Assembly of Hepatic Gap Junctions

TOPOGRAPHY AND DISTRIBUTION OF CONNEXIN 32 IN INTRACELLULAR AND PLASMA MEMBRANES DETERMINED USING SEQUENCE-SPECIFIC ANTIBODIES

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The subcellular distribution in rat liver and the topography in intracellular and plasma membranes of connexin 32, a major protein component of gap junctions, was studied using sequence-specific anti-peptide antibodies generated to extracellular and intracellular domains of the protein. The distribution of connexin 32 in liver analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting showed the relative protein levels in the subcellular fractions to be: lateral plasma membranes > Golgi membranes > sinusoidal plasma membranes > lysosomes. Low amounts of connexin 32 were detected in microsomes, endosomes, and bile canalicular plasma membranes. Six highly conserved cysteine residues are located in the amino acid sequences comprising the two extracellular loops of all connexins thus far isolated, and these loops are positioned to extend the channel in the lipid bilayers across the intercellular region of the gap junction. In the present work, the intramolecular disulfide bonds linking the extracellular loops in gap junctions were shown to be present in connexins located in plasma membranes, Golgi, and a microsomal fraction, and it was concluded that the disulfide linkages were formed in the endoplasmic reticulum. In addition, immature configurations of connexin 32, probably occurring during membrane insertion, were detected in liver microsomal fractions. The results contribute to charting of the biogenetic routes followed by connexins in hepaticocytes and the general mechanisms of gap junction assembly.

Gap junctions comprise strongly adherent regions of the plasma membrane of animal cells where direct intercellular communication occurs. Gap junctions are constructed of clusters of hexagonally arranged hemi-channels (connexons) coaxially aligned and attached to connexons contributed by the neighboring cell’s plasma membrane (for reviews, see Refs. 1–3). The precise alignment of connexons in the plasma membranes contributed by the two cooperating cells allows direct movement through the junctional channel of small molecules and ions thereby facilitating the integration of metabolic events in tissues and organs. The switching between the open and closed channel configuration in gap junctions is regulated by a variety of factors, including intracellular pH (4) Ca2+ (5, 6), and cAMP levels that influence the phosphorylation state of the channel protein (7).

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Connexon hemi-channels are constructed of six protein subunits, the connexins, that have been shown by sequencing of the cDNAs to be closely related members of a multigene family of integral membrane proteins (1, 3, 8, 9). Connexin 32, the major connexin in rodent liver, traverses the lipid bilayer four times with the amino and carboxyl termini located cytoplasmically. This arrangement of the protein sequence in the membrane generates two extracellular loops projecting into the “gap” and a single intracellular loop (10–14). The third transmembrane region is postulated to contribute to the channel wall of the gap junction (12). A similar topography in the membrane has been proposed for connexin 43 studied in cardiac gap junctions. (15–17). Further studies on the precise topography of connexin 32 in the membrane have indicated that the two extracellular loops, that are positioned to provide the structural scaffolding extending the channel across the intercellular gap, are cross-linked by intramolecular disulfide bonds (14).

The mechanism of assembly of polymeric membrane channels in general is poorly understood (18). In the context of the assembly of gap junctions, we have addressed in the present work aspects of their assembly in liver tissue by using a panel of sequence-specific antibodies to examine the topography of connexin 32 in membranes that originate from various subcellular fractions comprising the biogenetic pathway followed by proteins destined for the plasma membrane. The results indicate that the disulfide bridges that attach the two extracellular loops are generated early in the biogenetic pathway, probably in the endoplasmic reticulum, and that the topography in the membrane of connexin 32 is similar from the Golgi apparatus to the gap junction plaques.

EXPERIMENTAL PROCEDURES

Preparation of Subcellular Fractions—Liver plasma membrane fractions originating from the lateral, sinusoidal, and canalicular domains of the plasma membrane were prepared and characterized from Sprague-Dawley rats as described previously (19). A “residual” microsomal fraction was collected at the 37–49% (w/v) sucrose interface after removal, by flotation, of sinusoidal plasma membranes and contained smooth and rough-surfaced membrane vesicles deriving mainly from the endoplasmic reticulum (20). This microsomal fraction accounted for approximately 6–8% of the liver homogenate protein and the specific activities of 5'-nucleotidase (EC 3.1.3.5) leucylnaphthylamidase (EC 3.4.11.1) were 1.5–2-fold those measured in the homogenate compared to a 20–100-fold increase in plasma membranes (23). The endosomal fractions were prepared as described (21), endosome-depleted Golgi fractions prepared as described (22), and lysosomes prepared as described (24). Protein content of the fractions was measured using a dye-binding assay (25).

