Topology of the M, 27,000 Liver Gap Junction Protein

CYTOPLASMIC LOCALIZATION OF AMINO- AND CARBOXYL TERMINI AND A HYDROPHILIC DOMAIN WHICH IS PROTEASE-HYPERSENSITIVE*

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Hydropathy analysis of the M, 27,000 rat liver gap junction protein sequence deduced from a cDNA clone has suggested the presence of four transmembrane segments (Paul, D. L. (1986) J. Cell Biol. 103, 123–134). In the present report, several features of the molecular topology of the protein were investigated by microsequence analysis of peptides generated by treatment of isolated gap junctions with a variety of proteases. Under the experimental conditions used, the proteases had access only to the portion of the M, 27,000 protein that was originally (in vivo) the cytoplasmic surface of the gap junction. Microsequencing of the peptides resolved the amino termini of the protein is disposed at or near the cytoplasmic surface of the gap junction, and that this surface also contains a protease-hyposensitive hydrophilic sequence between residues 109 and 123, presumably connecting the second and third transmembrane segments. Immunocytochemical localization of binding of monoclonal antibody to the carboxyl terminus of the protein is also localized to the cytoplasmic surface of the gap junction. No protease sensitivity was found in the hydrophilic sequences thought to connect either the first and second transmembrane segments or the third and fourth segments, supporting the model's prediction that some sequences face the narrow intercellular gap which cannot be penetrated by proteases.

Gap junctions are plasma membrane specializations, which form transmembrane channels that connect the cytoplasms of adjoining cells and permit cell-cell communication via the transfer of small molecules. In the case of rat liver, the gap junction appears to be made up of two hexameric arrays of protein subunits, i.e. two hemichannels, or connexons, one within the bilayer of each cell (1–4).

A single approximately M, 27,000 protein has been found by most investigators to be a major component of each subunit (5–9). An increasing body of evidence indicates that the protein forms an integral part of the gap junction's structure (10–13) and plays a central role in this unique form of direct intercellular communication (14–16). Structural studies utilizing x-ray diffraction and electron microscopic image-processing techniques (1–4), while providing some structural details, have not yet provided adequate resolution of this membrane structure to visualize the disposition of the peptide backbone or other atomic features. In principle, recent publication of the deduced primary structure of the rat liver gap junction protein (11, 17) and a hydropathy analysis (18) based upon the sequence data (11) have provided an additional, independent source of structural information.

By any consideration of the properties or structure of gap junctions, this protein should contain one hydrophobic domain (contacting the hydrocarbon region of the bilayer) and three hydrophilic domains, the transmembrane pore and the regions of the protein present at the cytoplasmic surface and facing the extracellular space at the gap. As for other membrane proteins, biochemical and immunological approaches may be used to distinguish the various domains of the gap junction protein. Unlike the situation for other membrane proteins, which are accessible to hydrophilic or bulky reagents on either side of the membrane bilayer, the close juxtaposition of extracellular domains of gap junctions along the narrow "gap" (~1.5 nm) between cells renders these regions inaccessible to macromolecular probes such as antibodies or proteases (19, 20). Hence, treatment of isolated, intact gap junctions with these probes involves only the binding and cleavage sites available on what was the cytoplasmic surface of the gap junction in vivo. Indeed, gap junctions retain most of their morphological characteristics even after extensive treatment with proteases (19, 20). Moreover, the punctate pattern of antibody binding, which localizes to cell peripheries, as observed by indirect immunofluorescence (7, 9–11) has been shown by immunoelectron microscopy to represent binding at the cytoplasmic surface of gap junctions (10–13, 15, 19).

In the series of experiments using proteases and antibodies on isolated gap junctions reported here, we have capitalized upon the protective nature of the narrow, intercellular gap (19, 20) to identify those segments of the protein protruding from the cytoplasmic surface. The data support the model suggested by hydropathy plots (11) in which the M, 27,000 gap junction protein spans the plasma membrane bilayer four times. They directly demonstrate, as was recently proposed (19), that both the amino and carboxyl termini of the M, 27,000 rat liver gap junction protein are located at or near the cytoplasmic surface of the gap junction, and identify a region within the molecule which is extremely sensitive to proteolytic cleavage.

