Expression, Two-Dimensional Crystallization, and Electron Cryo-crystallography of Recombinant Gap Junction Membrane Channels

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INTRODUCTION

Gap junction membrane channels provide an intercellular pathway for passive diffusion of ions, metabolites, and molecules up to ~1000 Da (Loewenstein, 1981), thereby coordinating the metabolic and electrical activities of tissues. Previous low-resolution analyses of gap junction structure by X-ray scattering (Makowski et al., 1977) and electron microscopy (Unwin and Ennis, 1984) supported a model in which the intercellular channel is formed by the end-to-end association of two oligomers termed connexons. More recently, recombinant DNA technology revealed the existence of a diverse multigene family of gap junction membrane proteins termed connexins (reviewed by Beyer et al., 1990; Willecke et al., 1991; Kumar and Gilula, 1992, 1996; Bruzzone et al., 1996; Goodenough et al., 1996). Experiments using protease cleavage, immunolabeling, and hydrophathy analysis contributed to current models in which each connexin subunit traverses the membrane bilayer four times, placing the N- and C-termini on the cytoplasmic membrane surface (reviewed by Yeager and Nicholson, 1996; Yeager, 1998). Electron cryo-crystallography of a recombinant, C-terminal truncation mutant of 43-kDa α₁ cardiac connexin (α₁,Cx43)3 has enabled us to derive two-dimensional (2D) and three-dimensional (3D) maps that confirm this model and show that each connexon is formed by 24 closely packed α-helices (Unger et al., 1997, 1999).

EXPRESSION OF FULL-LENGTH AND C-TERMINAL TRUNCATED α₁ CONNEXIN IN BHK CELLS

A major challenge in the structure analysis of low-abundance polypeptide integral membrane proteins has been the overexpression of sufficient functional material. A powerful approach for the electrophysiological characterization of gap junction channels has been provided by the expression of connexins and formation of functional channels in a variety of cell types such as Xenopus oocytes (Dahl et al., 1987; Swenson et al., 1989; Suchyna et al., 1993; White et al., 1995; Morley et al., 1996; Oh et al., 1997; Cao et al., 1998) and transfected, communication-deficient mammalian cell lines [for example, SKHep1
cells (Eghbali et al., 1990; Fishman et al., 1990, 1991; Moreno et al., 1991a,b), HeLa cells (Traub et al., 1994; Verselis et al., 1994; Eligang et al., 1995; Cao et al., 1998), and mouse N2a neuroblastoma cells (Veenstra et al., 1994, 1995; Beblo et al., 1995; Brink et al., 1997). However, the low level of expression in these systems precludes biochemical isolation and structural characterization. Connexins have been overexpressed in SF9 insect cells infected with a baculovirus vector (Stauffer et al., 1991). However, high-resolution crystals could not be generated. By use of a stably transfected baby hamster kidney (BHK) cell line, connexins have been expressed under control of the inducible mouse metallothionein promoter by the addition of 100 µM zinc acetate to the culture medium (Fig. 1; Kumar et al., 1995). Although the level of expression was quite low, the structure analysis was nevertheless feasible with just microgram amounts of recombinant material because the gap junction channels aggregate into plaques that can be isolated by cell fractionation techniques.

THE EXPRESSED RECOMBINANT CONNEXINS ASSEMBLE GAP JUNCTION PLAQUES

In BHK cells transfected with either the full-length or the C-terminal truncated α1 connexin cDNA (α1,Cx-263T), indirect immunofluorescence microscopy showed that the antigen was localized to the cell surface as well as to intracellular sites (Fig. 2). The punctate fluorescence on the cell surface was consistent with the assembly of the recombinant protein into gap junction plaques. By freeze-fracture analysis, plaques of variable size were detected with closely packed particles on the P fracture face and complementary pits on the E fracture face (Fig. 3). The gap junctions observed in BHK cells expressing α1,Cx-263T had a freeze-fracture morphology similar to that observed with BHK cells transfected with the full-length α1 connexin cDNA. Taken together, the freeze-fracture and immunofluorescence results demonstrated that at least some of the recombinant α1 connexin molecules were targeted to the plasma membrane and assembled into bona fide gap junctions.

