THE PROTEIN COMPONENT OF MOUSE HEPATOCYTE GAP JUNCTIONS

Jean-Claude EHRHART and Jean CHAUVEAU†

Institut de Recherches Scientifiques sur le Cancer, BP No. 8, 94800-Villejuif, France

Received 28 March 1977

1. Introduction

A comprehensive view of the role that gap junctions (or communicating junctions [1]) could play on the structure and function of many animal tissues and organs has been established during the last ten years [2–4]. Yet little is known about the chemical nature of these membrane specializations.

Communicating junctions are remarkably resistant to the solubilization by 'mild' surfactants [5,6]. This characteristic has been exploited in the development of isolation methods [6–9]. The general features of the procedures appear well delineated [6–14]. However, there are strong limitations due to the lack of homogeneity of isolated fractions and, most accurately, to the proteolytic degradation of junctional protein which occurs during the isolation protocol [9,12,14]. Altogether, these difficulties have led to various estimations of the molecular weight(s) of the major junctional protein constituent(s) [6,7,9–14]. We isolated an enriched fraction of gap junctions from mouse liver heavy subfractions of plasma membranes. Electrophoresis analysis of such SDS-solubilized and reduced preparations consistently demonstrated the presence of only one major protein component with a mobility that corresponds to $34 \pm 1 \times 10^3$ daltons, in absence and in presence of trypsin.

2. Methods

2.1. *Preparation of the heavy subfraction of plasma membranes*

Liver plasma membranes were isolated from fed unperfused Swiss male mice (30 ± 5 g), essentially as described by Evans [15]. Following the rate-dependent zonal centrifugation in a Beckman 14 Ti rotor at 4000 rev/min for 45 min, crude plasmalemmal fractions were collected between 32% and 40% (w/w) sucrose. They were then layered onto a discontinuous gradient made of 45.5% and 51.5% (w/v) sucrose in 5 mM Tris–HCl buffer (pH 7.6). Heavy subfraction (1.19 g/cm^3) of plasma membranes was collected at the interface between 45.5% and 51.5% sucrose after centrifugation for 3 h in an SW 25.2 rotor.

2.2. *Isolation of junctional fractions*

Gap junctions were isolated by some modifications of the procedure of Dunia et al. [9] applied to calf eye lens. Heavy plasma membranes from 120 g of wet liver were digested in 20 ml of 0.1% hyaluronidase (Sigma, type I), 0.1% collagenase (Worthington, type CLS), 0.9% NaCl, 1 mM $NaHCO_3$ (pH 7.6) for 1 h at 20°C. In a number of experiments, 0.005% trypsin (Sigma, type III) was added to the suspension. The digested membranes were centrifuged at $165\,000 \times g_{av}$ for 30 min at 4°C. They were resuspended in 3 ml 1 mM $NaHCO_3$ (pH 7.6) and treated for 15 min at 20°C with sodium deoxycholate (Sigma) to a final concentration of 1% [5,9]. The insoluble fraction was subfractionated by centrifugation at 20 000 rev/min for 20 min at 4°C in a Beckman 40 rotor. Two pellets were obtained, each of them was washed and resuspended in 0.8 ml of ice-cold hydrogenocarbonate buffer, layered on top of a 4 ml buffered 20–60% (w/v) linear sucrose gradient [9] and centrifuged at 12°C for 16 h in an SW 50.1 rotor at 40 000 rev/min. The junctions were collected at a buoyant density of 1.15–1.16 g/ml in absence of trypsin, 1.13–1.15 in its presence.

†This paper is dedicated to the memory of Dr Jean Chauveau

2.3. *Electron microscopy*

Pellets were fixed for 1 h in 2% OsO_4 in Michaëlis buffer (pH 7.4). They were stained in block for 2 h in 0.5% uranyl acetate in Michaëlis buffer (pH 5) before dehydration and Epon embedding. Sections were stained with uranyl acetate and lead citrate [16]. The preparations were examined in a Siemens 1A Elmiskop microscope.

