with a lipid/protein molar ratio of 100:1, a value close to that found in most biological membranes [21]. The data show that the calculation of distances between sites within the monomer of a membrane protein of a cross-sectional diameter of 5 nm based only from FET efficiency measurements is largely speculative in most cases in native biological membranes. Because the contribution of FET to acceptors bound to neighbour protein monomers is highly dependent upon the lipid/protein molar ratio because of dilution of proteins in the lipid bilayer, reliable estimations of distances between functional sites in a protein monomer could *a priori* be obtained for any donor/acceptor pair with membrane proteins reconstituted with high lipid/protein molar ratios. In practice, however, the determination of the aggregation state of a protein in the membrane (native or reconstituted) is technically difficult, and the increase of light scattering of samples prevents one from working far beyond a lipid/protein molar ratio of 1000:1. Therefore, the uncertainty in calculations of distances between functional sites in a protein monomer from measurements of FET efficiency remains high, unless the aggregation state of the membrane protein can be demonstrated to be monomeric in the samples used for fluorescence measurements.

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Assembly of gap junction intercellular communication channels

W. Howard Evans
Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

Introduction

The integration and summation of cellular metabolic and mechanical activities in tissues and organs involves the mediation of gap junctions: arrays of intercellular channels that connect the cytoplasms of interacting cells and allow transfer of small molecules and ions. Gap junctions are generally considered to facilitate, for example, propagation of action potentials in cardiac myocytes [1,2] and Ca^{2+} oscillations in tracheal epithelial and glial cells [3,4]. On a longer time scale, strong evidence is accruing that gap junction-mediated communica-

tion in embryos is necessary for proper development [5]. Gap junctions are constructed of clusters of integral membrane proteins, now termed connexins, and these oligomeric assemblies are believed to correspond to the distinctive particles and their complementary pits seen in freeze-fracture replicas; they also appear as hexagonal arrays by quick-freeze deep-etch immunoelectron microscopy [2,6]. In current models of gap junction structure, six connexin subunits are radially arranged around a central 2 nm pore, thereby forming a connexon 'half-channel' assembly, and these align and interact with complementary connexons present in the plasma membrane of a neighbouring cell [7,8]. Lateral accretion of paired connexons leads to the formation of the morphologically familiar gap junction plaques, the plasma membrane organelles that constitute conduits that bypass the extracellular space and thus allow direct intercellular cross-talk.

The application of molecular cloning techniques in conjunction with detailed analyses, using site-specific antibodies, of proteolytic fragments of connexin protein derived from isolated gap junction plaques has shown that connexins traverse the lipid bilayer four times, with the N- and C-termini projecting into the cytoplasm. Following the characterization of connexins 32 and 26 in liver and connexin 43 in heart, a number of other connexins have been shown to be expressed in various tissues and cell types (Table 1). Although these other connexins have not been subject to the detailed membrane topographical studies applied to connexin 32 [9,10] or connexin 43 [7], the amino acid sequence identities derived by molecular cloning and evident in all connexins studied suggest strongly that connexins are a conserved family of integral channel-forming membrane proteins [11,12].

Progress is being made in relating the consensus primary structure of gap junctions to discrete functional aspects. Thus, it has been predicted that the third transmembrane region in connexins contributes towards the channel wall of gap junctions [13]. Specific sites of phosphorylation on the cytoplasmic C-terminal arm of connexin 43 have been identified [14–16], and knowledge of the mechanism by which Ca^{2+} and calmodulin regulate the opening/closing of gap junctions has been advanced by the identification of calmodulin-binding sequences located at the N- and C-termini of connexin 32 [9,17,18]. The functional importance of the intracellular loop, which varies substantially in amino acid sequence among connexins, has become clear from studies using various anti-peptide antibodies generated to specific amino acid sequences in this loop. When injected into living mouse embryos, these antibodies blocked intercellular communication, resulting in cessation of development [19]. At the extracellular face, the two disulphide-linked loops have emerged as crucial areas of the protein involved in the recognition and docking of connexins, a prelude to the genesis of the gap junction [10,20]. Knowledge of such molec-

Table 1

Various connexin types and tissue distribution in rodents

Number refers to molecular mass (kDa) deduced for each connexin. Connexins with similar amino acid sequences have also been cloned from human liver and heart, as well as chicken, dog and *Xenopus* tissues. For amino acid sequence comparisons, see [11,30].