Determination of Connexin 32 Distribution in Liver Subcellular Fractions—Equal amounts (100 µg of protein) of each of the charac-
terized subcellular fractions were analyzed by SDS-PAGE using 12.5% (w/v) acrylamide. After electrophoretic transfer to nitrocellulose sheets and exposure to GAP 9 and 10 anti-peptide antibodies (14) as well as to the antibodies to total liver gap junctions (see below), the antigens were visualized using 125I-protein A and autoradiography. Signal strength was determined by densitometry of the bands corresponding to monomeric and dimeric connexins in the autoradiographs.

**Proteolytic Digestion of the Subcellular Fractions**—The subcellular fractions, prepared from liver homogenates as described above, were treated with trypsin and analyzed by SDS-PAGE (26) followed by Western bloting (27). The amounts of protein used for trypsin digestion were 30 mg/g of homogenate (10) and 200 mg/g (all other fractions analyzed), and the ratio of trypsin to membrane protein was 1:25 (w/w) for the lateral plasma membranes and 1:5 (w/w) for the other fractions. Addition of trypsin (Sigma) to the fractions suspended in 50 mM NH4HCO3, pH 8.0, was made at 0.1–100 μg/mL, and 15-h intervals at a ratio of 70:20, and 10% of the total enzyme applied, respectively. Saponin (0.05%) was included when necessary during proteolytic digestion to permeabilize vesicular membranes present in the fractions examined. The reactions were stopped after 18 h by addition of a trypsin inhibitor (Sigma). After trypsin digestion, the fractions were divided into 2 aliquots, 10 μM N-ethylmaleimide added, and the fractions (0.5%) stabilized in Laemml buffer (26) in the presence of β-mercaptoethanol. The membranes fractions were then analyzed by SDS-PAGE under reducing and non-reducing conditions; the acrylamide concentration generally used in the gels was 15%. Proteins were electrophoretically transferred to nitrocellulose sheets (27), and probed with the sequence specific antibodies (Table 1).

**Treatment of Subcellular Fractions with Glycosidases**—To assess whether connexins were glycosylated, lateral and sinusoidal plasma membranes and a Golgi fraction (50 μg of protein) were suspended in 0.25 M sodium acetate, pH 6.0, 20 mM EDTA, 0.05% saponin, and 10 mM mercaptoethanol (50 μL) endoglycosidase F (0.15 unit) (Boehringer) was added (total volume, 100 μL) and the fractions centrifuged at 37°C for 15 min at 12,000 × g (Aepinövör) and the pellets dissolved in Laemml buffer (26). Membrane fractions were analyzed by SDS-PAGE in 12.5% acrylamide gels, transferred electrophoretically to nitrocellulose paper, and connexin 32 detected using GAP-9 antibodies. For assessment of O-glycosylation of connexin 32, the subcellular fraction (100 μg), suspended in 0.05% saponin in 200 mM sodium acetate, pH 5.0 (100 μL), were digested with 50 μL or units of neuraminidase for 1 h at 37°C. After pelleting and removal of the supernatant, the pellets were then suspended in 20 mM sodium cacodylate, pH 6.1, 0.05% saponin (100 μL), and 0.3 μL of O-glycosidase (Boehringer) added (28). After overnight incubation at 37°C, the reaction was halted by addition of 100 mM sodium carbonate, pH 9.0. The subcellular fractions centrifuged to yield pellets that were analyzed by SDS-PAGE. After electrophoresis and transfer to nitrocellulose paper, these were probed using an antibody to the total gap junction protein as described above.

