Conformational Maturation and Post-ER Multisubunit Assembly of Gap Junction Proteins

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For all previously well-characterized oligomeric integral membrane proteins, folding, multisubunit assembly, and recognition of conformationally immature molecules for degradation occurs at their organelle of synthesis. This cannot, however, be the case for the gap junction-forming protein connexin43 (Cx43), which when endogenously expressed undergoes multisubunit assembly into connexons only after its transport to the *trans*-Golgi network. We have developed two novel assays to assess Cx43 folding and assembly: acquisition of resistance of disulfide bonds to reduction by extracellularly added DTT and Triton X-114 detergent phase partitioning. We show that Cx43 synthesized at physiologically relevant levels undergoes a multistep conformational maturation process in which folding of connexin monomers within the ER is a prerequisite for multisubunit assembly in the TGN. Similar results were obtained with Cx32, disproving the widely reported contention that the site of endogenous β connexin assembly is the ER. Exogenous overexpression of Cx43, Cx32, or Cx26 allows these events to take place within the ER, the first example of the TGN and ER as alternative sites for oligomeric assembly. Our findings also constitute the first biochemical evidence that defective connexin folding is a cause of the human disorder X-linked Charcot-Marie-Tooth disease.

INTRODUCTION

Many integral membrane proteins, including most that form channels, are oligomeric. The well-documented paradigm is that folding and multisubunit assembly of such proteins takes place at the same compartment as their synthesis, namely the endoplasmic reticulum (ER). Failure to acquire the proper stoichiometry causes the nascent protein to be recognized as abnormal by the ER “quality control” system and be destroyed before transport through the Golgi (Hurtley and Helenius, 1989; Ellgaard and Helenius, 2003).

The best characterized exception to this dogma is connexin43 (Cx43), a very widely expressed member of the connexin family of gap junction-forming integral membrane proteins. Gap junctions are clusters of intercellular channels that transfer ions, second messengers, and other low-molecular-weight substances between the cytosols of adjoining cells (Goodenough *et al.*, 1996). Defects in gap junction formation and/or function are the cause of at least 10 human disorders, including the second most common inherited peripheral neuropathy, X-linked Charcot-Marie-Tooth (CMTX) disease (Wei *et al.*, 2004; Alldredge, 2008). The first step in gap junction assembly is the noncovalent oligomerization of

six connexin monomers into a connexon, also known as a hemichannel. Two connexons on apposing cell surfaces then dock head-to-head to form a complete intercellular channel. Using four mechanistically distinct blockers of ER-to-Golgi transport and two independent assembly assays, Musil and Goodenough (1993) demonstrated that oligomerization of endogenously expressed Cx43 into a connexon occurred only after exit of Cx43 from the ER. Additional experiments indicated that connexon assembly takes place within a late Golgi compartment that has the properties of, and will be referred to throughout this study as, the *trans*-Golgi network (TGN). This novel site of multisubunit assembly was observed in all cell types examined and was subsequently confirmed by another group using similar methods (Koval *et al.*, 1997).

The discovery of a post-ER oligomerization pathway for an integral membrane protein raises important questions as to how protein synthesis, folding, multisubunit assembly, and quality control are spatially and temporally coordinated in the secretory pathway. In the case of Cx43, one possibility is that folding and oligomerization take place within the same subcellular compartment, but instead of being the ER this organelle is the TGN. Connexins that fail to acquire a native state within the TGN could then be directly delivered to the lysosome or returned to the ER for destruction, perhaps via one or more of the post-ER quality control mechanisms utilized by monomeric proteins (Arvan *et al.*, 2002; Trombetta and Parodi, 2003). Indeed, trafficking of connexins to either the lysosome (VanSlyke *et al.*, 2000; Qin *et al.*, 2003) or back to the ER (VanSlyke *et al.*, 2000) without delivery to the cell surface has been described. Alternatively, connexins could begin the folding process in the ER, but achieve conformational maturation only upon oligomerization into a connexon within the TGN.

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Abbreviations used: BFA, brefeldin A; CCCP, carbonyl cyanide *m*-chloro-phenyl hydrazone; CHX, cycloheximide; CMTX, X-linked Charcot-Marie-Tooth disease; Cx26, Cx32, Cx43, connexin26, -32, and 43, respectively; DTT, dithiothreitol; N-LS, *N*-lauroyl sarcosine; TX-100 and -114, Triton X-100 and -114, respectively.

A related, medically relevant issue is how disease-causing mutations in connexins prevent their trafficking to the cell surface. For other types of abnormal proteins, intracellular retention can be a result of either an inability to attain a mature conformation, or of the acquisition of an intracellular retention motif and/or the loss of an anterograde transport determinant in an otherwise normally folded protein (Ellgaard *et al.*, 1999). To date, knowledge of the posttranslational folding of wild-type or disease-causing mutant forms of connexins is very limited because of the lack of biochemical assays or antibodies capable of detecting such conformational changes. Another major question in gap junction biosynthesis is whether post-ER assembly of connexins into connexons is a general feature of the connexin family. Although sharing the same four-transmembrane domain topology and a relatively high degree of overall homology, differences between their primary sequences have led to the categorization of connexins into subfamilies, with Cx43 as the prototypical α connexin and connexins 32 and 26 classified as β connexins. Based mainly on studies in which Cx32 was exogenously overexpressed in tissue culture cells (Kumar and Gilula, 1992; Kumar *et al.*, 1995) or ER microsomes (Falk *et al.*, 1997) and on experiments utilizing forms of Cx43 and Cx32 that were mutated to contain an ER retention-retrieval motif (Sarma *et al.*, 2002; Maza *et al.*, 2005), it has been repeatedly stated in the primary literature (Kumar *et al.*, 1995; Sarma *et al.*, 2002; Gemel *et al.*, 2004; Maza *et al.*, 2005) and in reviews (e.g., Kumar and Gilula, 1996; Saez *et al.*, 2003; Berthoud *et al.*, 2004; Laird, 2005; Solan and Lampe, 2005; Koval, 2006) that Cx32 assembles into connexons within the ER or the ERGIC (endoplasmic reticulum–Golgi intermediate compartment). If true, this would imply that the mechanisms that govern the assembly of Cx43 and of Cx32 are fundamentally different and provide an explanation for why α and β connexins do not co-oligomerize when expressed within the same cell (Koval, 2006).

To address these issues, we have used novel as well as previously established biochemical assays to assess the conformation and oligomerization of Cx43, Cx32, and/or Cx26 within the secretory pathway. We show that the site of connexon assembly of endogenously expressed, wild-type Cx32 is indistinguishable from that of endogenously expressed, wild-type Cx43 in that oligomerization of both Cx43 and Cx32 takes place after exit from the ER in a late Golgi compartment likely to be the TGN. In addition to providing the initial insights into the process of post-ER assembly of a multisubunit polytopic protein, we demonstrate that disease-linked mutant forms of the β connexin Cx32 are conformationally abnormal, implicating connexin misfolding as a cause of the first reported connexin-linked disease, CMTX.

