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Cell-free synthesis and assembly of connexins into functional gap junction membrane channels

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Several different gap junction channel subunit isotypes, known as connexins, were synthesized in a cell-free translation system supplemented with microsomal membranes to study the mechanisms involved in gap junction channel assembly. Previous results indicated that the connexins were synthesized as membrane proteins with their relevant transmembrane topology. An integrated biochemical and biophysical analysis indicated that the connexins assembled specifically with other connexin subunits. No interactions were detected between connexin subunits and other co-translated transmembrane proteins. The connexins that were integrated into microsomal vesicles assembled into homo- and hetero-oligomeric structures with hydrodynamic properties of a 9S particle, consistent with the properties reported for hexameric gap junction connexons derived from gap junctions *in vivo*. Further, cell-free assembled homo-oligomeric connexons composed of β_1 or β_2 connexin were reconstituted into synthetic lipid bilayers. Single channel conductances were recorded from these bilayers that were similar to those measured for these connexons produced *in vivo*. Thus, this is the first direct evidence that the synthesis and assembly of a gap junction connexon can take place in microsomal membranes. Finally, the cell-free system has been used to investigate the properties of α_1, β_1 and β_2 connexin to assemble into hetero-oligomers. Evidence has been obtained for a selective interaction between individual connexin isotypes and that a signal determining the potential hetero-oligomeric combinations of connexin isotypes may be located in the N-terminal sequence of the connexins.

Keywords: cell-free translation/connexons/gap junction channels/protein oligomerization/selective subunit assembly

Introduction

Gap junction (GJ) channels are oligomeric plasma membrane structures in which six protein subunits known as connexins (Cx) assemble by non-covalent interactions into a conducting membrane channel with a central aqueous pore. For gap junctional cell-to-cell communication, two of these single membrane channel structures (referred to as connexons), each provided by one of the adjacent cells, 'pair' to form the complete, double-membrane intercellular junction.

Although a general structure for GJ channels has been determined by electron microscopy and X-ray analysis (for reviews see Goodenough *et al.*, 1996; Kumar and Gilula, 1996; Yeager and Nicholson, 1996), detailed information about the utilization of channel subtypes and channel subunit composition is not known. Further, the mechanisms by which the cell is able to accomplish the complex process of GJ channel assembly have not yet been defined experimentally. It has been assumed that GJ channels, composed of identical Cx subunits (referred to as homo-oligomeric) are potentially assembled *in vivo*. This assumption has been supported by structural analyses of individual GJ plaques (Risek *et al.*, 1994; Sosinsky, 1995; Unger *et al*., 1997), and by the assembly of gap junctions in cell culture that are structurally identical to gap junctions *in vivo* after expressing a single Cx isotype in baculovirus-infected insect cells (Stauffer *et al.*, 1991; Buehler *et al.*, 1995) or in transfected tissue culture cells (Elfgang *et al.*, 1995; Kumar *et al.*, 1995). However, more than a dozen different GJ channel subunit isotypes have been cloned and sequenced from rodents, and many cells express more than one Cx isotype that can be localized to the same GJ plaque (Nicholson *et al.*, 1987; Risek *et al.*, 1994). This raises the possibility that, in addition to homo-oligomeric GJ channels, a large number of hetero-oligomeric GJ channel subtypes, composed of more than one Cx isotype, could also exist. The existence of hetero-oligomeric GJ channels has been suggested by some recent observations (König and Zamphigi, 1995; Stauffer, 1995; Jiang and Goodenough, 1996). The precise subunit composition, stoichiometry and organization within the oligomeric channel are likely to play a critical role in determining the properties of these hetero-oligomeric GJ channels. By analogy, many different subunits for vertebrate ligand- and voltage-gated ion channels have also been cloned; these oligomerize into many different channel subtypes that are believed to be specifically adapted to regulate precisely the function of the cell in which they are expressed (for a review, see Green and Millar, 1995). Normally, the possible combinations of subunit isotypes far exceeds the actual number of different channel subtypes that are assembled, suggesting that the assembly of subunits into a channel structure is unlikely to be a random process.

GJ channels are not typically expressed in great abundance, and the biochemical characterization of GJ proteins has been limited thus far to only a few organs, notably liver, heart and lens (see Kistler *et al.*, 1994 for an extensive listing of references), limiting the research on GJ channel assembly *in vivo*. Consequently, several types of GJ channel subunits have been expressed in mRNA-injected *Xenopus* oocytes or DNA-transfected tissue cul-

ture cells which are capable of assembling newly translated connexins in the surface membrane (Werner *et al.*, 1985; Dahl *et al.*, 1987; Swenson *et al.*, 1989; Elfgang *et al.*, 1995; Kumar *et al.*, 1995). However, oocytes and tissue culture cells represent complex heterologous cell systems that can create complications in the interpretation of the results obtained in these systems. Here, we present an alternative expression system for GJ channel subunit proteins. Cell-free protein synthesis, co-translational microsomal membrane integration, immuno-co-precipitation techniques, and reconstitution of newly synthesized channels into planar lipid bilayers have been combined to analyze the synthesis and potential assembly properties of connexins into GJ channels.

Results

Cell-free synthesis of wild-type and mutated GJ connexins

The principal structural components of gap junctions are the transmembrane proteins called connexins. At present, 13 different rodent connexins have been cloned and sequenced. All represent structurally conserved, non-glycosylated polytopic membrane proteins that traverse the membrane bilayer four times, oriented with their N- and C-termini facing the cytoplasmic side (Figure 1A; for review see Falk *et al.*, 1994; Bruzzone *et al.*, 1996; Kumar and Gilula, 1996).

To study the assembly of GJ subunit proteins (connexins) into GJ channels, three different Cx isotypes, α_1 (Cx43), β_1 (Cx32) and β_2 (Cx26), were expressed in cell-free translation/membrane integration systems consisting of translation-competent cell lyzates and endoplasmic reticulum (ER)-derived membrane vesicles (microsomes). In addition to the full-length proteins, N- and C-terminal truncated Cx polypeptides were generated (Figure 1A). As we reported previously, Cx polypeptides can be expressed efficiently in translation-competent cell lyzates. In the absence of microsomes, synthetic α_1, β_1 and β_2 Cx RNAs produce single translation products that migrate with an electrophoretic mobility on SDS–PAGE gels that correspond to their predicted molecular weights (Figure 1, lanes 1, 8 and 15; also Falk *et al.*, 1994).

As reported previously, wild-type connexins are aberrantly processed by signal peptidase concomitant with their integration into canine pancreatic microsomes, thereby increasing their electrophoretic mobility on SDS–PAGE gels (Figure 1B, lanes 4, 11 and 16; also Falk *et al.*, 1994; Zhang *et al.*, 1996). N-terminal amino acid sequencing of such processed α_1 and β_1 connexins revealed that the cleavage occurs at the border between the first transmembrane domain (M1) and the first extracellular domain (E1) (between amino acid residues 46/47 of α_1 and 40/41 of β_1, respectively; M.Falk and N.Gilula, manuscript in preparation); thus separating the N-terminal and M1 domain from the larger C-terminal portion of the polypeptides (designated $\alpha_1\Delta$N, $\beta_1\Delta$N and $\beta_2\Delta$N in Figure 1A). Since the Cx polypeptide sequence is not mutated and the cleavage occurs after the membrane integration process is initiated, the transmembrane topology was not expected to be altered in these Cx polypeptides, and therefore they should have the same transmembrane topology as connexins synthesized *in vivo*. The correct transmembrane

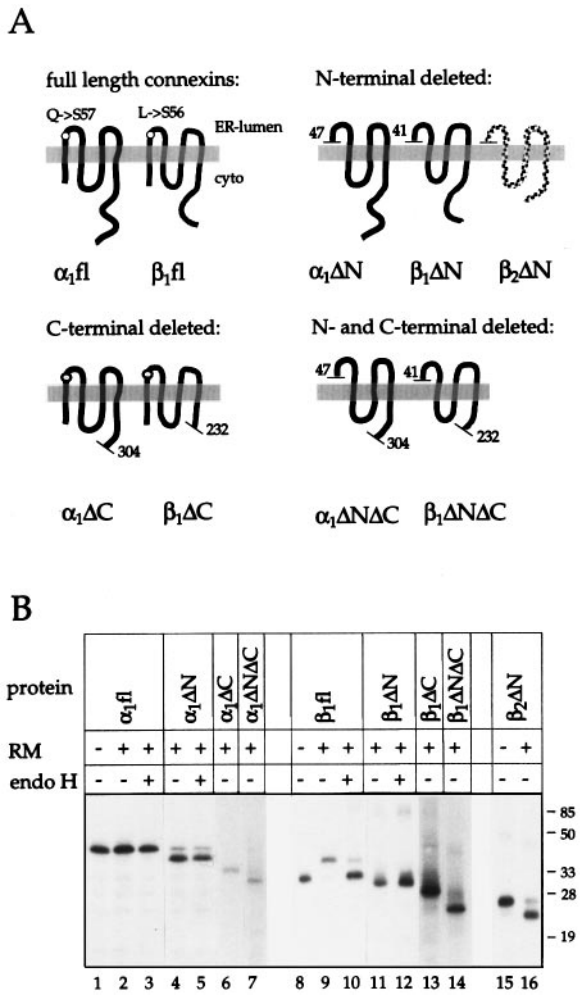


Fig. 1. Cell-free synthesis of GJ connexins. (**A**) Topology models and transmembrane organization of α_1 (Cx43), β_1 (Cx32) and β_2 (Cx26) Cx polypeptides used in this study. In addition to full-length (fl) connexins, N- and C-terminal-deleted (ΔN, ΔC) polypeptides were generated for this analysis of connexon assembly. The amino acid residues that were substituted and the numbers of the first and last residues in the N- and C-terminal truncated Cx constructs are indicated. The exact position of the proteolytic cleavage site of the β_2 Cx was not determined. The transmembrane topology of α_1, β_1 and β_2 Cx polypeptides integrated into microsomal vesicles was determined by N-glycosylation site tagging and protease protection assays. (**B**) Autoradiography of translation products analyzed by SDS–PAGE. Synthetic RNAs encoding Cx protein specific constructs were expressed in [^{35}S]methionine-containing reticulocyte lyzates in the absence (–) and presence (+) of microsomal vesicles (RM). Full-length α_1 and β_1 connexins were synthesized by substitution of a single amino acid residue in the first extracellular loop of the connexins (Q57→S in α_1, lane 2; and L56→S in β_1, lane 9), generating synthetic N-glycosylation sites. N-linked carbohydrates were added efficiently only to tagged, full-length β_1 connexins, reducing their electrophoretic mobility (lane 9). Endoglycosidase H (endo H) treatment removed the attached carbohydrates (lane 10). (Note that the endo H digest shown in lane 10 was incomplete.) N-terminal-deleted α_1, β_1 and β_2 Cx polypeptides were obtained by translating wild-type Cx sequences in the presence of microsomes (lanes 4, 11, and 16) (see Falk *et al.*, 1994, and Results for further details). Small amounts of non-membrane-integrated, full-length Cx polypeptides are visible as a faint band with a slower mobility than the major, cleaved product in these lanes. C-terminal-truncated connexins were obtained by linearizing the Cx-specific cDNAs at unique restriction sites located in their C-terminal tail (lanes 6, 7, 13 and 14). The position of marker proteins and their molecular masses (in kDa) are indicated on the right.

