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Connexin32 and X-Linked Charcot-Marie-Tooth Disease

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A common theme in the Symposium is that the identification of defective genes in hereditary neurological disease can give new insight into basic mechanisms of cellular structure and function. This paper deals with the genetic defect responsible for the X-linked form of Charcot-Marie-Tooth disease.

X-LINKED CHARCOT-MARIE-TOOTH DISEASE AND CONNEXIN32

Charcot-Marie-Tooth disease (CMT) causes progressive weakness, atrophy, and sensory loss in the feet, lower legs, and hands. Overall, CMT is a relatively common hereditary disorder, affecting about 1 in 2500 individuals, more than 100,000 Americans. The cause of the manifestations of CMT is degeneration of the peripheral nerves. In different families, the degenerative process can affect the axons, the myelin, or both.

CMT is genetically heterogeneous. It is classified first according to whether the pathology is primarily demyelinative or axonal, and second according to the specific genetic defect (Chance and Fischbeck 1994). The most common form, CMT1A, and a less common form, CMT1B, are caused by defects in the genes for myelin proteins PMP22 and P_0, respectively. The X-linked form of the disease (CMTX) is caused by mutations in the gap junction protein, connexin32 (Bergoffen et al. 1993b).

CMTX accounts for about 10% of patients with CMT and is probably the second most common cause of hereditary neuropathy after CMT1A. It is most often considered an X-linked dominant disease (Fischbeck et al. 1990; Deschênes et al. 1996). The disease is commonly passed from generation to generation, affecting both males and females as with autosomal dominant transmission, except that there is no father-to-son transmission. Heterozygous females are variably involved and in some families may be asymptomatic. Nerve conduction velocities tend to be slow, in an intermediate range, and nerve biopsies may show signs of both demyelination and axonal degeneration.

Several years ago, we and other workers set out to identify the CMTX disease gene by positional cloning, and the disease locus was eventually narrowed to a small segment of the proximal long arm of the X chromosome, at Xq13.1 (Bergoffen et al. 1993a). The interval to which CMTX was mapped contained three previously identified genes, including the gene for connexin32. We evaluated connexin32 as a candidate gene, first by determining whether or not it is expressed in peripheral nerve. Northern blots show that connexin32 is expressed in peripheral nerve, and that it follows the same pattern of expression as other myelin-related genes, decreasing in nerve that is degenerating after transection and increasing in nerve that is regenerating after a crush (Scherer et al. 1995). This pattern differs from that of nerve growth factor receptor and the housekeeping gene glyceraldehyde phosphate dehydrogenase. In cultured Schwann cells, connexin32 is expressed with other myelin genes when the cells are treated with forskolin, an agent that increases cAMP levels.

Connexin32 is encoded by a relatively simple gene with two promoters, and the translated sequence is contained in a single exon (Fig. 1). The first promoter, P1, drives expression in the liver, where connexin32 was first identified, and elsewhere in the body. The second promoter, P2, is nervous-system-specific and drives expression in peripheral nerve in a pattern similar to other myelin genes. Once we knew that the connexin32 gene is expressed in peripheral nerve and mapped to the same location as CMTX on the X chromosome, we set out to look for mutations in CMTX patients. We found that the large majority of CMTX patients have mutations in the translated portion of the gene (Bone et al. 1995). Since 1993, over 70 different mutations have been identified.

Many of the known CMTX mutations are shown in Figure 2, where connexin32 is shown in its likely topographic orientation, as an integral membrane protein with four transmembrane domains and amino and carboxyl termini within the cell. There are two extracellular loops with highly conserved cysteine residues that are thought to be involved in intramolecular disulfide bonds that ultimately stabilize

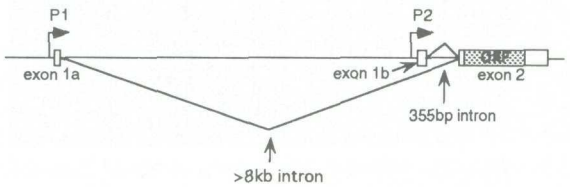

Figure 1. Structure of the human connexin32 gene. (ORF) Open reading frame.