MATERIALS AND METHODS

Cell Culture

Normal rat kidney (NRK) fibroblasts and mouse sarcoma S180 cells were cultured in DMEM plus 10% FBS and antibiotics (penicillin G and streptomycin) as previously described (Musil *et al.*, 1990). The rat C6 glioma cell line and two stably connexin43-transfected clones, Cx43-13 and Cx43-14 (generated as described in Zhu *et al.*, 1991), were cultured in low-glucose DMEM plus 10% FBS and antibiotics. Clonal lines of PC12J cells (a subclone of rat pheochromocytoma cells devoid of endogenous gap junction activity) stably expressing either wild-type human Cx32 or CMTX-linked Cx32 mutants were generated and maintained in RPMI 1640 medium supplemented with 10% horse serum, 5% FBS, antibiotics, and 400 μ g/ml G418 as described by Deschenes *et al.* (1997). Expression of endogenous Cx32 in the rat hepatoma cell line MH₁C₁ (a generous gift from R. Ruch, Medical College of Ohio) was enhanced by culturing for 48 h in the presence of 10 μ M dexamethasone as previously described (Ren *et al.*, 1994). HepG2 cells (provided by C. Enns,

Oregon Health & Science University) and NRK LA25 cells (kind gift of B. Nicholson, University of Texas Health Science Center-San Antonio) were cultured as previously described, respectively (Zhou *et al.*, 1999; Zhang *et al.*, 2008). HeLa cells stably expressing wild-type human Cx32 (Kleopa *et al.*, 2002) were a kind gift of S. Scherer (University of Pennsylvania School of Medicine) and were maintained in low-glucose DMEM, 10% FBS, antibiotics, and 1 mg/ml G418. Primary cultures of epithelial cells were prepared from E10 embryonic chick lenses as described in Le and Musil (1998). Three-day-old, newly confluent cultures were used for all experiments unless otherwise specified.

Antibodies and Reagents

Immunoprecipitations were conducted using affinity-purified polyclonal rabbit antibodies against either Cx43 (prepared as described in Musil *et al.*, 1990), Cx32 (Zymed, South San Francisco, CA, 71-0600 or Sigma, St. Louis, MO, C3470), or Cx26 (Zymed, 71-0500). Brefeldin A (BFA; used at 6 μ g/ml) was from Epicentre Biotechnologies (Madison, WI). Triton X-114 (TX-114; Boehringer-Mannheim) was pre-condensed as described by Bordier (1981). All other reagents were from Sigma, unless specified otherwise.

Transient Transfection

HepG2 cells were transfected 1 d after plating with plasmids encoding LCAM (kind gift of W. Gallin, University of Alberta, Edmonton) or Cx26 (either subcloned into pDNA3 or into pCH110 after excision of the LacZ coding sequence using HindIII/BamHI), using FuGENE 6 (Roche Applied Science, Indianapolis, IN) as specified by the manufacturer. Cells were analyzed 48 h later.

Metabolic Labeling

Labeling and pulse-chase analysis of cultured cells was conducted as previously described (VanSlyke *et al.*, 2000). In brief, cells were metabolically labeled with [³⁵S]methionine (EXPRE³⁵S³⁵S; PerkinElmer, Waltham, MA), in DMEM lacking methionine supplemented with 5% dialyzed FBS and 2 mM glutamine for labeling at 37°C or in reduced bicarbonate labeling medium (Earle's MEM lacking methionine and containing 15 mM HEPES, 0.35 g/l bicarbonate, 2 mM glutamine, and 5% dialyzed FBS) for labeling at 15 or 20°C. Chases were conducted either in fresh complete culture medium (for chase at 37°C) or in reduced bicarbonate labeling medium plus serum (for chase at 15 or 20°C), supplemented with 0.5 mM nonlabeled methionine. Incubations at 15 or 20°C were conducted in an ambient air (no CO₂) refrigerator maintained at the appropriate temperature. Intact embryonic day 10 (E10) chick lenses were isolated and labeled as described in Musil *et al.* (1990).

In Situ Dithiothreitol Treatment of Metabolically Labeled Cells

At the end of the pulse or chase period, cell cultures were rinsed twice in DMEM plus 20 μ g/ml cycloheximide (DMEM/CHX) and then incubated for 5 min in DMEM + CHX supplemented with (freshly prepared) 2 mM dithiothreitol (DTT). The medium was removed and replaced with PBS containing 50 mM iodoacetamide. After a 10-min treatment on ice, the cells were rinsed once with PBS at 4°C and resuspended in lysis buffer (5 mM Tris base, 5 mM EDTA, 5 mM EGTA, 10 mM iodoacetamide, and 2 mM PMSF, pH 8.0) supplemented with 0.6% SDS, 250 μ g/ml soybean trypsin inhibitor, and 200 μ M leupeptin. Cells were lysed at either 100°C for 3 min (Cx43) or at room temperature for 30 min (Cx32). Cx32-containing samples were immunoprecipitated as described in Musil *et al.* (2000) using polyclonal anti-Cx32 antibodies (Sigma, C3470). Cx43-containing samples were treated identically, except that after the last wash Cx43 (still bound to the protein A-Sepharose beads by the anti-Cx43 antibodies) was dephosphorylated using calf intestinal alkaline phosphatase (Roche, 713023) as described in VanSlyke and Musil (2005). Immunoprecipitates were removed from the protein A-Sepharose beads in SDS-PAGE sample loading buffer (5 min at 100°C for Cx43 or 5 min at 37°C for Cx32) before analysis on 8% (Cx43) or 12% (Cx32) SDS-PAGE gels in which the concentration of Bis was increased to 1.2% and the running gel buffer was modified to contain 50 mM Tris, 380 mM glycine, and 0.15% SDS. The dried gels were analyzed on a Bio-Rad Personal FX Imager (Richmond, CA) using Quantity One software.

In Situ DTT Treatment of Cell Surface-biotinylated Cells

S180 cells were biotinylated at 4°C with sulfo-NHS-LC-biotin as described by VanSlyke and Musil (2005). After the reaction was quenched, the intact cells were incubated with DTT followed by iodoacetamide as described above for [³⁵S]met-labeled cells. Biotinylated Cx43 was then precipitated with streptavidin agarose, dephosphorylated, and analyzed by Western blot as described by VanSlyke and Musil (2005). Unlike in NRK and other assembly-efficient cell types, very little cell surface Cx43 is assembled into gap junctions in S180 cells, maximizing the accessibility of the extracellular domains of Cx43 to extracellularly added DTT (VanSlyke and Musil, 2005).

Figure 1. Acquisition of resistance of Cx43 to reduction of disulfide bonds by extracellularly added DTT. (A–C) NRK cells were pulse-labeled with [35 S]methionine for 10 min at 37°C (A and C) or for 30 min at 15°C (B) and chased for the times indicated at either 37 (A), 15 (B), or 20°C (C). The intact cells were then subjected to in situ treatment with 2 mM DTT (+), or mock-treated in the absence of DTT (–), at the same temperature as the chase as described in *Materials and Methods*. Cx43 was immunoprecipitated from all samples and analyzed on nonreducing SDS-PAGE gels, as was Cx43 metabolically labeled for 10 min at 37°C in the continuous presence of 2 mM DTT, which prevents the formation of disulfide bonds (rightmost lane). (D) S180 cells were cell surface biotinylated at 4°C and then subjected to in situ treatment with either 2 mM (lanes 2 and 5) or 20 mM (lanes 3 and 6) DTT. Biotinylated Cx43 was isolated and then boiled in SDS-PAGE sample buffer in either the presence (lanes 1–3) or absence (lanes 4–6) of 2% β ME. Cx43 was detected by anti-Cx43 immunoblotting. All immunoprecipitates in A–D were treated with alkaline phosphatase before SDS-PAGE to eliminate phosphorylation-induced shifts in the electrophoretic mobility of Cx43.

Triton Solubilization, Chemical Cross-Linking, and Sucrose Gradient Velocity Sedimentation Analysis

Metabolically labeled cells (whole cell lysates or $100,000 \times g$ membrane pellets) were resuspended in incubation buffer (136.8 mM NaCl, 5.36 mM KCl, 0.336 mM Na_2HPO_4 , 0.345 mM KH_2PO_4 , 0.8 mM MgSO_4 , 2.7 mM CaCl_2 , and 20 mM HEPES, pH 7.5) and solubilized in the presence of either 1% Triton X-100 (TX-100) or TX-114 on ice for 30 min. The cell extract was separated into Triton-insoluble and -soluble fractions by centrifugation at $100,000 \times g$ for 50 min at 4°C as previously described (Musil and Goodenough, 1993; VanSlyke *et al.*, 2000), after which the Triton-soluble fraction was brought to a final concentration of 500 mM NaCl.

