Neurobiology of Disease **11,** 43–52 (2002)
doi:10.1006/nbdi.2002.0545

Diverse Trafficking Abnormalities of Connexin32 Mutants Causing CMTX

Sabrina W. Yum,* Kleopas A. Kleopa,† Susan Shumas,† and Steven S. Scherer†

**Division of Neurology, St. Christopher's Hospital for Children, MCP–Hahnemann University, Philadelphia, Pennsylvania 19134; and †Department of Neurology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6077*

Received March 12, 2002; revised July 8, 2002; accepted for publication August 1, 2002

Mutations in *GJB1,* the gene encoding the gap junction protein connexin32 (Cx32), cause X-linked Charcot–Marie–Tooth disease (CMTX). We compared the localization of CMTX mutants that affect different domains of Cx32, by expressing them in HeLa cells. Mutants were localized to the endoplasmic reticulum (M34K, N205I, and Y211x), in the Golgi apparatus without reaching the cell membrane (M34T, V38M, A40V, R75Q, R75P, R75W, and C217x), in the Golgi apparatus but also forming rare small gap junction-like plaques (M34I, M34V, and V37M), or mainly on the cell membrane, forming gap junction-like plaques (V35M, I213V, R219C, R219H, R220G, R230C, R230L, R238H, L239I, and S281x). Selected mutants expressed in cultured rat Schwann cells showed localization similar to that in HeLa cells. Thus, many CMTX mutants have trafficking abnormalities, whereas the carboxy-terminus mutants reach the cell membrane and probably cause disease through other mechanisms.

Key Words: **gap junctions; protein trafficking; neuropathy; Schwann cells.**

INTRODUCTION

The X-linked form of Charcot–Marie–Tooth disease (CMTX) is the second most common form of hereditary sensory and motor neuropathy (Dubourg *et al.,* 2001; Kleopa & Scherer, 2002). It is characterized clinically by progressive distal muscle weakness and atrophy, areflexia, and variable sensory abnormalities. Affected males have moderate to severe symptoms, whereas heterozygous females are usually mildly affected or even asymptomatic. Since the discovery that CMTX is caused by mutations in *GJB1* (Bergoffen *et al.,* 1993), the gene encoding the gap junction protein connexin32 (Cx32), more than 240 different mutations have been described (http://molgen-www.uia.ac.be/CMTMutations/DataSource/MutByGene.cfm). The mutations are diverse, including missense (amino acid substitutions), nonsense (premature stop), deletions, insertions, and frame-shift mutations, affecting every domain of the Cx32 protein. Although Cx32 is highly expressed by myelinating Schwann cells and oligodendrocytes, as well as other cell types, peripheral neuropathy is typically the sole clinical manifestation.

Cx32 is a member of a large family of highly conserved proteins, the connexins, all of which form gap junctions (Bruzzone *et al.,* 1996). Gap junctions are formed by two hemichannels (or connexons), each made up of six connexin molecules; a complete channel is formed when one hemichannel docks with another hemichannel in an apposed cell membrane (Bruzzone *et al.,* 1996). Gap junctions are usually found between adjacent cells, allowing the intercellular passage of ions and small molecules up to about 1000 daltons, and are thought to have diverse functions, including the propagation of electrical signals, metabolic cooperation, growth control, and cellular differentiation. In myelinating Schwann cells, Cx32 is localized to noncompact myelin in the incisures and the paranodes (Bergoffen *et al.,* 1993; Scherer *et al.,* 1995), leading to the hypothesis that gap junctions provide a direct/shorter pathway for the diffusion of

0969-9961/02 $35.00

ions and small molecules across the myelin sheath (Paul, 1995). Dye transfer studies on living myelinated fibers confirmed that incisures contain functional gap junctions (Balice-Gordon *et al.*, 1998).

The functional attributes of Cx32 mutants have been mainly studied in *Xenopus* oocytes. Naturally occurring nonsense mutations that truncate the intracellular tail/carboxy terminus after codon 211—C217x, R220x, and 281x—form functional gap junctions, although they may be biophysically different from those formed by wild-type Cx32 (Castro *et al.*, 1999; Rabadan-Diehl *et al.*, 1994; Ressot *et al.*, 1998). Similarly, some missense mutations that affect the distal carboxy terminus—R238H and C280G—form gap junctions with no known functional alternations, whereas missense mutations affecting the proximal intracellular tail/carboxy terminus—E208K and R215W—do not form functional gap junctions (Castro *et al.*, 1999). Because abnormal trafficking of Cx32 mutants appears to be a major phenotypic alteration in mammalian cells (Deschênes *et al.*, 1997; Kleopa *et al.*, 2002; Omori *et al.*, 1996), we analyzed the expression of 11 Cx32 mutants affecting the carboxy terminus in communication-incompetent HeLa cells. These 11 missense and nonsense mutants were compared to 12 other Cx32 missense mutants in different domains of the Cx32 protein. Unlike CMTX mutants affecting other portions of the protein (which exhibited a variety of trafficking defects), most mutations at the carboxy terminus formed gap junction-like plaques at the cell surface.

METHODS

Mutant Cx32 Expression Constructs

The *GJB1* mutations were introduced into the open reading frame of human *GJB1* cDNA (subcloned into a small plasmid; pBS) by PCR site-directed mutagenesis using the QuickChange kit (Stratagene, La Jolla, CA). To generate the mutants, oligonucleotide mutagenic primers were designed and incorporated using *Pfu-Turbo* DNA polymerase. The PCR products were digested by *Dpn*I endonuclease to eliminate the parental DNA template. The resulting DNA was used to transform XL-1 Blue bacteria, and minipreps were made from single colonies and sequenced at the Cell Center at the University of Pennsylvania. Inserts were isolated by double digestion with *Bam*HI and *Kpn*I and subcloned into pREP9, a eukaryotic expression vector (Invitrogen). A large-scale plasmid preparation was made from a single colony (Qiagen) and the *GJB1*/*Cx32* sequence was confirmed by direct sequencing.

Cell Cultures and Transfections

Communication-incompetent HeLa cells (Elfgang *et al.*, 1995) were obtained from Dr. Klaus Willecke (Institute for Genetics, University of Bonn, Germany). These HeLa cells do not show detectable levels of gap-junctional intercellular communication, nor do they express any connexins. They were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml penicillin/streptomycin) in a humidified atmosphere containing 5% CO_2 at 37°C. For permanent transfection, Lipofectin (GIBCO BRL) and plasmid DNA were incubated separately in OptiMem for 30 min in room temperature (RT), then combined for another 15 min. HeLa cells (approximately 80% confluent) were washed with OptiMem and then incubated with the combined Lipofectin/DNA solution for 6 h at 37°C. After 6 h the cells were washed once with Hanks' BSS (free of calcium or magnesium) and incubated for 3 days in DMEM at 37°C. After 3 days, 1 mg/ml G418 (GIBCO BRL) was added to the medium to select for permanently transfected cells. The cells were maintained in G418-supplemented medium, with medium changes every 3–4 days, until colonies with stable growth were obtained. The colonies were admixed after trypsinization and the "bulk selected," transfected cells were expanded in G418 for immunocytochemistry and immunoblotting. This protocol was repeated four or five times for each mutation. For comparison, both bulk selection and clonal selection were performed for some mutations (M34V, M34T, V35M, R219H, R220G, R230L) as well as WT Cx32. At least five independent clones were picked and expanded for each mutation 3 weeks after transfection and selection with G418. Untransfected HeLa cells were also treated with G418 and did not survive after 2 weeks.