2.4. *Limited proteolysis of isolated gap junctions*

Junctional fractions isolated in absence of trypsin were washed once with 40 mM Tris (pH 8) and resuspended in this buffer. Trypsin was added with a junctional protein/enzyme weight ratio comprised between 0–100 (high trypsin level) or 100–200 (low trypsin level). The incubations were carried out for 45 min at 38°C. The reaction was stopped by the addition of trypsin inhibitor (Sigma, type I-S) in quantity twice as much as that of trypsin present in the medium. After the volume had been completed to 2 ml with ice-cold buffer, the insoluble fraction was pelleted at 50 000 rev/min for 30 min (50 Ti rotor with adapters).

2.5. *Analytical electrophoresis*

Samples 13% polyacrylamide, 0.1% sodium dodecyl sulfate, 1.25 mm thick slab-gels (Studier's apparatus [17]) were prepared and run as described by Laemmli [18]. Alkylation of reduced samples was performed according to Dwyer and Blobel [19]. Molecular weight markers: bovine serum albumin (68 000), yeast alcohol dehydrogenase (37 000), carbonic anhydrase (29 000), β-Lactoglobulin (18 400) and cytochrome *c* (13 500) were run in parallel. Coomassie Blue-stained gels were obtained according to Lopez and Siekevitz [20], except that Dowex 1 X 4 was added to the destaining medium. Gels were dehydrated before being photographed.

3. Results and discussion

3.1. *Thin sections of purified communicating junctions*

The use of heavy fraction instead of the general plasma membrane as starting material, careful subfractionation of the deoxycholate-treated suspension and isopycnic centrifugation at 12°C have allowed the obtention of an enriched subcellular fraction of gap junctions from mouse liver in absence (fig.1A,B) and presence (fig.1C–E) of trypsin. The yield is small: 4–6 μg protein/g wet liver. Concentrated hyaluronidase–collagenase-treated junctional segments of various length may be seen in fig.1A. The main structural feature, the 2 nm 'gap', characteristic of the junctions in whole tissue, appears to be preserved (fig.1B). However, preparations are still contaminated by vesicular material. Although tryspin-treated junctions generate segments plus vesicles (fig.1C,D), the purity of the preparations is greatly increased as judged on morphological criteria (fig.1C). Whatever its segmental or vesicular nature, the junctional structure generally appears unaltered (fig.1E). Unfortunately, it is well known that unaffected electron-microscopic features of the communicating junctions do not constitute a criterium of structural integrity.

3.2. *SDS–Polyacrylamide gel electrophoresis*

Figure 2 C shows electropherograms of SDS-solubilized and reduced hyaluronidase–collagenase-treated gap junctions, isolated at three different deoxycholate/plasmalemmal phospholipid molar ratios higher than 2. Such a value is critical during the isolation procedure. This is in strict accordance with our previous studies on the differential solubilization of rat liver plasma membrane components by 0.26% deoxycholate [21,22]. In fig.2D, sample was further alkylated. Only one major protein band is observed with a mobility (molecular weight markers in fig.2A,B) corresponding to $34 \pm 1 \times 10^3$ daltons ($n = 14$). A number of minor polypeptides are detected in the $36–120 \times 10^3$ dalton region, the origin of which might be related to the vesicular contamination of best preparations. It cannot be excluded that some of these minor polypeptides are of junctional nature since the multitude of bands in the $27–100 \times 10^3$ dalton region of the heavy plasma membrane protein profile makes difficult any direct comparison. No significant quantity of material generally fails to enter the gel. No bands are observed with a molecular weight of $25–26 \times 10^3$ [9,12,14], $13–16 \times 10^3$ [9] or $9–11 \times 10^3$ daltons [7,13]. However, a faint diffuse band is always detected in the 15 000 dalton region. It arises from isopycnic centrifugation temperature conditions (12°C) which we meant to increase the purity of junctional fractions.