For cross-linking, the Triton-soluble fraction (or the TX-114 detergent-rich or aqueous phase) was diluted with two volumes of incubation buffer/500 mM NaCl and then incubated for 30 min on ice with either 1 mM EGS [ethylene glycolbis(succinimidylsuccinate); Pierce, Rockford, IL], freshly diluted from a 100 mM stock in DMSO, or with 100 $\mu\text{g}/\text{ml}$ DSP [dithiobis(succinimidyl propionate); Pierce], from a freshly prepared 12.5 mg/ml stock in DMSO. The cross-linking reaction was stopped by addition of glycine as detailed in Musil and Goodenough (1993). SDS was added to a final concentration of 0.6%, and the sample was either incubated for 30 min at room temperature (before immunoprecipitation of Cx32 or Cx26) or boiled for 3 min (before immunoprecipitation of Cx43). Mock cross-linked control samples were treated identically but received DMSO only. Samples were immunoprecipitated as described in VanSlyke *et al.* (2000), using Zymed 71–0600 to isolate Cx32. Cross-linked immunoprecipitates were analyzed on 4–11% (Cx43), 6–12.5% (Cx32), or 7–14% (Cx26) polyacrylamide gradient gels after incubation of samples in SDS-PAGE sample loading buffer for either 5 min at 100°C (Cx43) or 5 min at 37°C (Cx32 or Cx26).

For sucrose gradient velocity sedimentation analysis, the Triton-soluble fraction (or, as in Figure 2A, the TX-114 aqueous phase in 0.1–0.3% Triton or the detergent-rich phase) was loaded onto a linear gradient of 5–20% sucrose in incubation buffer and 0.1% Triton. The gradients were subjected to centrifugation for 12 h, fractionated, and analyzed for Cx43 as described in Musil and Goodenough (1993).

TX-114 Phase Partitioning

A modification of the technique described by Maher and Singer (1985) was used. TX-114-soluble fractions were warmed to 30°C for 3 min before centrifugation at $550 \times g$ for 3 min at room temperature to generate a detergent-rich pellet and a detergent-poor (aqueous) upper phase. The upper phase was transferred to a fresh tube on ice, brought to 1% TX-114, and subjected to a second round of partitioning. The two detergent-rich pellets were combined and diluted with incubation buffer/500 mM NaCl to the same volume as the detergent-poor fraction.

Detergent Disruption of Connexons

The TX-114 aqueous (e.g., connexon-containing) fraction was prepared from NRK cells labeled at 20°C. In the experiment shown in Figure 3B, the TX-114 aqueous fraction was incubated for 1 h at 25°C with 0.5% octyl-polyoxyethylene (8-POE) detergent. The sample was then fractionated at 4°C on a 5–20%

linear sucrose gradient containing 0.1% TX-114. Fractions in which monomeric Cx43 is known to migrate (5S; 8–11% sucrose) were collected at 4°C and pooled, as were fractions in which connexon-assembled Cx43 is recovered (9S; 14–17% sucrose). Both the 5S and 9S pools were brought to 1% TX-114 and analyzed by TX-114 phase partitioning at 30°C before immunoprecipitation of Cx43. In the experiment shown in Figure 4, a TX-114 aqueous fraction was cross-linked with EGS. One-half of the sample was incubated without (control), and the other half with, 1% *N*-lauroyl sarcosine (*N*-LS) in the presence of 20 mM DTT for 30 min at 25°C. The DTT was quenched by addition of 50 mM NEM, and both samples were subjected to centrifugation through a 3.5-ml cushion of 5% sucrose/0.1% TX-114/incubation buffer in a Beckman SW60 rotor (Fullerton, CA) at 49,000 rpm for 12 h at 4°C. The samples were collected from the tube bottom, brought to 1% TX-114, and subjected to phase partitioning at 30°C before immunoprecipitation of Cx43.

RESULTS

Cx43 Disulfide Bonds Undergo Conformational Maturation within the ER

All mammalian gap junction-forming connexin family members possess highly conserved cysteine residues in each of their two extracellular loops that participate in intramolecular (but not intermolecular) disulfide bond formation (John and Revel, 1991; Rahman *et al.*, 1993; Foote *et al.*, 1998; Sohl and Willecke, 2003). Mutagenesis of extracellular cysteine residues has established a strong correlation between the ability of connexins to form paired disulfide bonds and their capacity to mediate intercellular gap junctional coupling (Dahl *et al.*, 1991; Foote *et al.*, 1998). As is true of most proteins (Allore and Barber, 1984), [35 S]met-Cx43 migrates more slowly on SDS-PAGE in the absence of intramolecular disulfide bonds (e.g., if synthesized in the presence of the reducing agent DTT at 2 mM) than with them. Even after only a 10-min labeling period, [35 S]met-Cx43 migrated almost exclusively in an oxidized form (Figure 1A, cf. lane 1 with lane 7), indicating that disulfide bond formation is a rapid and highly efficient process.

The intramolecular disulfide bonds of many (although not all) newly synthesized and still conformationally immature proteins can be reduced in situ by subjecting living cells to a 5-min incubation with the membrane-permeant reducing agent DTT (2 mM). As the protein attains its properly folded state, many of these disulfide bonds become buried within the protein's tertiary structure and acquire resistance to this

treatment. The sensitivity of such a protein to reduction by extracellularly added DTT is therefore an assay of its folding status (Braakman *et al.*, 1992; Lodish *et al.*, 1992; Lodish and Kong, 1993; Opstelten *et al.*, 1993; Tatu *et al.*, 1993). Exposure of intact NRK cells to 2 mM DTT for 5 min immediately after a 10-min pulse caused the majority of immunoprecipitated [³⁵S]met-Cx43 to migrate on nonreducing SDS-PAGE in the same position as fully reduced [³⁵S]met-Cx43 (cf. lane 2 with lane 7), indicating that its disulfide bonds were sensitive to *in situ* reduction. The remaining pulse-labeled Cx43 continued to migrate as a fully oxidized species. After 30 min of chase, a greater fraction of the [³⁵S]met-Cx43 acquired resistance to *in situ* reduction (lane 4), becoming completely insensitive by 1 h (lane 6).

We next examined the sensitivity to *in situ* reduction of [³⁵S]met-Cx43 metabolically labeled and chased at 15°C, a condition that slows protein folding and blocks the transport of newly synthesized secretory pathway proteins to the Golgi in mammalian (but not amphibian) cells (Colman *et al.*, 1984; Saraste and Kuismanen, 1984; Schweizer *et al.*, 1990). It had previously been shown that Cx43 synthesized at this temperature can undergo all of the folding and assembly steps required to be fully functional when expressed in *Xenopus* oocytes (Musil and Goodenough, 1993). Figure 1B shows the near quantitative conversion of [³⁵S]met-Cx43 in NRK cells at 15°C from a DTT-sensitive to a DTT-resistant form, confirming the precursor-product relationship between these two species and establishing acquisition of a DTT-insensitive conformation as a pre-Golgi event (Cx43 also became DTT resistant at 37°C in the presence of the intracellular transport inhibitor BFA; data not shown). In principle, decreased accessibility of disulfide bonds to extracellularly added DTT could be caused by tight binding of Cx43 to another protein instead of by connexin folding. This possibility is, however, extremely unlikely given that endogenously expressed Cx43 is detected within the ER exclusively as a free monomer by either sucrose gradient sedimentation or chemical cross-linking when synthesized at 15–37°C (Musil and Goodenough, 1993).

