Cell-Free Synthesis for Analyzing the Membrane Integration, Oligomerization, and Assembly Characteristics of Gap Junction Connexins

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For gap junction channels to function, their subunit proteins, referred to as connexins, have to be synthesized and inserted into the cell membrane in their native configuration. Like other transmembrane proteins, connexins are synthesized and inserted cotranslationally into the endoplasmic reticulum membrane. Membrane insertion is followed by their assembly and transport to the plasma membrane. Finally, the end-to-end pairing of two half-channels, referred to as connexons, each provided by one of two neighboring cells, and clustering of the channels into larger plaques complete the gap junction channel formation. Gap junction channel formation is further complicated by the potential assembly of homo- as well as heterooligomeric connexons, and the pairing of identical or different connexons into homo- and heterotypic gap junction channels. In this article, I describe the cell-free synthesis approach that we have used to study the biosynthesis of connexins and gap junction channels. Special emphasis is placed on the synthesis of full-length, membrane-integrated connexins, assembly into gap junction connexons, homo- as well as heterooligomerization, and characterization of connexin-specific assembly signals. © 2000 Academic Press

Gap junctions are biological channels that function in the plasma membrane of neighboring cells to provide direct cell-to-cell communication. However, before gap junction channels can function, their subunit proteins, referred to as connexins, have to be synthesized and inserted into the cell membrane in their functional configuration, and the subunit proteins have to assemble into the intact channel structure. Synthesis, intracellular trafficking, and assembly are important steps in the maturation process of gap junction channels that have to be coordinated and regulated precisely for gap junction channels to function properly.

To study the biosynthesis and assembly properties of gap junction subunit proteins, we have used cell-free protein synthesis in translation-competent cell lysates supplemented with translocation-competent microsomal membranes, and combined them with biochemical, biophysical, and immunological techniques (1–5).

Expressing a protein in a cell-free system appears attractive since protein biosynthesis can be studied independently of the complex mechanisms occurring in a complete cell. In addition, the system is readily accessible to scientific manipulations. Other advantages of this method include speed, relative ease in interpreting results since only synthetic RNA species are present and will be translated, simple detection of the synthesized radiolabeled proteins on sodium dodecyl sulfate (SDS)–polyacrylamide gels, no detectable endogenous protease activity, and a wide range of co- and posttranslational protein modifications since the system is derived from eukaryotic cells. The system, however, is not suitable for the production of large quantities of protein. Yields of protein obtained with this method will be only in the picomole range. In addition, the standard cell-free translation system will not allow the study of processes occurring in intracellular compartments downstream of the endoplasmic reticulum (ER), although a modification of the method has been described in which the microsomal vesicles are replaced by detergent-permeabilized whole cells (6). Transport through the Golgi apparatus, as well as insertion into the plasma membrane, was observed in this modified system.

The cell-free translation system has been found in many cases to accurately reproduce the steps involved in translation, translocation, and co- and posttranslational protein modifications that occur *in vivo* and, therefore,

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has become a standard assay system to study protein membrane-translocation processes. Since secretory as well as transmembrane proteins were found to use the same translocation machinery in the ER membrane (7–9), the lysate system proved suitable to study both types of proteins.

Early experiments suggested that the oligomerization of membrane proteins would not occur in cell-free translation/membrane integration systems due to the low probability of multiple polypeptide insertions into a microsomal vesicle (10). Micrographs of thin-sectioned microsomes, however, showed that each microsomal vesicle has many ribosomes bound to its membrane surface, indicating that each vesicle has multiple protein insertion sites and such would potentially allow the integration of several polypeptides into the same vesicle. 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) (11), assembly of a human HLA-DR histocompatibility molecule (an $\alpha/\beta/\gamma$ heterotrimer) (12, 13), and assembly of the asialoglycoprotein receptor (an α/β heterooligomer) (14, 15) were reported to occur during cell-free expression in microsomes. These observations, combined with the expression of functional gap junction connexons [reported in Ref. (2)], demonstrated that the assembly of functional membrane structures consisting of several subunit proteins can take place in microsomes and that the cell-free translation system is suitable for studying protein oligomerization processes.

Expressing different connexin isotypes in cell-free translation systems proved very useful in studying their membrane integration and assembly into homo- and heterooligomeric connexons, and in characterizing signals within the connexin polypeptides that regulate their assembly.

In the following sections, I describe the most important cell-free expression and membrane integration techniques that have been used to study the membrane integration, oligomerization, and assembly characteristics of gap junction connexins. Another compilation of such methods, with an additional emphasis on the analysis of the membrane integration and transmembrane orientation of a membrane protein, has been described recently (16).

DESCRIPTION OF METHODS

1. Connexin Protein Synthesis and Membrane Integration

Cell-free translation systems consist of a translation-competent cell lysate, containing ribosomes, precursor tRNAs, an energy-generating system, factors involved in the translation process, a mixture of cold amino acids except in general methionine, and radioactively labeled methionine for protein detection. Upon addition of a synthetic RNA that encodes a protein the RNA will be translated into protein. If the RNA encodes a secretory or transmembrane protein and the lysate is supplemented with microsomes (see Section 1.2), the protein will cotranslationally translocate into the microsomes. The lysate can then be electrophoresed on an SDS–polyacrylamide gel and the translated proteins can be visualized by autoradiography using X-ray film or a phosphor imager system.

1.1. In Vitro Transcription

Before a desired protein can be translated a synthetic RNA transcript has to be synthesized. Several vectors are commercially available that have an SP6, T7, or T3 bacteriophage promoter cloned upstream of a multiple cloning site suitable for inserting the cDNA encoding the desired protein (compare Fig. 1). These bacteriophage promoters are highly specific for their RNA polymerases. Therefore, even cDNAs encoding proteins that are toxic for *Escherichia coli* can be cloned and amplified in these vectors. All three bacteriophage RNA polymerases are commercially available in very good qualities (also as complete transcription kits), allowing efficient synthesis of transcripts up to 5 to 10 kb long (17–19). Synthetic RNAs can be synthesized as capped as well as uncapped RNAs. While capping is required for transfection of mammalian cells (20), uncapped RNAs will also be translated efficiently in the lysate system. A somewhat higher translation efficiency of capped RNAs is in general compensated by the more efficient synthesis of uncapped RNAs. Poly(A) tails are also not required for efficient translation in lysate systems.

Materials and Equipment

Micropipets and 0.5- and 1.5-ml microcentrifuge tubes: tips and tubes must be autoclaved.

Reagents required for molecular biology, including connexin cDNAs and in vitro transcription vectors:

Adjustable heating block or bath.
Transcription kit containing $5\times$ transcription buffer, 0.1 M dithiothreitol (DTT), rNTPs, acetylated bovine serum albumin (BSA), and control DNA (Promega, Madison, WI).
Sterile, high-quality deionized water.
RNase inhibitor such as RNasin (Promega).
SP6, T3, and/or T7 RNA polymerase (Promega).
Agarose gel apparatus.
DNA-grade agarose for agarose gel electrophoresis.
$1\times$ TAE buffer: 40 mM Tris (pH 8.3), 20 mM acetic acid, 1 mM EDTA, prepared from $50\times$ stock solution.
Ethidium bromide.

Procedure

1. Although the vectors contain a transcription termination signal downstream of the cloning site, linear-

ization of the plasmid downstream of the inserted cDNA is desirable and substantially increases the yield of RNA copies. Standard procedures for synthetic RNA synthesis are supplied in Promega Biotech's Technical Manual *Transcription in Vitro Systems.*

2. A typical transcription reaction used for the synthesis of connexin cRNAs (1–3) was as follows:

10 μl 5X transcription buffer (Promega)

5 μl 0.1 M DTT (Promega)

10 μl 2.5 mM rNTPs (Promega)