Schwann cells were isolated from sciatic nerves of 3-day-old rat pups (Brockes *et al.*, 1979), purified by anti-Thy 1.1 immunoselection, and expanded for four to six passages on 10-cm plates coated with poly-l-lysine (Sigma) in DMEM supplemented with glutamine, 10% FCS, 2 μM forskolin (Calbiochem), and 10 ng/ml recombinant human secreted β isoform of neuregulin-1 (rhGGF2) (Marchionni *et al.*, 1993). All of the cultures used in these experiments were essentially free of fibroblasts. For transient transfection, some cells were plated into four-chamber glass slides (Nalge Nunc International) coated with poly-l-lysine and merosin. Transfection of 50–70% confluent Schwann cells was carried out using the same protocol as the one for HeLa cells described above, except that they

were incubated for 3 instead of 6 h in the transfection solution. The Schwann cells were not selected with G418 and were immunostained at 4 to 5 days post-transfection.

Immunocytochemistry

Cells were trypsinized, plated onto four-chamber glass slides (Nalge Nunc International), and incubated for 2–3 days to approximately 60% confluence. The cells were washed in PBS, then fixed in acetone (HeLa cells) or acid alcohol (Schwann cells) at −20°C for 10 min, and blocked with 5% fish skin gelatin in PBS containing 0.1% Triton for 1 h at RT. Primary antibodies were added in the same blocking solution and the samples incubated overnight at 4°C. After being washed in PBS, the secondary antibodies were added in the same blocking solution and incubated at RT for 1 h. Coverslips were mounted with Vectashield (Vector Laboratories, Inc.) and samples were photographed under a Leica fluorescence microscope with a Hamamatsu digital camera connected to a G4 Macintosh computer, using the Openlab 2.2 software. Confocal microscopy was carried out in selected samples using a Leica laser scanning confocal microscope.

For Cx32 staining, a rabbit antiserum (Chemicon; diluted 1:500) against the intracellular loop and the mouse monoclonal antibody 7C6.C7 (diluted 1:2) against the carboxy terminus were used. A mouse monoclonal antibody against calnexin (Affinity Bioreagents, Inc., diluted 1:100; for HeLa cells) and a rabbit antiserum against BiP (Affinity Bioreagents, Inc; diluted 1:100; for Schwann cells) were used to label the endoplasmic reticulum. A mouse monoclonal antibody against the 58-kDa protein (Sigma, diluted 1:50) was used as a Golgi marker. TRITC- and FITC-conjugated donkey anti-rabbit and anti-mouse antisera (Jackson ImmunoResearch), respectively, were used as secondary antibodies.

Immunoblotting

HeLa cells were grown to confluence on 100-mm plates and harvested in cold Dulbecco's PBS lacking calcium and magnesium (Life Technologies). The cell pellet was either stored at −80°C or lysed directly in ice-cold 50 mM Tris, pH 7.0, 1% SDS, and 0.017 mg/ml phenylmethylsulfonyl fluoride (Sigma), followed by a brief sonication on ice with a dismembrator (Fisher Scientific). Protein concentration was determined using the Bio-Rad kit (Bio-Rad Laboratories) according to the manufacturer's instructions. For each sample, after a 5- to 15-min incubation in loading buffer at RT, 100 μg of protein lysate was loaded onto a 12% SDS–polyacrylamide gel, electrophoresed, and transferred to an Immobilon polyvinylidene fluoride membrane (Millipore) over 1 h, using a semidry transfer unit (Fisher). The blots were blocked (5% powdered skim milk and 0.5% Tween 20 in Tris-buffered saline) overnight at 4°C and incubated for 24 h at 4°C in a rabbit antiserum against Cx32 (Zymed; diluted 1:1000). After being washed in blocking solution, the blots were incubated in peroxidase-coupled secondary antibody against rabbit (Jackson ImmunoResearch, West Grove, PA; diluted 1:2000) for 1 h at RT. After being washed in blocking solution and Tris-buffered saline containing 0.5% Tween 20, blots were visualized by enhanced chemiluminescence (Amersham) according to the manufacturer's protocols.

RESULTS

Cellular Localization of CMTX Mutants

To determine whether missense and nonsense CMTX mutants affecting the carboxy terminus have altered trafficking, we transfected 11 different mutations into communication-incompetent HeLa cells with a mammalian expression vector. For comparison, we similarly transfected cells with 12 mutations that affect different domains of the Cx32 protein, including mutants that affect the same amino acid, as well as wild-type human Cx32. The transfected cells were bulk selected and immunostained for Cx32 with a rabbit antiserum against the cytoplasmic loop and a mouse monoclonal antibody against an epitope in the carboxy terminus (Fig. 1). Both antibodies stained a subpopulation of cells in all cases, consistent with our prior studies that indicate only a fraction of the cells expressed Cx32 (Deschênes *et al.*, 1997; Kleopa *et al.*, 2002). Both Cx32 antibodies colabeled the same structures, except for the Y211x and C217x mutants, which did not show Cx32 immunostaining with the monoclonal antibody because their carboxy termini were truncated. Untransfected parental cells or cells transfected with an "empty" pREP9 expression vector showed no staining (data not shown). Cells transfected with wild-type human *GJB1/Cx32* cDNA formed gap junction-like plaques on their cell membranes at sites of apposition with other cells (Fig. 1X). Cells expressing wild-type Cx32 also had some intracellular Cx32-immunostaining, in the endoplasmic reticulum (ER) and Golgi. Immunoblot analysis confirmed that cells expressing wild-type Cx32 had an ~30-kDa band (Fig. 2); this band was absent in cells

