Synthesis and assembly of connexins *in vitro* into homomeric and heteromeric functional gap junction hemichannels

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The biogenesis of connexins and their assembly into functional gap junction hemichannels (connexons) was studied with the use of a cell-free transcription/translation system. Velocity sedimentation on sucrose gradients showed that a small proportion of connexin (Cx) 26 and Cx32 that were co-translationally translocated into microsomes were oligomers of Cx26 and Cx32. Chemical cross-linking studies showed that these corresponded to hexameric connexons. Reconstitution of connexons synthesized *in vitro* into liposomes induced permeability properties consistent with the view that open gap junction hemichannels were produced. By using an immunoprecipitation approach, a simultaneous translation of Cx26 and Cx32 incorporated into microsomes resulted in homomeric connexons. However, supplementation of the translation system *in vitro* with liver Golgi

membranes produced heteromeric connexons constructed of Cx32 and Cx26, and also resulted in an increased oligomerization especially of Cx32. All of the connexins analysed were inserted co-translationally into canine pancreatic microsomal membranes. In addition, Cx26 and Cx43, but not Cx32, were also inserted into microsomal membranes post-translationally. Analysis of various connexin constructs in which the cytoplasmic carboxy tails were transposed, the cytoplasmic tail of Cx43 was truncated or a reporter protein, aequorin, was attached to the C-terminus showed that tail length was not the major determinant of the post-translational membrane insertion of connexins.

Key words: connexin biosynthesis, gap junction assembly, oligomerization.

INTRODUCTION

Gap junctions are membrane channels that mediate direct intercellular communication in developing and differentiated organs and tissues. The cell-to-cell transfer of molecules and ions across gap junction channels exhibits size selectivity, and a number of potential biochemical messengers and metabolites, all below approx. 1 kDa, have been shown to pass through the channel pore [1]. Cell-to-cell communication via gap junctions allows the co-ordination and integration of metabolic activities [1,2]. Impaired gap junctional communication, often implicating mutations in the constituent proteins, has been associated with cardiac arrhythmia, tumorigenesis, Charcot–Marie–Tooth X-linked neuropathy, cataractogenesis and sensorineural deafness [3,4].

Gap junctions are constructed of clusters of hexamerically arranged hemichannels (connexons) co-axially aligned and interacting with connexons contributed by the neighbouring cell's plasma membrane. Connexon hemichannels are constructed of six protein subunits, the connexins, that oligomerize around a central aqueous channel. Connexins are members of a closely related multigene family of integral membrane proteins as shown by sequencing of cDNA species and topographical studies with site-specific antibodies and protease digestion [1,5]. Fourteen connexin isoforms have been identified in rodents, nine in humans, and many homologues in other species [5–8]. Connexins are polytopic transmembrane proteins containing four membrane-traversing α -helical domains. An approx. 20-residue N-terminus, a variable-length C-terminus and a 40–60-residue loop are located cytoplasmically. Two highly conserved sequences connecting the membrane-spanning domains form extracellular loops that are crucial in the docking of connexons to generate the gap junction intercellular channel. Communication across gap junction channels is thought to be regulated

by Ca^{2+} levels [9], pH [10] and phosphorylation of the carboxy tail of connexins [11,12].

Key questions about the biogenesis of gap junctions include the delineation of routes followed by connexins during their synthesis and assembly into functional gap junction and the identification of factors determining the formation of homomeric or heteromeric connexons, because most cells express more than one connexin isoform and both homomeric and heteromeric channels have been reported [13]. The connexin constitution of channels might determine their selectivity towards the size and charge of permeant molecules [14,15].

In this study we focused on the early stages of connexin synthesis by using a reconstitution approach to study their oligomerization into hexameric gap junction hemichannels. A cell-free coupled transcription–translation *in vitro* approach was used to study the biogenesis of connexin (Cx) 26, Cx32 and Cx43; the compositional and permeability properties of hexameric channels were determined.

