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Molecular Organization of Gap Junction Membrane Channels

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Gap junctions regulate a variety of cell functions by creating a conduit between two apposing tissue cells. Gap junctions are unique among membrane channels. Not only do the constituent membrane channels span two cell membranes, but the intercellular channels pack into discrete cell–cell contact areas forming *in vivo* closely packed arrays. Gap junction membrane channels can be isolated either as two-dimensional crystals, individual intercellular channels, or individual hemichannels. The family of gap junction proteins, the connexins, create a family of gap junctions channels and structures. Each channel has distinct physiological properties but a similar overall structure. This review focuses on three aspects of gap junction structure: (1) the molecular structure of the gap junction membrane channel and hemichannel, (2) the packing of the intercellular channels into arrays, and (3) the ways that different connexins can combine into gap junction channel structures with distinct physiological properties. The physiological implications of the different structural forms are discussed.

KEY WORDS: Intercellular communication; connexon structure; electron microscopy; image processing; connexin structure; gap junctions.

INTRODUCTION

One of the basic problems in cell biology is that when organisms evolve from the unicellular to the multicellular level they must also develop some means with which to establish communication between cells. Intercellular communication is vital for the function of the organism and its constituent tissues and has profound importance for cell survival, differentiation, metabolism, morphogenesis, and mutagenesis. One way that gap junctions may regulate different cellular roles in different tissues is to vary their gap junction membrane channel structures. These channel structures can be thought of as "variations on a theme" of a basic channel design.

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MOLECULAR STRUCTURE OF GAP JUNCTION MEMBRANE CHANNELS

Gap junction membrane channels possess a high degree of symmetry. Each *membrane channel* (also referred to as an *intercellular channel* or *junctional channel*) is composed of two oligomers with each of two adjacent tissue cells contributing one oligomer (see Fig. 1A). Each oligomer is called a *connexon* or *hemichannel* and each connexon is built from six subunits of a single member of the connexin family (Makowski *et al.*, 1977; Cascio *et al.*, 1995). Connexons are ~65 Å in diameter and are arranged on a hexagonal lattice. Depending on the isolation conditions and detergent extraction procedures used, the lattice constants range from 78–90 Å. As imaged by low-dose negative staining (Baker *et al.*, 1983, 1985) or frozen-hydrated EM, the membrane channel usually appears as a skewed, six-lobed unit (Unwin and Ennis, 1984; Gogol and Unwin, 1988; Sosinsky *et al.*, 1990). Images of hemichannels in projection show this same

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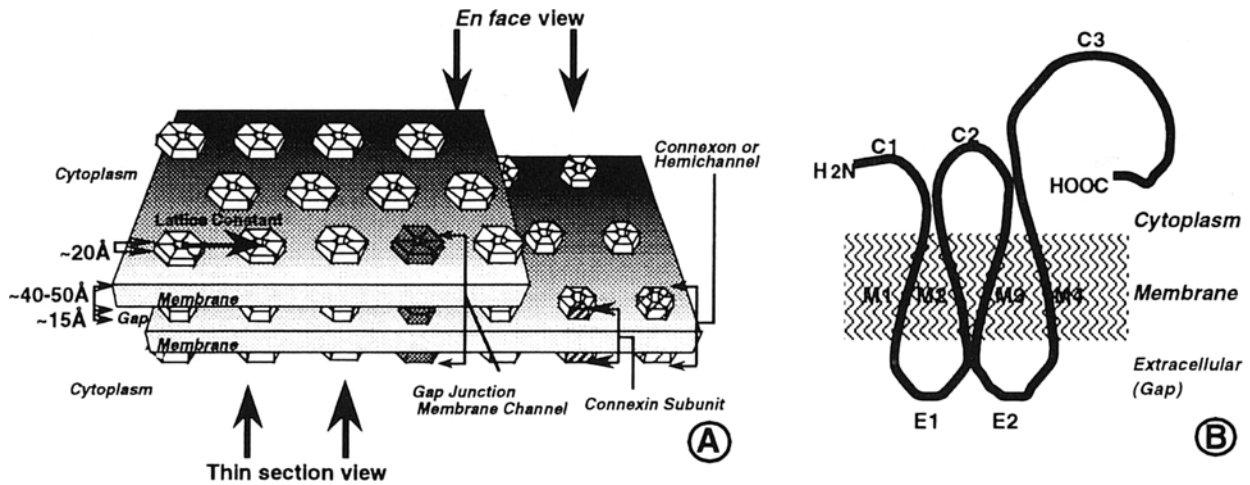

Fig. 1. Schematic illustration of current models for gap junction structure. (A) Each connexon is drawn as a hexamer of connexin subunits. Two apposing connexons dock to form a membrane channel. (B) Folding model of connexin structure containing four membrane-spanning domains (M1–M4), two extracellular loops (E1, E2), and three sequences localized to the cytoplasmic surface of the membrane [the *N*-terminus (C1), a cytoplasmic loop (C2), and the *C*-terminus (C3)]. Adapted from Ghoshroy *et al.* (1995) by permission of the *Journal of Membrane Biology.*

general appearance as whole channels (Ghoshroy *et al.*, 1995; Stauffer *et al.*, 1991). In negatively stained samples, there is often a stain-excluding region on the three-fold axis (Baker *et al.*, 1983, 1985). Polymorphism in the individual connexons arises from the interaction of the membrane channels and negative stain (Baker *et al.*, 1985; Sosinsky *et al.*, 1990). The two connexons pair to form a tight seal with a 20–30 Å gap between the apposing cell membranes. This structure creates a a hydrophilic pathway ~15 Å wide and ~180 Å long (Makowski *et al.*, 1977, 1984b).