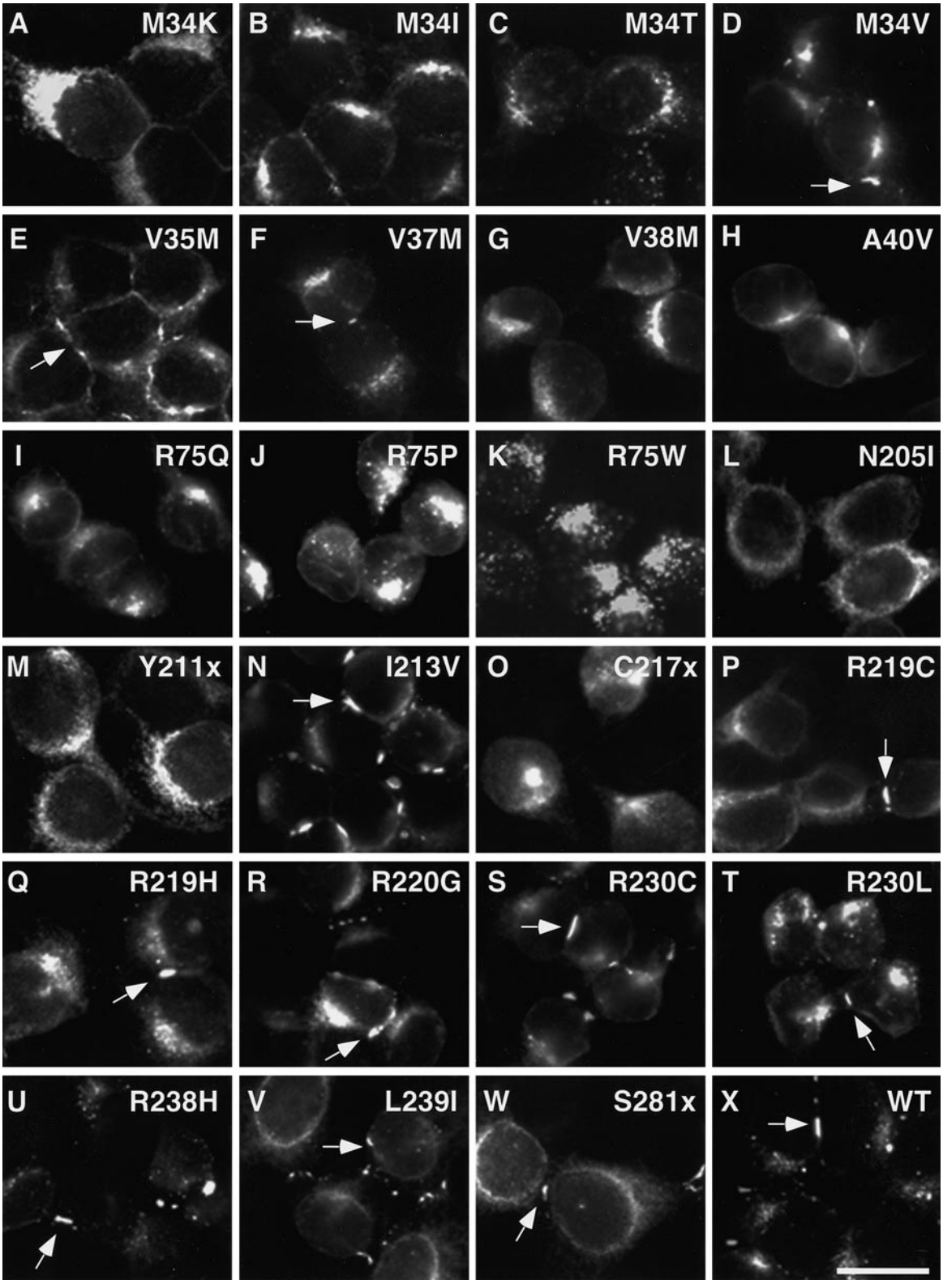


FIG. 1. Immunolocalization of Cx32 in transfected HeLa cells. These are digital images of transfected cells that were double labeled with a rabbit polyclonal antibody (TRITC) and a mouse monoclonal antibody (FITC) against Cx32; the staining with the rabbit antiserum is shown. Gap junction-like plaques (arrows) are seen in cells expressing wild-type Cx32 (X), most carboxy-terminus mutants (N, P–W), and to a lesser degree (rarer and / or smaller) in cells expressing the M34I (B), M34V (D), V35M (E), and V37M (F) mutants. In contrast, Cx32 immunoreactivity is confined to the ER in the M34K (A), N205I (L), and Y211x (M) mutants and to the Golgi in the M34T (C), V38M (G), A40V (H), R75Q (I), R75P (J), R75W (K), and C217x (O) mutants. Scale bar, 10 μm.

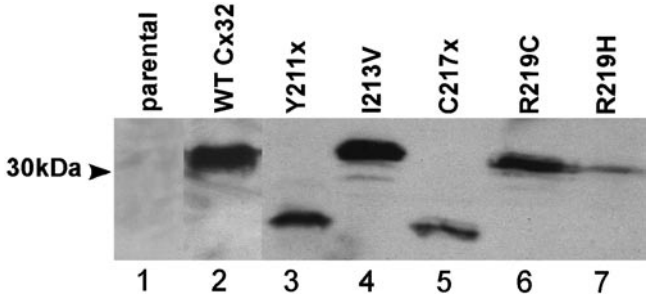


FIG. 2. Immunoblot analysis of Cx32 expression in transfected HeLa cells. Immunoblots were hybridized with a rabbit antiserum against Cx32. Untransfected ("parental") communication-incompetent HeLa cells (lane 1) or cells transfected with the vector alone (data not shown) did not express Cx32. In contrast, cells transfected with either wild-type Cx32 (lane 2) or the indicated nontruncated Cx32 mutants (lanes 4, 6, and 7) showed an ~30-kDa band. As expected, the two mutants with truncated carboxy terminus (lanes 3 and 5) produced a band that ran at a lower molecular mass. Lanes 1 and 2 were taken from different immunoblots.

expressing the pREP9 vector alone (data not shown) and in untransfected (parental) cells (Fig. 2).

