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Purification of bovine lens cell-to-cell channels composed of connexin44 and connexin50

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SUMMARY

Cell-to-cell channels composed of connexin44 and connexin50 were purified from plasma membranes of calf and fetal bovine lenses. The channels were treated with the nonionic detergents octyl-β-*D*-glucopyranoside and decyl-β-D-maltopyranoside, and the channel/detergent complexes purified by ion and gel filtration column chromatography. In negative staining, the channels appeared as annuli 11±0.6 nm (s.d., *n*=105) in diameter and as 16±0.8 nm (s.d., *n*=96) long particles which corresponded to top and side views of 'complete' cell-to-cell channels. The purified cell-to-cell channels were composed principally of a protein, called MP70, that appeared as a diffuse 55-75 kDa band in SDS-PAGE. Dephosphorylation with alkaline phosphatase transformed the diffuse 55-75 kDa band into two distinct bands of almost equal intensity. Immunoblotting showed the bands to be connexin44 and connexin50, respectively. The antibodies also recognized weaker bands composed of the unphosphorylated form of both connexins. The connexins appear to be processed independently 'in vivo'. The unphosphorylated form of connexin50 was present in channels and membranes from fetal, calf and adult bovine lenses, while unphosphorylated connexin44 only in channels purified from fetal lenses. Therefore, lens cell-to-cell channels are composed principally of equal amounts of phosphorylated connexins 44 and 50 that appear to be assembled in the same channel ('hybrid').

Key words: gap junction, connexin, lens, purification, cell-to-cell channel

INTRODUCTION

Lens fiber cells are connected by extensive networks of junctions that cover >60% of their surface. The junctions belong to two different types. The overwhelming majority are 11-13 nm thick and exhibit an asymmetric structure formed by tetragonal arrays adhering to a protein-free apposing membrane (Lo and Harding; Costello et al., 1985, 1989; Zampighi et al., 1989). The 11-13 nm thick junctions are composed of a 28 kDa protein called the 'major intrinsic protein' (MIP) (Bok et al., 1982; Zampighi et al., 1989). Proteins of the MIP family are expressed in plants, yeasts, mammalian erythrocytes and bacteria. These proteins function in the transport of water and neutral molecules through membranes. Expression in *Xenopus* oocytes has shown that MIP functions as a water channel (Zampighi et al., 1995). Therefore, the structure and function of MIP eliminate the 11-13 nm thick junctions in mediating lens cell communication.

Lens fiber cells are also connected through a thicker (16-18 nm) junction composed of a protein called MP70 (Kistler et al., 1985; Gruijters et al., 1987; Zampighi et al., 1989). This protein was identified as a connexin because its N terminus amino acid sequence was 40-50% identical with those of liver connexin32 and connexin26 and heart connexin43 (Kistler et al., 1988). Also, the N terminus sequence (including three degenerate positions) matched the deduced sequences of mouse lens connexin50 (White et al., 1992), rat lens connexin46 (Paul et al., 1991), bovine lens connexin44 (Gupta et al., 1994) and chick lens connexin56 (Rup et al., 1993). The identification of junctions composed of connexins indicated that lens fiber cells communicate via gap junctions channels.

However, the 16-18 nm thick lens junctions do not exhibit channels arranged in bidimensional plaques (crystalline or random), an important characteristic of gap junctions. This has complicated the demonstration that MP70 forms channels spanning the two plasma membranes and the intervening extracellular space, the hallmark of communication via gap junctions. An approach used to answer this question has been to treat isolated 16-18 nm thick junctions with nonionic detergent and to study the particles released into the solution by negative staining (Kistler and Bullivant, 1988). They showed that some particles released by the treatment exhibited sizes and shapes of 'complete' cell-to-cell channels in negatively stained preparations (Kistler et al., 1993). In this paper, we used this approach and developed a procedure that purifies milligram quantities of stable, monodisperse, and delipidated channels that, in negative staining appear as particles ~11 nm diameter and ~16 nm in length. We show that the 'complete' channels are composed of equal amounts of phosphorylated connexin44 and connexin50, probably assembled in the same channel.

MATERIALS AND METHODS

Calf and adult lenses and fetal bovine eyes were obtained from Pel-Freez (Rogers AK). Octyl-β-D-glucopyranoside (OG) was purchased from Bachem (Bubendorf, Switzerland), decyl-β-D-maltopyranoside (DeM) from Sigma Chemical Co. (St Louis, MO); calf intestine alkaline phosphatase (20 U/μl), endoglycosidase H (200 U/200 μl) and neuraminidase (1 U/100 μl) from Boehringer (Mannheim, Germany). HPTLC silica 60 gel plates (without fluorescent indicator) from Merck (Darmstadt, Germany) and phosphomolybdic acid from Sigma Chemical Co.

Buffers

Buffer A: 2 mM $NaHCO_3$, 3 mM EDTA, 100 μM PMSF, 0.08 μM aprotinin, 1 μM pepstatin, 1 μM leupeptin, pH 8.0.
Buffer B: 4 mM Tris-HCl, 5 mM EDTA, 1.5 mM NaN_3, pH 8.0.
Buffer 1: 10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 3 mM NaN_3, 1% OG, 10% glycerol, 5 mM DTT, pH 7.0.
Buffer 2: Buffer 1 plus 1 M NaCl.
Buffer 3: Buffer 1 plus 0.2% DeM instead of OG.
Buffer 4: Buffer 3 plus 1 M NaCl.
Buffer 5: 10 mM HEPES, 50 mM NaCl, 3 mM NaN_3, 0.1% DeM, 10% glycerol, pH 7.5.

