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Topology of the 32-kd liver gap junction protein determined by site-directed antibody localizations

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Synthetic peptides corresponding to sequences in the human liver gap junction protein were chemically synthesized and used for generation of peptide antisera to defined sequences in the protein. The antibodies were affinity purified and characterized by demonstrating that they specifically recognized both their corresponding synthetic peptide (as indicated by dot blot analysis) and the native 32-kd gap junction protein (by immunoblotting). The specificity of a subset of the different site-specific antibodies was subsequently confirmed by demonstration of their binding to specific gap junction fragments produced by treatment with a lysine-specific endoproteinase. Immunoelectron microscopy was used to localize the specific peptide antibody epitopes to either the cytoplasmic or extracellular surfaces of the gap junction. Results indicate a transmembrane orientation for the protein with the amino and carboxyl termini located on the cytoplasmic side of the membrane. Based on these data, a model is proposed for the transmembrane folding of the gap junction protein.

Key words: gap junction protein/immunoelectron microscopy/membrane channel/membrane protein topology/site-directed antibodies

Introduction

Gap junctions are extended collections of membrane channels which link end-to-end across the space between juxtaposed cells, creating continuous communication pathways for the diffusion of ions and small molecules between their interiors (Gilula *et al.*, 1972; Loewenstein, 1981). These channels are oligomeric proteins constructed of six circularly arranged identical subunits (Makowski *et al.*, 1977; Unwin and Zampighi, 1980). The subunits appear to be derived from a family of proteins related by sequence (Kumar and Gilula, 1986; Paul, 1986; Nicholson *et al.*, 1987; Beyer *et al.*, 1987; Kistler *et al.*, 1988), of which the most extensively characterized is the 32-kd polypeptide from mammalian liver (Henderson *et al.*, 1979; Hertzberg and Gilula, 1979; Young *et al.*, 1987). Recently, the complete amino acid sequence for this polypeptide (Paul, 1986; Kumar and Gilula, 1986) and two others – a 43-kd polypeptide from mammalian heart (Beyer *et al.*, 1987) and a 30-kd polypeptide from *Xenopus* embryos and liver (Gimlich *et al.*, 1988) have been deduced by recombinant DNA methods.

An enriched membrane fraction can be isolated from the rat liver that contains gap junction structures morphologically identical to those observed *in vivo*. These isolated gap junctions contain one major protein, calculated from the cDNA to be 32 kd in size (Kumar and Gilula, 1986; Paul, 1986) with the rat and human proteins being similar in sequence (Kumar and Gilula, 1986). The localization of this protein as a component of the gap junction has been demonstrated by electron microscopic immunolocalization procedures using both polyclonal and monoclonal antibodies (Dermietzel *et al.*, 1984; Paul, 1986; Young *et al.*, 1987; Zimmer *et al.*, 1987; Dermietzel *et al.*, 1987). Furthermore, the contribution this protein can make towards forming a functional channel has been demonstrated by the *in vitro* reconstitution of the purified protein into an artificial black lipid membrane system and the subsequent inhibition of its channel conductance by antibodies to the 32-kd junction protein (Young *et al.*, 1987).

While the antibodies produced against isolated junctions or electroeluted junction protein have been extremely useful, they have the disadvantage of being directed against multiple, unknown determinants within the junction protein. Monoclonal antibodies have also been produced to the rat liver gap junction (Stevenson *et al.*, 1986; Dermietzel *et al.*, 1987); however, the specific epitopes which they recognize have not been reported. In addition, all of the junction antibodies that have been produced appear to interact exclusively with determinants located on the cytoplasmic surface of the junctional membranes. Antibodies directed against the 'gap' portion of the polypeptide or against a transmembrane domain have not been reported. The availabilty of the entire amino acid sequence for the junction protein, the rapid synthesis of large numbers of diverse peptides (Houghten, 1985), and the application of peptide antibody procedures (Bulinski, 1986; Walter, 1986) offers the opportunity to overcome some of the apparent limitations.

In a previous study (Zimmer *et al.*, 1987), we applied an integrated approach utilizing protease digests and immunoelectron microscopic localizations with gap junction protein antibodies to obtain some information on the topological organization of the 32-kd liver protein in the intact junction. This information has been useful for understanding the integration of the primary amino acid sequence, obtained by cDNA analysis, into the intact junction membrane structure. The present study has been designed to extend the study on the topological organization of this protein by utilizing site-directed antibodies. For this study, virtually the entire length of the human liver gap junction protein has been synthesized as 18 peptides which have been used for the production of site-specific antibodies against defined regions of the protein. The synthesis of these peptides, the generation and characterization of the peptide antisera, and their use for the ultrastructural immunolocalization of gap junction epitopes is reported. These data have been integrated with

Table I. Synthetic peptides and peptide antibody reactivity

Peptide[a]	Residue assignment[b]	Sequence[b]	Relative reactivity[c] versus	
			Peptide[d]	Protein[e]
A	1–13	MNWTGLYTLLSGVC[f]	–	–
B	6–17	LYTLLSGVNRHSC	++	+
C	18–37	TAIGRVWLSVIFIFRIMVLV	–	–
D	38–53	VAAESVWGDEKSSFIC	+++	+
E	54–70	NTLQPGCNSVCYDQFFP	++++	+++
F	64–78	CYDQFFPISHVRLWS	+	+++
G	76–95	LWSLQLILVSTPALLVAMHV	–	–
H	96–110	CAHQQHIEKKMLRLEG	++++	++
I	103–115	CKKMLRLEGHGDPL	++++	++
J	111–125	HGDPLHLEEVKRHKVC	++	++++
K	126–145	HISGTLWWTYVISVVFRLLE	–	–
L	147–166	AVFMYVFYLLYPGYAMVRLV	+++	+
M	167–172	KCDVYP	++	+
N[g]	167–172	KCEAFP	+++	+
O	179–193	CFVSRPTEKTVFTVF	+	+
P	201–216	CIILNVAEVVYLIIRA	–	–
Q	217–234	CARRAQRRSNPPSRKGSG	++	+++
R	239–257	LSPEYKQNEINKLLSEQDGC	+	++++
S	262–280	ILRRSPGTGAGLAEKSDRC	+	++++
No.4[h]			+[i]	++

[a]Code letter used to identify these peptides throughout the paper.
[b]Based on sequence determined for a cDNA clone of the human liver gap junction protein as reported in Kumar and Gilula (1986).
[c]Diethylamine-eluted antibodies affinity purified against the native protein as described in Materials and methods.
[d]Based on the minimum concentration of peptide detected by affinity-purified antibodies (1:67) in a dot blot assay (see Materials and methods) where + = 1 μg peptide, ++ = 100 ng peptide, +++ = 10 ng peptide, ++++ = 1 ng peptide. – = no antisera reactivity to peptide detected (even though there was a positive response to KLH).
[e]Based on the relative strength of binding of affinity-purified antibodies (1:50) to Western blots of 2.4 μg purified 32-kd rat liver gap junction protein (see Materials and methods) where + = weakly reactive (generally to major band at 32 kd only), up to ++++ = very strong reactivity to both monomeric and multimeric bands. – = no antisera reactivity to protein detected.
[f]C denotes cysteine not present in the predicted sequence but added to either the N- or C-terminus in order to facilitate coupling to KLH.
[g]Rat sequence for this segment of the 32-kd liver junction protein.
[h]Rabbit antibodies raised against the electroeluted 32-kd rat liver gap junction protein (Warner *et al.*, 1984).
[i]Q peptide used for screening peptide reactivity.

a hydropathy analysis of three different gap junction protein sequences and the low resolution structural details to develop a model for gap junction protein folding.