IN SITU TWO-DIMENSIONAL CRYSTALLIZATION OF RECOMBINANT GAP JUNCTIONS

2D crystallization of membrane proteins is usually accomplished by reconstitution in which detergent-solubilized lipids are mixed with the detergent-solubilized and purified protein, and the detergent is removed by dialysis (Kühlbrandt, 1992; Jap et al., 1992; Engel et al., 1992). Gap junctions are formed by the aggregation of hundreds of channels in plaques that can be isolated by cell fractionation and sucrose gradient centrifugation. Since the protein is never removed from its native membrane, we refer to this approach as in situ crystallization (Yeager, 1994; Yeager et al., 1999). Detergent extraction removes lipids to concentrate the gap junction channels in the plane of the membrane to generate a tightly packed lattice. Optical diffraction patterns of the best 2D crystals often showed sharp spots to better than 10-Å resolution (Unger et al., 1997). A special advantage of such an expression system is the opportunity to generate mutants for detailed structure–function analysis. In order to focus on the transmembrane and extracellular architecture of the channel, the first mutant that we have examined, designated α1,Cx-263T, lacks most of the C-terminal domain of α1[Cx34].

FACTORS INVOLVED IN THE IMPROVEMENT IN RESOLUTION OF THE 2D CRYSTALS

Electron microscopy and image analysis of native gap junctions isolated from heart (Yeager and Gilula, 1992), liver (Makowski et al., 1977; Unwin and Ennis, 1984), and lens (Lampe et al., 1987) have been limited to 15- to 20-Å resolution. Examination of biological specimens in the frozen-hydrated state offers the possibility of higher resolution structure analysis. However, the best resolution achieved in the analysis of frozen-hydrated liver gap junctions was also ~15 Å (Unwin and Ennis, 1984; Gogol and Unwin, 1988). Our ability to record data to higher

![Schematic of pNUT vector containing the rat heart α1 connexin cDNA. The α1 Cx cDNA was inserted between the mouse metallothionein promoter (MT-1) and the polyadenylation sequence of human growth hormone (HGH 3′ UTR). Selection was based on the use of the dihydrofolate reductase gene (DHFR), which contained the polyadenylation site of the surface antigen gene from hepatitis B virus (HBV 3′ UTR) and was under control of an SV40 promoter (SV40 ori) (Simonsen and Levinson, 1983; Pulmiter et al., 1987). Adapted from Kumar et al. (1995).](image-url)
resolution from frozen-hydrated, recombinant gap
ejunction channels was probably dependent on sev-
eral factors that included the following.

(1) Molecular homogeneity. Liver tissue (Nicholson
et al., 1987) and cardiac tissue (Kanter et al., 1992)
are known to contain multiple connexin subtypes,
which may be present in the same gap junction
plaque. A special advantage of the stably transfect-
ed BHK cells is that only a specific connexin subtype is
expressed. Although BHK cells naturally express
full-length $\alpha_1\text{[Cx43]}$, Western immunoblots showed
that the full-length protein was detected at only very
low levels in overexposed immunoblots. Notably, the
carboxy-tail in native cardiac gap junctions is sensi-
tive to proteolysis (Manjunath et al., 1985; Yeager
and Gilula, 1992). By removing this protease-
sensitive site in the mutant, the specimen was
engineered to be more homogeneous.

(2) 2D crystallization. In previous studies of liver
and heart gap junctions, in situ 2D crystallization
was performed by treatment of gap junction plaques
with lubrol (Zampighi and Unwin, 1979), deoxycho-
late (Zampighi and Unwin, 1979; Yeager, 1994), and
dodecylmaltoside (Gogol and Unwin, 1988; Yeager,
1994). For the recombinant gap junctions, the vari-
ables that were tested to improve crystallinity in-
cluded detergent screens (Tween 20, Tween 80, Tween
85, and cymol), chaotropes (potassium iodide and
sodium thiosulfate), temperature (25 and 37°C), and
incubation in glycerol. The Tween detergents were
tested on the basis of their success in growing 2D
crystals of frog rhodopsin (Schertler and Hargrave,
1995). If the potassium iodide treatment was not
included, then the detergent extraction was not as
successful. In addition, potassium iodide could not be
replaced with sodium chloride. Best results were
obtained by adjusting the protein concentration to 1
mg/ml and incubating the membrane suspension in

FIG. 2. Phase-contrast (left) and indirect immunofluorescence micrographs (right) of BHK cells transfected with the full-length (A and
A’) or truncated $\alpha_1$ connexin ($\alpha_1\text{[Cx-263T]}$) cDNA (B and B’). Note the abundance of gap junction antigen on the cell surface at sites of
intercellular contact, as well as in undefined intracellular sites. BHK cells were grown on glass coverslips and processed as previously
described (Brissette et al., 1994; Kumar et al., 1995) using affinity-purified, polyclonal antibodies generated against a peptide
considering to residues 131–142 in $\alpha_1\text{[Cx43]}$ (Yeager and Gilula, 1992). A Zeiss Axiophot was used for immunofluorescence microscopy. Bar represents 7.5 µm.
2.8% Tween 20, 200 mM KI, 2 mM sodium thiosulfate, 140 µg/ml phenylmethylsulfonyl fluoride, 50 µg/ml gentamycin in 10 mM Hepes buffer (pH 7.5) containing 0.8% NaCl for 12 h at 27°C, followed by the addition of 1,2-diheptanoyl-sn-phosphocholine (DHPC) to 13 mg/ml and incubation for an additional 1 h at 27°C.