Gap junction channels contain one or more different proteins from a multigene family of homologous proteins called *connexins*. The connexin proteins are designated by the abbreviation Cx followed by the molecular weight in kilodaltons, e.g., Cx32. At present, approximately 14–16 different connexins have been identified from gene or cDNA cloning. Based on sequence homologies, the connexins share a common folding topology. Membrane protection studies using proteolysis and antibody labeling have shown that the protein chain traverses the membrane four times. The N and C termini are located on the cytoplasmic side of the cell membrane while the extracellular (or gap) side of the membrane contains two amino acid loops. Comparisons of the amino acid sequences of the connexins have shown that these proteins contain domains in which the primary sequence has been highly conserved and domains that have highly variable sequences. The conserved regions of the sequences are localized to the four membrane-spanning domains and the two extracellular loops while the variable regions of the amino acid sequences are found in the cytoplasmic domains (see Fig. 1B).

The connexon can be thought of as consisting of three functional domains: the transmembrane domains that form the channel, the extracellular domains that are important in cell–cell recognition and docking of the two connexons, and the cytoplasmic domains that influence or regulate the physiological properties of the channel. However, this is a simplistic view because the different domains do influence each other. For example, charge substitutions of amino acids at the border between the first transmembrane sequence and the first extracellular loop can reverse gating polarity. These charge substitutions also suppress another amino acid charge substitution located on the N terminus on the opposite side of the membrane (Verselis *et al.*, 1994). Nevertheless, this three-domain approach is useful in discussing the structure of the domains of the gap junction membrane channel.

Organization of the Transmembrane Domains

From hydrophobicity profiles of the sequence, the four membrane-spanning segments are thought to be α-helices. Based on the topological models, these four segments (named M1–M4) are about 20 amino acids in length. Examination of the sequence of protein

sequences of the four transmembrane sequences has led to the hypothesis that the putative M3 helices from six connexin subunits builds the wall of the pore (Milks *et al.*, 1988). This hypothesis is based on the M3 primary sequence in which a line of polar residues is surrounded by a high proportion of aromatic residues. If the M3 segment is α-helical, then the polar residues would face the inside of the channel while the aromatic residues would pack together to form the subunit–subunit interfaces (Milks *et al.*, 1988). Unwin's model of the channel gating mechanism is based on tilts and shifts in the packing of this four α-helical bundle, resulting in movements similar to that of a camera or lens iris (Unwin and Ennis, 1984; Unwin and Zampighi, 1980).

By comparing the ratio of the equatorial to the meridional reflections from gap junction fiber samples with simulated fiber diffraction patterns from protein structures with known α-helical and β-sheet compositions, Tibbitts *et al.* (1990), estimated that the connexin protein contained ~60% α-helical structure. The model that best fitted the gap junction diffraction data contained a four α-helical bundle with one of the helices tilted ~20° with respect to the membrane plane. This model is consistent with an analysis of circular dichroism spectra of sonicated gap junction plaques (Cascio *et al.*, 1990) and a solubilized connexon fraction (Cascio *et al.*, 1995). Based on the X-ray analysis, the lengths of the four α-helical hydrophobic segments should be ~30–45 Å. Since the thickness of the hydrocarbon part of the membrane bilayer has been calculated to be ~32 Å (Makowski *et al.*, 1977, 1984b; Tibbitts *et al.*, 1990), it is likely that some of the α-helical structure extends past the bilayer into the extracellular domains (Tibbitts *et al.*, 1990). Recently, the α-helical nature of the gap junction membrane channel in crystalline plaques containing intercellular channels made up of a carboxy terminal truncation mutant of Cx43 has been directly confirmed by high-resolution electron microscopy (Unger *et al.*, 1996).

Organization of the Cytoplasmic Domains

Several lines of evidence suggest that the structure at the cytoplasmic surfaces is disordered while the transmembrane and extracellular domains are highly ordered. Three-dimensional reconstructions from electron microscopy (Unwin and Zampighi, 1980; Unwin and Ennis, 1984; Sikewar and Unwin, 1988; Sikewar *et al.*, 1991) and X-ray diffraction analyses (Makowski, 1988; Tibbitts *et al.*, 1990) have all failed to demonstrate periodically ordered structure. However, it is possible that there may be some local order within the cytoplasmic domains, especially in connexins with large cytoplasmic termini.

More detailed evidence for disordered surface structure is as follows. X-ray diffraction measurements of the scattering profile of rodent gap junctions (containing Cx32 and Cx26) perpendicular to the membrane showed that the protein structure on the cytoplasmic surface extends out to about 90 Å from the middle of the gap (Makowski *et al.*, 1982; Makowski, 1988; Makowski *et al.*, 1984a). Removal of the cytoplasmic domains in rodent liver gap junctions by proteolysis also does not significantly change the high-resolution reflections in X-ray diffraction patterns. Electron micrographs of cross-sections of gap rodent liver junctions fixed with tannic acid show stain-excluding projections at the cytoplasmic surface which appear to correspond to cytoplasmic protein domains (Ghoshroy *et al.*, 1995; Sosinsky *et al.*, 1988) detected by X-ray diffraction. Structural protrusions extending out into the cytoplasm are seen in the original images (see Fig. 2). Fourier averages of images of cardiac gap junctions containing Cx43 at ~16 Å resolution are almost identical with liver gap junctions (Yeager and Gilula, 1992) in spite of ~11 kDa mass difference between Cx43 and Cx32 in the C-terminal tail. Endogenous proteolysis of Cx43 produces a connexin that is missing an ~13-kDa fragment, but the Fourier averages of images of the proteolyzed sample are almost identical to the unproteolyzed gap junction. Thin-sections of cardiac gap junctions contained a "fuzzy coat" which was absent if proteolysis of the cytoplasmic domains occurred during isolation (Manjunath *et al.*, 1985). Quasi-crystalline arrays of MP38 (a lens junctional protein which is the sheep homologue of mouse Cx50, (White *et al.*, 1992) form when the precursor protein, MP70, is proteolyzed to MP38 (Kistler, 1990, 1993). However, crystallization trials of MP70 have been unsuccessful (Lampe *et al.*, 1991). The cytoplasmic, carboxyl-terminal portion of MP70 is removed by proteolysis, furthering strengthening the hypothesis that the removal of the bulky cytoplasmic domains is important in determining crystallinity. A Fourier average of the units in the reconstituted MP38 gap junction membranes is similar in appearance to rodent liver (Cx32 or Cx26) or heart (Cx43) membrane channels. Furthermore, topographic images of the cytoplasmic surface of isolated rat cardiac and liver gap junction plaques obtained by atomic force microscopy

