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Conservation of a Cytoplasmic Carboxy-Terminal Domain of Connexin 43, a Gap Junctional Protein, in Mammal Heart and Brain

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Summary. According to the sequence of connexin 43, a cardiac gap junctional protein, the domain contained within residues 314–322 is located 60 amino acids away from the carboxy-terminus. Antibodies raised to a peptide corresponding to this domain label a unique 43-kD protein on immunoblots of both purified gap junctions and whole extracts from rat heart. Immunofluorescence investigations carried out on mammal heart sections reveal a pattern consistent with the known distribution of intercalated discs. Immunogold labeling performed with ultrathin frozen sections of rat heart or partially purified rat heart gap junctions demonstrate that antigenic determinants are associated exclusively with the cytoplasmic surfaces of gap junctions.

The antibodies were shown to cross-react with a 43-kD protein on immunoblots of whole extracts from human, mouse and guinea pig heart. However, no labeling was seen when heart of lower vertebrates such as chicken, frog and trout, was investigated. These results, confirmed by immunofluorescence investigations, were interpreted as a loss of antigenic determinants due to sequence polymorphism of cardiac connexin 43.

Proteins of M_r 43 and 41 kD, immunologically related to cardiac connexin 43, were detected in immunoblots of mouse and rat brain whole extracts. mRNAs, homologous to those of cardiac connexin 43 and of the same size (3.0 kb), are also present in brain. Immunofluorescence investigations with primary cultures of unpermeabilized and permeabilized mouse neural cells showed that the antigenic determinants recognized by the antibodies specific for connexin 43 are cytoplasmic and that the labeling observed between clustered flat cells, is punctate, as expected for gap junctions. Double labeling experiments demonstrated that the immunoreactivity is associated with GFAP-positive cells, that is to say, astrocytes.

Key Words gap junctions · connexin · heart · brain

Introduction

Since the pioneering work of Weidman (1952), it is known that cells in cardiac tissues are electrically coupled. This basic phenomenon explains the propagation of action potentials in heart. Numerous physiological studies dealing with electrical coupling, determination of conductance and transfer of action potentials between cardiac cells have followed. Among the most recent studies mention might be made of those by Noma and Tsuboï (1987), Rook, Jongsma and Van Ginneken (1988), Rook, Jongsma and deJongs (1989), and Weingart and Maurer (1988).

Barr, Dewey and Berger (1965), then Dreiffuss, Girardier and Forssmann (1966), have shown that the propagation of excitation in myocardium was dependent on the integrity of a particular membrane structure, the so-called "nexus." These structures, now termed "gap junctions," are formed between two cells by the close association of plasma membrane regions containing hexagonal arrays of particles (Revel & Karnovsky, 1967): the connexons. Ultrastructural, biophysical and physiological investigations suggest that the connexons of a membrane, tightly connected, in the extracellular space, to the apposed-membrane connexons are channel-making hexameric structures providing a direct route for the passage of ions and small molecules between the cytoplasms of adjacent cells (McNutt & Weinstein, 1970; Unwin & Zampighi, 1980; Loewenstein, 1981; Baker et al., 1983; Sosinsky et al., 1988). Thus the gap junctions are implicated in metabolic cooperation and electrical coupling between cells (Pitts & Finbow, 1986; Warner, 1988).

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Connexon subunits belong to a family of proteins: the connexins (*see* Beyer, Paul & Goodenough (1987) for nomenclature) related by their sequences but differing by their molecular weight (Nicholson et al., 1985, 1987; Kumar & Gilula, 1986; Paul, 1986; Beyer et al., 1987; Paul & Goodenough, 1987; Gimlich, Kumar & Gilula, 1988; Kistler, Christie & Bullivant, 1988). The complete amino acid sequence of human and rat liver connexin 32 (Kumar & Gilula, 1986; Paul, 1986), rat cardiac connexin 43 (Beyer et al., 1987), *Xenopus* connexin 30 and 38 (Gimlich et al., 1988; Ebihara et al., 1989), and rat liver connexin 26 (Zhang & Nicholson, 1989) have been deduced from cDNAs isolated by recombinant DNA methods.

Among the identified connexins, rat liver connexin 32 is the most extensively characterized (Henderson, Eibl & Weber, 1979; Hertzberg & Gilula, 1979; Nicholson et al., 1981; Hertzberg, 1984; Young, Cohn & Gilula, 1987; Green et al., 1988). Electron microscopy immunolabeling procedures confirmed the localization of this protein as a component of liver gap junctions (Dermietzel et al., 1984; Paul, 1986; Young et al., 1987; Zimmer et al., 1987; Milks et al., 1988; Traub et al., 1989). Incorporated into an artificial lipid membrane system, connexin 32 forms channels with properties similar to those of intact gap junctions (Young et al., 1987). Functional channels are also expressed when connexin 32 RNA-injected *Xenopus* oocytes are paired with themselves (Dahl et al., 1987; Swenson et al., 1989). Hydropathy plots of the connexin 32 sequence deduced from cDNA (Paul, 1986) suggested that this junctional protein has four transmembrane segments and that both amino- and carboxy-termini are located on the cytoplasmic side of the membrane. Topological analyses carried out by Zimmer et al. (1987), Hertzberg et al. (1988), and Milks et al. (1988) demonstrated the actual cytoplasmic localization of both protein termini. Two other hydrophilic domains, those contained within amino acids 98–124 and 164–189 and thought to connect the second and third, and the third and fourth transmembrane segments, were shown to be cytoplasmic for the first, and extracellular for the second (Goodenough, Paul & Jesaitis, 1988).

Using site-directed antibodies, Dupont et al. (1988), Beyer et al. (1989) and Yancey et al. (1989) demonstrated that connexin 43 molecules are components of heart gap junctions. The junctional nature of connexin 43 was also demonstrated by Swenson et al. (1989) who showed that large voltage-insensitive conductance develops when connexin 43 RNA-injected *Xenopus* oocytes are paired with themselves (or with connexin 32 RNA-injected oocytes). Both the amino- and carboxy-termini of connexin 43 are exposed on the cytoplasmic side of the plasma membrane (Manjunath et al., 1987; Beyer et al., 1989; Yancey et al., 1989). By combining proteolysis experiments with data from immunoblotting, Yancey et al. (1989) identified a third cytoplasmic region, a 4-kD loop located between membrane-protected domains. Finally, an antiserum to the conserved extracellular sequence of amino acids 164–189 of connexin 32 reacts with both liver and gap junction proteins on Western blots (Beyer et al., 1989). All these findings, added to those previously reported for connexin 32, support a topological model of connexins, with unique cytoplasmic domains but conserved transmembrane and extracellular regions.