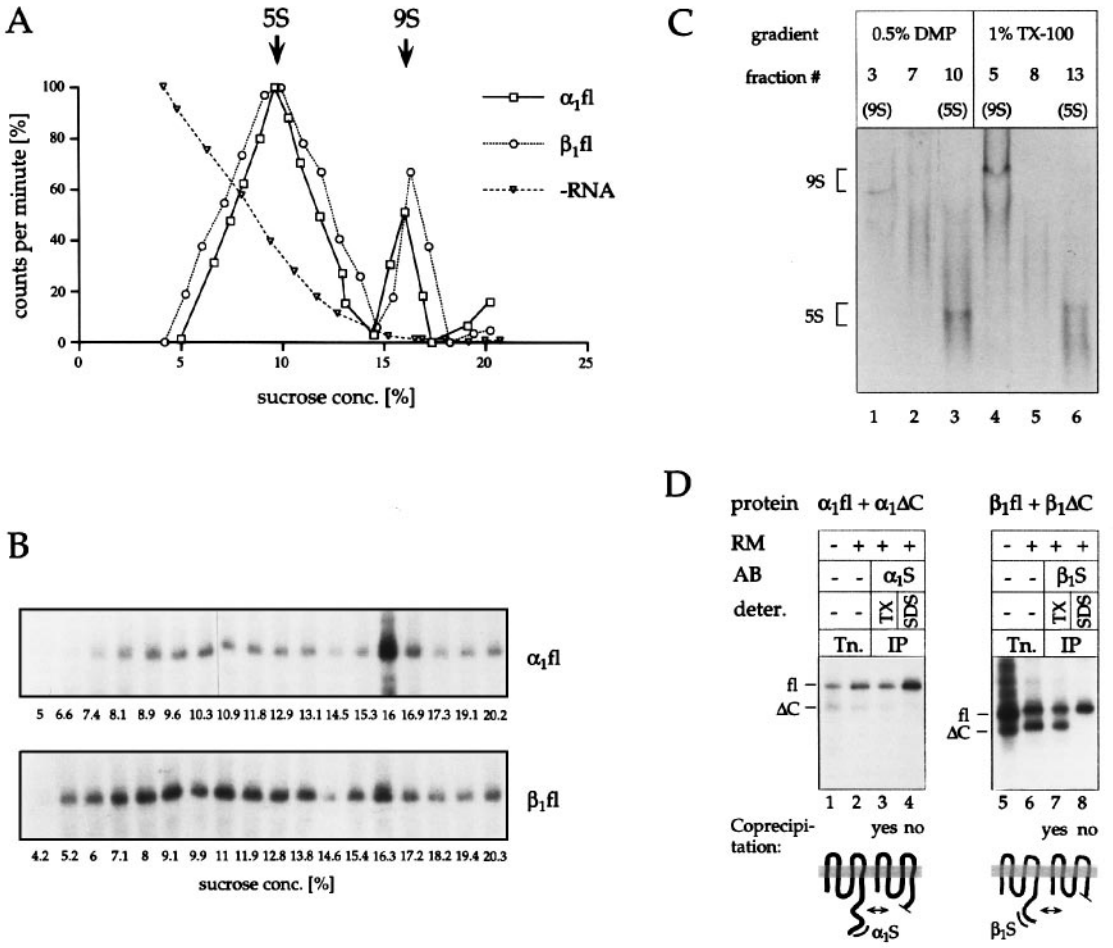


Fig. 2. Cell-free assembly of homo-oligomeric GJ connexons. (**A**) Sucrose gradient sedimentation analysis of GJ connexins. Cx polypeptides were translated in the presence of microsomes and [^{35}S]methionine. Microsomes were lyzed under conditions that kept protein-complexes intact (1% TX-100), after which proteins were sedimented through 5–20% sucrose gradients. Gradients were fractionated, and an aliquot of each fraction was analyzed by liquid scintillation counting. Counts per minute detected in each fraction are indicated on *y*-axis; sucrose concentrations for each fraction are indicated on *x*-axis. Peaks containing 5S particles (monomeric connexins) and 9S particles (assembled GJ connexons) are marked. Increased radioactivity in the bottom fractions of some gradients corresponded to incompletely lyzed microsomal material. (**B**) Immunoprecipitation of Cx polypeptides. Cx protein was immunoprecipitated from each gradient fraction, using antibodies specific for the individual Cx isotypes. Precipitates were analyzed by SDS–PAGE, and detected by autoradiography. (**C**) Native PAGE of 9S and 5S particles derived from cell-free-expressed β_1 Cx. Microsomes containing cell-free-expressed β_1 Cx were lyzed in 0.5% DMP or 1% TX-100, and the proteins were separated on sucrose gradients containing the respective detergents. Protein from 9S and 5S particle-containing fractions, and a fraction in between, was then separated on 4% polyacrylamide gels in the absence of SDS. Cx polypeptides were detected by autoradiography. (**D**) Immunoprecipitation of assembled Cx polypeptides. C-terminal-truncated α_1 and β_1 Cx polypeptides which hence lacked epitopes for the α_1S and β_1S antibodies (AB) were co-translated together with full-length connexins in the presence of microsomes (RM). Microsomes were lyzed in 1% TX-100 (TX) or 0.1% SDS, and the co-expressed membrane-integrated polypeptides were analyzed by immunoprecipitation (IP), SDS–PAGE and autoradiography. Translation reactions (Tn.) in the absence (–) and presence (+) of microsomes are shown for comparison of the amounts and sizes of the immunoprecipitated polypeptides with their corresponding translation products.

topology for Cx polypeptides integrated into microsomes was verified by an N-glycosylation site tagging approach for α_1 and β_1 connexins (M.Falk and N.Gilula, manuscript in preparation). Small amounts of non-membrane-integrated full-length Cx polypeptides, synthesized as a by-product by non-membrane-bound ribosomes in these reactions (visible as a fainter band above the major, cleaved product, in Figure 1B, lanes 4, 5, 11, 12 and 16; Figure 5C, panel 5; and Figure 6, panels 1, 2 and 4) could be removed by pelleting the membrane vesicles through 0.5 M sucrose cushions (see experiments shown in Figure 2D; Figure 5B and C, panels 1–4; Figure 6, panels 5–8; and Figure 7).

To produce membrane-integrated full-length Cx polypeptides in the cell-free system that correspond in size and topology to Cx polypeptides assembled *in vivo*, the Cx sequences were modified by a single amino acid residue. This amino acid exchange completely abolished the proteolytic processing described above. Glu57 was exchanged with serine in α_1 Cx, and Leu56 was exchanged with serine in β_1 Cx to change the wild-type amino acid sequence into N-glycosylation consensus sites (Hart *et al.*, 1978) asparagine–threonine–serine (designated as $\alpha_1$fl and $\beta_1$fl in Figure 1A). Translation in the presence of microsomes generated membrane-integrated full-length Cx polypeptides and no proteolytically processed Cx polypeptides were generated under these conditions (Figure 1B, lanes 2 and 9). Engineered N-linked carbohydrate side chains, added to the β_1 Cx polypeptides (decreasing their electrophoretic mobility) were removed upon the addition of endoglycosidase H, shifting their mobility back to the

mobility of full-length proteins (Figure 1B, lanes 3 and 10). Core glycosylation and the single amino acid residue exchange, evoked by the engineering of the N-glycosylation consensus sites, did not modify the membrane integration or natural transmembrane topology of the connexins as was concluded from an extensive topology analysis (M.Falk and N.Gilula, manuscript in preparation), nor did they affect the oligomerization of the connexins into GJ connexons. This result is consistent with previous studies reporting that the biosynthesis and function of other channel proteins was not affected by the introduction of engineered N-glycosylation consensus sites or the removal of natural N-linked carbohydrates (Smith *et al.*, 1986; Tamkun and Fambrough, 1986; van Koppen and Nathanson, 1990; Deal *et al.*, 1994; Bai *et al.*, 1996). No other method is presently available that allows the synthesis of full-length non-mutated Cx polypeptides in the presence of canine pancreatic microsomes.

To obtain C-terminal truncated Cx polypeptides that were membrane-integrated and properly oriented, wild-type and mutated α_1 and β_1 Cx cDNAs described above were linearized on natural restriction sites in their C-terminal domain, removing 78 amino acids (approximately one-half of the C-terminal domain of α_1) and 51 amino acids (approximately two-thirds of the C-terminal domain of β_1) (designated $\alpha_1\Delta C$, $\alpha_1\Delta N\Delta C$, $\beta_1\Delta C$ and $\beta_1\Delta N\Delta C$ in Figure 1A). Translation in the presence of microsomes generated Cx polypeptides that corresponded to their expected molecular weights after separation on SDS–PAGE gels (Figure 1B, lanes 6, 7, 13 and 14).