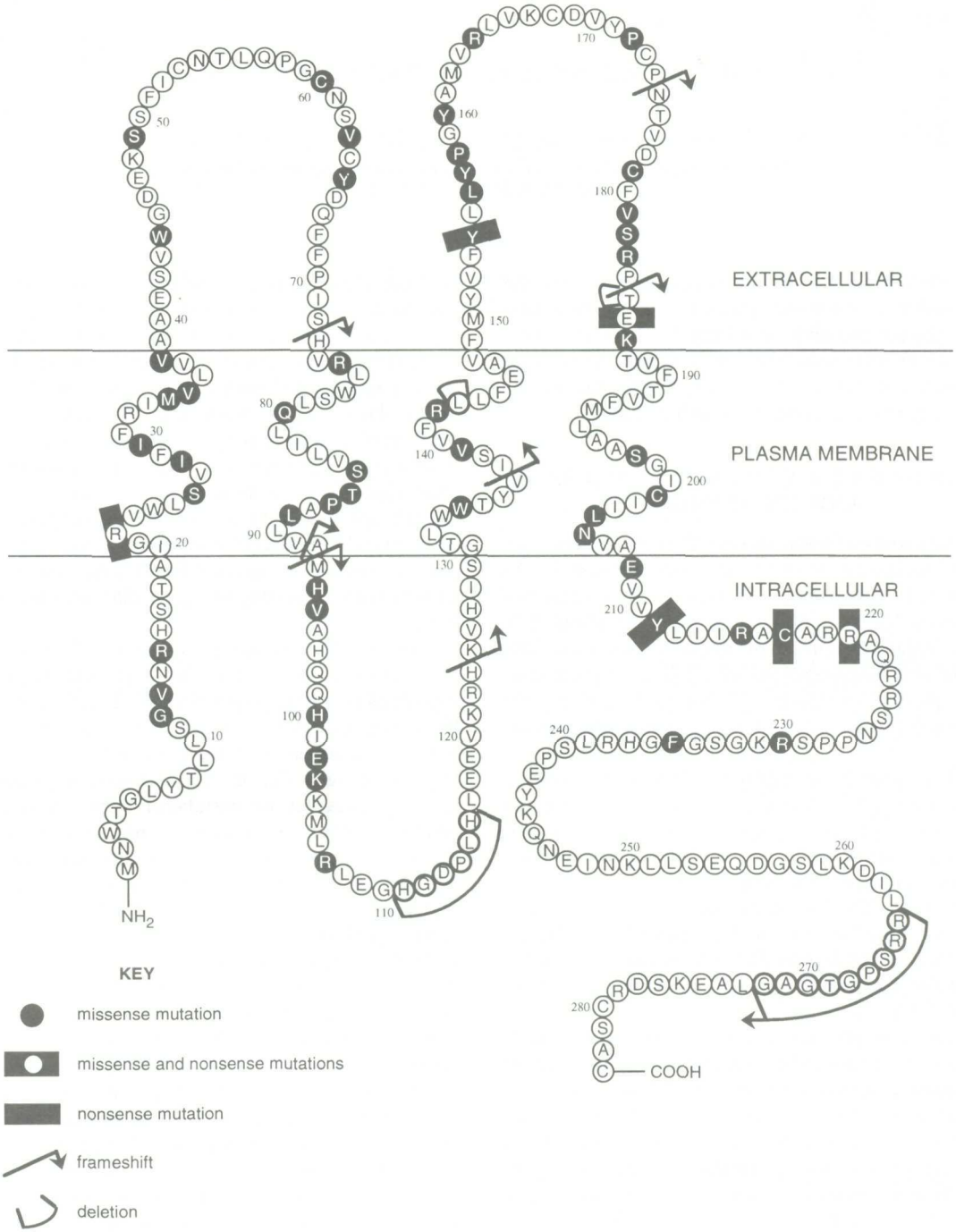

Figure 2. Diagram showing the topographic orientation of connexin32 with the locations of known CMTX mutations. 74 different mutations have been identified in 91 families.