Antibodies raised to the amino-terminus of rat connexin 43 cross-react with homologous proteins, thought to be junctional proteins, in heart of other vertebrates, and in various organs, in particular with a brain 41-kD protein (Dupont et al., 1988). In this paper it is shown by immunoblotting and immunolabeling that a carboxy-terminal domain of rat cardiac connexin 43 contained within amino acids 314–322 is cytoplasmic and that the epitopes of this domain are conserved in mammal heart and on the cytoplasmic faces of astrocytes gap junctions.

Materials and Methods

Biological Material

Rat heart gap junctions (GJ) were isolated as described by Manjunath, Goings and Page (1984) in the presence of PMSF (1 mM).[1] All the solutions contained 0.1 M iodoacetamide as recommended by Dupont et al. (1989). Gap junctions were also isolated without any protease inhibitor. The amounts of protein in isolated gap junction fractions were determined by densitometry from Coomassie blue stained electrophoresis gels (Dupont et al., 1988).

Whole organ fractions were prepared as described by Paul (1985). Organs were plunged, immediately after dissection, in Freon 22 cooled with liquid nitrogen, pulverized under liquid nitrogen and freeze-dried. Human samples were taken from the heart of a 52-year-old patient free of known cardiovascular disease who had been maintained under life support after a head trauma. Protein content of whole organ fractions was determined according to Lowry et al. (1951).

For immunofluorescence microscopy small fragments of vertebrate heart (man, rat, mouse, guinea pig, chicken, frog, trout) and of mouse cerebral cortex were either directly frozen in liquid nitrogen (for subsequent cryostat sectioning) or fixed with 4% formaldehyde in PBS (pH 7.2) for 1 hr at 4°C, infused over-

[1] *Abbreviations:* BDB, *bis*-diazobenzidine; BSA, bovine serum albumin; DAB, 3-3′ diaminobenzidine tetrahydrochloride; EDTA, ethylenediamine tetraacetic acid; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; GJ, gap junction; HEPES, (N-(2-hydroxyethyl) piperazine-N′-)2-ethanesulfonic acid); HPLC, high pressure liquid chromatography; HRP, horseradish peroxidase; Ig, immunoglobulin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenyl-methylsulfonyl fluoride; SDS, sodium dodecyl sulphate; and TRITC, tetraethyl rhodamine isothiocyanate.

night with 2.0 M sucrose and then frozen in liquid nitrogen (for subsequent thin sectioning according to Tokuyasu (1973, 1986)). For immunogold labeling on ultrathin frozen sections, rat heart samples were fixed with 4% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer for 1 hr at 4°C, infused overnight in 2.0 M sucrose and then frozen in liquid nitrogen.

Primary neural cell cultures were prepared from 15 days post-coitum embryo forebrain dissected free of meninges. Cells were dissociated with 0.25% trypsin, 2 mM EDTA in medium 199 (GIBCO-BRL) buffered with 25 mM HEPES as described by Rougon et al. (1983*a*) and Rougon, Noble and Mudge (1983*b*). Single cell suspensions were filtered through a 48-mesh nylon gauze and plated on poly-L-lysine (2 μg/ml) coated glass coverslips. The cells were maintained for 5–7 days in defined medium adapted for neural cells (Pettman, Louis & Sensenbrenner, 1979). Two days after plating the cultures were treated with 10^{-5} M cytosine arabinoside for 24 hr in order to kill fast-dividing cells. Astrocyte enriched cultures were prepared from the previous cell cultures (*see* Rougon et al., 1983*b*) by a modification of the method of McCarthy and de Vellis (1980). After the treatment with cytosine arabinoside the cultures were placed on an orbital shaker to remove cells growing on top of the cell monolayers and then were grown for one further day before use. These cell cultures were tested for the presence of neurons using anti-tetanus toxin antibodies (Raff et al., 1979) and no positive cell was observed.

Preparation and Purification of Site-Directed Antibodies

The peptide SAEQNRMGQY (Ser-10-Tyr) was synthesized in solid phase according to Merrifield (1963). The sequence SAEQNRMGQ corresponds to the predicted sequence of connexin 43 contained within residues 314–322 (Beyer et al., 1987); it is located 50 amino acids away from the carboxy-terminus. A tyrosyl residue was added to the carboxy-terminus of the peptide SAEQNRMGQ to facilitate the coupling to the carrier protein. Coupling of the synthetic peptide to BSA by means of BDB, immunication of rabbits in the popliteal lymph nodes and purification of antipeptide IgGs by affinity chromatography from immune sera were described in detail by Dupont et al. (1988) in a previous paper dealing with the characterization of other antibodies (anti-SALGKLLDKVQAY or anti-Ser-13-Tyr antibodies) directed to the amino-terminus of connexin 43.

Pre-immune sera were subjected to the same purification steps as immune sera (Dupont et al., 1988). Fractions collected from elutions with HCl-glycine were used in control experiments and will be referred to as "pre-immune sera affinity-purified fractions."

Polyacrylamide Gel Electrophoresis and Immunoblotting

Analysis of gap junctions and of freeze-dried whole organ fractions were carried out by SDS-PAGE under reducing conditions as reported by Dupont et al. (1988). Molecular weights were estimated by reference to standard proteins (BioRad). Gels were stained with Coomassie brilliant blue R-250.

For immunoblotting, samples fractionated by electrophoresis were transfered according to Towbin, Staehelin and Gordon (1979) onto nitrocellulose membranes (0.22 μm; Schleicher and Schuëll) at constant voltage (25 V) for 12–15 hr with 0.02% SDS in the electrode buffer. Transfer of proteins was checked by staining the nitrocellulose membrane with Ponceau S. Immunoreplicas were first saturated with the so-called BLOTTO solution (Johnson et al., 1984) then incubated overnight at 4°C with affinity-purified antipeptide IgGs (2 μg/ml in BLOTTO). Treatment of replicas with a secondary antibody (biotinylated-goat anti-rabbit F (ab') 2, Jackson Immunoresearch Lab.) then with peroxidase-labeled streptavidin (Jackson Immunoresearch Lab) before detection of peroxidase activity with 4-chloronaphtol was as described by Dupont et al. (1988). Control experiments were performed by omission of the incubation step with antipeptide antibodies and also by using pre-immune sera affinity-purified fractions instead of primary antibodies.

Northern Blots

The cDNA clone G1, coding for the rat cardiac connexin 43, was kindly provided by Dr. E. Beyer (*see* Beyer et al., 1987). The radiolabeled cDNA probe was prepared by nick-translation (Maniatis, Fritsch & Sambrook, 1982) using a labeling kit (Amersham) and (^{32}P)dCTP.