1 μl 1 mg/ml acetylated BSA

1 U/μl transcription volume RNasin (Promega)

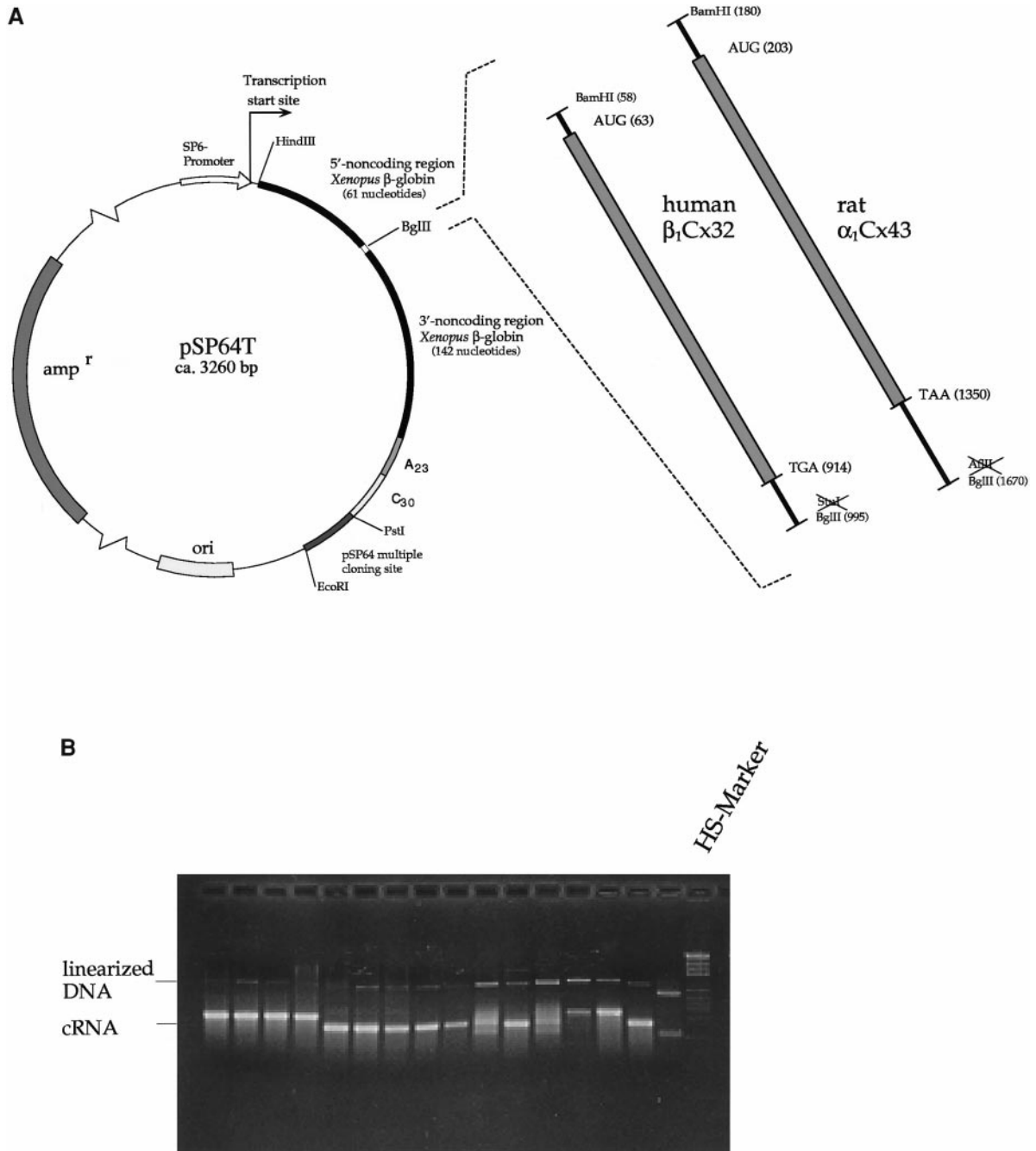0.3 U/μl transcription volume SP6-Polymerase (Promega)

FIG. 1. Cloning and synthesis of synthetic connexin RNAs. (A) Connexin-specific cDNAs were cloned into the transcription vector pSP64T. The resulting SP6 polymerase-driven RNA transcripts encode the 5′-noncoding region of globin in front of the connexin coding sequence. This was found to dramatically increase the translation efficiency of connexin polypeptides in translation-competent cell lysates. (B) Synthetic connexin transcripts derived from linearized plasmids described in (A), analyzed on a standard agarose gel, and visualized by ethidium bromide staining.

0.1–1 μg linearized plasmid DNA
water to 50 μl

Mix at room temperature and incubate at least 1 h at 40°C.

3. If the synthetic RNA is to be synthesized with a 5′ CAP structure, only the concentration of rGTP is decreased fivefold, and 1 μl 1 mM CAP analog [m^{7}G(5′)ppp(5′)G] (Pharmacia) is added to the transcription protocol given above.

4. Synthesized RNAs can be used without further purification in following translation reactions. However, if additional background bands appear on the gels, phenol/chloroform purification, followed by ethanol precipitation (21), is recommended.

5. Efficiency of RNA synthesis and quality of the RNA transcripts can be checked by electrophoresing a small aliquot (1–2 μl) on a freshly prepared standard agarose gel prepared with 1× TAE buffer. To recognize the newly synthesized RNA on the gel and to avoid confusion with the linearized DNA that was used as a template and is also present in the transcription reaction, a lane containing linearized plasmid alone should be electrophoresed as a control in parallel as well.

6. RNA can be visualized by standard ethidium bromide staining (21) (Fig. 1B).

7. Transcription reactions were divided into 10-μl aliquots and stored at −70°C until used. Shorter RNA transcripts (1–2 kb long) can be frozen and thawed several times without significant degradation. Longer transcripts (5–10 kb) are best used fresh to avoid degradation by the freeze/thaw cycle.

Tips

Bacteriophage RNA polymerases are active for several hours and yield of RNA transcripts increases with longer incubation times.

If several different transcripts will be synthesized, it is desirable to mix all components except the individual cDNAs to reduce pipetting steps, then aliquot the mix, and finally add cDNAs.

Great care has to be taken to avoid any possible contamination with RNases. Only autoclaved materials should be used, and tubes and pipet tips should not be touched with bare hands. All electrophoresis equipment has to be cleaned thoroughly immediately before use, and buffers should be prepared fresh to reduce cRNA degradation.

1.2. In Vitro Translation/Membrane Translocation

Two translation-competent cell lysates are commercially available. Both lysates are prepared from cells highly active in protein biosynthesis. Reticulocyte lysate is prepared from the red blood cells of rabbits that have been injected with phenylhydrazine to destroy their mature red blood cells, and wheat germ extract is prepared from sprouting wheat seeds. Both extracts are depleted of endogenous RNAs and produce only minimal amounts of endogenous proteins. However, it is always advisable to run a control translation reaction without adding any synthetic RNA with each new batch of lysate. Translation in wheat germ lysates is sensitive to the concentrations of potassium and magnesium ions and their concentrations have to be optimized for each RNA. For most RNAs optimal potassium ion concentrations range from 120 to 160 mM, and optimal magnesium ion concentrations range from 1.5 to 4 mM. Although both lysates can be prepared in the laboratory (22, 23), efficient connexin translation results have been obtained with Promega Biotech's nuclease-treated Rabbit Reticulocyte Lysate System. Protocols for standard translation reactions are provided in the Promega Biotech Technical Manuals *Rabbit Reticulocyte Lysate System* and *Wheat Germ Extract.* A standard protocol for connexin translation is given below.

Secretory as well as transmembrane proteins in general are cotranslationally translocated into the membranes of the ER. Several protocols have been developed to prepare ER membrane-derived vesicles (microsomes) from cells highly active in protein secretion. The microsomes are patches of rough ER membranes that vesiculate on their isolation. The microsomal preparation contains all the components required for cotranslational protein translocation, including signal recognition particle (SRP), ribosomes, and energy-supplying molecules. Furthermore, the microsomes have signal peptidase (24) and core glycosylation activity (25). N-Terminal signal peptides will be cleaved from secretory and certain transmembrane proteins, and certain asparagine residues located in the lumen of the microsomes can be glycosylated [compare Fig. 3 in Ref. (16)]. A translation reaction in the presence of microsomes should always be accompanied by a parallel translation reaction in the absence of microsomes to indicate the electrophoretic mobility of unmodified, full-length polypeptides.

Most common, and the source of commercially available microsomes (e.g., Promega Biotech), are microsomes prepared from the acinar cells of canine pancreas. This organ is low in RNase concentration and soft in texture, and the secretory acinar cells are very rich in rough ER membranes. The microsomes are depleted of endogenous RNAs and give only little background bands. However, it is always advisable to run a control translocation reaction without adding any synthetic RNA with each new batch of microsomes.

When purchased, microsomes are relatively costly. I have prepared microsomes from fresh canine pancreas following the protocol of Walter and Blobel (25) with great success [compare Fig. 2 in Ref. (16)]. The prepared microsomes had at least similar translocation

efficiency and low backgrounds comparable to those of purchased microsomes, and were very efficient in connexin protein integration. Inside-out microsomes were not detected in the preparations. Microsomes are quite stable when stored at −70°C (2–3 years). However, they should not be thawed and refrozen more than once or twice. Therefore, when first used, aliquoting them out into smaller samples is highly recommended.

Materials and Equipment

Reticulocyte lysate or wheat germ extract, including amino acid mixture minus methionine or cysteine, and control RNA (Promega).

Canine pancreatic rough microsomes (Promega).

Isotope: This example uses high quality [^{35}S]methionine (SJ1515, Amersham).

SDS–PAGE apparatus such as Bio-Rad (Hercules, CA) Mini-PROTEAN II.

10 and 12.5% standard SDS Laemmli acrylamide gels. *Caution:* Acrylamide is a cumulative neurotoxin. It is recommended that gloves be worn whenever it is handled.

Protein sample buffer containing 3% SDS, 0.5% 2-mercaptoethanol.

Mixture of prestained SDS gel marker proteins (Bio-Rad).