EXPERIMENTAL PROCEDURES

Gap junctions were isolated subsequent to treatment of rat liver plasma membranes with 20 mM NaOH as described earlier (7).
Conditions for treatment of gap junction proteins with various proteases were optimized using 1-2 μg of isolated gap junctions/reaction (~20 μl final volume). Incubations were carried out overnight at 37°C using a range of protease concentrations. Inhibition of proteinase A (Sigma) for control experiments was achieved by evaporating 3 μl of pepstatin A (0.5 mM) or 1 μl of aprotinin (0.1 mg/ml) in methanol, followed by drying in a microfuge tube, then adding proteinase A (100 μl, 0.1 mg/ml), and incubating at room temperature for 4 h prior to its addition to gap junctions.

Subsequent to incubation of gap junctions with protease, the reaction mixture was usually solubilized in SDS-containing buffer for polyacrylamide gel electrophoresis (8). In the case of proteins which retain activity in SDS, notably proteinase K and pronase, the reaction mixture was first diluted with 1 mM NaHCO3, and centrifuged, and the supernatant fraction was then chromatographed on Sephadex G-25, and the void volume was pooled and brought to dryness by lyophilization (Pierce Chemical Co.) dissolved in 100 μl of dimethylformamide (19). The sample was then briefly bubbled with nitrogen to remove dissolved oxygen and the thiols were mixed with trifluoroacetic acid added to 25%, as necessary, to achieve 95% solubility (20). Material eluted in the void volume,材料在聚丙烯酰胺凝胶电泳中被分离（21）。为了在电泳中优化分辨率，使用了不同浓度的蛋白酶。在电泳前，对溶酶体的活性进行了抑制，许多溶酶体仍处于活动状态。

For microsequence analysis, proteolytic treatments were scaled up using a Applied Biosystems model 470A sequencer; phenylthiohydantoin amino acids were identified as described above for the P3x63.Ag8 myeloma cells plus 1% hyposotamine/thymidine (Whittaker), 1% aminopterin (HANA), and 2% BSA (GIBCO). For microsequence analysis, proteolytic treatments were scaled up using a Applied Biosystems model 470A sequencer; phenylthiohydantoin amino acids were identified as described above for the P3x63.Ag8 myeloma cells plus 1% hyposotamine/thymidine (Whittaker), 1% aminopterin (HANA), and 2% BSA (GIBCO). For microsequence analysis, proteolytic treatments were scaled up using a Applied Biosystems model 470A sequencer; phenylthiohydantoin amino acids were identified as described above for the P3x63.Ag8 myeloma cells plus 1% hyposotamine/thymidine (Whittaker), 1% aminopterin (HANA), and 2% BSA (GIBCO). For microsequence analysis, proteolytic treatments were scaled up using a Applied Biosystems model 470A sequencer; phenylthiohydantoin amino acids were identified as described above for the P3x63.Ag8 myeloma cells plus 1% hyposotamine/thymidine (Whittaker), 1% aminopterin (HANA), and 2% BSA (GIBCO). For microsequence analysis, proteolytic treatments were scaled up using a Applied Biosystems model 470A sequencer; phenylthiohydantoin amino acids were identified as described above for the P3x63.Ag8 myeloma cells plus 1% hyposotamine/thymidine (Whittaker), 1% aminopterin (HANA), and 2% BSA (GIBCO).
RESULTS

Conditions for protease treatment of intact gap junctions were optimized with respect to buffer, pH, and protease concentration. Digestion of intact gap junctions with increasing concentrations of proteinase A (Fig. 1) led to degradation of the 27-kDa gap junction protein and the formation of two major peptides with apparent molecular masses of 10 and 18 kDa. An apparent intermediate digestion product, migrating slightly slower than the 18-kDa product on this gel, was transiently observed, suggesting the occurrence of a cleavage prior to formation of the 10- and 18-kDa products. Sensitivity to digestion by proteinase A is also seen for the minor 21-kDa protein, characteristic of rat liver gap junction preparations, which has a mobility on this gel system slightly greater than that of the 27-kDa protein.

Prolonged treatment of intact gap junctions with many different proteases again generated two characteristic fragments which, by SDS-polyacrylamide gel electrophoretic analysis, had apparent molecular masses of 10 and 17–18 kDa, respectively. Specifically, the peptides generated by treatment with trypsin, proteinase K, chymotrypsin, Staphylococcus aureus V8 protease, elastase, and subtilisin had apparent molecular masses of 10 and 17 kDa on SDS-polyacrylamide gel electrophoresis (Fig. 2, left panel). On occasion, the 17-kDa band was found to be diffuse (e.g., trypsin-treated gap junctions, lane A). The 10- and 17-kDa bands were also obtained after treatment of gap junctions with other proteases, including papain, clostripain, thermolysin, and pepsin (not shown). Only treatment with proteinase A (lane G) generated a band with an apparent molecular weight that was higher than the slower-migrating band of the routinely observed doublet (18 as compared to 17 kDa).