(3) Electron cryo-microscopy. The thermal stability and vibrational stability of cryo-microscopes have certainly improved over the past decade. For the projection density map, a resolution of 7Å was achieved using a conventional Philips CM12 electron microscope operating at 100 kV, which was equipped with a standard Gatan626 cryo-stage (Unger et al., 1997). For the 3D map, images of tilted crystals were recorded using a Philips 200-kV electron microscope equipped with a field emission 9 um. The improved stability and coherence of the 200-kV microscope compared with the CM12 was important for recording high-resolution images of tilted crystals. Nevertheless, high-resolution images could not be recorded from specimens tilted >35°. Factors that limit the resolution of images of tilted crystals include specimen flatness, beam-induced movement, and charging. The same 200-kV microscope and cold stage were used to collect higher resolution images of 2D crystals of AQP1 tilted to ~50° (Cheng et al., 1997). Since the thickness of the AQP1 and gap junction crystals was ~60 and ~150 Å, respectively, we presume that the inability to record high-resolution images from highly tilted gap junction crystals was related to the increased thickness of the gap junction specimens.

(4) Image processing. The MRC image-processing package developed by R. Henderson and colleagues (Henderson et al., 1986, 1990; Crowther et al., 1996) was used to analyze the images and to correct for lattice distortions and effects of the contrast transfer function. Although lattice unbending has previously been used for the analysis of endogenous gap junctions (Gogol and Unwin, 1988; Yeager and Gilula, 1992), the MRC software has undergone substantial improvements compared with the earlier versions of the program. In particular, the program MAKET-RAN was used to calculate a theoretical reference area based on the initial 3D density map of the structure, which improved the accuracy of the cross-correlation and unbending procedures.

Factors that probably did not significantly affect the improvement in resolution included the following.

(1) Crystal size. The isolated gap junction plaques were formed by a mosaic of crystalline areas. This mosaiacity as well as possible curvature of the

![FIG. 3. Electron micrographs of freeze-fractured BHK cells expressing either full-length (left) or truncated α1 Cx-263T connexin (right). Both replicas display plaques that exhibit particles on the P fracture face and corresponding depressions on the E face that are typical of mammalian gap junctions. The plaque from the cells expressing α1 Cx-263T appears to be more crystalline than that from the cells expressing full-length α1 [Gx43]. Cells were grown on plastic petri dishes and processed for freeze-fracture analysis as previously described (Kumar et al., 1995). Freeze-fracture replicas were examined with a Hitachi H600 electron microscope at an accelerating voltage of 75 kV. Bar represents 200 nm.](image-url)
plaques made it necessary to use areas with only a few hundred unit cells for image processing. The small size of the crystals was comparable to crystals derived from liver and heart tissue. At present, the crystals are still too small for electron diffraction.

(2) Specimen purity. The gap junction preparations were substantially contaminated by nonjunctional membranes and were probably comparable in purity to crude plasma membrane preparations derived from liver tissue (Sikerwar and Unwin, 1988). DHPC treatment was particularly useful for solubilizing nonjunctional membranes. Nevertheless, SDS gels of the enriched membrane preparations displayed a pattern of bands that was similar to that of crude preparations. Even in the enriched specimens, a Coomassie-stainable band that corresponded to the recombinant gap junction protein was not detectable. The yield of gap junctions was quite variable from preparation to preparation. Nevertheless, the crystals were quite reproducible with a variation in unit cell size of 76 to 79 Å. These observations suggest that the major limiting factors in producing crystals was the culturing of the BHK cells and expression of recombinant protein. In general, if negatively stained specimens showed at least five crystals per grid square, then frozen-hydrated specimens were prepared. Since specimens that had more than five crystals per grid square were rarely produced, the major limiting factors for the cryomicroscopy were the number of crystals on the grid as well as the difficulty of recording images from tilted crystals.