1 M sodium salicylate (Sigma, St. Louis, MO).

Equipment to dry SDS–polyacrylamide gels.

X-ray film, X-ray cassettes including intensifying screens.

Densitometer.

Procedure

1. Standard connexin translation/translocation reactions were 10–25 μl in volume and mixed as follows:

    25 μl reticulocyte lysate (Promega)
    2.5 μl amino acid mixture minus methionine
    2.5 μl [^{35}S]Methionine (15 μCi/μl)
    0.5–2 μg synthetic RNA
    water to 47.5 μl

2. Divide into two aliquots of 25 and 22.5 μl.

3. Add 2.5 μl [corresponding to 1 Eq/10 μl reaction volume; see Ref. (25) for definitions] microsomes to the smaller sample.

4. Incubate at 30–37°C for 30–60 min.

5. Connexin translation products were analyzed on 10 and 12.5% SDS Laemmli Bio-Rad minigels (acrylamide:bisacrylamide ratio, 29:1). Samples were solubilized in SDS sample buffer containing 3% SDS, 5% 2-mercaptoethanol and analyzed without heating to prevent nonspecific aggregation of connexin polypeptides, a phenomenon often observed with polytopic membrane proteins (Fig. 2A).

6. Following electrophoresis, gels were soaked for 10 min in 1 M sodium salicylate (Sigma) to enhance ^{35}S

and ^{3}H autoradiography, dried, and exposed to Kodak X-AR film at −70°C using an intensifying screen.

Tips

To reduce background, a highly purified methionine, such as SJ1515 (Amersham Biotech), should be used in the translation reactions. Translabel (ICN) was found to produce additional bands, and therefore, I do not recommend using it for this application.

To avoid nonspecific aggregation of connexin polypeptides that failed to integrate into microsomes during translation and are detected on top of the separating gel or in the stacking gel, shorter incubation times of only 30–45 min are recommended (Fig. 2B).

A few years ago combined transcription/translation systems were introduced that synthesize protein from an added cDNA in one step. These systems (e.g., TNT lysate from Promega) were designed primarily to test if a cloned cDNA or open reading frame produced a protein with the expected molecular weight. Such systems are probably better not used for the integration of connexins into microsomes since the combination of different reaction components into one mixture may complicate the interpretation of results and may generate less clean translation reactions.

1.3. Increasing Translation Efficiency

A problem sometimes encountered with soluble as well as membrane proteins is that the synthetic RNA
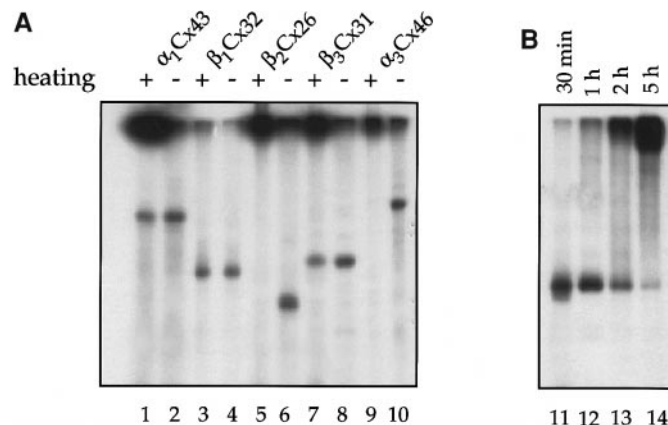

FIG. 2. Analysis of connexin polypeptides translated in reticulocyte lysates in the absence of microsomal membranes by SDS–PAGE analysis and autoradiography. (A) After translation was completed SDS–PAGE sample buffer was added to the translation reactions. Aliquots either were heated to 80°C for 3 min (+) and analyzed or were analyzed without heating (−). Connexin polypeptides aggregate while the samples are heated in sample buffer and shift their electrophoretic mobility to the top of the separating gel. Note the complete loss of monomeric β_2Cx26 and α_3Cx46. (B) Connexin translation reactions were incubated for 30 min, 1 h, 2 h, and 5 h at 37°C. SDS–PAGE sample buffer was added to the reactions and aliquots were analyzed by SDS–PAGE. Note the unspecific aggregation of non-membrane-integrated connexin polypeptides when translation reactions were incubated for extended times.

that encodes the desired protein is not translated efficiently in translation-competent cell lysates. This was also found for connexin polypeptides, and may be related to the length of the 5′-untranslated region and the structure of the translation initiation sequence adjacent to the translation start-AUG (26). In some instances optimization of the translation initiation sequence by site-directed mutagenesis can improve translation efficiency (M. Falk, unpublished data). However, connexin translation efficiency was not substantially increased by this approach. Another approach is based on cloning the 5′-untranslated region of an efficiently translated protein, such as globin, upstream of the cloned cDNA. This approach was chosen for the efficient expression of connexins in lysates (Fig. 1A). Cloning the connexin cDNAs into the transcription vector pSP64T (27) that encodes the 5′-noncoding region of *Xenopus* β-globin (28) immediately downstream of an SP6 promoter was found to dramatically increase the translation efficiency of the connexins as well as other cDNAs (1–3, 29). An advanced version of pSP64T, pSPUTK, is commercially available from Stratagene. Another approach to enhance translation efficiency is based on using the CAP-independent translation initiation sequences (IRES, internal ribosomal entry site elements; or CITE, CAP-independent translation enhancer) from picornaviruses [see Ref. (30) for review]. Several vector constructs using these sequences, such as Novagen's pCITE-1, are commercially available. In general it is advisable to ensure that no AUG codon in any of the three possible reading frames is encoded in the 5′-untranslated region upstream of the start-AUG to prevent ribosomes scanning along the cDNA from initiating at inappropriate, upstream AUG codons.

1.4. Synthesis of Full-Length, Membrane-Integrated Connexins in Cell-Free Translation Systems

We found that the translation of connexin polypeptides in standard cell-free translation systems supplemented with ER-derived microsomes resulted in a complete, but inappropriate proteolytic processing that affected all connexin polypeptides on their membrane integration (see Fig. 3) [also reported in Refs. (1, 3)]. A careful analysis of the cleavage reaction revealed that the relatively weak hydrophobic character of the first transmembrane spanning domain, which acts as an internal signal anchor sequence in connexins (M. Falk, unpublished results), is responsible for this inappropriate cleavage. Our results indicate that the connexin signal anchor sequence is falsely recognized and positioned as a cleavable signal peptide within the ER translocon, and that this mispositioning enabled signal peptidase to access the cleavage sites (3). These observations indicate that yet uncharacterized cellular factors are involved in the membrane integration process

of connexins that are absent or inactive in standard cell-free translation systems (1, 3).

Two different methods have been found that prevent the cleavage and allow the efficient synthesis of full-length, membrane-integrated connexin polypeptides with the connexin (Cx) authentic transmembrane configuration. A detailed description of methods used to determine the membrane integration and transmembrane topology of connexins is given in Falk (16).

Single-amino-acid exchanges, introduced by site-directed mutagenesis into the first extracellular loops of α_1Cx43, β_1Cx32, and β_2Cx26 (glutamine-57 to serine in α_1Cx43, leucine-56 to serine in β_1Cx32, and leucine-54 to serine in β_2Cx26), completely inhibited the cleavage reaction, most likely because of a steric hindrance between oligosaccharyltransferase (OST), the enzyme that recognizes the core glycosylation sequence and transfers core glycosyl groups from dolichol onto asparagine, and signal peptidase (2, 3; M. Falk, unpublished results). The single-amino-acid mutations result in the creation of core glycosylation sites within the connexin sequences and binding of OST to the
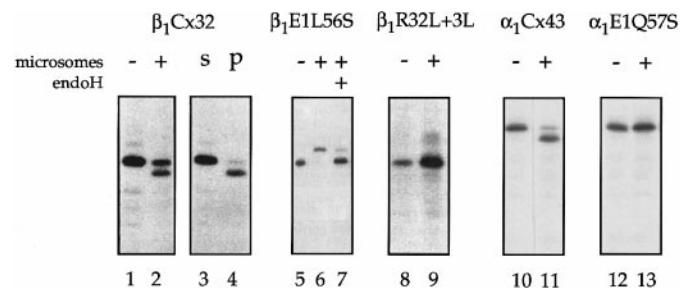

FIG. 3. Synthesis of full-length and cleaved membrane-integrated connexins. Connexin-specific cRNAs were translated in reticulocyte lysates in the presence (+) and in the absence (−) of microsomes and translation products were analyzed by SDS–PAGE and autoradiography. Translation of wild-type connexins in cell-free translation systems supplemented with pancreatic microsomes resulted in complete, aberrant processing by the ER resident protease signal peptidase that removed an N-terminal portion including the N-terminal domain, and the first transmembrane-spanning domain of connexins (lanes 2, 11). In the absence of microsomes no cleavage occurred (lanes 1, 3, 5, 8, 10, 12). The cleaved connexins pelleted (p) together with the microsomes (lane 4), while the full-length connexins stayed in the soluble lysate fraction (s) (lane 3). This result indicates that all membrane-integrated connexin polypeptides are cleaved. Full-length connexins also synthesized in the presence of microsomes (lanes 2, 11) are synthesized on non-membrane-bound ribosomes, and have failed to insert cotranslationally into the microsomal membranes. Full-length and membrane-integrated connexins were obtained when an N-glycosylation site was introduced into the first extracellular loop (L56S and Q57S amino acid exchanges, lanes 6 and 13). Note that the β_1Cx32 mutant is efficiently glycosylated while the α_1Cx43 mutant is not. Endoglycosidase H (endoH) removes the carbohydrate side chain (lane 7). The electrophoretic mobility of the deglycosylated polypeptides corresponds to the mobility of full-length connexins (lane 7). Full-length, membrane-integrated connexins were also obtained when the length and hydrophobicity of the first transmembrane-spanning domain were increased (R32L + 3L amino acid mutant, lane 9).

nascent connexin polypeptides [see Refs. (3, 16)]. However, core glycosyl groups were added only to the β_1Cx32 and β_2Cx26 polypeptides during translation, and not to the α_1Cx43 sequence (2, 3; M. Falk, unpublished results) (Fig. 3).

