Journal of Cellular Biochemistry 19:27–44 (1982)

Immunological Properties of Gap Junction Protein From Mouse Liver

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Hepatic gap junctions were purified as plaques from BALB/c mice and separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Antisera were raised in rabbits and rats against gap junction plaques as well as against protein bands of the following apparent molecular weights: 44K to 49K ("dimer" proteins), 26K, and 21K. Using an enzyme immunoassay, we found that the reactivities of the different antisera towards gap junction plaques decreased in the following order: anti-plaque antisera, anti-26K antisera, anti-"dimer" protein antisera, and anti-21K antisera.

The gap junction protein bands separated by SDS-polyacrylamide gel electrophoresis were transferred by blotting onto nitrocellulose paper and the immunological cross-reactivities were compared: the anti-26K antisera reacted with the dimer protein bands and the 26K band but did not cross-react with the 21K protein band. The rabbit anti-21K antiserum reacted weakly with the 21K protein. The missing immunological cross-reaction of the 26K and the 21K protein band can be most easily explained if both proteins were independent of each other.

No inhibition of metabolic cooperation between fibroblastoid mouse 3T6 cells was observed in the presence of Fab fragments prepared from rabbit anti-plaque antiserum or from rabbit anti 26K antiserum. When the total proteins of plasma membranes from mouse liver were separated by SDS-polyacrylamide electrophoresis, only the 26K protein reacted with rabbit anti 26K antiserum. This result opens the possibility for direct quantitation of gap junction protein in tissues and cell fractions.

Key words: anti-26K antiserum, anti-21K antiserum, liver plasma membranes, enzyme immunoassay, immunoblotting

Gap junctions consist of protein channels that connect the cytoplasmic compartments of adjacent cells [1, 2]. Metabolites of a molecular weight <900 daltons can pass through gap junctions between mammalian cells [3]. Gap junctions are found between all mammalian cells except most nerve fibers and skeletal muscle cells. The almost ubiquitous existence of these structures has led to many speculations about their biological functions. Besides a role in electrical cell coupling and in homeostasis, gap junctions have been suggested to mediate the cell-to-cell transfer

Received August 15, 1981; accepted October 19, 1981.

0730-2312/82/1901-0027$05.00

of small ions and molecules involved in morphogenesis, embryonic induction, and proliferation control [4–6].

Gap junction channels of adjacent cells adhere in structures called plaques so firmly that the adherent plasma membranes can be dissolved by urea and nonionic detergents leaving the gap junction plaques morphologically intact. Since no enzymatic test exists for monitoring the degree of purification during isolation of gap junctions, electron microscopy is used for this purpose. Recently purifications of gap junctions plaques from liver [7, 8] and from heart [9] have been described. Henderson et al [8] investigated two major polypeptides of purified mouse hepatic gap junctions after polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (SDS). The authors suggested that the minor protein component (21K) is a proteolytic degradation product of the more abundant species (26K). Using specific antisera, we have studied the immunological cross-reactivity of these proteins in order to evaluate their role in the structure of gap junctions. Moreover, we found that the anti-26K antiserum reacted specifically with the 26K protein when the total proteins of hepatic plasma membranes were assayed.

MATERIALS AND METHODS

Cells and Culture Conditions

Mouse 3T6 cells were obtained from the American Type Culture Collection. The derived 3T6-TG8 cells, defective in hypoxanthine phosphoribosyl transferase (HPRT, E.C. No. 2.4.2.8) activity, were received from Dr. Howard Green [10]. All cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum plus streptomycin and penicillin (50 μg, each, per ml) (standard medium). The cells were found to be negative for mycoplasma contamination using the fluorescence assay with the dye Hoechst 33258 [11].

Isolation of Gap Junction Proteins

The procedure for isolation of mouse hepatic gap junction plaques was very similar to the method described by Henderson et al [8]. From 100 livers (about 220 gm, fresh weight) of BALB/c mice (14 weeks old), we obtained routinely after centrifugation in the linear sucrose gradient (15–35%) 500 to 600 μg of purified gap junction protein (plaque fraction) using the Lowry method for protein determination [12] or the Bio-Rad microassay procedure [13]. The electrophoretic pattern after SDS-polyacrylamide gel electrophoresis is nearly identical to that described by Henderson et al [8]. Our yield of purified gap junction protein per gm mouse liver is about twofold more than the yield reported by this group [8]. This could be due to the contaminating membrane material in our preparations and/or due to differences in the mouse strains used for gap junction isolation.

Electron Microscopy

Thirty to 60 μg of protein (gap junction plaque fraction) were collected by centrifugation in the airfuge (Beckman) at 140,000g for 30 min and fixed with glutaraldehyde (2.5%, v/v) in 0.05 M cacodylate buffer (pH 7.2) for 3 h. The pellet was twice washed with 0.1 M cacodylate buffer, pH 7.2, and treated with osmium tetroxide (2%, v/v) for 3 h. Before each liquid change the pellet was centrifuged in

the Eppendorf centrifuge at 9,980g for 10 min. The pellet was then washed 3 times for 5 min with cacodylate buffer at room temperature and subsequently incubated for 30 min with tannic acid (1%) dissolved in cacodylate buffer (0.05 M, pH 7.0) [14]. Afterwards, the pellet was washed for 5 min in the same cacodylate buffer containing 1% sodium sulfate. Finally the pellet was dehydrated in ethanol, equilibrated in propylene oxide, and embedded in epon (Serva).

Gel Electrophoreses

Gel electrophoreses were carried out in the presence of SDS in polyacrylamide gels using the discontinuous buffer system described by Laemmli [15] in vertical slab gels. The separating gel contained 12.5% polyacrylamide and the stacking gel 5% polyacrylamide. The gels were 1.5 mm thick for analytical purposes or 3 mm thick for preparative purposes. For the transfer of proteins onto nitrocellulose paper, we initially used the composite polyacrylamide/agarose slab gel system reported by Renart et al [16]. Later on, we used 12.5% polyacrylamide gels prepared according to Laemmli [15] for transfer experiments. The gels were stained for 2 h with Coomassie brillant blue G 250 and destained for at least 10 h with an aqueous solution containing 30% methanol and 10% acetic acid. Alternatively staining with silver nitrate and destaining according to Merril et al [17] allowed detection of protein bands containing less than 1 μg protein. All relevant protein bands that could be stained with silver nitrate at low concentrations could also be seen at higher concentration after staining with Coomassie brillant blue.