EXPERIMENTAL

Cell-free synthesis of connexins

Plasmids, pBSKXG Cx26 [16], pcDNA Neo I Cx32 [17] and pCR3-Cx43 [18] containing cDNA encoding Cx26, Cx32 and Cx43 respectively were transcribed and translated *in vitro* by using reticulocyte lysate (TNT Quick; Promega) in a 25 μl reaction in the presence of ^{35}S -labelled (15 μCi) methionine and cysteine (Promix; Amersham, Little Chalfont, Bucks., U.K.). For velocity sedimentation assays and cross-linking studies, a larger-volume (75 μl) reaction was used. Plasmids (0.5 μg) and [^{35}S]Promix were added to the reticulocyte lysate and incubated for 90 min at 30 °C. Glutathione (5 mM) was added to compensate for the small amount of dithiothreitol (DTT) present in

Abbreviations used: Cx, connexin; DMS, dimethyl sulfoxide; DSP, dithiobispropionate; DTT, dithiothreitol; TEA, triethanolamine.

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Figure 1 Synthesis of Cx26 and Cx32 *in vitro* in the presence or absence of microsomes

Plasmids encoding Cx26 or Cx32 were transcribed and translated in the rabbit reticulocyte lysate system as described in the Experimental section. Connexins were separated by SDS/PAGE and detected by autoradiography. Connexins synthesized in the absence (—) of microsomes showed a band at the corresponding molecular mass position; however, connexins synthesized in the presence (+) of microsomes showed an additional faster-migrating band.

[³⁵S]Promix [19]. Reactions were stopped by the addition of Laemmli sample buffer [20] and products were analysed by SDS/PAGE and autoradiography.

Plasmids encoding chimaeric domain-swapped connexins pCR3-Cx26/43^T (the first 210 residues of Cx26 fused to the 156-residue carboxy tail of Cx43) and pCR3-Cx32/43^T (the first 209 residues of Cx32 attached to the 156-residue tail corresponding to the carboxy tail of Cx43) were prepared by using a two-step PCR as described [16,21]. Plasmid pCR3-Cx43^{A244} encoded a truncated mutant of Cx43 containing first 244 residues, thus bearing a cytoplasmic tail of only 16 residues that was similar in length to that of wild-type Cx26 [18]. pCR3-Cx43^{A244}Aeq encoded a chimaera comprising Cx43^{A244} attached to aequorin, a 21 kDa Ca²⁺-dependent photoprotein [22] fused to the carboxy tail. Domain boundaries of connexins were based on Clustal V sequence alignment and current topological models [1].

Co-translational and post-translational membrane insertion

To study co-translational membrane insertion, the transcription/translation reactions were supplemented with endoplasmic-reticulum-derived canine pancreatic microsomes (Promega) (1.25 equiv. for each 25 µl of reaction mixture) and incubated at 30 °C for 90 min. To study post-translational membrane insertion, reactions were performed in the absence of membranes. On completion of protein synthesis, DNase I (2 µg/25 µl; Boehringer Mannheim) and RNase I (9 units/25 µl; Promega) were added and incubation was continued at 30 °C for 30 min to degrade the plasmid and its transcript to prevent co-translational translocation into microsomal membranes. Microsomes (1.25 equiv./25 µl) were added and incubation was continued for a further 90 min at 30 °C to allow presynthesized connexins to insert into the membranes. To separate membrane-integrated connexins from free connexins, the translation mixture was layered on a 0.5 M sucrose cushion (500 µl) prepared in PBS, pH 7.4, and centrifuged at 150 000 *g* for 90 min. Membrane pellets were collected and resuspended in 0.1 M Na₂CO₃, pH 11.0, for 30 min and then centrifuged for a further 1 h at 150 000 *g*. The supernatants were discarded and the pellets were washed again in 0.1 M Na₂CO₃, pH 11.0, and centrifuged as before. Pellets were finally resuspended in Laemmli sample buffer and analysed by SDS/PAGE and autoradiography.