The 27-kDa band and its 47-kDa dimer were the most prominent features of the control, which consisted of isolated rat liver gap junctions (Fig. 2, lane F). In addition, the 21- and 16-kDa peptides often observed in such fractions (5–8, 24, 25) were detected. After proteolytic treatment, higher molecular weight aggregates were also seen, most notably in this gel for proteinase A (lane G), endoproteinase Lys-C (lane I) and subtilisin (lane J).

In many cases, a peptide migrating more rapidly than the 27-kDa native protein band, but more slowly than the 17–18-kDa digestion product, was also observed. A 23-kDa band was readily detected in the samples treated with trypsin, proteinase K, endoproteinase lys-C, and subtilisin (lanes A, B, I, and J, respectively) but was also observed as a minor component in samples treated with the other proteases. This band is likely to be an intermediate, since increasing incubation time or concentration of protease led to its loss, accompanied by an increase in the lower molecular weight peptides, as demonstrated above (Fig. 1) for proteinase A.

The ability of antibodies to the 27-kDa rat liver gap junction protein (9, 16) to bind to the 10- and 17–18-kDa proteolytic products was characterized using Western blots (Fig. 2, right panel). The data indicate that these polyclonal antibodies bind to the 17–18-kDa peptides, with no detectable binding to the 10-kDa fragment.

With several of the proteases, treatments were carried out on a preparative scale to generate peptides which could be electroeluted from the gels for microsequencing. The sequence data obtained for these peptides, summarized in Fig. 3, demonstrated that the faster migrating (10 kDa) band in each case contained the amino-terminal portion of the protein molecule. For proteinase K, S. aureus V8 protease and thermolysin, the only sequence observed in this faster-migrating band was that beginning with the amino-terminal methionine. These findings are consistent with the data of Zimmer et al. (19) for the 10-kDa band they obtained after treating rat liver gap junctions with either trypsin or endoproteinase Lys-C (19). With proteinase A, however, we observed a single, internal sequence, beginning with Leu at position 10. Moreover, in the case of chymotrypsin, microsequence analysis of the 10-kDa fragment indicated the presence of three distinct peptides. While about 50% of the sequenced material began with Met at position 1 (fragment denoted as C7; in Fig. 3), the remainder was nearly equally divided between peptides.
beginning with Thr at position 8 (CT2) and Ser at position 11 (CT3).

The data obtained using proteinase A and chymotrypsin provide evidence that the amino terminus of the protein is accessible to these proteases and is found, therefore, at or near the cytoplasmic surface of the gap junction. A control experiment was conducted with proteinase A to determine whether this accessibility was an artifact due to the presence of lipolytic or detergent-like activities in the protease preparation. Isolated gap junctions were incubated for 18 h with proteinase A active, relatively nonspecific protease, proteinase K, would be expected to cleave bonds at or near Leu 10 (as for proteinase A). However, this treatment did not in any way alter cleavage of the amino-terminal region of the protein by proteinase K, with the amino-terminal methionine-containing sequence being the only one detected in the lower (10 kDa) band.

Microsequencing of the slower-migrating peptides (apparent M, 17,000 for S. aureus V8 protease, thermolysin, and chymotrypsin, and M, 18,000 for proteinase A), indicated the presence of a protease-sensitive region from amino acids 109-116 for thermolysin (TH), and 116 for thermolysin (TH). Sequence analysis of the unidigested 27-kDa protein gave a single sequence as described earlier by others (24). No inconsistencies were found with the predicted protein sequence and our sequence analysis of the various peptides.
shown to bind at the cytoplasmic surface of the gap junction by immunoelectron microscopy (12) (McAbs). Binding to lower molecular weight peptides was not observed.

Immunocytological characterization of these antibodies by indirect immunofluorescence on frozen sections of rabbit liver (Fig. 5) indicated that they all bind with a punctate pattern surrounding individual hepatocytes. Focusing through the section indicated that antibody binding was not confined to the surface of the sections. As earlier (7, 9), no antibody binding was observed in the absence of primary antibody or with preimmune sera.