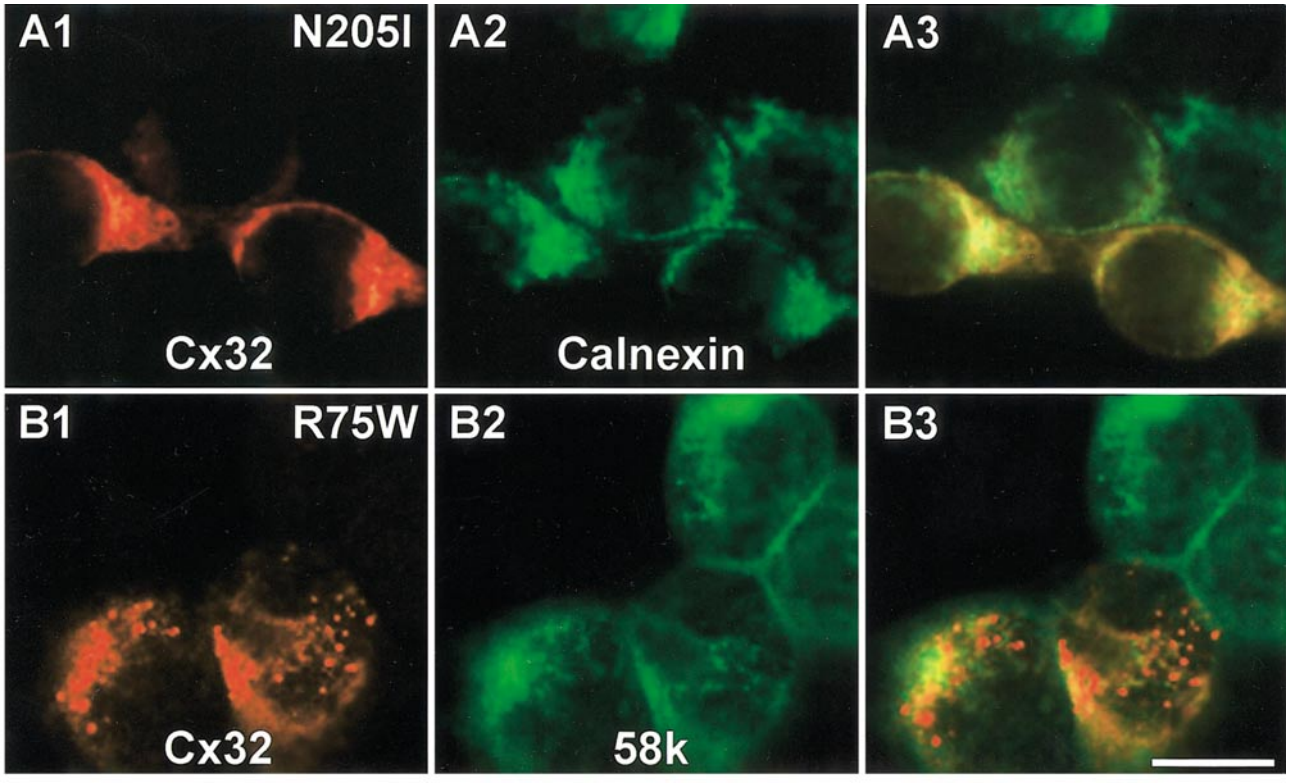
A total of 23 different CMTX mutants were similarly evaluated. Because cells expanded from the same clone had a variable level of Cx32 expression, and showed the same qualitative results as bulk-selected cells, we used the latter approach. Cells expressing the M34K, N205I, and Y211x mutants (Figs. 1A, 1L, and 1M) had diffuse intracellular Cx32 staining, without intracellular granules of Cx32 staining or gap junction-like plaques. Intracellular punctate / granular Cx32 immunoreactivity was seen in clones expressing the M34T, V38M, A40V, R75Q, R75P, R75W, and C217x mutants (Figs. 1C, 1G, 1H, 1I, 1J, 1K, and 1O), regardless of the level of protein expression; the mutants M34I, M34V, and V37M showed a similar expression pattern, but sometimes they formed smaller gap junction-like plaques (Figs. 1B, 1D, and 1F). In contrast, most Cx32 carboxy-terminus mutants (I213V, R219C, R219H, R220G, R230C, R230L, R238H, L239I, and S281x; Figs. 1N, 1P, 1Q, and 1R–1W), as well as the V35M mutant (Fig. 1E) were expressed in a wild-type-like pattern, with punctate cell surface immunostaining and numerous gap junction-like plaques.

Based on previous investigations (Deschênes *et al.*, 1997; Kleopa *et al.*, 2002; VanSlyke *et al.*, 2000), we suspected that the diffuse intracellular Cx32 staining corresponded to the ER and that the focal accumulations of granular Cx32 staining corresponded to the Golgi apparatus. Thus, we double labeled cells for Cx32 and markers of the ER (calnexin) or the Golgi (58-kDa protein). The M34K, N205I, and Y211x mutants colocalized with calnexin (Fig. 3A and data not shown), indicating that these mutants were localized in the ER. The M34I, M34T, M34V, V35M, V37M, V38M, A40V, R75Q, R75P, R75W, R183C, and C217x mutants predominantly colocalized with the 58-kDa protein (Fig. 3B and data not shown), although Cx32 staining also appeared to overlap with calnexin, especially in cells expressing the M34T, A40V, and C217x mutants (data not shown). Thus, these mutants appeared to be largely localized in the Golgi. These colocalizations were confirmed with laser scanning confocal microscopy (data not shown).

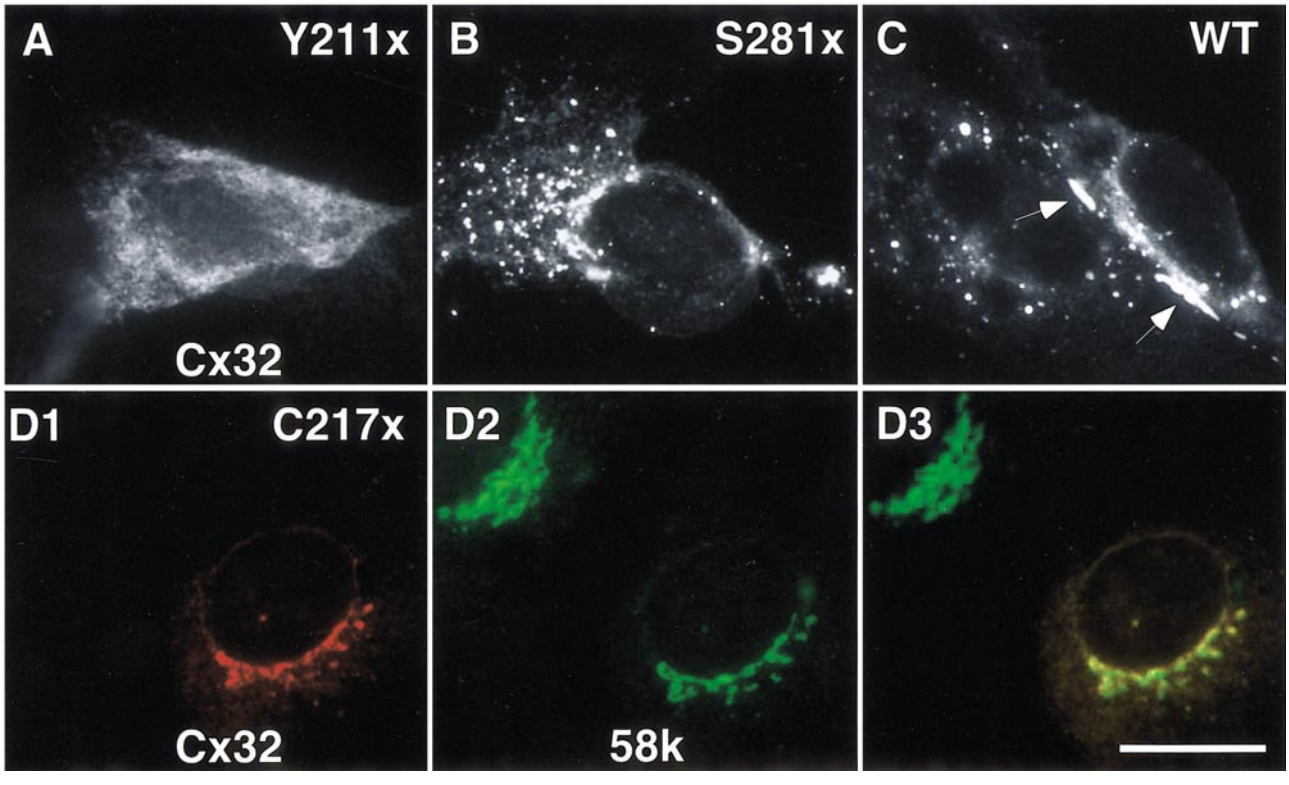
To confirm and extend these findings, we performed immunoblot analysis on the bulk-selected cells used in the above experiments. Some of these results are shown in Fig. 2. Cells expressing wild-type Cx32 or different missense Cx32 mutants showed an ~30-kDa band; this band was not detected in untransfected (parental) HeLa cells. As expected, cells expressing either the Y211x or the C217x mutants had a band that ran at a lower molecular mass, because the mutant protein is truncated. The intensity of these bands was not uniform among cells expressing different constructs, consistent with the observed variation in the proportion of transfected cells expressing the Cx32 protein; this ranged from ~50% in some constructs to less than 10% in others (data not shown).

