The 43-kD Polypeptide of Heart Gap Junctions: Immunolocalization (I), Topology (II), and Functional Domains (III)

S. Barbara Yancey (I), Scott A. John (II), Ratneshwar Lal (III), Brian J. Austin, and Jean-Paul Revel

Division of Biology, California Institute of Technology, Pasadena, California 91125

Abstract. Analysis by SDS-PAGE of gap junction fractions isolated from heart suggests that the junctions are comprised of a protein with an M_r 43,000. Antibodies against the electroeluted protein and a peptide representing the 20 amino terminal residues bind specifically on immunoblots to the 43-kD protein and to the major products arising from proteolysis during isolation. By immunocytochemistry, the protein is found in ventricle and atrium in patterns consistent with the known distribution of gap junctions. Both antibodies bind exclusively to gap junctions in fractions from heart examined by EM after gold labeling. Since only domains of the protein exposed at the cytoplasmic surface should be accessible to antibody, we conclude that the 43-kD protein is assembled in gap junctions with the amino terminus of the molecule exposed on the cytoplasmic side of the bilayer, that is, on the same side as the carboxy terminus as determined previously. By combining proteolysis experiments with data from immunoblotting, we can identify a third cytoplasmic region, a loop of some 4 kD between membrane protected domains. This loop carries an antibody binding site. The protein, if transmembrane, is therefore likely to cross the membrane four times. We have used the same antisera to ascertain if the 43-kD protein is involved in cell–cell communication. The antiserum against the amino terminus blocked dye coupling in 90% of cell pairs tested; the antiserum recognizing epitopes in the cytoplasmic loop and cytoplasmic tail blocked coupling in 75% of cell pairs tested. Preimmune serum and control antibodies (one against MIP and another binding to a cardiac G protein) had no or little effect on dye transfer. Our experimental evidence thus indicates that, in spite of the differences in amino acid sequence, the gap junction proteins in heart and liver share a general organizational plan and that there may be several domains (including the amino terminus) of the molecule that are involved in the control of junctional permeability.

THE cells of cardiac muscle (for review, see Page and Manjunath, 1986), and those in most tissues of organisms throughout the animal kingdom, are interconnected by channels that provide a low resistance pathway for the passage of ions and small molecules. These channels collectively form the membrane specialization known as the gap junction (Revel and Karnovsky, 1967) or nexus (Dewey and Barr, 1962; 1964). In the intact heart, gap junctions provide cell-to-cell electrical continuity. Such connections are believed important in supporting the coordinated contraction of myocytes, allowing the heart to perform as an effective pump (Barr et al., 1965; De Mello, 1977; Joyner, 1982; Spach and Kootsey, 1983; Fozzard and Arnsdorf, 1986). Presumably the synchronous beating of cardiac myocytes in cell culture is a reflection of the transmission of electrical signals via gap junctional channels (De Haan and Hirakow, 1972; Griepp and Bernfield, 1978; Griepp et al., 1978; De Haan et al., 1981; however see Sperelakis and Mann, 1977; Williams and De Haan, 1981). Rapid propagation of electrical signals and synchronization of activity are postulated roles of the gap junctions in other excitable tissues (Robertson, 1963; Bennett, 1977). In nonexcitable tissues, and probably in heart as well, gap junctions mediate the cell-to-cell traffic of metabolites and may carry signal molecules for the control of growth, embryonic development, and cellular differentiation (Pitts, 1978; Loewenstein, 1979; Warner et al., 1984; Revel, 1986; Fraser et al., 1987; Gilula et al., 1987).

Until recently, much of our understanding of the molecular organization of gap junctional channels has come from the characterization of the junctions from liver where it has been shown that cell–cell connections are formed by the end-to-end interaction of hemichannels (connexons) comprised of six protein subunits (Unwin and Zampighi, 1980). In the first models proposed, each protein was believed to be a polypeptide of M_r 28 kD (Makowski et al., 1977; Makowski, 1985). In the last few years, however, there has been a growing realization that gap junctions from different tissues may differ in their protein components (Nicholson et al., 1985; Manjunath and Page, 1986; Manjunath et al., 1987; Gruijters et al., 1987) and that even within a single cell more than one kind

Portions of this work have appeared in abstract form (John et al., 1988. *Proc. Int. Congr. Cell Biol.*; Lal et al. 1988. *Soc. Neurosci. Abstr.* 14:145; Yancey et al., 1988. *J. Cell Biol.* 107:555a. [Abstr.]).

of protein may be involved in their formation (Nicholson et al., 1987; Revel et al., 1986; 1987). Gap junctions isolated from heart appear to contain a major component that migrates, depending on the gel system, between ~43 and 47 kD (Manjunath et al., 1984), but has a true molecular mass of 43 kD as determined by DNA sequencing (Beyer et al., 1987); junctions from liver (Nicholson et al., 1987; Revel et al., 1986; 1987) are now known to be comprised of two proteins that migrate at ~28 and 21 kD but have an actual molecular mass of 32 and 26 kD, respectively (Paul, 1986; Kumar and Gilula, 1986; Nicholson and Zhang, 1988). The known heart and liver proteins are related to each other at the level of ~50% homology as first indicated by a comparison of the amino terminal sequences obtained by protein sequencing (Nicholson et al., 1981; 1985; 1987) and later confirmed when their complete amino acid sequences became known from cDNA cloning (Paul, 1986; Kumar and Gilula, 1986; Heynkes et al., 1986; Beyer et al, 1987; Nicholson and Zhang, 1988). More recently, another protein with sequence homology, but with an M_r of 70 kD, has been identified in the lens (Kistler et al., 1988). Other proteins, not related to those above, have also been proposed as components of vertebrate gap junctions, notably a polypeptide of 16 kD present in many different tissues including liver and heart (Finbow et al., 1985) and the main intrinsic polypeptide (MIP) of lens fiber cell membranes (Johnson et al., 1985). Of all these proteins, only the 32-kD protein from liver and the 70-kD protein from the lens have been unequivocally localized to gap junctions (Paul, 1986; Zimmer et al., 1987; Dermietzel et al., 1987; Gruijters et al., 1987; Milks et al., 1988). In addition, immunofluorescence staining of the 32- and 26-kD proteins of liver shows that they colocalize. Thus, the 26 kD also is likely a junction protein (Nicholson et al., 1987).

The specific properties of gap junction channels of particular organs may be due to differences in the proteins leading to a distinctive organization of the channels or to differential sensitivity to signals. The theoretical predictions about the organization of gap junction proteins in membranes which can be made on the basis of analysis of the sequence (Schultz, 1988) must be tested experimentally. Milks et al. (1988) have just published such a study for the 32-kD hepatic gap junction protein; the work we present here shows that the heart junction protein has the same general structural features. By immunocytochemistry with an antibody against the 43-kD protein from heart and an antipeptide antibody against its amino terminus, we demonstrate that this protein is distributed in the heart in the pattern one would expect if it were a major component of gap junctions. We show that it is specifically localized to gap junctions in fractions isolated from heart. We present direct evidence that the amino terminus is exposed at the cytoplasmic surface of the junction and that there is a cytoplasmic loop. Since the carboxy terminus of the molecule is already known to be cytoplasmic (Manjunath et al., 1987), the molecule could cross the membrane four times. By combining results produced by immunolocalization and biochemical dissection, we construct a model that confirms several aspects of the theoretical predictions which can be made on the basis of the cDNA-derived amino acid sequence (Beyer et al., 1987).

1. *Abbreviations used in this paper*: LY, Lucifer yellow; MIP, main intrinsic polypeptide; TR, Texas red.

The topological data is of particular interest when combined with physiological information on gap junctions obtained by measurement of their electrical properties and of the extent of dye coupling. The activity of the gap junction channels has been reported to be gated by changes in pH, pCa, membrane potential and second messengers (for reviews, see Spray and Bennett, 1985; Bennett and Spray, 1987). It is tempting to hypothesize that the tissue-specific differential gating of physiological activity may result from the biochemical diversity of the gap junctions. As a first step in testing this idea, we have asked if separate functional domain(s) in gap junction proteins can be recognized using antibodies raised against identified epitopes of these proteins. The antibodies we have characterized inhibit dye coupling between neonatal rat cardiac muscle cells in culture. Thus, a domain at the amino terminus and epitopes located in other parts of the 43-kD heart protein could independently be involved in controlling cell–cell communication via gap junctions.

Materials and Methods

Preparation of Membrane and Gap Junction–Enriched Fractions

Crude plasma membranes and gap junction enriched fractions were isolated from the hearts of rats (300–350 g male Sprague-Dawley from Simonsen Laboratories, Gilroy, CA; or Bantin and Kingman, Fremont, CA). Plasma membranes were prepared according to the methods described by Manjunath et al. (1985) and gap junctions according to Manjunath and Page (1986). Partially purified preparations were obtained by using sarkosyl concentrations <0.3% for membrane solubilization. In our hands, concentrations of 0.35% were needed to yield consistently high purification (but lower yields) of gap junctions. Protein recovery was estimated by densitometry of Coomassie stained gels.