Velocity sedimentation on sucrose/detergent gradients

After the synthesis of connexins in the reticulocyte lysate in the presence of microsomes, the translation mixture was incubated for 2 h in a solubilization buffer [20 mM EDTA/10 mM DTT/2% (w/v) n-dodecyl β-D-maltoside (Sigma)/20 mM triethanolamine (TEA) (pH 9.2)] [23]. Samples were centrifuged at 12 000 *g* at 4 °C for 15 min and the supernatants then loaded on preformed 10–40% (w/v) linear sucrose gradients prepared in 20 mM EDTA/10 mM DTT/0.2% n-dodecyl β-D-maltoside/20 mM TEA (pH 9.2). After centrifugation at 150 000 *g* for 18 h at 4 °C, ten 0.5 ml fractions were collected, dialysed against distilled water and concentrated under vacuum. Fractions were then resuspended in Laemmli sample buffer and analysed by SDS/PAGE and autoradiography to detect connexins. In separate experiments, the reticulocyte lysate and canine pancreatic microsomes were also supplemented with Golgi membranes (50 µg of protein) prepared from guinea-pig liver [24].

Chemical cross-linking

Microsomes containing connexins synthesized *in vitro* were sedimented through a sucrose cushion and solubilized in n-dodecyl β-D-maltoside as described above. One of the cross-linkers dimethyl suberimidate (DMS) or dithiobispropionate (DSP) (10 mM final concentration of each) (Pierce) was added for 12 h (DMS) or 1 h (DSP) [25–27]. The reactions were stopped by the addition of glycine (30 mM final concentration) and the samples were dialysed against distilled water and reduced in volume with a Centricon-10 concentrator (Amicon). The cross-linked products were analysed on SDS/PAGE gradient gels [5–20% (w/v) polyacrylamide]. Connexons cross-linked with DSP were solubilized in Laemmli sample buffer without or with 50 mM DTT to reduce the disulphide bonds generated by the cross-linker [27].

Reconstitution in liposomes

Microsomes containing connexons were reconstituted into liposomes containing cytochrome *c* [28]. Microsomes, with or without connexins synthesized *in vitro*, were pelleted through a sucrose cushion and solubilized in a buffer containing 50 mM KCl, 20 mM Tris/HCl, pH 7.4, 10 mg/ml L-α-phosphatidylcholine type II-S (Sigma), 20 mg/ml n-octyl β-D-glucopyranoside and 4 mg/ml cytochrome *c*. Reconstituted liposomes were formed after the removal of detergent by dialysis against a buffer containing 50 mM KCl and 20 mM Tris/HCl, pH 7.4. The permeability of liposomes and proteoliposomes containing connexons was measured by the ability of ascorbate to cross the phospholipid bilayer containing connexon hemichannels and thus reduce the cytochrome *c* present in the lumen of the liposomes. The reduction of cytochrome *c* was monitored spectrophotometrically at 417 nm.

Immunoprecipitation

Two connexin isoforms were transcribed/translated together *in vitro* in the presence of microsomes with or without supplementation with Golgi membranes (50 µg) from guinea-pig liver prepared as described [24]. Microsomal membranes containing connexons synthesized *in vitro*, after centrifugation through a sucrose cushion, were solubilized in 20 mM EDTA/10 mM DTT/20 mM TEA (pH 9.2)/2% n-dodecyl β-D-maltoside, a mild detergent in which hexameric connexons remain intact [23,24]. Samples were centrifuged at 12 000 *g* for 15 min at 4 °C and supernatants were diluted 1:10 to decrease the DTT con-

Figure 2 Separation of membrane-inserted and free connexins, and immunoprecipitation of full-length and truncated connexins

(a) Membrane-integrated connexins were separated from free connexins by centrifugation and analysed by SDS/PAGE. Membrane pellets of both Cx26 and Cx32 showed two products, whereas the supernatants showed only full-length products. (b) Cx32 translated in the presence of microsomes was immunoprecipitated with antibodies generated against a C-terminal sequence (GAP9) or an N-terminal sequence (GAP10). Lane 1 (Tn.) is the sample saved before immunoprecipitation.

centration. Antibodies generated against specific amino acid sequences located in the intracellular loop of Cx26 (residues 113–124, GAP28H) and different regions of Cx32 {C-terminus residues 264–283, GAP9 [29], TRX-CT (the cytoplasmic tail of Cx32 attached to thyrodoxin) [30] and an N-terminal region (residues 1–21, GAP10)} were added and left overnight at 4 °C, with shaking. Immunoglobulins bound to connexons or con-

nexins were precipitated with Protein A–Sepharose beads [31] and subsequently analysed by SDS/PAGE and autoradiography. Cx26 and Cx32 synthesized together in the presence of microsomes and Golgi membranes were also sedimented in 10–40 % (w/v) linear sucrose/detergent gradients as described above to separate monomeric and oligomeric connexins.

