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Dislocation and degradation from the ER are regulated by cytosolic stress

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A key step in ER-associated degradation (ERAD) is dislocation of the substrate protein from the ER into the cytosol to gain access to the proteasome. Very little is known about how this process is regulated, especially in the case of polytopic proteins. Using pulse-chase analysis combined with subcellular fractionation, we show that connexins, the four transmembrane structural components of gap junctions, can be chased in an intact form from the ER membrane into the cytosol of proteasome inhibitor–treated cells. Dislocation of endogenously expressed connexin from the ER was reduced 50–80% when the cytosolic heat shock response was induced by mild oxidative or thermal stress, but not by treatments that instead upregulate the ER unfolded protein response. Cytosolic but not ER stresses slowed the normally rapid degradation of connexins, and led to a striking increase in gap junction formation and function in otherwise assembly-inefficient cell types. These treatments also inhibited the dislocation and turnover of a connexin-unrelated ERAD substrate, unassembled major histocompatibility complex class I heavy chain. Our findings demonstrate that dislocation is negatively regulated by physiologically relevant, nonlethal stress. They also reveal a previously unrecognized relationship between cytosolic stress and intercellular communication.

Introduction

After insertion into the ER, nascent secretory proteins can either be exported to the Golgi or destroyed by ER-associated degradation (ERAD)* (McCracken and Brodsky, 1996). ERAD is carried out by the 26S proteasome, the multicatalytic protease complex that mediates the majority of protein degradation in the cytosol and nucleus (Plemper and Wolf, 1999). It has been demonstrated that ERAD substrates (with certain possible exceptions; Walter et al., 2001) gain access to the proteasome by being extracted from the ER via the Sec61 translocon in a process referred to as dislocation (Wiertz et al., 1996b; Pilon et al., 1997; Plemper et al., 1997; Zhou and Schekman, 1999). Major substrates for ERAD include proteins that are conformationally abnormal as a result of mutation, lack of assembly partners, or imperfect folding. To date, no mechanism has been described to regulate the dislocation of multiple unrelated proteins under physiologically relevant conditions.

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*Abbreviations used in this paper: ALLN, acetyl-leu-leu-norleucinal; BFA, brefeldin A; Cx32, connexin32; Cx43, connexin43; ERAD, ER-associated degradation; MHC1, major histocompatibility complex class I; NAC, *N*-acetyl cysteine.

Key words: endoplasmic reticulum; proteasome; heat-shock response; connexins; gap junctions

Connexins form a family of nonglycosylated, tetra-spanning proteins with both amino and carboxyl termini facing the cytosol. Connexins are the structural components in vertebrates of gap junctions, which are collections of intercellular channels that mediate the regulatable cell-to-cell transfer of low molecular mass substances including ions and second messengers (for review see Goodenough et al., 1996). Cell types differ greatly in the efficiency with which they assemble connexins into gap junctions under basal conditions, and have been operationally categorized as either assembly incompetent (no gap junctions), assembly inefficient (few gap junctions relative to the level of connexin expressed on the cell surface), or assembly efficient (Musil et al., 2000).

Pulse-chase analysis in tissue culture cells and in whole organs has revealed that both gap junction–assembled and –unassociated forms of connexins turn over remarkably quickly ($t_{1/2}$ = 1.5–5 h), much faster than most other types of plasma membrane proteins (Fallon and Goodenough, 1981; Musil et al., 1990; Beardslee et al., 1998). We have recently shown that ~40% or more of newly synthesized wild-type connexin43 (Cx43) and connexin32 (Cx32), and up to 100% of some mutant forms of Cx32, are rapidly degraded in the cell types examined even if their exit from the ER is blocked (VanSlyke et al., 2000). This ER-associated turnover is sensitive to chemical inhibitors of the proteasome but not of the lysosome, thereby characterizing connexins as substrates for ERAD. It is likely, but has not yet been

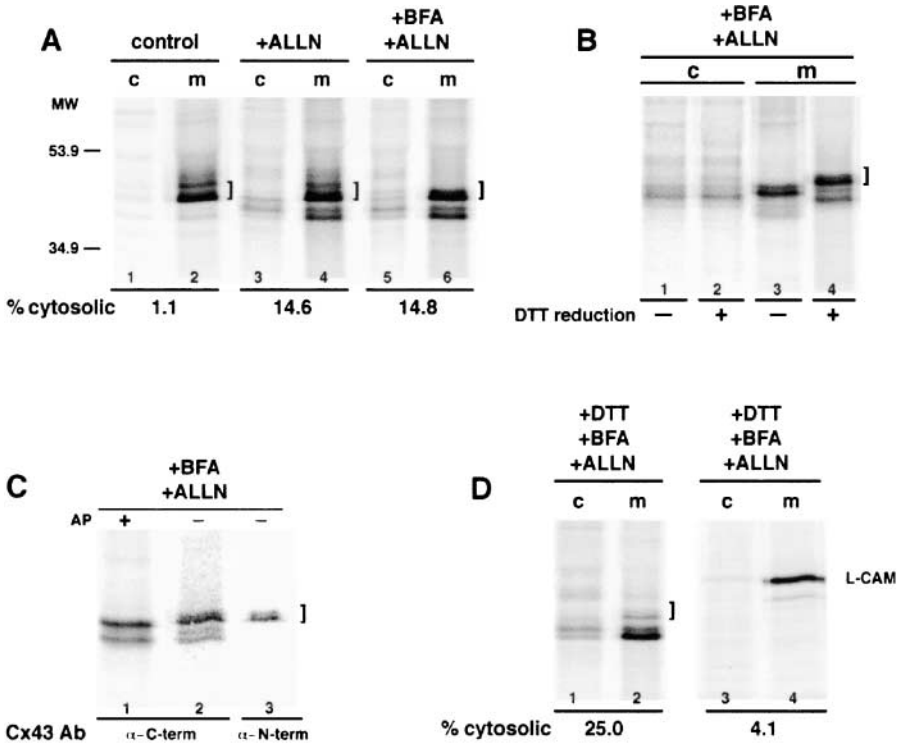

Figure 1. **Recovery of Cx43 in the cytosolic fraction of proteasome inhibitor-treated cells.** S180 or (for L-CAM only) S180L cells were metabolically labeled with [^{35}S]methionine for 4 h in the presence of the indicated additions. For A, B, and D, cells were then lysed and fractionated into cytosolic (c) and membrane (m) fractions before immunoprecipitation of Cx43 or (D, lanes 3 and 4) L-CAM. For B only, sample preparation was modified to maximize the difference in electrophoretic mobility between DTT-reduced (+) and unreduced (−) forms of Cx43 (as described in Materials and methods). (C) Nonfractionated cell lysates were immunoprecipitated with anti-Cx43 antibodies directed against the C terminus, either without (lanes 1 and 2) or with (lane 3) reimmunoprecipitation with antiserum specific for the Cx43 N terminus. The sample in lane 1 was treated with alkaline phosphatase before SDS-PAGE. Brackets indicate the position of reduced, full-length forms of membrane-associated Cx43; the upper band in A, lanes 2 and 4, is the plasma membrane–associated P_1 form of Cx43 (Musil et al., 1990).

definitively established, that degradation of connexins at the ER is a consequence of slow and/or inefficient folding.

In this work, we have studied connexin ERAD to investigate how dislocation from the ER is regulated. We present a novel model of how dislocation is influenced by the amount of misfolded proteins in the cytosol/nucleus, which compete with ER-localized ERAD substrates for association with 26S proteasomes.

Results

Intact connexins are recovered from the cytosolic fraction of proteasome inhibitor–treated cells

The subcellular distribution of Cx43 endogenously expressed in S180 cells was examined using a fractionation method previously shown to separate membrane-associated from cytosolic (dislocated) forms of the major histocompatibility complex class I (MHC1) heavy chain (Wiertz et al., 1996a) (Fig. 1). As expected for an integral membrane protein, ≥98% of the [^{35}S]methionine-labeled Cx43 in a postnuclear supernatant prepared from control S180 cells was pelleted by a 100,000-*g*, 1-h centrifugation (Fig. 1 A, lanes 1 and 2). However, if the proteasome inhibitor acetyl-leu-leu-norleucinal (ALLN) was included during the 4-h labeling period, ∼15% of the [^{35}S]met-labeled anti-Cx43 immunoprecipitable material was recovered in the 100,000-*g* supernatant (cytosolic) fraction as two to four ∼42–38-kD bands (Fig. 1 A, lane 3). All of the [^{35}S]met-Cx43 in the 100,000-*g* pellet, but none in the supernatant, contained intramolecular disulfide bonds, consistent with the known redox states of the lumen of the secretory pathway and the cytosol, respectively (Fig. 1 B). No additional Cx43 partitioned into the 100,000-*g* supernatant if cells were fractionated in the presence of 10 mM NaOH to strip peripheral proteins from membrane surfaces (unpublished data). We conclude that Cx43 that sedimented at 100,000 *g* was membrane integrated, whereas that recovered in the supernatant was cytosolic. Because disulfides form exclusively between the two ER-lumenal loops of the connexin molecule (Foote et al., 1998), their presence in membrane-associated [^{35}S]met-Cx43 is indicative of the acquisition of the correct, four transmembrane topology. Similar results were obtained if cells were labeled in the presence of the intracellular transport blocker brefeldin A (BFA), suggesting that cytosolic connexin was generated in a pre-Golgi compartment (Fig. 1 A, lanes 5 and 6).

Cx43 recovered from cells treated with ALLN and BFA migrated on SDS-PAGE as a combination of full-length (43 and 42 kD) and slightly smaller (∼39 and 38 kD) species. This pattern was simplified to a 42- and a 38-kD band when immunoprecipitates were treated with alkaline phosphatase (Musil et al., 1990), consistent with the onset of Cx43 phosphorylation in a premedial Golgi compartment (Laird et al., 1995) (Fig. 1 C, lane 1). Full-length Cx43 could be immunoprecipitated by antibodies directed against either the N or C terminus, whereas the lower molecular mass species were recognized only by the C-specific antibody (Fig. 1 C). Removal of the N terminus of topologically correct Cx43 to generate a 38-kD fragment has previously been observed after translation in vitro or after extreme overexpression in tissue culture cells (Falk et al., 1994; Zhang et al., 1996). This cleavage takes place within the ER lumen and has been attributed to signal peptidase acting on a site on Cx43 that becomes inaccessible when the molecule folds properly (Falk and Gilula, 1998). The presence of amino-clipped Cx43 in the 100,000-*g* supernatant suggests that the connexin had been translocated through the ER membrane prior to its release into the cytosol.

