Preparation of a Gap Junction Fraction from Uteri of Pregnant Rats: The 28-kD Polypeptides of Uterus, Liver, and Heart Gap Junctions Are Homologous

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ABSTRACT A procedure for the preparation of a gap junction fraction from the uteri of pregnant rats is described. The uterine gap junctions, when examined by electron microscopy of thin sections and in negatively stained preparations, were similar to gap junctions isolated from heart and liver. Major proteins of similar apparent molecular weight (M, 28,000) were found in gap junction fractions isolated from the uterus, heart, and liver, and were shown to have highly homologous structures by two-dimensional mapping of their tryptic peptides. An M, 10,000 polypeptide, previously deduced to be a proteolytic product of the M, 28,000 polypeptide of rat liver (Nicholson, B. J., L. J. Takemoto, M. W. Hunkapiller, L. E. Hood, and J.-P. Revel, 1983, Cell, 32:967–978), was also studied and shown by chymotryptic mapping to be homologous in the uterine, heart, and liver gap junction fractions. An antibody raised in rabbits to a synthetic peptide corresponding to an amino-terminal sequence of the liver gap junction protein recognized M, 28,000 proteins in the three tissues studied, showing that the proteins shared common antigenic determinants. These results indicate that gap junctions are biochemically conserved plasma membrane specializations. The view that gap junctions are tissue-specific plasma membrane organelles based on previous comparisons of M, 26,000-30,000 polypeptides is not sustained by the present results.

MATERIALS AND METHODS

Preparation of Gap Junctions: Female Sprague-Dawley rats (150–200 g wt) were used to prepare the subcellular fractions. Gap junctions were prepared from the uteri of 10–15 pregnant rats at term (days 21–22 after...
the nitrocellulose was washed twice with 0.1% Nonidet P-40. Nitrocellulose junctions were prepared from 10-12 rat hearts as described in references 14, reported elsewhere. After several washes using 0.1% Nonidet P-40 in Tris-HCl, pH 8.2. The resultant pellet was washed and suspended and stirred for 16 h at 4°C in 100 ml of 0.6 M NaCl, 6 mM Na3SO₄, 1 mM PMF, 1 mM NaHCO₃, pH 8.2, and then in 5 mM Tris-HCl, pH 9. The final pellet, suspended in 60 ml of 5 mM Tris-HCl, pH 10, 0.3% Na-sarcosinate, 1 mM PMFSF, was layered onto four discontinuous gradients constructed of 44.5% (10 ml) and 35% (wt/vol) sucrose solutions containing 0.6 M NaCl, 6 mM Na3SO₄, 1 mM PMF, 1 mM NaHCO₃, pH 8.2. The resulting pellet was washed by centrifugation (33,000 g for 15 min in 0.6 M KI, 6 mM Na3SO₄, 1 mM PMF, 1 mM NaHCO₃, pH 8.2). The amino-terminal sequence of the rat liver Mr 27,000 gap junction protein with a synthetic peptide corresponding to amino acid residues 7-21 of the globulin. Full details of the preparation and properties of this antiserum will be reported by Nicholson et al. (31). The peptide was coupled to porcine thyroglobulin, 14,000, purchased from Sigma Chemical Co., St. Louis, MO.

**Electron Microscopy:** Samples were routinely examined by negative staining using 1% Na silico tungstate, pH 7. Gap junctional pellets were processed and examined in a Phillips 300 electron microscope (21).

**Electrophoresis:** SDS PAGE was carried out in 15% acrylamide, 0.086% bis-acrylamide, using samples dissolved by boiling for 30 s in 2% SDS, 1% 2-mercaptoethanol, 100 mM Tris-HCl, pH 6.8 (24). The molecular weight markers used were as follows: myosin, 200,000; phosphorylase a, 94,000; catalase, 60,000; creatine kinase, 40,000; trypsin inhibitor, 21,000; and lysozyme, 14,000, purchased from Sigma Chemical Co., St. Louis, MO.

**Peptide Mapping:** This was carried out essentially as described by Elder et al. (25). The Coomassie Blue-stained polypeptide bands in the polyacrylamide gels were excised and iodinated with 115I (300 μCi per slice) using chloramine T (26), and digested overnight at 37°C using 50 μg diphenylcarbazyl chloride trypsin or chymotrypsin (Sigma Chemical Co.) dissolved in 0.05 M NH₄HCO₃, pH 8. The gel slices were removed and the digests lyophilized. In the mixing experiments, approximately equal amounts of radioiodinated peptides of the heart, liver, and uterus Mr 28,000 polypeptide were pooled and then applied to the plates. Samples were dissolved in electrophoresis buffer: acetic acid:formic acid:H2O (15:5:80) (vol/vol/vol) and electrophoresed at 600 V in cellulose sheets (Eastman Kodak No. 13255); progress was assessed using Kodak XRP-I X-ray film.

