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THE CARDIAC GAP JUNCTION PROTEIN (M_r 47,000) HAS A TISSUE-SPECIFIC CYTOPLASMIC DOMAIN OF M_r 17,000 AT ITS CARBOXY-TERMINUS

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The molecular weight of the heart gap junctional protein subunit was, until recently, believed to be about M_r 28,000-30,000, similar to that of other previously characterized gap junctional proteins. A larger polypeptide of about M_r 44,000-47,000, which undergoes proteolysis during isolation, has recently been proposed as the form of the heart junction protein in vivo. We show here that this entity has the same amino-terminal sequence as the previously characterized M_r 29,000-30,000 component. Thus, the cardiac junctional protein has, at its carboxy-terminus, a cytoplasmic domain of M_r 17,000; this domain is absent in the liver protein. These observations provide further evidence that gap junction proteins form a highly diversified family.

In recent years, proteins with molecular weights of ~28,000 have been consistently associated with gap junctions in a variety of different tissues either by criteria of coisolation (liver (1-5), heart (6-9), lens (10,11)) or immunological cross-reactivity (12,13). However, despite the uniform morphology of gap junctions in different tissues and species and the similar sizes of the protein components, detailed analyses of some of these proteins have revealed a striking diversity between junctional proteins in different tissues (8, 9, 13, 14, 19). Further variability in this apparent family of proteins has recently been suggested by the association of proteins of M_r 16,000 (15), 54,000 (16,27) and 44-47,000 (17-19) with gap junctions. The significance of the first two remains

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clouded due to discrepencies in the published data from different laboratories. However, in the latter case, we present here direct amino acid sequencing data which, in conjunction with earlier studies on the effects of protease inhibition (17-19), conclusively demonstrate the derivation of the previously characterized cardiac gap junction protein of M_r 29,000 from the M_r 44-47,000 protein by cleavage of a carboxy-terminal domain of 14,500-17,000 daltons. Localization of this domain to the cytoplasmic face of gap junctions is inferred from both its protease sensitivity and the correlation between its removal and the loss of morphologically identifiable structures on the inner gap junctional surface (17-20), structures which are not seen in liver gap junctions (18, 20, 21).

MATERIALS & METHODS

Isolation of Cardiac Gap Junctions—Cardiac gap junctions were isolated as described by Manjunath et al. (17, 19). The incorporation of solid PMSF as an enzyme inhibitor reduces contamination by proteins which are apparently precipitated by the ethanol normally used to dissolve PMSF. In order to generate partially degraded forms of the protein for sequence analysis, PMSF was not added until the 2 hr time point of KI extraction (18).

Sequence Analysis (a) Sample Preparation—Samples for sequence analysis were prepared by the novel method of Aebersold et al. (22), which is specifically adapted to handling small amounts of material (≥ 5 pmoles). Five preparations (each corresponding to the gap junction proteins isolated from eight rat hearts) were loaded on a mini Laemmli sodium dodecyl sulfate (SDS) polyacrylamide gel (9 cm long, 0.7 mm thick) using the precautions described by Hunkapiller et al. (23) to prevent amino-terminal blockage. Based on quantitative densitometry of Coomassie blue stained bands in identical trial preparations, 7-12 µg of each polypeptide were available for sequence analysis. After electrophoresis, the gel was immediately blotted for 3 hr at 50 volts in 4°C buffer (25 mM Tris HCl, pH 8.3, 10 mM glycine, and 0.5 mM dithiothreitol) to Whatman GF/F paper which had been previously activated with trifluoroacetic acid and derivatized with N-trimethoxysilylpropyl-N, N, N-trimethylammonium chloride (22). The latter reagent creates a positive surface which facilitates the binding of SDS solubilized proteins at neutral pH. Visualization of the bands for excision from the paper blot was achieved with the dye 3, 3'-dipentyloxacarbocyanine iodide and subsequent ultraviolet illumination at 254 nm (as little as 20-50 ng of protein in a band could be detected) (22). No direct assay of transfer efficiency was performed for the experiments described here, but a wide range of proteins (including liver gap junction protein and other membrane proteins) have yielded values from 30 to 100%.

(b) Analysis—Sequence analysis was performed directly on the protein bound to the paper strips according to Hewick et al. (24) as modified by Aebersold et al. (22). Analysis of PTH derivitives from each cleavage step was done as described by Hunkapiller et al. (23) on an IBM cyano column except that 5-7% (v/v) tetrahydrofuran was added to the A buffer and the pH adjusted to 5.1. Estimates of yields were obtained by comparison of peak heights with standard HPLC runs. Sequencing signals for the various heart proteins ranged from 4 pmoles (M_r 34,000) to 12 pmoles (M_r 47,000) with repetitive yields of 90-99% (variation in a single run of ±2%).