Incubating living cells with 2 mM DTT prevents the formation of disulfide bonds in nascent connexin molecules

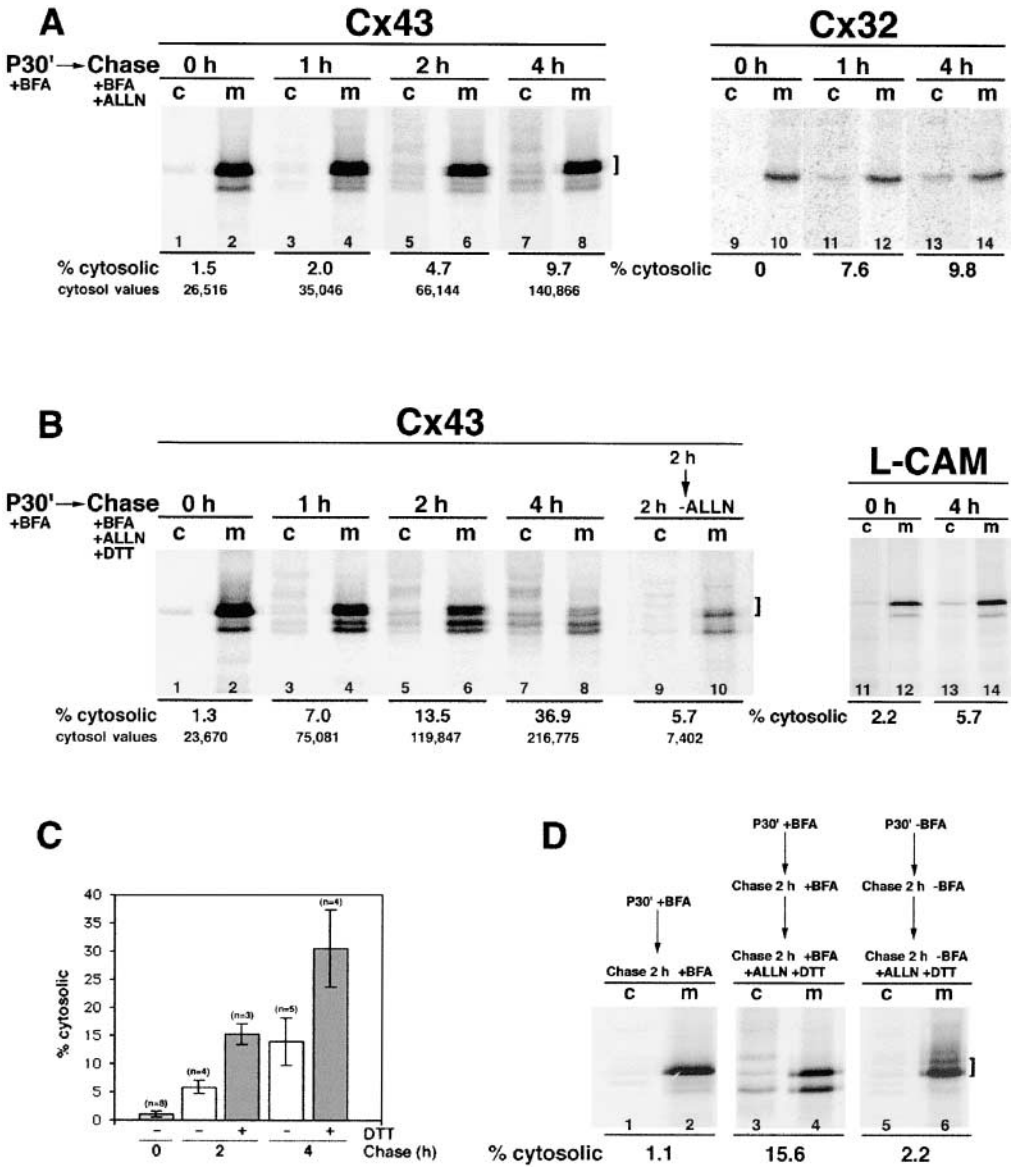

Figure 2. **Pulse-chase analysis of connexin dislocation.** Cells were labeled for 30 min with [^{35}S]methionine and chased as described below before fractionation into cytosolic (c) and membrane (m) fractions and immunoprecipitation of the indicated protein. Brackets indicate the position of full-length forms of Cx43. Raw cytosol values (PhosphorImager volume counts) are provided in (A and B) to demonstrate the time-dependent increase in the absolute amount of [^{35}S]met-Cx43 in the cytosol. (A) S180 (lanes 1–8) and LA25-Cx32 NRK (lanes 9–14) cells were labeled in the presence of BFA and then chased (+BFA and ALLN) for 0, 1, 2, or 4 h in the absence of DTT. (B) S180 (lanes 1–10) and S180L (lanes 11–14) cells were labeled in the presence of BFA and then chased (+BFA and ALLN) in the continuous presence of DTT, or for 2 h with BFA, ALLN, and DTT and then 2 h with BFA and DTT but without ALLN (lanes 9 and 10). (C) Graph summarizing multiple independent Cx43 pulse-chase dislocation experiments in S180s. (D) CHOs were labeled and then chased for 2 h, all in either the presence (lanes 1–4) or absence (lanes 5 and 6) of BFA. Cells were then either lysed immediately (lanes 1 and 2), or after an additional 2 h chase with ALLN, DTT, and BFA (lanes 3 and 4) or with ALLN and DTT in the absence of BFA (lanes 5 and 6) before immunoprecipitation of Cx43.

and enhances their proteasome-mediated degradation (Musil et al., 2000). Labeling S180 cells in the presence of DTT increased the fraction of [^{35}S]met-Cx43 recovered in the 100,000-*g* supernatant 1.5–2-fold in three out of three independent experiments and enhanced cleavage to the 39-/38-kD species (Fig. 1 D, lanes 1 and 2). Similar results were obtained in other cell lines that endogenously express Cx43, including CHOs, NRKs, and S180 transfectants stably expressing the cell adhesion molecule L-CAM (S180L cells) (unpublished data). In the latter cells, only very low levels (~4%) of [^{35}S]met-L-CAM were ever recovered in the 100,000-*g* supernatant (Fig. 1 D, lanes 3 and 4). We have previously demonstrated that L-CAM in these cells is degraded predominantly by post-ER pathways despite its relative rapid turnover ($t_{1/2}$ ~6 h) (VanSlyke et al., 2000).

Pulse-chase analysis reveals the origin and fate of cytosolic connexin

The precursor–product relationship between connexins integrated in the ER membrane and those recovered from the cytosol was demonstrated in a series of pulse-chase experiments conducted in the continuous presence of BFA to pre-

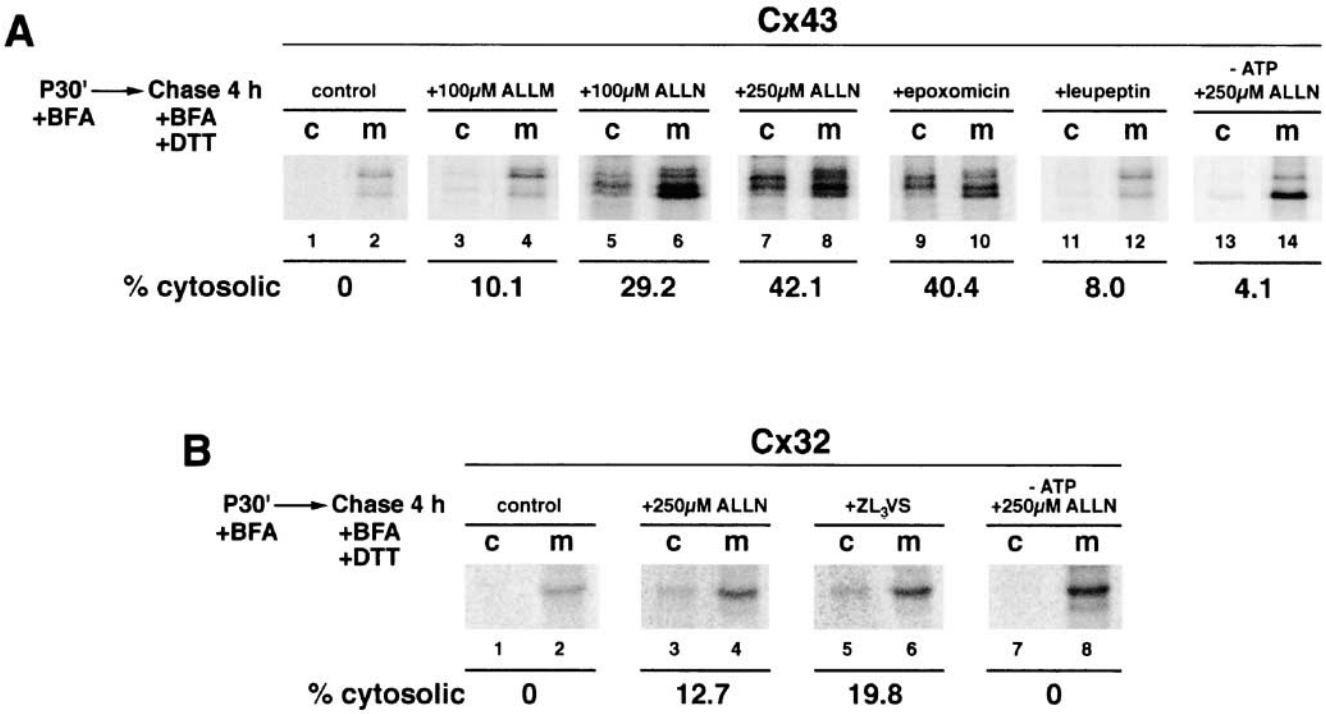

Figure 3. **Recovery of dislocated connexin in the cytosol requires inhibition of the proteasome and is dependent on ATP.** S180 (A) or LA25-Cx32 NRK (B) cells were labeled for 30 min and then chased for 4 h with DTT, all in the presence of BFA. Cytosolic (c) and membrane (m) fractions were prepared and Cx43 (A) or Cx32 (B) immunoprecipitated. Where indicated, the chase medium contained a proteasome inhibitor (100 μM or 250 μM ALLN; epoxomicin; ZL_3VS), an inhibitor primarily active against the lysosome (100 μM ALLM, or leupeptin), or ALLN plus an ATP-depleting mix (−ATP + 250 μM ALLN).

vent transport of the labeled connexin to post-ER organelles (Fig. 2, A–C). Immediately after the pulse, >98% of the labeled Cx43 in the postnuclear supernatant was recovered in the 100,000-*g* pellet (Fig. 2 A, lane 2). During a 1–4-h chase in the presence of ALLN, this disulfide-bonded, membrane-integrated [^{35}S]met-Cx43 was gradually released into the cytosolic fraction in a form lacking disulfides (Fig. 2 A, lanes 3, 5, and 7; unpublished data). Reduction of disulfides during dislocation has been demonstrated for the MHC1 heavy chain (Tortorella et al., 1998). Reasons for the relatively slow accumulation of [^{35}S]met-Cx43 in the cytosol are addressed in the Discussion.

The fraction of pulse-labeled Cx43 recovered in the cytosol more than doubled if DTT was added to the chase medium (Fig. 2 B, lanes 1–8), suggesting that dislocation was enhanced under conditions that prevented connexin conformational maturation. In contrast, only trace amounts of L-CAM expressed in S180L cells were released into the cytosolic fraction in either the absence (unpublished data) or presence (Fig. 2 B, lanes 11–14) of DTT.

The results of multiple independent pulse-chase experiments (Fig. 2 C) indicated that Cx43 could undergo dislocation several hours after synthesis. This was confirmed using a double-chase protocol (Fig. 2 D). Pulse-labeled cells were first chased for 2 h without DTT or ALLN. Presumably, some of the [^{35}S]met-Cx43 underwent dislocation during this period, but because of the absence of a proteasome inhibitor, it did not appreciably accumulate in the cytosolic fraction (Fig. 2 D, lanes 1 and 2). The cells were then chased for an additional 2 h, still in the continuous presence of BFA, with DTT and ALLN to maximize [^{35}S]met-Cx43 dislocation and recovery after dislocation, respectively. Approximately 15% of the labeled Cx43 that remained after the second chase was present in the cytosol (Fig. 2 D, lanes 3 and 4), similar to the percent of [^{35}S]met-Cx43 released into the cytosol from cells chased for 2 h with BFA, ALLN, and DTT immediately after the pulse (Fig. 2 C). However, if BFA was omitted from the pulse and chase media, much less (~2%) [^{35}S]met-Cx43 was released into the cytosol between 2 and 4 h of chase (Fig. 2 D; compare lanes 5 and 6 with lanes 3 and 4). The latter finding indicates that Cx43 loses the ability to be dislocated if permitted to traffic through the secretory pathway. Experiments conducted in the absence of DTT led to a similar conclusion (unpublished data). Either with or without DTT, we obtained no evidence for prolonged association of pulse-labeled Cx43 with the Sec61 translocon (unpublished data). This is in contrast to apoB100, which can remain associated with the translocon that mediated its insertion into the ER membrane for >1 h and then be dislocated via the same channel (Pariyarath et al., 2001).