Preparation of Antigen and Immunization of Rats and Rabbits

Purified gap junction plaques were suspended in 1 mM Na_2-EDTA, pH 7.5, at 40–60 μg/ml using a vortex mixer. The solution was treated with the same amount of Alugel S (2%, Serva) for 1 h at room temperature and subsequently emulsified with complete or incomplete Freund's adjuvant (Behringwerke). For the first injections of all animals, complete Freund's adjuvant was used. All subsequently injected solutions contained incomplete Freund's adjuvant. For preparative electrophoresis 100 μg of plaque fraction protein were distributed into each of three slots of a slab gel and separated as indicated above. On one side of the preparative slots 20 μg protein of the same plaque fraction were electrophoresed. After electrophoresis, these reference lanes were cut off the preparative lanes and stained with Coomassie brillant blue. Meanwhile the preparative polyacrylamide gel lanes were stored frozen at $-20°$ C, enwrapped in Saran wrap. When the stained protein bands of the reference lane were clearly visible, the reference and the preparative lanes were placed in parallel position, and the thawed polyacrylamide pieces containing the unstained protein bands were cut out as indicated in Figure 2. In this manner, the protein bands of the dimer proteins, the 26K protein, and the 21K protein band were collected. From the staining pattern with Coomassie brillant blue, we estimated that the relative amounts of proteins with regard to the mentioned protein bands were about 1:2:1, respectively. To reduce possible cross-contamination, a 3-mm wide piece of polyacrylamide gel was discarded between the collected pieces containing the 26K and the 21K band. The collected polyacrylamide gel pieces were homogenized and suspended in an equal volume of Tris-buffered saline (about 0.2 ml per gel piece). No Alugel was used under these conditions. The suspended mass was emulsified with the double volume of complete or incomplete Freund's adjuvant by

shaking for 12 h at room temperature. During the injections, the syringes had to be shaken frequently since the gel particles tended to settle down. All animals were immunized five times at intervals of four weeks. Rats and rabbits imunized with protein of the plaque fraction received 60 μg and 135 μg protein, respectively, at the first injections and subsequently 40 μg. Rats and rabbits immunized with cut-out protein bands received 100 μg and 125 μg at the first injections and 20 μg and 60 μg during later injections, respectively.

Rats (BD IX strain) and rabbits were immunized at each time point by 15 subcutaneous injections near the lymph nodes of the rear legs. After 5 months, the anesthetized animals were bled by heart puncture (rabbits) or through a catheter in the vena jugularis (rats). The serum was obtained by centrifugation of the blood clotted for 12 h at room temperature and stored at 4°C or −20°C in the presence of 0.02% sodium azide.

Preparation of Fab Fragments

The IgG fraction of different rabbit sera (4 ml) isolated by affinity chromatography on protein A-Sepharose (Pharmacia) was cleaved by incubation for 30 min at 37°C with 0.5 mg papain in 0.2 M sodium phosphate buffer (pH 8.0), 2mM EDTA, and 1 mM cysteine (total volume: 5 ml). The reaction was stopped by addition of iodoacetamide to a final concentration of 2 mM. The cleavage products were separated on a DEAE cellulose column (Whatman DE 52, 1.5 × 2.5 cm, equilibrated in 0.01 M potassium phosphate buffer, pH 8.0). The Fab fragments were collected in the void volume and concentrated by pressure dialysis on an Amicon YM membrane. Further purification was accomplished by molecular sieving through a Sephadex G100 column (1.5 × 25cm) in 0.01 M potassium phosphate buffer, pH 8.0. Protein yield: 45 mg (according to the absorption at 280 nm).

Enzyme Immunoassay

For the development of this assay, we adapted the procedures described by Engvall and Perlmann [18]. For preparation of the immunoadsorbents, 100 mg rat IgG (Miles) in 6.7 ml of 0.1 M sodium acetate buffer, pH 5.0, (protein concentration 15 mg/ml) were polymerized with 2 ml of glutaraldehyde (Sigma; 2.5 %) for 12 h and homogenized. Excess glutaraldehyde was inactivated by incubation with glycine (1 M) in 0.1 M sodium borate buffer (pH 8.5). The immunoadsorbent was washed five times with phosphate buffered saline (PBS) and stored in the presence of 1% bovine serum albumin (BSA) and 0.02% sodium azide.

For enrichment of specific rabbit anti rat IgG antibodies 45 mg of the rabbit anti-rat IgG fraction (Miles) were dissolved in 7.5 ml PBS and incubated for 2 h at room temperature with the immunoadsorbent described above. The polymerized material was centrifuged and washed five times in PBS. The IgG specific antibodies were eluted three times with 0.1 M glycine-HCl, pH 2.3 (each time 3 ml for 5 min at room temperature) and neutralized by addition of 0.3 ml Tris-HCl (1 M, pH 8.5). All eluates were pooled, centrifuged for 10 min at 3,000g, dialyzed three times for 1 h against 1:10 diluted PBS and lyophilized. Final protein yield: 0.96 mg.

For coupling to alkaline phosphatase, 0.5 mg of the affinity purified IgG protein was dissolved in 0.5 ml PBS containing 1.5 mg alkaline phosphatase (Sigma).

The solution was dialyzed for 2 h at 4°C against PBS and polymerized with glutaraldehyde (Sigma, 0.2% final concentration) during slow agitation. Then, the conjugate was dialyzed for 12 h at 4°C against PBS and stored in the presence of 1% BSA and 0.02% sodium azide. The conjugate of goat anti-rabbit IgG (Miles) to alkaline phosphatase was prepared without prior purification of the IgG specific antibodies.

The enzyme immunoassay was performed in microtiter plates (Greiner). Into each well, 100 μl of a solution of 3 μg/ml of plaque fraction protein in Tris-buffered saline were pipetted and incubated for 12 h at 4°C. Then the wells were washed twice with 0.1% (v/v) NP40 (Roth) in PBS. The remaining binding sites of each plastic well were saturated by incubation with 350 μl of BSA (3%) in Tris-buffered saline for 2 h at room temperature. The wells were washed 3 times with 0.1% NP40 in PBS, incubated with the serum diluted in 0.1% NP40 in Tris-buffered saline for 1.5 h at 37°C and washed again 4 times with 0.1% NP40 in PBS. Afterwards, 100 μl of the alkaline phosphatase conjugated antibodies (2 μl conjugate per ml solution of 1 mM $MgCl_2$, 0.04% sodium azide, 5% BSA, in Tris-HCl, [0.5 M, pH 8.0]) were pipetted into each well for incubation (1.5 h) at 37°C. All wells were washed four times with 0.1% NP40 in PBS and twice with PBS without NP40. Finally the wells were incubated with 100 μl (4 mg/ml) of *p*-nitrophenyl phosphate in sodium carbonate, 50 mM, pH 9.5, containing 2 mM $MgCl_2$ at 37°C. After 30 min, the extinction at 405 nm was recorded in a Titertek Multiscan spectrophotometer. To avoid nonspecific binding, it was important to wash the wells extensively.