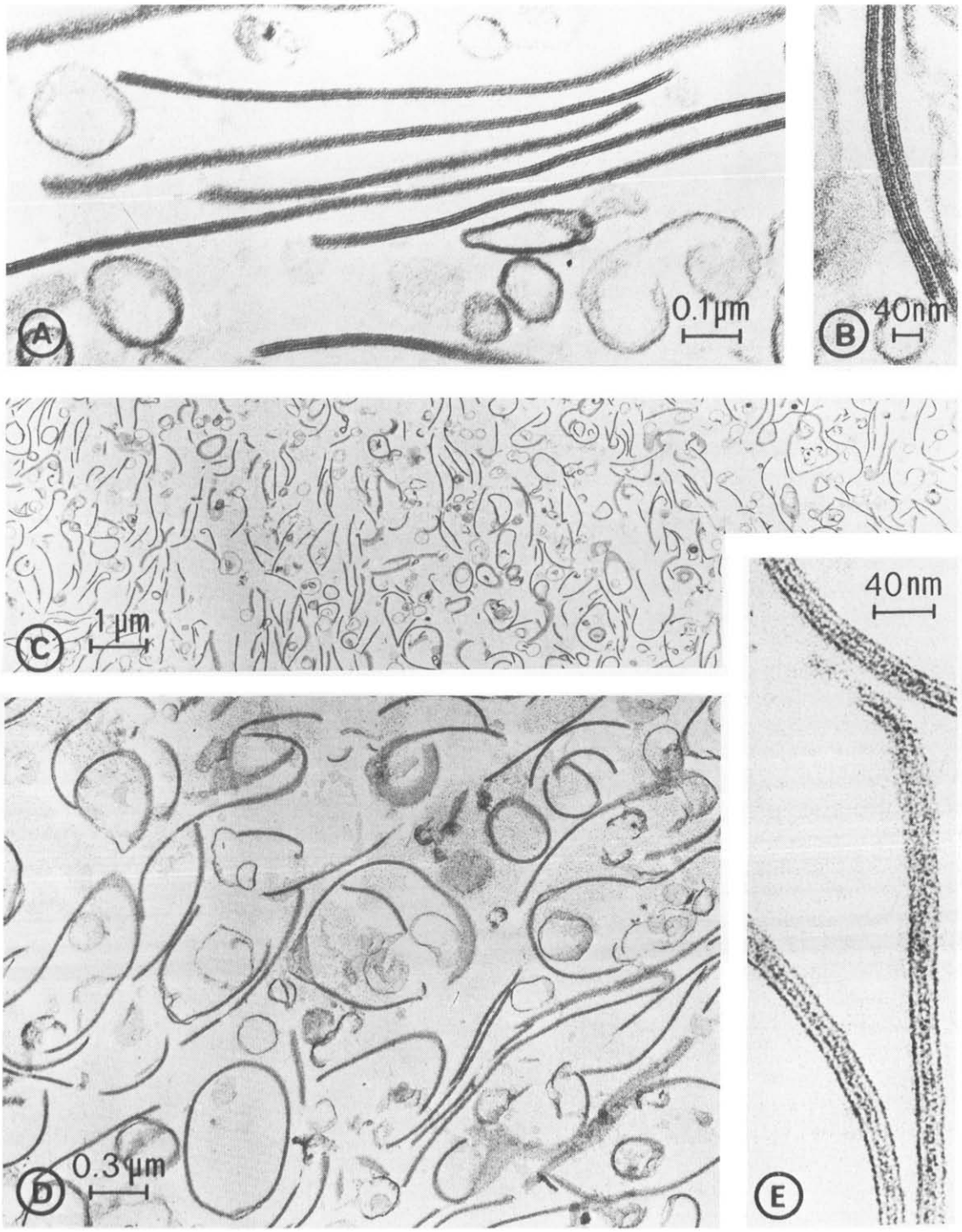

Fig.1. Electron micrographs of mouse hepatocyte gap junctions: (A,B) isolated in absence of trypsin; (C–E) isolated in presence of 0.005% trypsin.