To address the fate of dislocated Cx43, pulse-labeled cells were first chased for 2 h in the presence of BFA, DTT, and ALLN to accumulate [^{35}S]met-Cx43 in the cytosol. ALLN was then washed out and the incubation continued for 2 h more. Relative to cells chased for 4 h in the continuous presence of ALLN (Fig. 2 B, lanes 7 and 8), very little [^{35}S]met-Cx43 was recovered in the cytosolic fraction (Fig. 2 B, lanes 9 and 10). We conclude that dislocated Cx43 is rapidly destroyed upon restoration of proteasome function, indicating that it is a bona fide intermediate in the degradation pathway.

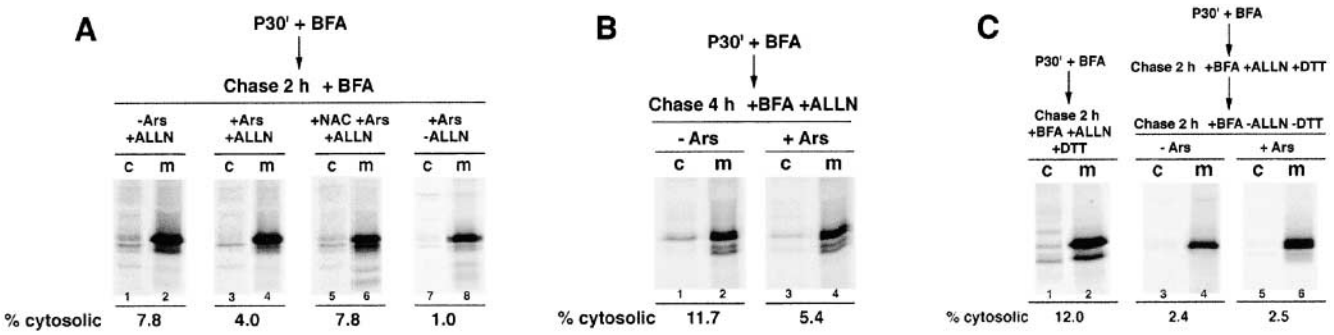

Figure 4. **The oxidant sodium arsenite reduces the amount of Cx43 released into the cytosol, but not its degradation after dislocation.** Cells were labeled for 30 min in the presence of BFA and chased as described below before fractionation and immunoprecipitation of Cx43. (A) CHOs were chased for 2 h in the presence of BFA and either ALLN alone (lanes 1 and 2), ALLN and arsenite (lanes 3 and 4), ALLN, arsenite, and the antioxidant NAC (lanes 5 and 6), or arsenite without ALLN or NAC (lanes 7 and 8). For lanes 5 and 6, cells were pretreated and pulsed in the presence of NAC. NAC alone had no significant effect on Cx43 dislocation (unpublished data). (B) S180s were chased for 4 h with BFA and ALLN in either the absence (lanes 1 and 2) or presence (lanes 3 and 4) of arsenite. (C) CHOs were chased for 2 h with BFA, ALLN, and DTT and then either lysed immediately (lanes 1 and 2) or chased for 2 h more in the absence of ALLN or DTT, either without (lanes 3 and 4) or with (lanes 5 and 6) arsenite.

Wild-type Cx32, like Cx43, is degraded in large part via ERAD (VanSlyke et al., 2000). Cx32 also underwent dislocation into the cytosol in an intact form when stably expressed in transfected NRK fibroblasts (LA25-Cx32 NRK cells) (Fig. 2 A, lanes 9–14). Because Cx32 does not shift in electrophoretic mobility upon phosphorylation and appears less susceptible to N-terminal clipping than Cx43 (Falk et al., 1994), it is recovered as a single, full-length species. Given that Cx32 and Cx43 are among the least closely related members of the connexin family, it is likely that en bloc dislocation is a general feature of vertebrate gap junction–forming proteins. Dislocated Cx43 (Fig. 3 A) and Cx32 (Fig. 3 B) accumulated in the cytosol if ALLN was replaced with mechanistically distinct inhibitors of the proteasome including ZL_3VS and epoxomicin, but not with the lysosome-selective blockers leupeptin and 100 μM ALLM. As reported for other ERAD substrates (McCracken and Brodsky, 1996; Wiertz et al., 1996b), depletion of ATP during the chase period greatly reduced the amount of [^{35}S]met-Cx43 (Fig. 3 A) and [^{35}S]met-Cx32 (Fig. 3 B) in the cytosol fraction even in the presence of ALLN.

An inducer of mild oxidative stress inhibits connexin dislocation

Incubation of cells with 1 mM diamide or NEM, compounds that shift the cellular redox potential towards a more oxidizing state, has been reported to inhibit the dislocation of two single-spanning ERAD substrates (Tortorella et al., 1998). Similar results were obtained with connexins, but were difficult to interpret because >95% of the cells were rapidly killed by either treatment (unpublished data). Therefore, we investigated whether an oxidizing agent with fewer nonspecific toxic sequelae, sodium arsenite (Bernstam and Nriagu, 2000), could also inhibit connexin dislocation. Pulse-labeled CHO cells were chased for 2 h in the presence of 80 μM sodium arsenite, BFA, and ALLN, the latter to inhibit the degradation of membrane-associated and any dislocated forms of Cx43. Sodium arsenite reduced the fraction of [^{35}S]met-Cx43 recovered in the cytosol by ~50% (Fig. 4 A, compare lanes 1 and 2 with lanes 3 and 4). As expected (Lee and Dewey, 1988), almost all of the treated cells remained viable and continued to proliferate (unpublished data). The effect of arsenite on dislocation was blocked by incubating cells with the antioxidant and glutathione precursor *N*-acetyl cysteine (NAC) (Fig. 4 A, lanes 5 and 6). Comparable results were obtained using S180 cells and/or if the chase period was extended to 4 h (Fig. 4 B).

[^{35}S]met-Cx43 that had accumulated in the cytosol during a 2-h chase with ALLN, BFA, and DTT turned over rapidly after ALLN washout, even in the presence of arsenite (Fig. 4 C, compare lanes 3 and 4 with lanes 5 and 6). The failure of arsenite to stabilize already dislocated connexin indicated that arsenite was not acting as an inhibitor of the proteolytic activity of the proteasome, in keeping with the reports of others (Westwood and Steinhardt, 1989). It is also consistent with the finding that no [^{35}S]met-Cx43 accumulated in the cytosolic fraction of cells chased in the continuous presence of arsenite but without ALLN (Fig. 4 A, lanes 7 and 8).

Nonoxidative cytosolic stress, but not ER stress, inhibits connexin dislocation

A major effect of oxidizing agents is aberrant oxidation and ensuing denaturation of proteins, particularly those in the normally reducing environment of the cytosol and nucleus (Beckmann et al., 1992; Senisterra et al., 1997). The increased level of abnormal protein in these locations overwhelms the binding capacity of the basal pool of cytosolic molecular chaperones and induces the heat shock response, a process in which expression of heat shock proteins is transcriptionally upregulated to assist in the renaturation/destruction of misfolded proteins (for review see Georgopoulos and Welch, 1993). As expected (Westwood and Steinhardt, 1989; Bernstam and Nriagu, 2000), the concentrations of arsenite that inhibited connexin dislocation triggered a robust, NAC-sensitive heat shock response in the cell types tested (CHO, S180, and NRK), as assessed by synthesis of the stress-specific form of HSP70 (HSP70i) (unpublished data). Therefore, we examined whether increasing the level of unfolded proteins in the cytosol by thermal instead of oxidative denaturation (Lepock et al., 1993) also reduced con-

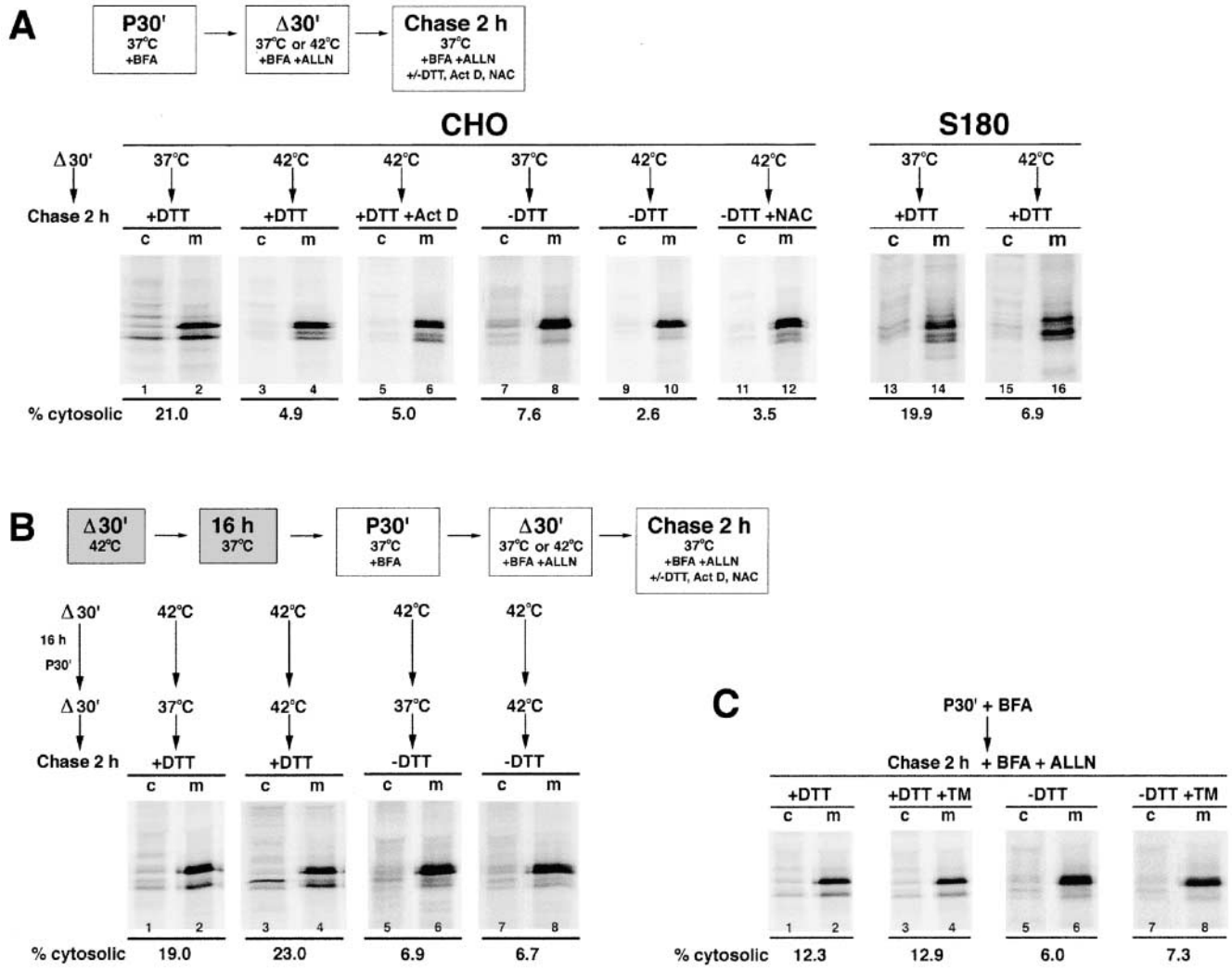

Figure 5. **Transient hyperthermia, but not induction of ER stress, inhibits connexin dislocation.** Cells were pulsed (at 37°C) and chased in the continuous presence of BFA as described below and (for A and B) indicated in the flow charts. Cytosolic (c) and membrane (m) fractions were then prepared and Cx43 immunoprecipitated. Δ denotes hyperthermic (42°C) or mock hyperthermic (37°C) treatment. (A) CHO (lanes 1–12) and S180 (lanes 13–16) cells were pulsed for 30 min and then subjected to a 30-min incubation at either 37 or 42°C as indicated. The cells were subsequently chased for 2 h at 37°C in the presence of ALLN, either with (lanes 1–6 and 13–16) or without (lanes 7–12) DTT. For lanes 5 and 6, actinomycin D was included in the chase. Results in lanes 11 and 12 were from cells pretreated, pulsed, and chased in the presence of NAC. (B) CHOs were thermally preconditioned by a 30-min incubation at 42°C before being returned to 37°C for 16 h in the absence of BFA (shaded boxes). The cells were then pulsed for 30 min, subjected to a 30-min incubation at either 37 or 42°C, and finally chased for 2 h at 37°C with ALLN in either the presence (lanes 1–4) or absence (lanes 5–8) of DTT as in A. (C) CHOs were pulsed for 30 min and then chased at 37°C for 2 h with ALLN and DTT (lanes 1 and 2), DTT and tunicamycin (TM) (lanes 3 and 4), no other additions (lanes 5 and 6), or tunicamycin (lanes 7 and 8). Tunicamycin was added 1 h before the pulse and present throughout the experiment.