Total cellular RNA was extracted from rat heart and rat and mouse brain according to Chirgwin et al. (1979) and quantified by spectrophotometry. RNA samples were fractionated by electrophoresis in 1% agarose/8% formaldehyde gels and capillary blotted (Maniatis et al., 1982) onto nylon membranes (Hybond N, Amersham). RNA was crosslinked to the membrane by ultraviolet exposure. High stringency blots were prehybridized in (5 × SSPE 50% formamide; 5 × Denhart's; 0.5% SDS; 250 μg/ml salmon sperm DNA) for 4 hr at 42°C, then hybridized overnight, at 42°C, in the same buffer, with the labeled probe. Blots were then washed in (2 × SSPE; 0.1% SDS) three times 5 min at room temperature and twice 35 min in (0.5 × SSPE; 0.1% SDS) at 65°C before exposure to Hyperfilm (Amersham) at −80°C with intensifying screens.

Immunolabeling

Except when mentioned, all the incubation steps were carried out at room temperature. Whatever the labeling technique used, the controls were performed either by omission of the incubation step with primary antibodies or by using pre-immune sera affinity-purified fractions instead of primary antibodies, or both.

Fluorescence Microscopy

Frozen-sectioning (5 μm) of unfixed heart and brain samples was performed at −20°C using a Reichert-Jung cryostat; thin frozen-sectioning (0.5–1 μm) of formaldehyde-fixed samples was performed at −100°C using a Sorvall ultracryotome. Myocardium sections were reacted from 30 min to 12 hr with affinity-purified antipeptide IgGs (3.75 μg/ml in PBS containing 0.2% gelatin), then for 1 hr with TRITC-labeled sheep anti-rabbit antibodies diluted in the same buffer. Brain sections were reacted for 12 hr and 90 min with antipeptide antibodies (3.75 μg/ml) and secondary antibodies, respectively. Preparations were examined with a Zeiss fluorescent microscope.

Cultured neural cells were permeabilized by treatment with an acetic acid-alcohol solution (5:95, vol/vol) for 7 min at −20°C. Unpermeabilized live cells or permeabilized cells were incubated with affinity-purified antipeptide antibodies at 3.75 μg/ml in HEPES-buffered medium 199 containing 10% of fetal calf serum for 90 min, then with FITC-coupled goat (Fab') 2 anti-rabbit antibodies (Jackson Immunoresearch Lab.) for an additional 30 min. After rinsing, coverslips were mounted on slides and examined by fluorescent microscopy.

For double-labeling experiments, astrocyte-enriched cell

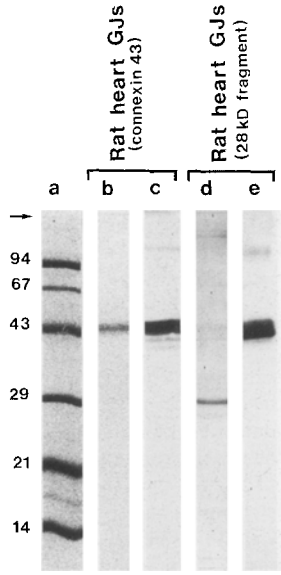

Fig. 1. Characterization by immunoblotting of affinity-purified anti-Ser-10-Tyr antibodies. Arrow indicates the top of separating gels and immunoreplicas. Lanes *a*, *b* and *d* show Coomassie blue stained gels of standard proteins (molecular weights in kD are indicated on the left), rat heart gap junctions (GJ) isolated in the presence of PMSF (connexin 43), and rat heart gap junctions isolated without protease inhibitor (28-kD fragment), respectively. Note in lane *d* that the proteolysis of gap junctions is not complete and that faint bands at 43 and 41 kD are seen. Lanes *c* and *e* show immunoreplicas of gels represented in lanes *b* and *d*, respectively. The 28-kD junctional fragment (lane *d*) generated by uncontrolled proteolysis during isolation of gap junctions without protease inhibitor does not react with anti-Ser-10-Tyr antibodies (lane *e*) whereas connexin 43 (lanes *c* and *e*), and the 41-kD fragment as well, (lane *e*) do. The bands at about 100 kD in lanes *c* and *e* are not seen in controls; they probably correspond to aggregated forms of connexin 43

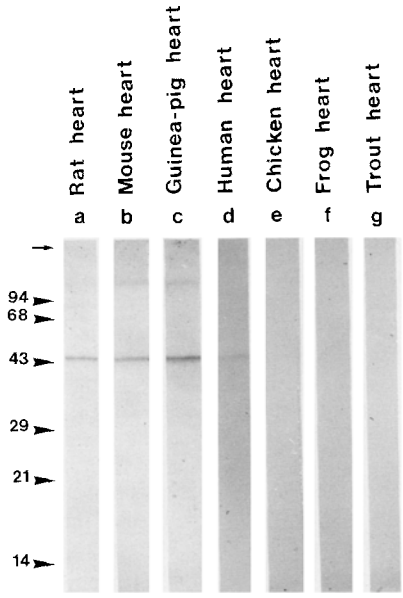

Fig. 2. Immunoblotting of whole heart fractions. Position of molecular weight standards is indicated on the left. Arrow indicates the top of separating gels and of immunoreplicas. Lanes *a–d* show immunoreplicas of whole fractions from rat, mouse, guinea pig and human heart, respectively. A unique 43-kD band is detected in these immunoreplicas. Lanes *e–g* show immunoreplicas of whole fractions from chicken, frog and trout heart. No labeling was ever seen in these replicas. Each electrophoresis well contained the same amount of proteins: 100 μg

cultures grown on coverslips were first permeabilized as described above then successively treated with: antipeptide antibodies (90 min), TRITC-labeled sheep anti-rabbit antibodies (30 min), rabbit anti-GFAP antibodies (30 min), and FITC-coupled goat anti-rabbit antibodies (30 min).

Electron Microscopy

Ultrathin frozen-sectioning of rat heart tissue prepared for immunogold labeling was performed according to Tokuyasu (1973, 1986) and stained according to Griffiths et al. (1983). Sections were incubated for 30 min with antipeptide antibodies (12 μg/ml) diluted in PBS containing 10% newborn calf serum, then for 30 min with goat anti-rabbit antibodies coupled to 10-nm gold particles (Janssen Pharmaceuticals). Preparations were examined with an Hitachi H600 electron microscope.

Partially purified rat heart gap junctions were incubated for 1 hr at 37°C with affinity-purified antipeptide antibodies at 15 μg/ml in PBS/hemoglobin, 0.1%/Tween 20, 0.05% (PBS/Hb/Tw). Gap junctions were carefully washed in PBS/Hb/Tw by centrifugation in Eppendorf centrifuge tubes. The final pellet was resuspended for 30 min in colloidal gold-conjugated-goat anti-rabbit IgG (Janssen Pharmaceuticals) diluted in PBS/Hb/Tw, then washed by centrifugation as above. The resulting pellet was then fixed as described by Gros, Nicholson and Revel, (1983). Ultrathin sections, stained with uranyl acetate and lead, were examined by electron microscopy.