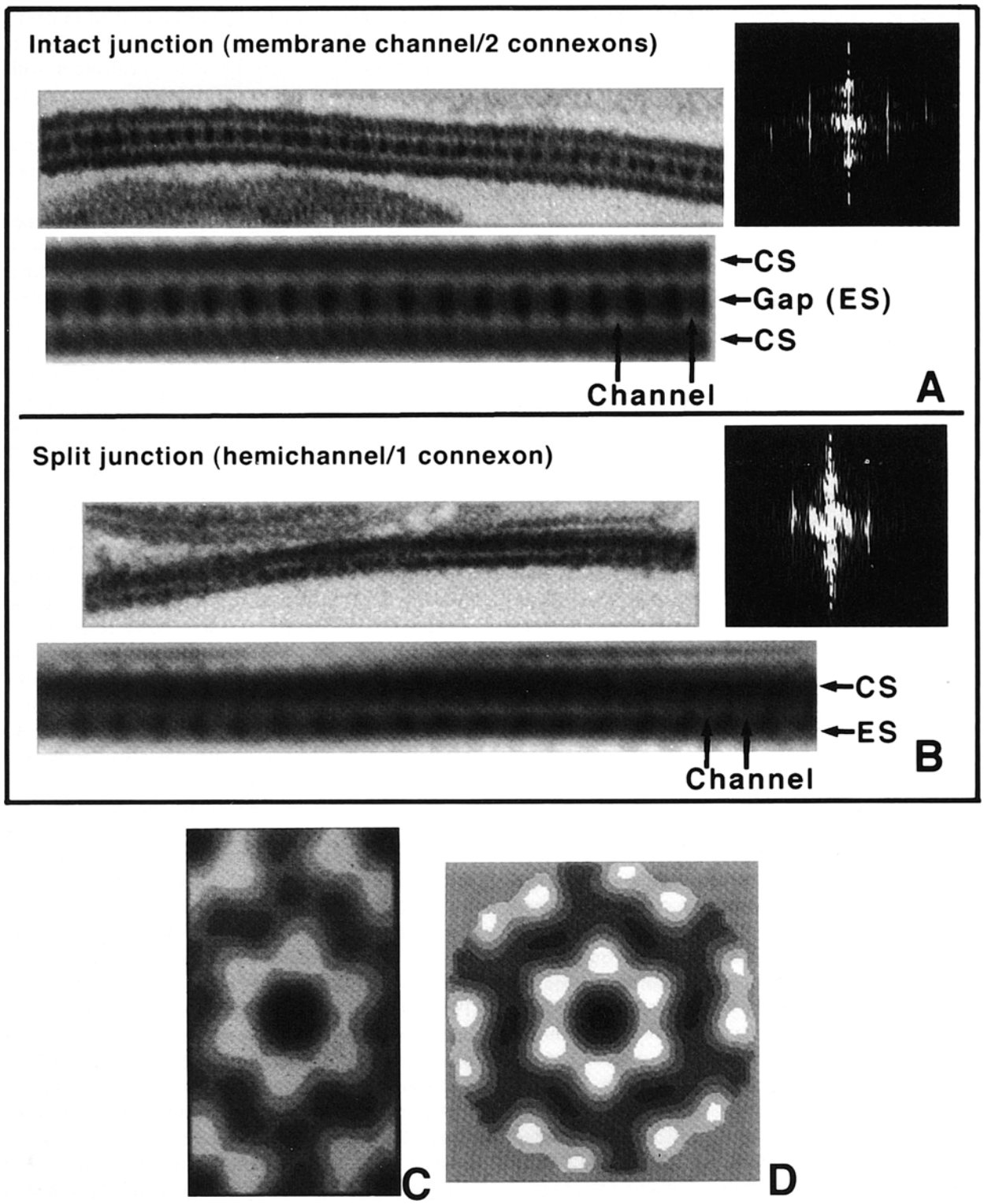

Fig. 2. Sections along the lattice axes showing the connexon structure perpendicular to the membrane plane. (A) An intact junction containing intercellular channels and (B) a split junction containing single connexons. The cytoplasmic surface is denoted by CS and the extracellular surface by ES. Adapted from Ghoshroy *et al.* (1995) by permission of the *Journal of Membrane Biology.* (C) Hexagonally symmetrized image averages of a single unit obtained from *en face* views of a frozen-hydrated plaque containing intercellular channels and (D) hemichannels.

(AFM) appear fuzzy and indistinct while AFM images of the extracellular surface show distinct lattice structure and subunit detail (Hoh *et al.*, 1991b, 1993; Lal *et al.*, 1995). It is interesting to note that in gap junction structures obtained by crystallographic methods (Baker *et al.*, 1983, 1985; Ghoshroy *et al.*, 1995; Gogol and Unwin, 1988; Makowski, 1988; Makowski *et al.*, 1977; Perkins *et al.*, 1996; Unwin and Ennis, 1984; Unwin and Zampighi, 1980) the pore at the cytoplasmic surface is a very strong feature. There are two explanations for the AFM observation: (1) the cytoplasmic domains block the entrance to the pore or (2) part of the protein sits at the mouth of the channel and controls gating as suggested by Makowski (1988).