**Preparation of Anti-peptide Antibodies**—These reagents (see Table 1) were synthesized and their specificity to the peptide sequences characterized as described by Bahman and Evans (14). These antibodies have been shown to specifically identify monomeric and dimeric forms of connexin 32 by Western blotting in liver homogenates and to immuno localize to punctate cell surface regions in cryosections (14). Furthermore, highly similar two-dimensional peptide maps of connexin 32 monomers and dimers have been demonstrated previously (29). In brief, peptides, synthesized by 9-fluorenylethylcarbonyl polyamide solid-phase chemistry, were coupled to Keyhole limpet hemocyanin via free amino groups by glutaraldehyde or via sulfanyl groups using m-leucinomethionyl-N-hydroxysuccinimide ester. Antibodies were generated in rabbits, and the specificity of the antibodies to each peptide immunogen was demonstrated by dot blot analysis (14). An antibody to purified rat liver gap junctions that was shown to bind to a sequence at the carboxyl terminus of connexin 32 was raised in a similar manner in rabbits (14). Affinity purification was carried out by immunabsorption of immunoglobin fractions on columns containing the peptides attached to either CNBr-activated Sepharose CL-4B (Pharmacia) or Sepharom Hemacart 1000E (Anachem) and elution under either acidic or alkaline conditions (14).

**RESULTS**

A number of studies (10–14) have proposed that connexin 32 in gap junctional plaques isolated from rat liver traverses the lipid bilayer four times, with the amino and carboxyl termini located in the cytoplasm thereby generating two extracellular loops and a single intracellular loop connecting the second and third transmembrane segments. Furthermore, intramolecular disulfide bonds link the two extracellular loops (14). In the present work, the subcellular distribution of connexin 32 in liver and the location where the disulfide bridges cross-linking the extracellular loops occur were studied using a panel of antibodies generated to non-membrane-embedded amino acid sequences (see Table I).

**Subcellular Distribution of Connexin 32 in Liver Subcellular Fractions**—The subcellular distribution of connexin 32 in plasma membranes originating from the sinusoidal, lateral, and canalicular domains and from Golgi, lysosomal, endosomal, and microsomal fractions was determined by SDS-PAGE (Fig. 1A), followed by Western blotting using antibodies to specific regions of the protein. Fig. 1B shows that antibodies to GAP 9 (see Table I for sequence and topographical location) bound specifically to monomeric (Mr 28,000) and dimeric (Mr 54,000) forms of connexin 32 in the subcellular fractions; some variation in the ratio of monomeric, dimeric, and occasionally trimeric forms of connexin 32 bound by the various antibodies utilized in the present work was observed. Two anti-peptide antibodies (GAP 9 and 10) and an antibody generated to purified gap junctions with a binding specificity to a sequence at the carboxyl-terminal tail were used to quantify the relative distribution of connexin 32 in the subcellular fractions. The predominance of connexin 32 in the lateral plasma membrane fraction, where the gap junctions are located, was clear (Fig. 2), but connexin 32 was also detected in Golgi, sinusoidal plasma membranes, and lysosomes. Estimations of connexin 32 amounts in the latter fraction were carried out using freshly prepared lysosomes to minimize the generation of autodegradation products. Low amounts of connexin 32 were detected in microsomes, endosomes, and bile canalicular plasma membranes.

**Cell Surface and Intracellular Connexins Are Not Glycosylated**—Connexins in gap junction plaques prepared by detergent or alkaline extraction of liver plasma membranes are not glycosylated (30, 31). A consensus glycosylation sequence (Asn-Trp-Thr) is located at residues 2–4 of connexin 32, a sequence proposed to be located at the cytoplasmic aspect of the gap junction membrane (10). It is generally thought that this consensus amino acid sequence is unlikely to be glycosylated owing to its disposition at the cytoplasmic aspect of the junctional membrane. The demonstration in the present work of connexin 32 in Golgi membranes allowed the opportunity to examine more rigorously whether glycosylated forms of connexins exist inside the cell on the biogenetic route by examination for N- or O-linked oligosaccharides in the subcellular fractions. The predominance of connexin 32 in the lateral plasma membrane fraction, where the gap junctions are located, was clear (Fig. 2), but connexin 32 was also detected in Golgi, sinusoidal plasma membranes, and lysosomes. Estimations of connexin 32 amounts in the latter fraction were carried out using freshly prepared lysosomes to minimize the generation of autodegradation products. Low amounts of connexin 32 were detected in microsomes, endosomes, and bile canalicular plasma membranes.