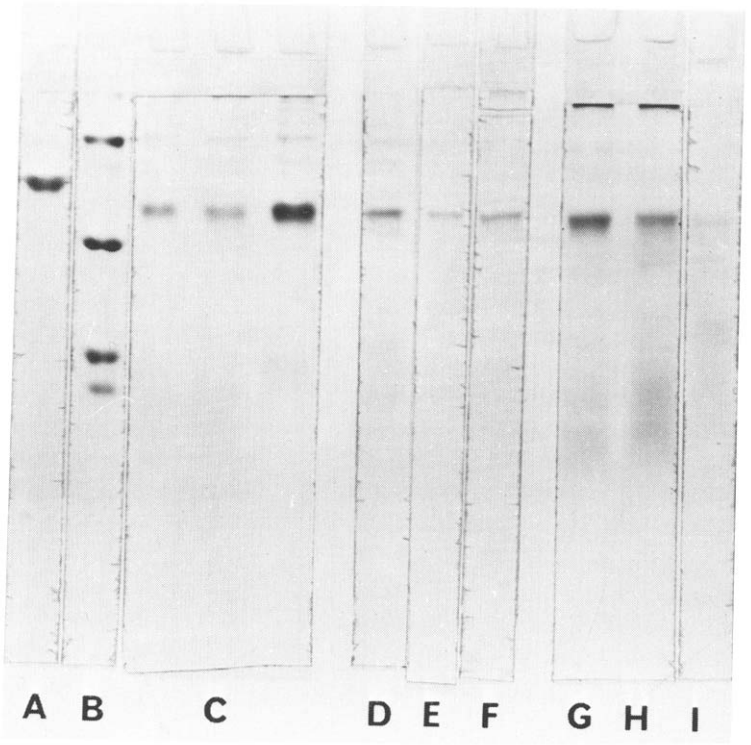

Fig.2. Electropherograms of molecular weight markers (A,B, see text) and of the following SDS-solubilized and reduced samples (isolated from 120 g wet liver): (C) gap junctions treated with hyaluronidase–collagenase alone at deoxycholate/plasmalemmal phospholipid molar ratios higher than 2 (3, 5 and 8 from right to left); (D) same as median C but sample was further alkylated; (E) trypsin–hyaluronidase–collagenase-treated gap junctions; (F) same as median C but with half concentration of both hyaluronidase–collagenase; (G–I) limited proteolysis of gap junctions isolated in absence of trypsin; (G) low trypsin level (see text); (H) high trypsin level; (I) very high trypsin level.

The electropherograms of junctional fractions isolated after centrifugation at 4°C but lacking of homogeneity never showed any band at 25–26 X 10^3 daltons whatsoever. Besides the major component and the polypeptides of higher molecular weight, the patterns were usually characterized by a 31 X 10^3 dalton band of variable intensity and three minor bands in the 13 X 10^3 dalton region. Only in one case of obtention of a highly enriched junctional fraction was the gel pattern reduced to the presence of the one major band.

Tryspin–hyaluronidase–collagenase-treated communicating junctions display a protein pattern which is shown in fig.2E. As demonstrated in absence of trypsin, one major protein band migrates at 34 ± 1 X 10^3 daltons (n = 6). The electropherogram of hyaluronidase–collagenase-treated gap junctions but with half the concentration of both enzymes (0.5 mg/ml for 1 h) also presents one major protein band at 34 X 10^3 daltons and no material at 25–26 X 10^3 daltons (fig.2F). While the junctional structure was virtually unchanged, the junctions were collected at a buoyant density (1.11–1.13 g/ml) much lower than under usual conditions. The use of hyaluronidase or collagenase alone did not allow the obtention of purified fractions.