Similar Expression Pattern of Cx32 Mutants in Schwann Cells

We wanted to determine whether our findings in HeLa cells could be reproduced in Schwann cells, the relevant cell type for the pathogenesis of CMTX. Because cultured Schwann cells express little if any Cx32 protein (Chandross *et al.*, 1996; Scherer *et al.*, 1995), we transfected cultured rat Schwann cells and evaluated the expression of wild-type Cx32 and three mutants by immunocytochemistry. Wild-type Cx32 (Fig. 4C) reached the cell membrane and formed occasional gap junction-like plaques. The S281x mutant similarly reached the cell surface (Fig. 4B) and formed rare gap junction-like plaques (data not shown). The Y211x mutant was retained in the ER (Fig. 4A), confirmed by colocalization with the ER marker BiP (data not shown), whereas the C217x mutant was retained in the Golgi, confirmed by double staining for the Golgi marker 58 kDa (Figs. 4D1–4D3). These results provide further evidence that the trafficking patterns of these mutants in cultured rat Schwann cells were like those observed in HeLa cells (Kleopa *et al.*, 2002). Furthermore, when two Cx32 mutants are expressed as transgenes in myelinating Schwann cells, their behavior is



3



4

FIG. 3. Localization of CMTX mutants in the ER and Golgi. These are digital images of transfected HeLa cells that were double labeled with a rabbit antiserum against Cx32 (A1 and B1; TRITC) and a mouse monoclonal antibody (FITC) against calnexin (A2; an ER marker) or 58 kDa (B2; a Golgi marker); A3 and B3 show the merged images. Cells expressing N205I (A1–A3) show overlapping calnexin and Cx32 staining. Cells expressing R75W (B1–B3) show overlapping 58 kDa and Cx32 staining. Scale bar, 10 μm.

FIG. 4. Localization of selected CMTX mutants in Schwann cells. These are digital images of transiently transfected Schwann cells that were labeled with a rabbit antiserum against Cx32 (A–C; TRITC) or double labeled for Cx32 (TRITC) and 58-kDa protein (FITC), a Golgi marker (D1–D3). The localization of the Y211x, C217x, and S281x mutants and wild-type Cx32 was similar to that in HeLa cells: Y211x was localized to the ER (A) and C217x was localized to the Golgi (D1–D3), whereas S281x (B) and wild-type Cx32 (C) were able to reach the cell membrane and form gap junction-like plaques at regions of cell–cell contact (arrowheads in C). Scale bar, 10 μm.