Results

Characterization of individual site-directed antibodies

Eighteen peptides were synthesized based on the human liver gap junction protein sequence (Kumar and Gilula, 1986). The position of each peptide in the protein and its amino acid sequence is listed in Table I. The peptides were assigned letters A–S based on their sequential position from the amino to the carboxyl terminus of the protein. One peptide (N) that was synthesized is identical to peptide M except that it contains a three-amino-acid change based on the rat sequence (Kumar and Gilula, 1986; Paul, 1986). In addition, eight of the peptides (A, B, E, F, G, H, I and J) contained regions of overlapping sequence. Combined, these peptides covered 91.2% of the entire amino acid sequence of the human (and rat) liver gap junction protein.

The peptides were used to immunize two rabbits per peptide and the elicited antisera were screened for reactivity with both the immunizing peptide and the junction protein. The relative reactivities of the peptide antibodies are shown in Table I. Five peptides (A, C, G, K and P) did not elicit peptide-specific responses, even though each of the antibodies did react strongly to the carrier protein (KLH), suggesting that these particular peptides are poor immunogens. In order to use the peptide antibodies for structural, and eventually functional, analysis of the gap junction, all the antibodies were affinity purified against the rat liver gap junction protein rather than their respective peptides. Many of the peptide antibodies that gave a strong response to their corresponding peptide (as determined by a dot blot assay) had a relatively weak interaction with gap junction protein (as assayed by immuno-blot analysis). These peptide antisera may be detecting a determinant (conformation or sequence) that is more accessible in the peptide than in the protein. However, a number of peptide antibodies (J, Q, R and S) gave a stronger signal to the gap junction protein than to the peptides. This may be due to the fact that the peptide antibodies were affinity purified against the gap junction protein, resulting in an enrichment for antibodies that recognize conformational determinants present in the protein but not in the peptides.

The reactivity of each responding rabbit to a screen of all of the peptides is shown in Figure 1. The peptides were adsorbed to nitrocellulose in the horizontal dimension. After blocking with BSA, the nitrocellulose was incubated with peptide antisera in the vertical dimension. The first lane shows a screen of all the peptides, together with antisera raised against the rat liver gap junction protein that has been used in previous studies (Warner *et al.*, 1984; Fraser *et al.*, 1987; Lee *et al.*, 1987; Young *et al.*, 1987; Zimmer *et al.*,

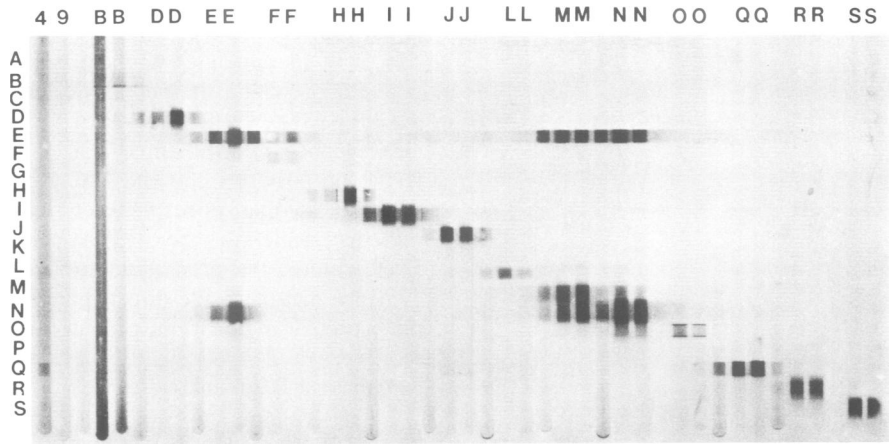

Fig. 1. Interaction of peptide antisera with peptides. Peptides A–S (20 μg/lane) were added in the horizontal dimension (left labels). Individual peptide antisera, shown in the vertical dimension, were then screened against all 19 peptides. Each antisera lane is labeled in duplicate along the top of the figure, representing the response of duplicate rabbits immunized successfully with the individual peptides. Five peptides (A, C, G, K and P) did not elicit peptide-specific responses, and therefore were not screened in this assay. The first two lanes on the left demonstrate screens of the peptides with rabbit antisera raised against the electroeluted 32-kd rat liver gap junction protein (No. 4) (Warner *et al.*, 1984) and the β-chain of fibrinogen (No. 9). See Materials and methods for experimental details.

1987). This antisera, which is referred to as No. 4, clearly recognized the Q peptide. In the next lane, antisera against the β-chain of rat fibrinogen (referred to as No. 9) did not recognize any of the peptides, and thus served as a non-specific control. The remaining lanes contained antisera from each pair of rabbits that produced a response. The specificity of these antisera to their corresponding peptides was readily apparent with most sera only reacting with the peptide used for immunization (although there was some cross-reactivity where peptides have overlapping sequences). However, the E peptide antisera as well as the N and M peptide antisera cross-reacted with each other's peptides, as well as their own. The lack of direct sequence correlation between these two peptide regions suggests that these antisera may be recognizing related conformational determinants on both peptides.

The peptide antibodies were also tested for their ability to recognize the rat liver gap junction protein by immunoblotting. Figures 2 and 3 contain an example of an immunoblot analysis using the four peptide antibodies (B, D, J and Q) that have been used for immunolocalizations in this study. In each case, the immune antibodies specifically recognized the rat liver gap junction protein that had been separated on SDS–polyacrylamide gels. A similar interaction was also observed using peptide S antibodies (Figure 3, panel A). In fact, all of the peptide antibodies that reacted with their corresponding peptide also interacted with the rat liver gap junction protein, albeit with different affinities (Table I). This is not surprising since the antibodies were purified against the gap junction protein.

Interaction of site-directed antibodies with protease-digested junctions

The site-specific properties of the peptide antibodies were initially determined by their application to specific proteolytic fragments. Figure 3 (panel B) contains a lysine-specific endoproteinase digestion of isolated intact gap junctions, followed by immunoblotting with different peptide antibodies. It was previously demonstrated (Zimmer *et al.*, 1987) that this protease generates two recoverable fragments when applied to isolated gap junctions: one at 17 kd, and another at 10 kd,

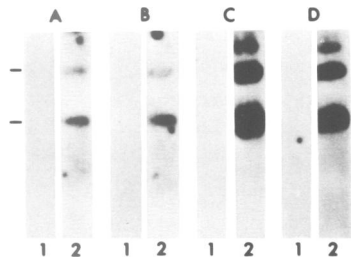

Fig. 2. Immunoblotting of isolated rat liver gap junction protein with peptide antibodies. Purified rat liver gap junction protein (17.5 μg/lane) was electrophoresed on an 18% SDS–polyacrylamide gel, transferred to nitrocellulose, and processed as described in Materials and methods. In each panel **lane 1** was incubated with preimmune IgG and **lane 2** with immune IgG. The panels illustrate the binding observed with B (1:50; **panel A**), D (1:50; **panel B**), J (1:100; **panel C**), and Q (1:50, **panel D**) peptide antibodies. The two markers on the left represent the positions of the junction protein monomer (32 kd) and dimer (64 kd).

