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Immunological Characterization of Rat Cardiac Gap Junctions: Presence of Common Antigenic Determinants in Heart of Other Vertebrate Species and in Various Organs

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Summary. Antibodies to the following synthetic peptide, SALGKLLDKVQAY, were purified by affinity chromatography and characterized by ELISA and immunoblotting. These antibodies, shown to be specific to the major protein constituent of isolated rat heart junctions: connexin 43, cross-reacted with a homologous protein in immunoreplicas of whole heart fractions of trout, frog, chicken, guinea pig, mouse and rat, suggesting a phylogenic conservation of connexin 43 in vertebrates. By immunoblotting of whole organ fractions it was also demonstrated that these antibodies cross-reacted with major proteins of M_r 32 and 22 kD in rat and mouse liver, of M_r 41 kD in rat cerebellum, of M_r 43 kD in uterus, stomach and kidney of rat, of M_r 46 and 70 kD in rat lens, suggesting that these proteins share common or related epitopes with the synthetic peptide and connexin 43.

Key Words gap junction · myocardium · immunoblotting · biotin-streptavidin · antipeptide antibodies

Introduction

Gap junctions (GJs) are specialized regions of the plasma membrane (Revel & Karnovsky, 1967) implicated in metabolic cooperation and electrical coupling between cells (Gilula, Reeves & Steinbach, 1972; Loewenstein, 1981; Pitts & Finbow, 1986). In myocardium, as in smooth muscle, GJs are responsible for the rapid propagation of action potentials from cell-to-cell (Dreiffuss, Girardier & Forssmann, 1966) and since the pioneer works of Weidman (1952, 1966) the physiology and the regulation of cardiac junctional conductance have been intensively investigated (Veenstra & Dehaan, 1986; Imanaga, Kameyama & Irisawa, 1987; Noma & Tsuboi, 1987; Jongsma et al., 1988).

In liver, the major junctional protein comprises 283 amino acids, in man and rat (Kumar & Gilula, 1986; Paul, 1986), with a real molecular mass of 32 kD. However, this JP behaves anomalously in polyacrylamide gels and depending on gel percentage it can run at either 27–28 kD (Nicholson et al., 1981; Hertzberg, 1984) or 32 kD (Green et al., 1988). This hepatic JP is referred to as connexin 32 in agreement with the nomenclature proposed by Beyer, Paul and Goodenough (1987). Reconstituted into lipid bilayers, connexin 32 forms channels with properties similar to those of intact GJs (Young, Cohn & Gilula, 1987). Models and hydrophobicity plots (Paul, 1986; Zimmer et al., 1987) suggest that this protein has four transmembrane segments and that both amino and carboxy terminals lie on the cytoplasmic side. A minor protein of M_r 21 kD is associated connexin 32 (Henderson, Eibl & Weber, 1979; Traub et al., 1982), and its amino-terminal twenty residues show 45% identity with the sequence of connexin 32 (Nicholson, 1987).

Gros, Nicholson and Revel (1983) identified a polypeptide of M_r 28 kD in rat heart GJs, the N-terminal partial sequence of which shows 30% homology with the N-terminus sequence of the liver connexin 32 (Nicholson et al., 1985). Later, Manjunath, Goings and Page (1985) and Manjunath et al. (1987) showed that the 28-kD cardiac polypeptide was in fact a proteolytic product of the native JP and that both the 28-kD polypeptide and the 44–47 kD native protein had the same N-terminal partial sequence. Recently, Beyer et al. (1987) sequenced a composite cDNA coding for the cardiac JP. They demonstrated that this protein comprises 382 amino acids with a molecular mass of 43 kD and named it *connexin 43*. The predicted sequence is in excellent agreement with the partial sequence observed in protein from isolated heart GJs (Nicholson et al., 1985). Comparisons of the complete predicted sequences for connexin 32 and 43 show two regions of high homology and suggest that both belong to the same protein family (Beyer et al., 1987).

Several groups have characterized by immunoblotting and/or immunofluorescence polyclonal or monoclonal antibodies specific for con-

nexin 32 and the related 21-kD JP (Traub et al., 1982; Dermietzel et al., 1984; Hertzberg & Skibbens, 1984; Paul, 1985, 1986; Janssen-Timmen et al., 1987; Zervos, Hope & Evans, 1985; Zimmer et al., 1987). Among these antibodies only two polyclonals were reported to cross-react with myocardial GJs. Antibodies characterized by Hertzberg and Skibbens (1984) cross-reacted with a protein of M_r 28 kD in immunoreplicas of alkaline heart extracts. Antibodies prepared by Zervos et al. (1985) were specific for the N-terminal region of connexin 32 and, besides recognizing liver GJs, they also labeled a protein of M_r 28 kD in isolated heart GJs.

Whatever the reasons are, no antibody has been shown to be specific for heart connexin 43. To further investigate cardiac GJs, we decided to raise and characterize antibodies capable of recognizing this cardiac JP. This paper reports the characterization of polyclonal antibodies raised to the peptide SALGKLLDKVQAY, the sequence of which is included in the N-terminal region of connexin 43 (Nicholson et al., 1985; Beyer et al., 1987). The results show that these antibodies specifically recognize rat heart connexin 43 and cross-react with homologous proteins present in heart of different vertebrate species and in a variety of organs. A preliminary report describing the preparation and characterization of these antipeptide antibodies has already appeared (Roustiau et al., 1986).

Materials and Methods

Preparation of Rat Heart Gap Junctions

Cardiac GJs were isolated as described by Manjunath et al. (1984) and Manjunath and Page (1986) under three conditions: (i) without protease inhibitors; (ii) with 1 mM phenylmethylsulfonyl fluoride (PMSF); (iii) with 1 mM PMSF and 0.1 M iodoacetamide (IAA), until the Sarkosyl step, included. The alkylating agent IAA added to the buffers to block free thiols and prevent artifactual oxidation or thiol/disulphide exchange (Freedman, 1984).