Results

SDS-PAGE analysis of isolated rat heart gap junctions are illustrated in Fig. 1 (lanes *b* and *d*). Coomassie blue stained gels show a single band of M_r 43 kD or a major band of M_r 28 kD associated with minor bands of 41 and 43 kD depending on whether the junctions are isolated in the presence of PMSF (lane *b*) or isolated without protease inhibitor (lane *d*), respectively. These results are in agreement with those previously published by Manjunath et al. (1985, 1987) who demonstrated that during heart gap junction isolation, uncontrolled proteolysis generates a 28-kD fragment from the intact cardiac junctional protein. Electron microscopy analyses (*not shown*) of gap junctions isolated with or without protease inhibitor give results identical to those reported by Gros et al. (1983) and Dupont et al. (1989). Immunoblots of gap junctions, carried

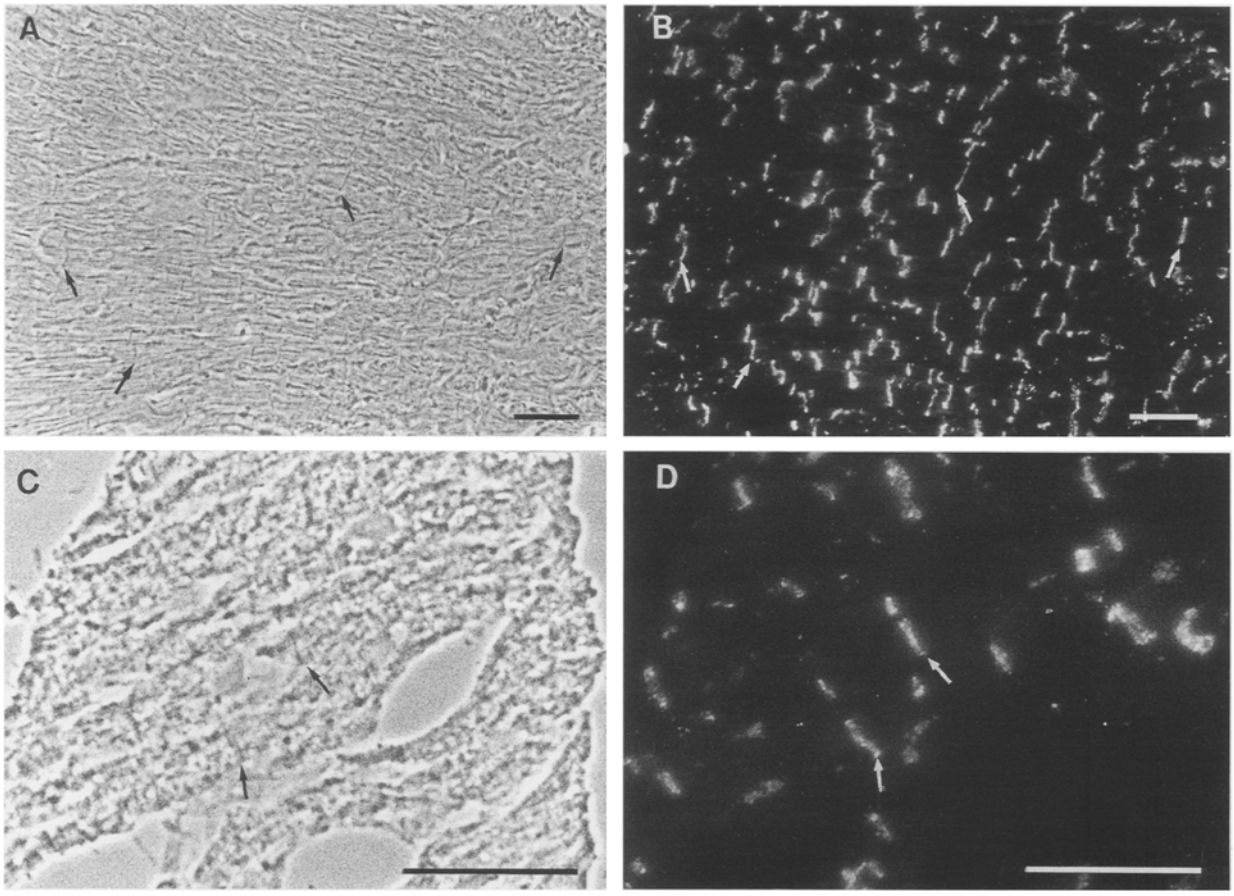

Fig. 3. Immunolabeling of mouse heart and human heart. (*A*) and (*C*) Phase contrast photographs of cryostat sections (4–5 μm) of mouse heart (ventricle) and human heart (ventricle), respectively. (*B*) and (*D*) Fluorescence photographs of the previous sections of rat and mouse heart, respectively. Sections were incubated with anti-Ser-10-Tyr IgGs, then with TRITC-labeled sheep anti-rabbit antibodies. The labeling is distributed in a pattern consistent with the known distribution of intercalated discs (arrows). Bar = 50 μm

out with antipeptide IgGs, are shown in lanes *c* and *e* of Fig. 1. Connexin 43 and the 41-kD proteolytic fragment are recognized by antipeptide antibodies (lanes *c* and *e*) whereas, under the same experimental conditions, the 28-kD fragment is not (lane *e*). No labeling is seen in replicas of control experiments (*see* Materials and Methods).

Antibodies were used to probe replicas of whole fractions of rat, mouse, guinea pig, human, chicken, frog and trout heart. A band of M_r 43 kD is detected in replicas of rat, mouse, guinea pig and human heart (Fig. 2, lanes, *a–d*). No labeling is seen in control experiments (*see* Materials and Methods) of these samples, nor in replicas of chicken, frog and trout heart (Fig. 2, lanes *e*, *f* and *g*).

Sections from unfixed or formaldehyde-fixed human, rat, mouse and guinea pig heart, incubated with antipeptide antibodies visualized with TRITC-labeled anti-IgGs, reveals a pattern consistent with the known distribution of intercalated discs (Fig. 3). In control experiments (*see* Materials and Methods) no labeling is seen. In chicken, frog and trout heart sections, treated with the same experimental conditions as mammal heart sections, no labeling is observed (*not shown*). These results were confirmed by using high concentrations of antipeptide antibodies (30 μg/ml) associated with long incubation times (12 hr).

