., Yang, F., Beech, W., Cole, G. M., and Frautschy, S. A. (1998). Effect of chloroquine and leupeptin on intracellular accumulation of amyloid- β (A β) 1–42 peptide in a murine N9 microglial cell line. *FEBS Lett.* 436, 439–444.
- Crespo, P. M., Iglesias-Bartolome, R., and Daniotti, J. L. (2004). Ganglioside GD3 traffics from the trans-Golgi network to plasma membrane by a Rab11-independent and brefeldin A-insensitive exocytic pathway. *J. Biol. Chem.* 279, 47610–47618.
- Fallon, R. F., and Goodenough, D. A. (1981). Five-hour half-life of mouse liver gap-junction protein. *J. Cell Biol.* 90, 521–526.
- Gaietta, G., D. T., Adams, S. R., Bouwer, J., Tour, O., Laird, D. W., Sosinsky, G. E., Tsien, R. Y., and Ellisman, M. H. (2002). Multicolor and electron microscopic imaging of connexin trafficking. *Science* 296, 503–507.
- Garcia-Mata, R., Bebek, Z., Sorscher, E. J., and Sztul, E. S. (1999). Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J. Cell Biol.* 146, 1239–1254.
- Gelman, M. S., Kannegaard, E. S., and Kopito, R. R. (2002). A principal role for the proteasome in endoplasmic reticulum-associated degradation of misfolded intracellular cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 277, 11709–11714.
- Goodenough, D. A., and Paul, D. L. (2003). Beyond the gap: functions of unpaired connexon channels. *Nat. Rev. Mol. Cell Biol.* 4, 285–294.
- James, J. L., Friend, D. S., MacDonald, J. R., and Smuckler, E. A. (1986). Alterations in hepatocyte plasma membrane in carbon tetrachloride poisoning. Freeze-fracture analysis of gap junction and electron spin resonance analysis of lipid fluidity. *Lab. Invest.* 54, 268–274.
- Jordan, K., Chodock, R., Hand, A. R., and Laird, D. W. (2001). The origin of annular junctions: a mechanism of gap junction internalization. *J. Cell Sci.* 114, 763–773.
- Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992). Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116, 1071–1080.
- Laing, J. G., and Beyer, E. C. (1995). The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. *J. Biol. Chem.* 270, 26399–26403.
- Laing, J. G., Tadros, P. N., Westphale, E. M., and Beyer, E. C. (1997). Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. *Exp. Cell Res.* 236, 482–492.
- Laird, D. W., Castillo, M., and Kasprzak, L. (1995). Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. *J. Cell Biol.* 131, 1193–1203.
- Lepock, J. R., Frey, H. E., Heynen, M. P., Nishio, J., Waters, B., Ritchie, K. P., and Kruuv, J. (1990). Increased thermostability of thermotolerant CHL V79 cells as determined by differential scanning calorimetry. *J. Cell. Physiol.* 142, 628–634.
- Lepock, J. R., Frey, H. E., and Ritchie, K. P. (1993). Protein denaturation in intact hepatocytes and isolated cellular organelles during heat shock. *J. Cell Biol.* 122, 1267–1276.
- Longva, K. E., Blystad, F. D., Stang, E., Larsen, A. M., Johannessen, L. E., and Madhus, I. H. (2002). Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. *J. Cell Biol.* 156, 843–854.
- Marques, C., Pereira, P., Taylor, A., Liang, J. N., Reddy, V. N., Szweda, L. I., and Shang, F. (2004). Ubiquitin-dependent lysosomal degradation of the HNE-modified proteins in lens epithelial cells. *FASEB J.* 18, 1424–1426.
- Mellman, I., Fuchs, R., and Helenius, A. (1986). Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* 55, 663–700.
- Miller, S. G., Camell, L., and Moore, H. H. (1992). Post-Golgi membrane traffic: brefeldin A inhibits export from distal Golgi compartments to the cell surface but not recycling. *J. Cell Biol.* 118, 267–283.
- Mizzen, L. A., and Welch, W. J. (1988). Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *J. Cell Biol.* 106, 1105–1116.
- Musil, L. S., Cunningham, B. A., Edelman, G. M., and Goodenough, D. A. (1990). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* 111, 2077–2088.
- Musil, L. S., and Goodenough, D. A. (1991). Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J. Cell Biol.* 115, 1357–1374.
- Musil, L. S., and Goodenough, D. A. (1993). Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell* 74, 1065–1077.
- Musil, L. S., Le, A. C., VanSlyke, J. K., and Roberts, L. M. (2000). Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J. Biol. Chem.* 275, 25207–25215.
- Nagy, J. I., Li, W. E., Roy, C., Doble, B. W., Gilchrist, J. S., Kardami, E., and Hertzberg, E. L. (1997). Selective monoclonal antibody recognition and cellular localization of an unphosphorylated form of connexin43. *Exp. Cell Res.* 236, 127–136.
- Nakamura, T. Y., Yamamoto, I., Kanno, Y., Shiba, Y., and Goshima, K. (1994). Metabolic coupling of glutathione between mouse and quail cardiac myocytes and its protective role against oxidative stress. *Circ. Res.* 74, 806–816.
- Qin, H., Shao, Q., Igdoura, S. A., Alaoui-Jamali, M. A., and Laird, D. W. (2003). Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. *J. Biol. Chem.* 278, 30005–30014.
- Rohmann, A., Laskawi, R., Hofer, A., Dermietzel, R., and Wolff, J. R. (1994). Astrocytes as rapid sensors of peripheral axotomy in the facial nucleus of rats. *Neuroreport* 5, 409–412.
- Saitongdee, P., Milner, P., Becker, D. L., Knight, G. E., and Burnstock, G. (2000). Increased connexin43 gap junction protein in hamster cardiomyocytes during cold acclimatization and hibernation. *Cardiovasc. Res.* 47, 108–115.
- Schmidt, A., Hannah, M. J., and Huttner, W. B. (1997). Synaptic-like microvesicles of neuroendocrine cells originate from a novel compartment that is continuous with the plasma membrane and devoid of transferrin receptor. *J. Cell Biol.* 137, 445–458.
- Segretain, D., and Falk, M. M. (2004). Regulation of connexin biosynthesis, assembly, gap junction formation, and removal. *Biochim. Biophys. Acta* 1662, 3–21.
- Turner, D. L., and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* 8, 1434–1447.
- Valiunas, V., Bukauskas, F. F., and Weingart, R. (1997). Conductances and selective permeability of connexin43 gap junction channels examined in neonatal rat heart cells. *Circ. Res.* 80, 708–719.
- VanSlyke, J. K., Deschenes, S. M., and Musil, L. S. (2000). Intracellular transport, assembly, and degradation of wild-type and disease-linked mutant gap junction proteins. *Mol. Biol. Cell* 11, 1933–1946.
- VanSlyke, J. K., and Musil, L. S. (2000). Analysis of connexin intracellular transport and assembly. *Methods* 20, 156–164.
- VanSlyke, J. K., and Musil, L. S. (2002). Dislocation and degradation from the ER are regulated by cytosolic stress. *J. Cell Biol.* 157, 381–394.