the gap junction structure. The transmembrane domains are also highly conserved across species and among different members of the connexin gene family. The CMTX mutations are mostly missense (amino acid substitutions), although deletions, frameshifts, and premature stop mutations are also seen. Mutations have been found in every portion of the protein.

CONNEXIN32 STRUCTURE AND FUNCTION

Connexin32, like other members of the connexin family, is a channel protein. Six identical subunits cluster in the plasma membrane to form a hemichannel or connexon. This structure docks with a similar structure in another membrane to form a complete channel.

The channel has a central pore that allows passage of ions and other small molecules up to 1000 daltons in size from one cell to another. An array of individual channels makes up a gap junction.

Connexin32 is found in a variety of cells and tissues, including liver, secretory epithelium, and brain. Until it was implicated in CMTX, connexin32 was not known to be expressed in peripheral nerve, nor were gap junctions known to play a role in peripheral nerve structure and function.

What effect do the CMTX mutations have on the function of connexin32? Roberto Bruzzone in David Paul's laboratory at Harvard addressed this question by introducing wild-type and mutant connexin32 into *Xenopus* oocytes and assaying the formation of gap junctions electrophysiologically (Bruzzone et al. 1994). Normal connexin32, but not mutant connexin32, forms functional gap junctions in this system. Furthermore, mutant connexin32 interferes with the formation of gap junctions by co-injected wild-type cDNA, suggesting a dominant-negative effect.

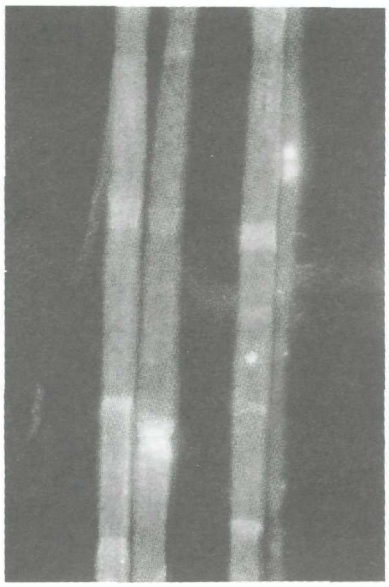

Figure 4. Teased fiber preparation of rat sciatic nerve, showing immunofluorescent staining of connexin32 at nodes of Ranvier and Schmidt-Lanterman incisures.

INTRACELLULAR TRAFFICKING OF MUTANT CONNEXIN32

We have gone on to investigate the fate of mutant connexin32 in stably transfected mammalian (PC12J) cells. Here the wild-type connexin32 protein forms aggregates at points of cell-cell contact (Fig. 3A). These are not seen in nontransfected cells. Immunohistochemistry of cells transfected with connexin32 cDNA derived from a CMTX patient shows that the mutant protein "hangs up" in the cytoplasm, most prominently in the endoplasmic reticulum and Golgi apparatus, and is not properly routed to the plasma membrane (Fig. 3B).