Assembly of cell-free synthesized connexins into GJ connexons

Electron diffraction analysis of isolated GJ plaques (Unwin and Zampighi, 1980; Yeager, 1995; Unger *et al.*, 1997) and crosslinking of isolated GJ connexons (Cascio *et al.*, 1995) have indicated that a GJ connexon is composed of six subunits. To determine if the GJ channel subunit proteins that were synthesized in the cell-free system assembled into oligomers, microsomes derived from an $\alpha_1$fl and a $\beta_1$fl translation reaction were solubilized in 1% Triton X-100 (TX-100), and the Cx translation products were separated by sucrose gradient sedimentation analysis as described in Materials and methods. After fractionation of the gradients, α_1 and β_1 Cx protein products were found in two distinct peaks centered at ~10% and 16% sucrose (w/v), corresponding to 5S and 9S particles, or monomeric connexins and assembled GJ connexons, respectively (Figure 2A, □ and ○). No comparable peaks were detected when a control translation reaction was analyzed that was not programmed with synthetic RNA (Figure 2A, ▽). Under these conditions, ~16% of the translation products were recovered as assembled connexons (9S particles), while ~84% were recovered as unassembled Cx subunits (5S particles). No paired double-membrane intercellular junctions (16S particles; Kistler *et al.*, 1994) were detected under these conditions. Sedimentation coefficients of assembled and monomeric connexins were determined by analyzing control proteins with known S-values and connexons that were expressed and purified from baculovirus-infected insect cells on parallel gradients. Aliquots of the individual fractions were analyzed by SDS–PAGE to verify that the radioactivity detected corresponded to labeled Cx protein (not shown). In addition, antibodies specific for the individual Cx isotypes were used to immunoprecipitate the corresponding Cx polypeptides from the gradient fractions (Figures 2B and 5A).

9S particles (fractions 3 and 5), 5S particles (fractions 10 and 13), and fractions in between (7 and 8) derived from gradients of a β_1 Cx translation reaction were further analyzed by native PAGE. This procedure made it possible to analyze the natural protein–protein interactions within the connexons without any chemical modification of the polypeptides. Protein complexes remained assembled during electrophoresis, and thus migrated according to their natural charge and shape. Fractions from the gradients that contained either 0.5% *n*-decyl-β-D-maltopyranoside (DMP), or 1% TX-100 are shown (Figure 2C). In both detergents, the electrophoretic mobility of the 9S particles was significantly reduced compared with that of the 5S particles, consistent with the predicted molecular weights and shapes for an assembled GJ connexon and a single β_1 Cx subunit (Figure 2C, lanes 1 and 4, and 3 and 6). Slight differences in the electrophoretic mobility of the 9S and 5S particles are probably due to the different detergent types. No distinct protein structures leading to a sharp protein band on the gel were detected in the control fractions 7 and 8 (Figure 2C, lanes 2 and 5).

The interaction of the Cx subunits that were assembled in the cell-free expression system was directly demonstrated by immuno-co-precipitation. For this purpose, C-terminal-truncated α_1 and β_1 Cx polypeptides were generated that no longer contained the specific epitopes recognized by the α_1S- and β_1S-specific monoclonal antibodies ($\alpha_1\Delta C$ and $\beta_1\Delta C$, Figure 2D). Co-translation of the full-length connexins ($\alpha_1$fl or $\beta_1$fl), together with their corresponding truncated connexins ($\alpha_1\Delta C$ or $\beta_1\Delta C$) in the presence of microsomes, resulted in the immunoprecipitation of the full-length connexins and also in the co-precipitation of the truncated Cx polypeptides. These samples were recovered after purification of the microsomes, solubilization in 1% TX-100, and immunoprecipitation with the α_1S- or β_1S-specific antibodies, respectively (Figure 2D, lanes 3 and 7). No co-precipitation was observed in control aliquots treated instead with 0.1% SDS (Figure 2D, lanes 4 and 8).

To investigate if the connexin-oligomerization was restricted to full-length Cx polypeptides, truncated Cx polypeptides were also translated in the cell-free system, and were analyzed by hydrodynamic analysis. Wild-type α_1, β_1 and β_2 connexins (designated $\alpha_1\Delta N$, $\beta_1\Delta N$ and $\beta_2\Delta N$) that were proteolytically cleaved in their first extracellular loop concomitant with their integration into microsomes (described above and in Falk *et al.*, 1994) also assembled into GJ connexons, or connexon 'core structures', with similar sedimentation coefficients (9S) and characteristics described for GJ connexons assembled from full-length Cx subunits (Figure 3 and Discussion).

Different detergents were used for the solubilization of the microsomes to determine if the type of detergent had an influence on the amount of assembled GJ connexons that were recovered. Microsomes derived from a translation reaction programmed with $\beta_1$fl cRNA were split into equal aliquots, lyzed either in *n*-octyl-β-D-glucopyranoside (OG), DMP, TX-100, ZWITTERGENT 3-12 (ZW. 3-12),

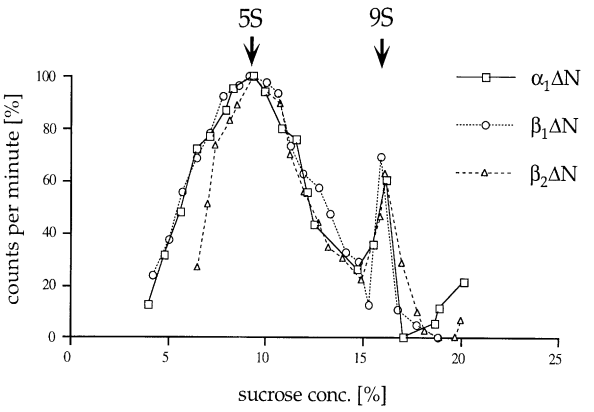


Fig. 3. Hydrodynamic analysis of N-terminal-processed connexins. α_1, β_1 and β_2 connexins that were proteolytically cleaved in their first extracellular loop upon integration into microsomal vesicles by the enzyme signal peptidase assembled into oligomeric structures with a sedimentation coefficient of ~9S, similar to that of full-length connexins, when translated in the presence of microsomes, lyzed in 1% TX-100 and analyzed by sucrose gradient centrifugation as described in the legend to Figure 2.

or in SDS, and the proteins were sedimented through linear 5–20% sucrose gradients containing the respective detergents. While assembled connexons were recovered in variable amounts together with unassembled connexins using either OG, DMP or TX-100, only unassembled subunits were recovered using ZW. 3-12 or SDS. Yields of >30% assembled connexons were recovered after solubilization of the microsomes in 2% OG (Table I).

Biophysical characterization of cell-free synthesized and assembled GJ channels

The potential functional properties of cell-free assembled GJ connexons were analyzed by fusing the microsomal vesicles containing cell-free expressed wild-type α_1, β_1 or β_2 connexins to preformed planar lipid bilayers, and subsequently determining single channel activities. Distinct channel activities were observed with all three Cx isotypes tested that were not observed in microsomes not containing cell-free-expressed connexins. Channels induced with microsomes that contained cell-free-synthesized β_2 Cx (Figure 4A) displayed a similar behavior (rapid open/closing transitions and short open-stages) and a unitary conductance of 52.4±2.6 pS that was similar to channels that were recorded previously by reconstituting recombinant β_2 GJ connexons purified from baculovirus-infected insect cells into planar lipid bilayers (56±7 pS) (Figure 4C; Buehler *et al.*, 1995). A channel induced with microsomes that contained cell-free-synthesized β_1 Cx showed a unitary conductance of 170 pS (Table II) which is similar to the conductance of ~180–200 pS, reported recently for β_1 GJ connexons that were purified under native conditions from rat liver, reconstituted into unilamellar phospholipid liposomes, and fused to planar lipid bilayers (Rhee *et al.*, 1996). A channel with a unitary conductance of 35.5 pS was recorded from membranes fused with microsomes containing α_1 Cx (Table II). However, no comparable data are available on the activity of reconstituted GJ connexons composed of α_1 Cx that would allow a comparison of our recording with previously published recordings. Therefore, the α_1 recording can only be indicative of channel activity exhibited by cell-free-expressed α_1 Cx. Control microsomes not incubated in a translation reaction before had endogenous channel activities of 11.5±2.5, 28±7 and 105±15 pS that in addition were frequently observed in the microsomes containing cell-free-synthesized connexins (and served as internal controls for microsome/synthetic bilayer fusion), but endogenous channels with conductances of 52.4±2.6 and 170 pS were never observed in the control microsomes (Figure 4B, Table II). In total, 70 trials were performed: 42 with microsomes containing cell-free-expressed α_1, β_1 and β_2 connexins, and 28 with control microsomes. Channel activities were recorded in 24 (68%) and 10 (36%) experiments, respectively. In five (24%) of the 21 active synthetic membranes that were fused with β_2 Cx-containing microsomes, there was defined β_2 GJ connexon activity, and at least one membrane fused with β_1 Cx-containing microsomes had apparent β_1 connexon activity (Table II).

Table I. Influence of detergent on the recovery of assembled connexons[a]

Detergent	Assembled connexons (9S particles; %)	Unassembled Cx polypeptides (5S particles; %)
2% OG	32.2	67.8
0.5% DMP	24.1	75.9
1% TX-100	16.8	83.2
1% ZW. 3-12	0	100
0.1% SDS	0	100

[a]Microsomes derived from a translation reaction programmed with β_1 Cx cRNA were split into equal aliquots and lyzed in the detergents listed in the Table. Proteins were sedimented through linear 5–20% sucrose gradients containing the respective detergents. Peak areas containing 9S particles (assembled connexons) and 5S particles (unassembled Cx polypeptides) were integrated and shown as relative percentages.

Regulated assembly of distinct hetero-oligomeric GJ connexons

The results presented above indicated that functional homo-oligomeric GJ connexons were assembled in the microsomal vesicles following translation of the Cx subunits in the cell-free translation system. To determine if connexins could also assemble into hetero-oligomeric connexons consisting of more than one Cx isotype, α_1 and β_1 connexins were translated together in the presence of microsomes. Microsomes were solubilized in 1% TX-100 and proteins were sedimented on sucrose gradients as described previously. Oligomeric complexes with a sedimentation coefficient of ~9S were obtained along with unassembled Cx polypeptides (5S particles), with co-translated full-length connexins ($\alpha_1$fl plus $\beta_1$fl, Figure 5A, □) and with cleaved connexins ($\alpha_1\Delta$N plus $\beta_1\Delta$N, Figure 5A, ○). To analyze the subunit composition of the non-junctional GJ channels assembled under these conditions, Cx polypeptides from the 9S and 5S particle-containing fractions were immunoprecipitated with α_1 and β_1 Cx monoclonal antibodies (α_1S and β_1S) in the presence of 1% TX-100. The presence of 1% TX-100 was shown previously to leave oligomeric Cx-complexes intact. No Cx polypeptides of the other Cx isotype were immuno-co-precipitated from the 9S and 5S fractions when $\alpha_1$fl and $\beta_1$fl connexins were co-translated (Figure 5B, panel 1).