The second method is based on increasing the hydrophobic character of the signal anchor sequence (the first transmembrane-spanning domain) of connexins. Increasing the length of the hydrophobic core of the first transmembrane-spanning domain in β_1Cx32 from 18 amino acids (valine-23 to alanine-40) in the wild-type protein to 21 amino acids and exchanging the central arginine-32 with an uncharged amino acid (leucine) completely abolished the cleavage as well (3) (Fig. 3).

2. Synthesis of Oligomeric Connexons in Microsomes

Since gap junction channels are oligomeric protein structures, the connexin subunits have to assemble before they can function. For almost all known oligomeric membrane proteins, including voltage- and ligand-gated ion channel subunits (31), assembly in the ER is a necessary prerequisite for their further transport through the secretory pathway [reviewed in Ref. (32)]. We have observed functional assembly of GJ connexons composed of α_1Cx43 or β_1Cx32 in isolated ER membrane vesicles (microsomes) following translation in cell-free translation systems supplemented with microsomes (2).

Substantially increasing the translation efficiency of connexins in lysates (see Section 1.3) was a crucial prerequisite to achieving multiple connexin polypeptide insertions into individual microsomes and with that the successful assembly of connexins into connexons. The increased translation efficiency also allowed subsequent analysis of the assembly characteristics of connexin isotypes and determination of specific assembly signals within the connexin polypeptide sequences (2, 4, 5; M. Falk, unpublished results).

2.1. Oligomerization Assayed by Hydrodynamic Analysis

Connexons assembled in the cell-free translation system were investigated by hydrodynamic analysis using linear 5–20% sucrose gradients. This technique is used in general for assaying assembly of protein subunits into oligomeric structures and was used for connexins by others before (33–35).

Materials and Equipment

Airfuge or tabletop ultracentrifuge (both Beckman Instruments).
0.25 M sucrose in $1\times$ PBS.
Detergents such as Triton X-100 (TX-100), Nonidet P-40 (NP-40), deoxycholate (DOC), and sodium dodecyl sulfate (SDS).

Membrane lysis buffer: 150 mM NaCl, 1% NP-40, 0.5% DOC, 50 mM Tris pH 7.6.
$1\times$ PBS.
Ultracentrifuge and SW55Ti rotor, or equivalent.
Sucrose for gradients.
Linear 5–20% sucrose gradients containing 150 mM NaCl, 50 mM Tris (pH 7.6), and the respective detergent used for solubilization.
Standard proteins with known sedimentation coefficient (*S* values) such as myoglobin, 2S; ovalbumin, 3.5S; BSA, 4.3S; and catalase, 11.5S.
Refractometer.
Scintillation fluid for aqueous solutions.
Liquid scintillation counter.

Procedure

Translation and oligomeric assembly analysis conditions were as follows [also see Ref. (2)]:

1. Translation reactions were generally 50–100 μl in volume for subsequent hydrodynamic oligomerization analysis. Volumes of 10–25 μl were used for the assembly analysis of connexins by immunoprecipitation (see Section 2.2).

2. To maximize translation efficiency rabbit reticulocyte lysates were programmed with large amounts of the appropriate cRNA (typically 2–4 μg of RNA, as estimated from an ethidium bromide-stained agarose gel, per 100 μl reaction volume).

3. To maximize connexin polypeptide membrane integration small amounts of microsomes were used in the translation reactions. Typical concentrations were 5 Eq/100 μl (25) reaction volume.

4. Translation reactions were incubated at 30°C for 1 to 3 h to allow complete posttranslational folding and association of the newly synthesized polypeptides before subsequent oligomerization analysis.

5. Non-membrane-integrated connexin polypeptides that are synthesized to a certain extent in the cell-free translation reactions as a by-product on non-membrane-bound, free ribosomes (Fig. 3) [also see Ref. (16)] and unincorporated radioactive label were removed prior to gradient analysis by pelleting the microsomes through 0.25 M sucrose cushions made in $1\times$ PBS, using an Airfuge ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) (15 min, 30 psi).

6. Microsomes were then solubilized in membrane lysis buffer containing 1% Triton X-100 (or other detergents) for 30 min at 4°C.

7. Detergent-insoluble material was precipitated by a high-speed centrifugation (15 min, 30 psi) using the Airfuge ultracentrifuge.

8. 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 respective detergent used for solubilization.

9. After centrifugation (16 h at 43,000 rpm, 4°C,

SW55Ti rotor; Beckman Instruments Inc.), gradients were fractionated by puncturing the bottom of the tube with a 26-gauge needle, and approximately twenty 0.25-ml fractions were collected.

10. Aliquots of the fractions (25 μl) were analyzed by liquid scintillation counting and SDS–PAGE. In general, between 2000 and 15,000 cpm were obtained per 5S peak fraction (unassembled connexin subunits).

11. Counts per minute recorded in each fraction were corrected by the background activity (fraction with the lowest cpm) and plotted as percentage activity per fraction (Fig. 4).

12. Aliquots of all fractions were also analyzed by immunoprecipitation (see Section 2.2) using connexin-specific antibodies.

13. To verify assembly of connexins into connexons, control aliquots of the translocation reactions were solubilized in 0.1% SDS to resolve the oligomeric assemblies and were analyzed in parallel as described above, except that the gradients were prepared with 0.1% SDS, and the gradients were run at 20°C to prevent precipitation of the SDS in the gradients.

14. The refractive index of each fraction was measured using a refractometer, and it was converted into the corresponding sucrose concentrations using a standard conversion table.

15. Standard proteins with known sedimentation coefficients (myoglobin, 2S; ovalbumin, 3.5S; BSA, 4.3S; catalase, 11.5S) and gap junction connexons consisting of α_1Cx43, β_1Cx32, or β_2Cx26 expressed and purified from baculovirus-infected insect cells (36) were analyzed on parallel gradients to compare the connexin-specific S values with corresponding sucrose concentrations.

Dependent on the detergent used for the solubilization of microsomes up to 32% of the membrane-integrated connexin polypeptides were recovered as hexameric connexons (2).

Cell-free expressed connexons were found to be functional by means of channel activity. Single-channel activities were characterized by electrophysiological analysis of channels obtained after fusion of microsomes containing cell-free expressed connexins with planar lipid membranes. This method requires a special experimental setup and is not described in this article. However, the experimental conditions are described in detail in Buehler *et al.* (37) and Falk *et al.* (2).

Tips

Several researchers have used chemical crosslinking to detect connexin/connexin interactions (33, 35). However, since this technique requires a chemical alteration of the polypeptides that possibly can produce misleading results, this technique was not further developed for the assembly analysis of cell-free expressed connexins.

Direct protein/protein interactions can be visualized by native PAGE without any chemical modification of the proteins. Since the electrophoresis is performed in the absence of SDS and reducing reagents, the protein/protein interactions stay intact. Although, this technique can be very difficult, a modification of the method described by McKay *et al.* (38) that they have used to determine assembly of integrin subunits produced convincing results with cell-free expressed connexins. The method is described in Refs. (2, 38).