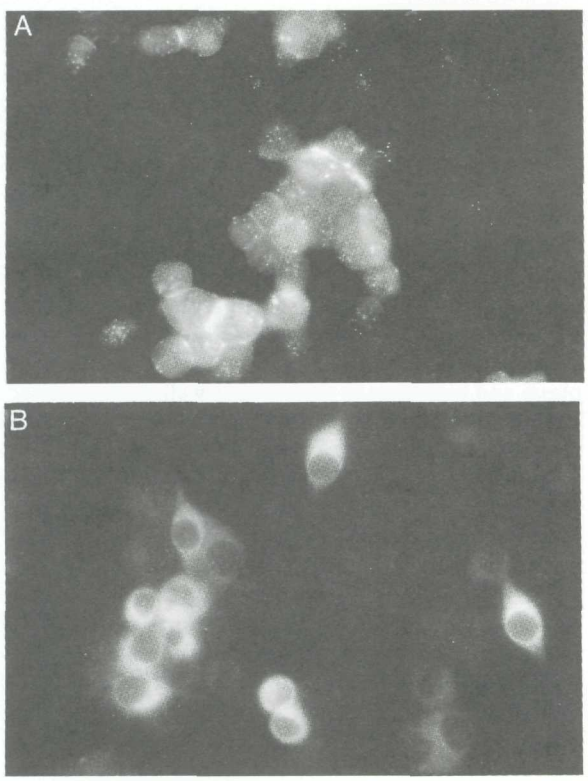

Figure 3. Immunofluorescence of connexin32 in stably transfected PC12J cells. (*A*) Wild-type connexin32, which localizes to the plasma membrane at points of cell-cell contact. (*B*) Mutant connexin32, which remains in the cytoplasm.

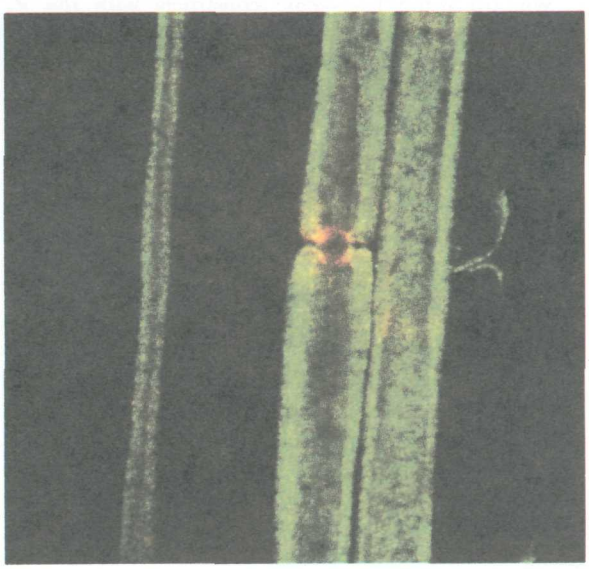

Figure 5. Confocal image of myelinated nerve fibers showing immunofluorescent staining of P_0 (*green*) and connexin32 (*orange*).