Synthesis of Diazobenzoxymethyl Paper

The synthesis of 1-(*m*-nitrobenzoxy)methylpyridinium chloride, the preparation of aminobenzoxymethyl paper (ABM-paper), and the diazotation to diazobenzoxymethyl paper (DBM-paper) were carried out as described by Alwine et al [19].

Blotting of Gap Junction Proteins Onto Paper

A method previously described [16, 20] was used with minor modifications: The polyacrylamide gels were incubated before protein blotting for 20 min at 37°C in 2% (w/v) periodic acid to oxidize the cross-links of the gel matrix caused by N,N′-diallyl tartardiamide. The gels were equilibrated twice for 20 min in phosphate buffer, 0.5 M, pH 7.5, at room temperature. For blotting of the protein bands onto appropriate paper, an apparatus similar as described by Southern [21] was used; blotting time: 12–24 h at room temperature. The following buffers were used: phosphate buffer, 0.5 M or 0.05 M and four fold concentrated electrophoresis buffer (1 M Tris and 7.7 M glycine, pH 8.3, 4% SDS). After transfer, the free binding sites on the paper were saturated by incubation with 1% BSA in PBS and incubated with the diluted antisera (1:25 for rabbit antisera, 1:50 for rat antisera) for 8–12 h at room temperature. For this purpose, the paper strip was placed in a plastic bag and rocked slowly on a platform. Air bubbles in the incubation solution were carefully removed. Afterwards, the paper was washed twice for 30 min with 0.3 M NaCl, and 0.1% Triton X-100 in PBS. The paper strips treated with rabbit antisera were washed briefly with PBS before being incubated with protein A. Paper strips treated with rat antisera were incubated for 3 h with rabbit anti-rat IgG (Miles) in PBS

(containing 0.1% BSA) since protein A does not bind to rat IgG. All paper strips were incubated for 2 h with ^{125}I-protein A (Amersham Buchler, specific activity: 30 mCi/mg, 0.0625 μCi per paper strip) in 5 ml PBS containing 0.05% Triton X-100 and in PBS containing 0.3 M NaCl and 0.1% Triton X-100. Each strip was rinsed in 10 ml PBS and dried between two layers of Whatman 3 MM paper, first for 10 min at 80°C and then for 10 min at room temperature. Finally, autoradiography was carried out with Kodak X-omat XR5 film in a Siemens cassette (18 × 24 cm) using an intensifying screen (Spezialfolie, Siemens) for 10–17 h at −70°C.

Cocultivation and Labelling of Cells for Detection of Metabolic Cooperation

3T6 cells (2×10^3) and 3T6-TG8 cells (2×10^4) were grown at 37°C in standard medium and cocultured in the well (about 2 cm^2 surface area) of a Costar plate containing a round glass coverslip (11 mm diameter). After 6 h, 20 h, and 24 h, the culture medium was replaced by standard medium containing Fab fragments of rabbit IgG (0.5 mg/ml). The medium added at 24 h contained in addition 2 μCi/ml [^{3}H] hypoxanthine (specific activity: 25.3 Ci/mmol, Amersham-Buchler). After 5 h of incubation with [^{3}H] hypoxanthine, the cells on the coverslips were washed three times with PBS and fixed for 10 min at room temperature with 1-methylpropanol (95%). Then, the cells were treated twice with trichloroacetic acid (10%, w/v) for 10 min at 4°C and washed three times each with water and with methanol. The coverslips were glued right-side-up with DePex (Gurr) onto microscopic slides, bathed in 0.5% gelatine solution, exposed to Kodak AR10 stripping film for five days, developed, and stained with toluidine blue.

In some cases, to avoid trypsinization of the initial cell cultures, the 3T6 and 3T6-TG8 cells were grown separately for three days in bacteriological Petri dishes in standard culture medium until they reached about 10% confluency. Under these conditions, the cells were well spread but only loosely attached to the plastic surface and rarely contacted each other. The culture medium contained Fab fragments and [^{3}H] hypoxanthine as indicated above. The cells were shaken loose in this medium, mixed at about the same ratio as described above and transferred into Costar wells containing glass coverslips. After 5 h of cocultivation at 37°C, the same procedure (for fixation, etc) as outlined above was followed. For evaluation of metabolic cooperation, we used the 90th percentile as suggested by Gaunt and Subak-Sharpe [22].

Electrical Measurements of Ionic Coupling

These electrical measurements of ionic coupling between 3T6-TG8 cells were carried out according to published procedures [23].

RESULTS

Purification and Polyacrylamide Gel Electrophoresis of Gap Junction Proteins

Figure 1 shows a cross-section through a typical preparation of purified gap junction plaques negatively stained by tannic acid treatment. The typical structures

Fig. 1. Electron micrograph of thin sections through a pellet of purified gap junction plaques. In addition to an excess of typical gap junction structures the preparation contained some nonjunctional membranes (Δ). Junctional plaques and nonjunctional membranes appear negatively stained due to the conditions of treatment with tannic acid (see Materials and Methods).

of gap junctions are most abundant, but cross-sections of contaminating membrane vesicles can be found. Figure 2 shows the separation after SDS-polyacrylamide gel electrophoresis of the plaque fraction protein. We assigned the protein bands according to the suggestion of Henderson et al [8] as 21K, 26K, dimeric protein bands (44K to 49K). We noted an additional weak protein band of 24K, which was discernible in most of our preparations of gap junction protein. The 24K protein band can also be seen in Figure 7 of the paper by Henderson et al [8] but was not discussed by these authors. We noticed the 24K protein band in particular in preparations of gap junction plaques that had been stored at 4°C for several weeks and that were reexamined by electrophoresis. Under these conditions, the 26K band had become weaker and the 24K band stronger compared with the first electrophoretic pattern. This result suggests that the 24K band could be a degradation product of 26K. If more protein of the plaque fraction was applied per gel slot the 24K band was often not well resolved from the 26K band (cf Figs. 7 and 8). At the beginning of the stacking gel and after entrance into the separating gel, a significant amount of protein was not resolved into bands.