α-HELICAL SECONDARY STRUCTURE OF RECOMBINANT GAP JUNCTIONS

The most prominent features in the two-dimensional projection density map were two concentric rings of six circular densities (Unger et al., 1997). The circular densities at a radius of 17 Å from the central sixfold axis of symmetry lined the lumen of the central aqueous pore, whereas the densities at 33 Å were on the perimeter of the channel in contact with membrane lipids. These circular densities had the characteristic appearance of transmembrane α-helices oriented roughly perpendicular to the membrane plane (Unwin and Henderson, 1975; Kühlbrandt and Downing, 1989; Havelka et al., 1993; Schertler et al., 1993; Jap and Li, 1995; Karrasch et al., 1995; Mitra et al., 1995; Walz et al., 1995; Hebert et al., 1997). The two rings of α-helices were separated by a continuous band of density at a radius of 25 Å, which arose from the superposition of projections of additional transmembrane α-helices and polypeptide density arising from the extracellular and cytoplasmic loops within each connexin subunit.

THE DODECAMERIC CHANNEL IS FORMED BY 48 TRANSMEMBRANE α-HELICES

Image analysis of tilted 2D crystals yielded a 3D map with resolution cut-offs of 7.5 Å in the x,y plane and 21 Å along the z plane (Unger et al., 1999). A side view of the map (Fig. 4) showed that the channel formed by the C-terminal truncation mutant had a thickness of ~150 Å, which compared with ~250 Å for gap junction channels formed by full-length α3[Cx43] (Yeager, 1998). The reduced length of the recombinant channel is consistent with the loss of the ~13-kDa cytoplasmic C-tail.

In the 3D map, regions corresponding to the transmembrane (M) and extracellular (E) portions of the molecule were clearly resolved (Fig. 4). The outer diameter within the membrane region was ~70 Å, and the diameter narrowed to ~55 Å within the extracellular gap (Fig. 4A). A vertically sectioned view of the map (Fig. 4B) showed that the aqueous channel narrowed from ~40 to ~15 Å in proceeding from the cytoplasmic to the extracellular side of the bilayer. Within the hydrophobic regions of the bilayers, map sections displayed roughly circular contours of density that are typical for α-helices preferentially aligned perpendicular to the membrane (Fig. 4C, top and bottom). Since there are 24 circular densities per connexon, the dodecameric channel is formed by 48 transmembrane α-helices.

MODELS FOR THE DOCKING OF CONNEXONS

The extracellular region of the map was double-layered with a continuous band of density at ~17-Å radius and six arcs of density at ~26-Å radius (Fig. 4C, middle). The extracellular density was in distinct contrast to the characteristic “signature” for α-helices in cross section (Fig. 4C, top and bottom). This difference indicated that at least a portion of the polypeptide within the extracellular gap was not folded as α-helices. In fact, site-specific mutagenesis of the extracellular loops suggests that the polypeptide in the extracellular loops may fold as β-sheets (Foote et al., 1998). The interior band of density formed a continuous wall of protein (Fig. 4B) that would serve as a tight electrical and chemical seal connecting the two cells.

A notable feature of the projection density map was the 30° displacement between the rings of α-helices at 17- and 33-Å radius, which places constraints on possible structural models for the intercellular channel. Assuming a roughly circular shape for the connexin subunit, the 30° displacement predicted that the two connexons forming the channel were likely to be rotationally staggered with respect to each other (Unger et al., 1997; Perkins et al., 1998). Because the connecting loops between the transmembrane α-helices were not revealed in the
3D map, there was ambiguity in assigning the molecular boundary of the connexin subunit. The 3D map was nevertheless consistent with reasonable models that involved rotational stagger, but some possible molecular boundaries did not involve rotational stagger. It is noteworthy that models with rotational stagger predict that each subunit in one connexon will interact with two connexin subunits in the apposing connexon. Such an arrangement may confer stability in the docking of the connexons.

**FUTURE PROSPECTS**

Higher resolution analysis is required to test the prediction that there is rotational stagger between the connexons. Other structural features yet to be determined are the molecular boundary and the assignment of the transmembrane α-helices to the four hydrophobic domains. In addition, a higher resolution map will reveal details about the folding and potential interactions of the extracellular loops that may provide insight into the high degree of stability between the docked connexons as well as how the extracellular loops confer selectivity in the docking process.

Preliminary functional studies of BHK cells that express α1Cx-263T demonstrate that oleamide, a sleep-inducing compound, blocks in vivo dye transfer. As previously demonstrated for other connexins (Guan et al., 1997), this behavior is an indication that oleamide causes closure of α1Cx-263T. These results have encouraged us to compare the 3D structure of channels grown in the presence and in the absence of oleamide, which may allow us to explore the conformational changes that are associ-
ated with oleamide-induced blockage of dye transfer. The structural details revealed by our analysis will be essential for delineating the functional properties of this important class of channel proteins.

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