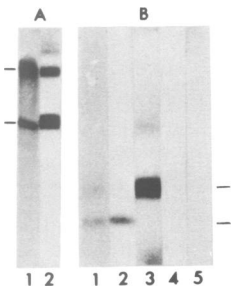

Fig. 3. Interaction of peptide antibodies with untreated and lysine-specific endoproteinase digests of intact gap junctions. The control (untreated; **panel A**) and endoproteinase Lys-C-treated (**panel B**) gap junctions were electrophoresed on 15% SDS–polyacrylamide gels. **Panel A** contains the Coomassie-blue-stained profile of the untreated gap junction protein (**lane 1**) and the immunoblot profile of the untreated protein after incubation with the S peptide antibodies (**lane 2**). **Panel B** contains the Coomassie-blue-stained profile of the Lys-protease fragments (**lane 1**) and the results of incubation of the digest with D (**lane 2**), Q (**lane 3**), R (**lane 4**), and S (**lane 5**) peptide antibodies. The two markers on the left of **panel A** represent the positions of the junction protein monomer (32 kd) and dimer (64 kd). The two markers on the right of **panel B** represent the positions of the 17-kd and 10-kd protease fragments.

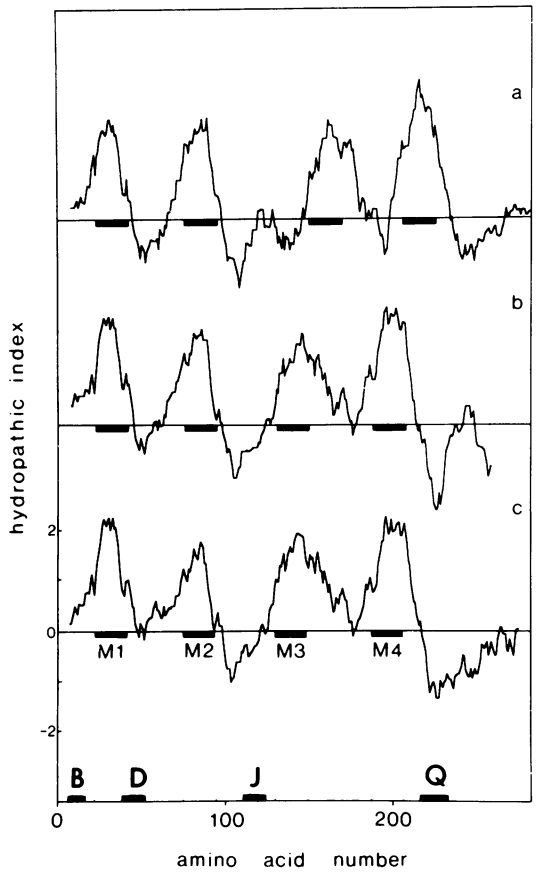

Fig. 4. Hydropathy plots (19 amino acid window) (Kyte and Doolittle, 1982) of gap junction polypeptides: (**a**) first 283 amino acid residues of the 43-kd polypeptide from rat heart (Beyer *et al.*, 1987); (**b**) 30-kd polypeptide from *Xenopus* embryos and liver (Gimlich *et al.*, 1988); (**c**) 32-kd polypeptide from human liver (Kumar and Gilula, 1986). B, D, J and Q denote the stretches 6–17, 38–53, 111–125 and 217–234 of the human liver sequence from which matching peptides were synthesized to derive the corresponding antibody probes. M1–4 denote the regions corresponding to the putative membrane-spanning α-helical segments (Figure 6); bars identify the positions of the homologous sequences from rat heart and *Xenopus* liver. The initial 230 residues contain ~45% (comparing the liver sequences with the heart) and 80% (comparing the human and *Xenopus* liver sequences) identical amino acids.

with the gap junction antibodies (No. 4) binding to the larger 17-kd fragment. It was shown by microsequencing of these two proteolytic fragments that the 10-kd fragment contained the same amino terminus found in the intact 32-kd protein, whereas the amino terminus of the 17-kd fragment contained the sequence following the lysine at position 124 in the gap junction protein sequence. However, in that study it could not be determined whether additional cleavage sites, if any, were present since the gap junction protein and its fragments appear to run anomalously on SDS gels (Green *et al.*, 1988). The orginal observations have been extended in this study by using the site-directed antibodies to determine and define the location of additional cleavage sites for the lysine-specific protease. Figure 3 shows binding of the peptide D antibodies to the 10-kd fragment, whereas the peptide Q antibodies interacted strongly with the 17-kd fragment generated by the action of this protease on gap junctions. Both the extreme C-terminal peptide S and peptide R antibodies did not recognize any proteolytic fragment. In addition, both F and J peptide antibodies bound to the 10-kd fragment (unpublished data). Together, these results indicate the presence of one cleavage site for the lysine-specific protease at position 124, with a second cleavage site at a lysine residue located between or within the peptide R and Q domains (i.e. at position 231, 244 or 250). It is, of course, possible that cleavage occurs at all three lysine residues as well as at lysines in positions 260 and 276. Nevertheless, this study does indicate that lysines between residues 124 and 231 are not accessible to the lysine-specific endoproteinase. Furthermore, the proteolytic fragment analysis provides evidence that these site-directed antibodies are specific for the particular region of the protein from which their peptide sequence was derived, since in all cases the antibodies bound to only one fragment.

Topological localization

On the basis of the above-determined specificities for these site-directed antibodies, the analysis was extended to examine the topological localization of specific regions of the junction protein using indirect immunoelectron microscopy on isolated gap junctions. The analysis was confined to selected regions in the human liver sequence that flanked the four hydrophobic domains (peptide regions B, D, J and Q) predicted from hydropathy plots (Figure 4). The antibodies were affinity purified against rat liver gap junction protein and used for electron microscopic immunolabeling of both intact and alkaline pH–urea split gap junctions with a secondary reagent consisting of goat anti-rabbit IgG conjugated to gold particles (Zimmer *et al.*, 1987). The results are shown in Figure 5 where each row of three micrographs represents the binding of different peptide antibodies. The left micrograph in each row shows the background labeling observed with preimmune antibodies. The middle and right figures demonstrate the labeling observed with peptide antibodies on intact and split gap junctions (to expose both the extracellular and cytoplasmic surfaces) respectively. J peptide antibodies labeled the intact junctions, which have only the cytoplasmic surfaces of the junctions exposed (Figure 5, upper row). This confirmed earlier results obtained with antibodies (No. 4) to the junction protein (Zimmer *et al.*, 1987) indicating that the region between the two pairs of hydrophobic domains is located on the cytoplasmic surface. The convex surfaces of the split junctions were labeled, suggesting that the concave face represented the exposed extracellular side. Next, the B and Q peptide antibodies were applied to the intact and split jucntions. The results (Figure 5, middle rows) were essentially the same as for the J peptide antibody, showing that the N- and C-terminal segments flanking the four hydrophobic domains were also located on the cytoplasmic side. In contrast, application of the D peptide antibodies to the intact and spit junctions labeled the concave side only in the split junctions, with no detectable labeling to the intact junctions (Figure 5, lower row). Therefore, this segment, between the first two hydrophobic domains, must be located on the extracellular or 'gap' surface.