Double Immunodiffusion Assay and Enzyme Immunoassay

As described under Materials and Methods, the plaque fraction and the different protein bands were used for immunization of rats and rabbits. In Figure 2, the areas on the polyacrylamide gel are indicated that were cut out unstained because they were supposed to contain the protein bands, by comparison with stained reference proteins. Most likely, the 24K band was at least in part cut out together with the 26K band. Figure 3 illustrates the results of double immunodiffusion assays according to Ouchterlony. A double precipitation band was obtained with serum from each of two rabbits and rats (not shown) immunized with purified plaques. At a 1:5 dilution, this double precipitation band was no longer visible. No other antisera produced precipitation bands with plaque fraction protein in the central well of the Ouchterlony plate. It is possible that the double precipitation band was due to differently aggregated complexes of gap junction plaques. Since the double diffusion assay was too insensitive to allow detailed immunological analysis, we developed an enzyme immunoassay as described under Materials and Methods. The

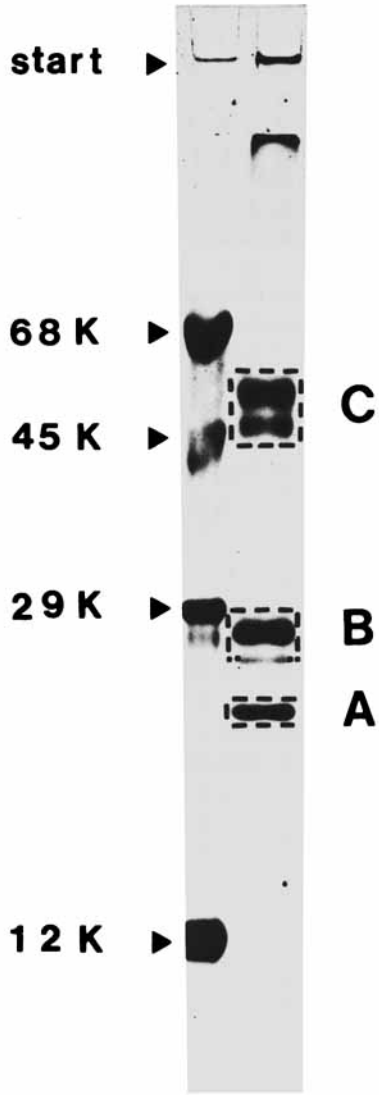

Fig. 2. Electrophoresis in SDS-polyacrylamide (12.5%) gels of purified gap junction plaques. Right lane: a typical preparation of gap junction plaques (cf Fig. 1) was dissolved in SDS-containing sample buffer, subjected to electrophoresis, and stained with Coomassie brillant blue (see Materials and Methods). The indicated areas represent the pieces of polyacrylamide gels that were cut out, homogenized, and used for immunization. They contain the following proein bands: A: 21K; B: 26K; C: dimer proteins 44K to 49K. Left lane: Molecular weight reference proteins, 68K: bovine serum albumin, 45K: ovalbumin, 29K: carbonic anhydrase, and 12K: cytochrome c.

plaque fraction protein was bound to the plastic surface of microtiter wells and treated with the antisera to be tested. Then a second antibody coupled to alkaline phosphatase was allowed to bind to the antiserum bound to the plaque fraction. Figure 4 shows the time dependency of the enzymatic reaction under our assay con-

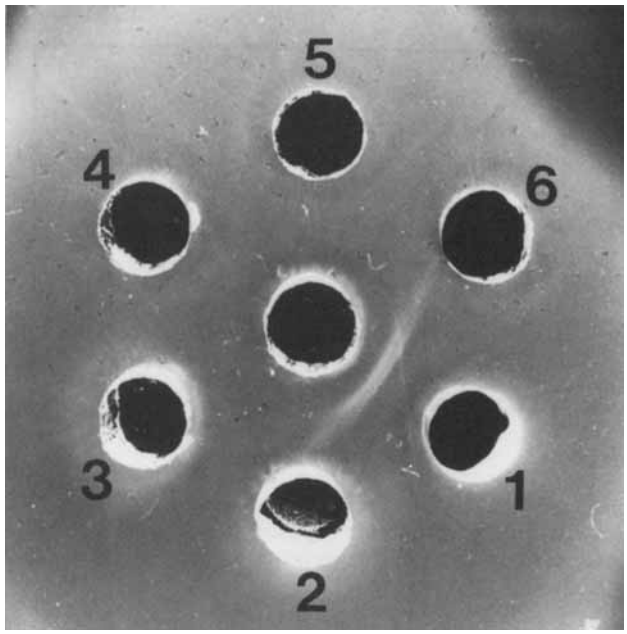

Fig. 3. Double immunodiffusion assay of different anti gap junction antisera. The central well of an Ouchterlony agar plate was filled with 50 μl of purified gap junction plaques suspended in 1 mM EDTA, pH 7.5, at a concentration of 0.6 mg protein per ml. Aliquots of the following undiluted sera were distributed into the peripheral wells: 1, rabbit anti-plaque antiserum; 2, rabbit anti-21K antiserum; 3, rabbit anti-26K antiserum; 4, rat anti-dimer protein antiserum; 5, rat anti-21K antiserum; and 6, rat anti-26K antiserum. The photograph was taken after incubation for 36 h at 4°C.

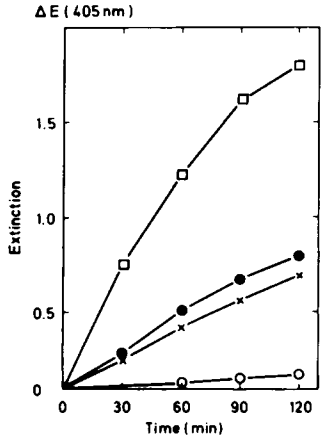

Fig. 4. Enzyme immunoassay: Time dependence of alkaline phosphatase reaction after binding of different antisera from rats to the plaque fraction of gap junctions. Experimental details of the enzyme immunoassay are described under Materials and Methods. Explanation of symbols: □, anti-plaque antiserum; x, anti-dimer proteins antiserum; ●, anti-26K antiserum; and ○, anti-21K antiserum.

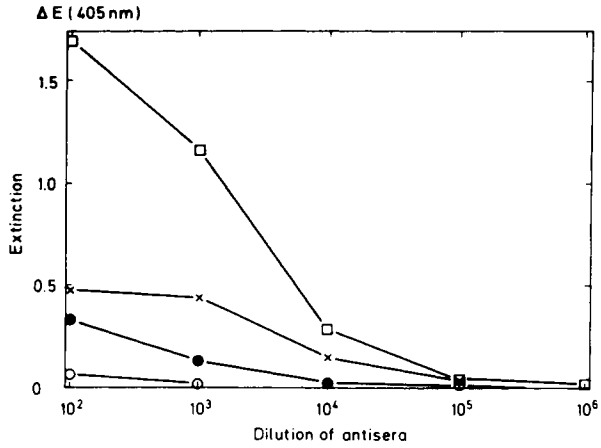

Fig. 5. Enzyme immunoassay: Binding of different antisera from rats to the plaque fraction of gap junctions. For experimental details of the enzyme immunoassay see Materials and Methods. Explanation of symbols: □, anti-plaque antiserum; x, anti-dimer proteins antiserum; ●, anti-26K antiserum; and ○, anti-21K antiserum.

ditions. We decided to choose the incubation time of 90 min for maximal color development near the end of the linear phase of the enzyme kinetics. Thus, all enzyme immunoassays were stopped after 90-min incubation time with the enzyme substrate.