If folding of Cx43 to a DTT-insensitive state takes place within the ER, then it would be expected that post-ER Cx43 pools would be completely resistant to *in situ* reduction. Indeed, Cx43 chased at a temperature at which anterograde transport through the secretory pathway is stalled within the TGN (20°C; Saraste and Kuismanen, 1984; Griffiths and Simons, 1986) was not reduced by extracellularly added DTT (Figure 1C, lane 4). To examine Cx43 on the plasma membrane, cultures were cell surface biotinylated under conditions previously shown to restrict conjugation of NHS-LC-biotin to Cx43 on the plasmalemma (Musil and Goodenough, 1991; VanSlyke and Musil, 2005; Figure 1D). Incubation of the intact cells with either 2 mM DTT (lane 5) or 20 mM DTT (lane 6) at 37°C had no effect on the migration of biotinylated Cx43 on nonreducing SDS-PAGE, indicating that the disulfide bonds had remained intact (cf. with lane 4; note that Cx43 in lanes 4–6 migrates more rapidly than cell surface biotinylated Cx43 subjected to reduction immediately before SDS-PAGE; lanes 1–3). This is in contrast to certain other proteins, which remain sensitive to DTT-induced reduction of disulfide bonds throughout the secretory pathway (Massague and Czech, 1982). Taken together, these results are consistent with the possibility that folding of Cx43 to a DTT-resistant conformation is a requirement for its proper assembly and transport to the cell surface. For additional support for this contention, see Figure 9.

A Novel Conformation-sensitive Assay of Cx43 Multisubunit Assembly

Current biochemical assays for connexon assembly measure the difference in molecular mass between monomeric and hexameric connexin species without providing any information as to the conformational state of the oligomer. Assessing the extent to which a detergent-solubilized connexon is properly folded is becoming increasingly important given the now frequent use of connexons reconstituted into lipid vesicles in the study of connexin channel permeability, gating, and structure (Kim *et al.*, 1999; Thimm *et al.*, 2005; Ayad *et al.*, 2006; Bao *et al.*, 2007; Oshima *et al.*, 2007). TX-114 is a nonionic detergent highly related to TX-100 that separates into a detergent-enriched (abbreviated hereafter as D) and a less dense, detergent-depleted (aqueous; abbreviated as A) phase when briefly heated above its cloud-point temperature of 20°C (Bordier, 1981). Partitioning in TX-114 has been extensively used to distinguish membrane-associated proteins (recovered in the D phase) from more hydrophilic species (recovered in the A phase) (Sanchez-Ferrer *et al.*, 1994). There are, however, proteins that behave anomalously. For example, Maher and Singer (1985) have reported that the fully assembled pentameric nicotinic acetylcholine receptor channel, a complex comparable in size to a Cx43 connexon that contains a total of 20 transmembrane domains, unexpectedly fractionated into the aqueous TX-114 phase. Several proteins have been reported to undergo conformation-induced changes in TX-114 partitioning (Seth *et al.*, 1985; Sandvig and Moskaug, 1987; Yoshimura *et al.*, 1987; Skern *et al.*, 1991; Martayan *et al.*, 1997; Day *et al.*, 2002). This includes the insulin receptor, a multimeric integral plasma membrane protein that partitions into the TX-114 detergent-enriched phase when unliganded but switches to the aqueous fraction upon insulin-induced changes in conformation (Florke *et al.*, 1993). Such studies raised the possibility that oligomerization into a connexon and the concomitant change in conformation might cause a switch in the partitioning of Cx43 in TX-114. This was investigated by metabolically labeling NRK cells with [³⁵S]methionine at 20°C, a temperature that allows trafficking of newly synthesized Cx43 to the site of connexon assembly (the TGN) but inhibits its transport to the cell surface and thus its incorporation into Triton-insoluble gap junctional plaques. The labeled cells were incubated with 1% TX-114 at 4°C (well below the detergent's cloud-point), and the solubilized material resolved by centrifugation on a 5–20% linear sucrose gradient. If the total TX-114-soluble lysate was loaded onto a sucrose gradient without prior phase partitioning, two peaks of [³⁵S]met-Cx43 immunoreactivity were resolved that have previously been shown to represent monomeric (5S) and connexon-assembled (9S) forms of the protein (Musil and Goodenough, 1993; Figure 2A, T). The lower two gradient profiles in Figure 2A depict the results obtained if the TX-114-soluble lysate was instead phase-partitioned at 30°C, and the resulting detergent-rich and aqueous fractions were analyzed separately on sucrose gradients. The detergent-rich (D) phase contained monomeric, 5S Cx43, as expected for a typical integral membrane protein, but no detectable Cx43 in the 9S position. Instead, the connexon-assembled, 9S Cx43 was nearly quantitatively recovered in the aqueous (A) phase.

A similar result was obtained if cells were labeled and lysed in TX-114 at 4°C as in Figure 2A and then assayed for connexon assembly using a second previously characterized method, chemical cross-linking with the homobifunctional reagent DSP (Figure 2B). Cross-linking of the total TX-114-

Figure 2. Cx43 connexons partition into the TX-114 aqueous phase. NRK cells were metabolically labeled with [35 S]methionine at 20°C to allow radiolabeled Cx43 to assemble into connexons. The cells were then lysed in 1% TX-114 at 4°C, and the solubilized material analyzed as described below. In A, half of the lysate was subjected to TX-114 phase partitioning at 30°C into detergent-rich (middle panel, D) and detergent-poor aqueous (bottom panel, A) phases before fractionation on 5–20% linear sucrose gradients. The other half of the lysate was fractionated on gradients without prior phase partitioning (top panel, T). Equal fractions were collected from the sucrose gradients and analyzed for Cx43 content by immunoprecipitation followed by SDS-PAGE. In B, one-half of the lysate was TX-114 phase-partitioned into detergent-rich (D) and detergent-poor aqueous (A) phases, and the other half remained unpartitioned (T). The samples were then subjected to chemical cross-linking with DSP (+) or mock cross-linked with DMSO only (–) as indicated, followed by immunoprecipitation of Cx43 and SDS-PAGE. In C, the entire lysate was cross-linked with DSP. One-half of the cross-linked lysate was then TX-114 phase-partitioned into detergent-rich (D) and detergent-poor aqueous (A) phases, whereas the other half remained unpartitioned (T). Cx43 was immunoprecipitated and analyzed by SDS-PAGE. cxon, connexon; m, monomer. Bands migrating at >220 kDa in this and subsequent figures are nonspecifically immunoprecipitated and probably represent binding of newly synthesized fibronectin to the protein A-Sepharose beads (Musil and Goodenough, 1993).

soluble lysate without prior phase partitioning yielded both monomeric Cx43 ($M_r = 40$ kDa) and a cross-linked ~200 kDa homo-oligomer that has previously been determined to be a connexon (Musil and Goodenough, 1993; lane 2). When an identically prepared TX-114 lysate was first phase-partitioned and then subjected to cross-linking, the detergent phase contained only monomeric Cx43 (lane 4), whereas Cx43 that could be cross-linked into a connexon was recovered exclusively in the aqueous phase (lane 6). Cx43 from TX-114 lysates that were cross-linked before phase separation behaved identically in that the monomeric and connexon-assembled forms partitioned into the detergent-rich and aqueous fractions, respectively (Figure 2C). Comparable results were obtained in other Cx43-expressing cell types (e.g., LA25 NRK cells; HEK293-Cx43 transfectants), if lysates were cross-linked with EGS instead of DSP, or if cells were radiolabeled at 37°C instead of 20°C to permit unrestricted transport to the plasma membrane (data not shown).