SDS-PAGE

Aliquots of the fractions were solubilized at room temperature in buffer (final concentration of 2% SDS, 5% beta-mercaptoethanol, 5% glycerol, 0.004% bromophenol blue, and 0.03 M Tris-HCL, pH 6.8) and analyzed on 15% polyacrylamide gels. The following low molecular weight standards (provided by Bio-Rad Laboratories, Cambridge, MA) were used: phosphorylase B, 97,400; BSA, 66,200; ovalbumin, 42,700; carbonic anhydrase, 31,000; soy bean trypsin inhibitor, 21,500; lysozyme, 14,400. The size of the fragments produced by exogenous proteases was obtained using a Turbo Pascal implementation of the program "Gel" described by Schaffer and Sederoff (1981), but is only an approximation of the true molecular mass because of the anomalous behavior of very hydrophobic polypeptides in SDS-PAGE (Isaac et al., 1985).

Preparation of Antibodies against Heart Gap Junctions

Both antisera were produced in New Zealand White rabbits.

Anti-43-kD Antibody. Purification of 43-kD protein: gap junctions were isolated from whole rat hearts as described by Manjunath and Page (1986), except that the preparations were scaled up to 32 rats and sarkosyl detergent treatment was varied to optimize the yield. The 43-kD protein to be used as immunogen was electroeluted from bands excised from 10% preparative gels after brief staining with Coomassie blue and partial destaining. Electroelutions were carried out in a Schleicher and Schuell Elutrap, using standard Tris-glycine electrophoresis buffer containing 0.1% dithiothreitol. The electroeluted protein was analyzed on 15% polyacrylamide gels and also on nitrocellulose blots for immunoreactivity with the anti-amino terminus antibody (see below). The protein was lyophilized before resuspension, emulsification, and injection.

Production of Antibody. One rabbit was injected with 40 μg of electroeluted protein mixed 1:1 with complete Freund's adjuvant. Approximately 25% of the emulsion (50–75 μl) was injected into each popliteal lymph node;

the remainder was injected intradermally at several sites along the back. At 4 wk and 6 mo after the first injection, 150–180 μg of electroeluted protein in incomplete Freund's adjuvant was injected, 50% subcutaneously in the neck and 25% intramuscularly in each hind leg near the popliteal nodes. Blood was taken 10 d after each boost and then at 1-wk intervals.

Affinity Purification. Specific antibodies were isolated from the immune serum by affinity purification against the 43-kD protein using the Olmsted technique (Olmsted, 1981). Components of the gap junction preparations were electrophoretically separated and blotted to nitrocellulose by semi-dry electrotransfer in CAPS buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 11) with 10% methanol (vol/vol) and 0.1% SDS. The blots were stained with Ponceau-S, and the 43-kD band was excised and cut into small squares. The squares were treated with blocking solution (3% BSA in TBS with 0.1% Tween 20), and then incubated with the antiserum. The bound antibody was eluted with 3 M KSCN, and then passed over an acrylamide buffer-exchange column (Beckman Instruments, Inc., Palo Alto, CA). Antibody concentration was determined by optical density measurements (1 mg is equal to 1.4 OD units at 280 nm).

Anti-Amino Terminus Antibody. Synthesis and conjugation of the peptide: a peptide corresponding to the amino terminal 20 residues of the mature heart 43-kD protein as determined by Nicholson et al. (1985), with a glycine and a cysteine residue added at the carboxy terminus for purposes of cross-linking, was synthesized on a peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) (S. Kent, California Institute of Technology). The peptide differs from the cDNA-derived sequence (Beyer et al., 1988) at position 1 of the protein sequence by having an Ala instead of a Gly. Fully reduced crude peptide (24 mg) was conjugated through its carboxy terminal cysteine to Keyhole limpet hemocyanin (6 mg) using the bifunctional cross-linker succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce Chemical Co., Rockford, IL).

Production of the Antibody. 1.25 mg of the conjugate were suspended in Freund's complete adjuvant (in a ratio of 1:2) and injected, ~66% intradermally at multiple sites on the back and 33% intramuscularly in the hind limb adjacent to the popliteal node. The rabbit was subsequently boosted with 500 μg of conjugate, prepared similarly, at 2 and 4 mo after the first injection and then 1 yr later. Titers >1 in 10,000 against the peptide were consistently obtained in bleeds 1–3 wk following each boost.

Affinity Purification. Crude peptide (5–7 mg) was conjugated via Schiff's base to the free aldehyde groups on the membrane filter of a MAC-25 cartridge (Memtek Corporation, Billerica, MA) in accordance with the manufacturer's instructions. After blocking, specific antibody was bound by passing 500 μl of serum through the filter. After several washes with PBS/0.1% Tween 20, the bound antibody was eluted with 0.1 M glycine at pH 2.2 and immediately equilibrated with PBS/0.01% sodium azide by passage over a Sephadex G50 spun column.

Characterization of Antisera

Western Blots. Components of the gap junction enriched fractions were separated on 15% polyacrylamide gels and transferred to nitrocellulose as described above. The blots were treated with blocking solution, then incubated with the primary antibody diluted in TBS with 0.1% Tween 20 (TBST), washed in TBST, and reacted with goat anti-rabbit IgG (Fc) conjugated to alkaline phosphatase (Promega Biotec, Madison, WI). Reaction with the enzyme substrate and color development were performed according to the instructions of the supplier. Strong signals were obtained with the anti-43-kD antiserum at 1:1,000 dilution and the anti-amino terminal antiserum at 1:5,000 dilution.

ELISA. Assays for binding of antisera to the peptide were performed using the ELISAmate kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The peptide (100 μg/ml of binding solution) was bound to the wells of microtiter plates, incubated sequentially with serial dilutions of the primary antiserum, peroxidase-labeled protein A, and the peroxidase substrate solutions supplied by the manufacturer.

Antibodies not Directed against Heart Gap Junctions

Besides preimmune serum, we also used a site-specific antiserum against the synthetic peptide corresponding to the amino acid sequence 234-245 of rat lens MIP (Gorin et al., 1984) and an affinity purified site-specific antibody against a synthetic peptide representing the 14 NH_2-terminal amino acids of the beta 1 subunit of a G protein from HL-60 myeloid cell membranes (Amatruda et al., 1988). By Western blot assay, the latter antibody reacts with high specificity with G protein in rat cardiac plasma membranes. Strong signals were obtained in Western blots with a 1:5,000 dilution of the antiserum and a 1:1,000 dilution of the affinity purified antibody.

Immunofluorescence and Immunogold Labeling with Silver Enhancement

Animals, similar to those used for isolation of gap junctions, were anesthetized with ether and perfused through the left ventricle with PBS. Papillary muscle and small pieces of tissue from atrial and ventricular muscle and from liver were frozen in Freon 22 at its melting temperature and then stored in liquid nitrogen. Cryosections (~10 μm thick) were fixed in absolute ethanol for 30 min at −20°C and then processed at room temperature for immunofluorescence or immunogold labeling with silver enhancement.

For immunofluorescence, the sections were washed in PBS, treated with 1% BSA in PBS for 20 min, and then incubated for 1 h with antisera or affinity purified antibody in PBS with 1% BSA (antisera at a dilution of 1:100, affinity-purified antibody at 80–100 μg/ml for the anti-43-kD antibody or 40 μg/ml for the anti-amino terminus antibody). For controls, preimmune sera were used at a dilution of 1:50 or sections were reacted with second antibody alone. After washes in PBS containing 1% BSA and 0.1% Tween 20, the sections were incubated with fluorescein-conjugated goat anti-rabbit IgG (Gibco Laboratories, Grand Island, NY) at a dilution of 1:500. Sections were photographed by epifluorescence on a Zeiss microscope equipped with the appropriate filters.

For immunogold labeling with silver enhancement, sections of longitudinally oriented papillary muscle were processed according to the protocol recommended by Boehringer Mannheim Biochemicals (Indianapolis, IN). The sections were blocked with 5% normal goat serum and 0.1% BSA in TBS, and then incubated sequentially with affinity-purified anti-43-kD antibody diluted to 20 μg/ml in 1% normal goat serum, 0.1% BSA in TBS, and 5 nm colloidal gold-labeled goat anti-rabbit IgG (Boehringer Mannheim Biochemicals) diluted 1/40 in 0.4% gelatin, 0.1% BSA in TBS. After washes in PBS, the sections were postfixed in 2% glutaraldehyde in PBS for 15 min, washed in PBS and then in distilled water. To make the gold visible in the light microscope, metallic silver was deposited on the gold particles according to the protocol supplied with the silver enhancement kit from Boehringer Mannheim Biochemicals.

Immunogold EM

All reactions were done at room temperature in microfuge tubes with frequent mixing to resuspend the membranes that otherwise tended to pellet. About 25 μg of a crude plasma membrane fraction or 15 μg of a gap junction enriched fraction were suspended in 200 μl of 5% BSA in PBS for a 15 min block before addition of the antisera. Either antiserum or preimmune serum was then added to give a final dilution of 1:20, 1:50, or 1:100 and then incubated for 90 min. Membranes were pelleted at 15,000 rpm in a microfuge (Wheaton Instruments, Millville, NJ), washed with PBS, and then incubated for 90 min in 5 nm colloidal gold-labeled goat anti-rabbit IgG, diluted 1:10, 1:20, or 1:40 in 5% BSA in PBS. After thorough washes in PBS, the pellets were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, postfixed in 1% osmium tetroxide, en bloc stained with uranyl acetate, dehydrated, and embedded in Epon. Thin sections were stained with lead citrate and uranyl acetate and examined on an electron microscope (model 420; Philips Electronic Instruments, Inc., Mahwah, NJ).