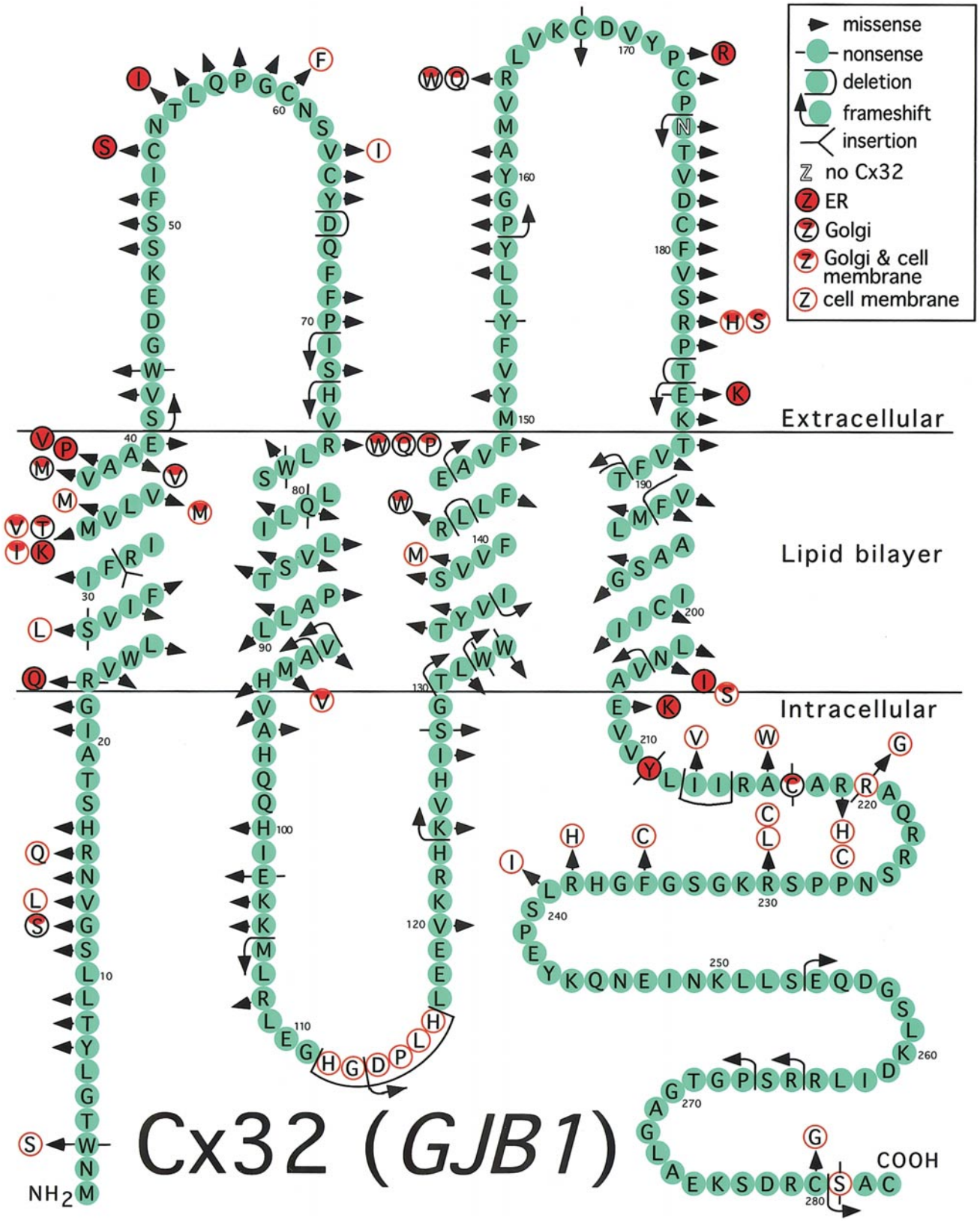


FIG. 5. Trafficking of CMTX mutants. The structure of the human Cx32 protein is depicted according to Yeager and Nicholson (1996). The consequences of the known CMTX mutations are shown schematically, along with the localization of 51 different Cx32 mutants in transfected mammalian cells (Deschênes *et al.*, 1997; Kleopa *et al.*, 2002; Martin *et al.*, 2000; Omori *et al.*, 1996; Yoshimura *et al.*, 1998), 23 of which are from the present study. Note that colocalization studies with ER and Golgi markers were not performed on some of the mutations that have been reported to be retained intracellularly (Omori *et al.*, 1996; Yoshimura *et al.*, 1998); these are depicted as being retained in the ER, but they may be retained in the Golgi.

identical to that seen in transfected cultured cells: the 175 frame-shift protein is undetectable (Abel *et al.*, 1999) and the R142W mutant accumulates in the Golgi (unpublished observations).

DISCUSSION

To date, our laboratory has studied the localization of 38 different CMTX mutants in mammalian cells (Deschênes *et al.*, 1997; Kleopa *et al.*, 2002), including the 23 reported here. As summarized in Fig. 5, the intracellular localization of 51 different CMTX mutants has been reported, some more than once. One striking finding of the current study is that most missense and nonsense mutations affecting the carboxy terminus do not result in intracellular retention of the mutant protein; this is in contrast to most missense mutations that affect the rest of the Cx32. Thus, mutants of the carboxy terminus—the most divergent region of connexins (Bruzzone *et al.*, 1996)—do not appear to disrupt the synthesis or assembly of Cx32 into hemichannels. This result contrasts with the finding that dominant mutants of aquaporin-2, a water channel, typically affect the carboxy terminus and result in retention in the Golgi (Deen *et al.*, 2000; Kuwahara *et al.*, 2001).

Because connexins are a highly conserved family of proteins with a known function, it is possible to surmise the effects of a few CMTX mutants, such as those affecting the six cysteines that form disulfide bonds between the two extracellular loops (Dahl *et al.*, 1992). The trafficking and functional consequences of different mutations affecting the same codon in Cx32 have not been systematically evaluated, except for the biophysical properties of different amino acid substitutions at T86 and P87 (Ri *et al.*, 1999), and the cellular localizations of the W3S and W3Y mutants (in the cell membrane and in the Golgi, respectively), although the latter is not a naturally occurring mutation (Martin *et al.*, 2000). We find that many mutations at the same residue have the same pattern of intracellular retention, suggesting that for some, the amino acid position may be more important than the nature of the amino acid substitution (Fig. 5). However, N205I is retained in the ER (Fig. 1L), whereas N205S reaches the cell membrane (Kleopa *et al.*, 2002). Similarly, M34K is retained in the ER and M34T in the Golgi apparatus, whereas M34I and M34V reach the cell membrane. Thus, the nature of both the affected residue and the substitution likely affect the trafficking of Cx32 mutants.

Intracellularly Retained Cx32 Mutants

The finding that most mutations in the transmembrane domains disrupt the trafficking of Cx32 is in line with previous studies. "Swapping" the transmembrane domains of Cx32 and synaptophysin, another protein with four transmembrane domains, results in the intracellular retention of the chimeric proteins (Leube, 1995). Further, missense mutants mainly accumulate in the ER or Golgi (Deschênes *et al.*, 1997; Kleopa *et al.*, 2002; Matsuyama *et al.*, 2001; Oh *et al.*, 1997; Omori *et al.*, 1996), although the reason for their apparent retention in one or the other compartment remains to be determined. Perhaps the most severely misfolded proteins are retained in the ER, whereas less severely misfolded mutants reach the Golgi.

Proteins that are retained in the ER are degraded, at least in part, by a process sensitive to proteasomal inhibition, but not to lysosomal inhibition (Kleopa *et al.*, 2002; VanSlyke *et al.*, 2000). Inhibiting proteasomes leads to aggresome formation (Johnston *et al.*, 1998; Notterpek *et al.*, 1999), but Cx32 immunoreactivity for the N205I (data not shown), E186K, E208K, and T55I remains in the ER and not in these protein aggregates (Kleopa *et al.*, 2002; VanSlyke *et al.*, 2000). These results contrast with the findings that wild-type peripheral myelin protein 22 kDa (PMP22) accumulates in degradation-resistant aggregates in cultured Schwann cells (Notterpek *et al.*, 1999). The two mutants studied here (N205I and R211x; data not shown), like the ER-retained mutants E186K, E208K, and T55I (Kleopa *et al.*, 2002; VanSlyke *et al.*, 2000), remain in the ER and do not accumulate in lysosomes even in the presence of lysosomal inhibitors.

Connexins are assembled into hemichannels in the Golgi (Musil & Goodenough, 1993), so that some mutants that appear to be retained in the Golgi may not assemble properly into hemichannels (Martin *et al.*, 2000) and therefore fail to reach the cell membrane. These mutants could be degraded by lysosomes or could be transported back from the Golgi to the ER and be degraded by proteosomes (VanSlyke *et al.*, 2000). Like other mutants that appear to be retained in the Golgi (Kleopa *et al.*, 2002; VanSlyke *et al.*, 2000), the R75Q and R75P mutants remained mostly outside of aggresomes after proteasomal inhibition (data not shown). In keeping with their predicted pathway of degradation (Kleopa *et al.*, 2002; VanSlyke *et al.*, 2000), some of the Golgi-retained mutants in this study (A40V, R75Q, and R75P) showed increased localization in chloroquine-inhibited lysosomes (data not shown).