nexin dislocation (Fig. 5). Cells were pulse labeled for 30 min and then incubated for 10–30 min at 42°C in the continuous presence of BFA. This mild heat treatment rapidly induced HSP70i expression, but did not kill the cells or prevent their proliferation (unpublished data). The labeled cells were then returned to 37°C and chased for 2 h under conditions (+ BFA, DTT, and ALLN) that maximized the accumulation of dislocated [^{35}S]met-Cx43 in unheated cells. Relative to mock-treated controls maintained at 37°C throughout the experiment, the 42°C treatment reduced the fraction of [^{35}S]met-Cx43 recovered in the 100,000-*g* cytosolic fraction by ~70–80% in both CHO and S180 cells (Fig. 5 A, compare lanes 1 and 2 with lanes 3 and 4, and lanes 13 and 14 with lanes 15 and 16). Similar results were obtained if actinomycin D was added to the chase medium (Fig. 5 A, lanes 5 and 6) at concentrations that completely blocked HSP70i induction. Therefore, inhibition of Cx43 dislocation in response to hyperthermia (or sodium arsenite; unpublished data) did not require new gene expression, suggesting that the cause of the heat shock response (accumulation of unfolded protein in the cytosol/nucleus) rather than its consequence (increased heat shock protein synthesis) was responsible for the effect. Transient hyperthermia also inhibited Cx43 dislocation in the absence of DTT (Fig. 5 A, lanes 7–10). Pretreatment with the antioxidant NAC did not block the ability of heat shock to induce HSP70i expression (unpublished data) or to inhibit Cx43 dislocation (Fig. 5 A, lanes 11 and 12), indicating that the effect of hyperthermia

(unlike that of arsenite; Fig. 4 A) does not involve changing the redox environment of the cytosol. As was the case with arsenite (Fig. 4 C), hyperthermia did not inhibit the degradation (after ALLN washout) of [^{35}S]met-Cx43 already released into the cytosol (unpublished data). The latter is in keeping with reports that nonlethal hyperthermia has little, if any, impact on the ability of the 26S proteasome to degrade soluble protein substrates (Fujimuro et al., 1997; Kuckelkorn et al., 2000).

Several hours after a cell has experienced a heat shock, the level of upregulated cytosolic chaperones is high. If cells in this state are subjected to a second heat treatment, these chaperones prevent the subsequent accumulation of unfolded proteins in a phenomenon referred to as thermotolerance (Mizzen and Welch, 1988; Lepock et al., 1990; Parsell and Lindquist, 1993). Cells were incubated at 42°C for 30 min and then returned to 37°C for 16 h to permit the buildup of heat shock proteins and the acquisition of thermotolerance. The cells were then pulse labeled (at 37°C), subjected to a second 30-min treatment at either 42°C or 37°C, and finally chased for 2 h in the presence of BFA, ALLN, and DTT at 37°C. In five out of five independent experiments, the fraction of [^{35}S]met-Cx43 released into the cytosol by thermotolerant cells was the same whether or not they were subjected to a second hyperthermic treatment (Fig. 5 B, compare lanes 1 and 2 with lanes 3 and 4) and was indistinguishable from the amount of [^{35}S]met-Cx43 recovered in the cytosol of cells never exposed to elevated temperature (Fig. 5 A, lanes 1 and 2). Qualitatively similar results were obtained if the chase was conducted in the absence of DTT (compare Fig. 5, A, lanes 7–10, with B, lanes 5–8). The finding that thermal preconditioning rendered Cx43 dislocation heat insensitive implies that mild hyperthermia reduces dislocation by a process linked to increased levels of unfolded protein in the cytosol instead of by causing nonspecific thermal damage to either the connexin molecule or the dislocation machinery.

By blocking N-linked glycosylation, tunicamycin prevents the proper folding of many newly synthesized glycoproteins, whereas cytosolic proteins (which do not undergo this modification) are unaffected. Consequently, tunicamycin induces the ER-specific unfolded protein response, but not HSP70i expression (unpublished data) or other aspects of the heat shock response (Shamu et al., 1994). Connexins are not glycosylated and assemble into functional gap junctions in the presence of tunicamycin (Wang and Mehta, 1995). Pulse-chase analysis demonstrated that tunicamycin did not reduce the amount of [^{35}S]met-Cx43 dislocated into the cytosol of CHO cells in either the presence or absence of DTT (Fig. 5 C). Similar results were obtained in S180 cells, or if cells were preincubated with tunicamycin for 6 h prior to metabolic labeling (unpublished data). We verified that under all conditions tested, tunicamycin effectively blocked N-linked glycosylation as assessed by its effect on a connexin-unrelated glycoprotein, cadherin (unpublished data). Unfolded proteins also accumulate in the ER (but not in the cytosol) with concomitant induction of the unfolded protein response when disulfide bond formation is blocked by DTT (Chapman et al., 1998). In all cell types examined, DTT increased rather than decreased connexin dislocation (Figs. 1, 2, and 5). The demonstration that connexin dislocation was inhibited by two mechanistically distinct inducers of protein unfolding in the cytosol (hyperthermia and the oxidant arsenite), but not by agents that specifically perturb folding within the ER (tunicamycin and DTT), indicates that connexin dislocation is selectively regulated by cytosolic stress.

Inducers of the heat shock response, but not ER stressors, inhibit connexin turnover and increase the formation of functional gap junctions

If dislocation from the ER plays a major role in connexin degradation, treatments that inhibit this process would be expected to decrease the rate at which newly synthesized connexins turn over. This prediction was confirmed in a series of pulse-chase studies that assessed the half-life of total cellular [^{35}S]met-Cx43 in CHO cells in the absence of either BFA or ALLN (Fig. 6, A–D). Pulse-labeled cells were incubated for 30 min at either 37 or 42°C and then returned to 37°C for the remainder of the chase. In the 37°C cells, immunoprecipitable [^{35}S]met-Cx43 was degraded at the rapid rate characteristic of connexins (Fig. 6 A, lane 3). Turnover was much slower in heat-treated cells, resulting in a 7.5-fold increase ($\pm$ 2.1; $n = 8$) in the amount of [^{35}S]met-Cx43 that survived a 6-h chase relative to control cells (Fig. 6, A, lane 4, and B). Connexin degradation was also reduced if cells were chased in the presence of 80 μM Na arsenite (Fig. 6 A, lane 5), albeit to a lesser extent (fold increase relative to control = 3.9 $\pm$ 0.93; $n = 7$) (Fig. 6 B). Arsenite and 42°C also slowed Cx43 turnover in S180 cells (unpublished data). In contrast, neither of the ER-specific stressors tested (tunicamycin and DTT) increased the amount of pulse-labeled connexin recovered after a 6-h chase by more than ~1.3-fold (Fig. 6 A, lanes 6 and 7); indeed, DTT significantly accelerated [^{35}S]met-Cx43 degradation if disulfide bond formation was completely blocked by including DTT in the pulse medium (Musil et al. 2000). Notably, hyperthermia and arsenite also reduced connexin turnover in CHO cells when exit from the ER was blocked with BFA, indicating that they suppressed connexin degradation by inhibiting ERAD (Fig. 6 E, compare lane 2 with lanes 3 and 4).

As was observed in dislocation assays (Figs. 4 and 5), pretreatment of cells with the antioxidant NAC completely blocked the ability of arsenite, but not of hyperthermia, to slow Cx43 degradation (Fig 6 C). Also in keeping with its effect on dislocation (Fig. 5 B), thermal preconditioning largely prevented a subsequent 42°C treatment from reducing Cx43 turnover (Fig. 6 D).

In unstressed cells, assembly of Cx43 into functional gap junctional plaques is closely correlated with its phosphorylation to a 46-kD species (referred to as Cx43-P_2) that takes place only after transport of Cx43 to the cell surface (Musil and Goodenough, 1991). Very little Cx43 is converted to the P_2 species in untreated CHO cells (Fig. 6 A, lane 2), as expected given the limited ability of this cell type to assemble gap junctions under basal conditions despite normal oligomerization of Cx43 into connexons and trafficking to the plasma membrane (Musil and Goodenough, 1993; Musil et al., 2000). However, note that after treatments that inhibit the turnover of [^{35}S]met-