Connexin	Cell/tissue location
Cx26	Hepatocytes, mammary epithelia, pinealocytes
Cx30.3	Skin
C.31	Skin, placenta
C.31.1	Keratinocytes, oesophageal decidual cells
Cx32	Hepatocytes, exocrine pancreas
Cx33	Testis
Cx37	Endothelium
Cx40	Kidney, heart (bundle of His and Purkinje fibres)
Cx43	Heart, smooth muscle, endothelium, fibroblasts, ovary, kidney, brain, endocrine pancreas, Leydig cells, lens ciliary epithelial cells
Cx45	Kidney, skin, intestine
Cx46, Cx50	Lens fibre cells

ular features now permits advances in unravelling how the connexin protein subunits are assembled into a gap junction. This article assesses and summarizes current approaches and recent progress.

Connexin intracellular trafficking routes

Gap junction proteins turn over extremely rapidly (in liver, $t_{1/2}=6-10$ h [21–23]), and in addressing their mechanisms of biogenesis, it is necessary to trace first the intracellular membrane trafficking routes of the constituent proteins, the connexins. Subcellular fractionation studies in liver have shown that the distribution of connexins between the various membrane compartments is variable [20], with ~40% of connexins located in the endoplasmic reticulum/Golgi membranes, as determined by analysis of characterized membrane fractions by SDS/PAGE and immunoblotting using various antibodies to connexins. This subcellular distribution provides clues as to a likely general route to the baso-lateral domain of the plasma membrane. Although extremely low connexin immuno-reactivity was detected in liver endosomal fractions, far higher levels of connexins, in various degrees of degradation, were detected in lysosomes using the various anti-peptide antibodies. These results suggest that gap junctions are internalized as plaques and then subject to autolysis, probably in the lysosomes and phagocytic structures [24,25]. By taking advantage of a broad panel of polyclonal antibodies generated to peptides corresponding to amino acid sequences in discrete extracellular and intracellular domains of connexin 32 [17], the orientation in the membrane of connexins as they progress along various intracellular stations, and the site of synthesis of the intramolecular disulphide bonds that connect the extracellular loops were established [20]. A 'mature' topography in connexins that accounts for the absence of carbohydrate residues, since a potential glycosylation site was cytoplasmically located, is achieved in the Golgi apparatus. In microsomal fractions, the site-specific antibodies detected, in enzymic digests, a number of folding states of connexin 32, and the results showed that the intramolecular disulphide linkages located in the extracellular loop were placed in position cotranslationally. The probable biogenetic pathways followed by connexin 32 in rat hepatocytes are depicted in Figure 1.

Where do connexins oligomerize?

A fundamental question in understanding channel assembly concerns the mechanism of oligomerization of the protein subunits and the cellular location of this event. Figure 2 shows two possible mechan-

Figure 1

Diagrammatic representation of the topography and likely intracellular trafficking route of connexin 32 in hepatocytes

Mature and immature connexin configurations are shown in the endoplasmic reticulum. The overall topography in the membrane of connexins is retained during trafficking along the biogenetic pathways.

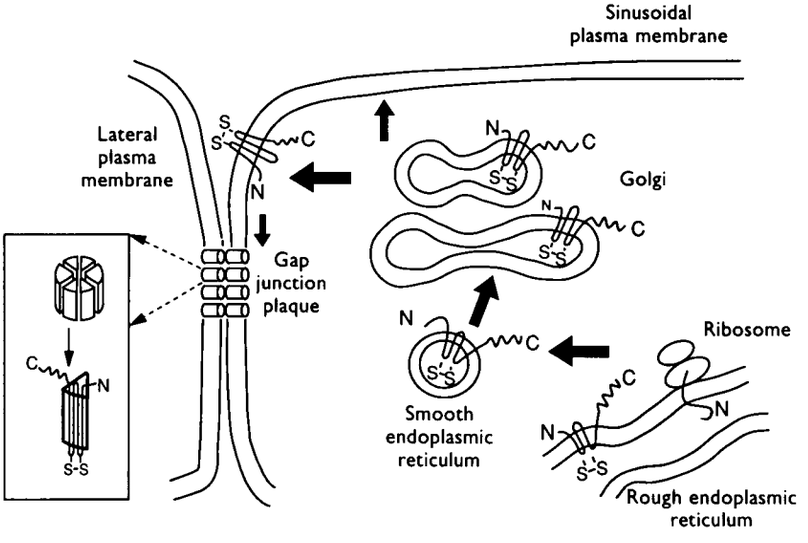
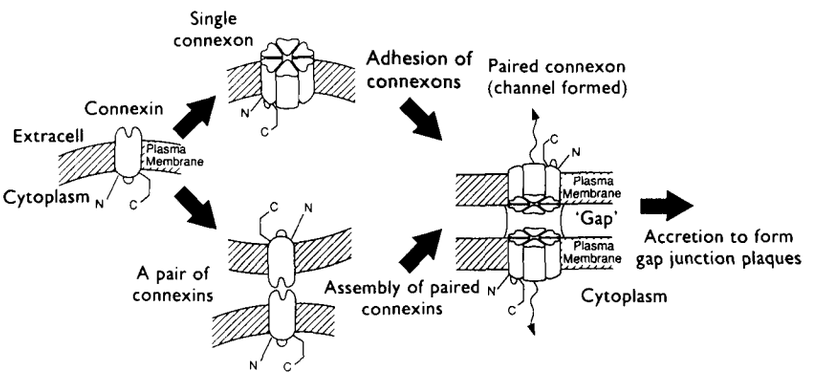