RESULTS

Transcription and translation of connexins *in vitro*

The products of synthesis of connexins *in vitro* showed a single band corresponding to Cx26 or Cx32 after SDS/PAGE and autoradiography (Figure 1). However, connexins synthesized in the presence of canine pancreatic microsomes showed an additional faster-migrating band (Figure 1). When the translational products were sedimented through a sucrose cushion to separate the membrane pellet from the supernatant containing free connexins, the pellet contained both products (Figure 2a, pellet), whereas the supernatant contained only full-length connexin (Figure 2a, Sup.). Antibodies generated against a sequence at the C-terminus for Cx32 (GAP9) immunoprecipitated full-length and truncated Cx32, whereas an antibody generated against a sequence at the N-terminus for Cx32 (GAP10) immunoprecipitated only full-length Cx32 (Figure 2b).

Post-translational membrane insertion

The co-translational and post-translational insertion into microsomal membranes of different connexins and a range of domain-swapped and chimaeric connexins was investigated (Figure 3). Wild-type Cx26, Cx32 and Cx43 were translocated co-translationally into microsomes (Figure 3, lanes 2, 5 and 8); however, post-translational insertion properties were clearly shown by Cx26 (lane 3) and Cx43 (lane 9) but not by Cx32 (lane 6). Analysis of connexins with transposed C-terminal tails revealed that Cx32/43^T was inserted into membranes co-translationally (Figure 3, lane 11) but not post-translationally (lane 12), whereas Cx26/43^T was able to insert into microsomes both co-trans-

Figure 3 Co-translational and post-translational insertion of wild-type connexins and connexins with switched tails in microsomal membranes

Transcription and translation *in vitro* of plasmids containing cDNA encoding wild-type and chimaeric connexins. For co-translational and post-translational studies, membranes were separated through a sucrose cushion, washed and then analysed for the presence of inserted connexins. Wild-type or chimaeric connexins were synthesized in the absence of microsomal membranes (—), or with microsomes added co-translationally (co) or post-translationally (po). Further details of the connexins with tail switches (Cx32/43^T, Cx26/43^T), truncated connexin (Cx43^{Δ244}) and the chimaera (Cx43^{Δ244}Aeq) are given in the Experimental section.

Figure 4 Separation of monomeric connexins from oligomeric connexons

Connexins synthesized *in vitro* (a) in the presence of microsomes or (b) supplemented with Golgi membranes were solubilized and fractionated on 10–40% (w/v) linear sucrose gradients containing 0.2% dodecyl maltoside. After separation, fractions were analysed by SDS/PAGE. The graphs were obtained by densitometric analysis of autoradiographs. The positions in the gradients of monomeric connexins and oligomeric connexons sedimenting are indicated: Cx26 (□) and Cx32 (■) in (a) and Cx26 (△) and Cx32 (▲) in (b) are shown.

lationally and post-translationally (lanes 14 and 15). Similarly, Cx43^{Δ244} (Figure 3, lanes 17 and 18) and a chimera, Cx43^{Δ244}Aeq (lanes 20 and 21), showed patterns similar to that of Cx43, because these connexins were inserted into microsomes both co-translationally and post-translationally.