Figure 5 shows a comparison of the different antisera from rats at several dilutions, starting with a serum dilution of 1:100. Undiluted sera and 1:10 dilutions of the sera inhibited the enzymatic reaction. From Figure 5, it can be concluded that the different antisera can be grouped according to decreasing binding activity to the plaque fraction in the following order: anti-plaque antiserum, anti-dimer proteins antiserum, anti-26K antiserum, and anti-21K antiserum. The anti-21K antiserum exhibits only at 1:100 dilution barely detectable binding activity to the plaque protein. In addition to the sera used in Figure 5, each antiserum was raised in a second rat. The titers for binding of antisera to the plaque protein differed between two rats by not more than 50%.

In addition to rats, rabbits were immunized with the same preparation of antigens (without dimer proteins). With different antisera from rabbits, we found the same order of binding to the plaque protein fraction as with antisera from rats: anti-plaque antiserum, anti-26K antiserum and anti-21K antiserum (Fig. 6). Furthermore, Figure 6 illustrates the binding activity to the plaque fraction of Fab fragments isolated from rabbit anti-plaque antiserum and from rabbit anti-26K and antiserum. These Fab fragments were used for attempts to inhibit metabolic cooperation between cultured cells (see below).

Transfer of Proteins to DBM-Paper or Nitrocellulose Paper and Comparison of Immunological Reactivity

We synthesized DBM-paper according to published procedures [19] and compared its binding activity for gap junction proteins with commercial ABM-paper

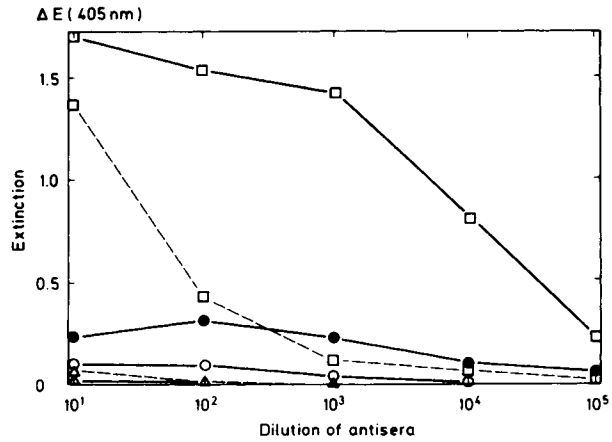

Fig. 6. Enzyme immunoassay: Binding of different antisera and Fab fragments from rabbits to the plaque fraction of gap junctions. For details of the enzyme immunoassay see Materials and Methods. Explanation of symbols: □ (drawn line), anti-plaque antiserum; □ (hatched line), anti-plaque Fab fragments prepared from the previous antiserum; ●, anti-26K antiserum; ○, anti-21K antiserum; △ (drawn line), control serum from nonimmunized rabbit; and △ (hatched line), Fab fragments from the previous control serum.

(Schleicher und Schüll, converted to DBM-paper in our laboratory) as well as with nitrocellulose paper (Sartorius, Type SM 11 336). In addition, we compared the protein transfer onto the paper in the presence of different buffer systems (data not shown). We found that the binding activity of gap junction proteins from SDS-polyacrylamide gels to our synthesized DBM-paper was superior compared with the commercial product. However, binding to nitrocellulose paper gave similar results as our synthesized DBM-paper. All protein bands covalently bound after blotting to DBM-paper were also seen on nitrocellulose paper. Since the nitrocellulose paper did not require the time-consuming processing before use, we decided to blot the gap junction protein bands onto this paper in fourfold concentrated electrophoresis buffer, which gave optimal results under our conditions.

Figure 7 shows a comparison of different rat antisera prepared against different protein bands of gap junction plaques. It is evident that anti-plaque antiserum, anti-dimer proteins antiserum, and anti-26K antiserum show a very similar binding pattern towards the separated protein bands. The different sera react with the 26K band, with the dimer protein band, and with the poorly resolved protein bands in range of 60K to 80K apparent molecular weight (trimers?) and with some protein material near the start of the stacking gel. Surprisingly, we never found cross-reactivity of the different antisera with the 21K band. Furthermore, the rat anti-21K antiserum showed no binding activity at all with any protein band. The missing reaction of the 21K band was not due to insufficient transfer onto nitrocellulose paper. We checked the nitrocellulose paper after blotting (before reaction with the antisera) with Amido-Schwarz 10 B (Merck) [24] and found that the 21K band had indeed been transferred to the nitrocellulose paper although apparently less efficiently than the 26K band. Figure 7 also demonstrates that in addition to the 21K band, other proteins near the beginning of the separating gel did not