If partitioning of Cx43 into the detergent-depleted (A) TX-114 phase is causally linked to its assembly into connexons, then it would be expected that 1) Cx43 fractionates into the detergent-rich phase under conditions that block its

oligomerization and 2) dissociation of connexons into individual subunits would cause Cx43 to shift from the A to the D fraction. To test the first prediction, NRK cells were metabolically labeled for 4–5 h at either 37°C in the presence of BFA or at 15°C, treatments that block the transport of newly synthesized proteins before the TGN and as a result prevent assembly of endogenous Cx43 into connexons (Musil and Goodenough, 1993). In both cases, [35 S]met-Cx43 partitioned nearly completely into the detergent-rich phase (Figure 3A, lanes 1–6). A similar result was obtained when cells were pulsed for 10 min at 37°C, a period previously shown to be too brief to allow significant amounts of the newly synthesized Cx43 to assemble into connexons (Musil and Goodenough, 1993; Figure 3A, lanes 7–9). To test the second prediction, we used the nonionic detergent 8-POE. Although somewhat more denaturing than either TX-100 or TX-114, 8-POE is considered to be a relatively mild solubilizing agent and preserves the conformation of most proteins (e.g., Gilboa *et al.*, 1998). Nonetheless, 0.5–1% 8-POE at 25°C caused Cx43 connexons to dissociate into monomers as indicated by two types of experiments: 1) Cx43 connexons recovered in the aqueous phase after TX-114 partitioning

Figure 3. Cx43 only partitions into the TX-114 aqueous phase when it is assembled into a connexon. (A) NRK cells were metabolically labeled under the conditions indicated. Cells were solubilized in 1% TX-114 at 4°C, and the lysates analyzed by TX-114 phase partitioning at 30°C before immunoprecipitation of Cx43 and SDS-PAGE. (B) NRK cells were labeled for 5 h at 20°C. A TX-114 aqueous (connexon-containing) fraction was prepared, treated with 8-POE detergent, and then centrifuged on a 5–20% linear sucrose gradient. Gradient fractions in which monomeric Cx43 is known to migrate (5S) were collected at 4°C and pooled, as were fractions in which connexon-assembled Cx43 is recovered (9S). Both pools were analyzed by TX-114 phase partitioning at 30°C before immunoprecipitation of Cx43. See *Materials and Methods* for experimental details. (C) NRK cells were labeled for 15 h at 20°C before solubilization in 0.5% TX-114 at 4°C. The lysates were incubated for 1 h at 25°C with no additions (lanes 1 and 2) or with 1% (final concentration) of octyl-polyoxyethylene (POE), N-lauroyl sarcosine (N-LS), or SDS. The samples were then cross-linked with DSP before immunoprecipitation of Cx43 and SDS-PAGE.

Figure 4. Partitioning of Cx43 connexons into the TX-114 aqueous phase is sensitive to the folding status of Cx43. NRK cells were labeled for 4 h at 20°C. A TX-114 aqueous (connexon-containing) fraction was prepared and cross-linked with EGS. One-half of the sample was incubated without (control), and the other half with, 1% *N*-lauroyl sarcosine (N-LS) in the presence of 20 mM DTT. After detergent exchange, the samples were subjected to TX-114 phase partitioning at 30°C before immunoprecipitation of Cx43. See *Materials and Methods* for details.

and then subjected to a 60-min incubation with 8-POE subsequently migrated exclusively in the 5S, monomeric position on sucrose density gradients (Figure 3B, lane 1 vs. 4) and 2) NRK cells labeled under conditions (15 h at 20°C) that maximize the recovery of connexons after lysis in TX-114 (Figure 3C, lane 2) yielded no connexons if the TX-114 lysates were treated for 1 h at 25°C with 8-POE before cross-linking (Figure 3C, lane 3). When the monomeric [³⁵S]met-Cx43 that resulted from 8-POE-induced dissociation of connexons was subsequently subjected to TX-114 phase partitioning, it was recovered exclusively in the detergent phase (Figure 3B, lane 3).

The size and surface-to-mass ratio of a connexon differs significantly from that of a connexin monomer. To address if this is the sole determinant of the partitioning behavior of Cx43 in TX-114, we used the harsh denaturing detergent *N*-lauroyl sarcosine (N-LS). Preliminary experiments demonstrated that, as expected, no oligomeric forms of Cx43 were recovered if connexon-containing TX-114 lysates were exposed to 1% N-LS for 1 h (Figure 3C, lane 4). In the experiment shown in Figure 4, NRK cells were radiolabeled at 20°C to accumulate [³⁵S]met-Cx43 connexons, after which the cells were lysed with TX-114 at 4°C and the aqueous (connexon) fraction cross-linked with EGS. The samples were then incubated at 25°C without additions (control) or with 1% N-LS in the presence of 20 mM DTT (followed by the alkylating agent NEM) to maximize the unfolding of Cx43. After detergent exchange by sedimentation through 5% sucrose/0.1% TX-114, the samples were partitioned in TX-114 at 30°C. All of the Cx43 from the control (Triton only) sample was recovered in the aqueous phase (lanes 1–3). In contrast, more than half of the connexons in the N-LS-treated sample now partitioned into the detergent-rich phase despite being maintained in a hexameric state by the EGS cross-links (lanes 4–6). Control experiments (not shown) demonstrated that N-LS pretreatment did not alter the subsequent partitioning of either monomeric Cx43 (in the D phase) or of soluble proteins such as BSA (in the A phase) after the samples had been restored to 1% TX-114. We

conclude that the partitioning behavior of Cx43 connexons is sensitive to the conformational state of the Cx43 molecule. The inability of N-LS exposure to quantitatively switch connexons to the D phase could be due to partial renaturation of Cx43 during overnight centrifugation in the absence of N-LS and/or to the presence of intra- and intermolecular EGS-induced cross-links that prevent the protein from unfolding completely. In preliminary studies, we have found that connexons composed of Cx26 partition exclusively into the TX-114 detergent phase, whereas Cx32 connexons are consistently recovered in both aqueous and detergent fractions (not shown). These findings rule out a simple model in which the TX-114 partitioning behavior of connexins can be explained by masking of hydrophobic (TX-114 binding) regions of connexin monomers at subunit interfaces during connexon assembly, and further support the contention that assembly-associated changes in the conformation of Cx43 underlie its partitioning behavior.

Intracellular Site of Connexon Assembly

The contention that Cx32, unlike Cx43, assembles into connexons within the ER is based on studies of cells transfected with constructs driving either very high-level expression of wild-type Cx32 (Kumar and Gilula, 1992; Kumar *et al.*, 1995) or mutant forms of Cx32 engineered to contain an ER retention motif (Sarma *et al.*, 2002; Maza *et al.*, 2005). To determine the site of assembly of endogenously expressed, wild-type Cx32, HepG2 hepatoma cells were metabolically labeled at 37°C in either the absence or presence of BFA before lysis at 4°C in TX-100 and chemical cross-linking. BFA is one of three inhibitors of ER-to-Golgi transport previously shown to block connexon assembly of endogenous Cx43 in multiple cell types (Musil and Goodenough, 1993; see Figure 5C). Although BFA did not reduce the amount of [³⁵S]met-Cx32 synthesized, anti-Cx32 immunoprecipitable complexes migrating at the position of a hexameric connexon were recovered only from the untreated cells (Figure 5A, lanes 1–4). Similar results were obtained with a second anti-Cx32 antibody (not shown).