In rat heart ultrathin frozen sections treated for immunoelectron microscopy, gold particles are associated with gap junctions, which are easily identifiable in the intercalated discs (Fig. 4). In sections incubated only with gold-labeled secondary antibodies no labeling associated with gap junctions is observed. Pellets of partially purified rat heart gap junctions were also treated for immunogold labeling. In ultrathin sections of these pellets, gold particles coat the cytoplasmic surfaces of the junctions, leaving the nonjunctional membranes free of label-

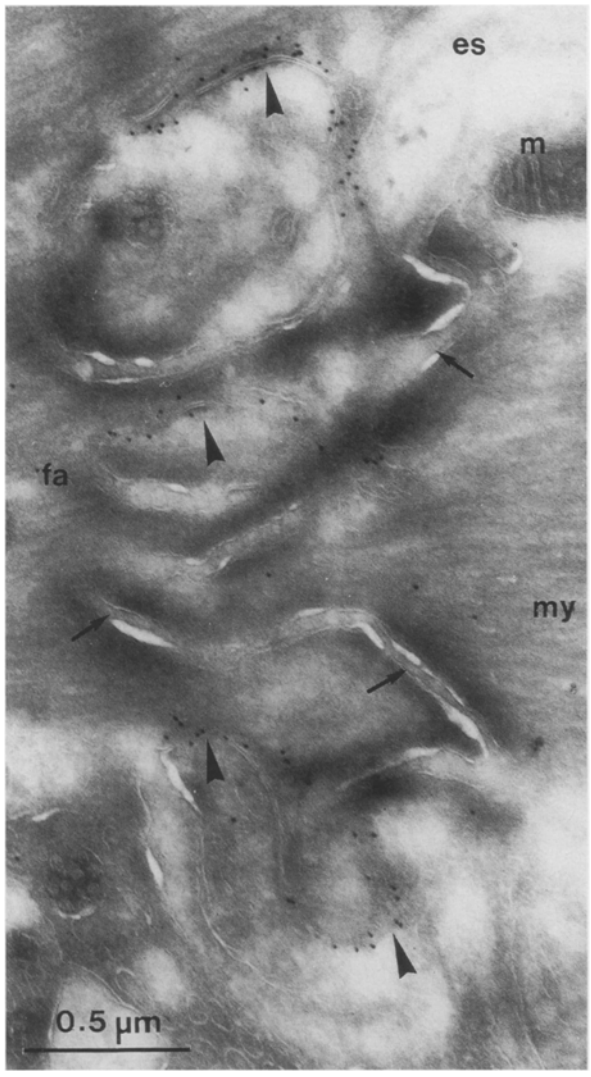

Fig. 4. Immunogold labeling of ultrathin frozen section of rat heart at the level of an intercalated disc. The section was incubated with anti-Ser-10-Tyr rabbit IgGs then with gold-labeled goat anti-rabbit antibodies. Arrows indicate the usual 20–30 nm wide extracellular space between the two plasma membranes of the intercalated disc. Gold (10 nm) particles are distributed over three regions (arrowheads) of the intercalated disc where the plasma membranes are in close association. The 2–3 nm narrow extracellular space in these membrane regions indicates that they correspond to gap junctions. *es*, extracellular space; *fa*, fascia adhesens; *m*, mitochondria; *my*, myofibrils. ×50,000

ing (Fig. 5). In control experiments (*see* Materials and Methods), junctional and nonjunctional membranes are both free of labeling (Fig. 5).

Cross-reactivities of the antibodies directed to the carboxy-terminal domain of cardiac connexin 43 were first investigated by immunoblotting of whole organ fractions. No labeling was seen in rat liver (*data not shown*), as expected from sequence comparison. In contrast, a major large band of M_r 41 kD associated with a band of M_r 43 kD whose labeling is very variable and sometimes absent, is detected in rat brain (Fig. 6*A*, lane *b*). In mouse brain, the M_r 43 kD band is always well labeled and it is always associated with a M_r 41-kD band (Fig. 6*A*, lane *c*). In both cases, a diffuse labeling at about 100 kD, probably corresponding to dimeric forms of the previously detected proteins, is sometimes seen. This band never appears in controls.

Brain RNAs were examined for messages homologous to cardiac connexin 43 mRNAs by Northern blot analyses. Under high stringency conditions of hybridization and washing a single band of 3.0 kb is seen both in rat heart and rat and mouse brain RNAs (Fig. 6*B*).

Frozen sections from unfixed (5 µm) or formaldehyde-fixed (0.5–1 µm) mouse cerebral cortex, incubated with cardiac connexin 43-specific antibodies, then with TRITC-labeled anti-IgGs show a very characteristic staining made of numerous tiny fluorescent dots quite close to each other (Fig. 7*A*). In some sections, areas devoid of labeling were observed, but no attempt was made to identify these brain regions. In control experiments, carried out either by using TRITC-labeled anti-IgGs only or by substituting pre-immune serum affinity-purified fractions (*see* Materials and Methods) for antipeptide antibodies, no labeling is seen (Fig. 7*B*). Immunohistochemistry investigations were continued with primary cultures of mouse neural cells (Figs. 8*A* and *C*). When live unpermeabilized cells are incubated with antipeptide antibodies then with TRITC-labeled anti-IgGs no staining is observed (Fig. 8*B*). Conversely, when cells are first permeabilized before treatment with antibodies, an intense, dotted staining is seen between flat clustered cells (Fig. 8*D*) thought to be glial cells. Neurons, growing on the top of glial cells, show a very weak and diffuse intracellular staining (Fig. 8*D*). When astrocytes-enriched cell cultures are permeabilized and treated for double labeling experiments a punctate staining due to the antipeptide antibodies is associated with GFAP-positive cells (Figs. 8*E* and *F*). The specificity of the immunoreactivity observed in cell cultures was checked as previously described for sections.

Discussion

In agreement with Manjunath et al. (1984) and Dupont et al. (1988), purified rat heart gap junctions contain one major protein of M_r 45–43 kD. By immunoblotting, antipeptide antibodies raised to a carboxy-terminal domain of rat connexin 43, contained within amino acids 314–322, specifically label the