This disorder in the cytoplasmic domains argues for flexibility of these domains within the intercellular channel. This flexibility may have functional importance. A recent paper by Morley *et al.* (1996) presents evidence that the cytoplasmic domain of Cx43 may act as an independent "particle" which can bind to the channel domain in a receptor–ligand interaction. Previous work had shown that shortening of the C-terminal domain of Cx43 to the length of the C-terminal domain in Cx32 changes the channel properties in expression systems so that its response to acidification resembles the response of Cx32 junctions (Liu *et al.*, 1993; Fishman *et al.*, 1991). The pH sensitivity of the Cx43 truncation mutant in paired *Xenopus* oocytes was restored when a fragment consisting of residues 259–382 of Cx43 (the difference in length between Cx43 and Cx32) was expressed as a separate protein. In addition, a less pH-sensitive connexin, Cx32, can be made more pH sensitive when the 259–383 fragment is co-expressed. The flexibility in the cytoplasmic tails may be necessary in order to interact with the channel in the same manner that flexibility in insulin is strictly necessary in order for it to bind to the insulin receptor (Derewenda *et al.*, 1981).

Organization of the Extracellular Domains

The binding of two apposing connexons must be sufficiently strong to create an insulated channel (Peracchia *et al.*, 1994). Each of the two extracellular loops contains ~40 amino acids with a set of three cysteines which are strictly conserved. These cysteines are believed to form disulfides within connexins but not between connexin subunits or to the partner connexon (Dupont *et al.*, 1989; John and Revel, 1991; Rahman *et al.*, 1993). In Cx32, one disulfide bond forms between E1 and E2 while two disulfide bonds are formed by two cysteine residues within one of the extracellular loops. Close inspection of the amino acid sequence for the extracellular loops, E1 and E2, suggests that there are significant stretches of hydrophobic residues. The E1 loop in Cx32 contains 12 hydrophobic residues out of 35 while 21 out of 43 amino acids in the E2 loop are hydrophobic. E2 contains a stretch of 11 consecutive hydrophobic amino acids, which is remarkable because this portion of the sequence is localized to the aqueous gap region. E2 has been shown to be a determinant in heterotypic channel formation (White *et al.*, 1994). Hydrophobic interactions predominate at the contact regions because urea (a chaotropic agent) is absolutely necessary to split the junction pair. Previously published work had shown that incubation of gap junctions in 8 M urea and high pH was an effective agent in splitting the membrane pair (as reviewed in (Ghoshroy *et al.*, 1995), but the results of these protocols were variable. Recent experiments in our laboratory have focused on determining the best conditions for splitting of the membrane pair (Ghoshroy *et al.*, 1995). We tried splitting gap junctions by changing one or more of seven different parameters: urea concentration, length of incubation, temperature of incubations, chelating agents, pH, reducing agents, and isolation protocol. To summarize, we found that there was a minimum urea concentration (4 M) that was effective in separating the membrane. Higher temperatures were also necessary for splitting the pair. We also found that there is an optimum incubation time. These conditions indicate that there is a breakdown of hydrophobic interactions during the urea incubation as has been demonstrated for water-soluble proteins (Timasheff, 1993). Less important was the pH of the incubation and the presence of DTT as a reducing agent. This would support the findings that the disulfide bridges are intra-connexin rather than inter-connexon or inter-connexin. Another critical ingredient in determining connexon–connexon interaction is the presence of EGTA. We found that including EGTA in our urea incubation significantly increased the numbers of split junctions. EDTA did not have the same effect. At certain pH's EGTA has a higher specificity for chelating Ca^{2+} than other ions. The binding of Ca^{2+} may contribute to interactions stabilizing the docking of apposing connexons. Connexins contain two aspartic and three glutamic residues that are absolutely conserved among the primary sequences of connexins (Peracchia *et al.*, 1994). A third aspartic acid is conserved in 14 out of 16 connexin sequences. These

residues are negatively charged at pH's higher than 3 and are likely sites for divalent cation binding (Chakrabarti, 1990). While Ca^{2+} has been implicated as a switch for a gating mechanism (Unwin and Ennis, 1983, 1984; Unwin and Zampighi, 1980), it is also possible that Ca^{2+} bridges may exist between the hemichannels, thereby stabilizing intercellular channel structure.

Although it appears that the cytoplasmic domains of the gap junction are flexible and, therefore, invisible to image-averaging techniques, the opposite is true of the extracellular surface (Sosinsky, 1992; Hoh *et al.*, 1991a,b). The transmembrane and extracellular domains of the connexon are the most ordered parts of the structure. As previously mentioned, estimates of α-helical content based on X-ray diffraction patterns by Tibbitts *et al.* (1990) suggest that some parts of the extracellular domains are α-helices in addition to the transmembrane domains. However, recent work by Dahl *et al.* (1994) with a synthetic peptide containing 12 amino acid sequences from the E2 loop of Cx32 shows that channels form in the bilayers. Given the short length of the peptide, Dahl *et al.* suggest that the only way channels could form would be if this peptide is in a β-sheet conformation. Mutagenesis experiments by Rosinski and Nicholson (1995) show that functional expression can be rescued by moving two cysteine residues along the extracellular loops in tandem, also suggesting an anti-parallel β-sheet conformation in the extracellular domains.

The rigidity of the extracellular surface domains makes the gap junction a suitable specimen for atomic force microscopy (AFM) (Hoh *et al.*, 1993). In essence, atomic force micrographs are high-resolution topographs and, therefore, AFM is a good tool for probing surfaces. In the original paper by Hoh *et al.* (1991b), the tip of an AFM cantilever was used to strip off the top bilayer of the double membrane structure, a process which the authors called "force dissection." The forces necessary for force dissection are at least ten times larger than the forces used for imaging surfaces. The images of the extracellular surface that was revealed showed a fairly regular hexagonal lattice. With improvements in AFM technology, more detailed images of the extracellular surface of force-dissected liver gap junctions were collected. The individual connexons protuded 14 Å from the extracellular surface and had a larger pore size (38 Å) than previously measured (~20 Å; see Unwin and Zampighi, 1980). The larger pore size could be due to parts of the protein being pushed away from the tip or to structural changes that occur when the two hemichannels are separated. These AFM images are intriguing because they show topological detail of the extracellular surface that was not evident in previous three-dimensional reconstructions (Unwin and Zampighi, 1980; Unwin and Ennis, 1984). Connexons in the best images showed a height modulation at their periphery suggesting a surface modulation. These images suggest that two apposing connexons fit into one another in the same fashion as intermeshing cogs. Such a topology would be an important factor in the molecular recognition of two hemichannels. The extracellular surface of connexons has been poorly characterized because the published three-dimensional reconstructions were of the whole channel (two docked connexons) which was then divided at the midpoint to show a single connexon. In our laboratory, a three-dimensional reconstruction of single connexon layers (split junctions) is in progress and initial indications confirm the extracellular surface topology seen in the AFM images (Perkins *et al.*, 1996).