How Do Mutants That Form Gap Junctions Cause CMTX?

Some CMTX mutants form functional gap junctions both in oocytes and in cultured mammalian cells, including at least two mutants (Arg238His and C280G) that have electrophysiological characteristics essentially identical to those of wild-type Cx32 (Bruzzone *et al.,* 1994; Castro *et al.,* 1999; Oh *et al.,* 1997; Rabadan-Diehl *et al.,* 1994; Ressot *et al.,* 1998). Most mutants that reach the cell membrane in mammalian cells form functional gap junctions in oocytes (S26L, V35M, V38M, 112–117 deleted, R220x, C280G, S281x). Conversely, most mutants that are completely retained intracellularly (G12S, V38M, C53S, R142W, E186K, E208K, and Y211x) usually do not form functional gap junctions in oocytes. We suspect that the mutants that reach the cell membrane of transfected cells have altered trafficking in myelinating Schwann cells. A strong precedent for this possibility comes from the analysis of dominant rhodopsin mutations that cause retinitis pigmentosa (http://www.retina-international.org/sci-news/rhomut.htm). One mutation deletes the last five amino acids from the carboxy terminus. In transfected cells, this mutant traffics normally and even binds opsin normally, but when it is expressed in the rods of transgenic mice, it "hangs up" in the inner segment and fails to reach the outer segment (Sung *et al.,* 1994). Similarly, carboxy-terminus Cx32 mutants may not reach their destination at the incisures and paranodes of the myelinating Schwann cells, but instead remain confined to other areas of the Schwann cell membrane. We are generating transgenic animals to study the mechanism of disease for these carboxy-terminus mutants.

How Does Altered Trafficking Cause Disease?

Dominant mutations in three other intrinsic membrane proteins of the myelin sheath—protein zero (P_0), proteolipid protein (PLP), and PMP22—cause inherited dys/demyelination (http://molgen-www.uia.ac.be/CMTMutations/DataSource/MutByGene.cfm). Intracellular retention of mutant protein has been documented for both PLP and PMP22, not only in cultured heterologous cells (Gow & Lazzarini, 1996; Naef & Suter, 1999), but also in myelinating Schwann cells (for PMP22; Naef *et al.,* 1997; Tobler *et al.,* 1999) and oligodendrocytes (for PLP; Roussel *et al.,* 1987). The dominant effects of P_0, PMP22, and PLP mutants remain to be determined, but they may well involve abnormal interactions between mutant and wild-type monomers, as P_0 forms tetramers (Shapiro *et al.,* 1996) and PMP22 forms dimers (Tobler *et al.,* 2002; Tobler *et al.,* 1999). One unresolved paradox in this regard is that the Cx32 mutants that are retained in the ER cause less profound demyelination than do the PMP22 mutants that are retained in the ER. This could be related to the fact that Cx32 mutants do not interact with the wild-type Cx32, because *GJB1* is subjected to X inactivation in myelinating Schwann cells (Scherer *et al.,* 1998). However, Schwann cells likely express at least one other connexin, Cx29 (Altevogt *et al.,* 2002; Sohl *et al.,* 2001), so it is possible that certain Cx32 mutants have "*trans*-dominant" interactions with Cx29.

ACKNOWLEDGMENTS

We thank Drs. Klaus Willecke and Bruce Nicholson for the HeLa cells. This work was supported by the National MS Society with an advanced postdoctoral fellowship award (to K.A.K.) and by NIH Grant NS42878 (to S.S.S.).

REFERENCES

Abel, A., Bone, L. J., Messing, A., Scherer, S. S., & Fischbeck, K. F. (1999) Studies in transgenic mice indicate a loss of connexin32 function in X-linked Charcot–Marie–Tooth disease. *J. Neuropathol. Exp. Neurol.* **58,** 702–710.

Altevogt, B. M., Kleopa, K. A., Postma, F. R., Scherer, S. S., & Paul, D. L. (2002) Connexin29 is uniquely distributed within myelinating glial cells of the central and peripheral nervous systems. *J. Neurosci* **22,** 6458–6470.

Balice-Gordon, R. J., Bone, L. J., & Scherer, S. S. (1998) Functional gap junctions in the Schwann cell myelin sheath. *J. Cell Biol.* **142,** 1095–1104.

Bergoffen, J., Scherer, S. S., Wang, S., Oronzi-Scott, M., Bone, L., Paul, D. L., Chen, K., Lensch, M. W., Chance, P., & Fischbeck, K. (1993) Connexin mutations in X-linked Charcot–Marie–Tooth disease. *Science* **262,** 2039–2042.

Brockes, J. P., Fields, P., & Raff, M. C. (1979) Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* **165,** 105–118.

Bruzzone, R., White, T. W., Scherer, S. S., Fischbeck, K. H., & Paul, D. L. (1994) Null mutations of connexin32 in patients with X-linked Charcot–Marie–Tooth disease. *Neuron* **13,** 1253–1260.

Bruzzone, R., White, T. W., & Paul, D. L. (1996) Connections with connexins: The molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238,** 1–27.

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

Chandross, K. J., Kessler, J. A., Cohen, R. I., Simburger, E., Spray, D. C., Bieri, P., & Dermietzel, R. (1996) Altered connexin expression after peripheral nerve injury. *Mol. Cell. Neurosci.* **7,** 501–518.

Dahl, G., Werner, R., Levine, E., & Rabadan-Dahl, C. (1992) Mutational analysis of gap junction formation. *Biophys. J.* **62,** 172–182.

Deen, P. M. T., van Balkom, B. W. M., & Kamsteeg, R.-J. (2000) Routing of the aquaporin-2 water channel in heath and disease. *Eur. J. Cell Biol.* **79,** 523–530.

Deschênes, S. M., Walcott, J. L., Wexler, T. L., Scherer, S. S., & Fischbeck, K. H. (1997) Altered trafficking of mutant connexin32. *J. Neurosci.* **17,** 9077–9084.

Dubourg, O., Tardieu, S., Birouk, N., Gouider, R., Leger, J. M., Maisonobe, T., Brice, A., Bouche, P., & LeGuern, E. (2001) Clinical, electrophysiological and molecular genetic characteristics of 93 patients with X-linked Charcot–Marie–Tooth disease. *Brain* **124,** 1958–1967.

Elfgang, C., Eckert, R., Lichternberg-Frate, H., Butterweck, A., Traub, O., Klein, R. A., Hulser, D. F., & Willecke, K. (1995) Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J. Cell Biol.* **129,** 805–817.

Gow, A., & Lazzarini, R. A. (1996) A cellular mechanism governing the severity of Pelizaeus–Merzbacher disease. *Nat. Genet.* **13,** 422–427.

Johnston, J. A., Ward, C. L., & Kopito, R. R. (1998) Aggresomes: A cellular response to misfolded proteins. *J. Cell Biol.* **143,** 1883–1898.