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Laemmli discontinuous buffer system (Laemmli, 1970) was used with 4.5% stacking gels and 12.5% separating gels. Gap junction pellets were solubilized for 30 min at room temperature in sample buffer made of 62.5 mM Tris HCl (pH 6.8), 40 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 10% glycerol. Free sulfhydryl groups were then alkylated for 30 min at room temperature by addition of the same volume of sample buffer in which 2-mercaptoethanol was replaced by 120 mM of N-ethylmaleimide. In some experiments 2-mercaptoethanol was omitted in the sample buffer. Molecular weights were estimated by reference to standard proteins (Bio Rad). Gels were stained with Coomassie Brilliant blue R-250 (Fairbanks, Steck & Wallach, 1971). The amounts of protein in the gel bands, after Coomassie-blue staining, were quantified by measuring the area of the peaks obtained by means of a Vernon PHI5 densitometer scan and by comparing them to the mean area of the peaks generated by known quantities of stained molecular weight markers.

Electron Microscopy

Negative Staining

Small aliquots of junctional fraction were negatively stained with phosphotungstic acid as described by Gros et al. (1983).

Thin Sectioning

Pellets of isolated junctions were fixed and stained "en bloc" with uranyl acetate as described by Gros et al. (1983) before being dehydrated in alcohol and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

Synthesis of Peptide and Purification of Antipeptide Antibodies

Peptide Synthesis

The peptide SALGKLLDKVQAY was synthesized in solid phase according to Merrifield (1963). The purity, controlled by high pressure liquid chromatography, was more than 87%.

Immunization of Rabbits

The synthetic peptide was coupled to bovine serum albumin (BSA) by means of bis-diazobenzidine as described by Briand, Muller and Van Regenmortel (1986). Coupling results in 5 to 7 molecules of peptide per molecule of BSA, as calculated from amino-acid analyses of peptide-BSA complexes and BSA (Briand et al., 1986). Two mg of peptide conjugate dissolved in 0.5 ml of PBS and emulsified with an equal volume of Complete Freund's adjuvant were injected in the popliteal lymph nodes of New Zealand rabbits (Sigel, Sinha & Van der Laan, 1983). Rabbits were boosted every month with 1 mg of peptide conjugate in 1 ml PBS emulsified with an equal volume of incomplete Freund's adjuvant. Fifteen days after every boosting, rabbits were bled from ear arteries. Immune sera were preserved at −80°C with 0.02% NaN_3, and 1% Zymophren (Specia Rhône-Poulenc).

Purification of Antipeptide Antibodies

The immune sera were incubated overnight at 4°C with cyanogen bromide-activated Sepharose 4B (Pharmacia S.A.) coupled to BSA. The resulting immune sera, extensively dialyzed (4°C) against a buffer made of 28 mM Tris, 35 mM NaCl 0.02% NaN_3 (pH 8.8), were applied to a Trisacryl M-DEAE (IBF) column. IgGs were eluted using the previous dialysis buffer and then incubated overnight at 4°C with AH-Sepharose 4B (Pharmacia S.A.) coupled to the synthetic peptide by means of 1-ethyl-3-(3

dimethyl-aminopropyl) carbodiimide-HCl (EDC). The next day the gel was poured into a 1 ml disposable plastic syringe, washed with 25 mM Tris containing 0.5 M NaCl (pH 8.8), and PBS and then eluted with 0.1 M HCl-glycine (pH 2.5). One-ml fractions, collected on ice, were neutralized with 1 M Tris base. Antipeptide IgGs were extensively dialyzed against PBS, diluted 1:2 with glycerol and stored at −20°C.

ELISA Technique

The Enzyme-Linked Immunosorbent Assay (ELISA) was used to test either pre-immune or immune sera (diluted 1:100 to 1:10,000) or fractions eluted from the affinity column. The antigen was either the synthetic peptide (2 μg/ml in 50 mM Na_2CO_3; pH 9.6) or isolated GJs (2 μg/ml of JP in 50 mM Na_2CO_3 buffer, pH 9.6). The procedure used was described in detail by Johnson and Thorpe (1982) and Sas et al. (1985). All solutions used contained 0.02% NaN_3.

Immunoblotting Technique

Electrophoresis and Electrotransfer

GJs were isolated and electrophoresed as described above. Rat intercalated discs were prepared according to Green and Severs (1983) in the presence of 0.1 mM PMSF. Whole organ fractions were prepared as described by Paul (1985). Organs were plunged, immediately after dissection, in Freon 22 cooled with liquid nitrogen, pulverized with a pestle under liquid nitrogen and then freeze dried. Protein content of the fractions was determined according to Lowry et al. (1951). 100 to 130 μg of protein were dissolved in sample buffer (62.5 mM Tris-HCL, pH 6.8; 20% SDS, 5% 2-mercaptoethanol; 10 mM EDTA; 5% glycerol; with or without 1 mM PMSF), sonicated, heated for 5 min at 95°C and loaded on gels. After electrophoresis, transfers onto nitrocellulose sheets (0.22 μm, Schleicher & Schüell) were performed at constant voltage (25 V) for 15 hr as described by Towbin, Staehelin, and Gordon (1979) except for the following points: the electrode buffer contained 0.02% SDS and the gels were soaked for 15 min in electrode buffer transfer.