DISCUSSION

The existence of four transmembrane segments in the 27-kDa rat liver gap junction protein was initially suggested by Paul (11) based upon hydropathicity analysis of the sequence

![Image](image_url)

**Fig. 4.** Specificity of binding of antibodies to the 27-kDa liver gap junction protein and its proteolytic products determined by Western blots. Digestions, gel electrophoresis, and transfer to nitrocellulose were as described in the legend to Fig. 2. Gap junction samples were treated with proteinase A (lanes a), thermolysin (lanes b) or untreated (lanes c). One replicate was stained with silver (Ag) and the remainder transferred to nitrocellulose and screened for antibody binding. The antibodies used were polyclonal, affinity purified rabbit antibody (PcAb); the two monoclonal antibodies to peptide 224–234 (26C, McAb1 and 92B, McAb2); and a rat monoclonal antibody (12) to the liver gap junction protein (McAbb).

encoded by a cDNA clone. Identification of protease-sensitive sites in the primary structure of the M, 27,000 rat liver gap junction protein has been used, in the present study and by others (19), to elucidate several of the basic topological features of the gap junction model. In the latter study (19), a single protease cleavage site was identified between the second and third putative transmembrane segments, localizing that hydrophilic portion of the protein to the cytoplasmic surface of the gap junction. It was inferred from other protease-digestion experiments that the carboxyl-terminal hydrophilic region of the protein is likewise cytoplasmically disposed (19, 24). However, while the amino terminus of the protein was also placed at the cytoplasmic surface, the experiments designed to test this assignment were unable to demonstrate the presence of predicted sites of either protease action or site-specific antibody binding (19). The data obtained in our study directly demonstrate cytoplasmic localization of the amino terminus of the 27-kDa rat liver gap junction protein, confirm the cytoplasmic localization of a hydrophilic domain of the protein connecting the putative second and third transmembrane segments, and demonstrate that some part of the hydrophilic carboxyl terminus of the protein, including amino acids 224–234, is also at the cytoplasmic surface of the gap junction.

Two major fragments (10 and 17–18 kDa, respectively) have been characterized in this study as the peptides generated by treatment of intact gap junctions with a wide variety of different proteases. Our findings are consistent with earlier reports that treatment of isolated gap junctions with trypsin yields two fragments (19, 24) and with the SDS-polyacrylamide gel profile of endoproteinase Lys-C generated fragments of rat liver gap junctions (19).

In the study of Zimmer et al. (19), more extensive degradation of the gap junction protein to fragments of approximately 6 kDa in size was reported with either pronase or proteinase K. We have made similar observations with pro-
nase and have attributed them to an inability to remove the protease from the gap junction membranes prior to solubilization in SDS; this enzyme retains proteolytic activity even in the presence of SDS. Our recovery of larger fragments (10 and 17 kDa) relative to those obtained by Zimmer et al. (19) following digestion with proteinase K, also known for retention of activity in SDS, may simply reflect our greater success in removing the enzyme from the gap junctions during washing steps prior to solubilization in SDS. We have noted that failure to remove or inhibit protease prior to addition of SDS can introduce new cleavage sites with specific proteases, such as a cleavage after Glu-169 by S. aureus V8 protease (not shown).

Microsequencing of the 10-kDa peptides demonstrated that they are derived from the amino terminus of the protein, and that they are relatively resistant to proteolysis near their amino-terminal ends. No cleavage was detected within this part of the protein in our study using proteinase K, S. aureus V8 protease, or thermolysin, and by others using trypsin, endoproteinase Lys-C, or endoproteinase Arg-C (19). We have, however, mapped three cleavage sites between Tyr-7 and Ser-11, one site for proteinase A and two partial sites for chymotrypsin, indicating that this region is exposed at the cytoplasmic surface of the gap junction. Microsequencing of the 17-18-kDa peptides, which begin in the region of the protein including amino acids 109-124, indicates that these peptides represent the carboxyl-terminal portion of the protein.

The fact that the 13-amino terminal residues are primarily hydrophobic in nature suggests that they may, in part, be buried in the lipid bilayer. Hence, while protease specificity could be a factor, partial occlusion of the amino terminus by the lipid bilayer may account for both the observed paucity of proteolytic cleavage sites and the reported inability of antibodies to a synthetic peptide, corresponding to amino acids 7-21, to bind to gap junctions by immunocytochemical criteria (19).