**Topography of Connexin 32 in Plasma Membranes, Golgi,
TABLE I
Properties of antibodies to connexin 32 used in the present work

| Antibody | Peptide assignment | Sequence | Topographical specificity |
|----------|-------------------|----------|--------------------------|
| GAP (5 + 6)* | 182–192 (5) 162–176 (6) | SRPTEKTVFTN (5) MVRLVKCEAFPCFNT (6) | Extracellular loop 2 |
| GAP 7 M | 43–59 | VVGDEKSSFICNTLQPGY | Extracellular loop 1 |
| GAP 9 | 264–283 | REPSPGALAEKSDRCSAC | Cytoplasmic (COOH terminus) |
| GAP 10 | 1–20 | MNWTGVYTLKSAGVNRHSTAIG | Cytoplasmic (NH2 terminus) |
| GAP 11 | 151–187 | YVFYLLPYAMVRLVKCEAF PCPNTVDCFVSRTPEK | Extracellular loop 2 |
| DES 1d | 102–112 116–124 | EKKMLRLEGHGHLEEVKRHK | Cytoplasmic loop |
| WGJ | NA* | See text | Cytoplasmic (COOH terminus) |

* Based on the rat liver cDNA sequence (9).
+ See Ref. 14 for discussion.
1 Both peptides were coupled together to keyhole limpet haemocyanin.
* A composite peptide composed of two discontinuous sequences.
- NA, not applicable.

Fig. 1. A, analysis by SDS-PAGE of polypeptide composition of lateral plasma membranes (LAT P.M.), sinusoidal plasma membranes (SIN P.M.), canalicular plasma membranes (CANT P.M.), lysosomes (LYSOS), Golgi, endosomes (ENDOS), and microsomal “residual” fraction (MCS). Equal amounts of protein were resolved in 10% acrylamide gels and stained with Coomassie Blue. In B, autoradiogram of a Western blot of an SDS-PAGE gel corresponding to the subcellular fractions analyzed in A exposed to antibodies to GAP 9 (see Table I for GAP 9 sequence). The bands corresponding to monomeric (M, 28,000) and dimeric (M, 54,000) forms of connexin 32 are indicated.

and Microsomes—In the procedure developed to establish the presence of intramolecular disulfide linkages in connexin 32 using the sequence-specific antibodies, trypsin digestion of gap junctions produced two major tryptic fragments (13,000 and 10,000) resolved in SDS-PAGE under reducing conditions and a 23,000 tryptic product in which the above fragments had remained attached by disulfide bonds when migrating in non-reducing gels (14). Trypsin treatment of lateral and sinusoidal plasma membranes and Golgi produced a 23,000 fragment in non-reduced gels that was identified by the specified sequence-specific antibodies (Fig. 4). Under reducing conditions, the gels showed major tryptic fragments of 10,000 (comprising the amino terminus, the first extracellular loop, and a portion of the intracellular loop) and 13,000 (comprising the second extracellular loop and a portion of the cytoplasmic tail) to be present (Fig. 4). Under non-reducing conditions, the two tryptic fragments remained attached by intramolecular cystine bridges.

Similar results were obtained using the same procedure with the microsomal fraction. Fig. 5a shows that using GAP 10 (generated to an amino-terminal sequence) and GAP 11 (generated to the second extracellular loop) antibodies, 23,000 tryptic fragments were identified only under non-reducing conditions, and 10,000–13,000 fragments under reducing conditions. However, in contrast to the other subcellular fractions analyzed, these antibodies also detected a ladder of peptides (10,000–13,000) in the microsomal fraction under non-reduc-
Distribution and Topography of Connexin 32 in Liver

Analysis by SDS-PAGE and Western blotting of the effects of exposure of sinusoidal and lateral plasma membranes and Golgi membranes to (a) O-glycosidase and (b) endoglycosidase F on the electrophoretic mobility of monomeric (M, 28,000) and dimeric (M, 54,000) connexin 32. After transfer to nitrocellulose and exposure to an antibody to purified gap junctions (WGJ), autoradiography was carried out. Control lanes (−) omitted the enzymes. See Fig. 1 for abbreviations.

Analysis by SDS-PAGE of the disulfide bonds in connexin 32 in Golgi, SIN. P.M. and LAT. P.M. Subcellular fractions were treated with trypsin and electrophoresed either in the presence (+) or absence (−) of β-mercaptoethanol. After electrophoretic transfer, the peptides were identified by autoradiography using the specified sequence-specific antibodies. The positions of disulfide-linked (M, 23,000) and the reduced tryptic peptides (M, 13,000 and 10,000) are indicated. Bands of higher relative mobility represent undigested protein or dimeric products of the tryptic fragments. See Fig. 1 for abbreviations.