Immunoreactions

Nitrocellulose sheets were incubated first for 8 hr at 4°C with the saturation solution (BLOTTO) made of 40 mM Tris (pH 7.5), 0.1% Tween 20 and 4% nonfat dry milk (Johnson et al., 1984), then overnight at 4°C with affinity-purified antipeptide IgGs (15 μg/ml) in BLOTTO. After four washes in BLOTTO, replicas were incubated for 1 hr, at room temperature, with biotinylated-goat antirabbit $F(ab')_2$ (Jackson Immunoresearch Laboratories) diluted 1:1,000 in BLOTTO, then washed again four times in BLOTTO and incubated for 1 hr at room temperature with peroxidase-labeled streptavidin (Jackson Immunoresearch Laboratories) (Gesdon, Ternynck & Avrameas, 1979; Clark, Tani & Damjanov, 1986) diluted 1:2,500 in BLOTTO containing 0.5 M NaCl. Replicas were washed three times in 40 mM Tris (pH 7.5), 0.1% Tween 20, 0.5 M NaCl and three more times in 40 mM Tris (pH 7.5), 0.5 M NaCl. Peroxidase activity was detected with a substrate solution made of 0.015% H_2O_2, 0.05% 4-chloronaphtol, 16.5% methanol, 40 mM Tris (pH 7.5), 0.5 M NaCl.

Inhibition Experiments

For these experiments, the protocol was the same as that previously described but antipeptide IgGs, diluted in BLOTTO, were preincubated for 15 hr at 4°C, before use, with various concentrations of either the peptide SALGKLLDKVQAY (0.03 to 6 μM) or the unrelated peptide RIDPANGNTKYDPKFQC (0.03 to 200 μM) (a gift from Drs. Mazza and Moinier, Centre d'Immunologie de Marseille-Luminy). After immunolabeling, nitrocellulose strips were soaked in xylene (Seshi, 1986) to make them transparent, and the optical density of labeled bands was determined using a Vernon PHI5 densitometer scan.

All solutions used for immunoreaction and inhibition experiments contained 0.02% merthiolate (Fluka).

Results

Isolation and Analyses of Myocardial Gap Junctions

Figure 1*A* is a micrograph of a thin section in a final pellet of GJs isolated in the presence of 1 mM PMSF and 0.1 M IAA. The junctional nature of double layered membranes is confirmed by observations at higher magnification (Fig. 1*B*) and by negative staining (Fig. 1*C*). Analysis of micrographs shows very little contamination of the final pellet (Fig. 1*A*). Only GJs isolated in the presence of 1 mM PMSF conserved on their cytoplasmic surfaces a fuzzy layer as described by Manjunath et al. (1984, 1985). Electron-microscopy analysis of GJs isolated without inhibitor gives results identical to those previously reported (Gros et al., 1983; Manjunath et al., 1984, 1985).

SDS-PAGE analyses of isolated GJs are illustrated in Figs. 2*A* and 3*b*. Fractions of GJs isolated without PMSF contain a single protein of M_r 28 kD or M_r 50 kD (dimer of the 28-kD protein) according to whether analyses are performed under reducing or nonreducing conditions, respectively. Fractions of GJs isolated in the presence of 1 mM PMSF, with or without 0.1 M IAA, contain a single protein of M_r 43 kD which is connexin 43. These results are in agreement with those previously published by Gros et al. (1983), Manjunath et al. (1984, 1985, 1987), and Manjunath and Page (1986).

Purification and Characterization of Antipeptide Antibodies

By ELISA tests pre-immune sera (diluted 1:100 to 1:10,000) fail to bind to either the synthetic peptide or isolated GJ fractions. On the other hand, these same tests show that immune sera IgGs eluted from the affinity column with HCl-glycine contain anti-