Kleopa, K. A., & Scherer, S. S. (2002) Inherited neuropathies. *Neurol. Clin. North Am.,* **20,** 679–709.

Kleopa, K. A., Yum, S. W., & Scherer, S. S. (2002) Cellular mechanisms of connexin32 mutations associated with CNS manifestations. *J. Neurosci. Res.* **68,** 522–534.

Kuwahara, M., Iwai, K., Ooeda, T., Igarashi, T., Ogawa, E., Katsushima, Y., Shinbo, I., Uchida, S., Terada, Y., Arthus, M. F., Lonergan, M., Fujiware, T. M., Bichet, D. G., Marumo, F., & Sasaki, S. (2001) Three families with autosomal dominant nephrogenic diabetes insipidus caused by aquaporin-2 mutations in the C-terminus. *Am. J. Hum. Genet.* **69,** 738–748.

Leube, R. E. (1995) The topogenic fate of the polytopic transmembrane proteins, synaptophysin and connexin, is determined by their membrane-spanning domains. *J. Cell Sci.* **108,** 883–894.

Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Berminghan-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., & Gwynne, D. (1993) Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362,** 312–318.

Martin, P. E. M., Mambetisaeva, E. T., Archer, D. A., George, C. H., & Evans, W. H. (2000) Analysis of gap junctions assembly using mutated connexins detected in Charcot–Marie–Tooth X-linked disease. *J. Neurochem.* **74,** 711–720.

Matsuyama, W., Nakagawa, M., Moritoyo, T., Takashima, H., Umehara, F., Hirata, K., Suehara, M., & Osame, M. (2001) Phenotypes of X-linked Charcot–Marie–Tooth disease and altered trafficking of mutant *Connexin 32 (GJB1). J. Hum. Genet.* **46,** 307–313.

Musil, L. S., & Goodenough, D. A. (1993) Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell* **74,** 1075–1077.

Naef, R., Adlkofer, K., Lescher, B., & Suter, U. (1997) Aberrant protein trafficking in *Trembler* suggests a disease mechanism for hereditary human peripheral neuropathies. *Mol. Cell. Neurosci.* **9,** 13–25.

Naef, R., & Suter, U. (1999) Impaired intracellular trafficking is a common disease mechanism of PMP22 point mutations in peripheral neuropathies. *Neurobiol. Dis.* **6,** 1–14.

Notterpek, L., Ryan, M. C., Tobler, A. R., & Shooter, E. M. (1999) PMP22 accumulation in aggresomes: Implications for CMTIA pathology. *Neurobiol. Dis.* **6,** 450–460.

Oh, S., Ri, Y., Bennett, M. V. L., Trexler, E. B., Verselis, V. K., & Bargiello, T. A. (1997) Changes in permeability caused by connexin 32 mutations underlie X-linked Charcot–Marie–Tooth disease. *Neuron* **19,** 927–938.

Omori, Y., Mesnil, M., & Yamasaki, H. (1996) Connexin 32 mutations from X-linked Charcot–Marie–Tooth disease patients: Functional defects and dominant negative effects. *Mol. Biol. Cell* **7,** 907–916.

Paul, D. L. (1995) New functions for gap junctions. *Curr. Opin. Cell Biol.* **7,** 665–672.

Rabadan-Diehl, C., Dahl, G., & Werner, R. (1994) A connexin-32 mutation associated with Charcot–Marie–Tooth disease does not affect channel formation in oocytes. *FEBS Lett.* **351,** 90–94.

Ressot, C., Gomes, D., Dautigny, A., Pham-Dinh, D., & Bruzzone, R. (1998) Connexin32 mutations associated with X-linked Charcot–Marie–Tooth disease show two distinct behaviors: Loss of function and altered gating properties. *J. Neurosci.* **18,** 4063–4075.

Ri, Y., Ballesteros, J. A., Abrams, C. K., Oh, S., Verselis, V. K., Weinstein, H., & Bargiello, T. A. (1999) The role of a conserved proline residue in mediating conformational changes associated with voltage gating of Cx32 gap junctions. *Biophys. J.* **76,** 2887–2898.

Roussel, G., Neskovic, N. M., Trifilieff, E., Artault, J.-C., & Nussbaum, J.-L. (1987) Arrest of proteolipid protein through the Golgi apparatus in jimpy brain. *J. Neurocytol.* **16,** 195–204.

Scherer, S. S., Deschênes, S. M., Xu, Y.-T., Grinspan, J. B., Fischbeck, K. H., & Paul, D. L. (1995) Connexin32 is a myelin-related protein in the PNS and CNS. *J. Neurosci.* **15,** 8281–8294.

Scherer, S. S., Xu, Y.-T., Nelles, E., Fischbeck, K., Willecke, K., & Bone, L. J. (1998) Connexin32-null mice develop a demyelinating peripheral neuropathy. *Glia* **24,** 8–20.

Shapiro, L., Doyle, J. P., Hensley, P., Colman, D. R., & Hendrickson, W. A. (1996) Crystal stucture of the extracellular domain from P_0, the major structural protein of peripheral nerve myelin. *Neuron* **17,** 435–449.

Sohl, G., Eiberger, J., Jung, Y. T., Kozak, C. A., & Willecke, K. (2001) The mouse gap junction gene connexin29 is highly expressed in sciatic nerve and regulated during brain development. *Biol. Chem.* **382,** 973–978.

Sung, C.-H., Makino, C., Baylor, D., & Nathans, J. (1994) A rhodopsin gene mutation responsible for autosomal dominant retinitis pigmentosa results in a protein that is defective in localization to the photoreceptor outer segment. *J. Neurosci.* **14,** 5818–5833.

Tobler, A. R., Liu, N., Mueller, L., & Shooter, E. M. (2002) Differential aggregation of the Trembler and Trembler J mutants of peripheral myelin protein 22. *Proc. Natl. Acad. Sci. USA* **99,** 483–488.

Tobler, A. R., Notterpek, L., Naef, R., Taylor, V., Suter, U., & Shooter, E. M. (1999) Transport of *Trembler-J* mutant peripheral myelin protein 22 is blocked in the intermediate compartment and affects the transport of the wild-type protein by direct interaction. *J. Neurosci.* **19,** 2027–2036.

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

Yeager, M., & Nicholson, B. J. (1996) Structure of gap junction intercellular channels. *Curr. Opin. Struct. Biol.* **6,** 183–192.

Yoshimura, T., Satake, M., Ohnishi, A., Tsutsumi, Y., & Fujikura, Y. (1998) Mutations of connexin32 in Charcot–Marie–Tooth disease type X interfere with cell-to-cell communication but not cell proliferation and myelin-specific gene expression. *J. Neurosci. Res.* **51,** 154–161.