The location of the amino terminus at or near the cytoplasmic surface would explain the lack of detectable N-linked glycosylation of the 27-kDa rat liver gap junction protein which, based upon the presence of the sequence Asn-Trp-Thr, would be expected to occur at Asn-2 (26). The enzymatic machinery necessary for this type of processing is found in the lumen of the endoplasmic reticulum and Golgi apparatus, exposure to which occurs only at the extracellular domains of membrane and secretory proteins (27).

Once the amino terminus is fixed at the cytoplasmic surface, one may infer localization at the cytoplasmic surface for the carboxyl terminus of the protein (see below) as well as for a hydrophilic loop connecting the second and third transmembrane spans. Data described here for four proteases (Fig. 5) have demonstrated a series of five protease-sensitive sites from amino acids 109-123, within the predicted span of a cytoplasmically disposed loop connecting transmembrane segments 2 and 3 (amino acids 94-132). Cleavage by endoproteinase Lys-C or trypsin after Lys-124 (19) is consistent with these findings.

It is noteworthy that the fragment obtained with proteinase A (cleavage between residues 108 and 109) appears to be significantly larger than that generated by S. aureus V8 protease (which cleaves between residues 109 and 110) based upon their mobilities on SDS polyacrylamide gels (Fig. 2). While these two peptides begin within one amino acid of each other, the overall size difference implies an additional cleavage site(s) close to the carboxyl terminus of the protein. The carboxyl terminus of the protein must, therefore, also be located on the cytoplasmic surface of the gap junction, a conclusion reached earlier by others (19, 24) using a similar rationale. The presence of cleavage sites close to the carboxyl terminus of the protein could also account for production of the 23-kDa peptide (Fig. 2) as well as the proteinase A-generated peptide migrating somewhat more slowly than the 18-kDa product (Fig. 1) as intermediates in formation of the 10- and/or 17-18-kDa peptides.

Our polyclonal antibodies bind to all of the 17-18-kDa COOH-terminal generated peptides in this study (Fig. 2), an observation similar to that described by other investigators (19).

Further evidence for cytoplasmic localization of the carboxyl terminus comes from characterization of monoclonal antibodies to a peptide corresponding to amino acids 224-234 in the predicted sequence of the protein. As in the case of protease, accessibility of bulky immunoglobulins and fluorescein-conjugated second antibodies is restricted to the cytoplasmic surface of the gap junction. As with the polyclonal antibodies, these monoclonal antibodies bind to the 17-18-kDa carboxyl terminus-derived proteolytic peptide on Western blots (Fig. 4). The patterns revealed by indirect immunofluorescence on frozen sections of rabbit liver (Fig. 5) are indistinguishable from those analyzed here and earlier with a number of polyclonal antibodies to the 27-kDa rat liver gap junction protein (7, 9-13, 19). Hence, binding of these site-specific monoclonal antibodies demonstrates that at least this part of the hydrophilic carboxyl terminus is cytoplasmically disposed and suggests that the polyclonal antibodies also bind to this portion of the protein. Given the relative paucity of hydrophobic amino acids in the remainder of the protein (12 of 59 amino acids in the sequence from residue 235 to the predicted carboxyl-terminal cysteine 283) and their distribution, it is unlikely that this part of protein could insert itself in a lipid bilayer. It is likely, based upon the data presented in Figs. 4 and 5, that the rat monoclonal antibody developed by others (12) also binds to the carboxyl terminus region of the gap junction protein.

Phosphorylation of the 27-kDa rat liver gap junction protein has been observed (29, 30) and appears to be involved in channel regulation (29). The constraints thus far imposed upon our topological model of the gap junction protein suggest the cytoplasmic localization of a phosphorylatable serine (residue 233), which is preceded by two basic amino acids (residues 230 and 231) as is required for phosphorylation by the cAMP-dependent protein kinase (31). The presence of additional serine and tyrosine residues which are potential substrates for other protein kinases has been noted (17) in this cytoplasmically disposed carboxyl terminus of the protein.

Collectively, our data provide strong support for the model suggested by hydropathy plots (11) and provide several constraints upon models being developed based upon cryoscopic and image processing techniques. Additional experiments will be necessary to further delineate the topological features of the gap junction protein, especially the assignment of residues to the extracellular domain of the protein. The data described in this report do, however, reinforce the emerging model of a gap junction protein spanning the bilayer four times with both the amino and carboxyl termini localized at the cytoplasmic surface.

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