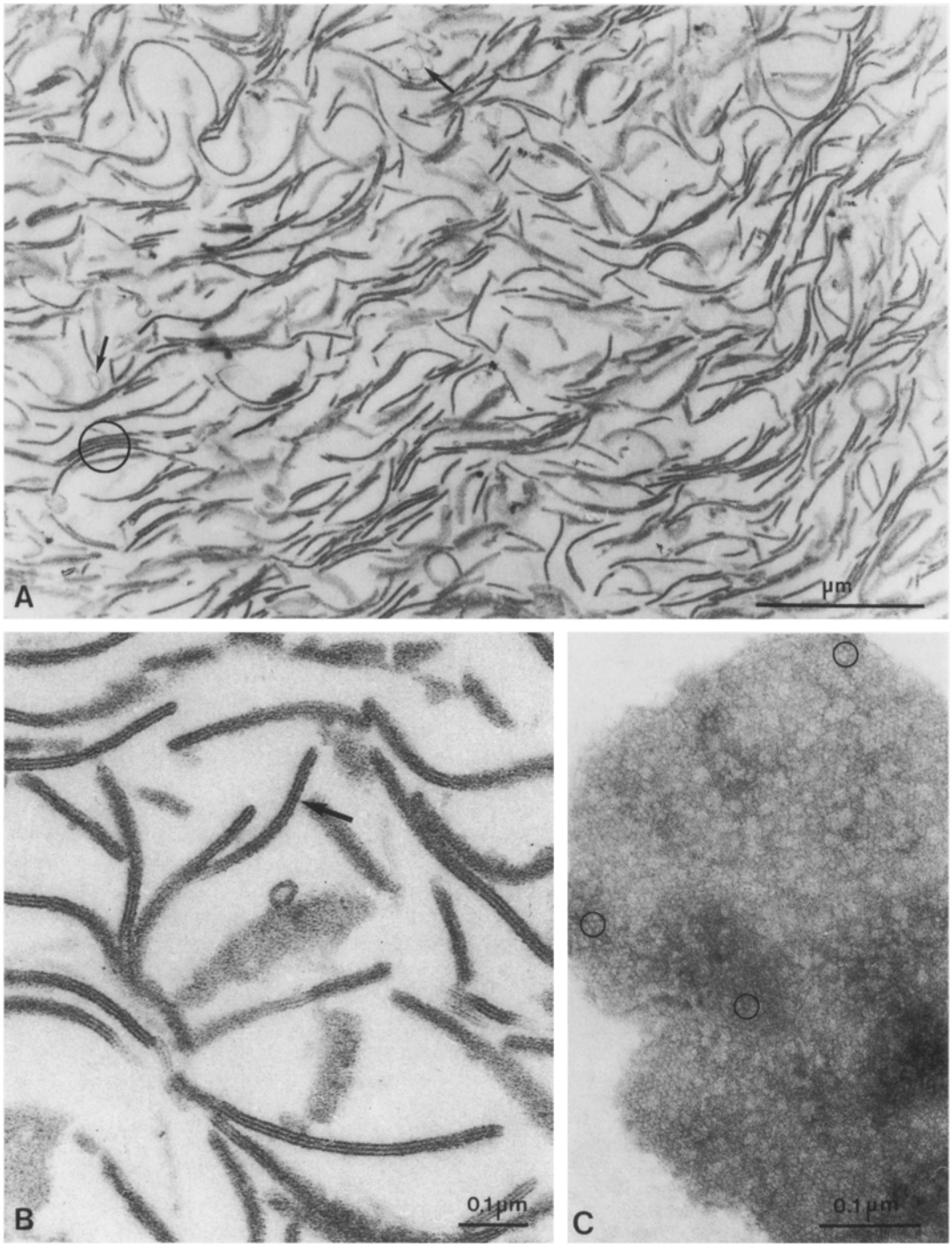

Fig. 1. Heart gap junctions isolated in the presence of 1 mM PMSF and 0.1 M IAA. (*A*) In thin sections, the final pellet of the isolation protocol is seen to be composed of gap junctions (circle) and of a very few single membranes (arrows); ×25,000. (*B*) At higher magnification the paired membranes of the junctions are seen to be separated by a 2–3 nm "gap." The presence of connexons spanning the extracellular space between membranes is suggested by the periodic densities visible in the "gap" where the plane of section is appropriate (arrow); ×105,000. (C) Isolated junction negatively stained reveals a lattice of closely packed connexons. The hexagonal symmetry of the lattice can be recognized in some region of the junction (circles); ×150,000

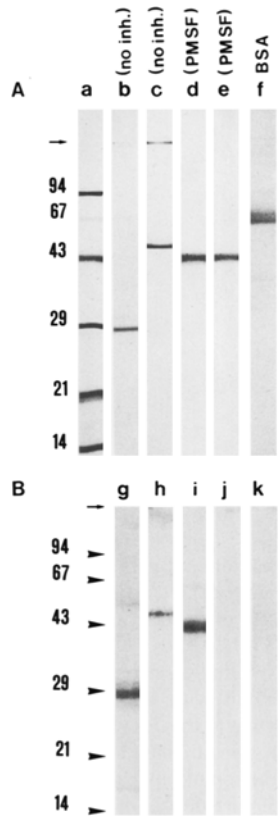

Fig. 2. Characterization of the affinity-purified antipeptide antibodies by immunoblotting. (*A*) Analysis by SDS-PAGE and Coomassie-Blue staining of isolated heart gap junctions and BSA (lane *f*). All the samples were reduced before analysis except that of lane *c*, which is unreduced. Lane *a*: standard proteins (mol wt in kD are indicated on the left). Lane *b*: heart gap junctions isolated without PMSF or IAA (*no inh.*) Lane *c*: as in *b* but the sample was neither reduced, nor alkylated before loading in the gel (*no inh.*). Lanes *d* and *e*: duplicates of heart GJs isolated in the presence of 1 mM PMSF (*PMSF*). Identical results are obtained with GJs isolated in the presence of PMSF and IAA (*see* Fig. 4, lanes *b* and *c*). Lane *f*: BSA (1 μg of BSA was located in the gel). (*B*) Immunoreplicas of the previous gels. Positions of molecular weight standards are indicated by head arrows. Arrow shows the top of replicas. All the nitrocellulose replicas, except that of lane *j*, were treated with antipeptide antibodies. Replica *j* was treated with pre-immune serum. Pre-immune serum fails to label GJs (lanes *e* and *j*). Antipeptide antibodies fail to detect BSA (lanes *f* and *k*)

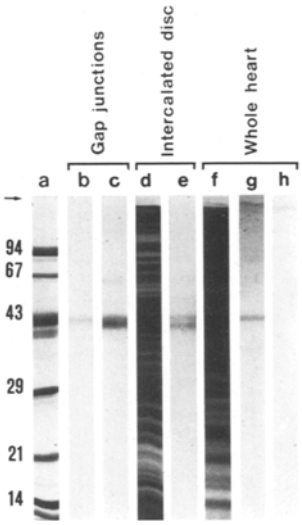

Fig. 3. Immunoblotting of cardiac fractions. Lanes *a*, *b*, *d* and *f*: Coomassie-blue stained gels of standard proteins in *a*; gap junctions isolated in the presence of 1 mM PMSF and 0.1 M IAA in *b*; intercalated discs isolated according to Green and Severs (1983) in *c*; whole heart fraction in *d*. Lanes *c*, *e* and *g*: immunoreplicas of samples shown in lanes *b*, *d* and *f*, respectively. Lane *h*: control experiment. Immunoreplica of total heart fraction, untreated with antipeptide antibodies, but revealed according to the described protocol as other replicas

bodies that bind specifically to the synthetic peptide and to the fractions of GJs isolated in the presence of 1 mM PMSF or without inhibitor. Antipeptide antibodies-containing fractions were pooled and further tested by immunoblotting. The 28-kD junctional polypeptide, its 50-kD dimer and the 43-kD native protein, are all immunolabeled after transfer onto nitrocellulose replicas as shown in Fig. 2*B*. Pre-immune serum fails to detect electroblotted junctional polypeptides (Fig. 2*B*); purified antipeptide antibodies fail to label electroblotted BSA (Fig. 2*B*) or actin (1 μg loaded in the gel; *not shown*). Inhibition experiments with electro-blotted 28-kD junctional polypeptide show that immunodetection of this polypeptide is inhibited by 50% when antipeptide antibodies are pre-incubated with 1 μM of synthetic peptide before use. Preincubation with 200 μM of an unrelated synthetic peptide (RIDPANGNTKYDPKFQC) fails to inhibit immunolabeling.