PACKING INTERACTIONS BETWEEN CONNEXONS AND/OR INTERCELLULAR CHANNELS

Gap junction membrane channels form two-dimensional arrays *in vivo*. Why these membrane channels pack together into discrete cellular structures is an interesting question. One would expect that it would be more advantageous for a cell to have open cell–cell channels all over the surface of the plasma membrane in order to transport metabolites and ions rather than in discrete areas.

Freeze-fracture electron micrographs show that gap junctions in intact tissues have variable numbers of closely packed membrane channels (Goodenough and Revel, 1970; Hirokawa and Heuser, 1982). Gap junctions may contain aggregates of small numbers of intercellular channels or thousands of intercellular channels. In freeze-fracture electron micrographs of intact tissues, most gap junction arrays are identified by their close-packing arrangement. Freeze-fracture of gap junction plaques in various tissues shows different packing morphologies (Shivers and McVicar, 1995; see Fig. 3). The packing of the gap junction channels range from a close-packed, liquidlike morphology to morphologies where the intramembranous particles are ordered into microdomains or rows within the arrays separated by lipid areas (Shivers and McVicar, 1995).

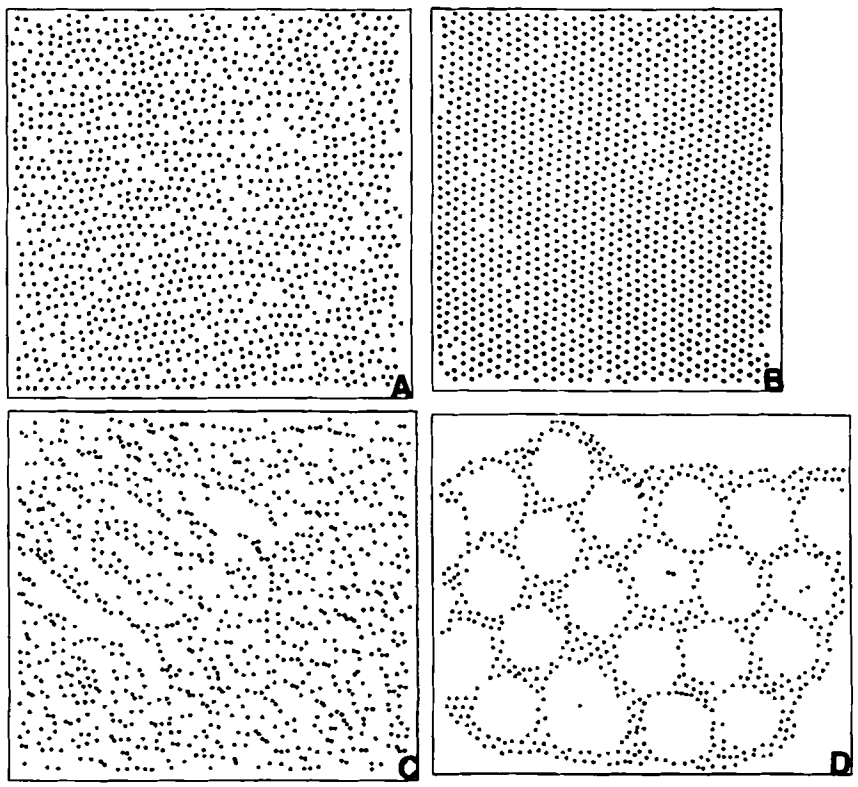

Fig. 3. Packing of channels in intact tissues and isolated plaques. This figure shows various packing morphologies in four different images. The positions of the intercellular channels were plotted in micrographs either by computer pattern recognition algorithms (A and B, adapted from Sosinsky *et al.*, 1990) or by eye (C and D). (A) Intercellular channel positions in a freeze-fracture electron micrograph obtained from rodent liver (see Sosinsky, 1992 for original image). Note the hexatic phase appearance of the channels in the plaque. (B) Packing of the membrane channels in isolated detergent-treated rat liver gap junction plaques. Notice that despite the more crystalline arrangement there are dislocations and absences of units in the lattice. (C) Packing of intercellular channels in a freeze-fracture electron micrograph of gap junctions in cultured bovine brain epithelial cells (see Shivers and McVicar, 1995 for original micrograph). The membrane channels form loosely packed rows as opposed to the more liquidlike arrangement in (A). (D) One of the more unusual packing arrangement of gap junction channels. Adapted from a freeze-fracture electron micrograph of gap junctions in the atrioventricular node of the golden hamster heart. (See Skepper and Navarantnam, 1986 for original micrograph.)

In one of the most unusual gap junction morphologies, freeze-fracture electron micrographs of the atrioventricular node of the golden hamster heart show an arrangement of the intercellular channels in which narrow rows of particles are arrayed in circular patterns around uniformly sized particle-free areas of membranes (Skepper and Navarantnam, 1986). Guerrier *et al.* (1995) showed that Cx32 and Cx43 are located in different regions of the plasma membrane of thyroid epithelial cells. Laser scanning confocal microscopy revealed that Cx32 gap junctions were scattered over the lateral membrane domain, while Cx43 gap junctions formed a meshed network superimposable with tight junctions in the subapical regions of cells. Before that work, no interaction between gap junctions and other cellular junctions or cytoskeleton had been demonstrated.