RESULTS AND DISCUSSION

It has previously been shown (17, 18) that the inclusion of the serine protease inhibitor PMSF during the isolation of cardiac gap junctions yields fractions having a single broad band of M_r 44,000-47,000 in SDS polyacrylamide gel electrophoresis (Fig. 1, lane b). If PMSF is omitted in the early stages of the preparation the fractions thus produced have less of this high molecular weight species and correspondingly more smaller components [M_r 34,000, 31,000, and 29,000 (18)]. The smallest of these polypeptides (M_r 29,000) represents a relatively stable end product of a protease-mediated degradation. By adjusting the time at which PMSF was added during the KI extraction, roughly equivalent amounts of the "native" M_r 44,000-47,000 protein and its apparent degradation products could be isolated from a single preparation (Fig. 1,

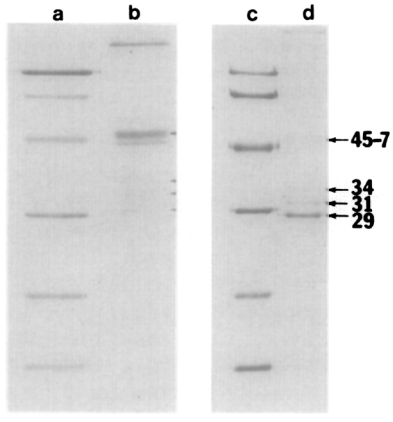

Fig. 1. SDS polyacrylamide gels of rat cardiac gap junctional fractions isolated under varying conditions of protease inhibition by PMSF.

Lanes a and c - Bio-Rad low molecular weight markers: phosphorylase a (M_r 94,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 29,000), soybean trypsin inhibitor (M_r 21,000) and lysozyme (M_r 14,000).

Lane b - Rat cardiac gap junctional fraction prepared with PMSF present throughout. While minor amounts of material are present at M_r 34,000, 31,000 and 29,000 (arrows), most of the protein is in a diffuse band at M_r 44,000-47,000 (arrowhead). Some contaminating proteins of M_r 42,000, 55,000 and >150,000 are either inconsistently present in junctional fractions or are associated with non-junctional sources (see ref. 17-19).

Lane d - Gap junction fraction similar to that used in sequence analysis. The omission of PMSF from the initial homogenate and washes and the first 2 hr of KI extraction produces fractions with a predominance of lower molecular weight species. Breakdown below M_r 29,000 is rarely seen. Major junctional bands are indicated in thousands of daltons.

lane d). These were blotted as described in Materials and Methods and the bands excised for sequence analysis.

All four proteins, including the M_r 44,000-47,000 band, gave identical amino-terminal sequences (Table 1) 13 to 29 residues in length. This sequence is identical to that already published (9) for the M_r 28,000-30,000 protein isolated from cardiac gap junctions by previous approaches. The results of the sequencing not only confirm that the M_r 29,000 polypeptide previously associated with highly purified cardiac gap junction fractions (7-9) is a degradation product of the M_r 44,000-47,000 protein, but also that the

TABLE 1

Sequence Analysis of Components in Cardiac Gap Junctional Fractions Obtained at Different Stages of Proteolysis

	Molecular Weight of Component				
Step #	28,000+¶	44-47,000*¶	34,000*	31,000*¶	29,000*
1	Ala [A]	A;G;H	A;G	A;G	++
2	Asp [D]	D	D	D	D
3	Trp [W]	W	W	W	W
4	Ser [S]	S	S	S++	**
5	Ala [A]	A	A	A	A
6	Leu [L]	L	L	L	L
7	Gly [G]	G	G	G	G
8	Lys [K]	K	K	K	K
9	Leu [L]	L	L	L	L
10	Leu [L]	L	L	L	L++
11	Asp [D]	D	D	D	D
12	Lys [K]	K	K	K	**
13	Val [V]	V	V	V	V
14	Gln [Q]	Q	Q	S; Q	
15	Ala [A]	A		A	
16	Tyr [Y]	Y		Y	
17	Ser [S]	S++		T++	
18	Thr [T]	T++		**	
19	Ala [A]	A		A	

+Data from B. Nicholson et al., J. Biol. Chem. 260, 6514 (1985)
*Components from preparation shown in Figure 1
¶Sequence could be read further e.g., M_r 28,000 protein to residue 29
M_r 44-47,000 proteins to residue 24
M_r 31,000 protein to residue 23

++Assignment uncertain
**No detectable signal

protease-sensitive M_r 17,000 domain is at the carboxy-terminus of the protein. The correlation made earlier between the cleavage of this region and the loss of fuzzy material on the cytoplasmic surfaces of the junctional membranes suggests strongly that the carboxy-terminal tail is exposed to the cytoplasm. This is consistent with the observation that all known serine proteases are too large in diameter (25) to penetrate the narrow gap between the extracellular faces of gap junctional membranes and can only cleave protein exposed at the cytoplasmic face. While the simplest interpretation of the data is that the cleaved domain is wholly cytoplasmic, it remains possible that a portion of it, small enough to escape detection in our SDS gel analyses (i.e., $\leq M_r$ 5000), could be embedded in the lipid bilayer. The lack of structures on the cytoplasmic surfaces of liver gap junctions (18, 20, 21) and the failure to isolate hepatic junctional protein components of $M_r > 28,000$ (2-4) strongly suggests that an equivalent C-terminal domain is absent in liver gap junctions. The recently isolated cDNA clone encoding the liver protein (26) has now confirmed this.