Immunodetection of Gap Junctional Protein in Rat Intercalated Discs and Whole Heart Fractions

Antipeptide IgGs were used for immunodetection of gap junctional protein onto replicas of intercalated discs and whole heart fractions (Fig. 3). In both cases a protein of M_r 43 kD is labeled. A protein of M_r 41 kD is also usually detected on intercalated discs replicas. When the incubation step with antipeptide antibodies is omitted no labeling is seen (Fig. 3, lane *h*). In parallel experiments, preimmune and immune sera were submitted to the different steps of purification described in Materials and

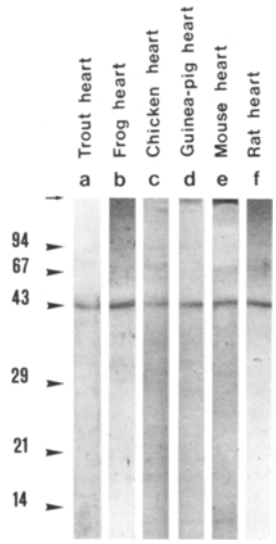

Fig. 4. Immunoblotting of whole heart fractions of various vertebrate species. Whole heart fractions were prepared as described in Materials and Methods. Protein content of the fractions was determined according to Lowry et al. (1951). One hundred μg of each fraction were submitted to SDS-PAGE and electroblotted onto nitrocellulose. Replicas were incubated with affinity-purified antipeptide IgGs and revealed according to the described protocol. Positions of the molecular weight standards are indicated on the left (arrowheads). Arrow shows the top of replicas. Lanes *a*, *b*, *c*, *d*, *e* and *f* correspond to nitrocellulose replicas of whole heart fractions of trout, frog, chicken, guinea pig, mouse and rat (reference), respectively

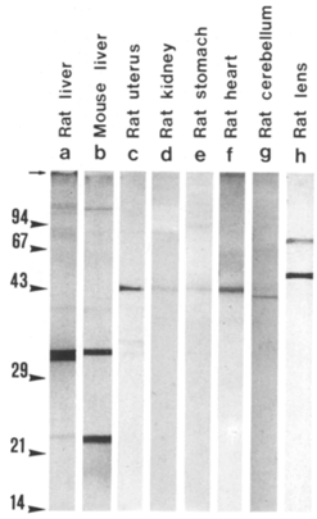

Fig. 5. Immunoblotting of whole fractions of various organs. Whole fractions of various organs were prepared as described in Materials and Methods. Protein content of the fractions was determined according to Lowry et al. (1951). 100 to 130 μg of each fraction were submitted to SDS-PAGE and electroblotted onto nitrocellulose. Replicas were incubated with affinity-purified antipeptides IgGs and revealed according to the described protocol. Positions of the molecular weight standards are indicated on the left (arrowheads). Arrow shows the top of replicas. Lanes *a*, *b*, *c*, *d*, *e*, *f*, *g*, and *h* correspond to immunoreplicas of the following whole fractions: rat liver, mouse liver, rat uterus, rat kidney, rat stomach, rat heart (reference), rat cerebellum and rat lens, respectively. Homologous major proteins are detected at 32 and 22 kD in rat and mouse liver, at 43 kD in rat uterus, stomach and kidney, at 41 kD in rat cerebellum and at 46 and 70 kD in rat lens

Methods. In both cases, fractions collected after elution with 0.1 M HCl-glycine were diluted to 1 : 5 and 1 : 10 and tested by immunoblotting. While eluates coming from immune serum label a protein of M_r 43 kD, eluates from pre-immune serum fail to detect any band.

The immunoblotting technique used for rat heart was applied to whole heart fractions of trout, frog, chicken, guinea pig and mouse. For all those vertebrate species, the labeling of the M_r 43 kD protein is conserved (Fig. 4). Omission of the incubation step with antipeptide antibodies leads to an absence of labeling onto replicas.

Cross-reactivities of Affinity-Purified Antipeptide Antibody

A variety of rat and mouse organs, liver, kidney, stomach, cerebellum, uterus and lens, were electroblotted onto nitrocellulose replicas and submitted to immunodetection (Fig. 5). Antipeptide IgGs cross-react with proteins of M_r 32 and 22 kD in rat and mouse liver (the labeling of the 22-kD protein being always weaker in rat than in mouse), with a major protein of M_r 43 kD in uterus, kidney and stomach from rat and with a protein of M_r 41 kD in rat cerebellum. In rat lens the major labeled protein has a M_r of 46 kD but another band, of variable intensity and sometimes poorly defined, is also detected at about 70 kD. Specificity of the cross-reactions was demonstrated by the absence of labeling when the incubation step with antipeptide antibody is omitted.

Discussion

According to Hopp and Woods (1981), hydrophilic regions of proteins are antigenic. Using the predictions formulated by the previous authors, antibodies were raised against the synthetic peptide SALGKLLDKVQAY, the sequence of which corresponds to the most hydrophilic part of the N-terminal region of connexin 43 (Beyer et al., 1987). The equivalent region of the heart 28-kD junctional polypeptide, which is a degradation product of con-

nexin 43, also contains this sequence (Nicholson et al., 1985; Manjunath et al., 1987). ELISA tests showed that affinity-purified antipeptide antibodies bind to one or several proteins present in fractions of isolated cardiac GJs. Immunoblotting experiments carried out with isolated GJs more precisely demonstrated that antibodies specifically recognized the junctional polypeptide of M_r 28 kD (and its 50-kD dimer) and connexin 43. When the immunolabeling technique was applied to nitrocellulose replicas of intercalated discs or whole rat heart a band of M_r 43 kD was labeled which is thought to be the main constituent of cardiac GJs, i.e., connexin 43. However, several studies have shown that antisera against peptides may cross-react with other proteins related or unrelated to the protein under investigation (Walter & Doolittle, 1983). Thus, the presence in the heart homogenates of nonjunctional proteins showing common or related epitopes with connexin 43 cannot be excluded, but the results obtained with a variety of organs, in particular with the liver, are quite consistent with the findings of other authors (*see* the discussion following) and make this possibility rather unlikely. In intercalated disc replicas a band of M_r 41 kD was usually detected. This band, which was not seen in whole heart replicas, most likely corresponds to a degradation product of the 43-kD protein.