What are the functional implications of these packing arrangements? Membrane channels in intact and physiologically coupled junctions were shown to be much more widely dispersed than in uncoupled noncommunicating junctions or in isolated gap junction plaques (Raviola *et al.*, 1980; Hirokawa and Heuser, 1982). Therefore, the packing of the junc-

tional channels is important since it may be indicative of whether two apposing cells are communicating. In freeze-fractured junctions obtained from living cells, the mean center-to-center connexon spacing is ~95 Å and in more disordered quasi-hexagonal arrays this distance increases to ~110 Å. Analysis of the pair correlation function (a measure of the interaction distance between the channels) from freeze-fractured electron micrographs of gap junctions showed that this lateral interaction is repulsive in nature (Abney *et al.*, 1987). The connexons are associated in closely packed arrays despite the repulsive forces between coupled cells (Braun *et al.*, 1987). The membrane–membrane repulsive forces could either be electrostaic repulsion between charged molecules in the membrane bilayers or nonspecific steric repulsion between glycoproteins at the cell surface (Bruinsma *et al.*, 1994). Although gap junction formation is influenced by the expression of cell adhesion molecules (Meyer *et al.*, 1992) gap junctions do not seem to have any interactions with cellular cytoskeletal components. While some electron micrographs have provided tantalizing evidence that bridges or substructure exist in the lipid matrix between the connexons (Baker *et al.*, 1983, 1985; Peracchia and Peracchia, 1985; Rash and Yasumura, 1992), these features have not been imaged in any of the published three-dimensional reconstructions (Sikewar *et al.*, 1991; Sikewar and Unwin, 1988; Unwin and Ennis, 1984; Unwin and Zampighi, 1980).

When isolated from rodent liver cells (Benedetti and Emmelot, 1968; Fallon and Goodenough, 1981; Hertzberg, 1984; Hertzberg and Gilula, 1979), the connexons in gap junction plaques form hexagonal crystalline domains with lattice constants ranging from 76 to 90 Å (Makowski *et al.*, 1982, 1984a). The magnitude of the lattice constant (distance between adjacent connexons) and the degree of crystalline order depends on the isolation conditions and specimen preparation. Long-range order in the hexagonal lattice is generally associated with substantial short-range disorder (Caspar *et al.*, 1977; Sosinsky *et al.*, 1990). The combination of short-range packing disorder with long-range hexagonal order appears to be an inherent feature of gap junction plaques. For gap junctions in tissues, the root-mean-square variation of connexons from ideal hexagonal lattice positions is about twice that of isolated plaques (Sosinsky, 1992). The distance between the membrane channels increases as the number of lattice repeats increases. This loss of translational order with increasing separation shows that the connexon lattices are not true crystals. In a true crystal, the distance between unit repeats is constant. In these gap junction lattice images, the hexagonal lattice orientation is maintained over large distances. The term "hexatic phase" (Nelson and Halperin, 1979) has been applied to solid phases intermediate between liquid and crystals (such as a melting crystal) that conserve hexagonal order without long-range translational order. An example of a well-studied hexatic phase solid is closely packed polystyrene latex spheres (Murray and Van Winkle, 1987) where the interactive forces between the identical particles are repulsive in nature. Therefore, gap junctions are a naturally occurring hexatic phase structure, reflecting the fact that gap junctions in living cells are dynamic and fluid structures.

The packing density in isolated gap junction plaques may be altered by the removal of lipids from the spaces between the membrane channels. The protein-to-lipid ratio is completely dependent on the procedure used to isolate the junctions (Malewicz *et al.*, 1990). Lattice constants for gap junction specimens treated with detergents are reproducibly smaller than in untreated membranes (Caspar *et al.*, 1977; Gogol and Unwin, 1988) or gap junctions isolated by an alkali extraction of the plasma membrane fraction (Ghoshroy *et al.*, 1995). Analysis by Henderson *et al.* (1979) showed that treatment with detergents selectively removes phospholipids, but that the cholesterol composition remains relatively invariant. This observation indicates that the channels are stabilized by cholesterol rather than phospholipid. In addition, the cholesterol content of isolated gap junctions is quite high compared to other membranes (Malewicz *et al.*, 1990). The detergent solubilities and buoyant densities of Cx32 gap junctions from the plasma membrane of thyroid epithelial cells were different from Cx43 gap junctions, implying a difference in the molecular environment (i.e., lipid-to-protein ratio) of the two types of gap junctions. Thus, the gap junction lattice is fairly fluid, capable of large changes in lattice constants or nearest-neighbor distances, while the structure of the individual unit is maintained. The lattice structure is influenced by cell adhesion molecules, lipid interactions, and the repulsive forces between junctional channels and cells.

The means by which connexins assemble into connexons has become an increasingly important area of investigation. The review by Laird in this series describes in depth the pathways in which gap junction proteins are assembled into connexons. However, there

are some interesting questions as to how mature hemichannels aggregate and dock with a partner connexon from another cell that remain to be investigated. The turnover time of gap junction proteins is fairly fast (~5 hours as estimated by Fallon and Goodenough, 1981). Therefore, gap junction plaques should not be assumed to be static structures. Musil and Goodenough (1993) have shown that oligomerization occurs in the trans-Golgi network and the connexon is transported to the plasma membrane. There should be connexons in the plasma membrane that have not yet been incorporated into gap junction plaques. Johnson and his co-workers have postulated the existence of "formation plaques" (Preus *et al.*, 1981). They hypothesized that once two cells adhere, they produce regions with clustered 90–110 Å particles and decreased numbers of other particles. The interaction of these intramembranous particles across an initially wide extracellular space brings the membranes closer together, and when a critical particle density or proximity is reached, the particles aggregate into small gap junctions. Additional gap junction membrane channels are added until the gap junction plaques reach their mature size. Biochemical studies have shown that unpaired connexons exist in the plasma membrane (DeVries and Schwartz, 1992; Evans, 1994; Musil and Goodenough, 1993). It is interesting to note that Cx46 forms open hemichannels under certain physiological conditions in single *Xenopus* oocytes (Paul *et al.*, 1991). However, because it is not possible to distinguish between intramembranous particles in freeze-fracture electron micrographs, isolated hemichannels in the plasma membrane of living cells or different connexin-containing gap junctions have not been directly imaged or isolated.