2.2. Oligomerization Assayed by Coimmunoprecipitation

Connexin assembly into gap junction connexons is a complicated process that has to be regulated precisely. This is especially critical in cells that express more then one connexin isotype. In the cell-free expression system connexins can assemble into homo- as well as heterooligomeric connexons. However, different connexin isotypes do not assemble randomly with any other connexin isotype, and the number of possible heterooligomeric combinations is restricted, probably by connexin polypeptide intrinsic signals (2, 4) [re-
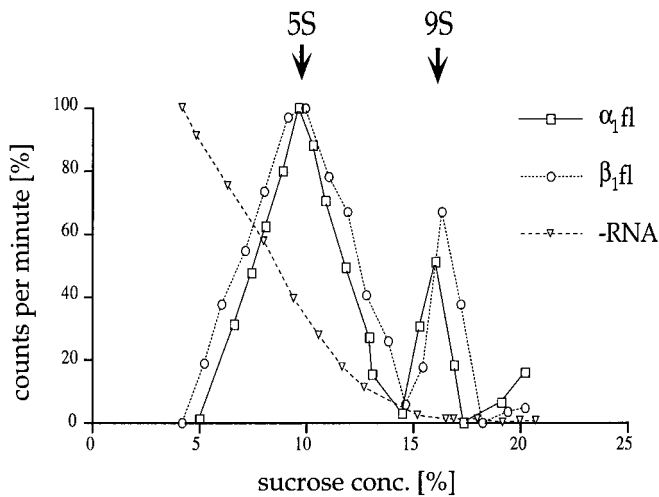

FIG. 4. Assembly of homooligomeric connexons in a cell-free translation system. Reticulocyte lysates were supplemented with microsomes and programmed with α_1Cx43 RNA and β_1Cx32 RNA, and without (−) RNA in control. After translation was completed microsomes were harvested and lysed in nonionic detergent, and assembly of connexins was analyzed by hydrodynamic analysis on linear sucrose gradients. Gradients were fractionated from the bottom, and sucrose concentration, radioactivity, and connexin protein content was determined in each fraction. Radioactivity recovered from each fraction was plotted versus the sucrose concentration after subtracting the lowest counts from each fraction. 9S particles represent assembled connexons, while 5S particles represent unassembled connexin polypeptides. More than 30% of the connexin polypeptides were recovered as assembled hexameric gap junction connexons. Reproduced, by permission of Oxford University Press from Falk *et al.* (2).

viewed in (5)]. Our oligomerization results obtained in the cell-free system are consistent with results obtained in other heterologous expression systems (36, 37, 39–42) or *in vivo* (44).

Connexin specific monoclonal and antipeptide antibodies directed against different regions of α_1Cx43, β_1Cx32, and β_2Cx26 that displayed no detectable cross-reactivity with other connexin isotypes were used for the immunoprecipitation of connexin polypeptides from *in vitro* translation reactions. Oligomerization of connexin polypeptides into homo- and heterooligomeric complexes was analyzed by immunoprecipitation following general methods described in Harlow and Lane (45) (Fig. 5). Connexins were translated in different combinations together with other connexin isotypes, connexin mutants, or non-connexin transmembrane proteins. Connexin polypeptides were immunoprecipitated either from complete translocation reactions or from microsomes that were pelleted through sucrose cushions as described in Section 2.1, followed by their resuspension in 0.25 M sucrose made in 1× PBS before immunoprecipitation buffer (see below) was added.

Materials and Equipment

Connexin specific antibodies (Zymed Laboratories Inc.).

Immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris, pH 7.6, and either no detergent, nonionic detergent such as TX-100, or ionic detergent (SDS), respectively.

Protein A–Sepharose beads (Pharmacia).

Laboratory rocker or shaker.

Procedure

1. Microsomes were solubilized for 30 min on ice in immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris, pH 7.6, 1% TX-100, which lysed the microsomal vesicles without disrupting the oligomeric connexin complexes.

2. Insoluble material was precipitated by high-speed centrifugation using the Airfuge ultracentrifuge (see Section 2.1).

3. Aliquots of the supernatant corresponding to 10–25 μl translocation reaction, a connexin-specific antibody against α_1Cx43, β_1Cx32, or β_2Cx26 (1–2 μl of a 1 mg/ml solution), and preswollen protein A–Sepharose (50 μl of a 1:10 slurry) (where required) were incubated together in 1 ml of immunoprecipitation buffer for 2 h at room temperature or at 4°C overnight with shaking.

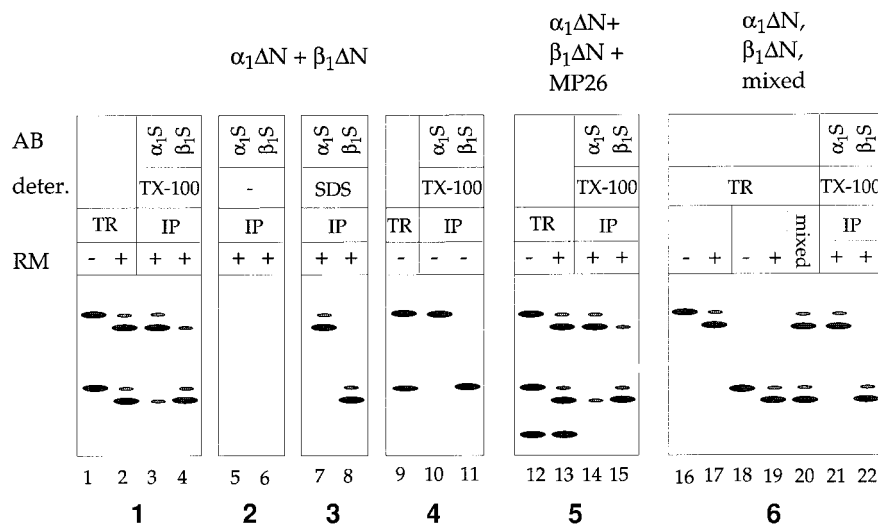4. Beads were sedimented by centrifugation and washed two times with immunoprecipitation buffer

FIG. 5. Analysis of connexin assembly conditions. Schematic representation of the protein band patterns obtained after cotranslation of different connexin isotypes, immunoprecipitation, and SDS–PAGE. N-Terminal truncated α_1Cx43 and β_1Cx32 polypeptides (wild type) were cotranslated in either the absence (−) (panel 4) or the presence (+) (panels 1–3, 5, 6) of microsomes (RM), and either not lysed in detergent (panel 2) or lysed in 1% TX-100 (panels 1, 4–6) or 0.1% SDS (panel 3). Alternatively, the connexins were translated individually in separate vials, and the reactions were mixed before lysis in 1% TX-100 (panel 6). In addition, N-terminal truncated connexins were cotranslated together with nonconnexin membrane proteins, such as MP26 (panel 5). Potential protein interactions and assembly were analyzed by immunoprecipitation (IP) using connexin-specific monoclonal antibodies (α_1S, β_1S). Non-membrane-integrated, full-length connexin polypeptides were not removed before IP, and served as an internal control to detect specific protein–protein interactions. Membrane-integrated connexin polypeptides assembled and were coprecipitated (lanes 3, 4, 14, 15). Non-membrane-integrated connexins in these reactions and in lanes 10 and 11 were not coprecipitated. Also, no coprecipitation occurred when microsomes were lysed in SDS (lanes 7, 8), or when translation reactions were mixed after translation was completed (lanes 21, 22). Nonconnexin membrane proteins were not coprecipitated (lanes 14, 15). No immunoprecipitation occurred when microsomes were not lysed in detergent (lanes 5, 6). Aliquots of the translation reactions (TRs) were analyzed in parallel, and translation products served as protein size controls.

prior to the addition of 10 μl SDS protein sample buffer.

5. Precipitated antigens and associated polypeptides were detected by SDS–PAGE and autoradiography (see Section 1.2).

As controls, aliquots of the translocation reactions or resuspended microsomes were solubilized in immunoprecipitation buffer containing 0.1% SDS or no detergent (see Section 3).

Tips

One to two micrograms of protein A–Sepharose beads (enough for approximately 10 immunoprecipitation reactions) was soaked for 1 h on ice in 1 ml immunoprecipitation buffer and then washed twice with 1 ml immunoprecipitation buffer before resuspension in 0.5 ml immunoprecipitation buffer. The fine end of a yellow micropipet tip was cut off, and 50-μl aliquots of the protein A–sepharose slurry were taken out of the stock immediately after vortexing. This procedure guaranteed an equal distribution of the protein A–Sepharose beads into the individual immunoprecipitation reactions.

Antibodies were generally used in combination with protein A–Sepharose beads (Sigma). Very clean immunoprecipitations were also obtained with connexin-specific antibodies covalently bound to protein G–Sepharose beads (Pharmacia, Piscataway, NJ). To link the antibodies to the beads 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.

3. Characterization of Assembly Signals within the Connexin Sequences

Coexpression of different connexin isotypes within a cell requires that their assembly into gap junction channels is regulated precisely by some intrinsic signals. One approach to characterize such signals is to generate deletion mutants of the proteins and cotranslate them with the full-length proteins. Interaction and assembly are then investigated by coimmunoprecipitation analysis. We have generated progressively truncated C-terminal connexin truncation mutants and have translated them together with glycosylation mutants (full-length proteins, see Section 1.4). Interaction

and assembly of the different proteins were analyzed by hydrodynamic (Section 2.1) and immunoprecipitation analyses (Section 2.2) (2, 4). A similar approach has been used to investigate the assembly of the potassium channel subunits into the tetrameric potassium channel (46–48).