Comparative analysis of immunoreplicas of whole heart extracts from trout, frog, chicken, mouse, guinea pig and rat showed that antipeptide antibodies label a protein of M_r 43 kD in all hearts investigated. Thus hearts of several vertebrate classes, including mammals, birds, amphibians and fishes, all contain 43-kD proteins sharing epitopes with the peptide SALGKLLDKVQAY. Although the identity of these proteins with cardiac connexin 43 has to be considered with certain reservations (*see above*), the hypothesis of a phylogenic conservation of connexin 43 in vertebrate heart is reasonable. A similar situation was found in liver by Hertzberg and Skibbens (1984) who showed that protein immunologically related to rat liver connexin 32 was present in liver homogenates of several vertebrate species. The phylogenic conservation of this JP was confirmed later by the comparison of amino-acid sequences of connexin 32 from man and rat (Kumar & Gilula, 1986), sequences which differ by only four residues.

In both rat and mouse whole liver extracts, antipeptide antibodies cross-reacted with two proteins of M_r 32 and 22 kDa which are thought to be the two JPs identified in liver. The mobility of the labeled bands in gels is in agreement with the recent investigations of Green et al. (1988) and Nicholson et al. (1987). The staining of the 22-kDa component is always weaker in rat than in mouse, and this is consistent with the fact that this protein makes up less than 10% of rat liver junctional fractions but as much as 35% in mouse liver GJs (Nicholson et al., 1987). Comparison of published data show that the peptide used to elicit antibodies and the equivalent sequences of liver JPs share four (with connexin 32) and three (with the 21-kD JP) identical aminoacids. This raises the question of whether the presence of these three or four common amino acids may explain the cross-reactives observed. Antigenic epitopes are usually regarded as at least six amino acids in length, but recent work suggests a different interpretation of characteristics of epitopes. Thus, some residues in an epitope can be replaced by any of the 19 amino acids without impairing antigenic activity, whereas others cannot be substituted at all (Geysen, Meloen & Barteling, 1984; Getzoff et al., 1987). Not every residue in a so-called continuous epitope is in contact with the antibody and the linear peptide is, in fact, antigenically discontinuous. A similar situation might explain the recognition of the putative two liver JPs by the antipeptide antibodies used in the present investigation.

By immunoreplicas analyses of lens, binding of antipeptide antibodies was shown to be specific for two polypeptides of M_r 46 and 70 kD. A protein of M_r 70 kD, associated with GJs isolated from lens, was identified by Kistler, Kirkland and Bullivant (1985), and immunolocalization experiments have confirmed its junctional nature (Gruitjers et al., 1987). More recently, Kistler, Christie and Bullivant (1988) have demonstrated that the amino-terminal sequence of this JP shows 50% homology with connexin 43, but no homology with MIP 26, a lens membrane protein the junctional nature of which is still a matter of controversy (Bok, Dokstader & Horwitz, 1982; Fitzgerald, Bok & Horwitz, 1983; Paul & Goodenough, 1983; Sas et al., 1985; Revel & Yancey, 1985). The antigenic peptide SALGKLLDKVQAY and the equivalent sequence of the lens JP share seven identical amino acids, a situation which might explain the recognition of this protein by the antipeptide antibodies. Paul and Goodenough (1987) have sequenced to 1.6-kb lens cDNA which hybridizes by Northern analysis to a single 3.0-kb message. Preliminary studies showed that the predicted amino-acid sequence for the protein coded by this cDNA has regions of "highly conserved structure shared with both hepatic and myocardial gap junctions," but the molecular mass of this protein has not yet been published. The results presented in this paper, which showed that connexin 43 and lens protein(s) share similar antigenic epitopes, are in agreement with the work of Kistler et al. (1988) and Paul and Goodenough

(1987), but several questions remain unanswered: is the 46-kD lens protein a degradation product of the 70-kD JP or is the 70 kD JP an aggregation product of the 46-kD protein or are the 46 and 70 kD proteins homologous proteins coded by two different genes?

Binding of antipeptide antibodies was also demonstrated to be specific for one major polypeptide of M_r 43 kD in uterus, stomach and kidney and of M_r 41 kD in cerebellum. These results show that proteins with similar antigenic epitopes and related to the peptide SALGKLLDKVQAY and connexin 43 are widely distributed among vertebrate organs. Does this suggest that the labeled proteins represent JPs? There is not conclusive evidence to support this assumption, more especially as little is known about the JPs of the organs investigated. However, the results of hybridization experiments of either liver connexin 32 cDNA or heart connexin 43 cDNA to RNA extracted from brain, kidney, stomach and uterus suggest homologies between JPs of these organs (Paul, 1986; Beyer et al., 1987). Such a hypothesis has been previously expressed on the basis of cross-reactions observed between anti-liver GJ antibodies and heart, uterus, stomach, kidney and brain extracts (Hertzberg & Skibbens, 1984; Dermietzel et al., 1984; Zervos et al., 1985). In these latter studies the M_r of the labeled proteins was reported to be 26–28 kD, i.e., a M_r much lower than that observed in the present investigation (41–43 kD). The discrepancy between these results and ours could be explained either by an uncontrolled proteolytic degradation of organ extracts or by the presence of more than one homologous protein, both in organ extracts and whole organ fractions. Which of these propositions is valid is not known at present.