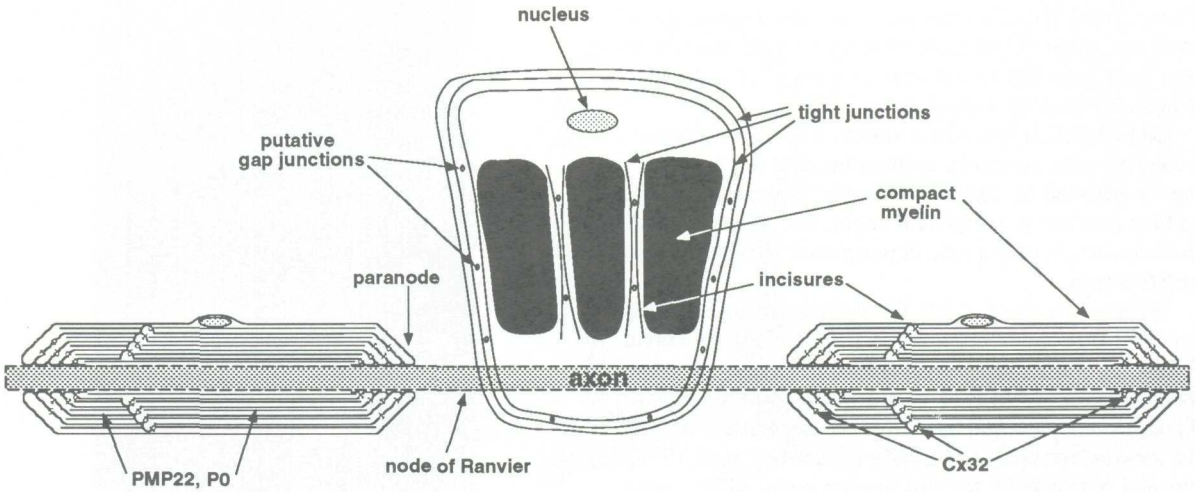

Figure 6. Schematic diagram of myelinated nerve, with the Schwann cell "unwound." Shown are the relative distributions of connexin32 and other myelin proteins and the location of putative gap junctions.

We have looked at seven different mutations and have found different effects on intracellular trafficking. In one case, the mutant cDNA is expressed at the mRNA but not the protein level; perhaps this protein is degraded too rapidly to give signal by immunofluorescence and Western blot. In several cases, some plasma membrane signal is seen. However, with most mutations, particularly those affecting residues within and adjacent to the transmembrane domains, the protein accumulates in the cytoplasm. It may be that misrouted connexin32 protein has a toxic effect on Schwann cells and axons, beyond the direct effects of loss of the protein's function. We plan to sort out toxic or dominant-negative effects in vivo by studying transgenic mice.

We are using transgenic constructs with the P_0 promoter to introduce two different mutant forms of connexin32 into mice. These mice are being bred with connexin32 knockout mice to observe the effects of the mutations on a null background. The knockout mice were produced by Dr. Klaus Willecke in Bonn, and have a relatively mild phenotype, consistent with a dominant-negative or toxic effect of the mutant protein on peripheral nerve.

CONNEXIN32 FUNCTION IN NORMAL NERVE

What is connexin32 doing in peripheral nerve? We can begin to answer that question by looking to see where connexin32 is normally located. We have done immunohistochemistry and have found connexin32 to be present in noncompacted myelin around the nodes of Ranvier and at Schmidt-Lanterman incisures (Bergoffen et al. 1993b; Scherer et al. 1995). This is a different distribution from other proteins implicated in hereditary neuropathy, PMP22 and P_0, which are located in compact myelin. The incisures and paranodes where connexin32 is located are residual folds of noncompacted Schwann cell cytoplasm that remain after the Schwann cell wraps itself around the axon to form the myelin sheath.

Figure 4 shows a teased fiber preparation demonstrating connexin32 staining in noncompacted myelin at the nodes and incisures. Figure 5 is a confocal image of myelinated nerve fibers with P_0 stained green in the compact myelin, and connexin32 stained orange at the node of Ranvier. There is no overlap between the distributions of P_0 and connexin32. Figure 6 shows the locations of connexin32 and other myelin proteins in peripheral nerve. David Paul proposed that connexin32 forms "reflexive" gap junctions at these sites, connecting different parts of the same Schwann cell rather than two different cells, and forming a path for diffusion of ions and other small molecules across the myelin sheath. This path perhaps provides nutritional support to the innermost layers of Schwann cell cytoplasm and indirectly to the axon, as well.

Preliminary results with injection of different dyes into myelinating Schwann cells of peripheral nerve indicate that such a path does indeed exist (R. Balice-Gordon et al., unpubl.). Low-molecular-weight dyes pass rapidly through incisures to the inner layers of Schwann cell cytoplasm, whereas high-molecular-weight dyes do not. We suspect that a loss of this pathway and perhaps additional toxic effects of misrouted protein account for the myelin loss and axonal degeneration of X-linked Charcot-Marie-Tooth disease.

The connexin32-CMTX story demonstrates how, when the interests of basic research and clinical medicine are coincident, we are brought to a better understanding not only of disease mechanisms, but also of the essentials of normal nervous system structure and function.

ACKNOWLEDGMENTS

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