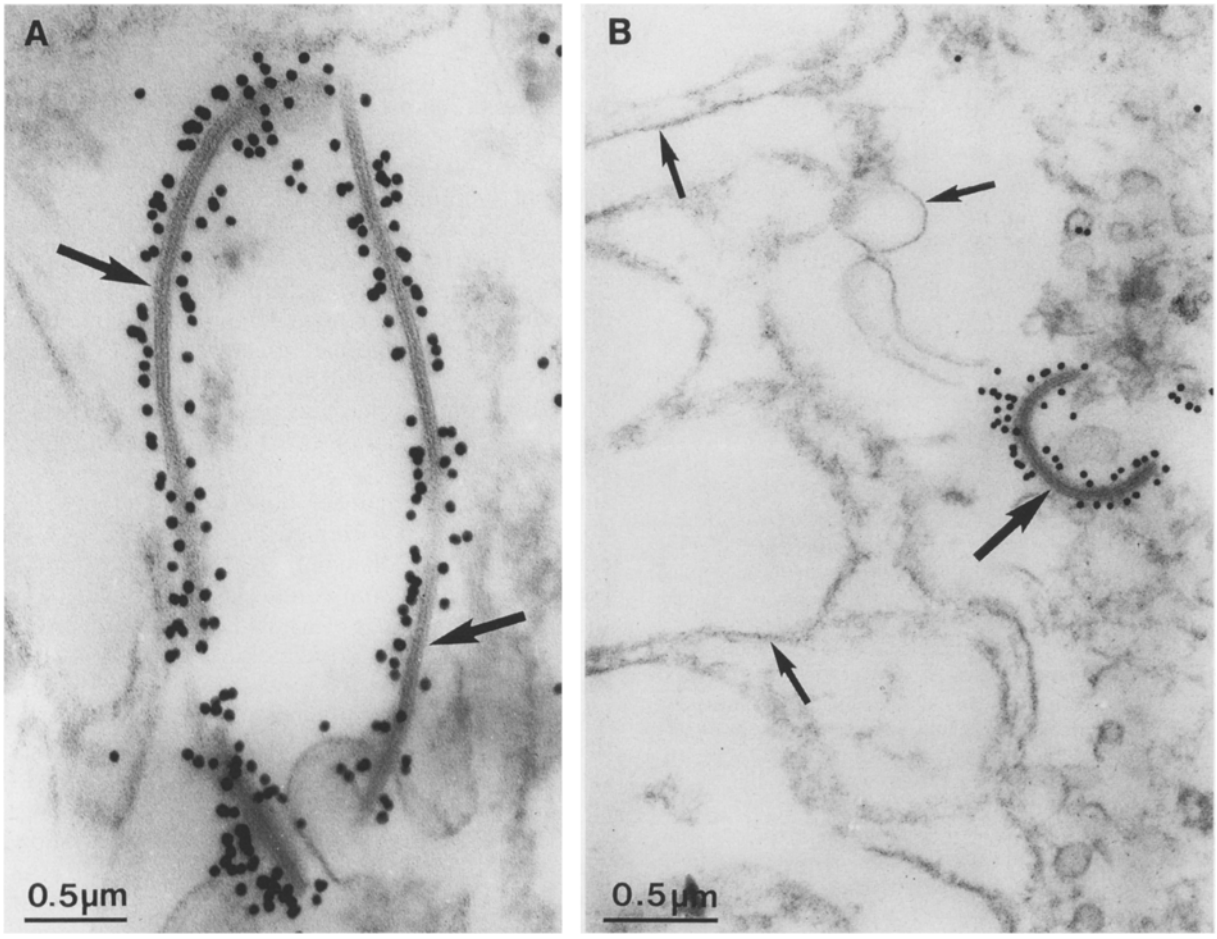

Fig. 5. Immunogold labeling of isolated rat heart gap junctions. A pellet of partially purified gap junctions was incubated with anti-Ser-10-Tyr rabbit IgGs, then with gold-labeled goat anti-rabbit antibodies before ultrathin sectioning. In sections, gap junctions (large arrows) are identified as double membranes separated by a 2–3 nm narrow space. Both cytoplasmic surfaces of the junctions are coated with 10-nm gold particles (*A*) and (*B*); contaminating single membranes (small arrows in *B*) of the pellet are free of labeling. (*A*) ×140,000; bar = 0.1 μm. (*B*) ×80,000; bar = 0.2 μm

43-kD protein constitutive of isolated rat heart gap junctions (Fig. 1). Immunofluorescence investigations show that the epitopes recognized by the site-directed antibodies are located within the intercalated discs (Fig. 3). *In situ* immunogold labeling, performed with ultrathin frozen sections of rat heart strongly suggest that antigenic determinants are associated with gap junctions (Fig. 4). Finally, immunogold labeling carried out with partially purified gap junctions contaminated with single membranes clearly demonstrates that epitopes are exclusively localized on gap junctions (Fig. 5). These results demonstrate that connexin 43, sequenced by Beyer et al. (1987), is a cardiac gap junctional protein and confirm the investigations by Dupont et al. (1988), Yancey et al. (1989) and Beyer et al. (1989).

In the absence of protease inhibitors during the isolation of cardiac gap junctions, uncontrolled proteolysis occurs resulting in the cleavage of connexin 43 into a 28-kD proteolytic fragment (Manjunath et al., 1985). Microsequencing and immunoblotting showed that the native protein and its proteolytic by-product possess the same amino-termini (Manjunath et al., 1987; Dupont et al., 1988) and consequently, that the cleaved M_r 17–15 kD domain is located at the carboxy-terminal side of the protein. Antibodies raised to the amino-terminus of connexin 43 label the 28-kD fragment on Western blot (Dupont et al., 1988). Under the same conditions of analysis, the antibodies raised to the connexin 43 domain contained within residues 314–322 do not recognize the 28-kD fragment (Fig. 1) suggesting that antigenic determinants were exposed to endogenous proteolytic enzymes and cleaved away. Because of the narrowness of the cleft between the two gap junctional membranes it is unlikely that

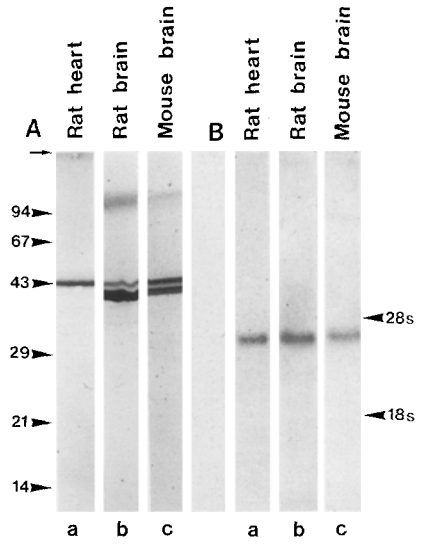

Fig. 6. Expression in rat and mouse brain of a protein homologous to the cardiac connexin 43. (*A*) Immunoreplicas of whole fractions of rat heart (lane *a*), rat and mouse brain (lanes *b* and *c*). Molecular weight markers are indicated on the left. Arrow indicates the top of immunoreplicas. A large major band of 41 kD, associated with a M_r 43-kD band whose labeling is very variable, is labeled in rat brain immunoreplicas. In mouse brain, the M_r 43-kD band is always well labeled and it is associated with a M_r 41-kD band. (*B*) Hybridization of rat heart connexin 43 cDNA to total RNAs from rat heart (lane *a*) and rat and mouse brain (lanes *b* and *c*). Northern blots were probed at high stringency with cDNA clone G1 (Beyer et al., 1987). RNA (5 μg) were loaded per well. Arrowheads on the right indicate the positions of 28 S and 18 S rRNA subunits. Lane *a* shows a single band of 3.0 kb corresponding to the heart connexin 43 mRNAs and previously described by Beyer et al. (1987). Lanes *b* and *c* show that the cardiac probe also hybridizes at high stringency to a 3.0-kb single band in rat and mouse brain Northern blots

proteolytic enzymes penetrate into the junctions. Consequently, the epitopes recognized by the antibodies are cytoplasmic. This hypothesis is confirmed by the immunogold labeling of the cytoplasmic surfaces of isolated gap junctions (Fig. 5).