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References

Beyer, E.C., Paul, D.L., Goodenough, D.A. 1987. Connexin 43: A protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* **105**:2621–2623

Bok, D., Dockstader, J., Horwitz, J. 1982. Immunocytochemical localization of the lens main intrinsic polypeptide (MIP 26) in communicating junctions. *J. Cell Biol.* **92**:213–220

Briand, J.P., Muller, S., Van Regenmortel, M.H.V. 1986. Synthetic peptides as antigens: Pitfalls of conjugation methods. *J. Immunol. Meth.* **78**:59–69

Clark, R.K., Tani, Y., Damjanov, I. 1986. Suppression of nonspecific binding of Avidin-Biotin Complex (ABC) to proteins electroblotted onto nitrocellulose paper. *J. Histochem. Cytochem.* **34**:1509–1512

Dermietzel, R., Liebstein, A., Frixen, V., Janssen-Timmen, V., Traub, O., Willecke, E. 1984. Gap junctions in several tissues share antigenic determinants with liver gap junctions. *EMBO J.* **3**:2261–2270

Dreyffuss, J.J., Girardier, L., Forssmann, W.G. 1966. Etude de la propagation de l'excitation dans le ventricule de rat au moyen de solutions hypertoniques. *Pfluegers Arch.* **292**:13–33 (*in French*)

Fairbanks, G., Steck, J.L., Wallach, D.F.M. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2602–2617

Fitzgerald, P., Bock, D., Horwitz, J. 1983. Immunocytochemical localization of the main intrinsic polypeptide (MIP) in ultrathin frozen sections of rat lens. *J. Cell Biol.* **97**:1491–1499

Freedman, R.B. 1984. Native disulphide band formation in protein biosynthesis: Evidence for the role of protein disulphide isomerase. *Trends Biochem. Sci.* **9**:438–441

Gesdon, J.L., Ternynck, T., Avrameas, S. 1979. The use of Avidin-Biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* **27**:1131–1139

Getzoff, E.D., Geyson, M.H., Rodda, S.T., Allexander, H., Tainer, J.A., Lerner, R.A. 1987. Mechanisms of antibody binding to a protein. *Science* **235**:1191–1196

Geyson, M.H., Meloen, R.H., Barteling, S.J. 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino-acid. *Proc. Natl. Acad. Sci. USA* **81**:3998–4002

Gilula, N.B., Reeves, O.R., Steinbach, A. 1972. Metabolic coupling, ionic coupling and cell contacts. *Nature (London)* **235**:262–265

Green, C.R., Harfst, E., Gourdie, R.G., Severs, N.J. 1988. Analysis of the rat liver gap junction protein: Clarification of anomalies in its molecular size. *Proc. R. Soc. London B* **233**:165–174

Green, C.R., Severs, N.J. 1983. A simplified method for the rapid isolation of cardiac intercalated discs. *Tissue Cell* **15**:17–26

Gros, D., Nicholson, B.J., Revel, J.P. 1983. Comparative analysis of the gap junction protein from rat and liver: Is there a tissue-specificity of gap junction? *Cell* **35**:539–549

Gruitjers, W.T.M., Kistler, J., Bullivant, S., Goodenough, D. 1987. Immunolocalization of MP 70 in lens fiber 16–17 nm intercellular junctions. *J. Cell Biol.* **104**:565–572

Henderson, D., Eibl, H., Weber, K. 1979. Structure and biochemistry of mouse hepatic gap junctions. *J. Mol. Biol.* **132**:193–218

Hertzberg, E.L. 1984. A detergent-independent procedure for the isolation of gap junctions from rat liver. *J. Biol. Chem.* **259**:9936–9943

Hertzberg, E.L., Skibbens, R.U. 1984. A protein homologous to the 27,000 dalton liver gap junction protein is present in a wide variety of species and tissues. *Cell* **39**:61–69

Hopp, T.P., Woods, K.R. 1981. Prediction of protein antigenic determinants from amino-acid sequences. *Proc. Natl. Acad. Sci. USA* **78**:3824–2828

Imanaga, I., Kameyama, M., Irisawa, H. 1987. Cell-to-cell diffusion of fluorescent dyes in paired ventricular cells. *Am. J. Physiol.* **252:***H*223–*H*232

Janssen-Timmen, V., Traub, O., Dermietzel, R., Rabes, H.M., Willecke, K. 1987. Reduced number of gap junctions in rat hepato-carcinomas detected by monoclonal antybody. *Carcinogenesis* **7:**1475–1482

Johnson, A., Thorpe, R. 1982. Immunochemistry in Practice. Blackwell Scientific Publications, Oxford

Johnson, D.A., Gantsch, J.W., Sportsman, J.R., Elder, J.M. 1984. Improved technique utilizing non-fat dry milk for analysis of proteins and nucleic acids transfered to nitrocellulose. *Gene. Anal. Techn.* **1:**3–8

Jongsma, H.B., Rook, M.B., Van Ginneken, A.C.G., de Jong, B. de 1988. Cardiac gap junctions: Estimation of the single channel conductance. *In:* Recent Studies of Ion Transport and Impulse Propagation in Cardiac Muscle. W.R. Giles, editor. pp. 294–303. Alan Liss, New York

Kistler, J., Christie, D., Bullivant, S. 1988. Homologies between gap junction proteins in lens, heart and liver. *Nature (London)* **331:**721–723

Kistler, J., Kirkland, B., Bullivant, S. 1985. Identification of a 70,000 D protein in lens membrane junctional domains. *J. Cell Biol.* **101:**28–35

Kumar, N.H., Gilula, N.B. 1986. Cloning and characterization of human and red liver cDNAs coding for a gap junction protein. *J. Cell Biol.* **103:**767–776

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature (London)* **227:**680–685

Loewenstein, W.R. 1981. Junctional intercellular communication: The cell-to-cell membrane channel. *Physiol. Rev.* **61:**829–913