**Immunoblotting:** Partially purified gap junction fractions isolated from the rat liver, heart, and uterus were resolved in 15% SDS polyacrylamide gels, which were transferred to nitrocellulose (10.1 μm) (Schleicher & Schuell Co., Dassel, Germany) in a BioRad apparatus at 10 V for 16 h (27). The efficacy of transfer and Mr of the immunoreactive proteins was monitored by simultaneous transfer of a series of pre-stained marker proteins (Bethesda Research Laboratories, Gaithersburg, MD). Nitrocellulose sheets were washed with 0.1% Nonidet P-40 in 2% bovine serum albumin in a Tris-buffered saline (pH 7.5) and electrophoresed at 600 V in cellulose sheets (Eastman Kodak No. 13255); progress was assessed using Kodak XRP-I X-ray film.

**RESULTS**

**Morphology and Biochemical Composition of Uterus Gap Junctions**

In the fractionation of uteri removed from pregnant rats, material banding at the 33–44.5% interface was found to contain large numbers of gap junctions together with amorphous material when examined in the electron microscope (Fig. 1). The features of the intact gap junctions isolated from uterus were indistinguishable from those of gap junctions isolated from liver (9, 10, 18) and heart (14, 15, 29) tissues. In negatively stained preparations (Fig. 2) hexagonal arrays were evident, and the center-to-center spacing of 80–84 Å was measured to be that in liver and heart gap junctions. While attempting to modify conditions to increase the morphological purity of the uterine gap junctions, it was found that the junctions showed a tendency to change into amorphous structures. Addition of proteolytic inhibitors failed to prevent or reduce this, and often it was possible to observe gap junction structures in the process of breakdown (Fig. 3). The high susceptibility of uterine gap junctions to breakdown during isolation may reflect the presence of large amounts of tissue proteases in the uterus during parturition. The yield of the gap junctions obtained from pregnant uteri was about 1.5 μg protein/g wet tissue weight. When the same procedure was used with a similar number of nonpregnant uteri, a very low amount of material was recovered and examination of this material by electron microscopy indicated that gap junctions were present, with a similar morphology to those prepared from pregnant animals.

**Peptide Mapping**

The M₉ 28,000 and M₉ 10,000 polypeptide bands of gap junction fractions isolated from the liver, heart, and uterus were excised from polyacrylamide gels, radioiodinated, digested with trypsin or chymotrypsin, and the products then analyzed by two-dimensional mapping. Fig. 5 compares the tryptic maps of the M₉ 28,000 and M₉ 10,000 polypeptides. Two conclusions emerge. First, the tryptic maps were similar, indicating that the M₉ 28,000 polypeptides in liver and heart gap junction fraction occurred rapidly during handling and it was noted that the intensity of low molecular weight components, including the M₉ 10,000 polypeptide, increased with time.
the $M_r 10,000$ polypeptide now studied may correspond to a proteolytic fragment (31). Fig. 7 compares the peptide maps produced by chymotryptic digestion of the $M_r 10,000$ polypeptide in the gap junction fractions. Compared to the tryptic maps, a greater number of iodinated peptides were separated and the results again indicated the similarities of the chymotryptic digests of the $M_r 10,000$ polypeptide of liver, heart, and uterus. However, the chymotryptic maps of the heart $M_r 10,000$ polypeptide also showed one and possibly more further peptides to be present (Fig. 7, panel B), suggesting that minor differences may indeed exist between the gap junction polypeptides. Within the limits of the method, however, the results
FIGURE 2  Gap junction fraction prepared from rat uterus, negatively stained with 1% Na silicotungstate, pH 7.0. × 150,000. (Inset) Higher magnification (× 300,000) showing hexagonal lattice.

Indicate that the $M_r$ 28,000 polypeptide and a possible $M_r$ 10,000 degradative product in liver, heart, and uterine gap junction fractions are highly homologous polypeptides.

Immunological Similarities

To show that the $M_r$ 28,000 polypeptide analyzed in the present work by peptide mapping was indeed the same gap junction polypeptide as that used by other investigators (9, 11, 31), we used an antibody raised against a peptide corresponding to an amino-terminal region of the sequence determined on the $M_r$ 27,000 gap junction protein of rat liver (31). Fig. 8 shows that the antibodies bound specifically to the $M_r$ 28,000 polypeptide in fractions isolated from liver, heart, and
The morphological properties of the gap junctions prepared from the uterus of pregnant rats were similar to those of junctions obtained from cardiac (14, 15, 29) and liver tissue (9, 10, 18). In thin sections and in negatively stained preparations, no differences were discerned between gap junctions in the fractions isolated from rat liver, heart, and uterus of pregnant animals. Compared to the extensive morphological studies on junctional complexes in liver and heart tissue, the limited studies carried out with the smooth muscle of the uterus. The results confirm the cross-reactivity of this antibody with the rat liver gap junction polypeptides (20) and show that these polypeptides are immunologically homologous in the rat heart and uterus.