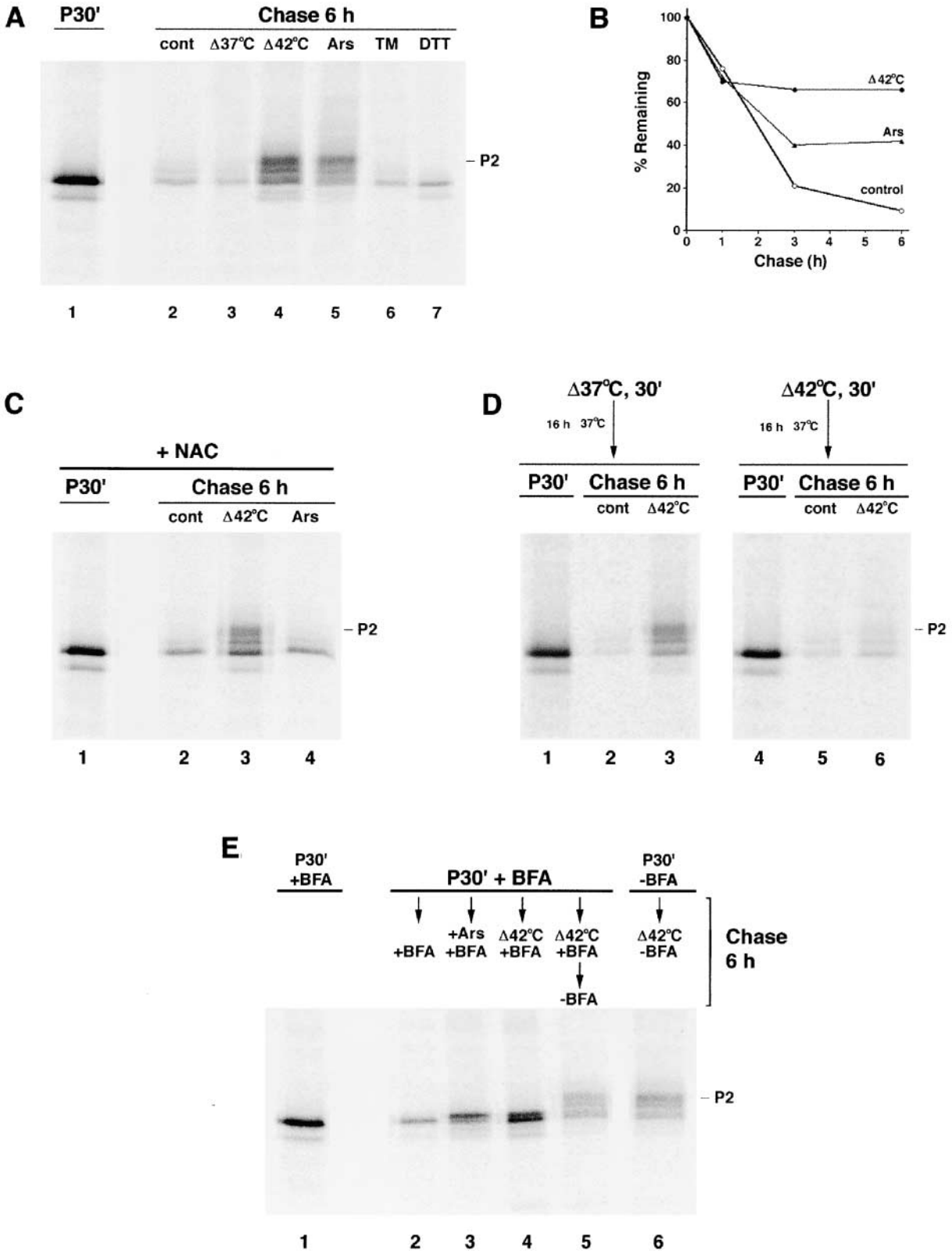

Figure 6. **Induction of cytosolic stress, but not ER stress, decreases Cx43 turnover.** CHO cells were labeled at 37°C for 30 min and chased as described below before immunoprecipitation of total cellular Cx43. Position of the Cx43-P_2 species is indicated (P2). (A) Cells were lysed immediately after the pulse (lane 1), or after a 6-h chase with no additions (control, lane 2), sodium arsenite (lane 5), tunicamycin (lane 6), or DTT (lane 7). Results in lanes 3 and 4 were from cells incubated for 30 min at either 37 (mock hyperthermic treatment, lane 3) or 42°C (lane 4) immediately after the pulse and then chased for 5.5 h at 37°C. (B) Plot from a typical experiment of the time course of pulse-labeled Cx43 turnover when chased as in lane 2, 4, and 5 of A. (C) Cells were labeled and chased as described in A, except in the continuous presence of the antioxidant NAC. (D) Cells were thermally preconditioned by a 30-min incubation at 42°C (lanes 4–6) or mock treated at 37°C (lanes 1–3) and returned to 37°C for 16 h. Cells were then labeled and lysed immediately after the pulse (lanes 1 and 4), after a 6-h chase at 37°C (lanes 2

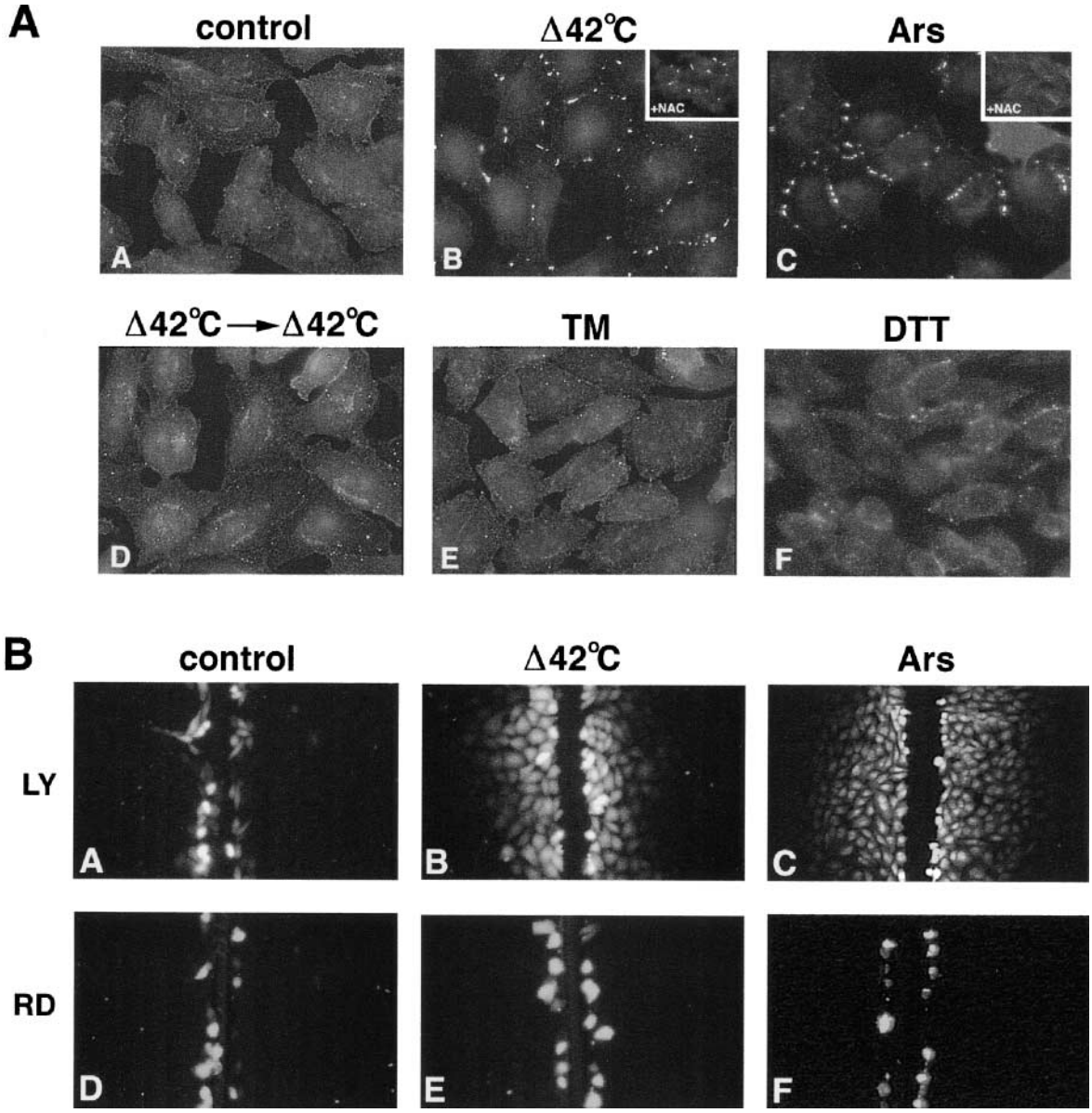

Figure 7. **Induction of cytosolic stress upregulates gap junction formation and function in assembly-inefficient CHO cells.** (A) CHOs were incubated for 3 h at 37°C with no additions (panel A), or with Na arsenite (panel C), tunicamycin (panel E), or DTT (panel F). Cells in panel B were heated at 42°C for 30 min and then returned to 37°C for an additional 2.5-h incubation. Cells in panel D were treated identically as in panel B, but after being thermally preconditioned by a 30-min incubation at 42°C followed by 16 h at 37°C. (Insets in B and C) Cells treated as in the corresponding main panel, except after a 1-h preincubation with NAC and in its continuous presence. All cells were then fixed and immunostained with anti-Cx43 antibodies. (B) Monolayers of CHO cells were incubated either for 3 h at 37°C with no additions (control), for 30 min at 42°C before being returned to 37°C for an additional 2.5-h incubation (Δ 42°C), or for 3 h at 37°C with Na arsenite (Ars). Gap junction–mediated intercellular transfer of Lucifer yellow (LY, panels A–C) was then assessed. Panels D–F are the same fields as in panels A–C, respectively, but visualized with rhodamine optics; note that junction-impermeant rhodamine dextran (RD) remained confined to cells at wound edge.

Cx43, but not its movement to the cell surface (42°C-BFA; arsenite −BFA −NAC), a substantial fraction of the labeled Cx43 was converted to the P_2 form as assessed by its electrophoretic mobility (Fig. 6 A, lanes 4 and 5, C, lane 3, and D, lane 3) and sensitivity to alkaline phosphatase (unpublished data). [^{35}S]Met-Cx43 confined to the ER by BFA during heat treatment was shifted to the P_2 form during a subsequent chase at 37°C if, and only if, BFA was removed (Fig. 6 E, compare lanes 4 and 5). The latter finding demonstrated that cytosolic stress conferred upon Cx43 in the ER the potential to be converted to the P_2 form, even though the P_2 phosphorylation event itself

and 5), or after a 30-min incubation at 42°C followed by a 5.5-h chase at 37°C (lanes 3 and 6). (E) Cells were labeled in the presence (lanes 1–5) or absence (lane 6) of BFA at 37°C. Cells were then either immediately lysed (lane 1), or chased as follows: for 6 h with BFA (lane 2), for 6 h with BFA and Na arsenite (lane 3), for 30 min at 42°C with BFA followed by 5.5 h at 37°C with BFA (lane 4), for 30 min at 42°C with BFA followed by 5.5 h at 37°C in the absence of BFA (lane 5), or for 30 min at 42°C followed by 5.5 h at 37°C, all in the absence of BFA (lane 6).