BFA inhibits anterograde trafficking of newly synthesized proteins from the ER to the Golgi without preventing retrograde transport of Golgi constituents back to the ER. Although BFA does not perturb the oligomerization of a wide variety of proteins known to be assembled within the ER (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989; Hille *et al.*, 1990; Collins and Mottet, 1991), it was theoretically possible that the lack of Cx32 connexon assembly in BFA-treated cells was due to BFA-induced mixing of ER and Golgi contents instead of the block in exit from the ER. The former process can be selectively inhibited by incubating cells with BFA in the presence of monensin (Barzilay *et al.*, 2005). Control experiments in which acquisition of endoglycosidase H resistance of the N-linked glycoprotein LCAM was used as an assay of ER-Golgi mixing confirmed that addition of 10 μM monensin efficiently inhibited Golgi-to-ER retrograde transport in BFA-treated HepG2 cells (Supplemental Figure 1S). As was observed with BFA alone, [³⁵S]met-Cx32 synthesized in the presence of both BFA and monensin remained monomeric (Figure 5A, lane 7). We conclude that the lack of Cx32 assembly in BFA-treated HepG2 cells is a direct result of blocking trafficking of [³⁵S]met-Cx32 out of the ER. The low level of endogenous Cx32 expression in all cell lines examined precluded the use of other established blockers of intracellular transport (e.g., carbonyl cyanide *m*-chloro-phenyl hydrazone [CCCP]; 15°C) because of their inhibitory effect on protein synthesis.

Figure 5. Intracellular site of connexon assembly of endogenously expressed and exogenously overexpressed Cx32. The indicated cell lines were metabolically labeled with [35 S]methionine for 4–5 h, in either the absence (– BFA) or presence (+ BFA) of 6 μ g/ml of the ER-to-Golgi transport inhibitor brefeldin A (BFA). To minimize the assembly of radiolabeled connexons into (Triton-insoluble) gap junctions in cells expressing high levels of exogenous Cx32, labeling of PC12, HeLa, and NRK cell transfectants in the absence of BFA was conducted at 20°C. All other labelings were performed at 37°C. The samples in lanes 5, 6, and 7 of panel A were prepared from cells transiently transfected with a plasmid encoding LCAM (see Supplemental Figure 1S) and metabolically labeled in either the presence of BFA (lane 6), BFA plus 10 μ M monensin (lane 7), or with no additions (lane 5). After labeling, all cells were lysed in TX-100, and the solubilized material was either cross-linked with EGS (+) or mock cross-linked with DMSO only (–). Anti-Cx32 or (5C, lanes 9–12) anti-Cx43 immunoprecipitates were prepared and analyzed by SDS-PAGE. To minimize nonspecific background on the gels at M_r >100 kDa, the concentration of anti-Cx32 antibody used for the MH1C1 immunoprecipitates was reduced to below that required to efficiently recover connexin monomers. Immunoprecipitation of uncrosslinked samples with saturating amounts of anti-Cx32 antibody verified that the input levels of [35 S]met-Cx32 in the samples analyzed in lanes 1–3 in panel B were equivalent (not shown).

We next examined the site of assembly of exogenously expressed Cx32 in stable HeLa and NRK cell transfectants. In both cell types, Cx32 formed intercellular gap junctions as assessed by anti-Cx32 immunolocalization (data not shown), as expected from the findings of others (Mitic *et al.*, 1999; Zhou *et al.*, 1999; Kleopa *et al.*, 2002). Unlike in HepG2 cells, however, the ability of [35 S]met-Cx32 to be cross-linked into a connexon was not blocked when exit of newly synthesized protein from the ER was prevented by BFA (Figure 5C, lanes 1–8). NRK cells endogenously express Cx43, previously shown to oligomerize into connexons in a BFA-inhibitable manner (Musil and Goodenough, 1993). The presence of Cx32 did not alter this sensitivity (lanes 9–12), indicating that the same cell can simultaneously support the assembly of (exogenous) Cx32 in the ER and (endogenous) Cx43 within the TGN. Cx32 also formed connexons within the ER in stable PC12 cell transfectants (Figure 5B, lanes 4–6), but not in MH1C1 hepatoma cells in which Cx32 is native (lanes 1–3).

Taken together, these results demonstrate that assembly of Cx32 into connexons can be initiated in either the TGN or the ER. One possible explanation for these findings is that the site of oligomerization is dictated not by the connexin species, but by the level at which the connexin is synthesized such that overexpression results in premature assembly within the ER. This was tested using C6 glioma cells, which endogenously synthesize low levels of Cx43, and two stably Cx43-transfected clones that express Cx43 at levels either threefold (Cx43-14 cells) or 30-fold (Cx43-13 cells) higher than the untransfected parental cells (Zhu *et al.*, 1991; Charles *et al.*, 1992; Naus *et al.*, 1992). Anti-Cx43 immunocytochemistry verified relatively uniform expression of Cx43 within each cell line, with little cell-to-cell variation (Supplemental Figure 2S). Assembly of [35 S]met-Cx43 was inhibited by the inclusion of BFA in the labeling medium in both the parental (Figure 6A, lane 4) and low-expressing (lane 6) cells. In contrast, connexons could readily be cross-linked from the high-expressing cell line even in the presence of

Figure 6. High-level stable overexpression of Cx43 switches the site of onset of connexon assembly to the ER. (A) Stable C6 cell transfectants expressing Cx43 at levels threefold (Cx43-14) or 30-fold (Cx43-13) over that of untransfected parentals (C6) were labeled with [35 S]methionine for 4 h at 37°C, in either the absence (– BFA) or presence (+ BFA) of BFA. TX-100-soluble lysates were prepared and either cross-linked with EGS (+) or mock cross-linked (–) before immunoprecipitation of Cx43 and analysis by SDS-PAGE. (B–E) Characterization of connexon formation within the ER in Cx43-13 cells. (B) C6 Cx43-13 cells were pulse labeled for 15 min without (lanes 1 and 2) or with (lanes 3 and 4) a subsequent 2-h chase, all at 37°C and in the continuous presence of BFA. Cx43 was immunoprecipitated from Triton-soluble lysates either with (+) or without (–) prior cross-linking with EGS. (C) C6 Cx43-13 cells were labeled for 3.5 h at 37°C in the presence of BFA and lysed in TX-100, and the solubilized material was fractionated on 5–20% linear sucrose gradients. Fractions known to contain monomeric (5S; 8–11% sucrose) and connexon-assembled (9S; 14–17% sucrose) forms of Cx43 were collected and then subjected to cross-linking with 500 μ g/ml DSP before immunoprecipitation of Cx43. (D) Cx43-13 cells labeled for 3 h at 37°C in the presence of BFA were lysed in 1% TX-114 at 4°C. After chemical cross-linking with EGS, one-half of the lysate was TX-114 phase-partitioned at 30°C into detergent-rich (D) and detergent-poor aqueous (A) phases, and the other half remained unpartitioned (T). Immunoprecipitated Cx43 was analyzed by SDS-PAGE. (E) Cx43-13 cells were pulsed for 1 h at 37°C in the presence of BFA either with (+ DTT) or without (– DTT) 2 mM DTT. Triton-soluble lysates were prepared, cross-linked with EGS (+) or left uncross-linked (–), and Cx43 immunoprecipitates were analyzed by SDS-PAGE.

BFA (lane 10), indicating that the intracellular site at which most Cx43 connexon assembly is initiated had been shifted to within the ER. Connexons were also recovered from another stably transfected cell line that expresses ~30-fold higher levels of Cx43 than NRK cells (21MT-2 mammary tumor cells; Tomasetto *et al.*, 1993) when ER exit was blocked using either BFA or CCCP (Musil and Goodenough, 1993; Supplemental Figure 3S).