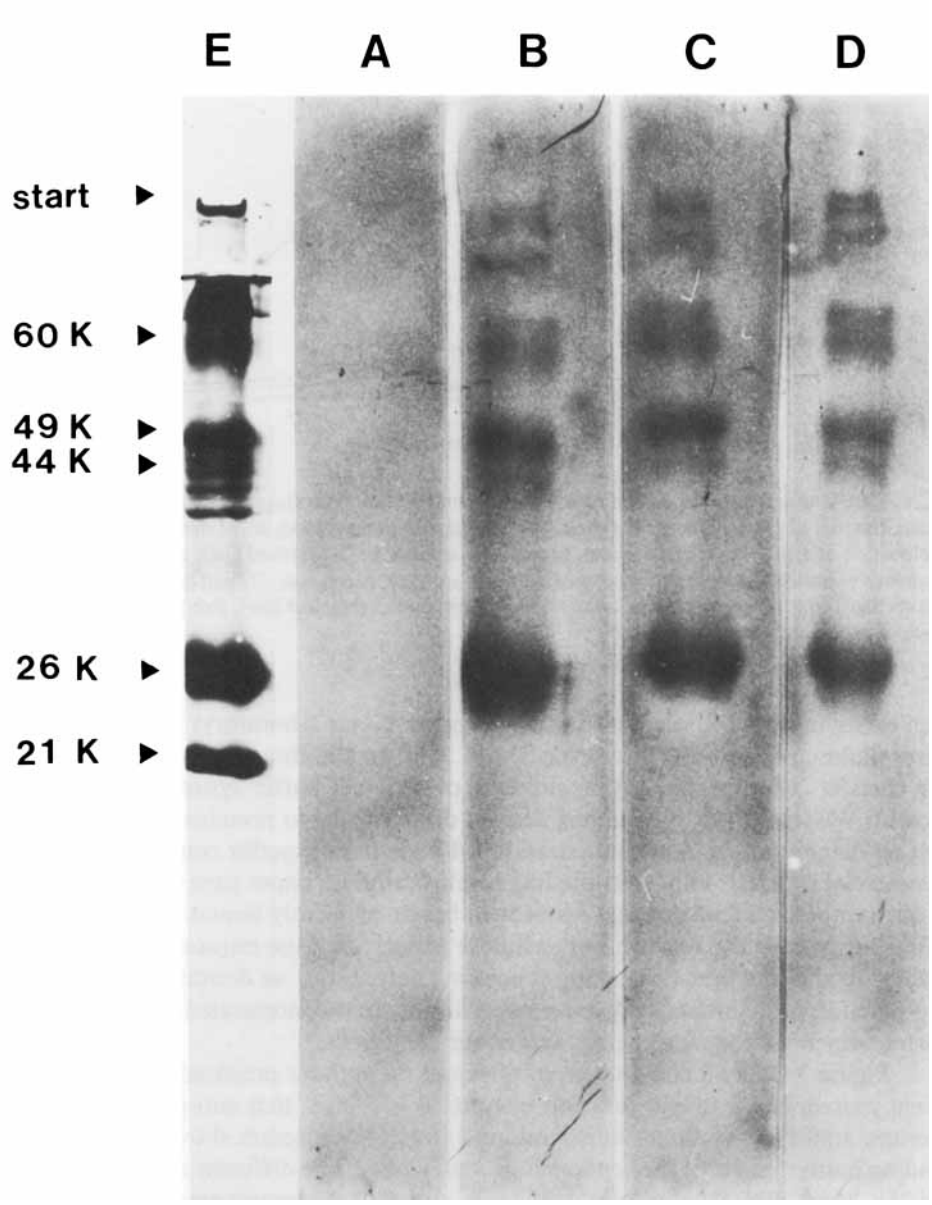

Fig. 7. Immunological reactivity of gap junction proteins with different antisera from rats. Purified gap junction plaques were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose paper. The nitrocellulose paper was incubated with the appropriate rat antiserum (see below), with rabbit anti-rat IgG, and with ^{125}I-protein A, before being autoradiographed. For experimental details see Materials and Methods. The following antisera were used: lane A: anti-21K antiserum, lane B: anti-26K antiserum, lane C: anti-dimer protein antiserum, and lane D: anti-plaque antiserum. Lane E shows a parallel electrophoretic run of plaque proteins stained with Coomassie brillant blue before transfer. The apparent molecular weights of the proteins were determined by coelectrophoresis of appropriate protein standards.

react with the different antisera. We conclude that the different antisera from rats appear to react with the same protein(s) from purified gap junction plaques and do not recognize the 21K band. Control sera taken from rats before immunization did not show any reaction with gap junction protein after blotting (results not shown).

Figure 8 illustrates the reactivity of different antisera prepared in rabbits against gap junction antigens. Again it was found that anti-plaque antiserum and anti-26K antiserum did not react with the 21K band, although reaction at the position of the aggregated proteins could be seen. It should be noted that in these gels, the 26K and the 24K band were not resolved. Therefore, we could not discriminate these proteins. It is likely that the anti-26K antiserum also contained antibodies