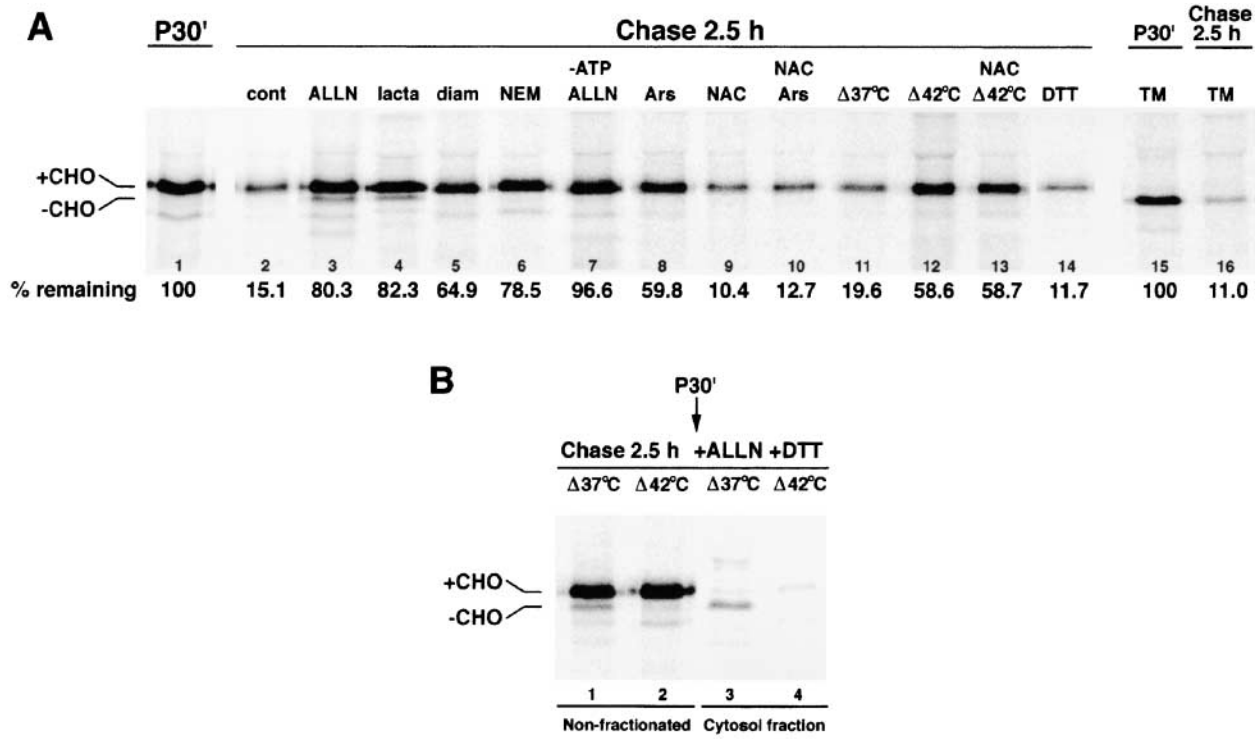

Figure 8. **Induction of cytosolic stress decreases MHC1 heavy chain dislocation and degradation.** Daudi cells were pulse-labeled for 30 min at 37°C and then treated as described below. The position of core glycosylated (+CHO) and nonglycosylated (−CHO) forms of the heavy chain is indicated. Both arsenite and 42°C induced HSP70i synthesis (unpublished data). (A) Total cellular MHC1 heavy chain was immunoprecipitated from 1.25×10^6 cells/sample after a 0 (lane 1) or 2.5-h chase with no additions (lane 2), 250 μM ALLN (lane 3), 20 μM lactacystin (lane 4), 1 mM diamide (lane 5), 1 mM NEM (lane 6), 250 μM ALLN plus an ATP-depleting mix (lane 7), 80 μM sodium arsenite (lane 8), 20 μM NAC (lane 9), 20 μM NAC plus 80 μM Na arsenite (lane 10), or 2 mM DTT (lane 14). Results shown in lanes 11–13 were from cells incubated for 30 min at either 37 (mock hyperthermic treatment; lane 11) or 42°C (lanes 12 and 13) immediately after the pulse and then chased for 2 h in unsupplemented chase medium at 37°C. For lanes 9, 10, and 13, cells were preincubated, pulsed, and chased in the continuous presence of NAC. For lanes 15 and 16, cells were pretreated (3 h) and labeled in the presence of 4 μM tunicamycin and then lysed (lane 15) or chased for 2.5 h with tunicamycin (lane 16). (B) After pulse-labeling, cells were incubated for 30 min at either 37 (Δ 37°C) or 42°C (Δ 42°C) in the presence of ALLN and DTT and then chased for an additional 2 h at 37°C in the same medium. The samples (10^7 cells/sample) were then halved for immunoprecipitation of heavy chain from either the whole cell lysate (lanes 1 and 2) or from a 100,000-*g* cytosolic supernatant fraction (lanes 3 and 4).

takes place only after transport of Cx43 to the cell surface.

As previously reported (Musil et al., 2000), anti-Cx43 immunostaining of control CHO cells revealed little of the punctate cell–cell interface staining characteristic of gap junctional plaques (Fig. 7 A, panel A). Essentially the same pattern was obtained immediately after a 10–30-min incubation at 42°C (unpublished data). However, if the heated cells were then returned to 37°C, a striking increase in gap junctional staining became evident within 3 h (B). Gap junctions were also upregulated if cells were incubated with 80 μM sodium arsenite for the same period (Fig. 7 A, panel C), or if oxidative stress was induced instead by hydrogen peroxide or menadione (unpublished data). As was the case with both Cx43 dislocation (Figs. 4 and 5) and degradation (Fig. 6), the effect of oxidants (Fig. 7 A, panel C, inset), but not of hyperthermia (Fig. 7 A, panel B, inset), on gap junction formation was blocked by pretreatment with NAC, whereas the ability of hyperthermia to upregulate gap junctional staining was abolished by thermal preconditioning (Fig. 7 A, panel D). Despite continued cell viability, neither tunicamycin (Fig. 7 A, panel E) nor DTT (Fig. 7 A, panel F) increased the number of immunodetectable gap junctional plaques. Similar results were obtained with other gap junction assembly-inefficient cell types (unpublished data).

As evaluated by the extent of cell-to-cell diffusion of Lucifer yellow to rhodamine dextran–negative cells in scrape-loading dye transfer assays, CHO cells under basal conditions were poorly coupled (Fig. 7 B, panel A) (Musil et al., 2000). Both hyperthermia (Fig. 7 B, panel B) and arsenite (Fig. 7 B, panel C) increased the intercellular spread of Lucifer yellow by an average of 5.1-fold (± 1.08; $n = 9$). Qualitatively similar results were obtained with biocytin, another gap junction–permeable compound with physical properties distinct from those of Lucifer yellow (unpublished data). The increase in dye transfer in cells subjected to cytosolic stress was prevented by the gap junction blocker 18β-glycyrrhetinic acid, indicating a bona fide increase in gap junction–mediated intercellular communication (unpublished data). Taken together, the results shown in Figs. 6 and 7 indicate that Cx43 spared from dislocation at the ER by cytosolic stress assembles into functional gap junctional plaques.

The effect of heat shock response inducers on dislocation is not restricted to connexins

Lastly, we asked whether cytosolic stress also reduced the dislocation and degradation of the MHC1 heavy chain, a connexin-unrelated, single transmembrane domain protein that is one of the best-characterized substrates for dislocation. MHC1 heavy chain is synthesized as an ~43-kD core glycosylated species. In cells expressing the cytomegalovirus US2/11 proteins or (less efficiently) in uninfected cells defective in MHC assembly such as Daudi B lymphoblasts, it is then dislocated from the ER and deglycosylated to a 40-kD form that can be recovered in the 100,000-*g* cytosolic fraction of proteasome inhibitor–treated cells (Wiertz et al., 1996a; Hughes et al., 1997). As expected (Tortorella et al., 1998), pulse-chase analysis showed that heavy chain turned over rapidly in Daudi cells in a process sensitive to proteasome blockers such as ALLN and lactacystin (Fig. 8 A, lanes 1–4). During a 2.5-h chase in the presence of either compound (Fig. 8 A, lanes 3 and 4), but not in their absence (Fig. 8 A, lane 2), a fraction of the 43-kD form of heavy chain was converted to an ~40-kD species. As determined by electrophoretic migration relative to nonglycosylated heavy chain synthesized in the presence of tunicamycin (Fig. 8 A, lane 15) and sensitivity to endoglycosidase H (unpublished data), the 43-kD band consisted of core glycosylated heavy chain, whereas deglycosylated heavy chain comprised the lower band. Addition of 1 mM diamide (Fig. 8 A, lane 5), 1 mM NEM (Fig. 8 A, lane 6), or ALLN and an ATP-depleting mixture (Fig. 8 A, lane 7) to the chase medium also reduced [^{35}S]met-heavy chain turnover, but did not result in the recovery of the 40-kD degradation intermediate, in keeping with the previously reported ability of these agents to block heavy chain dislocation (Wiertz et al., 1996b; Tortorella et al., 1998). Notably, mild hyperthermia (30 min at 42°C) (lane 12) or 80 μM sodium arsenite (lane 8) also reduced heavy chain degradation and conversion to the 40-kD form in four out of four independent experiments, the latter, but not the former, in an NAC-sensitive manner (Fig. 8 A, lanes 10 and 13). In contrast, neither of the ER-specific stressors examined (DTT and tunicamycin) decreased heavy chain turnover (Fig. 8 A, lanes 14 and 16).

To more directly demonstrate that cytosolic stress inhibited heavy chain dislocation, pulse-labeled cells were heat shocked for 30 min and then chased for 2.5 h at 37°C in the presence of DTT (to maximize the amount of heavy chain misfolding; Wiertz et al., 1996b) and ALLN to inhibit the degradation of any dislocated heavy chain generated. Heavy chain was then immunoprecipitated from either whole cell lysates (Fig 8 B, lane 2) or from a 100,000-*g* cytosolic supernatant fraction prepared as described for detection of dislocated connexins (Fig. 8 B, lane 4). Virtually no 40-kD band was recovered in either the whole cell lysate or in the cytosolic fraction of the heat-treated cells, in contrast to cells maintained at 37°C throughout the experiment in which ~11% of the [^{35}S]met-heavy chain in the postnuclear supernatant was recovered in the cytosol fraction in the 40-kD form (Fig. 8 B, lane 3). We conclude that dislocation and degradation of MHCI heavy chain, like that of connexins, is regulated by inducers of the cytosolic heat shock response.

Discussion

En bloc dislocation of connexins

Our pulse-chase data demonstrate that cells possess the machinery to eject intact connexins from the ER, and that Cx43 dislocated in the presence of ALLN is rapidly degraded if proteasome function is restored. To our knowledge, this is the first demonstration that a membrane-integrated polytopic protein can be dislocated in an intact form and then undergo efficient proteasomal destruction. Detectable accumulation of connexin in the cytosol required cells to be incubated with a proteasome inhibitor (Figs. 1 and 3). Therefore, we cannot rule out the possibility that under inhibitor-free conditions, degradation of connexins begins before dislocation is complete, as has been reported for certain other ER substrates (Plemper et al., 1998; Pariyarath et al. 2001). Even if this were the case, the extent to which connexin is released into the 100,000-*g* supernatant fraction of proteasome inhibitor–treated cells would still be indicative of the relative efficiency of connexin dislocation under various conditions.

How does cytosolic stress reduce dislocation?

Dislocation from the ER is an ATP-dependent process that, for the substrates examined, is blocked when the activity of the cim5p 19S cap ATPase is inhibited (Mayer et al., 1998; Hill and Cooper, 2000). These findings have contributed to the prevailing hypothesis that 19S cap ATPases, possibly in conjunction with other proteins, are responsible for physically extracting the substrate from the ER membrane during dislocation (Mayer et al., 1998; Plemper et al., 1998). It has been suggested that 26S proteasomes may be transiently recruited to the ER from their steady state cytosolic/nuclear location by ERAD substrates themselves (Hirsch and Ploegh, 2000). In this view, 26S proteasomes (which diffuse freely in the cytosol and nucleus; Reits et al., 1997) recognize and bind to cytoplasm-facing regions of ERAD substrates via the 19S proteasomal cap. Once docked, cap ATPases participate in extracting the substrate from the ER via the translocon, thereby gaining access to the 20S proteasomal core.