DISCUSSION

The morphological properties of the gap junctions prepared from the uterus of pregnant rats were similar to those of junctions obtained from cardiac (14, 15, 29) and liver tissue (9, 10, 18). In thin sections and in negatively stained preparations, no differences were discerned between gap junctions in the fractions isolated from rat liver, heart, and uterus of pregnant animals. Compared to the extensive morphological studies on junctional complexes in liver and heart tissue, the limited studies carried out with the smooth muscle of the
uterus indicated that although normally relatively small numbers of gap junctions were present, there was a sixfold increase in the gap junction area prior to parturition (8, 34), and this was accompanied by an increase in electrical conductivity (35). The present work used uteri removed at term and shows that the isolated junctions were morphologically similar to those investigated in other tissues (5). Minute amounts of gap junctions were also recovered from uteri removed from non-pregnant rats; these had a similar morphology, suggesting no differences between those gap junctions induced during preg-
nancy and gap junctions present in normal myometrium.

Analysis of the polypeptide composition of the uterine gap junctions showed that a major Mr 28,000 component was present. A polypeptide of similar electrophoretic mobility was also a major component in liver and heart gap junctions. Other polypeptides identified in minor amounts were possibly contaminants, especially in uterine gap junction fractions that were partially purified, but the presence of proteolytic degradation products as well as polymeric aggregates must also be considered. The inclusion of proteolytic inhibitors during the preparation of the gap junction fractions helped to minimize breakdown, but persistent action of endogenous proteases was still evident. It was noted that the gap junction polypeptide in heart and uterus fractions was extremely susceptible to breakdown, with a diffusely staining Mr 26,000-28,000 band preceding conversion into lower molecular weight components, with the Mr 10,000 polypeptide being especially prominent. The proclivity of the Mr 28,000 polypeptide to aggregate (10) may also complicate the situation. Morphological analysis of uterine gap junctions also indicated that deterioration of the characteristic gap junction heptalaminar structure was another likely consequence of proteolysis.

There is a large body of evidence showing that an Mr 26,000-29,000 polypeptide is present in gap junction fractions prepared by detergent extraction of rodent liver plasma membranes (9-11, 31). An alternative method for the preparation of liver gap junctions, involving extraction of liver plasma membranes with 20 mM NaOH, also showed an Mr 27,000 polypeptide to be a major component (32). Further supporting the candidacy of the Mr 26,000-28,000 polypeptides as major gap junction constituents are studies showing that antibodies raised against an Mr 26,000 polypeptide of mouse liver gap junctions immunolocalized to gap junction plaques (36). In contrast is a report that an Mr 16,000 polypeptide, often co-existing with an Mr 28,000 polypeptide, comprises gap junctions in mammalian liver and other tissues (16). The relationship of this component with the Mr 26,000-28,000 components awaits clarification.

Analysis of heart gap junction fractions showed a major Mr 28,000-30,000 component to be present (14, 15). Studies by Manjunath et al. (37) claim that an Mr 29,500 polypeptide comprises the intramembrane component of rat heart gap junctions, with an associated Mr 14,500-17,500 polypeptide that may correspond to a fuzzy layer, removable by serine proteases, on their gap junctions. The present results indicate that an Mr 28,000 polypeptide is the major component.

Comparative analyses of the Mr 26,000-29,000 gap junction polypeptide have been carried out with liver and lens, and liver and heart. Comparison of lens and liver gap junctions by tryptic mapping (2), analysis of cyanogen bromide peptides (13), and amino-terminal analysis (19) indicated that the major polypeptides were not homologous, despite variable results on the immunological cross-reactivity of the junctions (38, 39). These results, combined with the documentation of major morphological differences between eye lens fiber junctions and gap junctions in other vertebrate tissues (5, 40-42) have led to the view that lens fiber junctions comprise a different class of intercellular contact not necessarily involved in intercellular communication. Gros et al. (29) compared the composition of rat liver and heart gap junctions and showed that an Mr 30,000 cardiac gap junction polypeptide and an Mr 28,000 liver gap junction polypeptide were not homologous on the basis of two-dimensional tryptic maps, but they reported that a short amino-terminal sequence of amino acids of heart and liver Mr 28,000 polypeptides were similar, a result more in line with the present report.

Homology between the Mr 28,000 liver gap junction investigated in the present work and by others (9, 11, 31) was evident by showing that antibodies raised against a synthetic peptide corresponding to an amino-terminal amino acid sequence of this protein also cross-reacted, by immunoblotting with this protein (20). The present results also show that the anti-peptide antibodies also cross-reacted with an Mr 28,000 polypeptide present in heart and uterus gap junctions. The overall results now show that the major gap junction polypeptide of at least three rat tissues comprise a family of homologous proteins. However, the extent of amino acid sequence homology will only be clarified when the complete primary amino acid sequence is determined.

In conclusion, the present study describes the isolation of gap junction fractions from uteri of pregnant rats, and compares an Mr 28,000 polypeptide with a polypeptide of similar electrophoretic mobility that comprises the major component of liver and heart junctions. This study shows that gap junctions are highly conserved plasma membrane organelles from tissue to tissue. Immunoreactivity between the gap junction polypeptides of these tissues has also been shown. The results are in accord with (a) the demonstration that co-culture of heterologous cells resulted in gap junction–mediated intercellular communication (43-45) and (b) studies which show that gap junctions in several tissues shared common antigenic determinants (28, 33, 39).

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