Procedure

Progressively C-terminal deleted connexin polypeptides were generated by linearizing the connexin-specific cDNAs at authentic restriction sites located within the connexin sequence, using standard molecular biology approaches (21). Synthetic RNAs resulting from these cut cDNAs were translated into connexins extending from the start-methionine to the location of the restriction site.

Immunoprecipitation reactions were performed as described in Section 2.2.

For protein cotranslation, two to three different synthetic RNAs were added together to the lysate supplemented with microsomes. Since translation efficiencies of individual RNAs differ, appropriate concentrations were added to obtain approximately equal amounts of translation products.

In general, 10 μl translation reaction was sufficient per immunoprecipitation reaction.

Cotranslation reactions were always immunoprecipitated with antibodies against each of the translated proteins.

Five-microliter aliquots of each cotranslation reaction in the absence (taken from the mixture before the addition of microsomes) and in the presence of microsomes were always analyzed without immunoprecipitation for comparison of the amounts and sizes of the immunoprecipitated polypeptides with the corresponding translation products (Fig. 5, panel 1).

For better interpretation of the coimmunoprecipitation results, aliquots of the translocation reactions were also solubilized in immunoprecipitation buffer containing no detergent (Fig. 5, panel 2). No immunoprecipitation occurred under these conditions due to the relatively large size of the microsomes. Aliquots were also treated with SDS instead of TX-100. Oligomeric connexin complexes were disrupted under these conditions, resulting in no coimmunoprecipitation (Fig. 5, panel 3).

As an example, when two different connexins were cotranslated, 70 μl translation volume was required:

5 μl translation reaction control minus membranes.

5 μl translation reaction control plus membranes.

10 μl for immunoprecipitation with antibodies against connexin X in 1% TX-100.

10 μl for immunoprecipitation with antibodies against connexin Y in 1% TX-100.

10 μl for immunoprecipitation with antibodies against connexin X in 0.1% SDS.

10 μl for immunoprecipitation with antibodies against connexin Y in 0.1% SDS.

10 μl for immunoprecipitation with antibodies against connexin X without detergent.

10 μl for immunoprecipitation with antibodies against connexin Y without detergent.

As additional controls, connexins were further co-translated with nonconnexin membrane proteins. While assembly was observed between compatible connexin isotypes in these reactions, no coprecipitation of nonconnexin membrane proteins was observed (Fig. 5, panel 5).

No coassembly occurred when connexins were cotranslated in the absence of microsomes (Fig. 5, panel 4).

Finally, connexins were translated individually in separate vials, and reactions were mixed prior to lysis in 1% TX-100. No co-immunoprecipitation was observed under these conditions (Fig. 5, panel 6).

Immunoprecipitated proteins were detected by SDS–PAGE and autoradiography as described in Section 1.2 [Also compare Fig. 6 in Ref. (2).]

CONNEXIN BIOSYNTHESIS STUDIED USING CELL-FREE TRANSLATION SYSTEMS

Gap junction channel research *in vivo* is limited by many factors. Consequently, alternative systems have been used to study the structure and function of gap junction channels. Expression of gap junction connexins in different heterologous systems proved extremely beneficial for studying the structure and function of gap junction channels. Cell-free expression in translation-competent cell lysates in the presence of ER-derived microsomal vesicles, described in this report, is one such method. As with all results obtained in heterologous systems the possibility exists that results may not accurately reflect the *in vivo* situation. Therefore, it is advisable to verify an obtained result in other systems. We have compared the connexin synthesis, assembly, and oligomerization results obtained in the cell-free translation system with results obtained in other heterologous expression systems, such as transfected cells in culture or baculovirus-infected insect cells, and with the situation *in vivo* (1, 2, 35–37, 41, 49; unpublished results), and found that the cell-free translation system produced relevant results that reflect the *in vivo* situation. A substantial portion of our knowledge on the synthesis, assembly, and oligomerization characteristics of gap junction connexins described below has been obtained using this system.

Studies from several laboratories, including our own, have shown that connexins, as other transmembrane proteins, are synthesized and inserted cotranslationally into the ER membranes. Their functional transmembrane topology—with four transmembrane-spanning domains, two extracellular loops, and
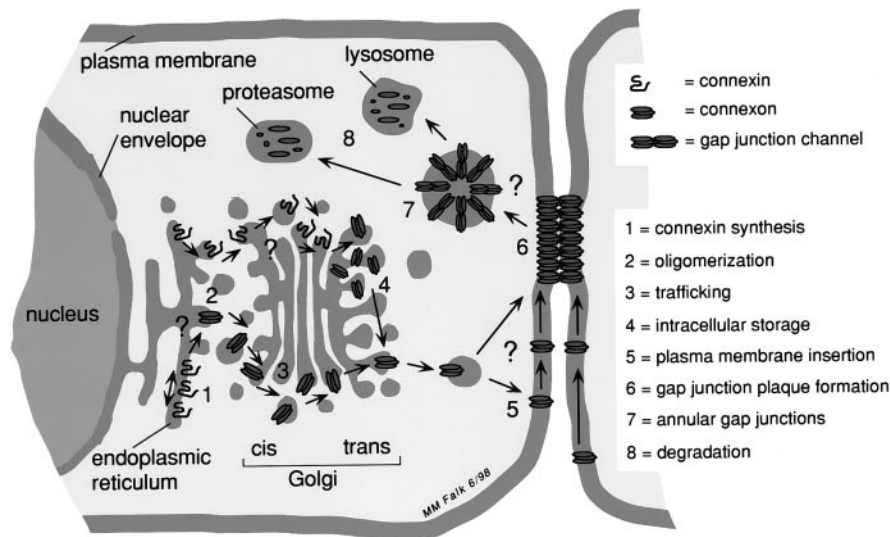

FIG. 6. Schematic representation of the synthesis, assembly, and degradation of gap junction membrane channels based on cell-free, *in vitro,* and *in vivo* analyses. Connexin synthesis at the endoplasmic reticulum membrane (1), oligomerization into gap junction connexons (hemichannels) (2), trafficking along the secretory pathway (3), intracellular storage within the Golgi apparatus (4), insertion of the gap junction connexons into the plasma membrane (5), formation of the gap junction plaque composed of many individual gap junction channels (6), proposed internalization of the plaque via formation of annular gap junctions (7), and degradation of gap junction channels via lysosomal and/or proteasomal pathways (8) are marked as steps 1 to 8. Some areas of current discussion have been highlighted with a question mark. Evidence has been obtained for oligomerization within the membranes of either the endoplasmic reticulum or the Golgi apparatus. How and where gap junction connexons are inserted into the plasma membrane and how the gap junction plaque is formed are unclear. Annular gap junctions may play a role in gap junction plaque degradation by lysosomal and/or proteasomal pathways. Rrprinted from *Curr. Opin. Struct. Biol.* **8,** M. Yeager, V. Unger, and M. M. Falk, pp. 517–524, 1998, with kind permission from Elsevier Science.
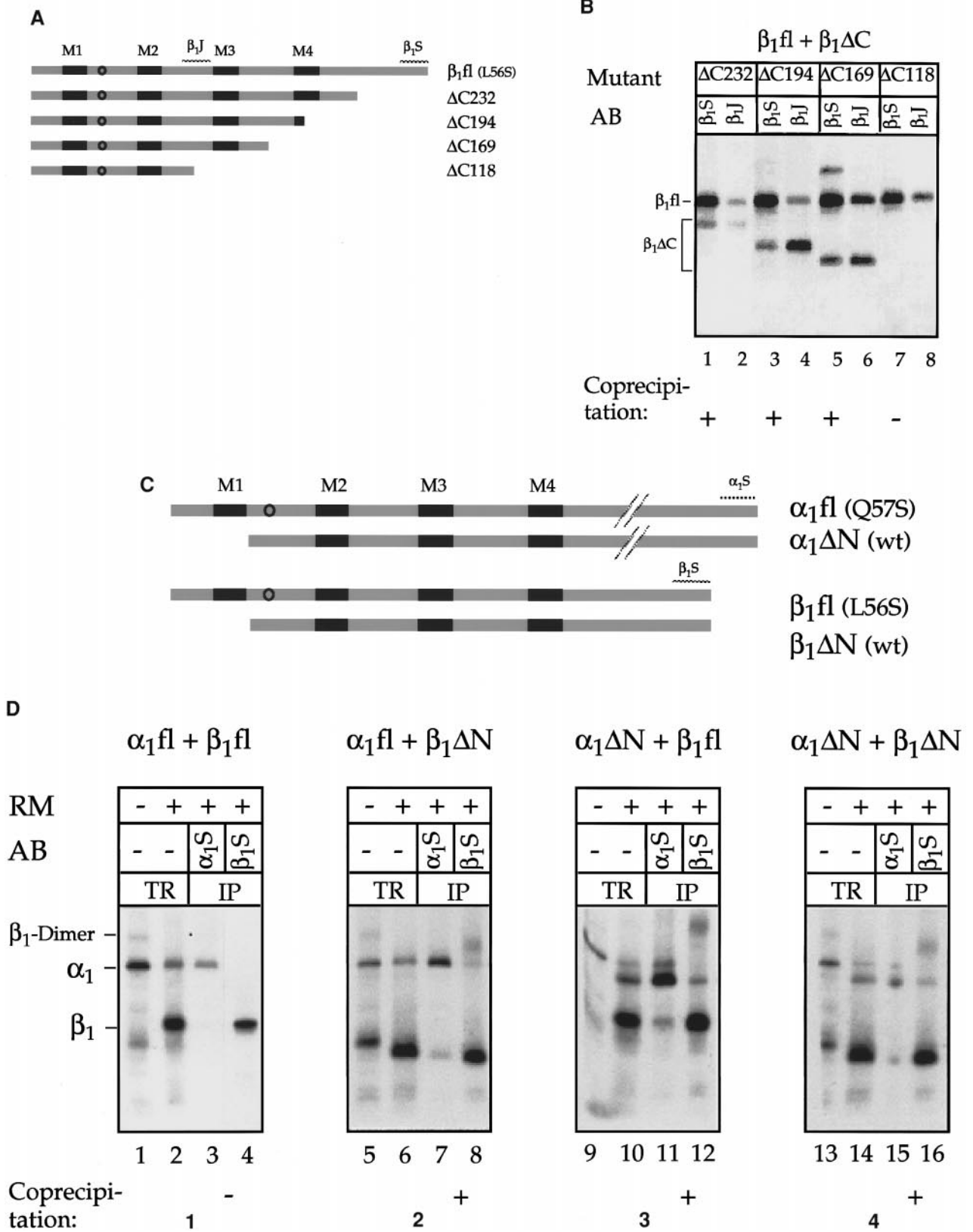
cytoplasmically located amino and carboxy termini—is achieved during their ER membrane integration (1, 3) [reviewed in (5)] (Fig. 6, step 1).