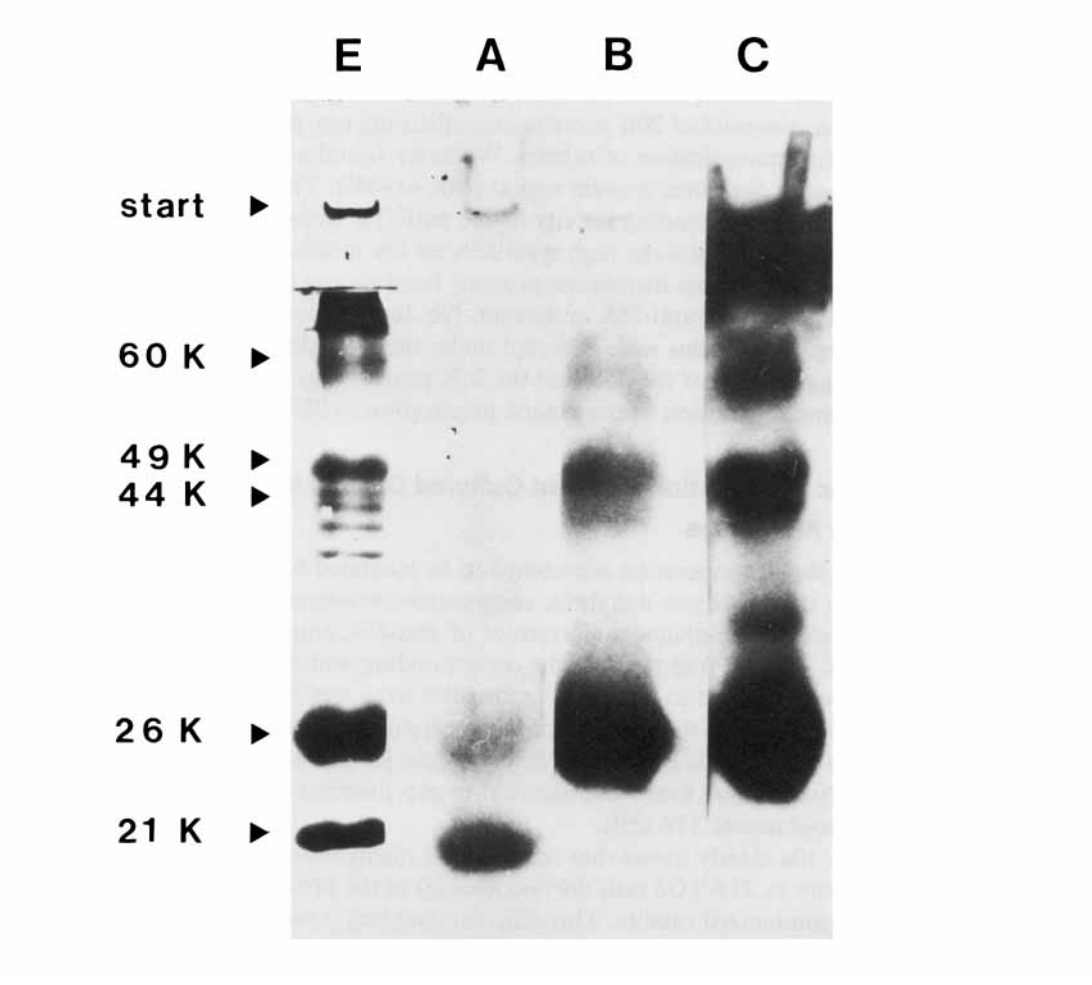

Fig. 8. Immunological reactivity of gap junction proteins with different antisera from rabbits. The proteins of purified gap junction plaques were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose paper. The assay procedure is identical to the one described in the legend to Figure 7 except that the incubation with ^{125}I-protein A was carried out without a second antiserum since protein A binds directly to rabbit IgG. Lanes A, B, C are autoradiographs of incubations with anti-21K antiserum, anti-26K antiserum, and anti-plaque antiserum, respectively. Lane E shows a parallel electrophoretic run of plaque proteins stained with Coomassie brillant blue before transfer.

against the 24K band. Very recently, it has been reported that the 26K and the 24K band of gap junction proteins from rat liver are likely to be structurally related according to their proteolytic peptide pattern [25]. When we examined preparations of plaques that had been stored for two to four weeks at 4°C, we noticed, after Coomassie brillant blue staining of polyacrylamide gels, a decrease of the 26K band and the appearance of two relatively broad protein bands of 16K to 18K and of about 10K. Both proteins react strongly with anti-26K antiserum (results not shown) and thus are likely to be proteolytic degradation products of the 26K protein. Goodenough [26] previously described the 10K protein ("connexin") and Revel et al [26] reported some evidence that it might be derived from the 26K protein. In no instance did we observe degradation products of a molecular weight close to the 21K band that reacted with the anti-26K antiserum. These results argue against the suggestion by Henderson et al [8] that the 21K band may be derived from the 26K band. The rabbit anti-21K antiserum reacted with the anti-21K protein band (Fig. 8). The weak reaction of the anti-21K antiserum with the 24K band was probably due to trace amounts of 26K protein contaminating our preparation of the 21K protein used for immunization of rabbits. We never found a reaction of the anti-21K antiserum with the dimer protein region (44K to 49K). This could have been due, however, to the low binding activity of the anti-21K antiserum.

Figure 9 illustrates the high specificity of the rabbit anti-26K antiserum. Of all SDS-solubilized plasma membrane proteins from mouse liver, only one, the 26K band, reacts with the anti-26K antiserum. No dimer proteins (44K to 49K) and further aggregated proteins were dedected under these conditions. This result suggests that the aggregation of the 26K and the 21K protein may be an artifact found after isolation and dissolution of the plaque preparation in SDS.

Metabolic Cooperation Between Cultured Cells in the Presence of Anti-Gap Junction Antibodies

Metabolic cooperation is assumed to be mediated by gap junctions [27]. We wanted to study whether metabolic cooperation between cells could be inhibited by the presence of anti-plaque antiserum or of anti-26K antiserum. For these experiments, the Fab fragments of the corresponding antisera were used. Fab fragments in contrast to whole IgG molecules were supposed not to cross-link cell surface antigens and thus not to stimulate capping, degradation, and the endogenous biosynthesis of these surface antigens [28]. We assumed that gap junction proteins from mouse liver were identical to gap junction proteins in cultured fibroblastoid mouse 3T6 cells.

Fig. 10a clearly shows that we observed highly efficient cell-to-cell transfer of radioactivity in 3T6-TG8 cells (90% coupling) in the presence of Fab fragments from nonimmunized rabbits. This efficient coupling is not significantly reduced in the presence of Fab fragments isolated from anti-plaque antiserum (Fig. 10b) or from anti-26K antiserum (data not shown). We also checked this effect after very short coculturing (60 min) of the cells, assuming that under these conditions relatively little inactivation of the Fab fragments and little resynthesis of gap junction protein could have occured. Again, no significant inhibition of metabolic cooperation was found in the presence of anti-plaque Fab or anti-26K Fab fragments. Furthermore, no inhibition of electrical coupling [23] between 3T6-TG8 cells was observed in the presence of these Fab fragments (data not shown).

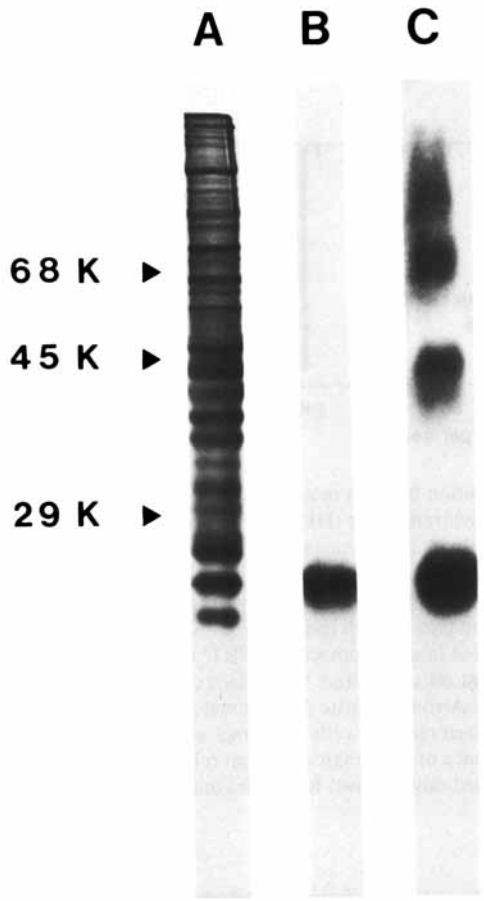

Fig. 9. Immunological reactivity of hepatic plasma membrane proteins with rabbit anti-26K antiserum. Plasma membrane proteins (35 μg) obtained during the routine purification from mouse liver [8] were separated on SDS-polyacrylamide gels [15] and analyzed after transfer onto nitrocellulose paper. Lane A, protein bands on the gel stained with Coomassie brillant blue; lane B, autoradiograph after incubation with rabbit anti-26K antiserum and ^{125}I-protein A according to standard conditions; lane C, autoradiograph of electrophoretically separated plaque proteins after transfer onto nitrocellulose paper. The molecular weights indicate the position of protein markers on a reference gel (not shown): 68K, bovine serum albumin; 45K, ovalbumin; and 29K, carbonic anhydrase.