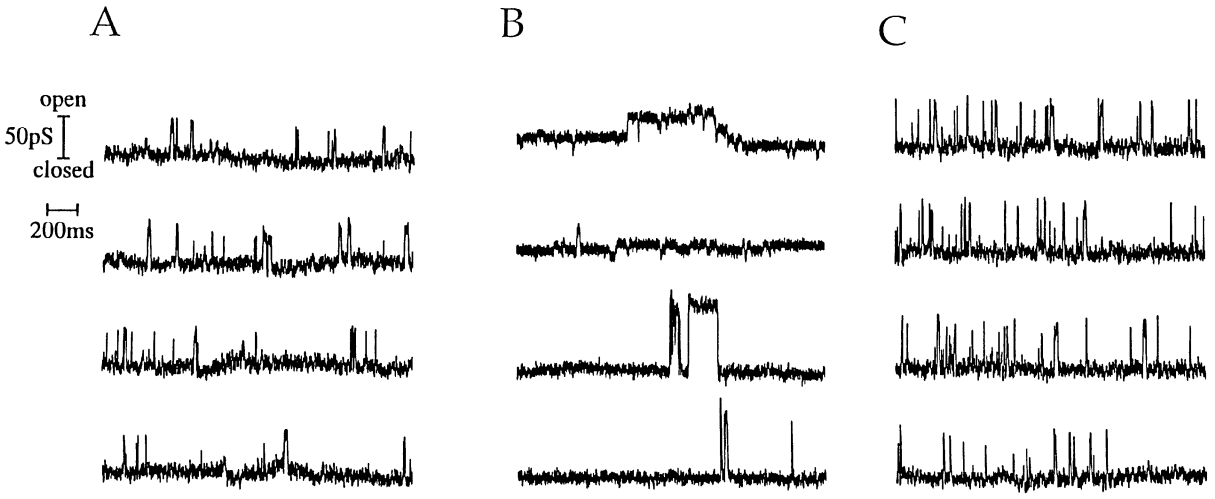


Fig. 4. Single channel recordings of cell-free-assembled GJ connexons. Cell-free-synthesized GJ channels were identified by comparison with the activities observed from baculovirus-derived GJ connexons. Selected recordings obtained after the fusion of microsomes containing cell-free-synthesized wild type β_2 Cx (in A), of control microsomes not used in a translation reaction before (in B), and after reconstitution of recombinant connexons purified from β_2 expressing baculovirus-infected insect cells (in C), are shown. (**A**) Microsomes containing cell-free-expressed β_2 Cx displayed a 49.8 pS channel activity in this selected recording in 200 mM KCl, 20 mM $MgCl_2$, 10 mM HEPES, pH 7.4, and 100 mM sucrose in the subphase. The membrane contained POPE/POPS mixture (4:1) and the voltage was held at +50 mV. (**B**) Control microsomes showed endogenous channel activities of 10, 28, and 120 pS in these selected recordings in 200 mM KCl, 5 mM $CaCl_2$, 10 mM HEPES, pH 7.4, and 100 mM sucrose in the subphase. The membranes were formed from soybean lipid extracts, and the voltage was held at +100 mV (upper two traces) and +50 mV (lower two traces). (**C**) β_2 Cx channels isolated and reconstituted from infected insect cells. The channel exhibited a unitary conductance of 56 pS in this selected recording in 200 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, pH 7.4. The membrane contained POPE/POPS mixture (4:1) and the voltage was held at +60 mV.

However, when co-translated cleaved α_1 and β_1 connexins were immunoprecipitated under these conditions, the other Cx isotype was co-immunoprecipitated from the 9S particle-containing fractions using either α_1 or β_1 antibodies (Figure 5B, panel 2). Antibody specificities for the individual Cx isotypes were maintained in these immunoprecipitation reactions. This result suggested that the cleaved connexins ($\alpha_1\Delta$N and $\beta_1\Delta$N) did not retain the discriminating assembly properties that was observed with their corresponding full-length proteins. Additional co-translation and immunoprecipitation experiments revealed that co-translation consistently resulted in the co-immunoprecipitation of the other co-translated Cx isotype when either one (Figure 5C, $\alpha_1$fl plus $\beta_1\Delta$N in panel 2, and $\alpha_1\Delta$N plus $\beta_1$fl in panel 3) or both Cx isotypes (Figure 5A, $\alpha_1\Delta$N plus $\beta_1\Delta$N in panel 4) were N-terminal processed. No co-precipitation was observed when $\alpha_1$fl plus $\beta_1$fl were co-translated (Figure 5C, panel 1). A similar lack of Cx subunit assembly discrimination was also found with other Cx isotypes. Co-translation of N-terminal-truncated α_1, β_1 and β_2 connexins ($\alpha_1\Delta$N, $\beta_1\Delta$N plus $\beta_2\Delta$N), followed by immunoprecipitation with a Cx-specific antibody, resulted in the precipitation of all three Cx isotypes (Figure 5C, panel 5).

Specificity of connexin assembly

Several results indicated that the specific Cx–Cx interactions observed with the full-length and truncated Cx polypeptides in the immunoprecipitation assays described above reflected specific protein–protein interactions that were restricted to the Cx isotypes. (i) The observed Cx–Cx interactions were restricted to membrane-incorporated Cx polypeptides only. In the experiment shown in Figure 5C, panel 5, non-membrane-integrated full-length Cx proteins, generated as a by-product in the *in vitro* translation reactions, were not removed from the reactions before immunoprecipitation. While the antibodies recognized and precipitated both the N-terminal-cleaved as well as the full-length forms of their antigen, predominantly the membrane-integrated N-terminal-cleaved polypeptides (with the faster mobility on SDS gels) of the other co-translated Cx isotypes were co-precipitated (Figure 5C, panel 5). (ii) When the microsomal vesicles were solubilized in 0.1% SDS before immunoprecipitation with Cx-specific antibodies, previously assembled Cx–Cx complexes were disassembled and the antibodies precipitated only their corresponding antigens (shown for $\alpha_1\Delta$N, $\beta_1\Delta$N plus $\beta_2\Delta$N co-translated in the presence of microsomes in Figure 6, panel 1). (iii) No antigens were precipitated when microsomes were not lyzed with detergents, which is consistent with the previous report (Anderson and Blobel, 1981) that the large size of the microsomes prevented the immunoprecipitation of intact microsomal vesicles under the experimental conditions applied (shown for co-translated $\alpha_1\Delta$N, $\beta_1\Delta$N plus $\beta_2\Delta$N in Figure 6, panel 2). (iv) Non-membrane-integrated connexins, co-expressed in the absence of microsomes, did not associate with each other, and they were not co-immunoprecipitated (shown for co-translated $\alpha_1\Delta$N, $\beta_1\Delta$N plus $\beta_2\Delta$N in Figure 6, panel 3). (v) α_1, β_1 and β_2 connexins did not assemble into hetero-oligomeric complexes when the individual connexins were translated in the presence of microsomes in separate vials and the translation reactions were mixed before the lysis of the microsomes in 1% TX-100. Under these conditions, the antibodies precipitated only their corresponding antigens (shown for $\alpha_1\Delta$N, $\beta_1\Delta$N and $\beta_2\Delta$N in Figure 6, panel 4). This result indicated first that the assembly process occurred only between Cx polypeptides that were incorporated into the same microsomal vesicle, leading to their co-immunoprecipitation. Second, a head-

Table II. Single channel activities recorded from planar lipid bilayers that were fused to microsomes containing cell-free assembled GJ connexons

Connexons	Active membranes	Inactive membranes	Average recorded conductances (pS)[a]
α_1 (Cx43)	1	4	35.5
β_1 (Cx32)	2	4	170
β_2 (Cx26)	21[b]	10	52.4±2.6[c]
Control microsomes	10	18	11.5±2.5[c]
	28±7[c]		
	105±15[c]		

[a]200 mM KCl, for complete solution composition, see Materials and methods.
[b]Number of successful fusion events increased when microsomes were incubated in the presence of translation mixture, explaining the lower ratio of active versus inactive membranes in control experiments.
[c]The average conductance of 11.5±2.5 pS represented individual channel activities of 9, 13 and 14 pS; the average conductance of 28±7 pS represented individual channel activities of 21, 22, 28, 30, 32 and 35 pS; the average conductance of 52.4±2.6 pS represented individual channel activities of 49.8 and 55 pS; the average conductance of 105±15 pS represented individual channel activities of 90, 100 and 120 pS. Average conductances of 11.5±2.5, 28±7 and 105±15 pS were frequently observed in microsomes containing cell-free-expressed connexins and in control microsomes not used in a translation reaction, indicating that they represent channel activities endogenous to the microsomes. The 105±15 pS channel showed a very stable open state (low noise) atypical of connexin-induced channel activity (Buehler et al., 1995; Rhee *et al.*, 1996) that might be due to endogenous maxi-K^+ channels reported to exist in plasma membranes of pancreatic acinar cells (Peterson and Findlay, 1987).

to-head pairing of connexins via an interaction of their extracellular loop domains, as is observed in complete double-membrane GJ channels, did not occur under the described conditions of the translation reactions. Further, the treatment in 1% TX-100 used to release the connexons from the microsomes subsequently also prevented such a pairing of the assembled connexons. (vi) Other polytopic non-connexin membrane proteins such as lens fiber MP26, the AChRα7 subunit, tight junction occludin, or synaptic vesicle synaptophysin that were co-translated in parallel in the presence of microsomes did not interact and assemble with Cx polypeptides, and they were not co-precipitated after lysis of the microsomes in 1% TX-100 (tested for α_1 plus β_1 full-length and $\alpha_1\Delta$N, $\beta_1\Delta$N plus $\beta_2\Delta$N; and shown for $\alpha_1$fl plus $\beta_1$fl in Figure 6, panels 5–8).