Figure 2 G–I shows electropherograms of gap junctions isolated in absence of trypsin and treated at junctional protein/trypsin weight ratios of 150, 50 and 5 respectively. A diffuse band appears in the 10 000 dalton region at low trypsin level (fig.2G). Isolation of gap junctions in the presence of trypsin

was carried out under these low trypsin level conditions (150 ± 20). Significant degradation of the 34×10^3 dalton protein component only occurs under high trypsin levels (fig.2H,I) which appear to disorganize the junctional membrane lipid-embedded protein assembly.

Whatever the various treatments we used may be, it is conclusively demonstrated that only one SDS-solubilized and reduced junctional major protein component is present which migrates at $34 \pm 1 \times 10^3$ daltons. Our observations are in sharp contrast with those of Goodenough [6,7,13], Duguid and Revel [12], on mouse liver junctions. However, they might corroborate the results of Dunia et al. [9], but only when these authors isolated eye lens junctions after trypsin and deoxycholate treatment. In this case, we can make a reserve about the presence of their distinct 14×10^3 and 16×10^3 dalton bands.

From our experiments, it is not clear whether or not the 34×10^3 dalton polypeptide(s) is(are) characteristic of the heavy subfraction of isolated plasma membranes. Furthermore, it cannot be completely ruled out that some junctional 'peripheral' protein constituents could be eliminated. In absence of trypsin, deoxycholate solubilization has been preceded by a hyaluronidase–collagenase treatment, whose action upon the junctional molecular architecture is not easily predictable [14].

Acknowledgements

I thank Mrs Y. Florentin and E. Guerry for their technical assistance in preparing the electron micrographs.

References

[1] Simionescu, M., Simionescu, N. and Palade, G. E. (1975) J. Cell Biol. 67, 863–885.
[2] Bennett, M. V. L. (1973) Fed. Proc. 32, 65–75.
[3] Staehelin, L. A. (1974) Int. Rev. Cytol. 39, 191–283.
[4] Weinstein, R., Merk, F. and Alroy, J. (1976) Adv. Cancer Res. 23. 23–89.
[5] Benedetti, E. L. and Emmelot, P. (1968) J. Cell Biol. 38, 15–24.
[6] Goodenough, D. A. and Stoeckenius, W. (1972) J. Cell Biol. 54, 646–656.
[7] Goodenough, D. A. (1974) J. Cell Biol. 61, 557–563.
[8] Bloemendal, H., Zweers, A., Vermorken, F., Dunia, I. and Benedetti, E. L. (1972) Cell Diff. 1, 91–106.
[9] Dunia, I., Sen Ghosh, C., Benedetti, E. L., Zweers, A. and Bloemendal, H. (1974) FEBS Lett. 45, 139–144.
[10] Evans, W. H. and Gurd, J. W. (1972) Biochem. J. 128, 691–699.
[11] Gilula, N. B. (1974) J. Cell Biol. 63, 111a.
[12] Duguid, J. R. and Revel, J. P. (1975) Cold Spring Harbor Symp. Quant. Biol. 40, 45–47.
[13] Goodenough, D. A. (1970) J. Cell Biol. 68, 220–231.
[14] Benedetti, E. L., Dunia, I., Bentzel, C. J., Vermorken, A. J. M., Kibbelaar, M. and Bloemendal, H. (1976) Biochim. Biophys Acta 457, 353–384.
[15] Evans, W. H. (1970) Biochem. J. 166, 833–842.
[16] Monneron, A., Blobel, G. and Palade, G. E. (1972) J. Cell Biol. 55, 104–125.
[17] Studier, F. W. (1973) J. Mol. Biol. 79, 237–248.
[18] Laemmli, U. K. (1973) J. Mol. Biol. 80, 575–599.
[19] Dwyer, N. and Blobel, G. (1976) J. Cell Biol. 70, 581–591.
[20] Lopez, R. M. and Siekevitz, P. (1973) Anal. Biochem. 53, 594–602.
[21] Ehrhart, J. C. and Chauveau, J. (1975) Biochim. Biophys. Acta 375, 434–445.
[22] Ehrhart, J. C. and Chauveau, J. (1975) Biochimie 57, 987–989.