Properties previously used to confirm that the ~200-kDa, anti-Cx43 immunoprecipitable oligomer recovered from cells endogenously expressing Cx43 is an authentic connexon include 1) a precursor-product relationship with monomeric Cx43 as established by pulse-chase analysis and 2) its migration on a 5–20% linear sucrose gradient in the 9S position (Musil and Goodenough, 1993). Connexons formed in C6 Cx43-13 cells in the presence of BFA met both of these criteria (Figure 6, B and C). Moreover, they were recovered in the detergent-depleted (aqueous) fraction after phase partitioning in TX-114 (Figure 6D). The latter finding further supports the contention that the partitioning behavior of

Cx43 is determined by its oligomeric state and not by its intracellular location. [35 S]met-Cx43 synthesized in the continuous presence of levels of DTT that prevent intramolecular disulfide bond formation (see Figure 1A, lane 7) could not be cross-linked to a connexon in BFA-treated C6 Cx43-13 cells (Figure 6E, lane 4). Because connexons can form within the ER in Cx43-13 cells, this lack of connexon assembly cannot be attributed to an inhibitory effect of DTT on connexin transport. Instead, this finding is in keeping with a direct requirement for disulfide bonds in wild-type connexon assembly.

Next, we examined the site of connexon assembly of another β -type connexin, Cx26. When HepG2 cells were transiently transfected with a plasmid that directs high-level expression of Cx26 from the robust CMV promoter, Cx26 could be cross-linked into a connexon when synthesized in either the absence or presence of BFA (Figure 7, lanes 1–4). Similar results were obtained in cells in which BFA-induced Golgi-to-ER fusion was blocked by monensin (data not shown), indicative of assembly within the ER. In contrast,

Figure 7. High-level transient overexpression of Cx26 switches the site of onset of connexon assembly to the ER. HepG2 cells were transiently transfected with one of two Cx26-encoding plasmids: a CMV-driven construct with an intact polyA tail (lanes 1–4) or a SV40-driven construct in which the polyA tail had been deleted that expressed ~60-fold lower levels of Cx26 protein (lanes 5–8). On day 2 after transfection, the cells were labeled with [³⁵S]methionine for 4 h at 37°C in either the absence (– BFA) or presence (+ BFA) of BFA. TX-100-soluble lysates were prepared and either cross-linked with EGS (+) or mock cross-linked (–) before immunoprecipitation of Cx26 and analysis by SDS-PAGE.

connexon assembly was completely blocked by BFA in HepG2 cells expressing ~60-fold lower levels of Cx26 after transfection with a Cx26-encoding plasmid driven by the SV40 promoter in which transcripts were made less stable by deletion of the polyA tail (lanes 5–8). Thus each of the connexins examined (Cx43, Cx32, and Cx26) has the capacity to assemble into connexons either within the ER or only after ER exit depending on its level of expression.

All of the aforementioned experiments were conducted using established cell lines. To rule out the formal possibility that oligomerization of connexons within the TGN is somehow associated with long-term clonal cell culture, we examined the assembly of Cx43 in 3-d-old primary cultures of embryonic chick lens epithelial cells (Le and Musil, 2001). As in other endogenously expressing cell types, [³⁵S]met-Cx43 in chick lens cell monolayers could not be cross-linked into a connexon when synthesized in the presence of BFA (Figure 8, lane 4), whereas connexons were readily recovered under conditions that allowed transport to the TGN (37 and 20°C;

lanes 3 and 5). Similar results were obtained when [³⁵S]met-Cx43 was analyzed from intact lenses metabolically labeled immediately after dissection from E10 chick embryos (lanes 6 and 7).

Defective Folding of Disease-causing Cx32 Mutants

In principle, the lack of transport of CMTX disease-linked mutant forms of Cx32 to the cell surface could be due to either 1) an inability to attain a mature conformation or 2) the acquisition of an intracellular retention motif and/or the loss of an anterograde transport determinant in an otherwise properly folded protein (Hong, 1998; Ellgaard *et al.*, 1999). Distinguishing between these two mechanisms is of therapeutic significance given that relieving ER retention can rescue the cell surface localization and function of the latter class of mutant membrane proteins (Kupersmidt *et al.*, 2002; Chang *et al.*, 1999; Taschenberger *et al.*, 2002). This was demonstrated for connexins by Minogue *et al.* (2005), who reported that truncating the sequence encoding the cataract-causing Cx46fs380 mutant immediately 5' of the defective codon restored the connexin's ability to be transported to the plasma membrane and form gap junctions. We have previously shown that each of three CMTX-linked Cx32 point mutants stably expressed in PC12 cells exhibits a distinct trafficking defect: E208K Cx32 accumulates only in the ER, whereas the E186K and R142W mutants are both detected in the Golgi region but differ in that R142W Cx32 can enter the endosome/lysosome system and E186K Cx32 undergoes retrograde transport back to the ER. None of these mutants can be cross-linked to a connexon (VanSlyke *et al.*, 2000).

To investigate whether these trafficking and assembly abnormalities could be due to defects in protein folding, we assessed the conformational state of wild-type and mutant forms of Cx32 using the techniques described in Figure 1 for Cx43. We found that the disulfide bonds in newly synthesized, pulse-labeled wild-type Cx32 were initially sensitive to reduction by extracellularly added DTT but rapidly acquired resistance to *in situ* DTT treatment, similar to what was observed for Cx43 (Figure 9A). Because of the efficiency of this process, virtually all of the wild-type Cx32 labeled under near steady-state conditions (4-h pulse) in PC12 cells at 37°C was insensitive to extracellularly added DTT (Figure 9B; note that the bands in lanes 1 and 2 comigrate). Control experiments demonstrated that all three CMTX-linked Cx32 mutants formed intramolecular disulfide bonds when ex-

Figure 8. Post-ER assembly of connexons in lens cells in primary culture and in intact lens. Three-day-old cultures of primary embryonic chick lens epithelial cells (lanes 2–5) or freshly dissected intact E10 chick lenses (lanes 6 and 7) were metabolically labeled with [³⁵S]methionine for 4 h at either 37°C with no additions (lanes 2, 3, and 6), 37°C in the presence of BFA (lanes 4 and 7), or 20°C without BFA (lane 5). The cells were lysed in 1% TX-100 at 4°C, after which the soluble lysates were either cross-linked with DSP or mock cross-linked with DMSO before immunoprecipitation of Cx43 and analysis by SDS-PAGE. Lane 1, cross-linked Cx43 from NRK cells labeled for 4 h at 37°C.

Figure 9. Folding of wild-type and CMTX-linked mutant forms of Cx32. (A) Wild-type Cx32 stable NRK cell transfectants were pulse-labeled with [35 S]methionine and chased for the times indicated at 37°C. The samples in lanes 2, 4, 6, and 7 were prepared from cells subsequently subjected to in situ treatment with 2 mM DTT at 37°C. Cells were lysed in SDS, Cx32 was immunoprecipitated, and the immunoprecipitates were analyzed on SDS-PAGE under either non-reducing (lanes 1–6) or reducing (R; lane 7) conditions. (B) PC12 cell lines stably expressing either wild-type (WT) Cx32 or the indicated disease-causing Cx32 point mutants were metabolically labeled for 4 h at 37°C before (even lanes) in situ treatment with 2 mM DTT at 37°C. Cx32 immunoprecipitates were analyzed on a single non-reducing SDS-PAGE gel in the order shown. The decrease in electrophoretic mobility of the Cx32 mutants after in situ DTT treatment indicates the presence of DTT-sensitive disulfide bonds.

pressed in this cell type (Supplemental Figure 4S). Unlike for wild-type Cx32, however, in situ treatment with DTT caused the electrophoretic mobility of R142W Cx32 (cf. lane 3 with lane 4) and E208K Cx32 (cf. lane 7 with lane 8) to decrease. Results for E186K Cx32 were intermediate between those obtained with WT Cx32 and the other two mutants, suggesting that at least some of the disulfide bonds in a fraction of the labeled E186K Cx32 molecules had become resistant to in situ DTT treatment (cf. lane 5 with lane 6). Similar conclusions were drawn from studies using an alternative method to assess disulfide bond integrity in which a decrease in electrophoretic mobility denotes DTT resistance (Supplemental Figure 5S). Taken together with our finding that oligomerization of wild-type Cx32 in PC12 transfectants does not require ER exit (Figure 5B), these data implicate abnormal folding rather than faulty trafficking as the reason why these mutants fail to assemble into connexons.