Potential signals involved in regulating hetero-oligomeric connexon assembly

The results described above suggested that the N-terminal-truncated Cx polypeptides may have lost a specific signal located in the N-terminal portion of the polypeptides that is involved in regulating the assembly of Cx isotypes into hetero-oligomeric connexons. To further investigate this hypothesis, C-terminal-truncated Cx polypeptides were constructed (described above) and co-translated in the presence of microsomes. A potential interaction of the proteins was analyzed by immunoprecipitation after solubilization of the membranes with 1% TX-100. Cx-specific antibodies directed against the intracellular loop domain of either α_1 or β_1 Cx (designated α_1J and β_1J in Figure 7) were used in this study. When C-terminal-truncated α_1 and β_1 connexins with intact N-termini ($\alpha_1\Delta$C plus $\beta_1\Delta$C) were co-translated they were not co-precipitated (Figure 7, lanes 3 and 4), suggesting that they did not co-assemble similar to their full-length parent polypeptides $\alpha_1$fl and $\beta_1$fl described above. However, when N- plus C-terminal-truncated α_1 and β_1 connexins ($\alpha_1\Delta$NΔC plus $\beta_1\Delta$NΔC) were co-translated they co-precipitated (Figure 7, lanes 7 and 8), suggesting that they co-assembled similar to their N-terminal-truncated parent polypeptides $\alpha_1\Delta$N and $\beta_1\Delta$N described above.

Discussion

The experiments presented in this report provide evidence that the synthesis and assembly of a GJ channel can be studied in a cell-free expression system. The Cx subunit proteins can be synthesized efficiently in translation-competent cell lyzates supplemented with pancreatic microsomes, and their transmembrane topology corresponds to the transmembrane topology of naturally expressed connexins organized in GJ plaques in the plasma membranes of adjacent cells. Following translation, Cx polypeptides that were integrated into the same microsomal vesicle oligomerized into conducting, hexameric GJ connexons, similar to the GJ channel structures that have been isolated from *in vivo* gap junctions. Functional connexon assembly was indicated by: (i) the formation of particles with a sedimentation coefficient of 9S, identified by hydrodynamic analysis; (ii) their electrophoretic mobility on native PAGE gels; (iii) the induction of channels, similar in behavior and conductance to channels characterized previously from GJ connexons assembled *in vivo* consisting of β_2 and β_1 connexins (Buehler *et al.*, 1995; Rhee *et al.*, 1996); and (iv) the specific assembly of homo- and hetero-oligomers consisting of α_1, β_1 or β_2 connexins, identified by immunoprecipitation analysis. Cell-free-expressed GJ connexons were indistinguishable by the techniques described above from connexons assembled in heterologous expression systems, or endogenous connexons purified from tissues and organs. The present study represents the first description of the successful synthesis and assembly of functional GJ channels in a cell-free expression system. In addition, some evidence has been obtained for a regulated assembly of connexin isotypes that may restrict the possible number of different hetero-oligomeric connexons. This apparent regulation may be dependent on the N-terminal portion of the Cx polypeptides.

A large portion of the structural information on proteins that transmit signals across membranes has been obtained by expressing and analyzing these proteins in coupled cell-free translation/membrane integration systems. However, it was thought that the oligomerization of membrane proteins did not occur in cell-free translation/membrane integration systems due to a low probability for multiple insertions into a microsomal vesicle (Anderson and Blobel, 1981). Later, functional expression and assembly of a *Shaker* type K^+-channel (*Shaker* H4, an oligomeric structure consisting of four identical copies of a protein traversing the membrane bilayer six times; Rosenberg and East, 1992), assembly of a human HLA-DR histocompatibility molecule (an $\alpha/\beta/\gamma$-heterotrimer; Kvist *et al.*, 1982; Qu and Green, 1995), and assembly of the asialoglycoprotein

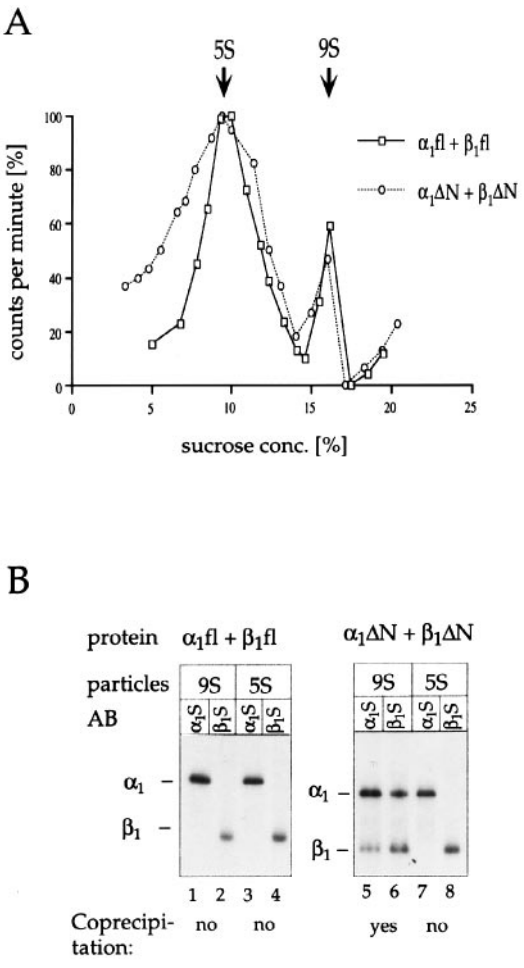
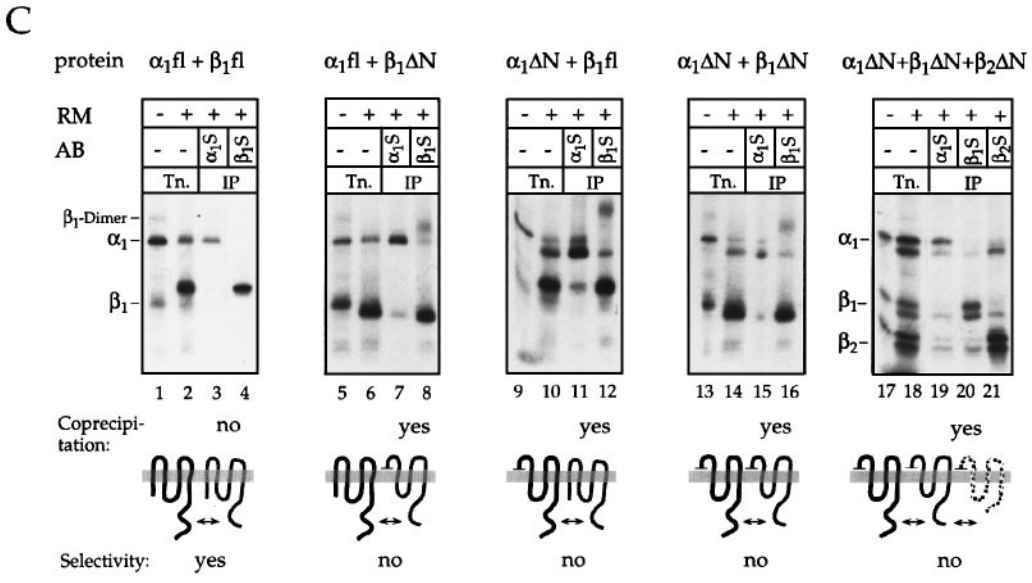


Fig. 5. Assembly of connexins into hetero-oligomeric GJ connexons. (**A**) Co-translated full-length or N-terminal-deleted α_1 and β_1 connexins were analyzed on 1% TX-100-containing sucrose gradients as described in the legend to Figure 2. (**B**) Gradient fractions containing 9S particles (assembled connexons) and 5S particles (unassembled connexins) were immunoprecipitated with α_1 or β_1 Cx antibodies respectively, and the precipitates were analyzed by SDS–PAGE and autoradiography. Antibodies used for the immunoprecipitation of the 9S particles composed of N-terminal-truncated connexins ($\alpha_1\Delta N$, $\beta_1\Delta N$) co-precipitated in addition the co-translated Cx isotype (lanes 5 and 6), indicating that the oligomers assembled under these conditions were hetero-oligomeric GJ connexons. Immunoprecipitation of the 9S particles composed of full-length connexins resulted only in the precipitation of their antigens (lanes 1 and 2), indicating that the oligomers assembled under these conditions were homo-oligomeric GJ connexons. No co-precipitation occurred with 5S particles of either type (lanes 3, 4, 7 and 8). (**C**) Different combinations of full-length and N-terminal-truncated α_1, β_1 and β_2 connexins were co-translated in the presence of microsomes (RM). Microsomes were lyzed in 1% TX-100, and the assembly of the co-translated membrane-integrated polypeptides was analyzed by immunoprecipitation (IP) with monoclonal Cx antibodies (AB), SDS–PAGE, and autoradiography. N-terminal-truncated α_1, β_1 and β_2 connexins assembled with each other and were co-precipitated (panels 4 and 5, lanes 15, 16, 19, 20 and 21). They also assembled with co-translated full-length Cx polypeptides (panels 2 and 3, lanes 7, 8, 11 and 12), while full-length α_1 and β_1 Cx proteins did not co-assemble, and they were not co-precipitated (panel 1, lanes 3 and 4), as observed in (B). Translation reactions are shown for comparison as described in the legend to Figure 2D. Note small amounts of a non-functional β_1-dimer aggregate synthesized in the translation reactions (visible in lanes 1, 5, 9, 13 and 18) that was resistant to treatment with sample buffer and was precipitated with the β_1 Cx antibodies (detected in lanes 8, 12 and 16).



receptor (an α/β hetero-oligomer; Sawyer and Doyle, 1990), although probably not into a functional receptor molecule (Yilla *et al.*, 1992), were reported to occur during cell-free expression in this system. These observations, combined with the expression of GJ connexons reported here, provide evidence that the assembly of functional membrane structures consisting of several subunit proteins can take place in the cell-free expression system, and that the system can be used to study their assembly process.