Proteolysis of Isolated Junctions

Protease digestions were carried out on isolated junctions in suspension at 37°C. The proteases endoproteinase Lys-C, endoproteinase Glu-C, and L-1-*p*-tosylamino-2-phenylethyl chloromethyl (TPCK) trypsin (Boehringer Mannheim Biochemicals) were all used in 0.1 M phosphate buffer, pH 8. The total incubation volume was 30 μl. For controls, parallel incubations were carried out on isolated junctions treated with buffer alone. For digestions to go to completions, proteases were added at time 0, a second addition was made after 2 h, and the incubations were continued overnight (total time 16 h). The final enzyme to substrate ratio was 1:20–1:10. For partial proteolysis, the reaction was stopped 30 min after adding the protease.

Suspensions of junctions in buffer containing protease were spun at 15,000 *g* for 10 min. The supernatant was removed and the junction pellet washed by resuspending in 0.1 M phosphate buffer and spinning a second time. To inactivate any remaining proteases, the junctional pellet was treated with 10 μl of 0.01 M HCl in water before solubilization of the junctions in 4 × solubilization buffer (8% SDS, 20% mercaptoethanol, 0.125 M Tris-HCl, pH 6.8). Water was then added to the solubilized junctions to bring the concentration down to the equivalent of 1 × solubilization buffer for analysis of the junctions by SDS-PAGE.

Functional Studies

Tissue Culture. Neonatal rat heart muscle cells were cultured by a procedure modified from Griepp and Bernfield (1978). Briefly, hearts of 1-d-old rats were dissected, minced, and incubated at 37°C for 10 min in a Ca- and Mg-free Earle's balanced salt solution. The minced tissue was dissociated by repeated treatment with 0.5% trypsin (hog pancreas; ICN Biomedicals, Cleveland, OH) in Ca- and Mg-free Earle's balanced salt solution, and mechanical stirring. The dissociated cells were filtered through 43-μm Nytex mesh to remove undissociated pieces. Cells were maintained in medium 199 (Gibco Laboratories; with 25 mM Hepes, Earl's salts, and L-glutamate) to which K-free Earle's balanced salt solution, antibiotics, FCS, and horse sera were added. The pH of the medium was maintained at 7.4 with 15 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonate-Hepes buffer. The medium also contained 10 μM cytosine-D arabinofuranoside (Sigma Chemical Co., St. Louis, MO) to inhibit the growth of fibroblasts in the muscle cell cultures. Most of the fibroblasts from the original cell suspension were removed by first plating on glass dishes. Myocytes, but not fibroblasts, were detached by gentle shaking, 1.5 h after initial plating. Fractions enriched in myocytes were then replated at low density. Cells were maintained in an incubator in humidified air at 37°C. After 36–48 h, cells were observed to beat rhythmically. Myocytes kept in culture for 4–6 d were used in the present study. During an experimental period, the medium was changed every 2–3 h. The pH of the medium was checked regularly and maintained at 7.4 with TES-Hepes buffer.

Intracellular Injection of Dyes and Antibodies. The dyes used for labeling in our experiments were: Lucifer yellow Ch (LY) (457 mol wt; Aldrich Chemical Co., Milwaukee, WI), Texas Red (TR) (625 mol wt; Molecular Probes Inc., Junction City, OR), and Rhodamine-labeled dextran (molecular weight: 10,000; Molecular Probes Inc.). All dyes were dissolved in 0.15 M lithium chloride (LiCl) buffered to pH 7.6 with 10 mM Hepes. The concentration (wt/vol) of these solutions were the following: 5% for LY, 0.2% for TR, and 30% for rhodamine-labeled dextran. TR and LY were selected as tracers because of their distinct and nonoverlapping excitation and emission peaks: they could be detected separately using appropriate filters.

For dye coupling experiments, aliquots of the antibodies were freshly thawed and filtered using Centrex disposable microfilter units (0.2 μm pore nylon mesh; Schleicher & Schuell, Keene, NH). The supernatant was diluted 1:4 in filtered (0.22 μm filter) solutions of 0.15 M LiCl buffered to pH 7.6 with 10 mM Hepes. All of the antibodies used reacted well with their specific antigens at roughly the same concentration. Dilutions of the various antibodies that gave strong signals in Western blots were 1:5,000 for the antiserum and 1:1,000 for the affinity purified antibody to the G protein, 1:5,000 for the antiserum to the amino terminus of the 43-kD gap junction protein, and 1:1,000 for the antiserum to the electroeluted 43-kD protein.

For intracellular injections, microelectrodes were drawn from Omega Dot borosilicate tube (outer diameter 1.2 mm; A-M Systems Inc., Everett, WA). The electrodes were connected to electrometers containing active bridge circuits (8800 total clamp; DAGAN Corp., Minneapolis, MN) and usually had a resistance of 25–50 megohms when filled with 0.15 M LiCl. For most studies, isolated pairs of cells were selected that had spontaneous rhythmic synchronous contractions. Occasionally, somewhat larger groups of cells were used. Action potentials and resting membrane potentials were monitored. Only those cells that showed a sizeable resting potential (−50 to −70 mV) were used in our studies. Spontaneous action potentials were frequently observed. The electrode impalements were accompanied by a large shift in the membrane potential. Each dye or antibody was injected through separate microelectrodes. Dyes were injected by passing 3–10 nA hyperpolarizing (negative) current pulses at 1 Hz for 20–30 s or ~5 nA negative DC currents for 2–5 min. Occasionally, dyes would diffuse passively (probably when an electrode tip diameter was large). Antibodies were injected by passing 20–30 nA negative current pulse at 1 Hz for 30–40 s or ~80–100 nA negative DC current for 2–5 s. These parameters for antibody injections are comparable to those used by other investigators (Hertzberg et al., 1985). Under these conditions, there was no detectable change in the injected cell's volume. Dye injection and spread were monitored on a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) with 75 W xenon arc lamp illumination using epifluorescence optics with appropriate excitation and barrier filters (LY: exciter filter, 485 nm, barrier filter, 520 nm, and an additional kp 560 filter; TR: exciter filter, 540 nm, barrier filter, 590 nm). Optical images were stored on videotapes through a silicon intensified tube camera (RCA, Lancaster, PA) for further analysis. The extent of dye spread was recorded on ultrafast color films (sr-v 3200; Konika USA Inc., Englewood Cliffs, NJ) using a camera (UFX-II; Nikon Inc., Garden City, NY), 3–5 min after the injection of dyes. Phase optics were also available on the same microscope.

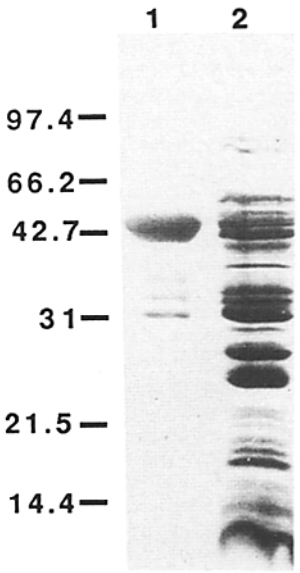


Figure 1. Coomassie-stained SDS polyacrylamide gel of gap junction fractions. The position of the low molecular weight standards (Bio-Rad Laboratories) are indicated on the left with molecular weights in thousands. Lane *1*, a highly purified gap junction fraction typical of those obtained with the scaled-up protocol. The fraction contains a major component migrating at about 43 kD. In this overloaded lane, one also sees bands at 35, 33, and 31 kD that have previously been shown to represent products of the proteolysis of the 43-kD protein during isolation. Only minor contaminants can be detected. Lane *2*, a partially purified gap junction preparation such as used for testing the specificity of the antibodies.

Results

Both Antibodies Bind Specifically to the 43-kD Protein and the Major Products of Its Proteolysis

The purity of the protein we obtain is usually good, showing only a single major band at 43 kD even in overloaded gels (Fig. 1). When testing the specificity of the antibodies on Western blots, however, it is advantageous to use partially purified preparations (Fig. 1).

The antipeptide antiserum was raised against a synthetic peptide representing the amino terminal 1–20 residues of the protein sequence; by ELISA, this antiserum binds with high titer to the peptide (data not shown). Both the antipeptide antiserum and affinity purified antibody derived from it (Fig. 2) bind to bands at M_r 43, 35, 33, and 31 kD, which represent the native protein and its three major breakdown products (Manjunath et al., 1984). Proteolytic cleavage during isolation occurs at the carboxy terminus and all products seen by SDS-PAGE retain the amino terminus of the parent molecule (Manjunath et al., 1987).

Blots probed with the polyclonal antiserum raised to the electroeluted 43-kD protein or the affinity purified anti–43-kD antibody reveal staining patterns (Fig. 2) identical to those seen with the antipeptide antibody. Thus, at least one of the epitopes recognized by the anti–43-kD antibody is part of the smallest breakdown product (31 kD), which still contains the amino terminus of the molecule. The anti–43-kD antiserum does not bind to the amino terminal peptide as assayed by ELISA (data not shown).