SPATIAL ORGANIZATION OF CONNEXIN ISOFORMS

Physiological studies using both the paired *Xenopus* oocyte expression system and communication-incompetent cell lines have shown that some, but not all, connexons composed of different connexins form intercellular channels (see the review by Willecke in this series for a discussion on connexin expression systems). In each case, the physiological profile, as measured by voltage or pH changes, of the *heterotypic channel* (each connexon composed of a different connexin) is distinctly different from the *homotypic channel* (both connexons composed of the same connexin). Heteromeric connexons can only occur if different connexins mix in hemichannels. Presumably, these channels can form and function because the extracellular loop portions of the sequence are conserved among the different connexins. For example, in the *Xenopus* expression system, Cx32 is able to form channels with Cx26, Cx32, and Cx46, but not Cx37, Cx40, Cx43, and Cx31.1 (White *et al.*, 1995). Factors important in the pairing of heterotypic combinations of connexins are reviewed by White and Bruzzone in this series. In essence, intercellular membrane channels can be formed with different and specific pore properties by combining the constituent proteins in different ways. Here, I focus on the structural aspects of heterotypic and heteromeric channels.

In our own work, we became interested in determining the composition of gap junction channels through a mass analysis using scanning transmission electron microscopy (STEM) (Sosinsky, 1995b). The pixel intensities in a scanning transmission electron microscope (STEM) image are directly proportional to the electron scattering from object in the image. With an appropriate internal standard such as tobacco mosaic virus, the mass of a macromolecular complex can be calculated from the pixel values. By applying image analysis methods to micrographs obtained from a STEM, it is possible to identify individual connexons in a gap junction plaque and determine their masses (Sosinsky, 1995a,b).

I chose to analyze rodent liver junctions because preparations for purifying rodent liver junctions in sufficient quantities (e.g., 0.1–1 mg/ml) have been well worked out and because there are only two isoforms in the preparations that can combine to form a channel. Rodent hepatocyte gap junctions contain both Cx32 and Cx26. Connexins 26 and 32 co-localize both *in situ* and in isolated gap junction plaques (Zhang and Nicholson, 1989; Traub *et al.*, 1989). Functional heterotypic junctions are obtained when the mRNAs for Cx32 and Cx26 are expressed in paired *Xenopus* oocytes (Barrio *et al.*, 1991) and show a physiological profile different from the homotypic pairings.

Competition experiments involving co-injection of Cx32 and Cx26 mRNAs in *Xenopus* oocytes show that heterotypic junctions of Cx32 and Cx26 form with equal probability to homotypic junctions (Nicholson *et al.*, 1993). Gel electrophoresis of preparations of mouse liver gap junctions confirmed that this sample contained Cx32 and Cx26 in a ratio of 2:1, so if heterotypic gap junctions occurred *in vivo*, I would most likely find them in this sample. The rat liver gap junc-

tion sample served as a control for a system made primarily of one connexin (Cx32:Cx26 10:1 as assayed by SDS-PAGE). I found that in the STEM images obtained from the mouse sample, the distribution of connexon masses could be classified into three types of histograms (a) sharply unimodal (rms variation ~5–6% of the mean connexon mass) (b) bimodal (rms variation from the mean of each mass peak ~5–6%), and (c) broader unimodal (rms variation of the mean connexon mass ~10–11%). In addition, when the STEM images were displayed in color (Sosinsky, 1995b), segregation of channels with different masses into spatially distinct regions became apparent. This observation suggests that like channels segregate into separate domains. All but one gap junction image from rat liver gave unimodal connexon mass distributions. The three types of mass histograms can be interpreted as gap junction plaques containing (a) single populations of homotypic membrane channels, (b) two populations of homotypic channels, and (c) a population containing heterotypic channels.

In order to determine if the individual connexons contain more than one type of connexin, images of urea split mouse junctions were obtained. These images resulted in either bimodal mass distributions or sharp (~5% of the average value) unimodal mass distributions. A peak fitting analysis of these histograms of single connexons revealed bimodal mass distribution in three out of five images and unimodal distributions in the other two images. When masses from all five images were combined, the total population appeared bimodal and was consistent with the expected 2:1 Cx32:Cx26 ratio. The curve fitting showed that these histograms were well fit by two Gaussian mass populations, but a third Gaussian mass population corresponding to a putative population of heteromeric connexons was either within the estimated errors of the analysis or vanished during the reiterative fitting procedure. Mass histograms obtained from split rat junctions were all unimodal. Thus, each connexon in isolated gap junction plaques appears to be made from only one type of connexin.