In a study on the intracellular assembly of α_1 connexin (Cx43) expressed endogenously in a cell culture line (normal rat kidney, NRK) Musil and Goodenough (1993) found that connexins did not assemble following synthesis in the ER membranes, but rather, after they exited from

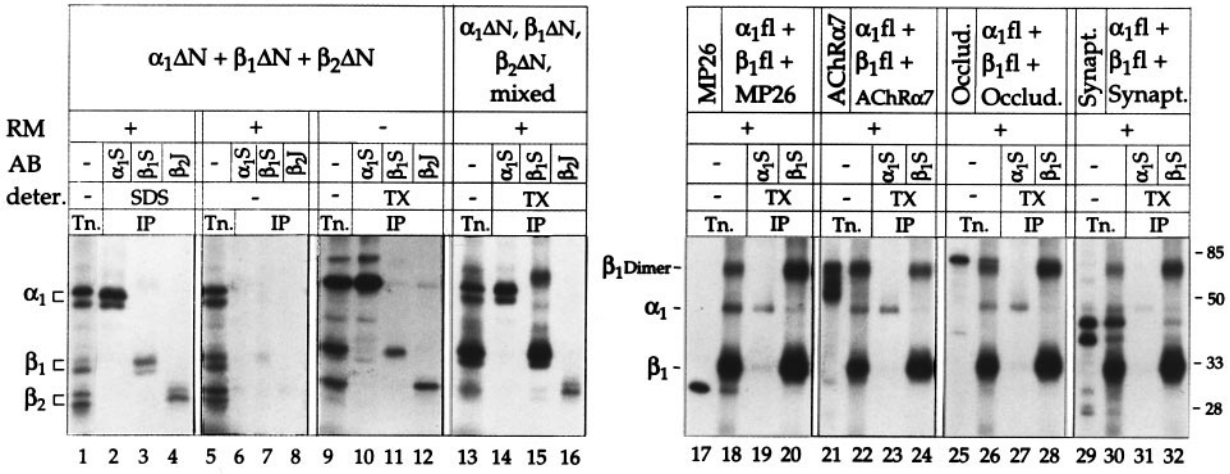


Fig. 6. GJ connexon assembly is dependent on specific interactions between the Cx subunits. N-terminal-truncated α_1, β_1 and β_2 connexins were co-translated either in the absence (–) (panel 3) or presence (+) (panels 1, 2 and 4) of microsomes (RM), and either not lyzed in detergent (panel 2), or lyzed in 1% TX-100 (panel 3) or 0.1% SDS (panel 1); alternatively, the connexins were translated individually in separate vials, and the reactions mixed before lysis in 1% TX-100 (panel 4). In addition, full-length α_1 and β_1 connexins were co-translated together with non-connexin membrane proteins in the presence of microsomes (panels 5–8). Potential protein interactions and assembly was analyzed by immunoprecipitation (IP), SDS–PAGE, and autoradiography, after lysis of the microsomes in 1% TX-100 (panels 5–8). Non-membrane-integrated Cx polypeptides were not removed before IP in panels 1, 2 and 4, and therefore they served as an internal control to detect specific protein–protein interactions. No assembly was observed with other co-translated non-Cx membrane proteins. Note that all gels shown in this figure were overexposed to better demonstrate the specificity in Cx polypeptide interaction. Translation reactions (Tn.) in the presence of microsomes (+) of non-Cx membrane proteins alone, or co-translated with Cx proteins, are shown as described in the legend to Figure 2D. A β_1 dimer also described there was detected in lanes 1, 5, 9, 10, 13, 14, 18, 20, 21, 22, 24, 26, 28, 30 and 32. Position and molecular masses (in kDa) of marker proteins are indicated on the right.

the ER. This observation appears to be inconsistent with the oligomerization of connexins in microsomes (ER-derived membrane vesicles) that have been observed in this study. Hypothetically, it is possible that in the absence of downstream transport components (as occurs in the cell-free system), connexins oligomerize in the ER membranes since they can not exit from this compartment. However, this possibility would be inconsistent with the observations on the expression of β_1 connexin (Cx32) in transfected baby hamster kidney (BHK) cells in which the ER membranes were observed to contain GJ connexons within GJ plaques without affecting the viability of these cells (Kumar *et al.*, 1995). For almost all other known oligomeric membrane proteins, assembly in the ER is an absolute prerequisite for their subsequent transport through the secretory pathway (reviewed in Hurtley and Helenius, 1989). Therefore, the unique observation reported by Musil and Goodenough (1993) may not necessarily reflect the general oligomerization behavior of connexins expressed *in vivo*, as discussed in Kumar and Gilula (1996) and Bruzzone *et al.* (1996). Consequently, additional experimentation in the future will be required to determine the site for connexin oligomerization *in vivo*.

Sedimentation coefficients of ~9S, similar to those determined for cell-free assembled GJ connexons composed of α_1 and β_1 Cx have been described previously for GJ connexons prepared from NRK tissue culture cells expressing α_1 (Cx43) (Musil and Goodenough, 1993), lens fibers expressing α_3 (Cx46) and α_8 (Cx50) (Kistler *et al.*, 1994), and rat livers expressing primarily β_1 (Cx32), respectively (Cascio *et al.*, 1995). In addition, comparable S-values have been described for other oligomeric protein channels, such as the AChR channel and its subunits (Blount and Merlie, 1988), and hexameric synaptophysin (Thomas *et al.*, 1988). Both have similar overall shapes

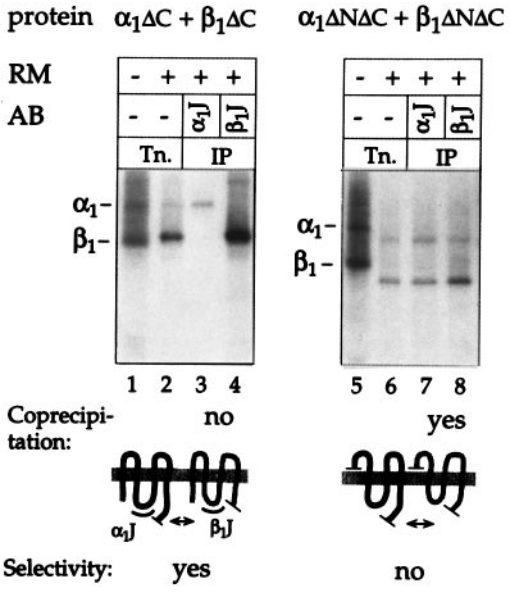


Fig. 7. Restricted hetero-oligomeric connexon assembly in the presence of the N-terminal portions of the connexins. C-terminal-truncated, or C- plus N-terminal-truncated α_1 and β_1 Cx polypeptides were co-translated in the presence of microsomes (RM). Microsomes were lyzed in 1% TX-100, and the assembly of the co-translated membrane-integrated polypeptides was analyzed by immunoprecipitation (IP) with Cx-specific antibodies (α_1J and β_1J) directed against the intracellular loop regions, SDS–PAGE, and autoradiography. Note that co-assembly of α_1 and β_1 connexins was only observed when the N-terminal regions were truncated (lanes 7 and 8 versus lanes 3 and 4). Deletion of large C-terminal portions had no influence on the observed assembly selectivity. Translation reactions are shown in comparison as described in the legend to Figure 2D.

and comparable molecular masses to the GJ connexons that we have analyzed.

Interestingly, Cx polypeptides with a cleavage between their first transmembrane domain (M1) and first extracellular loop domain (E1) (designated $\alpha_1\Delta N$, $\beta_1\Delta N$ and $\beta_2\Delta N$ in this report) assembled also into GJ connexons that had similar sedimentation coefficients and characteristics that were observed for GJ connexons assembled from full-length Cx subunits (Figure 3). These results suggest that the N-terminal domain and the first membrane-spanning domain (M1) of connexins may not be essential for the assembly of connexins into the GJ channel 'core' structure. A comparable result has been reported previously for K^+ channels. K^+ channel subunit mutants with various numbers of deleted C-terminal membrane-spanning domains still assembled into the tetrameric K^+ channel 'core' structure, as was determined by sedimentation analysis (Li *et al*., 1992; Shen *et al*., 1993; Babila *et al*., 1994). Alternatively, since the N-terminal-truncated Cx polypeptides were generated by proteolytic cleavage occurring concomitantly with their integration into microsomal vesicles (see Results, and Falk *et al*., 1994), and not by mutational deletion of the Cx cDNA sequence, it is possible that the cleaved N-terminal polypeptide portion remained associated with the larger C-terminal polypeptide portion, resembling a complete membrane-integrated Cx polypeptide with its first extracellular loop merely cleaved. The latter hypothesis may be supported by our finding that the connexons assembled from cleaved β_1 and β_2 connexins had similar conductances to those reported previously for connexons composed of wild-type β_1 and β_2 connexins (Buehler *et al*., 1995; Rhee *et al*., 1996). However, it has not yet been determined if the full-length connexins in this study that contain a single amino acid residue exchange in their first extracellular loop domain (E1) and an N-linked carbohydrate side chain in the case of β_1 Cx have similar or different biophysical properties.

The efficiency of connexin assembly into GJ connexons or gap junctions has not been characterized previously. However, assembly efficiencies of some ion channels have been studied previously, and it was reported to be a slow and inefficient process, when compared with the assembly of other oligomeric protein structures such as complex viral surface proteins. Only ~20–30% of the *Torpedo* and mouse AChR subunits assemble within 90 min into pentameric AChRs, and ~30% of the synthesized α subunits assemble with the appropriate β subunits into rat brain Na^+ channels within 2 h after heterologous expression of the subunits in tissue culture cells (reviewed in Green and Millar, 1995). Previous results (Musil and Goodenough, 1993) suggest that the GJ channel assembly could represent a similarly inefficient process. In that study, much more unassembled Cx subunits (5S particles) were recovered from α_1 (Cx43)-expressing tissue culture cells or cRNA-injected *Xenopus* oocytes than assembled GJ connexons (9S particles) (an ~80:20 ratio). In this study, we recovered ~17% assembled GJ connexons under comparable detergent conditions (1% TX-100; Table I), indicating that GJ connexon assembly can occur with a similar efficiency in the cell-free expression system.