DISCUSSION

This study is the first to investigate the process by which a channel protein that undergoes post-ER multisubunit assembly attains its native, functional state. For all other previously described oligomeric proteins, the quality control machinery recognizes unassembled monomers as incompetent to exit the ER. In the case of connexins, however, it must be able to discriminate between two forms of connexin monomer: one incapable of ever reaching a mature, fully folded state (e.g., those that fail to acquire resistance to in situ reduction of intramolecular disulfide bonds) and another that has attained an intermediate level of folding in the ER. The former type of molecule (exemplified by the E208K, R142W, and E186K Cx32 mutants) is identified as abnormal,

retained intracellularly, and destroyed, whereas the latter is permitted to undergo connexon assembly upon transport to the TGN and is delivered to the cell surface. These findings may shed light on the biosynthesis of proteins suspected, but not yet proven, to be capable of oligomerization in post-ER compartments (e.g., HIV-1 Vpu and gp160; Otteken *et al.*, 1996; Hussain *et al.*, 2007).

Folding of Connexins within the ER

We show that within the ER, connexin disulfide bonds become posttranslationally resistant to reduction by extracellularly added 2 mM DTT, a previously established assay for folding of certain other secretory pathway proteins. That this conformational change is an important, most likely necessary, step in gap junction biosynthesis is supported by three findings: 1) both an α (Cx43) and a β (Cx32) connexin acquire resistance to in situ reduction by DTT; 2) all connexin molecules detectable on the cell surface are DTT-resistant, and 3) three of three of the CMTX-linked Cx32 mutants studied in which disulfide bonds form but remain DTT-sensitive are incompetent to assemble into connexons and are not delivered to the plasma membrane. Given that two of the aforementioned Cx32 mutants are capable of being transported to the Golgi, acquisition of DTT resistance does not appear to be a requirement for ER exit. These abnormal molecules must instead be recognized by post-ER quality control mechanisms that either transport them back to the ER (E186K Cx32) or to the lysosome (R142W Cx32) for degradation.

Site of Connexon Assembly

A major new finding of this study is that the multisubunit assembly of an integral plasma membrane protein can take place in more than one intracellular compartment. The site at which connexon formation is initiated is not dictated by connexin species, cell type, or whether the connexin is natively expressed by the cell. Instead, the level of connexin synthesis appears to be the major determinant of the location of oligomerization. In all cases examined, assembly within the ER of Cx43 or 32 synthesized endogenously or at endogenous levels (e.g., C6–14 cells) was near or below the limit of detection. Appreciable assembly in the ER of Cx43, Cx32, and Cx26 occurred only after connexin overexpression in either stable or transient transfectants. The simplest explanation for our findings is that oligomerization into connexons, similar to the later stages of gap junction formation (Valiunas *et al.*, 1997; Castro *et al.*, 1999), is largely a concentration-driven self-assembly process. Under physiologically relevant conditions, the concentration of Cx43, Cx32, and Cx26 subunits required for connexon oligomerization may not be achieved until the TGN. Diez *et al.* (1999) examined the oligomeric state of native Cx32 and Cx26 solubilized under non-denaturing conditions from subcellular fractions prepared from guinea pig liver. Both connexins migrated on sucrose gradients in the position expected for a bona fide, discrete connexon from membranes derived from either gap junctions or the Golgi, but not from membranes enriched in the ER or the ER-Golgi intermediate compartment. Although not interpreted as such by the authors, these data are consistent with the conclusion that β as well as α connexins undergo assembly into connexons within the Golgi in vivo. The dominantly inherited deafness-associated Cx26 mutant D66H has been reported to accumulate in a perinuclear compartment (Marziano *et al.*, 2003) demonstrated by Thomas *et al.* (2004) to be the TGN. Expression of wild-type Cx26, Cx32, and Cx30 (all β connexins) results in its redistribution to the cell surface, consistent with the possibility

that the wild-type and mutant proteins formed heteromeric connexons within the TGN that were responsible for the dominant-negative effect of D66H Cx26 on wild-type Cx26 function (Thomas *et al.*, 2004). Delaying connexon assembly until the TGN may serve to prevent premature formation of gap junctions within the ER cisternae, which has been observed after massive overexpression of Cx32 in BHK transfectants (Kumar *et al.*, 1995). In light of our findings, results obtained from studies in which connexins are exogenously expressed at high levels must be interpreted cautiously with regard to the mechanism or functional consequences of connexon assembly.

Mechanism of Connexon Assembly

Given the relatively large size of the gap junction pore and the evidence that multiple transmembrane domains contribute to its lining (Fleishman *et al.*, 2004), oligomerization into a connexon is likely to involve significant conformational changes within each connexin subunit. The alternative possibility would be that connexin monomers become fully folded within ER, but are normally prevented from co-oligomerizing within that compartment by a tightly bound "spacer" protein that is removed only after arrival in the TGN. Our data do not support such a model for several reasons. First, neither chemical cross-linking nor velocity sedimentation in isocratic sucrose gradients provide any evidence for association of wild-type Cx43, Cx32, or Cx26 with another protein before connexon assembly. Second, our demonstration that each of the connexins studied can be induced to assemble into connexons within the ER shows that the TGN is not unique in its ability to support subunit oligomerization. Third, although connexin monomers newly liberated from such a putative spacer protein would be expected to coassemble with each other in a step-wise manner to form subcomplexes containing 2–5 connexin subunits, such assembly intermediates are not observed for Cx43. It could be argued that the amount of spacer protein available to prevent ER assembly is limited, leading to connexon formation within the ER when its capacity is exceeded by exogenous overexpression of connexins. We have shown, however, that although overexpression of Cx32 in LA25 NRK cells causes it to assemble within the ER, oligomerization of endogenous Cx43 within the same cells remains confined to the TGN (Figure 5B). This would necessitate the existence of multiple, connexin-specific spacer proteins, at least some of which would have to be synthesized by cells that do not constitutively express their client connexin (e.g., Cx26 in HepG2 cells). We favor the alternative explanation that formation of a connexon involves conformational changes within the individual connexin monomers initiated by their coassociation into complexes containing six connexin subunits. Given that conformational changes have been invoked as the reason for the anomalous partitioning of certain other integral membrane proteins in TX-114 (Florke *et al.*, 1993), it is possible that this is also the basis for the shift in TX-114 partitioning of Cx43 upon its assembly into connexons. To our knowledge, Cx43 is the first integral membrane protein that has been shown to undergo a posttranslational switch in TX-114 partitioning as a result of oligomerization into a channel.

Connexin Misfolding and Disease

Lastly, our studies allow us to add CMTX to the growing list of diseases linked to point mutations that cause protein misfolding. Before this work, the extent to which any CMTX-linked Cx32 point mutant was conformationally abnormal could not be assessed. Our previous finding that the

three Cx32 mutants investigated are not aggregated and have near wild-type rates of turnover (VanSlyke *et al.*, 2000), combined with the report that E186K Cx32 and R142W Cx32 can associate with coexpressed wild-type Cx32 in *Xenopus* oocytes (Bruzzone *et al.*, 1994), raised the possibility that they may be retained within mammalian cells because of relatively minor structural defects that expose a retention motif or mask an anterograde transport signal. The results presented here indicate instead that these proteins fail to properly undergo the first known step in connexin folding (acquisition of resistance to *in situ* reduction of disulfide bonds) and that this defect is responsible for their lack of connexon assembly even when expressed under conditions in which ER exit is not required for multisubunit oligomerization. Strategies to correct E208K, E186K, and R142W Cx32 must therefore focus on restoring proper folding instead of on disabling the mechanisms responsible for their intracellular retention.

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