Discussion

Properties of the antibodies and junction protein

This study reports the development, characterization and application of peptide antibodies generated against synthetic peptides based on the known amino acid sequence of the

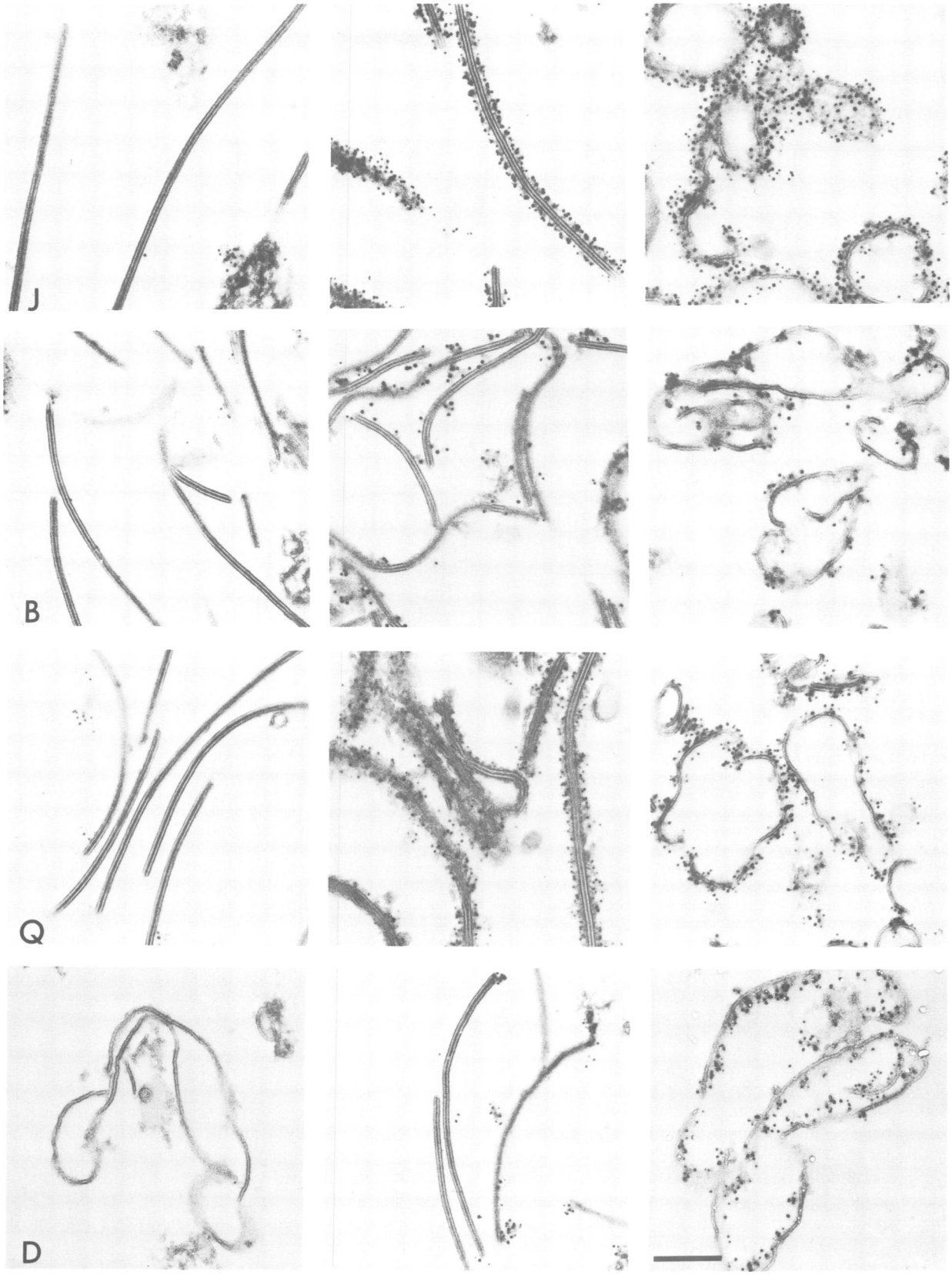

Fig. 5. Electron microscopic immunolabeling of intact and alkali−urea split gap junctions with site-directed antibodies. Isolated intact rat liver gap junctions were split using an alkaline pH−urea procedure (Zimmer *et al.*, 1987) and then both intact and split junction membranes were incubated in either affinity-purified preimmune IgGs or gap junction protein affinity-purified IgGs as described in Materials and methods. Each row illustrates the binding observed with the following peptide antibodies: row J, J peptide antibodies; row B, B peptide antibodies; row Q, Q peptide antibodies; and row D, D peptide antibodies. In each row, the left micrograph represents the binding of preimmune antibodies to either intact (J, B and Q) or split (D) junctions. The binding of peptide antibodies is shown to both intact (center micrographs) and split (right micrographs) junctions for each of the four peptide antibodies. All magnifications × 76 000 (bar in the bottom right micrograph represents 0.1 μm).