The results from my analysis of whole and split mouse gap junction plaques are consistent with the view that only connexins of the same type group together into connexons. However, the connexons may pair with connexons of either similar or different types. In the nervous system, the differential expression of gap junction isoforms may be related to a higher organization of channel selectivity. Experiments with rabbit retinas with three different glial cell types have shown that gap junction mediated dye transfer occurs not only between astrocytes but also from astrocytes (containing Cx32) to oligodendrocytes (containing Cx43) and Müller cells. These findings indicate that these gap junction structures probably are heterotypic in nature (Robinson *et al.*, 1993). Characterization of gap junction packing in glial cells demonstrated that astrocyte–astrocyte gap junctions had a crystalline appearance while gap junctions between oligodendrites and astrocytes had a close packed appearance (Massa and Mugnaini, 1982). As previously mentioned, Cx32 and Cx43 in polarized epithelial cells localize to different cell contact areas (Guerrier *et al.*, 1995). An analysis of images of rat epidermis obtained by laser scanning confocal microscopy in combination with double immunofluorescence labeling with antibodies specific to Cx43 and Cx26 shows domains of segregated Cx43 and segregated Cx26 as well as regions of overlap of the two connexins (Risek *et al.*, 1994). However, due to the resolution of the technique, plaques containing interspersed homotypic channels cannot be differentiated from plaques containing heterotypic junctions. The resolution provided by the STEM is much greater and allows for single connexon mass measurements. The measurements I obtained showed that segregation of homotypic junctions occurs and that mixtures of the connexin proteins in whole channels are primarily due to pairings of different homomeric connexons, perhaps reflecting their original spatial tissue organization. For example, in rat liver, Cx32 is expressed throughout the entire liver, whereas Cx26 is preferentially expressed in the periportal regions (Traub *et al.*, 1989), and in mouse liver, Cx26 is interspersed throughout the entire liver.

Do heteromeric connexons exist in looser packed plaques or as individual channels? The size of gap junctions in cells may be an important consideration for cellular structure as well as three-dimensional localization. Visualization of gap junctions using freeze-fracture electron microscopic techniques is problematic. Gap junctions are only identified in freeze-fracture micro$_{\text{L}}$raphs because of their close packing arrangement. Therefore, a minimum number of closely packed intramembranous particles is necessary before gap junctions can be identified in freeze-fracture electron micrographs. My mass analysis did not provide evidence for only heteromeric connexons within gap junction plaques. However, as previously stated, the composition of isolated gap junctions is extremely dependent on the isolation protocol. There are two types of gap junction protocols. The first

selects for tightly packed gap junction plaques using detergents (sarkosyl; see Fallon and Goodenough, 1981) or high pH (Hertzberg, 1984) to solubilize the plasma membrane fraction. An alternative protocol for isolating gap junctional proteins is to use different detergents (octylglucoside or POE) to solubilize the plasma membrane fraction and then use antibodies specific to the connexins to select for the structures which contain gap junction proteins (Kordel *et al.*, 1993; Jiang and Goodenough, 1996; Kistler *et al.*, 1993; Konig and Zampighi, 1995). Based on sedimentation analysis, the fractions obtained by this second protocol contain structures which are probably solubilized connexons. In both the liver connexon fraction studied by Harris and colleagues (Kordel *et al.*, 1993) and the lens fiber connexon fraction obtained by Jiang and Goodenough (1996), these fractions contained more than one connexin which are co-immunoprecipitated under nondenaturing conditions by either of the connexin antibodies. Lens fiber junctions display unusual voltage gating profiles atypical of homomeric connexons. Lens fibers express two connexins, Cx45.6 (homologue to rodent Cx50 and ovine MP70) and Cx56 (homologue to rodent Cx46). Double staining of frozen sections with antibodies to Cx45.6 and antibodies to Cx56 co-localize in the same junctional plaques. Because lens connexons are easily detergent solubilized (Lampe *et al.*, 1991, L. Musil, personal communication), it is assumed that the packing of intercellular channels in lens junctions is much looser than for liver gap junctions. Recently, Konig and Zampighi (1995) have isolated a preparation of bovine lens intercellular channels and demonstrated that these structures contain both Cx45.6 and Cx50. Stauffer has isolated a fraction containing Cx32 and Cx26 when both isoforms are co-expressed in insect cell expression system (Stauffer, 1995), suggesting the existence of heteromeric connexons. These observations suggest that there are heteromeric connexons. If this is true, then this leads to an interesting hypothesis that the best ordered plaques are selective for homomeric or heterotypic membrane channels, whereas looser packed gap junction structures may contain heteromeric connexons (see schematic in Fig. 4).

In conclusion and returning to the original discussion of the molecular channel structure, an interesting question that has yet to be considered is that if heteromeric connexons exist, how would connexins of different molecular sizes pack into channel structure. We may find that there is a selectivity among the connexins not only for heterotypic pairs, but also for heteromeric pairings which is dependent on the length and composition of the C-terminal tail.

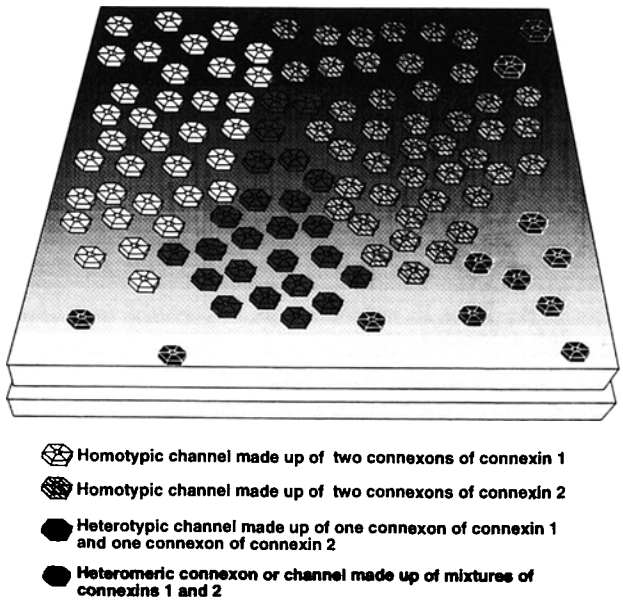

Fig. 4. Model for the arrangement of intercellular channels containing two different connexins. Intercellular channels containing two different protein isoforms can form homotypic junctions (all one protein), homomeric heterotypic junctions (two hemichannels each of which is made entirely of one protein), and heteromeric junctions (one or more hemichannels containing mixtures of connexins). As described in the text, evidence is presented for a model in which like channels self-associate into tighter packed arrays whereas connexons containing mixtures of two connexins may be found in either loosely packed areas or as isolated channels.

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