Immunolocalization (I)

Immunoreactive Protein Is Found in both Ventricle and Atrium. Sections through frozen, ethanol-fixed tissue were incubated separately with each antibody. In theory, all regions of the antigen molecule are rendered accessible by the sectioning process. In atrium and ventricle (papillary muscle,

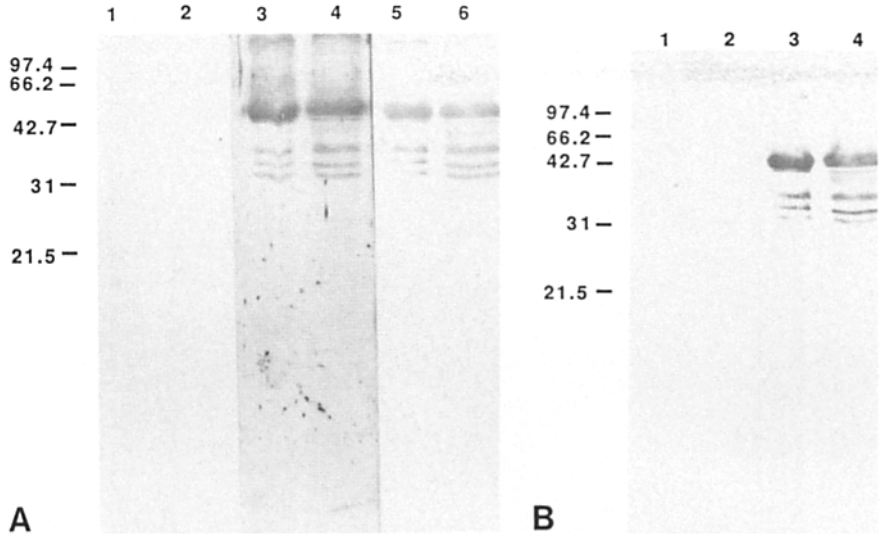


Figure 2. Immunoblots of gap junction preparations. Components of the gap junction fractions were separated by electrophoresis as in Fig. 1 and blotted to nitrocellulose. Highly purified fractions corresponding to Fig. 1 (lane *1*) were run in lanes *1*, *3*, and *5*. Partially purified gap junctions corresponding to Fig. 1 (lane *2*) were run in lanes *2*, *4*, and *6*. (*A*) Blot probed with preimmune serum (lanes *1* and *2*) and the anti-43-kD antiserum before (lanes *3* and *4*) and after affinity purification (lanes *5* and *6*). (*B*) Blot probed with preimmune serum (lanes *1* and *2*) and the anti-amino terminus antiserum (lanes *3* and *4*). The affinity-purified antibody gives the same results (not shown). No binding is seen with either preimmune serum. The pattern of labeling is the same regardless of which antiserum or affinity-purified antibody is used. All recognize the 43-kD polypeptide and the major products of its proteolysis at 35, 33, and 31 kD. In addition, all detect a sharp band migrating just below the 43-kD protein. This band is not always resolved, and we assume that it represents an intermediate stage of proteolysis.

septum, or wall), the label is distributed in the pattern consistent with the known distribution of gap junctions. This is most clearly seen in sections of papillary muscle (Fig. 3 *A* and *B*) in longitudinally sectioned muscle bundles. Intercalated disks form the transverse boundaries between cardiac myocytes and are the sites where gap junctions are predominantly localized (Page and McCallister, 1973; Page, 1978; Sommer and Johnson, 1979). Unfortunately, in sections of rat heart, the disks are not clearly defined under phase-contrast, differential interference contrast optics, nor after staining with iron-hematoxylin. The very distinct labeling pattern obtained with both antibodies nevertheless strongly suggests that it is the disks that are delineated. No reactive protein is seen in sections treated with preimmune serum (Fig. 3 *C*), nor with second antibody alone (data not shown).

Both affinity purified antibodies also bind to protein present in sections taken from the atrium (data not shown). The pattern of distribution is more complex than in ventricle since by examination of atrial muscle in the electron microscope the gap junctions are found both at the level of the intercalated disk and in areas of the sarcolemma where the cells are laterally apposed (Page, 1978). No immunoreactive protein was detected with either the anti-amino terminus or the anti-43-kD antisera when staining of liver cryosections was attempted (data not shown).

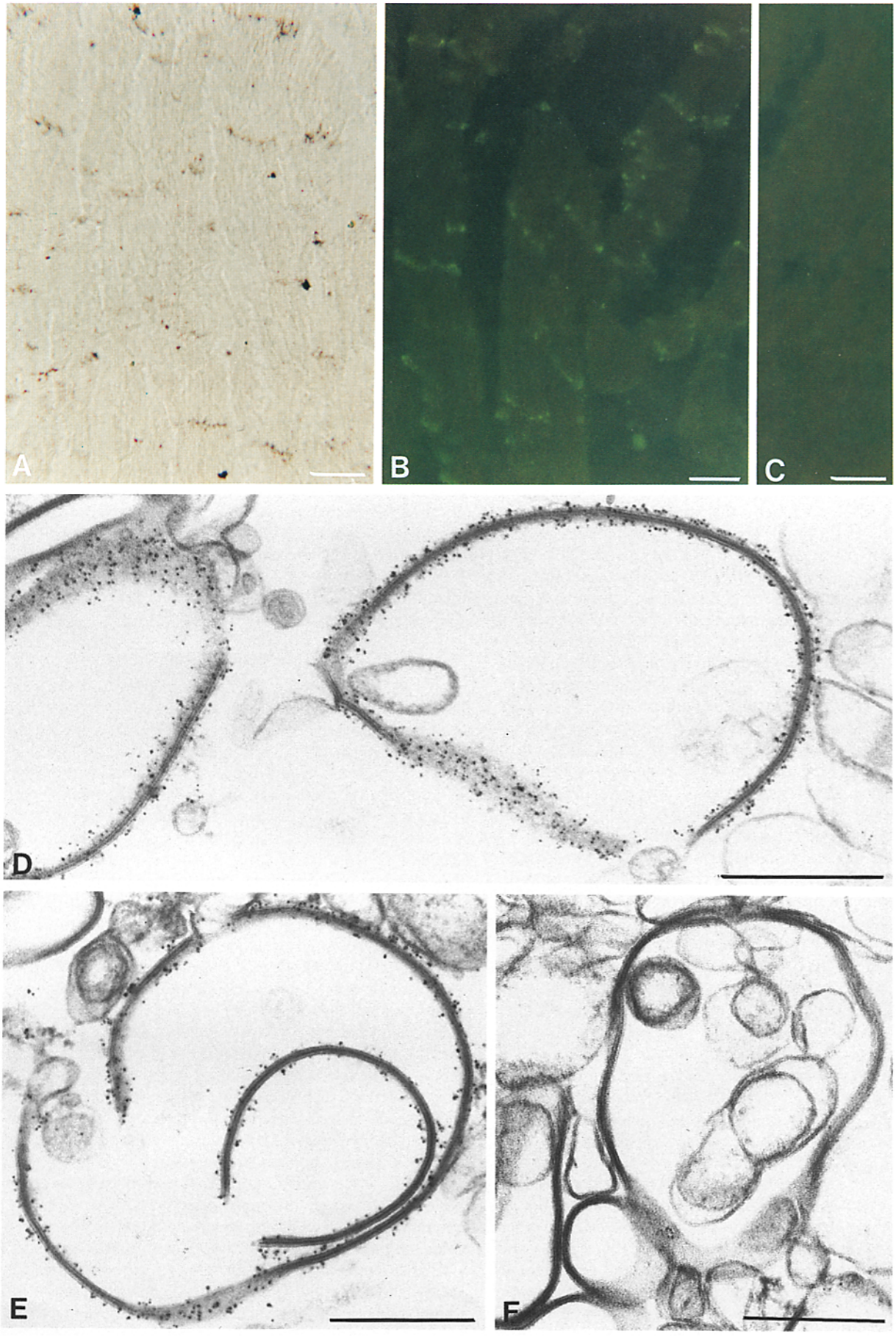
Both Antibodies Bind to Cytoplasmic Domains of the Gap Junction Protein. Crude plasma membrane and gap junction enriched fractions (which show contaminating proteins by SDS-PAGE, see above) were reacted with antisera under conditions in which the gap junctions were intact, so that only regions of the protein exposed at the cytoplasmic faces of the junction would be accessible to antibody. In thin sections of such preparations, the pentalaminar profiles of gap junctions makes them readily distinguishable from other membranous structures. In fractions reacted with either antiserum as the primary antibody and then with gold labeled second antibody, only the gap junctions are specifically labeled (Fig. 3, *D* and *E*). The few gold particles elsewhere in the sections are distributed in no definite pattern, as is the case in sections reacted with the preimmune serum (Fig. 3 *F*). No differences are discernable in the distribution of antigen detected by the anti-amino terminus antiserum and that detected by the anti-43-kD antiserum. In either case, the amount of label varies from junction to junction and within different regions of the same junction.

Topology of the Heart Junction Protein (II)

To characterize further the cytoplasmic domains of the heart junction protein, we have used proteolytic enzymes believed to be too large to enter either the 2-nm gap separating the membranes or the aqueous channels formed by the connexons (Goodenough and Revel, 1971). The junctions remain intact after proteolysis and can be sedimented by centrifugation: the proteases and fragments of the polypeptide released by cleavage are not sedimented and are removed by decantation. Only those parts of the polypeptide protected from cleavage by association with the membrane are recovered and detected by SDS-PAGE and immunoblotting.