Structural and biochemical analysis performed in wild-type connexin-expressing cells (1, 50, 51), as well as tissue culture cell lines endogenously expressing connexins (52, 53) or transfected with connexin cDNA (1, 49, 54), showed connexin polypeptides in the ER membranes, the Golgi membranes, and the plasma membranes of these cells, indicating that connexins follow the general intracellular transport route referred to as the secretory pathway [originally reviewed in Ref. (55)] (Fig. 6, steps 1–5). Furthermore, no gap junction channel assembly and/or gap junction plaque formation was observed in cells that were treated with drugs known to interfere with the secretory pathway (33, 50, 53).

Since gap junction channels are oligomeric protein structures, the connexin subunits have to assemble before they can function. We and others have observed functional assembly of gap junction connexons composed of α_1Cx43 or β_1Cx32 in isolated ER membrane vesicles (microsomes) following translation in a cell-free translation system (2, 56) (Fig. 6, step 2). On the other hand, an *in vitro* study performed by Musil and Goodenough (33), in which they investigated the location of α_1Cx43 assembly expressed endogenously in tissue culture cells, however, suggested that connexins may not assemble following their synthesis in the ER membranes, but later, after their exit from the ER in late Golgi membranes (Fig. 6, steps 1–4, alternative ER–Golgi trafficking). George *et al.* (57, 58) finally suggest that oligomerization occurs in the intermediate compartment, a specialized extension of the ER through which proteins in transit from the ER to the Golgi pass. Hypothetically, it seems possible that in the absence of downstream transport components in the cell-free system, connexins oligomerize already in the ER membranes since they can not exit this compartment. Heterologous expression studies of β_1Cx32 in stable transfected baby hamster kidney (BHK) cells have shown that gap junction channels assembled into gap junction plaques can be present in the ER membranes without affecting the viability of these cells (49). Although additional experimentation may be re-

quired to determine the actual site of connexin oligomerization *in vivo,* connexin oligomerization characteristics in the cell-free system were indistinguishable from the oligomerization characteristics of connexins in cells. The gap junction connexons assembled in the cell-free translation system had properties and sedimentation coefficients (9S) identical to those reported for hexameric connexons derived from gap junctions assembled in cells (33–35).

How gap junction connexons are directed to and localized in the plasma membrane (Fig. 6, step 5) is not known. However, an interaction of α_1Cx43 with the cytoskeletal proteins ZO-1 and α-spectrin was recently described (59). A linkage between α_1Cx43 polypeptides and α-spectrin, via ZO-1, implies that distinct elements of the cytoskeleton are involved in the trafficking and plasma membrane localization of gap junction connexins.

Once assembled connexons reach the plasma membrane (60), they are believed to register and pair via tight interactions of their extracellular loop domains to form the complete, double-membrane intercellular channel that then arranges in larger clusters (Fig. 6, step 6). This process is enabled or, at least facilitated, by the action of cell-adhesion molecules, e.g. cadherins (61, 62, and references cited therein). Degradation of the structure is believed to occur via internalization of complete or partial plaques containing complete gap junction channels (Fig. 6, step 7) in a complex process that involves lysosomal and probably proteasomal pathways (61, and references cited therein) (Fig. 6, step 8).

The process of connexin assembly into gap junction connexons is a complicated process. Homooligomeric connexons composed of only one connexin isotype are believed to exist *in vivo* since many cell types express only one known connexin isotype. This assumption has been supported by structural analyses of individual gap junction plaques (63, 64) and by the assembly of gap junctions in cell culture that are structurally identical to gap junctions *in vivo* after expressing a single connexin isotype in baculovirus-infected insect cells (36, 37) or in transfected tissue culture cells (39, 43, 49). However, more than a dozen different gap junction channel subunit isotypes have been cloned and se-

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FIG. 7. Characterization of assembly signals within the connexin polypeptides. (A) Schematic representation of the C-terminal truncated β_1Cx32 polypeptides used in the assembly analysis in (B). The L56S amino acid residue exchange in the first extracellular loop domain E1 is marked with a circle. (B) Full-length β_1Cx32 was cotranslated with individual C-terminal truncated β_1Cx32 polypeptides and interaction and assembly were investigated using β_1Cx32 specific monoclonal antibodies (β_1S, β_1J). Coprecipitation of the truncated β_1Cx32 polypeptides ΔC232 to ΔC169, but not ΔC118, was observed, indicating that a carboxy-terminal portion including the third transmembrane spanning domain (M3) may be required for successful connexin subunit recognition and assembly. (C) Schematic representation of full length and N-terminal truncated α_1Cx43 and β_1Cx32 used in the assembly analysis in (D). The Q57S and L56S amino acid residue exchanges are marked with a circle. (D) Coimmunoprecipitation analysis of full-length and N-terminal truncated α_1Cx43 and β_1Cx32 polypeptides cotranslated in different combinations. N-Terminal truncated α_1Cx43 and β_1Cx32 polypeptides assembled with each other (panel 4) and with full-length α_1Cx43 and β_1Cx32 polypeptides (panels 2, 3), but not with each other (panel 1), indicating that a "selectivity" signal is located in the N-terminal portion of the connexin polypeptides. Adapted, from Falk *et al.* (2) by permission of Oxford University Press.

quenced from rodents, and many cells express more than one connexin isotype that can be localized to the same gap junction plaque (63, 65). This suggests that in addition to homooligomeric gap junction connexons, a large number of heterooligomeric gap junction connexons, composed of more than one connexin isotype, could exist as well. Evidence for the existence of heterooligomeric gap junction connexons has been obtained recently by studying connexin subunit assembly *in vitro,* in cell-free and heterologous expression systems (2, 40–42), and *in vivo,* in lens fiber cells (44). By studying connexin assembly in the cell-free system we also found that different connexin isotypes do not assemble randomly, but interact selectively, allowing the assembly of only homooligomeric and certain types of heterooligomeric connexons (2, 4) [reviewed in Ref. (5)].

The precise connexin subunit composition, stoichiometry, and organization within the connexon are likely to play a critical role in determining the properties of such heterooligomeric gap junction connexons. In an attempt to characterize appropriate signals in the connexin polypeptides we have cotranslated different connexin isotypes and amino- and carboxy-terminal truncated connexins in cell-free translation systems, and investigated interaction and assembly of the subunit proteins by hydrodynamic and immunoprecipitation analyses. Our results are taken together in Fig. 7. The results suggest that an "assembly" signal regulating principal connexin subunit recognition may be located in the C-terminal portion (preferentially third transmembrane spanning domain) of the connexin polypeptides (Figs. 7A, 7B), while a "selectivity" signal regulating specific assembly of heterooligomeric connexons is located in the amino-terminal portion (NH_2-terminal, TM1, or E1 domain) of the connexin polypeptide sequence (Figs. 7C, 7D) (2, 4) [reviewed in Ref. (5)]. The high-resolution structure of gap junction channels (43) clearly indicates that individual connexin polypeptides interact with one or two transmembrane-spanning domains of the adjacent connexin polypeptides within the connexon. Therefore, it appears likely that specific signals regulating selective connexon assembly are located within the transmembrane domains and/or immediately adjacent connexin sequences.