A previous comparative analysis (Dupont et al., 1988) of immunoreplicas of whole heart fractions from rat, mouse, guinea pig, chicken, frog and trout showed that antibodies raised to the amino-terminus of rat connexin 43 label a protein of M_r 43 kD in all heart investigated suggesting a phylogenic conservation of this connexin 43 terminus. In immunoreplicas of whole heart fractions from human, rat, mouse and guinea pig, probed with antibodies anti-314–322, a band of M_r 43 kD corresponding to connexin 43 is also labeled. These results suggest a similar phylogenic conservation of the cytoplasmic domain contained within residues 314–322 in cardiac connexin 43 of mammals. In contrast, under the same experimental conditions, no labeling was seen on Western blots of whole heart fractions from chicken, frog and trout indicating a probable loss of epitopes. The immunofluorescence experiments lead to the same conclusions. Although these results need to be confirmed by sequencing they suggest a sequence polymorphism within connexin 43 molecules of vertebrate hearts.

Analyses carried out by Northern blots show that rat and mouse brain RNAs contain mRNAs homologous to those of cardiac connexin 43. If these mRNAs are translated, proteins homologous to cardiac connexin 43 should be expressed in rat and mouse brain. This is indeed the case since proteins of M_r 43, and 41 kD are detected on im-

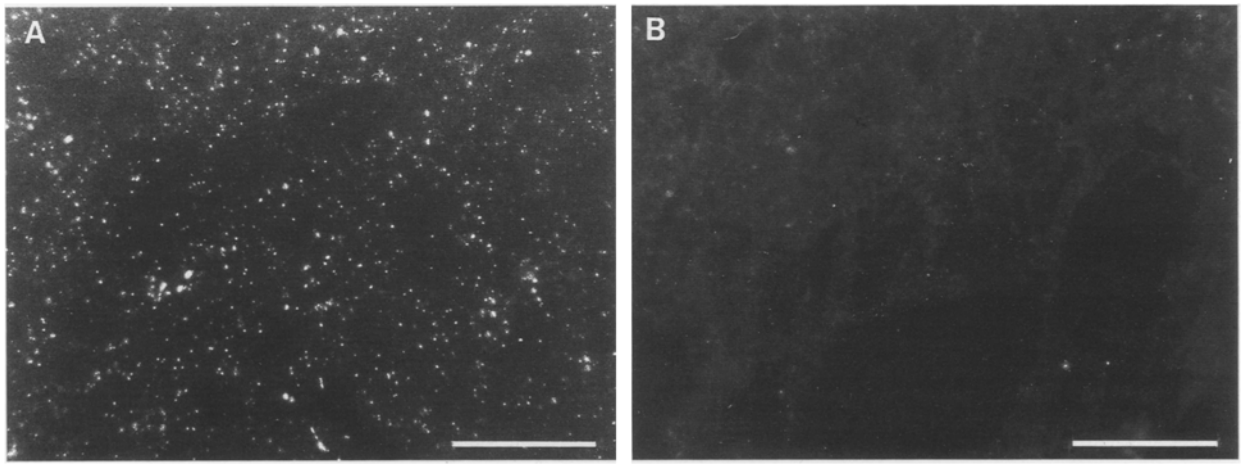

Fig. 7. Immunolabeling of frozen sections (0.5–1 μm) of paraformaldehyde-fixed mouse brain. (*A*) Fluorescence photograph of a section stained with anti-Ser-10-Tyr IgGs visualized with TRITC-labeled sheep antirabbit antibodies. (*B*) Control experiment. Fluorescence photograph of a section stained only with secondary antibodies. TRITC-labeled sheep anti-rabbit antibodies were used at the same concentration and for the same incubation time as in *A*. Both photographs *A* and *B* were processed from negatives which were exposed for the same time (60 sec) to sections examined by fluorescence microscopy. Bar = 30 μm