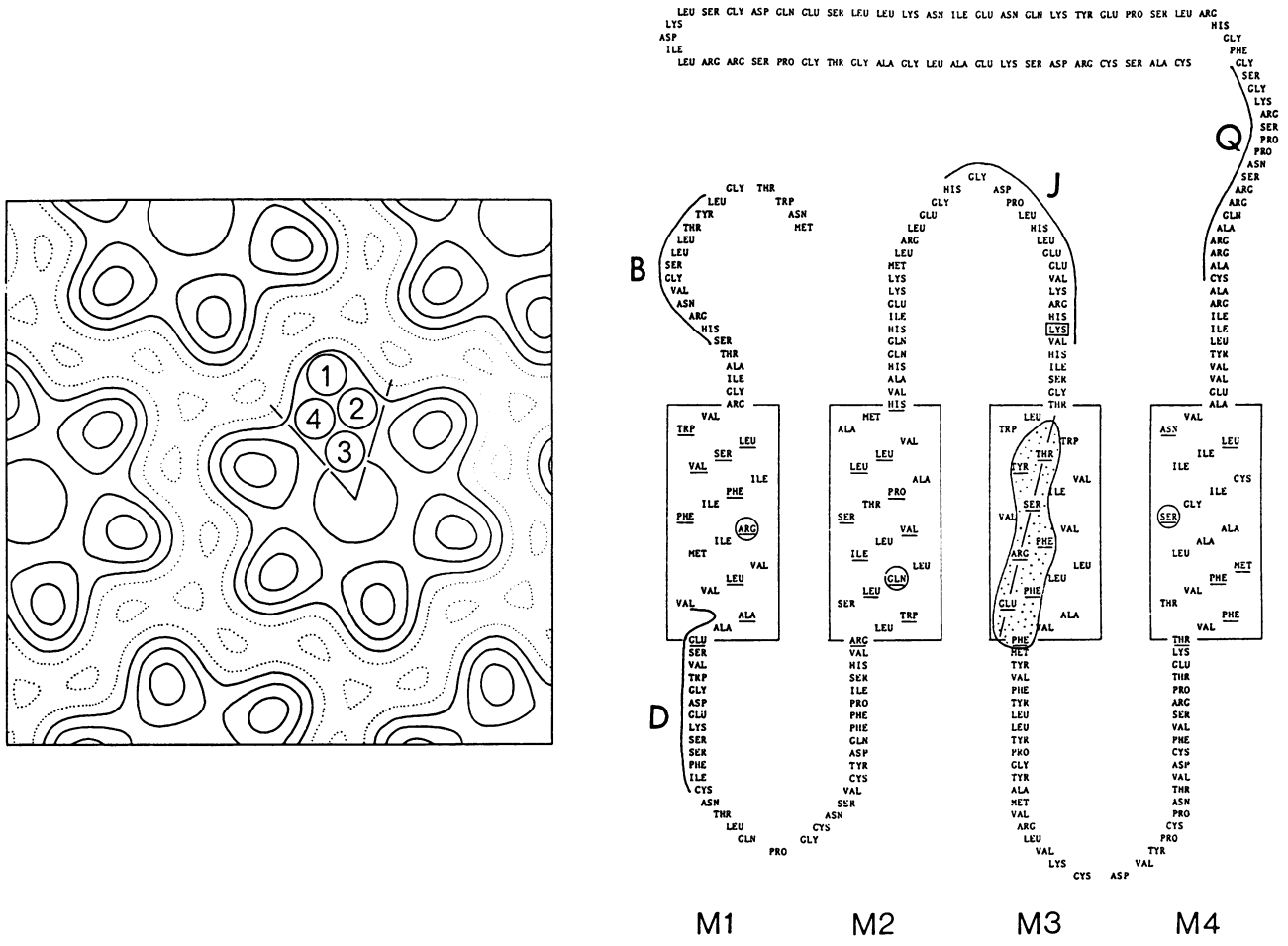

Fig. 6. (a) Section within the hydrophobic core of the lipid bilayer of the protein oligomer and central ion channel, determined by electron image analysis of isolated gap junctions (Unwin and Ennis, 1984). The four circles, representing α-helical segments within one subunit, have a diameter corresponding to 10 Å. The numbers indicate the arrangement of the membrane-spanning segments M1–4 (viewed from the cytoplasmic side) assuming that M3 forms the wall of the channel and that the structure is organized according to the chirality of the four-α-helical bundle motif (Weber and Salemme, 1980). (b) Topology of the human liver gap junction polypeptide (cytoplasmic surface uppermost), according to the immunochemical and structural results. M1, M2, M3 and M4 denote the four putative α-helical segments spanning the hydrophobic core of the bilayer; helices M2 and M4, which are exposed to similar environments, mostly contain similar amino acids in equivalent positions. B, D, J and Q denote the location of the sequences specific for the antibody probes used in the EM immunolabeling studies (Figure 5); the boxed residue (LYS 124) corresponds to the known cleavage site of a lysine-specific protease (Zimmer *et al.*, 1987). Amino acids within the putative α-helical segments which are conserved between all three sequences are underlined; they include the line of polar and bordering aromatic residues (shaded) proposed to be involved in forming the wall of the channel, and polar residues (circled) which model-building studies suggest are likely to be important in stabilizing the three-dimensional structure by hydrogen-bond interactions. The heart sequence contains a glycine instead of a threonine (THR 130) at the top of the line of polar residues. The basic and acidic residues within this line (ARG 142 and GLU 146) could form an intra-helical ion-pair, but are also in a position to interact with equivalent residues on the helices of neighboring subunits. The *Xenopus* liver and rat heart sequences have respectively 82 and 49% homology with the human liver sequence within the putative α-helical segments, 71 and 57% homology in extracellular domains, and 79 and 25% in matching regions of the cytoplasmic domains.

32-kd human (and rat) liver gap junction protein. The peptide antibodies were shown to recognize not only their specific peptide(s) but also the antigenic determinant(s) present in the native rat liver junction protein. The ability to screen the peptide antibodies against defined segments of almost the entire sequence of the gap junction protein using the synthetic peptides, as well as against proteolytic fragments derived from the native junction protein itself, made it possible to establish the specificity of the peptide antibodies to specific regions of the protein.

In order to use the peptide antibodies for structural, and eventually functional, analysis of the gap junction, all the antibodies were affinity purified against the rat liver gap junction protein, and subsequently depleted of carrier protein (KLH) reactivity. This is important since it was observed that some peptide antibodies initially affinity purified against their corresponding peptide reacted weakly with the gap junction protein. By using the gap junction protein as the affinity absorbent, antibodies with high affinity for the protein were enriched in the preparation. Furthermore, by removing antibodies against KLH, it was less likely that peptides or protein regions which may share antigenic determinants with KLH would cross-react.

Rabbit antibodies prepared against rat liver gap junction protein have been used previously to study the structure and function of the gap junction (Warner *et al.*, 1984; Young *et al.*, 1987; Zimmer *et al.*, 1987). In this study it has been demonstrated that this polyclonal reagent, No. 4, reacts specifically with one peptide, Q. In addition, it is known that these antibodies recognize at least two antigenic

determinants, one of which may be conformational (Zimmer *et al.*, 1987). Consequently, it is not known whether binding of antibodies to the region of peptide Q on the gap junction protein or binding of antibodies to the conformational determinant(s) is responsible for the observed effects on gap junction function (Warner *et al.*, 1984; Fraser *et al.*, 1987; Lee *et al.*, 1987).

Since all of the 14 positive peptide antibodies, covering ~63% of the entire amino acid sequence of the rat liver gap junction protein, recognized the native junction protein, this confirms that this protein contains the sequence deduced from the cDNA (Kumar and Gilula, 1986). The recognition of the junction protein (Figures 2 and 3) by peptide antibodies specific to regions close to both the N-terminus (peptide B) and the extreme C-terminus (peptide S) is consistent with the predicted mol. wt of 32 000 daltons for the human liver gap junction polypeptide. Furthermore, these data indicate that the full-length product is present in the purified gap junction fraction isolated from rat livers.

In the electron microscopy experiments, the antibody concentrations used were an order of magnitude higher than those used for the immunoblots, and thus there is a possibility of cross-reactivity to other peptides. However, this is not the case for 12 of the peptide antibodies described in this report since the peptide antibodies at 1:5 dilution only reacted with their corresponding peptide to generate a profile similar to that shown in Figure 1 (unpublished data). Furthermore, it is important to note that, at these high antibody concentrations, peptide E antibodies also cross-reacted with peptides H, I, M and N and peptide J antibodies cross-reacted with peptide S. Since peptide E antibodies were not used in this study for immunolocalizations, and since the peptide J sequence has been shown to be located in the cytoplasm by its access to proteolytic enzymes (Zimmer *et al.*, 1987), the interpretation of the immunolocalization data is not complicated by this cross-reactivity.