Separation of connexins and connexons

After solubilization of microsomes incorporating the co-translationally translocated connexins, and their analysis by velocity sedimentation in sucrose/detergent gradients, differences were observed between the ratios of monomeric to oligomeric connexins when liver Golgi membranes were added to the microsomes. In the presence of microsomes, most of the Cx32 and Cx26 sedimented as monomers, with a small peak (approx. 15% of the total) corresponding to the position in the gradient of oligomers (Figure 4a). However, supplementation with Golgi membranes resulted in a larger increase in oligomeric Cx32, with

Figure 5 Cross-linking of connexins synthesized *in vitro*

Connexins transcribed/translated *in vitro* in the presence of microsomes were solubilized and cross-linked with DMS (lanes 4 and 6) or DSP (lanes 8 and 9) to analyse cross-linked oligomers. Cx32 cross-linked with DSP was also treated with DTT to reduce the disulphide bonds generated by the cross-linker (lane 9). Lanes 3, 5 and 7, aliquots of samples saved after protein synthesis. Reactions without any plasmid were also treated in the absence or presence of DMS cross-linker (lanes 1 and 2).

approximately half oligomerized; only a small increase in oligomeric Cx26 was observed (Figure 4b).

Connexin cross-linking and evidence for hexameric connexons

Cx26 or Cx32 transcribed and translated *in vitro* in the presence of microsomes and cross-linked with DMS yielded a 180 kDa band (Figure 5, lanes 4 and 6). With DSP, a thiol-cleavable cross-linker, a 180 kDa band corresponding in position to a Cx32 hexamer was also observed (Figure 5, lane 8) that was absent from a control experiment that used DTT to reduce the cross-linked disulphide bonds (Figure 5, lane 9).

Immunoprecipitation demonstrates homomeric and heteromeric connexons

When two connexin isoforms were transcribed and translated together in the presence of microsomes, homomeric connexons constructed from Cx26 or Cx32 were produced (Figure 6a). However, repetition of this experiment in the presence of microsomes supplemented with liver Golgi membranes resulted in the respective antibodies immunoprecipitating both connexins (Figure 6b), indicating that heteromeric connexons were present. Sedimentation of the products through 10–40% (w/v) linear sucrose/detergent gradients showed that 55% of the connexins were in the oligomeric form. Importantly, this experiment confirmed that the oligomeric connexons were present in the 9 S fraction [30–37% (w/v) sucrose] used for immunoprecipitation (Figure 6c).

Reconstitution in liposomes

To investigate whether hydrophilic channels across lipid bilayers were provided by the generation of oligomeric connexons demonstrated by cross-linking analysis, the *in vitro* products were

Figure 6 Immunoprecipitation of two connexin isoforms synthesized together in the presence of microsomes

Cx26 and Cx32 were transcribed and translated together in the cell-free synthesis system in the presence of microsomes (a) or in the presence of microsomes supplemented with Golgi membranes (b). Membranes containing connexons were solubilized and immunoprecipitated with antibodies specific for Cx26 [GAP28H] or Cx32 [GAP9 and TRX-CT (the cytoplasmic tail of Cx32 attached to thyrodoxin)] as described in the Experimental section. A control aliquot of translation mixture saved before immunoprecipitation was also run (Tn. 26+32). On completion of protein synthesis, one half of the translation mixture from (b) was also sedimented through a 10–40% (w/v) linear sucrose gradient and fractions collected were analysed for the presence of connexins by SDS/PAGE and autoradiography (c).

incorporated into liposomes. We measured the reduction of cytochrome *c* in liposomes on the addition of ascorbate to a suspension of cytochrome *c*-loaded proteoliposomes. Proteoliposomes containing connexons synthesized *in vitro* showed a 40–50% increase in the rate of reduction of intraliposomal cytochrome *c* compared with liposomes containing microsomal proteins (Figure 7). No significant reduction of intraliposomal cytochrome *c* was observed when ascorbate was added to plain liposomes composed only of phospholipids.

DISCUSSION

In this study it is shown that a number of wild-type and chimaeric connexins were synthesized *in vitro* in a cell-free translation system and co-translationally translocated into microsomes. Approx. 15% of co-translationally integrated Cx26 and Cx32 oligomerized to generate functional connexon hemichannels that provided permeability in liposomes for small molecules such as ascorbate.