Another variant between the cardiac junctional protein and those of liver and lens, evident from the multiple sequence analyses performed here and previously (9), is the absence in the heart protein, of an amino-terminal methionine. Alanine is consistently detected at this position, although high backgrounds (e.g. glycine), typical of the first cleavage step, make an unequivocal assignment difficult. Nonetheless, it is clear that even the M_r 47,000 protein has undergone some proteolysis either during processing within the cell (e.g. cleavage of a signal peptide) or during isolation (by a protease resistant to inhibition by EDTA, iodoacetamide, TAME (9) or PMSF). Particularly notable is the exact alignment of the amino-terminii of the processed heart gap junction protein and the unprocessed protein in liver (2,26).

It has previously been shown that the heart and liver junctional proteins, while related (9, 12), display considerable divergence in their primary sequences (14) and also differ with respect to disulfide linkages: cardiac, but not liver, gap junctions contain disulfide linkages between channel subunits and connexons both in their cytoplasmic domain and in the lipid bilayer of the membrane (19). The data presented here demonstrate a further difference between these proteins—the presence in the cardiac protein of an M_r 17,000 cytoplasmic domain at the carboxy-terminus which is not found

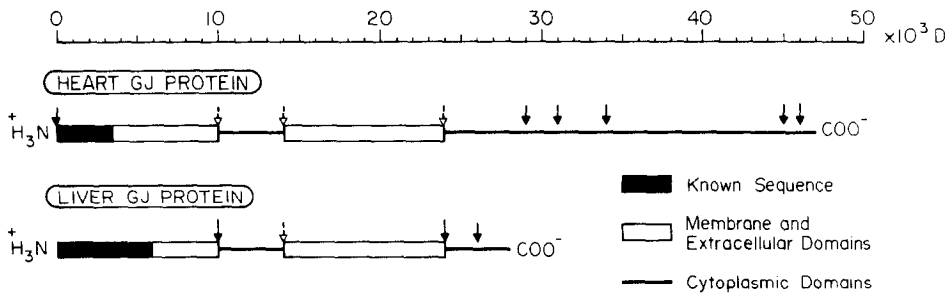

Fig. 2. Models of the cardiac and liver gap junctional proteins based on proteolysis susceptibility of the proteins in intact junctional plaques and limited sequence analysis. Both proteins have similar structures with two M_r 10,000 membrane-protected domains in the amino-terminal 28,000 daltons. The cardiac protein, however, contains an additional protease labile M_r 17,000 cytoplasmic domain at the carboxy-terminus.

Scales are indicated in thousands of daltons. Solid bars indicate the extent of reliable protein sequence. Open bars represent domains protected from proteolysis, presumably by virtue of their location in the narrow gap (2 nm) between or within adjacent cell membranes. Sites of proteolysis are marked by arrows, although in some instances (open, dotted arrows) the absolute location has not been mapped.

in the liver junctional protein. Some commonality between the gap junctional proteins studied to date does, however seem evident in the general structure of their amino terminal domains. Both heart and liver proteins each contain a hydrophobic domain beginning at residue 23. Furthermore, tryptic or chymotryptic proteolysis of either heart or liver junctions yields two membrane-protected M_r 10,000 domains (8).[1] The organizations of the liver and heart junctional proteins are compared and contrasted in the models of the proteins shown in Fig. 2.

It would be interesting to determine if a cytoplasmic domain similar to that found in heart is also found in other tissues. Perhaps the association of a fuzzy cytoplasmic coat, reminiscent of that in the heart, with gap junctions in cerebellum (28), may be an indication of the presence of such a domain. A testable hypothesis is that this cytoplasmic domain is responsible for the physiological uniqueness of cardiac junctions or possibly of excitable tissues in general. Its cytoplasmic location is well suited to modulating channel permeability. The sensitivity of cardiac gap junctional conductance to transjunctional voltage gradients and changes in pH is already known to be substantially different from that of other systems such as liver (29). The important

[1]Model building studies on the lens protein sequence (11) suggest that a similar pattern could be produced in this tissue were it not for the absence of appropriate cleavage sites resulting in a predominant M_r 20,000 proteolysis product.

possibility of functional differences between gap junctions from different tissues may be approachable by a more detailed comparison of differences between the various proteins which can form the cell-to-cell channels in these tissues.

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