We propose that under basal conditions, 26S proteasomes are recruited to the ER by newly synthesized connexin molecules targeted for ERAD, most likely by an inability to fold efficiently. After binding, the proteasome participates in extracting the connexin from the ER membrane for destruction. Incubating cells with DTT increases the misfolding of connexins, rendering them even higher affinity substrates for 19S cap binding and leading to an increase in the rate and absolute amount of connexin dislocation. Alternatively or in addition, DTT might facilitate dislocation by altering the activity of redox-dependent components of the dislocation machinery (Tsai et al., 2001). Unlike DTT or tunicamycin, hyperthermia and sodium arsenite increase the level of abnormal protein primarily in the cytosol and nucleus as evidenced by the fact that they enhance the expression of cytosolic chaperones to a much greater extent than they upregulate chaperones induced by unfolded proteins in the ER (Kim and Lee, 1986; Kozutsumi et al., 1988; Brostrom

et al., 1996). The relatively mild conditions that we have employed do not cause a marked, persistent depression of either ATP levels or protein synthesis, and damage is expected to be limited to a subset of particularly susceptible proteins (Lepock et al., 1993). We propose that 26S proteasomes become associated with such misfolded proteins in their immediate vicinity in the cytosol and nucleus, thereby reducing the number of unoccupied proteasomes available to be recruited to, and mediate dislocation from, the ER. This would be in keeping with the demonstration that proteasomes can become highly concentrated at cytoplasmic or nuclear sites of unfolded protein accumulation (Cummings et al., 1998; Garcia-Mata et al., 1999; Wigley et al., 1999), a redistribution that may be responsible for reduced degradation of substrates in other (presumably proteasome depleted) regions of the cytoplasm (Bence et al., 2001). Connexin molecules thus spared from ERAD are able to attain a conformation that permitted them to traverse the secretory pathway, as evidenced by the ability of Cx43 to undergo a phosphorylation event that takes place only after transport to the cell surface (Fig. 6) (Musil and Goodenough, 1991; Musil et al., 2000). Although a role for additional stress-induced processes cannot be ruled out, we propose that the resulting increase in the amount of connexin on the cell surface leads to enhanced gap junction formation. Because gap junction assembly is a cooperative process (Castro et al., 1999), even a relatively modest increase in the number of connexin molecules on the cell surface could account for the large stimulation in gap junction formation and function that we have observed in CHO cells. Notably, connexin dislocation, degradation, and gap junction assembly are unaffected by antioxidant-neutralized arsenite, tunicamycin, and heat treatment of thermotolerant cells, consistent with their failure to induce protein misfolding in the cytosol.

When the proteasome's peptidase activities are blocked, proteasomal substrates accumulate throughout the cell, but are particularly abundant in the cytosol and nuclear matrix where the majority of proteasome-dependent degradation normally takes place. The resulting increase in the level of unfolded protein in the cytosol is believed to be responsible for the induction of expression of HSP70i and other heat shock proteins that is observed within 1–2 h of proteasome inhibitor addition (Zhou et al., 1996). We would predict that proteasomes gradually become occupied with such cytosolic substrates and consequently become less available to mediate dislocation, although to a lesser extent than after treatments that directly induce protein unfolding in the cytosol (e.g., hyperthermia or arsenite). Such a phenomenon could contribute to the relatively slow rate at which connexins accumulate in the cytosol in our dislocation experiments, which by necessity are conducted in the presence of proteasome inhibitors. A reduction in dislocation efficiency would also account for the observation that connexin spared from degradation by ALLN accumulates mainly within the secretory pathway instead of in the cytosol or in aggresomes (Musil et al., 2000; VanSlyke et al., 2000). Interestingly, proteasome inhibitors have also been reported to reduce the efficiency of dislocation of several other ERAD substrates, including the MHC1 heavy chain (Yu et al., 1997; Story et al., 1999; Mancini et al., 2000).

If the level of unfolded protein in the cytosol is an important determinant of how well 26S proteasomes are recruited to the ER, and if the proteasome plays a general role in the dislocation of ERAD substrates, then cytosolic stress would be expected to inhibit the dislocation and degradation of at least some connexin-unrelated proteins. We have shown this to be true for the MHC1 heavy chain endogenously expressed in Daudi cells (Fig. 8). Although our data fully support the proposed model, we acknowledge that we have not directly demonstrated that cytosolic stresses act by titering 26S proteasomes away from the ER. We also recognize that a variety of nonproteasome proteins have been implicated in dislocation, most of which appear to contribute to the process in a substrate-specific manner. The extent to which cytosolic stress affects the dislocation, degradation, and fate of a particular ERAD substrate may well be influenced by its dependence on such additional components.

Cytosolic stress as a mechanism to upregulate gap junction–mediated intercellular communication

An important aspect of our study is that only mild stresses that induced a robust heat shock response but did not cause widespread cell death enhanced gap junctions. What might be the physiological relevance of this phenomenon? Ischemia-reperfusion injury is a common sequelae of stroke and myocardial infarction and a potential complication of surgery. Cells nearest the occluded vessel that have undergone irreversible damage (and would therefore be expected to have shut off gap junctional communication; Loewenstein, 1981) are separated from unaffected tissue by an intermediate zone. Cells in this intervening region express elevated levels of heat shock proteins, most likely induced by accumulation of unfolded proteins in the cytosol resulting from ATP depletion during ischemia and/or generation of reactive oxygen species after reperfusion (Plumier et al., 1996; Sharp et al., 2000). It is possible that this cytosolic stress reduces ERAD of newly synthesized connexin molecules and thereby upregulates gap junction assembly in these cells. Stress-induced intercellular channels could then mediate enhanced delivery of reduced glutathione and other antioxidants from less affected surrounding tissue to enhance cell survival. Such a scenario would be in keeping with reports that gap junctional transfer of GSH can restore the function of oxidatively stressed cultured cardiac myocytes (Nakamura et al., 1994).

Materials and methods

Metabolic labeling

Adherent cells, cultured as described in Musil et al. (2000), were labeled with [^{35}S]methionine and chased as in VanSlyke et al. (2000). Daudi cells were labeled in reduced bicarbonate labeling medium (Musil and Goodenough, 1993) supplemented with 5% dialyzed FCS at 2.5–5 $\times$ 10^6 cells/ml in a 37°C water bath, and chased in complete RPMI 1640 with 0.5 mM methionine in a 37°C CO_2 incubator. Where indicated in the text, the pulse and/or chase medium was supplemented with: 6 ug/ml brefeldin A (BFA; GIBCO BRL); 2 mM dithiothreitol (DTT); 70 μM cycloheximide (CHX); 100 or 250 μM ALLN (N-acetyl-leu-leu-norleucinal; Calbiochem); 10 μM epoxomicin (Calbiochem); 20 μM ZL_3VS (carboxybenzyl-leucyl-leucyl-leucyl-vinylsulphone, a gift of M. Bogyo, University of California, San Francisco, San Francisco, CA); 20 μM lactacystin (Calbiochem); 200 μM leupeptin; 100 μM ALLM; 22.5 mM 2-deoxyglucose plus 13.5 μM antimycin A (to deplete ATP); 80 μM sodium arsenite; 8 μM actinomycin D; 20 mM *N*-acetyl cysteine (NAC); or 4 μM tunicamycin (TM; Calbiochem). Cells were

preincubated in NAC for 30–60 min prior to the pulse. Unless otherwise specified, all reagents were from Sigma-Aldrich.

Heat shock treatments

Cells were heat shocked in either Leibowitz's L15 medium (adherent cells) or reduced bicarbonate medium (Musil and Goodenough, 1993) (Daudi cells), both supplemented with 0.2 mM methionine and 10% FCS. Hyperthermic treatments were conducted in water baths in which the cell growing surface of the tissue culture dish was maintained at 41.5–42°C or (for mock heat treatment) 36.5–37.5°C as assessed by continuous monitoring with a thermocouple thermometer. Thermal preconditioning was conducted identically. Preincubation of CHO cells for 10 min at 42°C followed by a 16-h recovery at 37°C allowed them to proliferate after an otherwise lethal heat treatment (10 min at 45°C), demonstrating acquisition of thermotolerance.

Preparation of cytosolic and membrane fractions

60-mm cultures of adherent cells were rinsed twice with 4°C PBS and then scraped from the dish in 750 ul of homogenization buffer (10 mM Tris, 1 mM EDTA, 10 mM iodoacetamide, 0.2 mM PMSF, 20 μM ALLN, pH 7.5). Daudi cells (1×10^7 cells/sample) were pelleted and resuspended in 750 ul of the same buffer. After a 20-min incubation on ice, the cells were passed eight times through a 25-g needle. The resulting lysate was subjected to a 5-min centrifugation at 5,000 rpm (7,000 rpm for Daudi cells) at 4°C, after which the pellet was resuspended in 250–500 ul of homogenization buffer and re-extracted by a second round of trituration and centrifugation. The supernatants of the first and second spins were combined, adjusted to 0.25 M NaCl, and separated into cytosolic (supernatant) and membrane (pellet) fractions by centrifugation at 100,000 *g* (45,000 rpm in a TLA100.3 Beckman ultracentrifuge) for 1 h at 40°C. The pellet was then resuspended in 1 ml of homogenization buffer with 0.25 M NaCl.

Analysis of the disulfide bonded state of Cx43

Metabolically labeled S180 cells were incubated for 10 min at 4°C with 50 mM iodoacetamide in PBS to alkylate free thiol groups. Cx43 was then immunoprecipitated from cytosolic and membrane fractions as described above, except that one-half of each sample was eluted from the protein A-Sepharose beads in immunoprecipitation buffer containing 0.6% SDS with 20 mM DTT (reducing), whereas the other half was eluted without DTT (nonreducing). After a 30-min incubation at 25°C, the eluate was removed and supplemented with iodoacetic acid (from a freshly made 0.25-M stock in 0.25 M Tris-HCl, pH 8.0, 0.25 M KOH) to a final concentration of 50 μM. After a 15-min incubation at room temperature, the samples were placed on ice and concentrated by methanol/$CHCl_3$ precipitation. The precipitates were resuspended in nonreducing SDS-PAGE sample loading buffer containing 5% SDS, heated to 100°C for 3 min, and then analyzed on 10% SDS-PAGE gels in which the concentration of Bis was increased to 0.4% and the running gel buffer was modified to contain 50 mM Tris, 380 mM glycine, and 0.15% SDS.

Immunoprecipitation and SDS-PAGE

For analysis of whole cell lysates, cells were solubilized in 0.6% SDS as reported in VanSlyke and Musil (2000). For cytosolic and membrane fractions, SDS was added to a final concentration of 0.6%. Samples containing Cx43, L-CAM, or MHC1 heavy chain were boiled for 3 min, whereas Cx32 samples were incubated for 30 min at room temperature. Immunoprecipitation and analysis by SDS-PAGE was as described in VanSlyke et al. (2000) using antibodies specific for Cx32 (Goodenough et al., 1988), L-CAM (W. Gallin; University of Alberta, Edmonton, Alberta, Canada), Cx43 (AP7298; Musil et al., 1990), or MHC1 heavy chain (HC10; Stam et al., 1986). In Fig. 1 C, lane 3 only, an antiserum directed against amino acids 1–20 of rat Cx43 was employed (designated AT-2, a gift of B. Nicholson [SUNY Buffalo, Buffalo, NY]) (Yancey et al., 1989).

Microscopy

CHO cells grown on glass coverslips were fixed in 2% paraformaldehyde in PBS and processed for immunocytochemical detection of Cx43 as previously described (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).

Quantitation

For analysis of immunoprecipitates, dried gels were quantitated on a PhosphorImager (model 445 SI; Molecular Dynamics, Inc.) utilizing IPLab Gel software (Signal Analytics Corp.). Percent cytosolic was calculated as c/c+ m × 100, where c and m are PhosphorImager volume counts of immunoprecipitates prepared from the 100,000-*g* supernatant (c, cytosol) and pellet (m, membrane) fractions. Scrape-loading/dye transfer assays were quantitated using the averaged fluorescence plot profile method as detailed in Le and Musil (2001). Unless indicated otherwise, all experiments were repeated a minimum of three times and representative experiments shown.

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References

Beardslee, M.A., J.G. Laing, E.C. Beyer, and J.E. Saffitz. 1998. Rapid turnover of connexin43 in the adult rat heart. *Circ. Res.* 83:629–635.

Beckmann, R.P., M. Lovett, and W.J. Welch. 1992. Examining the function and regulation of hsp 70 in cells subjected to metabolic stress. *J. Cell Biol.* 117: 1137–1150.

Bence, N.F., R.M. Sampat, and R.R. Kopito. 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science.* 292:1552–1555.

Bernstam, L., and J. Nriagu. 2000. Molecular aspects of arsenic stress. *J. Toxicol. Environ. Health B Crit. Rev.* 3:293–322.