Proteolytic cleavage of isolated gap junctions with TPCK trypsin produces small fragments migrating at M_r 14.5 and 15–17 kD (Fig. 4 *A*). Use of endoproteinase Glu-C under conditions in which the enzyme cleaves at the carboxy side of both glutamate and aspartate residues produces a similar digestion profile but the components recovered are larger. There is a well-resolved fragment of M_r 15.1 kD and a more diffuse band at M_r 17–19 kD (Fig. 4 *A*). Use of endoproteinase Lys-C, which cleaves the polypeptide chain on the carboxy side of lysine residues, produces two major membrane-embedded fragments seen as a well-resolved band of M_r 15.6 kD and a second broader band of M_r 19–23 kD (Fig. 4 *A*). These results indicate the presence of two membrane-protected domains, represented by the two major proteolytic fragments, which are separated by an enzyme accessible cytoplasmic region, forming a cytoplasmic loop.

To determine the position of these cleavage sites, isolated junctions were treated with proteases as described above and immunoblots of the resulting SDS-PAGE gels were probed with the antiserum raised against the amino terminal se-



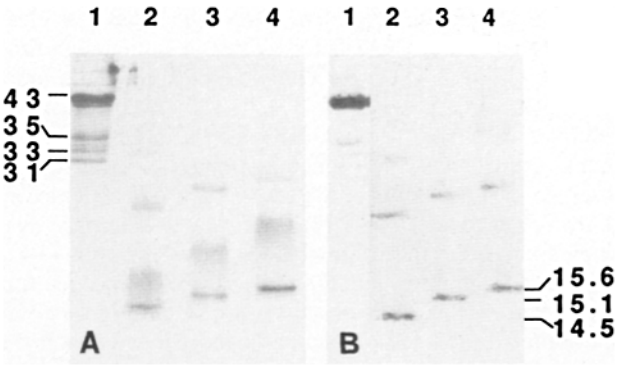


Figure 4. Analysis of proteolysis degradation products of heart junctions. (*A*) Coomassie-stained gel of heart gap junction fractions (15% acrylamide). The control (lane *1*) shows the prominent band at 43 kD that represents the parent molecule and the three easily formed breakdown products (35, 33, and 31 kD). In lane *2*, the junctions were treated with TPCK trypsin; in lane *3*, with endoproteinase Glu-C; and in lane *4*, with endoproteinase Lys-C. All three digests show a similar pattern: two membrane-protected domains appearing as a lower, well-resolved band (14.5, 15.5, and 15.6 kD) and a less well-resolved upper band (16–23 kD). The faint bands seen in a ladder are believed to be aggregation products (see text and Fig. 5). (*B*) Western blots of heart gap junction fractions after proteolysis, using the anti-amino terminal antiserum. Gap junctions were treated with the same endoproteases as in *A*. Lane *1* shows a control; the other lanes show the result of treatment with TPCK trypsin (lane *2*), endoproteinase Glu-C (lane *3*), and endoproteinase Lys-C (lane *4*). The immunoblot shows that the lower, well-resolved (membrane-protected) fragment is stained in each case; i.e., carries the amino terminal sequence. The ladder of bands seen in the Coomassie-stained gel (Fig. 4 *A*) is also revealed by the site-directed antiserum. Therefore, all these bands carry the amino terminus and are probably aggregation products.

quence. The lower, well-resolved band in each of the digests reacted positively while the upper band showed no reactivity (Fig. 4 *B*). Therefore, only the 15.6-, 15.1-, and 14.5-kD bands contain the amino terminal domain.

Since the anti-amino terminus antiserum could still bind after exhaustive digestion, it was possible to investigate the size of the cytoplasmic loop by examining the products of partial enzymatic digestion. By sizing the fragments that reacted with the antibody on immunoblots (Fig. 5), one obtains a nested series of fragments retaining the amino terminus, the smallest having an M_r of 14.5 kD (trypsin digestion) and the largest having an M_r of 18.6 kD (endoproteinase Lys-C digestion). The difference between the smallest and largest fragments yields a minimum size for the cytoplasmic loop of ~4.1-kD.

The anti-amino terminus antiserum also binds to some higher molecular weight species (Fig. 4 *B*). Presumably, these are aggregation products, dimers, and trimers of the amino terminus bearing peptides. Aggregation is a property commonly found in integral membrane proteins (Findlay, 1987), and has been repeatedly observed in the case of gap junction proteins (Henderson et al., 1979; Finbow et al., 1980). The removal of polar residues from the 43-kD protein may exacerbate this problem by increasing the chance of hydrophobic interactions.

Antibody Binding Sites

Two different antisera have been used in these studies. The first is an antiserum raised against the amino terminus, and hence its binding site is predetermined; the second is an antiserum raised against electroeluted 43-kD protein. A binding site of the affinity purified anti-43-kD antibody is detectable between two proteolytic cleavage sites. The lower band of M_r 15.1 kD seen after digestion with endoproteinase Glu-C, reacted positively on immunoblots. However, the lower band of M_r 14.5, produced by TPCK trypsin digestion, failed to bind this antibody. In addition, none of the upper, diffuse bands (15–17 kD) were detected by this antibody (Fig. 6).

Thus, an antibody binding site, or a portion of a discontinuous binding site, is present in the cytoplasmic loop, localized within an 0.6-kD region, ~15 kD from the amino terminus. There is also evidence (our unpublished observations) for at least one additional epitope on the carboxy terminal tail of the molecule.

Functional Studies (III)

Experimental Protocol. We used a two dye protocol to test junction permeability because preliminary studies showed that if only one dye were used, as is usually done (Warner et al., 1984; Hertzberg et al., 1985), false positives (no transfer) could result after antibody injection. We therefore first injected a tracer dye (LY or TR) to insure that the chosen cells were coupled before injection of the antibody. Only those cell pairs which showed uniform and complete dye spread were selected for further studies. To test whether these two dyes (LY, TR) would both label each of the cells

Figure 3. Immunolocalization of the 43-kD protein. (*A*) Indirect immunogold-silver stained section of longitudinally oriented papillary muscle reacted with affinity-purified anti-43-kD antibody. (*B* and *C*) Indirect immunofluorescence views of similar sections reacted with (*B*) affinity-purified anti-amino terminus antibody, and (*C*) preimmune serum (from anti-43-kD rabbit). No binding is seen with the preimmune serum. The distribution of the immunoreactive protein detected by either antibody is consistent with the known location of most gap junctions in the region of the intercalated disk, the site where myocytes interact end-to-end. At the level of resolution obtained, one cannot distinguish "end" from "lateral" junctions that occur at the level of the disk. (*D*, *E*, and *F*) Immunogold labeling of 43-kD protein in intact gap junctions. Thin sections of partially purified gap junction fractions reacted with (*D*) anti-43-kD antiserum; (*E*) anti-amino terminus serum; and (*F*) preimmune serum (from rabbit immunized with amino terminal peptide). Binding of the primary antibody is detected with colloidal gold-labeled second antibody. In preparations probed with either of the antisera (*D* and *E*), the immunogold labeling is restricted to the gap junctions that are easily distinguishable by their pentalaminar profile. The density of label is variable from junction to junction and occasional profiles of gap junctions show no label. Single membrane structures (i.e., nonjunctional membranes) are not labeled. No components in the preparations are labeled when preimmune serum is used as primary antibody. Bars, (*A*, *B*, and *C*) 50 μm; (*D*, *E*, and *F*) 0.3 μm.

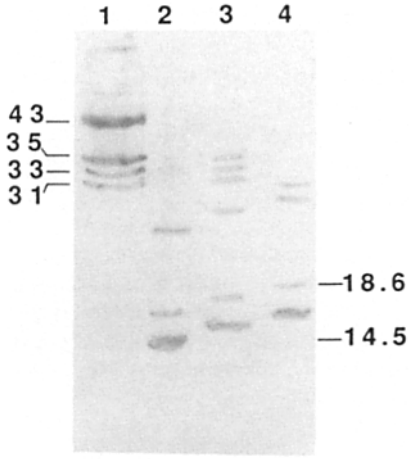


Figure 5. Immunoblot analysis of partial proteolytic degradation products of heart gap junctions using the amino terminal antiserum. Isolated gap junctions were treated with the same endoproteinases as in Fig. 4. However, the reaction was stopped after 30 min to obtain partial proteolysis only. The smallest fragment produced had an M_r of 14.5 kD after trypsin digestion (lane *2*), and the largest fragment recovered (18.6 kD) was formed after endoproteinase Lys-C digestion (lane *4*). One can thus estimate the size of the cytoplasmic loop to be ~4.1 kD.

of individual pairs, we injected the same cell or each of the two cells of a pair with these dyes using separate microelectrodes. In over 50 cell pairs tested, if the first dye transferred, the second one did also. In only five cases the first dye transferred, but not the second. In all of these cases, small cell clumps rather than pairs were used, and it is possible that the electrodes were inserted in cells other than those aimed for, because of the presence of undetected overlapping processes. The standard procedure was to inject one dye (e.g., LY) into one cell of a pair (or small cluster) to assay for normal dye coupling. Antibody was then injected in either the same cell or one to which the first dye had spread. After a delay of 5–7 min, the other dye (e.g., TR) was injected into either cell of the pair, using another microelectrode. Observing the transfer (or lack thereof) of the second dye permitted one to assess the effectiveness of the antibody injected. For an experiment to be scored as positive, the second dye should not enter (or leave), the cell that had received the antibody.