Analysis by SDS-PAGE and Western blotting of the polypeptides released by trypsin treatment of a microsomal fraction in the absence (−) or presence (+) of β-mercaptoethanol. In a, the microsomal fraction was exposed to the various anti-peptide antibodies (see Table I). In b, subcellular fractions (lane 1, sinusoidal plasma membrane; lanes 2 and 3, Golgi; lane 4, microsomes; lane 5, lateral plasma membranes). The positions in the autoradiographs of the major non-reduced (M, 23,000 and 17,000) and reduced (M, 10,000–12,000 and 13,000) trypptic products are indicated.

ANTIBODIES WERE USED TO ANALYZE THE TRYPTIC PRODUCTS, A SIMILAR, BUT MORE COMPLEX SERIES OF PEPTIDES WERE DETECTED WITH A 17,000 PRODUCT OF UNKNOWN ORIGIN APPEARING IN NON-REDUCED GELS. THE DES-1 ANTIBODY, GENERATED TO A SEQUENCE IN THE ENDOPLECTIC RETICULUM CONTAINED CONNEXIN 32 WITH A MODIFIED MEMBRANE TOPOGRAPHY TO THAT ESTABLISHED IN THE OTHER SUBCELLULAR FRACTIONS NOW STUDIED, AS WELL AS IN GAP JUNCTIONS (14).

Assessment of the Subcellular Distribution of Connexins Provides Clues to the Intracellular Biogenetic Routes—The present study, using characterized subcellular fractions originating from the three main functional domains of the hepatocyte’s plasma membrane, the Golgi apparatus, lysosomes, endosomes, and the endoplasmic reticulum, shows that connexin 32, the major gap junction protein in rat liver, although present at high relative protein levels at the lateral plasma membrane where the gap junctional plaques are located, is also located in other hepatocyte membranes. This distribution of connexins of identical mass in the membrane fractions is interpreted to reflect the trafficking of connexins in the cell prior to their assembly into gap junctional plaques at the hepatocyte’s lateral plasma membrane (Fig. 6).

The relative distribution of connexin 32 in the liver membranes using antibodies with specificities toward three different regions of the protein showed that although high levels of connexin 32 were found in lateral plasma membranes, significant amounts were also found in sinusoidal plasma membranes and Golgi with lower amounts in a microsomal fraction. Since the basolateral region of the plasma membrane identify sequences in the carboxyl-terminal tail (WGJ, GAP 9) failed to bind to the major tryptic fragments indicating that the corresponding regions had been removed by the trypsin treatment. The data indicated, however, that trypsin treatment of microsomes generated peptides that varied in length; fragments of 10,000–12,000 range were observed under both reducing and non-reducing gels (Fig. 5a). When DES-1 antibodies were used to analyze the tryptic products, a similar, but more complex series of peptides were detected with a 17,000 product of unknown origin appearing in non-reduced gels. The DES-1 antibody, generated to a sequence in the intracellular loop that was removed by trypsin treatment of gap junctions (14), showed that this antigenic site was also similarly ablated from connexin 32 resident in plasma membrane and Golgi fractions (Fig. 5b, lanes 1–3 and 5). In contrast, the DES-1 antibody recognized an approximately 12,000 fragment under reducing conditions in microsomes (Fig. 5b, lane 4), thus confirming that this fraction originating from the endoplasmic reticulum contained connexin 32 with a modified membrane topography to that established in the other subcellular fractions now studied, as well as in gap junctions (14).

FIG. 3. Analysis by SDS-PAGE and Western blotting of the effects of exposure of sinusoidal and lateral plasma membranes and Golgi membranes to (a) O-glycosidase and (b) endoglycosidase F on the electrophoretic mobility of monomeric (M, 28,000) and dimeric (M, 54,000) connexin 32. After transfer to nitrocellulose and exposure to an antibody to purified gap junctions (WGJ), autoradiography was carried out. Control lanes (−) omitted the enzymes. See Fig. 1 for abbreviations.