The synthesis of connexins in the presence of microsomes resulted in the appearance of an additional band of slightly

Figure 7 Permeability of reconstituted connexons in liposomes

The graph shows the change in absorbance with time when ascorbate was added to plain liposomes (▲), liposomes reconstituted with proteins from microsomal membranes (□) or liposomes reconstituted with connexons synthesized *in vitro* in microsomes (■). The arrow indicates the time at which ascorbate was added. The change in absorbance (slope of curve) represents the permeability of liposomes to ascorbate. The graph is representative of five experiments.

higher electrophoretic mobility, as first shown by Falk et al. [32]. Proteins corresponding to both bands were inserted into microsomes, because when microsomes were pelleted through a sucrose cushion, the two proteins were detected in the pellet, whereas the supernatant contained only full-length connexins. The results with antibodies specific for the N- and C-termini of Cx32 add further support to the conclusion that during co-translational membrane insertion, a membrane-dependent proteolytic cleavage occurs near the connexin's N-terminus, an event confined to cell-free translation systems [32]. Characterization of the cleavage site by increasing the length and hydrophobic character of the signal anchor sequence has shown that this aberrant cleavage, occurring *in vitro*, can be prevented; it was demonstrated that the signal anchor sequence in connexins was incorrectly positioned in the endoplasmic reticulum translocon, allowing a signal peptidase to access the cleavage sites [33,34].

Co-translational and post-translational membrane insertion determinants in connexins

The present results show that although all wild-type and chimaeric connexin constructs analysed were inserted into microsomes co-translationally, post-translational insertional properties were shown by Cx26 and Cx43 but not by Cx32. By using a non-membrane-associated protein, aequorin, as a control, it was found that no co-translational or post-translational insertion into microsomal membranes occurred (results not shown). To study the specific domains within Cx26 and Cx43 responsible for the post-translational membrane insertion properties observed *in vitro*, a range of chimaeric and domain-swapped connexin constructs were examined. Constructs with transposed tails, e.g. Cx32/43^T, behaved like Cx32; in other words this connexin retained its co-translational insertion properties and showed no evidence of post-translational insertion, leading to the conclusion that the length of the C-terminus is not the main determinant conferring the ability to insert post-translationally into membranes. Similarly, increasing the length of the C-terminus of

Cx26 (Cx26/43^T) did not affect its co-translational or post-translational insertion properties when compared with wild-type Cx26. Two further variants, Cx43^{Δ244} and the chimaera Cx43^{Δ244}Aeq, both of which behaved like wild-type Cx43 in displaying co-translational and post-translational insertion properties, demonstrated that decreasing the length of the C-terminus or replacing it with a hydrophilic protein such as aequorin did not alter their inherent properties of co-translational and post-translational insertion, thus reinforcing the above findings. The results complement and extend previous work in which the C-terminal tails of Cx32 and Cx26 were switched without effect on their co-translational and post-translational properties [35]. These results also demonstrate for the first time that Cx43, the most widely distributed of the connexin protein family, is able to insert post-translationally into membranes. It is therefore concluded that amino acid sequences within Cx26 and Cx43, other than cytoplasmic C-terminal domains, are responsible for this property of post-translational insertion into microsomal membranes.

For most proteins, membrane integration is dictated by a signal sequence that is located either at the N-terminus or consists of two or more regions within a protein sequence [36]. In some cases, however, membrane translocation is also regulated by domains other than the signal sequence [37]. The cystic fibrosis transmembrane conductance regulator (CFTR), a multi-pass channel-forming membrane protein, also utilizes two distinct translocation pathways: a co-translational pathway directed by the first transmembrane domain and a ribosome-dependent post-translational pathway directed by the second transmembrane domain [37]. It was suggested that this post-translational insertion property conferred by the second transmembrane domain of CFTR provides a second opportunity for membrane proteins to translocate into membranes if the first co-translational translocation attempt fails [37]. However, connexins lack a conventional signal sequence; it is therefore possible that amino acid sequences in or close to transmembrane domains have a role in post-translational insertional properties, a rare feature among eukaryotic membrane proteins [38–42]. It remains unclear whether post-translational insertion observed with Cx26 and Cx43 is an event confined to *in vitro* systems in eukaryotes or whether it is a feature of alternative trafficking pathways that deliver connexins to the plasma membrane studied in liver tissue and in living cultured cells [21,43].