Both Anti–Gap Junction Antibodies Block Dye Transfer

Dye coupling between isolated cardiac muscle cells was blocked by site-specific antibody directed against the amino terminal sequence of the heart junction protein (Fig. 7, *A–C*). The pattern of injection with the second dye and antibody was changed in different experiments without changing the result. Blockage of second dye transfer was observed in 27 dye-coupled cell pairs after antibody injection (Table I). In three other coupled cell pairs, the effect of antibody on dye transfer was uninterpretable, either because of incomplete filling of the cell injected with the second dye (transfer might not have been detectable even if it had taken place) or because there was only weak transfer of the second dye. Such instances of partial blockage of dye transfer were rare but were considered uninterpretable because it was not clear whether the antibody had failed to act, or not enough time had been given to allow for complete closure of the junctional channels.

Similar results were obtained with the antibody raised against electroeluted gap junction protein (Fig. 7, *D–F*). Blockage of the transfer of the second dye was observed in 24 dye-coupled cell pairs (Table I). In another six dye-coupled pairs, the intracellular injection of the anti–43-kD antiserum did not prevent second dye transfer. In three further instances, the effect of antiserum on dye transfer was uninterpretable (for reasons similar to those mentioned in the previous paragraph). In either case, there was blockage of dye transfer by these antibodies whether or not the antibodies and second dye were injected in same cells or in different cells of a pair.

Only Gap Junction Specific Antibodies Block Dye Transfer

Preimmune Serum. In one set of control experiments, preimmune serum against the site-specific polypeptide was injected in one cell of a dye-coupled cell pair (Fig. 8, *A–C*). No effect of preimmune serum on transfer of the second dye was observed in 22 coupled cell pairs. In another three coupled cell pairs, the effect of preimmune serum was uninterpretable (for reasons similar to those mentioned above).

Antibodies against Other Membrane Proteins. We tested whether inhibition of dye coupling observed in our study was because of the presence of antibodies specific to the cardiac gap junction protein. For this set of experiments, we selected antipeptide antibody against rat lens MIP and one against cardiac G protein. MIP has no amino acid sequence homology with heart gap junction protein (Gorin et al., 1984; Beyer et al., 1987), and the antibody against it does not cross react with the heart junction protein (our unpublished observations). This antibody should therefore serve as a control similar to preimmune serum. We did not use an antibody against the 32-kD liver gap junction protein because this protein has partial sequence homology with heart junction protein (Nicholson et al., 1985), and at least one antibody to the

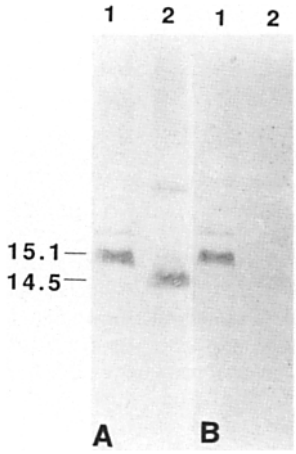


Figure 6. Immunoblot analysis showing an antibody binding site of the anti–43-kD antiserum. Blots of the products of digestion with endoproteinase Glu-C are seen in lane *1*, and the pattern of antibody binding to products of TPCK trypsin treatment is displayed in lane *2*. Immunoblot *A* was probed with the anti–amino terminal antiserum, and immunoblot *B* was probed with the affinity purified anti–43-kD antibody. The lower band (M_r 14.5 k) is not detected in *B*, indicating that the binding site for this antibody is between 14.5 and 15.1 kD. In *A* (lane *2*) the upper band represents a dimer of the M_r 14.5 kD fragment.

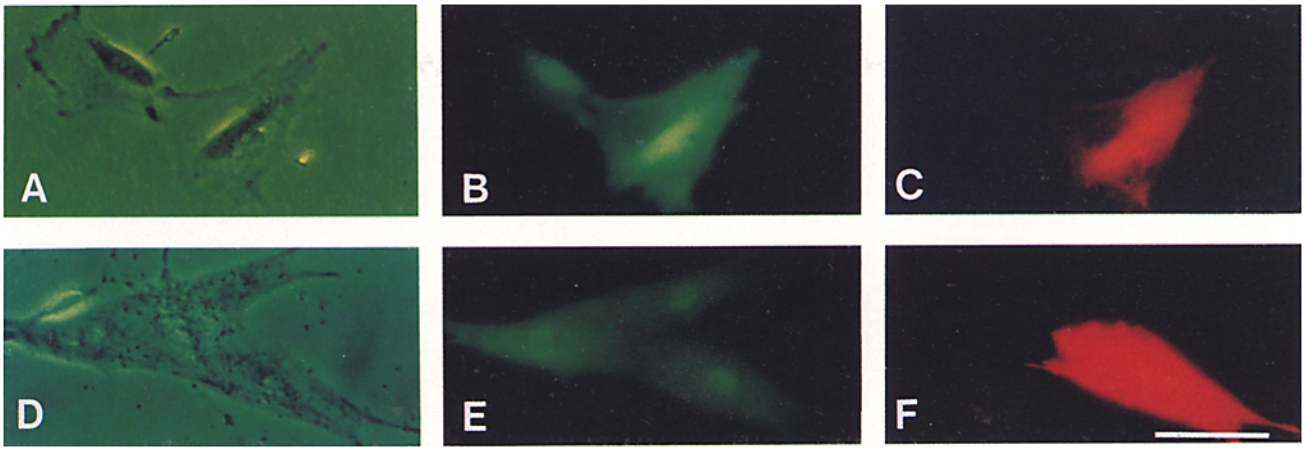


Figure 7. Antibodies specific to 45-kD heart gap junction protein block dye transfer. The left column (*A* and *D*) consists of phase-contrast photomicrographs of isolated pairs/clusters of cardiac muscle cells. The middle column (*B* and *E*) consists of photomicrographs of LY-labeled cells corresponding to those in the left column, respectively. Single cells in *B* and *E* were injected with LY. Subsequent transfer of LY to other cell(s) in pairs/clusters indicates normal dye coupling. The right column (*C* and *F*) shows the effect of antibodies on the transfer of the second dye (TR). TR was injected 5–7 min after injections of antibodies. (*A*, *B*, and *C*) Antibody against a synthetic peptide corresponding to the first 20 amino acids of the 45-kD cardiac protein was injected into the bottom cell, and TR was then injected into the same cell (*A*). Note that the antibody prevented the transfer of TR to the other cell of the coupled pair (compare *B* to *C*). (*D*, *E*, and *F*) Antibody raised against electroeluted cardiac gap junction protein was injected into the left cell (*D*). Subsequent injection of TR into the lower right cell (*D*) was localized to that cell only. The blockage of dye transfer to other coupled cells (compare *E* to *F*) by the antibody also suggests that the upper right cell was not directly coupled to the lower right cell but through the left cell. Bar, 50 μm.

32-kD protein has already been shown to affect dye coupling in adult rat cardiac myocytes (Hertzberg et al., 1985). Fig. 8 (*D–F*) shows that antibody against MIP has no effect on transfer of the second dye. A similar observation was also made for another 20 dye-coupled cell pairs. In three dye-coupled cell pairs, the second dye transfer appeared blocked (Table I).

Table I. Number of Cell Pairs Tested for the Effect of Various Antibodies and/or Reagents on Transfer of a Second Dye (TR or LY)

Antibodies/ Reagents	Second dye		
	Transfer	No transfer	Uninterpretable
Site-specific antipeptide antibody	0	27	3
Antibody to electroeluted 43-kD band	6	24	3
Preimmune serum	22	0	3
Site-specific anti-MIP antibody	21	3	0
Site-specific anti-G protein antibody	8	1	0
Dextran	0	21	0

The sequence of injection was dye 1, antibody, dye 2. Which dye was used first, or what cell was injected was random. The cell pairs listed in the uninterpretable category are those that had incomplete filling of the donor cell with the second dye or a weak transfer of it to the recipient cell(s).

We also tested whether the blockage of dye transfer observed in our study was due to the effect of antibodies on the gap junction channel itself or could be an indirect result of antibody binding to other membrane proteins. In these experiments, we used an antibody that recognizes a G protein present in rat cardiac plasma membrane. Intracellular injection of this antibody had no effect on the transfer of second dye (Fig. 8, *G–I*) in eight coupled cell pairs. In only one instance, the transfer of the second dye was inhibited by injection of the antibody against G protein.

Dye Exchange Is Via Structures of Restricted Permeability. The dye transfer observed was not due to the presence of cytoplasmic bridges that might be present in the case of dividing cells. This was tested by using rhodamine-labeled dextran (10,000) which is too large to pass through gap junction channels. Typically, when labeled dextran was injected into one cell of an LY injected coupled cell pair it remained in the injected cell. A similar result was obtained in 21 coupled cell pairs studied (Table I).

Time Course of Antibody Action

When dye and antibody were coinjected there was always at least a partial transfer of dye. We chose to wait 5–7 min between antibody injection and administration of the second dye because this schedule permitted complete blockage with the appropriate antibodies. Partial transfer resulted if this delay was reduced. Presumably, time is needed for the antibody injection to have an effect, either because any needed conformational changes take place slowly or because the reagents diffuse to the site of action only slowly.