While the conditions used for splitting the gap junctions are chemically harsh, involving the use of alkaline pH and urea, it is unlikely that these conditions would cause transmembrane segments to become completely reoriented or eliminated from the membrane. Indeed, the method used for isolation of gap junctions involves the use of sodium hydroxide (Hertzberg, 1984). Furthermore, the splitting procedure that has been applied is reversible; i.e. the single membranes can reassociate quite readily upon lowering the pH if the single membranes are not transformed into vesicles or small fragments by harsh physical treatments (unpublished observations). Thus, it is quite likely that regions that are localized to one surface would remain on that surface, and be accessible to the reagents applied.

Transmembrane topology of the junction protein

Recently, the complete amino acid sequence for a mammalian heart gap junction protein (Beyer *et al.*, 1987) and a 30-kd gap junction protein from *Xenopus* embryo and liver (Gimlich *et al.*, 1988) have also been deduced by recombinant DNA methods. Inspection of these three sequences indicates that the charged and polar C-terminal portions vary substantially, while the 1–230 N-terminal portions are similar. This is reflected in the strikingly similar hydropathy plot (Figure 4) for all the gap junction sequences using the algorithm of Kyte and Doolittle (1982). Four hydrophobic stretches can be identified, each ~20 amino acids in length.

In order to determine whether these domains span the membrane, electron microscopic immunolabeling of intact and alkaline pH–urea split gap junctions was carried out with four peptide antibodies that would recognize different domains of the protein as predicted by the hydropathy plots. The results of these studies suggested that the regions containing the B, J and Q sequences were on the cytoplasmic surface of the gap junction, while the D peptide sequence was exposed on the extracellular surface. In addition, lysine-specific protease digestion of isolated gap junctions and immunoblotting of the resulting fragments with the peptide antibodies indicated that antibodies to peptides R and S do not detect any additional proteolytic fragments, consistent with these regions being accessible to the protease, and consequently located on the cytoplasmic surface.

The fact that the determinants for the N- and extreme C-termini have been localized to the cytoplasmic surface is compatible with the observation that transmembrane proteins devoid of a cleavable signal peptide usually have their N-terminus on the cytoplasmic side of the membranes (Engleman and Steitz, 1981). The gap junction protein in contrast to many other transmembrane proteins (Kreil, 1981) does not possess a sequence characteristic of signal peptides and also is not glycosylated. In addition, it is clear that the polypeptide must traverse the bilayer an even number of times, at least twice but more likely four or six times. The possibility of four transmembrane segments is supported by the proteolytic fragmentation data indicating that the peptide J and S sequences are located on the cytoplasmic surface, and the immunolabeling localization of the N-terminus to the cytoplasmic surface as well as the localization of the peptide D sequence to the extracellular surface. Furthermore, the cross-sectional area of the transmembrane portion of the protein subunit, from electron image maps is ~500 Å^2 (Figure 6a) (Unwin and Ennis, 1984). This would be enough space to accommodate four closely packed α-helical rods, but inconsistent with a larger even number such as six or a smaller even number such as two. Therefore, we conclude that the correct number of traverses is most likely four (as suggested, but not proven by the hydropathy plots).

Figure 6b shows this topology based on the human liver sequence (Kumar and Gilula, 1986), assuming that the membrane-spanning segments correspond to the peaks in the hydrophobic domains and are α-helices. The partitioning of residues between the hydrophobic core of the bilayer (80 residues) and the hydrophilic space on the extracellular side (71 residues) agrees with the details in electron image maps, which show matter extending 15–20 Å from the membrane surface (Unwin and Ennis, 1984). However, there are a greater number of residues on the cytoplasmic side than the structural results suggest, possibly because the C-terminal domain is disordered and therefore does not contribute significantly to the coherent diffraction. The transmembrane segments are terminated mainly by charged residues, and on the extracellular side there is only a weak net negative charge (−1), contributing a minimal electrostatic barrier for end-to-end association between oligomers in the two apposed membranes. Both extracellular loops are of similar lengths and contain a string of three cysteine residues, with the two end cysteines of each string placed 11 residues apart. The loops may contain similar conformational elements since some peptides from the different loops have been observed to cross react with each other.

The location of the known proteolytic sites are consistent with this topology. It has been shown previously that trypsin- and lysine-specific endoproteinase cleave at the lysine at residue 124 (Zimmer *et al.*, 1987), and the present study locates further cleavage site(s) for the lysine-specific endoproteinase beyond residue 230. Since it is unlikely that these proteases can cleave protein in the extracellular 'gap' or membrane in intact isolated gap junctions, these regions must be exposed to the cytoplasm as predicted by the topological scheme. In addition, the inaccessibility of the two lysine residues (Lys 167 and Lys 187) between these two cleavage sites to cleavage by proteases would support locating this segment on the extracellular surface. Also, it has been pointed out that a number of serine, threonine and tyrosine residues are in consensus sequences for phosphorylation (Kumar and Gilula, 1986), and the topology predicts that most of these sites are located on the cytoplasmic surface of the membrane as would be required.

Hypothetical folding model

Structural data on several known membrane proteins have shown that regions spanning the lipid bilayer are usually α-helical (Henderson and Unwin, 1975; Michel *et al.*, 1986). We presume that the four hydrophobic domains, ~20 amino acids each in length, revealed by the hydropathy analysis and labeled M1–M4 (Figure 6b), correspond to such α-helical segments.

Helix M3 is unique, containing a line of polar amino acids bordered by an unusually high proportion of aromatic residues. These polar and aromatic residues appear to have an important functional role since they are conserved between all three sequences. It is plausible that the polar amino acids form the wall of the channel by the association of six copies of helix M3. The bulky aromatic residues would lie alongside to facilitate the close packing of the surrounding subunits and to maintain the required 60° sector angle. The line slopes up to the right, but would align more nearly parallel to the axis of the channel if the helix were tilted to the left–the sense required for it to be part of a close-packed α-helical assembly (Crick, 1953).

The folding pattern for this protein could correspond to the recurring structural motif found among soluble proteins, consisting of an antiparallel arrangement of four α-helices connected sequentially and angled slightly to form a left-handed bundle (Weber and Salemme, 1980). It should be noted that the two helices which then would be in the most equivalent environments (helices M2 and M4; see Figure 6a) contain a similar pattern of polar, aliphatic and aromatic residues; i.e. there is a degree of structural symmetry which is characteristic of the four-α-helical bundle motif (Hendrickson and Ward, 1977; McLachlan *et al.*, 1980). Furthermore, a strongly modulated surface would be created by the close-packing of such bundles in a ring, compatible with the details seen in electron image maps (Unwin and Ennis, 1984).

In conclusion, a scheme is presented for the topological organization of the gap junction protein from the human liver which is consistent with the known structural and biochemical features of the gap junction. The homology of this polypeptide with the sequence of other potential gap junction polypeptides (Beyer *et al.*, 1987; Gimlich *et al.*, 1988) suggests a common folding pattern for the gap junction protein family. The model described in this study provides a basis for future experimentation on studying the structure–function relationship of the gap junction protein.