Lowry, O.H., Nira, J.R., Farr, A.L., Randall, R. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193:**264–275

Manjunath, C.K., Goings, G.E., Page, E. 1984. Cytoplasmic surface and intramembrane components of rat heart gap junctional proteins. *Am. J. Physiol.* **246:***H*865–*H*875

Manjunath, C.K., Goings, G.E., Page, E. 1985. Proteolysis of cardiac gap junctions during their isolation from rat hearts. *J. Membrane Biol.* **85:**159–168

Manjunath, C.K., Nicholson, B.J., Teplov, D.B., Hood, L.E., Page, E., Revel, J.P. 1987. The cardiac gap junction protein (M_r 47,000) has a tissue-specific cytoplasmic domain of M_r 17,000 at its carboxy-terminus. *Biochem. Biophys. Res. Commun.* **142:**228–234

Manjunath, C.K., Page, E. 1986. Rat heart gap junctions as disulfide-bonded connexon multimers: Their depolymerization and solibilization in deoxycholate. *J. Membrane Biol.* **90:**43–57

Merrifield, R. 1963. Solid-phase peptide synthesis. 1. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **85:**2149–2154

Nicholson, B.J., Dermietzel, R., Teplov, D.B., Traub, O., Willecke, K., Revel, J.P. 1987. Two homologous protein components of hepatic gap junctions. *Nature (London)* **329:**732–734

Nicholson, B.J., Gros, D., Kent, S.B., Hood, L.E., Revel, J.P. 1985. The M_r 28,000 gap junction protein from rat heart and liver are different but related *J. Biol. Chem.* **260:**6514–6517

Nicholson, B.J., Hunkapiller, M.W., Grim, L.B., Hood, L.E., Revel, J.P. 1981. The rat liver gap junction protein: Properties and partial sequence. *Proc. Natl. Acad. Sci. USA* **78:**7594–7598

Noma, A., Tsuboi, N. 1987. Dependence of junctional conductance on proton, calcium and magnesium ions in cardiac paired cells of guinea-pig. *J. Physiol. (London)* **382:**193–211

Paul, D. 1985. Antibody against liver gap junctions 27 kD protein is tissue-specific and cross-reacts with a 54 kD protein. *In:* Gap Junction. M.V.L. Bennett and D.C. Spray, editors. pp. 107–122. Cold Spring Harbor Laboratory, Cold Spring Harbor, Maine

Paul, D. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.* **103:**123–134

Paul, D., Goodenough, D.A. 1983. Preparation, characterization and localization of antisera against bovine MP 26, an integral protein from lens fiber plasma membrane. *J. Cell Biol.* **96:**625–632

Paul, D., Goodenough, D.A. 1987. A novel gap junction protein present in rat lens fibers. *J. Cell Biol.* **105:**227*a*

Pitts, J.D., Finbow, M.E. 1986. The gap junction. *J. Cell Sci. Suppl.* **4:**239–266

Revel, J.P., Karnovsky, M.J. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33:***C*7–*C*10

Revel, J.P., Yancey, B.S. 1985. Molecular conformation of the major intrinsic protein of lens fiber membranes: Is it a junction protein? *In:* Gap Junctions. M.V.L. Bennett and D.C. Spray, editors. pp. 33–49. Cold Spring Harbor Laboratory, Cold Spring Harbor, Maine

Roustiau, S., El Aoumari, A.H., Dupont, E., Briand, J.P., Gros, D. 1986. Les jonctions gap du myocarde de rat: Caractérisation d'un anticorps antipeptide spécifique du polypeptide jonctionnel. *Biol. Cell* **57:**35*a* (*in French*)

Sas, D.F., Sas, J.M., Johnson, K.R., Menko, S.A., Johnson, R.G. 1985. Junctions between lens fiber cells are labelled with a monoclonal antibody shown to be specific for MP 26. *J. Cell Biol.* **100:**216–225

Seshi, B. 1986. Cell blotting: Techniques for staining and microscopical examination of cells blotted on nitrocellulose paper. *Anal. Biochem.* **157:**331–342

Sigel, M.B., Sinha, Y.N., Van der Laan, W.P. 1983. Production of antibodies by inoculation into lymph nodes. *Meth. Enzymol.* **93:**3–12

Towbin, M., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellular sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76:**4350–4354

Traub, O., Janssen-Timmen, U., Drüge, P.M., Dermietzel, R., Willecke, K. 1982. Immunological properties of gap junction protein from mouse liver. *J. Cell. Biochem.* **19:**27–44

Veenstra, R.D., Dehaan, R.L. 1986. Measurement of single channel currents from cardiac gap junctions. *Science* **233:**972–974

Walter, G., Doolittle, R.F. 1983. Antibodies against synthetic peptides. *In*: Genetic Engineering: Principles and Methods. J.K. Setlov and A. Hollaender, editors. Vol. 5, pp. 61–91. Plenum, New York

Weidmann, S. 1952. The electrical constants of Purkinje fibers. *J. Physiol. (London)* **118:**348–360

Weidmann, S. 1966. The diffusion of radiopotassium across intercalated discs of mammalian cardiac muscle. *J. Physiol. (London)* **187:**323–342

Young, J.D.E., Cohn, Z.A., Gilula, N.B. 1987. Functional assembly of gap junction conductance in lipid bilayers: Demonstration that the major 27 kD protein forms the junctional channel. *Cell* **48:**733–743

Zervos, A.S., Hope, J., Evans, W.H. 1985. Preparation of a gap junction fraction from uteri of pregnant rats: The 28 kD poly-

peptides of uterus, liver and heart gap junctions are homologous. *J. Cell Biol.* **101:**1363–1370

Zimmer, D.B., Green, C.R., Evans, H.W., Gilula, N.B. 1987. Topological analysis of the major protein in isolated intact rat liver gap junctions and gap junction-derived single membrane structures. *J. Biol. Chem.* **262:**7751–7763

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