Connexon assembly *in vitro*

In the present studies it was observed that although most of the connexins synthesized *in vitro* were in monomeric form, chemical cross-linking approaches demonstrated hexamers of Cx26 and Cx32. The fact that limited oligomerization occurs suggests that connexins begin to self-associate in the endoplasmic reticulum. Furthermore, no oligomerization was observed when connexins that had been translated in the absence of microsomes were fractionated on the sucrose gradient, indicating that connexins detected at this 9 S position [30–37% (w/v) sucrose] were oligomeric connexins and were unlikely to correspond to connexin aggregates (results not shown).

The increased oligomerization, especially that of Cx32, observed on supplementation of microsomes with Golgi membranes and the appearance of heteromeric connexons suggests that Golgi membranes provide unknown biochemical factors or structural properties, e.g. differences in membrane width of endoplasmic reticulum and Golgi membranes [44] that enable efficient oligomerization and selectivity in connexon composition. It was noted that the effect of Golgi membranes was more

pronounced with Cx32 than with Cx26. This observation, taken with the ability of Cx26 to insert post-translationally into membranes, agrees with results showing that Cx26 might transit to the plasma membrane via a non-classical route [21,24,43,45].

Assembly into functional hemichannels

Importantly, the connexins translated *in vitro* were able to oligomerize into hexamers and showed channel-forming properties, because they were permeable to ascorbate (140 Da). The low permeability shown by liposomes reconstituted with proteins from microsomes might be accounted for by the presence of low levels of endogenous connexins or possibly other channel-forming proteins in canine pancreatic microsomes. The higher rate of ascorbate uptake in microsomes containing connexons provides strong evidence that the channel-forming properties were due to connexons synthesized *in vitro*. In separate experiments (results not shown), liposomes reconstituted with connexons synthesized *in vitro* also showed permeability to sucrose, because they sedimented more deeply into the linear sucrose gradient than did plain liposomes and liposomes reconstituted with proteins from microsomal membranes [46].

During intracellular trafficking of connexons to the plasma membrane it is unknown whether they are closed, because this configuration would help limit leakage between the luminal and cytosolic environments. The connexon hemichannels synthesized *in vitro* and reconstituted in liposomes or in lipid bilayers were open, as demonstrated in the present study and as shown by their electrical properties [33]. Liposome model systems constructed of a single type of phospholipid are artifacts and synthetic connexons might lack the association with components required to keep the connexon hemichannels closed in the cell membrane, as occurs, for example, with the translocon channel that traverses the endoplasmic reticulum [47]. These possibilities are supported by reports in which open gap junction channels were observed in single cells showing permeability properties for small molecules [48] and membrane conductance observed in single oocytes transfected with chimaeric connexins [49]. Permeable channels were also observed when gap junctions purified from liver, heart and lens were reconstituted into liposomes [46,50,51].

Immunoprecipitation results indicating homomeric and heteromeric assembly further reinforced the finding that connexins can oligomerize during their co-translational translocation into microsomal membranes. However, the present results show that whereas connexins form homomeric connexons in microsomes, heteromeric assembly was facilitated in the presence of Golgi membranes. This suggests that the reticulocyte lysate system used was probably not competent to provide all the requirements necessary for the assembly of heteromeric connexons. Heteromeric connexons were also observed by immunoprecipitation of detergent extracts of labelled guinea-pig hepatocytes (results not shown) and in gap junctions isolated from guinea-pig liver [24]. The connexin subunit composition of gap junctions has been reported for various organs, with homomeric connexons present in mouse liver [52] and heteromeric assemblies in insect cells overexpressing recombinant Cx26 and Cx32 [53], rat hepatocytes with induced expression of Cx26 [54] and bovine lens fibres [55].

In conclusion, the results show that connexons synthesized *in vitro* in a cell-free translation system can be reconstituted into liposomes, where they show properties compatible with the formation of hexameric hemichannels. Homomeric and heteromeric hemichannels synthesized *in vitro* might prove useful for further studies of the permeability and regulatory properties of gap junctions.

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