DISCUSSION

This is the first investigation of mouse hepatic gap junctions using antisera directed against purified total gap junction plaques and against the separated protein components. An unexpected result of our studies was that antisera against the 26K protein did not react with the 21K protein. If the 21K protein were indeed a degradation product of the 26K protein as suggested by Henderson et al [8], one would have expected to find some immunological cross-reactivity.

From our results, we conclude that most likely the 21K and the 26K proteins are two independent proteins. Recently, the 26K protein has been postulated to be the main component of hepatic gap junctions [29]. The 21K protein may be a con-

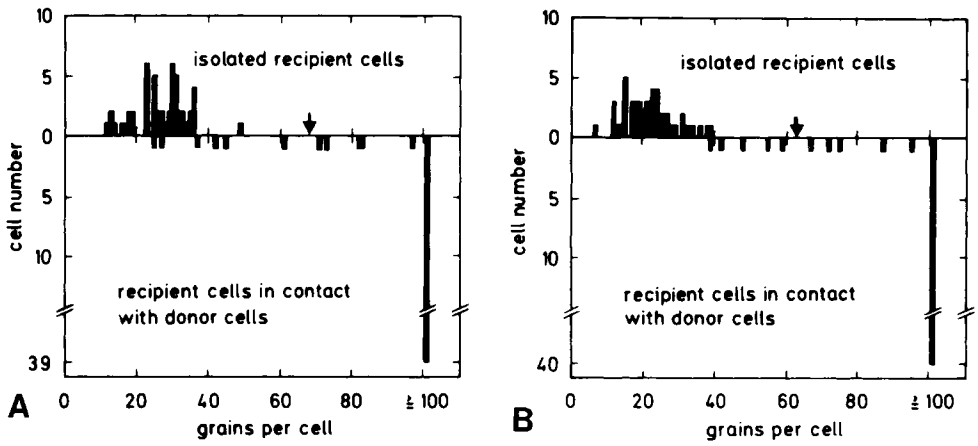

Fig. 10. Metabolic cooperation between mouse 3T6 cells and the derivated 3T6-TG8 cells defective in hypoxanthine phosphoribosyl transferase (HPRT). Both cell lines were cocultured on glass cover slips for 24 h. They were labelled with [^{3}H] hypoxanthine, washed, fixed, and autoradiographed (see Materials and Methods). The photographic silver grains over the cells were counted under the microscope. The upright part of each histogram shows the distribution of grains over isolated HPRT$^-$ (recipient) cells. The inverted part of each histogram shows the distribution of grains over those HPRT$^-$ cells that were located in close contact to HPRT$^+$ (donor) cells. Functional metabolic cooperation is evident when recipient cells in contact with donor cells show on the average more grains per cell than isolated recipient cells. Arrows indicate the minimal grain number taken as threshold value for metabolic cooperation between recipient cells in contact with donor cells [22]. Metabolic cooperation was determined in the presence of Fab fragments from rabbit antisera. Panel A: Fab fragments from the serum of a nonimmunized rabbit; panel B: Fab fragments from anti-plaque antiserum.

taminating protein of mouse gap junction plaques that at present cannot be separated without the use of SDS-polyacrylamide electrophoresis. The independent nature of the 26K and the 21K protein is supported by preliminary analyses of the N-terminal amino acids [O. Traub and K. Beyreuther, Institute of Genetics, University of Cologne, unpublished results]. Whereas methionine was found at the N-terminus of the 26K protein, neither methionine nor any other amino acid could be detected at the N-terminus of the 21K protein. Possibly the N-terminus of the 21K protein is blocked. Although there is a strong tendency for aggregation of the 21K and 26K when analyzed by SDS-polyacrylamide electrophoresis of purified plaques, little or no aggregation was seen when the total proteins of liver plasma membranes were analyzed with the anti-26K antiserum.

If the 21K protein would be part of the 26K protein one would have to postulate that the highly immunogenic sites present on the 26K protein should be located on the hypothetical protein (about 5K apparent molecular weight) that had to be cleaved off to yield the 21K protein of low immunogenicity. We tried to demonstrate this hypothetical cleavage protein by SDS electrophoresis on polyacrylamide gels under conditions where the reference proteins, aprotinin (6.5K) and insulin B (3.5K), were well resolved. No protein band was found after Coomassie brillant blue or silver nitrate staining in the expected range of molecular weights, and no reaction was seen after blotting analysis with rabbit anti-26K an-

tiserum (data not shown). It is possible, of course, that the hypothetical cleavage protein is further degraded and therefore no longer detectable at its initial size.

The anti-plaque antisera may be mainly directed against the cytoplasmic sites of gap junction proteins. The cell surface sites of gap junction plaques are presumably at least at the time of antigen injection not exposed but covered by the corresponding adherent connexon structures. Probably proteolytic degradation of purified gap junctions plaques starts at the former cytoplasmic side of the proteins. In this context, it should be noted that the anti-plaque antisera from rabbits never reacted with the 21K band, although the purified 21K band was weakly immunogenic when injected into rabbits. This finding could indicate that the 21K protein is covered in purified gap junction plaques. Electron micrographs of purified gap junction plaques show no indication for separation of the connexon hemichannels. Therefore, it is not too surprising that our anti-plaque antiserum and the anti-26K antiserum do not inhibit metabolic cooperation between mouse fibroblastoid 3T6 cells. No preferential binding of anti-plaque antiserum or anti-26K antiserum (compared with control sera from nonimmunized rabbits) to cell surfaces of mouse 3T6 cells could be demonstrated by the use of anti-rabbit IgG labelled with fluorescein isothiocyanate (data not shown). Perhaps one can obtain antibodies that recognize the cell surface part of connexons if the gap junction plaques were first separated into sheets of hemichannels before being used for immunization.

Alternatively, our failure to obtain antibodies against that part of gap junction proteins that is exposed to the cell surface could be explained by immunological tolerance. It is possible that the immune systems of rabbits and rats do not recognize the cell surface part of mouse gap junction proteins as foreign because it is very similar to their own corresponding protein structures. Recently, it was reported that the peptide maps of hepatic gap junction from mouse, rat, and calf were homologous [26]. The anti-26K antisera characterized in this paper should be very useful for quantitative analysis of the biosynthesis and turnover of gap junction protein in different tissues and cell fractions.

ACKNOWLEDGMENTS

We are indebted to Professor D. Hülser (Stuttgart) for his electrical measurements of ionic coupling between cells and for the discussions related to this work. The technical assistance of Beate Arendt, Hasan Özdogan, Ingeborg Rademacher, and Dorothea Schünke is gratefully acknowledged. The work was supported by grants of the Ministerium für Wissenschaft und Forschung (Düsseldorf) and in part by a grant of the Deutsche Forschungsgemeinschaft (SFB 102) to K.W.

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