FIG. 4. Analysis by SDS-PAGE of the disulfide bonds in connexin 32 in Golgi, SIN. P.M. and LAT. P.M. Subcellular fractions were treated with trypsin and electrophoresed in the presence (+) or absence (−) of β-mercaptoethanol. After electrophoretic transfer, the peptides were identified by autoradiography using the specified sequence-specific antibodies. The positions of disulfide-linked (M, 23,000) and the reduced tryptic peptides (M, 13,000 and 10,000) are indicated. Bands of higher relative mobility represent undigested protein or dimeric products of the tryptic fragments. See Fig. 1 for abbreviations.

FIG. 5. Analysis by SDS-PAGE and Western blotting of the polypeptides released by trypsin treatment of a microsomal fraction in the absence (−) or presence (+) of β-mercaptoethanol. In a, the microsomal fraction was exposed to the various anti-peptide antibodies (see Table I). In b, subcellular fractions (lane 1, sinusoidal plasma membrane; lanes 2 and 3, Golgi; lane 4, microsomes; lane 5, lateral plasma membranes). The positions in the autoradiographs of the major non-reduced (M, 23,000 and 17,000) and reduced (M, 10,000–12,000 and 13,000) trypptic products are indicated.

FIG. 6. Schematic representation of the topography and probable intracellular routing of connexin 32 in a rat liver hepatocyte. The scheme shows the topography of individual connexins; the position in the cell where oligomerization into hexameric connexons occurs is unknown. It is assumed that docking of connexons to form gap junctional plaques occurs predominantly, if not exclusively, in the lateral plasma membrane. RER and SER, rough or smooth endoplasmic reticulum. Mature and immature connexins are shown in the endoplasmic reticulum.
accounts for approximately 4–6% of the hepatocyte's membrane area whereas the endoplasmic reticulum–Golgi accounts for approximately 50% (32), the present subcellular distribution data show that substantial amounts of connexin 32 occur in intracellular membranes. This estimate increases if the lysosomal levels of connexin 32 are taken into account. Although connexins located in the endoplasmic reticulum and the Golgi apparatus of normal liver hepatocytes are not evident using morphological or immunocytochemical approaches, the present results are in accord with the high turnover rate of gap junctions in liver (33). Connexin 32 belongs to a limited category of membrane proteins functional at the plasma membrane that are not glycosylated, and the present studies reinforce this conclusion by showing that neither N- or O-linked glycosylated precursors occur in intracellular membranes, especially the Golgi apparatus where differences in electrophoretic mobility caused by glycosylation would be highlighted. A potential N-glycosylation site is identified at the cytoplasmic amino terminus in connexin 32; consensus sequences for O-glycosylation are less clear (34).

The present study indicates that the topographical arrangement in the membrane of connexin 32 that can account for the lack of glycosylation of sequences near the amino terminus is invariant during transit through the Golgi apparatus with the intracellular loop and the carboxyl and amino termini being located cytoplasmically and the two disulfide-linked loops projecting into the lumen of the membrane vesicles. Although no post-translational modifications resulting from glycosylation were detected in connexin 32, connexins have been shown to be phosphorylated; in hepatocytes, for example, connexin 32 is phosphorylated at residue serine 233 (35). Connexin 32 is also labeled by tritiated palmitic and myristic acids (36) and contains a CAAX sequence at the cytoplasmic tail (residues 280–283).

Very low relative amounts of connexin 32 were detected in the canalicular plasma membrane, for lateral diffusion of proteins into this plasma membrane domain is prevented or retarded by tight junctions. Although connexin 32 was detected in lysosomes, extremely low amounts were detected in endosomes, suggesting that gap junction plaques are directly internalized from the lateral plasma membrane into lysosomes or phagocytic structures. Analysis, using the present antibodies, of rat liver lysosomal fractions subjected to repeated freezing and thawing has indicated the loss of the carboxy-terminal tail concurrent with the breakdown of connexin 32 into two predominant polypeptides of approximately 10,000 and 13,000 similar to that induced by trypsin treatment of the connexin-containing membranes (results not shown). Electron microscopic evidence for internalized of gap junctions observed as vesicular structures has been presented, and these are most prevalent in steroidogenic epithelia (37). In the absence of kinetic measurements of the rate appearance of radiolabeled connexin 32 in lateral and sinusoidal plasma membranes, the precise point of insertion of connexins/connexon into the plasma membrane of the hepatocyte remains to be determined.