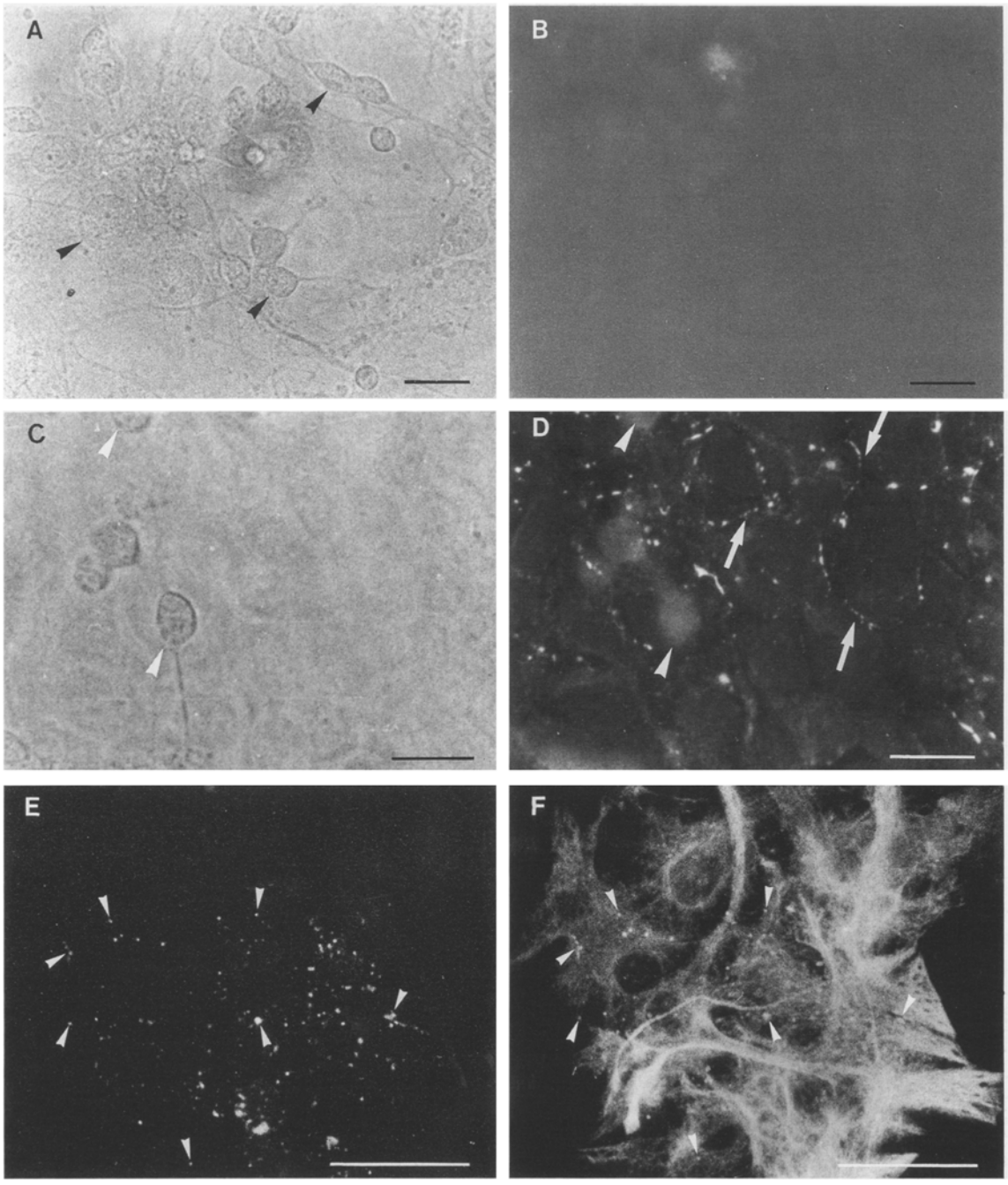

Fig. 8. Immunolabeling of mouse neural cells in culture. (*A*) Phase contrast photograph of cultured mouse neural cells. Cells were dissociated from 15 days post-coitum mouse embryo forebrain and maintained in culture for five days. Neurons are indicated by arrowheads. (*B*) Fluorescence photograph of cells shown in *A*, treated with anti-Ser-10-Tyr IgGs, then with FITC-labeled goat antibodies. No labeling is seen. Bar = 15 μm. (*C*) Phase contrast photograph of mouse neural cells permeabilized with a solution of acetic acid/alcohol at −20°C. Neurons are indicated by arrowheads. (*D*) fluorescence photograph of permeabilized cells shown in *C* and treated as in *B*. Note the dotted labeling between clustered flat cells (arrows). Neuron cell bodies show a very weak and diffuse fluorescence (arrowhead). Bar = 15 μm. (*E* and *F*) Double labeling experiments with permeabilized astrocyte-enriched mouse neural cells. *E*: fluorescence photograph taken on the channel TRITC, showing the distribution of the labelings (arrowheads) due to the antipeptide antibodies, in a cell cluster. *F*: fluorescence photograph of the previous field (*E*) taken on the channel FITC. The cells are GFAP-positive and they show intense punctate labelings (arrowheads) superimposed on the GFAP fluorescence. The distribution of the punctate labelings is identical to that shown in *E*. Bar = 15 μm

munoblots of whole brain extracts probed with antibodies specific for the cytoplasmic domain of cardiac connexin 43. First, these results confirm previous findings by Dupont et al. (1988) who demonstrated the presence in rat brain of a 41-kD protein sharing antigenic determinants with the amino-terminus of cardiac connexin 43. Second, epitopes recognized by the antibodies characterized in the present investigation, and associated with brain homologous proteins are cytoplasmic, as demonstrated by immunolocalization experiments carried out with unpermeabilized and permeabilized neural cells. Third, the pattern of immunoreactivity of cultured neural cells is consistent with the distribution of gap junctions between cells and this immunoreactivity was confirmed by the staining of cerebral cortex sections. These results suggest that the neural proteins, homologous to cardiac connexin 43, are gap junctional polypeptides.

The detection of two bands (but sometimes only one in rat), close to each other, on immunoblots of whole brain extracts is not sufficient evidence for assuming that each of them is a native junctional protein. One single 3.0-kb message was seen on brain Northern blots, and consequently, a single protein is expected to be expressed. Two hypotheses may explain these results. Gap junctional proteins are known to be very sensitive to proteolysis and it would be reasonable to suppose that the lower band of M_r 41 kD is a by-product of the 43-kD protein generated during the sample preparation. However, this hypothesis is not consistent with the following observations: (*i*) no degradation product (below 43 kD) was ever seen in the immunoblots of whole heart fractions processed in the same way as brain samples; (*ii*) the 41-kD band is recognized both by antipeptide antibodies directed to the amino-terminus of connexin 43 (Dupont et al., 1988) and by antipeptide antibodies, directed to the carboxy-terminus of connexin 43 (D.W. Laird & J.P. Revel, *personal communication*). Consequently, as a second hypothesis, the difference of M_r between the 43- and 41-kD proteins might possibly be attributed to the degree of post-translational modifications affecting the native protein.

Earlier studies by Hertzberg and Skibbens (1984) and more recent ones by Dudek et al. (1988), Nagy et al. (1988), and Shiosaka et al. (1989) showed by immunoblotting and immunohistochemistry the presence in brain and in neural cell cultures of proteins homologous to the M_r 27–28 kD liver gap junctional protein (i.e., connexin 32). These results have confirmed those of Paul (1986) who had characterized in brain 1.6-kb mRNAs homologous to liver connexin 32 mRNAs. The present investigations, taken in conjunction with the above results, provide evidence for the probable existence in brain of several junctional proteins.

Neurons as well as glial cells have been reported to be coupled through gap junctions (Sotelo & Korn, 1978; Llinas, 1985; Mugnaini, 1986; Dudek et al., 1988) and it may be expected that immunostaining associated with one or the other of these cell types or both might be observed. In brain sections, the staining pattern observed by light microscopy by Nagy et al. (1988), using antibodies against 27–28 kD liver gap junctional proteins, is similar to that reported here (*see* Fig. 7). However, the nature of cells with which immunoreactivity is associated cannot be deduced from observations made from 1- or 5-μm thick brain sections, at least under the experimental conditions used. In contrast, in neural cell cultures, the cellular phenotypes are more easily identifiable. In these cultures, a weak and diffuse fluorescence is observed within the neuron cell bodies. This kind of labeling can hardly be related to that which is expected for gap junctions and its origin is as yet unknown. The most obvious labeling observed is a punctate labeling, located between flat clustered cells, as expected for gap junctions. To determine precisely the nature of the cells with which this immunoreactivity is associated, double labeling experiments were performed on astrocyte-enriched cell cultures, free of neurons, using anti-GFAP and antipeptide antibodies. Observation clearly demonstrates that the labeling due to the antibodies directed to the cytoplasmic domain of connexin 43 is associated with GFAP-positive cells, that is to say, astrocytes. Recently, Dudek et al. (1988) and Shiosoka et al. (1989) have shown that proteins immunologically related to liver junctional proteins were also present in astrocytes. Thus, these glial cells, would represent the second cell type after hepatic cells containing at least two junctional proteins.

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