Brostrom, C.O., C.R. Prostko, R.J. Kaufman, and M.A. Brostrom. 1996. Inhibition of translational initiation by activators of the glucose-regulated stress protein and heat shock protein stress response systems. *J. Biol. Chem.* 271: 24995–25002.

Castro, C., J.M. Gomez-Hernandez, K. Silander, and L.C. Barrio. 1999. Altered formation of hemichannels and gap junction channels caused by C-terminal connexin-32 mutations. *J. Neurosci.* 19:3752–3760.

Chapman, R., C. Sidrauski, and P. Walter. 1998. Intracellular signaling from the endoplasmic reticulum to the nucleus. *Annu. Rev. Cell Dev. Biol.* 14:459–485.

Cummings, C.J., M.A. Mancini, B. Antalffy, D.B. DeFranco, H.T. Orr, and H.Y. Zoghbi. 1998. Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat. Genet.* 19:148–154.

Falk, M.M., and N.B. Gilula. 1998. Connexin membrane protein biosynthesis is influenced by polypeptide positioning within the translocon and signal peptidase access. *J. Biol. Chem.* 273:7856–7864.

Falk, M.M., N.M. Kumar, and N.B. Gilula. 1994. Membrane insertion of gap junction connexins: polytopic channel forming membrane proteins. *J. Cell Biol.* 127:343–355.

Fallon, R.F., and D.A. Goodenough. 1981. Five-hour half-life of mouse liver gap-junction protein. *J. Cell Biol.* 90:521–526.

Foote, C.I., L. Zhou, X. Zhu, and B.J. Nicholson. 1998. The pattern of disulfide linkages in the extracellular loop regions of connexin 32 suggests a model for the docking interface of gap junctions. *J. Cell Biol.* 140:1187–1197.

Fujimuro, M., H. Sawada, and H. Yokosawa. 1997. Dynamics of ubiquitin conjugation during heat-shock response revealed by using a monoclonal antibody specific to multi-ubiquitin chains. *Eur. J. Biochem.* 249:427–433.

Garcia-Mata, R., Z. Bebok, E.J. Sorscher, and E.S. Sztul. 1999. Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J. Cell Biol.* 146:1239–1254.

Georgopoulos, C., and W.J. Welch. 1993. Role of the major heat shock proteins as molecular chaperones. *Annu. Rev. Cell Biol.* 9:601–634.

Goodenough, D.A., D.L. Paul, and L. Jesaitis. 1988. Topological distribution of two connexin32 antigenic sites in intact and split rodent hepatocyte gap junctions. *J. Cell Biol.* 107:1817–1824.

Goodenough, D.A., J.A. Goliger, and D.L. Paul. 1996. Connexins, connexons, and intercellular communication. *Annu. Rev. Biochem.* 65:475–502.

Hill, K., and A.A. Cooper. 2000. Degradation of unassembled Vph1p reveals novel aspects of the yeast ER quality control system. *EMBO J.* 19:550–561.

Hirsch, C., and H.L. Ploegh. 2000. Intracellular targeting of the proteasome. *Trends Cell Biol.* 10:268–272.

Hughes, E.A., C. Hammond, and P. Cresswell. 1997. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proc. Natl. Acad. Sci. USA.* 94:1896–1901.

Kim, K.S., and A.S. Lee. 1986. The effect of extracellular Ca2+ and temperature on the induction of the heat-shock and glucose-regulated proteins in hamster fibroblasts. *Biochem. Biophys. Res. Commun.* 140:881–887.

Kozutsumi, Y., M. Segal, K. Normington, M.J. Gething, and J. Sambrook. 1988. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature.* 332:462–464.

Kuckelkorn, U., C. Knuehl, B. Boes-Fabian, I. Drung, and P.M. Kloetzel. 2000. The effect of heat shock on 20S/26S proteasomes. *Biol. Chem.* 381:1017–1023.

Laird, D.W., M. Castillo, and L. Kasprzak. 1995. Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. *J. Cell Biol.* 131:1193–1203.

Le, A.C., and L.S. Musil. 2001. A novel role for FGF and extracellular signal-regulated kinase in gap junction-mediated intercellular communication in the lens. *J. Cell Biol.* 154:197–216.

Lee, Y.J., and W.C. Dewey. 1988. Thermotolerance induced by heat, sodium arsenite, or puromycin: its inhibition and differences between 43 degrees C and 45 degrees C. *J. Cell. Physiol.* 135:397–406.

Lepock, J.R., H.E. Frey, M.P. Heynen, J. Nishio, B. Waters, K.P. Ritchie, and J. Kruuv. 1990. Increased thermostability of thermotolerant CHL V79 cells as determined by differential scanning calorimetry. *J. Cell. Physiol.* 142:628–634.

Lepock, J.R., H.E. Frey, and K.P. Ritchie. 1993. Protein denaturation in intact hepatocytes and isolated cellular organelles during heat shock. *J. Cell Biol.* 122:1267–1276.

Loewenstein, W.R. 1981. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol. Rev.* 61:829–913.

Mancini, R., C. Fagioli, A.M. Fra, C. Maggioni, and R. Sitia. 2000. Degradation of unassembled soluble Ig subunits by cytosolic proteasomes: evidence that retrotranslocation and degradation are coupled events. *FASEB J.* 14:769–778.

Mayer, T.U., T. Braun, and S. Jentsch. 1998. Role of the proteasome in membrane extraction of a short-lived ER- transmembrane protein. *EMBO J.* 17:3251–3257.

McCracken, A.A., and J.L. Brodsky. 1996. Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. *J. Cell Biol.* 132:291–298.

Mizzen, L.A., and W.J. Welch. 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., and D.A. Goodenough. 1991. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J. Cell Biol.* 115:1357–1374.

Musil, L.S., and D.A. Goodenough. 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., B.A. Cunningham, G.M. Edelman, and D.A. Goodenough. 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., A.C. Le, J.K. VanSlyke, and L.M. Roberts. 2000. Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J. Biol. Chem.* 275:25207–25215.

Nakamura, T.Y., I. Yamamoto, Y. Kanno, Y. Shiba, and K. Goshima. 1994. Metabolic coupling of glutathione between mouse and quail cardiac myocytes and its protective role against oxidative stress. *Circ. Res.* 74:806–816.

Pariyarath, R., H. Wang, J.D. Aitchison, H.N. Ginsberg, W.J. Welch, A.E. Johnson, and E.A. Fisher. 2001. Co-translational interactions of apoprotein B with the ribosome and translocon during lipoprotein assembly or targeting to the proteasome. *J. Biol. Chem.* 276:541–550.

Parsell, D.A., and S. Lindquist. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* 27:437–496.

Pilon, M., R. Schekman, and K. Romisch. 1997. Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *EMBO J.* 16:4540–4548.

Plemper, R.K., and D.H. Wolf. 1999. Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends Biochem. Sci.* 24:266–270.

Plemper, R.K., S. Bohmler, J. Bordallo, T. Sommer, and D.H. Wolf. 1997. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature.* 388:891–895.

Plemper, R.K., R. Egner, K. Kuchler, and D.H. Wolf. 1998. Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *J. Biol. Chem.* 273: 32848–32856.

Plumier, J.C., H.A. Robertson, and R.W. Currie. 1996. Differential accumulation of mRNA for immediate early genes and heat shock genes in heart after ischaemic injury. *J. Mol. Cell. Cardiol.* 28:1251–1260.

Reits, E.A., A.M. Benham, B. Plougastel, J. Neefjes, and J. Trowsdale. 1997. Dynamics of proteasome distribution in living cells. *EMBO J.* 16:6087–6094.

Senisterra, G.A., S.A. Huntley, M. Escaravage, K.R. Sekhar, M.L. Freeman, M. Borrelli, and J.R. Lepock. 1997. Destabilization of the Ca2+-ATPase of sarcoplasmic reticulum by thiol-specific, heat shock inducers results in thermal denaturation at 37 degrees C. *Biochemistry.* 36:11002–11011.

Shamu, C.E., J.S. Cox, and P. Walter. 1994. The unfolded-protein-response in yeast. *Trends Cell Biol.* 4:56–60.

Sharp, F.R., A. Lu, Y. Tang, and D.E. Millhorn. 2000. Multiple molecular penumbras after focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 20:1011–1032.

Stam, N.J., H. Spits, and H.L. Ploegh. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J. Immunol.* 137:2299–2306.

Story, C.M., M.H. Furman, and H.L. Ploegh. 1999. The cytosolic tail of class I MHC heavy chain is required for its dislocation by the human cytomegalovirus US2 and US11 gene products. *Proc. Natl. Acad. Sci. USA.* 96:8516–8521.

Tortorella, D., C.M. Story, J.B. Huppa, E.J. Wiertz, T.R. Jones, I. Bacik, J.R. Bennink, J.W. Yewdell, and H.L. Ploegh. 1998. Dislocation of type I membrane proteins from the ER to the cytosol is sensitive to changes in redox potential. *J. Cell Biol.* 142:365–376.

Tsai, B., C. Rodighiero, W.I. Lencer, and T.A. Rapoport. 2001. Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell.* 104:937–948.

VanSlyke, J.K., and L.S. Musil. 2000. Analysis of connexin intracellular transport and assembly. *Methods.* 20:156–164.

VanSlyke, J.K., S.M. Deschenes, and L. Musil. 2000. Intracellular transport, assembly, and degradation of wild-type and disease-linked mutant gap junction proteins. *Mol. Biol. Cell.* 11:1933–1946.

Walter, J., J. Urban, C. Volkwein, and T. Sommer. 2001. Sec61p-independent degradation of the tail-anchored ER membrane protein Ubc6p. *EMBO J.* 20:3124–3131.

Wang, Y., and P.P. Mehta. 1995. Facilitation of gap-junctional communication and gap-junction formation in mammalian cells by inhibition of glycosylation. *Eur. J. Cell Biol.* 67:285–296.

Westwood, J.T., and R.A. Steinhardt. 1989. Effects of heat and other inducers of the stress response on protein degradation in Chinese hamster and *Drosophila* cells. *J. Cell. Physiol.* 139:196–209.

Wiertz, E.J., T.R. Jones, L. Sun, M. Bogyo, H.J. Geuze, and H.L. Ploegh. 1996a. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell.* 84:769–779.

Wiertz, E.J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, and H.L. Ploegh. 1996b. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*. 384:432–438.

Wigley, W.C., R.P. Fabunmi, M.G. Lee, C.R. Marino, S. Muallem, G.N. DeMartino, and P.J. Thomas. 1999. Dynamic association of proteasomal machinery with the centrosome. *J. Cell Biol.* 145:481–490.

Yancey, S.B., S.A. John, R. Lal, B.J. Austin, and J.P. Revel. 1989. The 43-kD polypeptide of heart gap junctions: immunolocalization, topology, and functional domains. *J. Cell Biol.* 108:2241–2254.

Yu, H., G. Kaung, S. Kobayashi, and R.R. Kopito. 1997. Cytosolic degradation of T-cell receptor alpha chains by the proteasome. *J. Biol. Chem.* 272:20800–20804.

Zhang, J.T., M. Chen, C.I. Foote, and B.J. Nicholson. 1996. Membrane integration of in vitro-translated gap junctional proteins: co- and post-translational mechanisms. *Mol. Biol. Cell.* 7:471–482.

Zhou, M., and R. Schekman. 1999. The engagement of Sec61p in the ER dislocation process. *Mol. Cell.* 4:925–934.

Zhou, M., X. Wu, and H.N. Ginsberg. 1996. Evidence that a rapidly turning over protein, normally degraded by proteasomes, regulates hsp72 gene transcription in HepG2 cells. *J. Biol. Chem.* 271:24769–24775.