Alternatively, the number of assembled GJ connexons initially could have been much larger, but completely assembled connexons could have been disrupted partially or completely by the detergent treatment used to solubilize the microsomal vesicles. This hypothesis is supported by our finding that the number of connexons recovered was critically influenced by the type and concentration of detergent used for the solubilization of the microsomal vesicles (between 0% and 32%; Table I). For example, a dependence between detergent type and recovery of assembled oligomeric protein structures was reported previously to influence the recovery of assembled tetrameric inositol triphosphate receptors and cell-free-expressed K^+ channels (Mignery et al., 1990; Shen et al., 1993). A complete or partial disruption of previously assembled GJ connexons by detergent treatment was further suggested by the distribution of Cx polypeptides over a wide range of fractions in the gradients (Figure 2B), indicating that in addition to monomeric connexins and fully assembled connexons, partially assembled channel-substates, consisting of Cx dimers, trimers, tetramers and pentamers, were also present. In addition, it would not be unexpected if partially assembled channel-substates were synthesized in the cell-free expression system if, for example, less than six Cx polypeptides were integrated into the same microsomal vesicle.

In this context it is interesting to note that complete, double-membrane intercellular junctions, composed of two connexons paired head-to-head via an interaction of the extracellular loops of the Cx subunits was not observed in the present study (see controls presented in Figure 6). Since the connexins are oriented with their extracellular loops inside the microsomes, a pairing between a connexon assembled in one microsome will not be able to pair head-on with a connexon assembled in a different microsome. Lysis of the microsomes in non-ionic detergent, such as TX-100, released the connexons from the microsomes, but simultaneously prevented their pairing into complete intercellular junctions. In addition, the full-length β_1 connexins synthesized in this study have an N-linked carbohydrate side chain added to their first extracellular loop that is predicted to interfere with the head-on pairing of two connexons assembled from such mutated connexons, as long as the carbohydrates are not enzymatically removed. However, the use of other specific detergents promises that the synthesis of complete GJ channels in the cell-free system may be possible.

The assembly of a complex protein structure consisting of several subunits requires that the individual subunits recognize each other, for instance through high-affinity interactions based on distinct structural motifs that allow a selective discrimination between the subunits. The data obtained in the hydrodynamic and immunoprecipitation analyses indicated that different Cx isotypes can assemble into homo-oligomeric and into hetero-oligomeric GJ connexons. However, assembly into hetero-oligomeric connexons was only observed when the Cx isotypes were compatible with each other. Specifically, α_1 and β_1 Cx did not associate with each other when co-translated as full-length proteins; they assembled only into separate, homo-oligomeric connexons, and not into hetero-oligomeric connexons composed of α_1 and β_1 connexins. On the other hand, when α_1 and β_1, or α_1, β_1 and β_2 connexins were co-translated as N-terminal-truncated proteins (N-terminal domain and first transmembrane domain presumably removed concomitantly with their membrane

integration) they associated with each other and assembled into hetero-oligomeric GJ connexons. Removal of large portions of the C-terminal domain did not influence the selective association and assembly behavior of α_1 and β_1 connexins. These results indicated that GJ channel subunits did not assemble in all possible combinations into hetero-oligomeric connexons, but that their interaction was restricted, allowing only the assembly into certain permitted, hetero-oligomeric connexon subtypes. Many control experiments (presented in Figure 6) demonstrated that the discriminating assembly behavior of the N-terminal-truncated Cx polypeptides represented a specific interaction of these molecules, and was restricted to membrane-integrated Cx polypeptides. While some recent papers suggest that hetero-oligomeric GJ connexons could exist (König and Zamphigi, 1995; Sosinsky, 1995; Stauffer, 1995; Jiang and Goodenough, 1996), a selective interaction between individual Cx isotypes was never reported previously. Since the full-length α_1 and β_1 connexins used in this cell-free assembly study were mutated at a single amino acid residue, although in a region that most likely does not play a direct role in polypeptide contact and pairing, it could be possible that the observed selective assembly characteristics may not reflect the 'natural' behavior of wild-type connexins. However, a selective interaction of α_1 and β_1 Cx isotypes that supports our results has also been suggested by the recent findings obtained in a parallel study analyzing the subunit composition of GJ connexons isolated from GJ plaques formed in insect cells after infection with α_1, α_3, β_1 and β_2 Cx-expressing baculoviruses (N.M.Kumar, S.Gosh, R.Safarik, E.Monosow, G.Klier, M.M.Falk and N.B.Gilula, manuscript in preparation).

The apparent lack of discrimination between different subunit isotypes observed with the N-terminal-truncated Cx polypeptides, suggested that distinct structural elements in the cleaved N-terminal portion of the Cx polypeptides may initiate a selective subunit recognition, and hence determine which types of subunits can co-assemble to form distinct hetero-oligomeric GJ channel subtypes. Such 'assembly signals', critical for mediating subunit interactions have been identified in the N-terminal domains and in the first transmembrane domain of several ion-channel subunit proteins that have been reported to consist of specific amino acid stretches or individual amino acid residues (reviewed in Green and Millar, 1995). Deletion of these amino acids, or alternatively, disruption of their authentic structure caused by the proteolytic processing, could result in the loss of similar signals in the GJ channel Cx subunits.

The many advantages of the cell-free expression system related to protein expression and modulatory processes should facilitate the rapid identification of such potential molecular signals that conduct the regulated assembly of GJ connexins.

Materials and methods

cDNA clones, deletion and N-glycosylation constructs

For the *in vitro* transcription of efficiently translated synthetic Cx RNAs, rat α_1 (Cx43), human β_1 (Cx32) and rat β_2 (Cx26) were cloned into the transcription vector pSP64T (Krieg and Melton, 1984) as described in Falk *et al.* (1994).

Construction of N-glycosylated Cx constructs will be described in more detail in a separate report (M.Falk and N.Gilula, manuscript in preparation). Briefly, N-glycosylation sites (consensus sequence NXS or NXT; Hart *et al.*, 1978) were introduced into the first extracellular loops of α_1 and β_1 Cx by an overlap extension polymerase chain reaction (PCR) as described in Falk *et al.* (1992), but using proofreading *Pfu* polymerase (Stratagene, La Jolla, CA). Sites were selected in such a way that only a single amino acid residue mutation was required to construct the entire consensus site. Glu57 was exchanged with serine in α_1 Cx and Leu56 was exchanged with serine in β_1 Cx to change the wild-type amino acid sequences into the N-glycosylation consensus sites NTS. These constructs were designated $\alpha_1$fl and $\beta_1$fl. All constructs were verified by DNA sequencing.

C-terminal-truncated Cx polypeptides were generated by linearizing the wild-type and N-glycosylated α_1 and β_1 Cx cDNAs at natural restriction sites: *Nhe*I at position 1114, removing 78, and *Ava*I at position 758, removing 51 amino acid residues from the C-terminus of α_1 and β_1 Cx, respectively. Constructs were designated $\alpha_1\Delta$N, $\alpha_1\Delta$NΔC, $\beta_1\Delta$N and $\beta_1\Delta$NΔC. To compensate for the reduced translation efficiency of the resulting run-off transcripts that were missing their stop-codons, the amounts of the corresponding synthetic RNAs were increased appropriately in the translation reactions.

A pSP64T-based plasmid pCh2934-SP encoding the chicken brain AChRα7 subunit was generously provided by R.Schöpfer (University College London, London, UK). A Bluescript-based plasmid containing the synaptic vesicle synaptophysin cDNA was generously provided by R.Scheller (Howard Hughes Medical Institute, Stanford, CA, USA). A Bluescript-based plasmid containing the mouse tight junction occludin cDNA was generously provided by S.Tsukita (Kyoto University, Kyoto, Japan). Synaptophysin and occludin cDNAs were recloned into the *in vitro* transcription vector pSP64T to increase *in vitro* translation efficiency. Rat lens membrane protein MP26 was cloned by N.M.Kumar (our unpublished results) and the cDNA was recloned into pSP64T. All molecular biology enzymes used were purchased from New England Biolabs (Beverly, MA) and Promega Biotech (Madison, WI).

In vitro transcription, translation/membrane translocation

All plasmids used for *in vitro* transcription were linearized, phenol–chloroform extracted and ethanol-precipitated before RNA synthesis. Transcription reactions were performed with a Riboprobe transcription kit (Promega Biotech, Madison, WI) using either T7 or SP6 RNA polymerase. cDNAs were degraded using RNase-free DNase I (Boehringer-Mannheim, Indianapolis, IN). The quality and amount of synthesized RNA was determined by analyzing aliquots on agarose gels. Synthetic RNAs were used as soon as prepared or they were stored in aliquots at –70°C, and then used normally without further purification to avoid degradation of full-length RNAs.

Cell-free translation reactions were performed as described previously (Falk *et al.*, 1994). Nuclease-treated rabbit reticulocyte lyzates were purchased from Promega Biotech and programmed with large amounts (typically 0.5–1 μg of RNA, as estimated from an ethidium bromide-stained agarose gel, per 25 μl reaction volume) of the appropriate cRNAs, and translated in the presence of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL). Main aliquots of the translation reactions were supplemented co-translationally with canine pancreas rough microsomes, which were either obtained from Promega Biotech or prepared as described by Walter and Blobel (1983). Typical concentrations were 0.5 Eq/10 μl reaction volume (Walter and Blobel, 1983). Oxidizing conditions were maintained in our translation reactions by the addition of oxidized glutathione, described to promote appropriate folding of membrane proteins (Marquardt *et al.*, 1993; Qu and Green, 1995). Oxidized glutathione [3 mM final concentration (GSSG; Sigma, St Louis, MO)] was added co-translationally to the reactions to compensate for small amounts of DTT present in the reticulocyte lyzate, microsomal membranes and [^{35}S]methionine (Marquardt *et al.*, 1993). Translation reactions were incubated at 30°C for 30–60 min, and additional GSSG was added to a final concentration of 5 mM. Reactions were generally incubated additionally for 1–2 h at 30°C to allow complete post-translational folding and association of the newly synthesized polypeptides.

To separate non-membrane-integrated polypeptides that were synthesized on free ribosomes in the *in vitro* translation reactions, microsomes were pelleted through a 0.5 M sucrose cushion made in 1×PBS, using an Airfuge ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA), as described in Falk *et al.* (1994). To remove N-linked carbohydrates from the N-glycosylated Cx constructs, 10 μl aliquots of the corresponding translocation reactions were incubated with 1 unit endoglycosidase H

(Boehringer-Mannheim) for 2 h at room temperature before immunoprecipitation.