Materials and methods

Synthesis of peptides

Eighteen peptides (Table I) were synthesized according to the amino acid sequence of the human liver gap junction protein predicted from the nucleotide sequence of a cDNA clone (Kumar and Gilula, 1986). One peptide (N) was synthesized based on the rat liver gap junction specific sequence (Kumar and Gilula, 1986; Paul, 1986). Six of the peptides were synthesized with a cysteine added to either their amino (H and I) or carboxyl (A, B, J and R) termini in order to facilitate attachment to the carrier protein. All of the peptides were synthesized using the simultaneous multiple-peptide synthesis procedure developed by Houghten (1985). Purity of each peptide was assessed by high performance liquid chromatography.

Coupling of synthetic peptides to carrier protein

Fifteen of the synthetic peptides were coupled to the carrier protein, keyhole limpet hemocyanin (KLH), through either their natural or added cysteine, using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as the coupling reagent (Lui *et al.*, 1979). The protocol used for MBS-coupling has been described previously by Green *et al.* (1982).

Four of the peptides (C, G, K and L) did not contain any natural cysteine residues nor was a cysteine added to their synthesized sequences. These peptides were coupled to KLH with glutaraldehyde using the procedure of Avrameas and Ternyck (1969).

Preparation of rat liver gap junctions

Rat liver plasma membranes and intact gap junctions were prepared using the alkali extraction procedure of Hertzberg (1984) as modified by Zimmer *et al.* (1987). When indicated, intact gap junctions were split using 8 M urea at alkaline pH (Zimmer *et al.*, 1987). Briefly, isolated gap junctions were pelleted and resuspended in 10 mM Tris buffer (adjusted to pH 12 with NaOH) containing 8 M urea for 1 h at 37°C.

Preparation of peptide antisera

Rabbits were immunized according to the following schedule: on day 0, the peptide-KLH (200 μg) in complete Freund's adjuvant (1:1) was injected s.c.; on day 14, 200 μg in incomplete Freund's adjuvant (1:1) s.c.; and finally, 200 μg with 4 mg alum i.p. on day 21. Animals were bled weekly beginning on day 28. Rabbits were boosted with a mixture of 50 μg peptide–KLH and 4 mg alum, and bled weekly beginning 7 days later.

Affinity purification of peptide antibodies

All affinity purifications were carried out using Acti-Disks (Amerace, Hackettstown, NJ): either Acti-Disks containing prebound Protein A, or glutaraldehyde-activated Acti-Disks to which electroeluted 32-kd rat liver gap junction protein was covalently bound. Junction protein was bound by the following method. Electroeluted 32-kd protein was dialyzed overnight against 10 mM sodium bicarbonate buffer with 0.01% SDS. After dialysis, phosphate-buffered saline was added 1:1 (v/v) so that the final solution contained 0.01 M sodium phosphate (pH 7.4) with 0.15 M sodium chloride. The Acti-Disk was equilibrated with 0.01 M sodium phosphate (pH 7.4) with 0.15 M sodium chloride for 30 min. The dialyzed eluted junction protein with buffer was then added to the closed Acti-Disk system and allowed to cycle for 1 h at room temperature. The unoccupied active sites were blocked by recycling 10 ml of 0.1 M glycine (pH 7.4) for 1 h. The 32-kd protein–Acti-Disk was then extensively washed with 0.01 M sodium phosphate buffer (pH 7.2).

Preimmune immunoglobulins were purified from sera obtained from rabbits prior to immunization by affinity adsorption to a protein A–Acti-Disk. Sodium phosphate was added to the sera to a final concentration of 0.01 M (pH 7.2) and filtered through a 0.45-μm filter before addition to the Acti-Disk. Phosphate-buffered sera were recycled for 30 min through the Acti-Disk in a closed system. After collecting the flow-through, the Acti-Disk was washed with 0.01 M sodium phosphate buffer (pH 7.2). Bound immunoglobulins were then eluted from the Acti-Disk with 0.1 M glycine (pH 2.7). The antibody peak was pooled together, dialyzed overnight against 7.5 mM KCl, 0.5 mM Tris (pH 7.5) at 4°C, and then concentrated 10-fold, first with Aquacide II (Calbiochem, La Jolla, CA) and then in a Speed Vac concentrator (Savant, Farmingdale, NY). The protein concentration was determined using a BCA protein assay (Pierce, Rockford, IL) and the final concentration adjusted to 1.5 mg/ml.

Peptide antibodies were affinity purified using a 32-kd junction

protein–Acti-Disk. Immune sera were prepared and recycled through the Acti-Disk as already described for the preimmune sera. Bound immunoglobulins were then eluted in a two-step procedure with 0.15 M NaCl followed by 0.03 M diethylamine (DEA) in 0.15 M NaCl (pH 11.8). The two IgG antibody peaks (NaCl-eluted and DEA-eluted) were processed further and stored separately. The pooled IgG peaks were then passed separately over a KLH–Acti-Disk and the flow-through collected, dialyzed, concentrated and standardized as described for the preimmune IgG. Unless specified, DEA-eluted affinity-purified antibodies were used in all experiments.

Enzymatic digestions

Gap junction protein concentrations were determined by a BCA protein assay and the protein concentration of the intact gap junction preparation was adjusted to 1.0–1.5 mg/ml prior to digestion. Endoproteinase Lys-C (Boehringer-Mannheim) was added from a 2 mg/ml stock in two equal doses, one at time 0 and the other after 1–2 h, resulting in a final enzyme/substrate ratio of 1:25. All digestions were done in 0.1 M phosphate buffer (pH 8.0) at 37°C for 16 h. The reactions were terminated by the addition of SDS–gel electrophoresis sample buffer, and immediately loaded onto SDS–polyacrylamide gels for analysis.

Polyacrylamide gel electrophoresis

SDS–PAGE was carried out in these studies according to the procedure of Laemmeli (1970) as modified in Zimmer *et al.* (1987).

Immunoblotting

Peptide antibodies were screened for reactivity and specificity against peptides, carrier protein (KLH) and rat liver gap junction protein by a variety of immunoblotting studies. Relative reactivities were determined by dot blot assays. Solubilized peptides (1 μg to 1 ng) were dotted onto nitrocellulose strips, allowed to dry, and then blocked with 3% BSA in Tris-buffered saline (TBS) for 1 h. The strips were then incubated with their corresponding peptide antibody (affinity purified against the rat liver gap junction protein: 1:67 dilution in TBS) for 2 h. After extensive washing with TBS, the immunoblots were incubated with an [^{125}I]protein A secondary reagent (Amersham Corp.; 1:2000 dilution in TBS) and the binding visualized by autoradiography. The presence of anti-KLH antibodies in both the peptide responding and non-responding antisera (1:10 dilution) was confirmed by an identical dot blot assay against 1 μg of KLH. All of the rabbits generated a response to the carrier protein KLH regardless of whether or not they produced an anti-peptide response.