The hypothetical number of different gap junction channel subtypes is further broadened by the possibility that different types of connexons pair into heterotypic gap junction channels, as is suggested by the coculture experiments of tissue culture cells that express different connexin isotypes (39). Future experimentation including expression of connexins in cell-free translation systems will provide us with a better understanding of the oligomerization characteristics of the different gap junction channel subunits.

REFERENCES

1. Falk, M. M., Kumar, N. M., and Gilula, N. B. (1994) *J. Cell Biol.* **127,** 343–355.
2. Falk, M. M., Buehler, L. K., Kumar, N. M., and Gilula, N. B. (1997) *EMBO J.* **10,** 2703–2716.
3. Falk, M. M., and Gilula, N. B. (1998) *J. Biol. Chem.* **273,** 7856–7864.
4. Falk, M. M., Buehler, L. K., Kumar, N. M., and Gilula, N. B. (1998) *in* Gap Junctions (Werner, R., Ed.), pp. 135–139, IOS Press, Amsterdam.
5. Yeager, M., Unger, V., and Falk, M. M. (1998) *Curr. Opin. Struct. Biol.* **8,** 517–524.
6. Jadot, M., Hofmann, M. W., Graf, R., Quader, H., and Martoglio, B. (1995) *FEBS Lett.* **371,** 145–148.
7. High, S., and Laird, V. (1997) *Trends Cell Biol.* **7,** 206–210.
8. Borel, A. C., and Simon, S. M. (1996) *Biochemistry* **35,** 10587–10594.
9. Mothes, W., Heinrich, S. U., Graf, R., Nilsson, I. M., von Heijne, G., Brunner, J., and Rapoport. T. A. (1997) *Cell* **89,** 523–533.
10. Anderson, D. J., and Blobel, G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5598–5602.
11. Rosenberg, R. L., and East, J. E. (1992) *Nature* **360,** 166–169.
12. Kvist, S., Wiman, K., Claesson, L., Peterson, P. A., and Dobberstein, B. (1982) *Cell* **29,** 61–69.
13. Qu, D., and Green, M. (1995) *DNA Cell Biol.* **14,** 741–751.
14. Sayer, J. T., and Doyle, D. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4854–4858.
15. Yilla, M., Doyle, D., and Sawyer, J. T. (1992) *J. Cell Biol.* **188,** 245–252.
16. Falk, M. M. *in* Methods in Molecular Biology, Connexin Channels (Bruzzone, R., and Giaume, C., Eds.), Humana Press, Totowa, NJ (in press).
17. Falk, M. M., Grigera, P. R., Bergmann, I. E., Zibert, A., Multhaup, G., and Beck, E. (1990) *J. Virol.* **64,** 748–756.
18. Falk, M. M., Sobrino, F., and Beck, E. (1992) *J. Virol.* **66,** 2251–2260.
19. Zibert, A., Maass, G., Strebel, K., Falk, M. M., and Beck, E. (1990) *J. Virol.* **64,** 2467–2473.
20. Mayer, T., Tamura, T., Falk, M. M., and Niemann, H. (1988) *J. Biol. Chem.* **263,** 14956–14963.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY.
22. Jackson, R. J., and Hunt, T. (1983) *Methods Enzymol.* **96,** 50–74.
23. Erickson, A., and Blobel, G. (1983) *Methods Enzymol.* **96,** 38–50.
24. Evans, E. A., Gilmore, R., and Blobel, G. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 581–585.
25. Walter, P., and Blobel, G. (1983) *Methods Enzymol.* **96,** 84–93.

26. Kozak, M. (1989) *J. Cell Biol.* **108,** 229–241.

27. Krieg, P. A., and Melton, D. A. (1984) *Nucleic Acids Res.* **12,** 7057–7070.

28. Williams, J. G., Kay, M., and Patient, R. K. (1980) *Nucleic Acids Res.* **8,** 4247–4258.

29. Kahn, T. W., Beachy, R. N., and Falk, M. M. (1997) *Curr. Biol.* **7,** R207–R208.

30. Jackson, R. J., Howell, M. T., and Kaminski, A. (1990) *Trends Biochem. Sci.* **15,** 477–483.

31. Green, W. N., and Millar, N. S. (1995) *Trends Neurosci.* **18,** 280–287.

32. Hurtley S. M., and Helenius, A. (1989) *Annu. Rev. Cell Biol.* **5,** 277–307.

33. Musil, L. S., and Goodenough, D. A. (1993) *Cell* **74,** 1065–1077.

34. Kistler, J., Goldie, K., Donaldson, P., and Engel, A. (1994) *J. Cell Biol.* **126,** 1047–1058.

35. Cascio, M., Kumar, N. M., Safarik, R., and Gilula, N. B. (1995) *J. Biol. Chem.* **270,** 18643–18648.

36. Stauffer, K. A., Kumar, N. M., Gilula, N. B., and Unwin, N. (1991) *J. Cell Biol.* **115,** 141–150.

37. Buehler, L. K., Stauffer, K. A., Gilula, N. B., and Kumar, N. M. (1995) *Biophys. J.* **68,** 1767–1775.

38. McKay, B. S., Annis, D. S., Honda, S., Christie, D., and Kunicki, T. J. (1996) *J. Biol. Chem.* **271,** 30544–30547.

39. Elfgang, C., Eckert, R., Lichtenberg-Fraté, H., Butterweck, A., Traub, O., Klein, R. A., Hülser, D. F., and Willecke, K. (1995) *J. Cell Biol.* **129,** 805–817.

40. Konig, N., and Zampighi, G. A. (1995) *J. Cell Sci.* **108,** 3091–3098.

41. Stauffer, K. A. (1995) *J. Biol. Chem.* **270,** 6768–6772.

42. Brink, P. R., Cronin, K., Banach, K., Peterson, E., Westphale, E. M., Seul, K. H., Ramanan, S. V., and Beyer, E. C. (1997) *Am. J. Physiol. Cell Physiol.* **273,** C1386–C1396.

43. Unger, V. M., Kumar, N. M., Gilula, N. B., and Yeager, M. (1999) *Science* **283,** 1176–1180.

44. Jiang, J. X., and Goodenough, D. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1287–1291.

45. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY.

46. Li, M., Jan, Y. N., and Jan, L. Y. (1992) *Science* **257,** 1225–1230.

47. Shen, N. V., Chen, X., Boyer, M. M., and Pfaffinger, P. J. (1993) *Neuron* **11,** 67–77.

48. Babila, T., Moscucci, A., Wang, H., Weaver, F. E., and Koren, G. (1994) *Neuron* **12,** 615–626.

49. Kumar, N. M., Friend, D. S., and Gilula, N. B. (1995) *J. Cell Sci.* **108,** 3725–3734.

50. De Sousa, P. A., Valdimarsson, G., Nicholson, B. J., and Kidder, G. M. (1993) *Development* **117,** 1355–1367.

51. Rahman, S., Carlile, G., and Evans, W. H. (1993) *J. Biol. Chem.* **268,** 1260–1265.

52. Musil, L. S., and Goodenough, D. A. (1991) *J. Cell Biol.* **115,** 1357–1374.

53. Laird, D. W., Castillo, M., and Kasprzak, L. (1995) *J. Cell Biol.* **131,** 1193–1203.

54. Deschenes, S. M., Walcott, J. L., Wexler, T. L., Scherer, S. S., and Fischbeck, K. H. (1997) *J. Neurosci.* **17,** 9077–9084.

55. Pfeffer, S. R., and Rothman, J. E. (1987) *Annu. Rev. Biochem.* **56,** 829–852.

56. Ahmad, S., Diez, J. A., George, C. H., and Evans, W. H. (1999) *Biochem. J.* **339,** 247–253.

57. George, C. H., Kendall, J. M., Campbell, A. K., and Evans, W. H. (1998) *J. Biol. Chem.* **273,** 29822–29829.

58. George, C. H., Kendall, J. M., and Evans, W. H. (1999) *J. Biol. Chem.* **274,** 8678–8685.

59. Toyofuku, T., Yabuki, M., Otsu, K., Kuzuya, T., Hori, M., and Tada, M. (1998) *J. Biol. Chem.* **273,** 12725–12731.

60. Li, H., Liu, T.-F., Lazrak, A., Peracchia, C., Goldberg, G. S., Lampe, P. D., and Johnson, R. G. (1996) *J. Cell Biol.* **134,** 1019–1030.

61. Laird, D. W. (1996) *J. Bioenerg. Biomembr.* **28,** 311–318.

62. Fujimoto, K., Nagafuchi, A., Tsukita, S., Kuraoka, A., Ohokuma, A., and Shibata, Y. (1997) *J. Cell Sci.* **110,** 311–322.

63. Risek, B., Klier, F. G., and Gilula, N. B. (1994) *Dev. Biol.* **164,** 183–196.

64. Sosinsky, G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9210–9214.

65. Nicholson, B., Dermietzel, R., Teplow, D., Traub, O., Willecke, K., and Revel, J.-P. (1987) *Nature* **329,** 732–734.