Hydrodynamic analysis

The oligomerization of newly synthesized connexins was analyzed by velocity sedimentation on sucrose gradients. Microsomes, corresponding to a 50 µl translation reaction, containing *in vitro*-synthesized [^{35}S] methionine-labeled connexins were purified from the translation reactions by pelleting the vesicles through 0.5 M sucrose cushions as described above, and then solubilized in 1% TX-100, 2% OG, 0.5% DMP or 1% ZW. 3-12, for 30 min at 4°C. Microsomes were washed twice in 0.25 M sucrose, 1×PBS, before solubilization in detergent to remove unincorporated label, and the detergent-insoluble material was precipitated by high-speed centrifugation (15 min, 30 p.s.i; Airfuge). Supernatants were loaded on top of 5 ml linear 5–20% (w/v) sucrose gradients containing 150 mM NaCl, 50 mM Tris, pH 7.6, and the corresponding detergent used for solubilization. After centrifugation (16 h at 43 000 r.p.m., 4°C, SW55Ti rotor; Beckman Instruments Inc.), gradients were fractionated by puncturing the bottom of the tube with a 26-gauge needle, and ~0.25 ml fractions were collected. 25 µl aliquots of the fractions were analyzed by liquid scintillation counting and SDS–PAGE. In general, between 1000 and 10 000 c.p.m. were obtained per peak fraction. c.p.m. recorded in each fraction were corrected by the background activity (fraction with the lowest c.p.m.), and were plotted in percent activity per fraction. Aliquots of all fractions were also analyzed by immunoprecipitation using Cx-specific antibodies. Control aliquots of the purified microsomes containing cell-free-expressed connexins were solubilized in 0.1% SDS, and analyzed in parallel as described above, except that the gradients were prepared with 0.1% SDS, and the gradients were run at 20°C. The refractive index of each fraction was measured using a refractometer, and it was converted without further correction into the corresponding sucrose concentrations using a standard conversion table. Standard proteins with known sedimentation coefficients (myoglobin, 2S; ovalbumin, 3.5S; BSA, 4.3S; catalase, 11.5S) and GJ connexons consisting of α_1, β_1 or β_2 Cx expressed and purified from baculovirus-infected insect cells (Stauffer *et al.*, 1991; N.M.Kumar, S.Gosh, R.Safarik, E.Monosow, G.Klier, M.M.Falk and N.B.Gilula, manuscript in preparation), were analyzed on separate gradients to compare the Cx-specific S-values with corresponding sucrose concentrations.

Reconstitution and single channel recordings in planar lipid bilayers

Single channel recordings from planar lipid bilayers were obtained as described in Buehler *et al.* (1995). Membranes were formed from soybean lipid type II-S (Sigma Chemical Co., St Louis, MO) or from a 4:1 (wt/wt) mixture of synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) (Avanti Polar Lipids, Alabaster, AL) on the tip of a patch pipette (Suarez-Isla *et al.*, 1983).

Microsomes corresponding to ~50 µl translation reactions were purified from the reactions programmed with wild-type α_1, β_1 or β_2 Cx cRNAs by pelleting them through 0.5 M sucrose cushions as described above. Microsomes were washed in 0.2 M KCl buffer and resuspended in 20–50 µl of the same buffer in Airfuge tubes by gentle stirring using a micro stir-bar (20 min at 4°C). Resuspended microsomes were stored on ice until used. Microsomes were fused to bilayers by adding 5–10 µl of solution to the bilayer subphase (150 µl bath solution containing 200 mM KCl, 10 mM HEPES, pH 7.5) in the presence of 20 mM $MgCl_2$ or 5 mM $CaCl_2$ and a sucrose gradient (100 mM sucrose in subphase, no sucrose in pipette). Washing the microsomes proved necessary since adding as little as 2 µl of translation reaction mixture (reticulocyte lyzate) to the bilayer subphase routinely disrupted preformed membranes or induced baseline instabilities resulting in short single channel recordings of $<$1 min. Forming membranes in the presence of such trace amounts of translation mixtures inhibited the formation of bilayers altogether. To improve fusion rates, microsomes were washed once and resuspended in 10 µl of buffer. The tip of the pipette was then filled with KCl buffer, and a few microlitres of microsome solution was carefully added on top of the KCl buffer to create a pipette solution with a high concentration of microsomes close to the preformed membrane. The layered addition of microsomes inside the pipette did not interfere with subsequent bilayer formation on the pipette tip. The microsome solution was diluted up to 10-fold to decrease the rate of membrane disruption. Control microsomes were not incubated with a translation mixture. This resulted in a lower fusion rate (Table I), but in more stable membranes, allowing longer single channel recordings of endogenous channels for up to 10 min.

Single or multi-level currents were amplified with an EPC/7 patch–clamp amplifier (List Medical, Darmstadt, Germany) and stored on tape (DAT Sony two-channel recorder; Unitrade Co., Philadelphia, PA). Channel recordings were analyzed using pClamp acquisition and analysis software (Axon Instruments, Foster City, CA) on a Gateway2000 486/DX50 computer. Sampling rate was 0.1 ms at 2 kHz (corner frequency, 8-pole Bessel filter, Frequency Devices, Haverhill, MA). Mean conductance values for single channels were obtained from Gaussian fits of all points amplitude histograms, or channel transitions were analyzed visually allowing for a few ms plateaus on either side of the transition for an event to be included.

Antibodies and immunoprecipitations

Connexin-specific monoclonal and anti-peptide antibodies directed against C-terminal and intracellular loop regions of α_1, β_1 and β_2 Cx that displayed no detectable cross-reactivity with other Cx isotypes were used for the immunoprecipitation of Cx polypeptides from *in vitro* translation reactions. Murine antipeptide monoclonal antibodies Mα_1S09, directed against the C-terminal residues 366–381 of rat α_1 (Cx43); Mβ_1SD4, directed against the C-terminal residues 262–280 of human β_1 (Cx32); and Mβ_2JD5, directed against the cytoplasmic loop residues 112–125 of rat β_2 (Cx26), were prepared and purified from mouse ascites by saturated ammonium sulfate precipitation and dialysis against PBS. A mouse hybridoma cell line producing a β_1 (Cx32)-specific monoclonal antibody M12.13 (Goodenough *et al.*, 1988) that binds to a peptide corresponding to the cytoplasmic loop residues 111–125 of β_1 Cx was generously provided by D.A.Goodenough (Harvard Medical School, Boston, MA). An α_1 anti-peptide antibody, directed against the cytoplasmic loop residues 131–142 of rat α_1 Cx, was generated previously, and designated α_1J.

Antibodies were either used in combination with protein A–Sepharose beads (Sigma), or covalently bound to protein G–Sepharose beads (Pharmacia, Piscataway, NJ). 1 ml of protein G bead slurry was washed and incubated in 10 mM sodium phosphate buffer, pH 7.3, with 15 mg of monoclonal antibodies for 1 h at room temperature with continuous rocking. Antibody–bead complexes were washed twice in 0.1 M sodium borate, pH 9.0, and bound antibodies were covalently crosslinked to the protein A with 20 mM dimethyl pimelimidate.2HCl (DMP, Pierce, IL), in 0.1 M sodium borate, pH 9.0 for 30 min at room temperature with continuous rocking. The reaction was quenched by washing twice with 0.2 M ethanolamine, pH 8.0, for 1 h. Antibody–bead complexes were resuspended in 1×PBS, 0.01% Thimerosal and stored at 4°C until used.

Oligomerization of Cx polypeptides into homo- and hetero-oligomeric complexes was analyzed by immunoprecipitation. Connexins were co-translated in different combinations together with other Cx isotypes, Cx mutants or non-connexin transmembrane proteins. Cx polypeptides were immunoprecipitated either from complete translocation reactions or from microsomes that were pelleted through 0.5 M sucrose cushions as described above, followed by their resuspension in 1×PBS, 0.25 M sucrose. Microsomes were solubilized for 30 min on ice in immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris, pH 7.6, 1% TX-100, that lyzed the microsomal vesicles without disrupting the oligomeric Cx complexes. Insoluble material was precipitated by high-speed centrifugation. Aliquots of the supernatant corresponding to ~10 µl translocation reaction, a Cx-specific antibody against α_1, β_1 or β_2 Cx, and preswollen protein A–Sepharose (where required) were incubated together in 1 ml of immunoprecipitation buffer for 2 h at room temperature, or at 4°C overnight with shaking. Beads were sedimented by centrifugation and washed twice with immunoprecipitation buffer before the addition of SDS protein sample buffer. Precipitated antigens and associated polypeptides were detected by SDS–PAGE and autoradiography. As controls, aliquots of the translocation reactions or resuspended microsomes were solubilized in immunoprecipitation buffer containing 0.1% SDS or no detergent.

Gel electrophoresis and autoradiography

Native PAGE. Selected fractions of sucrose gradients used to separate 9S and 5S particles of wild-type β_1 Cx were analyzed on 1.5-mm thick 4% polyacrylamide gels in the absence of SDS, following the method described by McKay *et al.* (1996), except that the gel was prepared without a stacking gel, and the gel-matrix was prepared with the addition of 2% OG. 15–40 µl of the gradient-fractions were mixed with glycerine and bromophenol blue, and then loaded onto the gels. Novex MultiMark pre-stained Multi-colored Protein Standard, 4–250 kDa range, was

analyzed in parallel to control separation of the proteins during electrophoresis. Gels were enhanced, dried and exposed as described below.

SDS–PAGE. SDS–PAGE was performed on Bio-Rad mini-gels using 10 and 12.5% Laemmli gels (acrylamide:bisacrylamide ratio, 29:1). Samples were solubilized in SDS sample buffer containing 3% SDS, 5% β-mercaptoethanol, and analyzed without heating to prevent aggregation of Cx polypeptides. Following electrophoresis, gels were soaked for 10 min in 1 M sodium salicylate (Sigma) to enhance ^{35}S autoradiography, dried, and exposed to Kodak X-AR film at –70°C. All autoradiographs shown were directly reproduced from the original films, and were not processed with computer software.

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