Figure 2

Two possible assembly mechanisms for gap junctions

In the upper pathway, connexon half-channels are first assembled and two connexons, contributed by aligned cells, dock to form an intercellular channel. In the lower pathway, individual connexins from different cells interact and these then accrete to form gap junctions. For further details, see text.



isms that can account for the formation of gap junctions at the cell surface. In the first, two connexins, one each contributed by aligned cells, associate to form the first intracellular contact; the lateral accretion of these connexin pairs to form paired hexameric connexons would lead to the creation of a direct intercellular channel, followed by further accretion in the membrane by lateral mobility of the paired connexons to form gap junction plaques. In a second mechanism to be considered the recognition/docking event at the plasma membrane involves the adhesion of connexon 'half-channels' contributed by two aligned cells. Were this mechanism to be operational, it poses a number of subsidiary questions. Where does oligomerization of connexins into connexon half-channels occur? For example, this process might occur at non-junctional regions of the plasma membrane, or earlier in the biosynthetic pathway in the Golgi apparatus or the endoplasmic reticulum, two locations where connexins have been detected by subcellular fractionation studies in liver [20]. If connexon half-channels are assembled early in the biogenetic pathway, then it would be necessary for these channels to be maintained in a closed configuration before final assembly into a gap junction so as not to compromise the cytoplasmic/lumenal or extracellular/cytoplasmic interfacial environments traversed. The nature of the molecular mechanisms that account for a closed channel configuration is a further issue that arises for it may regulated, for example, by Ca^{2+} or calmodulin. A related aspect in gap-junction channel assembly concerns the question of whether individual connexons are constructed of the same or dissimilar connexin subunits, because many cells express two or possibly more connexin types. Varying the connexin make-up of gap junctions may provide a structural mechanism for regulating channel porosity, and this may impinge directly on the physiological reasons why different tissues express different connexins (Table 1).

To address these questions, techniques were devised that distinguish in cells and subcellular fractions between gap-junction plaques, connexons and individual connexins. These techniques, in conjunction with access to connexin-specific antibodies, and the preparation by transfection of cells over-expressing specific members of the connexin superfamily, are now allowing clarification of the details of how gap junctions are assembled. For example, liver subcellular fractions, solubilized under selected conditions with specific detergents that do not dissociate connexon assemblies, in conjunction with the clear separation of connexons from connexins by rate-zonal centrifugation, indicate that connexons are already assembled in the Golgi apparatus. In the endoplasmic reticulum, both connexins and connexons are present. These results suggest that the second assembly mechanism shown in Figure 2 is most likely to be operational, and identifies the endoplasmic reticulum as a probable key locus where oligomerization of connexins to form connexon half-channels occurs. Using C6 glioma cells that were over-expressing connexins 26, 32 or 43 and which were treated with the fungal metabolite Brefeldin A, it was shown that oligomerization of connexins was reversibly prevented, suggesting that

a more precise intracellular locus of the oligomerization process was a specialized subcompartment in the endoplasmic reticulum (E. Kalopothakis, G. Carlile and W. H. Evans, unpublished work). This subcompartment, positioned between the endoplasmic reticulum and the *cis*-Golgi [26,27], is being increasingly recognized as a key controller of vesicular movement between the endoplasmic reticulum and the Golgi, and as a key player in the quality control of proteins destined for targeting to various cellular locations [28].

The data presented above thus summarizes our current understanding of the mechanism of assembly of the gap junction. Unravelling the locus and mechanism of assembly of connexins is an essential step towards understanding the regulation of intercellular signalling across gap junctions. These approaches can provide important inputs into deciphering the effects of connexin-binding ligands such as calmodulin, post-translational modifications such as phosphorylation and acylation. The regulation of the channels by the extremely rapid turnover rates that allow connexin make-up and the extent of intercellular signalling across the gap junctions to be fine-tuned also need to be explained [29].

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