Peptide antibody specificity for its immunizing peptide was assessed by a cross-immunoblot analysis using an Miniblotter II (Immunetics, Cambridge, MA). Solubilized peptides A–S (20 μg/lane) were first adsorbed to prewetted (in TBS) nitrocellulose for 2 h. The nitrocellulose was then blocked in 3% BSA in TBS (pH 7.5) for 1 h. The nitrocellulose was rotated 45° and remounted in the Miniblotter such that the wells were now perpendicular to the peptide lanes. The peptide antisera (1:10 dilution in TBS) from each responding rabbit were then added to an individual wells and incubated against all the peptides overnight at 4°C. Previously generated antisera to the electroeluted rat liver 32-kd gap junction protein (called No. 4), which has been shown to block channel conductance (Warner *et al.*, 1984; Young *et al.*, 1987), and to the β-chain of fibrinogen (called No. 9) were also screened (1:10 dilution in TBS) against all of the peptides in separate wells of the miniblotter. Antibody binding was detected by an alkaline-phosphatase-conjugated goat anti-rabbit IgG secondary reagent (1:7500 dilution in TBS) as suggested by the manufacturer (Promega Biotech).

Western transfers were carried out using a modification of the procedure of Towbin *et al.* (1979). Polyacrylamide gels were immediately placed in transfer buffer and the apparatus assembled. Transfer to 0.2-μm nitrocellulose paper (Schleicher and Schuell) was for 90–120 min with 55 V (constant voltage). Immunoblots were then blocked with 3% BSA in TBS for 1 h, washed in TBS and incubated with affinity-purified peptide antibodies (1:50 in TBS) overnight at 4°C. Antibody binding was visualized by autoradiography as described above.

Electron microscopic gold immunolabeling

Isolated rat liver gap junctions were incubated for 1 h at 37°C in either 10 mM Tris buffer (pH 12) for intact junctions or 10 mM Tris buffer (pH 12) containing 8 M urea for split junction membranes. The junctions were then pelleted, washed twice with 75 mM KCl, 5 mM Tris buffer (pH 7.5), pelleted again and incubated in either affinity-purified preimmune immunoglobulins or affinity-purified peptide antibodies (1:5) for 2 h at room temperature. After washing twice in buffer, antibody binding was visualized by incubation with goat anti-rabbit IgG conjugated to 5-nm gold particles (Janssen Pharmaceuticals) for 2 h at room temperature. Following extensive washing, the samples were processed for electron microscopy as described previously (Zimmer *et al.*, 1987).

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References

Avrameas,S. and Ternyck,T. (1969) *Immunochemistry*, **6**, 53–66.
Beyer,E.C., Paul,D.L. and Goodenough,D.A. (1987) *J. Cell Biol.*, **105**, 2621–2629.
Bulinski,J.C. (1986) *Int. Rev. Cytol.*, **103**, 281–302.
Crick,F.H.C. (1953) *Acta Crystallogr. A.*, **6**, 689–697.
Dermietzel,R., Leibstein,A., Frixen,U., Janssen-Timmen,U., Traub,O. and Willecke,K. (1984) *EMBO J.*, **3**, 2261–2270.
Dermietzel,R., Yancey,B., Janssen-Timmen,U., Traub,O., Willecke,K. and Revel,J.P. (1987) *J. Histochem. Cytochem.*, **35**, 387–392.
Engleman,D.M. and Steitz,T.A. (1981) *Cell*, **23**, 411–422.
Fraser,S.E., Green,C.R., Bode,H.R. and Gilula,N.B. (1987) *Science*, **237**, 49–55.
Gilula,N.B., Reeves,O.R. and Steinbach,A. (1972) *Nature*, **235**, 262–265.
Gimlich,R.L., Kumar,N.M. and Gilula,N.B. (1988) *J. Cell Biol.*, **107**, in press.
Green,C., Harfst,E., Gourdie,R.G. and Severs,N.J. (1988) *Proc. R. Soc. Lond.*, **233**, 165–174.
Green,N., Alexander,H, Olsen,A., Alexander,S., Shinnick,T.M., Sutcliffe,J.G. and Lerner,R.A. (1982) *Cell*, **28**, 477–487.
Henderson,D., Eibl,H. and Weber,K. (1979) *J. Mol. Biol.*, **132**, 193–218.
Henderson,R. and Unwin,P.N.T. (1975) *Nature*, **257**, 28–32.
Hendrickson,W.A. and Ward,K.B. (1977) *J. Biol. Chem.*, **252**, 3012–3018.
Hertzberg,E.L. (1984) *J. Biol. Chem.*, **259**, 9936–9943.
Hertzberg,E.L. and Gilula,N.B. (1979) *J. Biol. Chem.*, **254**, 2138–2147.
Houghten,R.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5131–5135.
Kistler,J., Christie,D. and Bullivant,S. (1988) *Nature*, **331**, 721–723.
Kriel,G.A. (1981) *Annu. Rev. Biochem.*, **50**, 317–348.
Kumar,N.M. and Gilula,N.B. (1986) *J. Cell Biol.*, **103**, 767–776.
Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.
Laemmli,U.K. (1970) *Nature*, **224**, 149–154.
Lee,S., Gilula,N.B. and Warner,A.E. (1987) *Cell*, **51**, 851–860.
Loewenstein,W.R. (1981) *Physiol. Rev.*, **61**, 829–913.
Lui,F.-T., Zinnecker,M., Hanaoka,T. and Katz,D.H. (1979) *Biochemistry*, **18**, 690–697.
Makowski,L., Casper,D.L.D., Phillips,W.C. and Goodenough,D.A. (1977) *J. Cell Biol.*, **74**, 629–645.
McLachlan,A.D., Bloomer,A.C. and Butler,P.J.G. (1980) *J. Mol. Biol.*, **136**, 203–224.
Michel,H., Weyer,K.A., Gruenberg,H., Dunger,I., Oesterhelt,D. and Lottspeich,F. (1986) *EMBO J.*, **5**, 1149–1158.
Nicholson,B., Dermietzel,R., Teplow,D., Traub,O., Willecke,K. and Revel,J.P. (1987) *Nature*, **329**, 732–734.
Paul,D. (1986) *J. Cell Biol.*, **103**, 123–134.
Stevenson,B., Siliciano,J., Mooseker,M. and Goodenough,D. (1986) *J. Cell Biol.*, **103**, 755–766.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Unwin,P.N.T. and Ennis,P.D. (1984) *Nature*, **307**, 609–613.
Unwin,P.N.T. and Zampighi,G. (1980) *Nature*, **283**, 545–549.
Walter,G. (1986) *J. Immunol. Methods*, **88**, 149–161.
Warner,A.E., Guthrie,S.C. and Gilula,N.B. (1984) *Nature*, **311**, 127–131.
Weber,P.C. and Salemme,F. (1980) *Nature*, **287**, 82–84.
Young,J.D.E., Cohn,Z.A. and Gilula,N.B. (1987) *Cell*, **48**, 733–743.
Zimmer,D.B., Green,C.R., Evans,W.H. and Gilula,N.B. (1987) *J. Biol. Chem.*, **262**, 7751–7763.

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