Connexin 32 Topography in Intracellular and Plasma Membranes—The data presented indicate that the topographical arrangement in the membrane of connexin 32 in gap junction plaques probably occurs cotranslationally during insertion of the protein into the endoplasmic reticulum membrane. Although it is possible that the topography deduced in lateral plasma membrane fractions is contributed in part by connexin 32 in gap junctional plaques, essentially similar peptide digest products indicating the presence of an intramolecular disulfide bridge were obtained with sinusoidal plasma membranes and Golgi membranes. According to this topography, the amino acid sequences in the two loops located at the external surface of the non-junctional sinusoidal region of the plasma membrane are inviolate to tryptic attack. In gap junctions, the amino acid sequences comprising the two extracellular loops were also not hydrolyzed by a range of proteolytic enzymes, and when gap junctions transformed into "halves" were used, no hydrolysis of the amino acid sequences in the external loops was observed (14). Connexin 32 contains two potential tryptic cleavage points (Lys-48, Arg-74) in the first extracellular loop and 2 lysines (residues 167, 185) and 2 arginines (residues 164, 183) in the second extracellular loop. In microsomes populations of polypeptides with and without disulfide bonds were present pointing to the conclusion that disulfide-bond formation was occurring in the endoplasmic reticulum. The disulfide isomerase that catalyzes the formation of disulfide bonds in proteins is located in the lumen of the endoplasmic reticulum (38). The presence of a ladder of proteolytic products produced by treatment of microsomes with trypsin also indicated that a number of folding states of connexin sequences in the endoplasmic reticulum membrane. A 17,000 tryptic product identified by DES-1 antibodies in non-reducing gels could not be assigned topographically. A further difference between connexin 32 detected in the microsomal fractions and in membranes comprising later stages in the biogenetic route was the action of trypsin on the intracellular loop assessed using the DES-1 antibodies. This antibody-binding site corresponds to residues 115–123 (14) and was removed after trypsin treatment of gap junctions (14). Trypsin treatment of Golgi and plasma membrane fractions in the present work also removed this binding site, but it was detected by DES-1 antibodies in connexin 32 present in the microsomal fraction. This observation may be accounted for by the presence in the heterogenous microsomal fraction of connexin populations containing different configurations with the appropriate intracellular loop region being embedded in the lipid bilayer. Alternatively, these amino acid sequences in the loop are protected from proteolysis by their interaction with other accessory proteins that feature in ensuring correct insertion and folding of proteins in the membrane (40).

Assembly and Possible Functions of the Extracellular Loops of Connexin 32—The general conservation of the membrane topography of connexins (3, 8, 39) suggest that trafficking and assembly in non-hepatic tissues or cultured cells is similar. Disulfide bridges linking the extracellular loops have been demonstrated in other connexins e.g. connexin 43 (41), and the 6 cysteine residues and their flanking amino acids in the two extracellular loops are conserved in many other connexins (3). The present results show that these disulfide linkages are put in place in the endoplasmic reticulum, but the cellular locus of oligomerization of connexins into connexon hemichannels is currently unknown. Studies with other oligomeric proteins, e.g. acetyl choline receptor (42) and influenza hemagglutinin trimers (43), suggest that oligomerization immediately follows correct assembly in the endoplasmic reticulum. In the case of connexon assembly, the generation of "open" connexon channels in intracellular membranes would introduce problems in the equilibration of intracellular environments. It is likely, therefore, that if connexons are formed in intracellular membranes, channel opening is delayed until the gap junctions are assembled. The formation of functional gap junctional plaques constructed of connexin 43 has been shown to be triggered by phosphorylation (44).

The disulfide-linked extracellular loops of connexin 32 are resistant to proteolytic attack during transit to the plasma membrane, thus ensuring the recognition of connexins in
aligned cells in readiness for the establishment of the intercellular channel. Modeling of the 24 extracellular amino acid loops present in a gap junctional channel consisting of two hexameric connexons would be aided by the determination of the precise position of the disulfides. Fifteen possible arrangements of the 6 cysteine residues (3 in each loop) can occur in the connexin external loops, and the two most likely arrangements have been proposed based on an analysis of the flanking amino acid sequences (14). Mutational analysis of connexin proteins should aid in the elucidation of the roles of the disulfide structural features. Modification of cysteine residues interrupts and is thus crucial for intracellular trafficking (45) emphasizing the functional importance of this structural feature of connexin proteins.

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