Discussion

Taken together, the results of our study illustrate the follow-

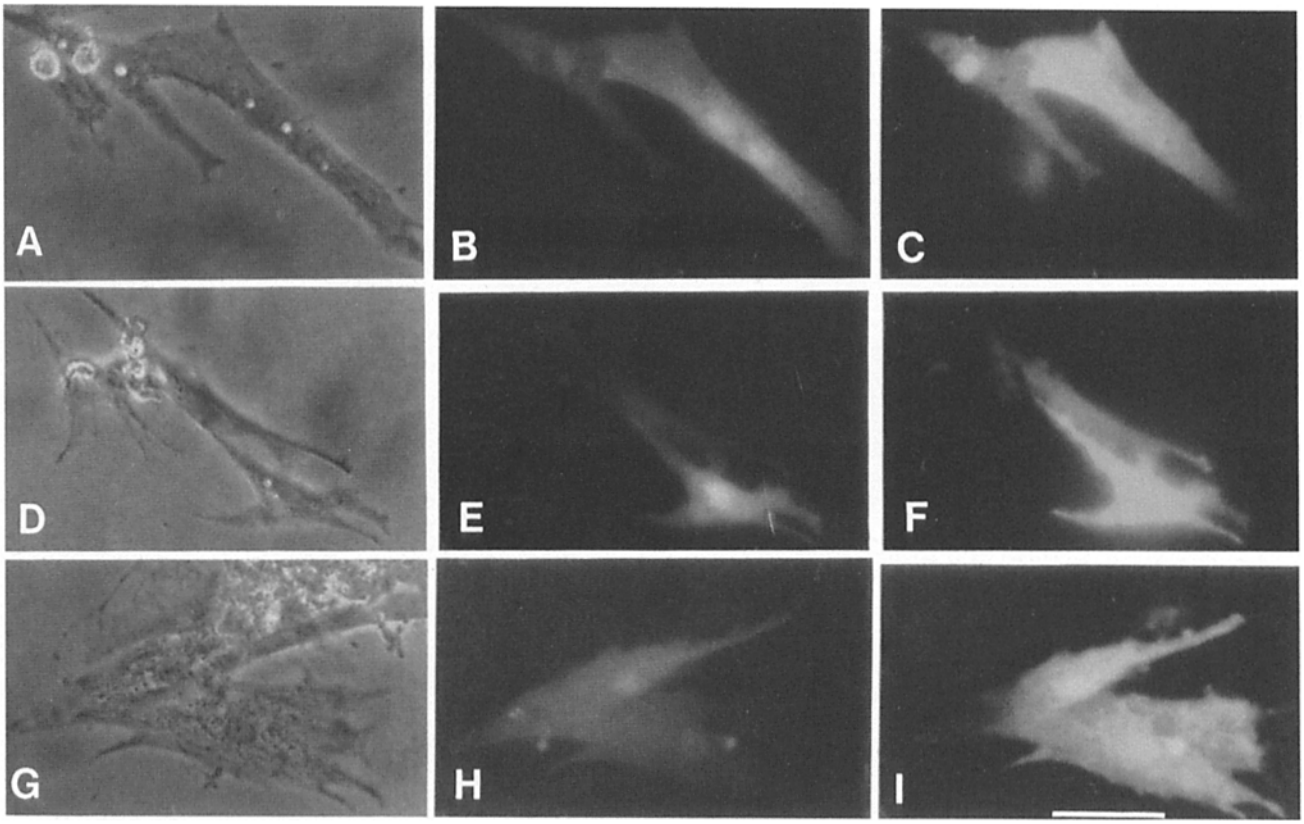


Figure 8. Control antibodies do not block dye transfer. Phase-contrast and fluorescence photomicrographs are arranged as in Fig. 1. (*A*, *B*, and *C*) Preimmune serum was injected into the left cell (*A*). Subsequent injection of TR into the right cell, which has just received the preimmune serum, was transferred to the other cell of the coupled pair (compare *B* to *C*). A fluorescent spot on the lower left is due to uptake of TR by a floating debris. (*D*, *E*, and *F*) Anti-peptide antibody against rat lens MIP was injected into the bottom left cell (*D*). TR was subsequently injected into the same cell. Note that the antibody did not prevent the transfer of TR to the other cell of the pair that had been injected with the antibody (compare *E* to *F*). (*G*, *H*, and *I*) Site-specific antibody to the amino terminus of the beta 1 subunit of G protein was injected into the bottom cell (*G*). This antibody did not prevent the transfer of TR (injected into the top cell) to the bottom cell that had just received the antibody (compare *H* to *I*). Bar, 50 μm.

ing points: (*a*) the 43-kD protein associated with gap junctions isolated from rat heart is, in fact, assembled in gap junctions in both the atrium and the ventricle; (*b*) the amino terminus of the molecule is exposed on the cytoplasmic side of the bilayer, that is, on the same side of the membrane as the carboxy terminus; (*c*) a polypeptide loop that binds affinity purified anti-43 kD antibody is also exposed to the cytoplasm. The protein, if it is transmembrane, is therefore likely to cross the membrane four times. (*d*) Both the amino terminus, and domains elsewhere on the cytoplasmic face of the junction can control junction permeability in the presence of specific antibodies.

The 43-kD Protein Is Part of Heart Gap Junctions

In immunocytochemical studies of sections of atrium and ventricle, the anti-43-kD and the anti-amino terminus antibodies show the same distinct pattern of distribution of immunoreactive protein consistent with the known distribution of gap junctions in the two tissues. In longitudinally sectioned papillary muscle from the ventricle, the pattern of immunostaining strongly suggests that the 43-kD protein is concentrated at the region of the intercalated disk, the locus of numerous gap junctions. That the protein detected in tissue sections is indeed a component of gap junctions is shown by the specific binding of both antibodies to isolated gap junctions.

Topology of the Heart Gap Junction Protein

Since the anti-amino terminus antibody binds to intact gap junctions, where only cytoplasmic domains of the molecule are accessible for antibody binding, the determinants recognized by this antibody must be exposed at the cytoplasmic face of the junction. The complete amino acid sequence of the 43-kD protein, obtained by cDNA sequencing (Beyer et al., 1987), shows no other regions of the molecule homologous with the sequence of the amino terminal peptide that we used as antigen. It has previously been shown that the protein has a domain of about 17 kD at its carboxy terminus, which is lost by proteolysis during isolation (Manjunath et al., 1987) and must therefore be exposed on the cytoplasmic face of the junction. Since amino and carboxy termini are on the same side of the membrane, the protein, if transmembrane, must be folded in a configuration such that it crosses the membrane an even number of times. One can a priori imagine domains where portions of the polypeptide enter the membrane and then come out on the same side, rather than

crossing the membrane; however, no such regions have yet been proposed in the case of other membrane proteins. Reverse turns usually involve polar residues and occur mostly at the surfaces of proteins (Schultz and Schirmer, 1979). The 43-kD protein is shown to be an integral membrane protein by its solubility characterisitics. It remains membrane bound after exposure to high salt buffer during the isolation protocol and in the presence of 0.33% sarkosyl and 0.1% sodium deoxycholate, but is solubilized by SDS. It can only be degraded partially by exogenous proteases and has an hydrophobicity profile (Kyte and Doolittle, 1982) suggesting the presence of four domains, characteristic of protein transmembrane regions. However, while the circumstantial evidence is strong, we present here no direct experimental evidence for the protein to be transmembrane since we have only studied the distribution of cytoplasmic determinants.

Since the anti–43-kD antiserum also binds to intact gap junctions, at least one of the determinants recognized by this antiserum must be exposed on the cytoplasmic face of the junction. On Western blots, the affinity purified antibody recognizes the 15.1-kD amino terminal fragment produced by endoproteinase Glu-C but not the corresponding fragment seen after digestion with trypsin. Therefore, at least one of the determinants recognized by this antibody must lie on a cleavable loop between two domains that remain unaffected by proteolysis, presumably because they were protected by association with the membrane.

The presence of the three cytoplasmic domains indicates that the heart 43-kD protein could cross the membrane four times (Fig. 9). The hydropathicity plot (Kyte and Doolittle, 1982) based on the sequence shows four strongly hydrophobic regions of appropriate length. Analysis of the sequence using the method of Klein et al. (1985), predicts four transmembrane helices: amino acid residues 20–40, 77–100, 154–173, and 203–234. Similarly, the Rao and Argos (1986) algorithm predicts four transmembrane helices: amino acid residues 19–41, 77–97, 149–184, and 207–233. If the polypeptide completely spans the membrane, presumably as alpha helices ~20 amino acids long, there must be two extracellular domains. The predicted extracellular domains are regions which contain numerous residues that should in theory be cleavable by the proteases used in our experiments. The fact that they are not cleaved supports the idea that these residues are found in domains inaccessible to our reagents, quite possibly the narrow gap between extracellular faces of apposed junctional membranes which is not permeable to proteins (Goodenough and Revel, 1971). The aqueous environment in the gap would be favorable for the putative extracellular loops that are shown by sequence analysis to contain highly conserved charged and other hydrophilic residues. Milks et al. (1988) present data showing the presence of such an extracellular loop in liver junctions, supporting the above arguments by homology.

Characterization of the Cytoplasmic Loop

The size of the cytoplasmic loop has been estimated to be ~4.1 kD. This represents a minimum value that is dependent on the location of accessible cleavage sites. Additional uncertainties about the size of the loop are introduced by the lack of precision of molecular mass estimates based on SDS-PAGE (see Materials and Methods, SDS-PAGE). The exact position of the cytoplasmic loop as determined by our data is therefore not absolutely certain. However, the estimated size of the amino terminus bearing protease resistant domain is such that it would seem reasonable to place the cytoplasmic loop between the second and third predicted transmembrane segments. This region contains one of the most hydrophilic regions of the molecule and is predicted to carry an antibody binding site (Hopp and Woods, 1981), located in the general portion of the sequence where we identify such a site or part of one.

The Amino Terminus

A surprising result of our studies is the apparent accessibility of the amino terminus to antibodies but not to proteolytic enzymes. The possibilities to account for this puzzle include (*a*) the proteolytic enzymes do remove some amino terminal sequence but enough of the sequence remains to allow binding of the anti–amino terminus antiserum; (*b*) the active site of the enzyme cannot sufficiently approach the amino terminus for cleavage while the antigen binding site is present at the end of an arm of the antibody molecule and is therefore less sterically hindered from binding. The definitive explanation will have to wait for conclusive determination of the amino terminal sequence of the molecule after digestion with specific proteases.

How Many Proteins Are Present in Heart Gap Junctions?

Our results indicate that the 43-kD band seen on gels represents protein that is a component of gap junctions in cardiac muscle, but is it the only protein present? An occasional profile of a gap junction in our preparations showed no labeling with the colloidal gold-linked antibody. These junctions could originate in vascular smooth muscle or endothelial cells since we have observed no staining in sections that could be ascribed to these sources. It could also be that the binding sites for the first or second antibody are sometimes physically inaccessible during the incubation because of clumping of the membranes. The overall intensity of labeling was increased when the concentrations of first and second antibody were increased. The results of dye coupling experiments suggest that binding antibodies to the 43-kD protein is essential and sufficient to control transfer of small molecules between cardiac myocytes. We cannot now rule out, however, the possibility that more than one protein is involved in the formation of the gap junctions as has been shown for the liver (Nicholson et al., 1987). Other proteins, however, would have to act cooperatively with the 43-kD polypeptide.

It has been suggested (Hertzberg and Skibbens, 1984; Evans et al., 1988), that the liver 32-kD protein (then called 27 k) is a component of gap junctions in heart. This conclusion has not been supported, however, by the results of Northern blot screening with the cDNA probe for the message that encodes the liver protein (D. Paul, 1986; our unpublished observations). We have also eliminated the possibility that we are detecting the liver protein with our antibodies. Neither of our antibodies cross react with either of the proteins that have been identified with liver gap junctions (our unpublished observations) nor do they immunolabel tissue sections from liver.

A species of mRNA is found in lens fiber cells and in heart that encodes a protein of 43-kD homologous at its amino ter-

minus with the protein we have isolated from heart (Beyer et at., 1988). Our anti-amino terminus antibody, however, does not show cross-reactivity with protein in homogenates of lens fibers (Kadle et al., manuscript in preparation). Given this lack of cross-reactivity and the similarities in distribution of immunoreactive protein we observe with the anti-43-kD and anti-amino terminus antibodies, it is unlikely that we are detecting the 46-kD protein in the heart. Its location remains to be defined.

Comparison of the Topology Proposed for Different Junction Proteins

The analysis of the primary sequence of junctional proteins has suggested that the different molecules that have been recognized share a number of features, in particular that there are four transmembrane segments, separated by two highly conserved extramembrane loops on one side and three domains on the other, including the amino and carboxy termini. A recently published experimental paper by Milks et al. (1988) amplifies the previously published results of several investigators (Hertzberg et al., 1988; Zimmer et al., 1987) and shows that these predicted features are in fact correct in so far as the 32-kD liver protein is concerned. We show here (Fig. 9) that this is the case for the heart 43-kD protein as well. Thus, it appears that different gap junction proteins might well have a similar organization with respect to the membrane.

Effect of Antibodies on Dye Coupling

Our results show that intracellular microinjections of the anti-amino terminus antiserum and the anti-43-kD antiserum both block cell-cell communication as measured by blockage of dye transfer between neonatal cardiac myocytes in culture. On the other hand, preimmune serum, antiserum against a noncardiac putative gap-junction protein (MIP), and affinity purified antibody against a non-gap junction membrane protein (G protein) have little or no effect on dye coupling. These data support the notion that 43-kD protein from cardiac tissue is a functional part of heart gap junctional channels. Since injected antibodies have access only to the cytoplasm, the observed blockage of dye coupling suggests that some of the domains of this protein responsible for gating are exposed to the cytoplasm. The finding that a site-specific antibody raised against the first 20 amino acids of the heart junction protein is effective in interfering with dye transfer, reinforces the idea that the amino terminal portion of the molecule is exposed to cytoplasm as already indicated by the fact that this antibody binds to intact gap junctions. The polyclonal antibody raised to electroeluted junction protein reacts with epitopes located in the cytoplasmic loop and also with some at the carboxy tail (our unpublished observations) but does not recognize the amino terminus of the molecule. Since both antibodies block dye transfer, our results would suggest that there may be several domains of the molecule that are involved in regulating gap junction channel permeability. An alternate view would be that it suffices to interfere with any domain: binding of antibody to any exposed part of the polypeptide produces a sufficient conformational change such that it always blocks transfer. If this were indeed the case, antibodies (at least whole antibodies) would be useless in functional mapping of the gap junction protein. To fully answer this question, it will be necessary to use antibodies directed against several other particular epitopes, as well as other approaches to sort out the various possibilities.

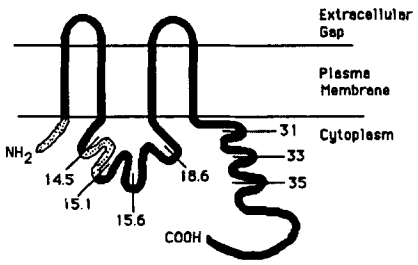


Figure 9. Topology of the heart 43-kD protein. This schematic diagram illustrates the arrangement of the heart 43-kD protein that can be deduced from the data presented. The numbers refer to fragment size in kilodaltons. There are three cytoplasmic and two extracellular domains. Cognate antibody binding sites are shown by stippling.

The lack of blockage of dye coupling in presence of an antibody against G protein further suggests that blockage of dye coupling in cardiac tissue is not because of a generalized effect of antibody binding to membrane. Jaslove and Brink (1987) have discussed potential artefacts that might arise when testing for cell-cell communication by dye coupling. In our experiments, dye transfer cannot reflect the rupture of junctional membranes because it was blocked by specific antibodies only. Blockage of dye transfer by changes in intracellular pH or pCa is unlikely because we should have observed similar blockage in control injections. Finally, the dyes used were not taken up from the extracellular medium. We did test for such a possibility by introducing a large amount of LY and TR dyes in the extracellular bath (data not shown).

Dye Transfer Versus Electrical Coupling

With a few exceptions (Kaczmarek et al., 1979; Muller, 1981; Warner and Lawrence, 1982; Murphy et al., 1983; and Bodmer et al., 1988), electrical coupling is always accompanied by dye coupling, and dye coupling has been a criterion to assess coupling via gap junctions (for review, see Spray and Bennett, 1985). There are a few instances, however where dye coupling but not electrical coupling has been found (Kuhnt et al., 1979; Zieglgansberger and Reiter, 1974). Dye coupling is not a very sensitive measure of cell-cell communication and often a weak electrical coupling is not revealed by dye coupling (Murphy et al., 1983). In fact, we chose to use dye coupling because in these early studies we wanted to make sure that we would be looking only at large, clear-cut effects, rather than examining fine modulations of permeability that might be more difficult to interpret. Since we assayed dye coupling only, it is possible that our experimental protocol might not have been effective in blocking of electrical coupling between cardiac myocytes. Hertzberg et al. (1985) have shown that antibodies against a liver gap junction protein reduces both electrical and dye coupling between cells of cardiac, liver, and neural tissues (see also Warner et al., 1984).

Conclusions

We conclude from our studies that the 43-kD polypeptide localized in isolated cardiac gap junctions is involved in

cell–cell communication mediated by gap junctions. Our experimental data supports the models of the junction protein that can be derived from an analysis of primary sequence; its arrangement in the membrane is similar to that of other junction proteins. Our work also suggests that the amino terminus as well as other regions of the molecule are possible functional domains somehow involved in gating the junctional channels. Subject to the caveats discussed above, antibodies against particular domains of a junctional protein could be a potential tool for understanding the gating properties, tissue specificity, and physiological function of cell–cell communication.

The authors want to express their gratitude to Dr. D. Laird and J. Hoh for their critical review of the manuscript. We, and Dr. Yancey in particular, want to express appreciation to Dr. R. Gomer (formerly at the University of California, San Diego, and now at Rice University) for his help and advice in the production of the anti-43-kD antiserum. We also appreciate the support provided by Dr. B. Nicholson in preparing some of the antipeptide antibodies and thank Dr. T. Amatruda for providing anti-G protein antibody. A. Battaglia and J. Backstrom gave us excellent technical assistance.

This research was supported by National Institutes of Health grant HL 37109 and the Markey Foundation. S. John's work on Topology of the Heart Junction Protein was supported by a Proctor and Gamble Fellowship, and R. Lal's Functional Studies were made possible by contributions from the Albert Billings Ruddock Fund.

Received for publication 6 October 1988 and in revised form 8 February 1989.

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