Isolation of fiber cell plasma membranes

Plasma membranes were isolated from fetal, calf and adult bovine lenses as described previously (Ehring et al., 1990; Zampighi et al., 1982; Zampighi et al., 1989). The procedure used 100-200 lenses from fetal, 3-month-old calves, or the cortical region of adult lenses. Decapsulated lenses were homogenized in 300 ml of Buffer A. The homogenate was diluted, filtered and centrifuged at 2,000 ***g*** for 10 minutes at 4°C. The pellets were resuspended in Buffer B and centrifuged at 2,000 ***g*** for 10 minutes. The pellets were resuspended in Buffer B with 4 M urea, centrifuged at 27,000 ***g*** for 20 minutes and then resuspended in Buffer B with 7 M urea an d centrifuged at 50,000 ***g*** for 90 minutes. The urea-extracted pellets were washed twice in Buffer B and stored at −70°C in 200 μl aliquots (5-10 mg/ml). The procedure yielded 0.56 mg, 0.72 mg and 0.17 mg of plasma membrane protein per gram of fetal, calf and adult lens, respectively.

Solubilization of cell-to-cell channels

Plasma membranes were resuspended in Buffer 1 without OG at a protein concentration of 2 mg/ml. Solubilization was performed by adding solid OG to the suspension up to a final concentration of 25 mg OG per mg of membrane protein (~3.5%). The solution was bath-sonicated 3 times for 10 seconds each, left on ice for 30 minutes, and centrifuged in a Beckman TLA-100 Ultracentrifuge (rotor TLA 100.2) at 120,000 ***g***, at 4°C for 60 minutes. The supernatant was diluted (1+1 v/v) with Buffer 1 and called 'OG solubilized proteins'.

Purification of cell-to-cell channels

The OG-solubilized proteins were applied to a MonoS (HR5/5) FPLC column (Pharmacia Fine Chemicals) equilibrated with Buffer 1. The 'flow-through peak' (area shaded in Fig. 1A, first panel) was collected and concentrated 10 times in a Centriprep30 microconcentrator (Amicon, Danvers, MA). The concentrate was diluted (1+9) with Buffer 3. This step replaced most of the OG with DeM, a detergent that stabilized the channels and permitted further purification. The fraction was applied to a MonoQ (HR 5/%) FPLC column (Pharmacia Fine Chemicals) equilibrated in Buffer 3. The column was eluted at a flow rate of 1 ml/minute with a continuous gradient formed by Buffers 3 and 4. The protein peak eluting at 350-450 mM NaCl (area shaded in Fig. 1A, second panel) was collected and concentrated to a final volume of 500 μl in a Centriprep100 microconcentrator, and then injected into a Superose 6 (HR 10/30) FPLC column (Pharmacia) equilibrated in Buffer 3. Fractions eluting from 6.5 ml to 10.5 ml contained 'purified cell-to-cell channels' (shaded area in Fig. 1A, third panel).

Starting with plasma membranes, the procedure took less than a day and yielded 0.5-1 mg of purified cell-to-cell channels per 10 mg of membrane protein. Protein concentration was determined by either the BCA (Pierce Chemicals, Rockford IL) or the Lowry (1951) assays.

SDS-PAGE and western blotting

SDS-PAGE was performed according to the procedure of Laemmli (1970) and immunoblotting according to the procedure of Towbin et al. (1979). The proteins were transferred onto nitrocellulose membranes in a semi-dry Milliblot-Electroblotter (millipore, Bedford, MA) at 250 mA for 30 minutes. Blocking was done with 4% bovine serum albumin in phosphate buffered saline (PBS) for 30 minutes at 45°C. The blots were probed with a monoclonal anti-MP70 (connexin50) or a polyclonal rat anti-connexin46 diluted in phosphate buffered saline. Primary antibodies were visualized with biotinylated horse anti-rat IgG or goat anti-rabbit IgG using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Coomassie Blue stained SDS-PAGE gels of purified cell-to-cell channel fractions were quantified using a Molecular Dynamic Computing Densitometer Model 3000. The purified cell-to-cell channel fractions (area shaded in Fig. 1A, third panel) were treated with alkaline phosphatase (see below). A ratio of 1 to 1.2 of connexin50 to connexin44 was estimated in three different fractions.

Antibodies

Connexin44 was probed with a polyclonal antibody against residues 411-416 of rat connexin46 (Paul et al., 1991), an antibody that also recognizes bovine connexin44 (Gupta et al., 1994). Connexin50 was probed with the monoclonal 6-4-B2-C6 against MP70 (Kistler et al., 1985), an antibody that also recognizes mouse connexin50 (White et al., 1992).

Dephosphorylation

Dephosphorylation was performed by adding 25 U alkaline phosphatase to 2.5 nmoles of purified cell-to-cell channels. The samples were incubated for 30 minutes at room temperature and chromatographed in a Superose 6 column to remove the enzyme from the solution.

Deglycosylation

Deglycosylation was performed by adding 90 mU endoglycosidase H and 100 mU neuraminidase in 50 mM sodium acetate buffer pH 5.2 to 0.25 nmoles of purified cell-to-cell channels. The samples were incubated for 12 hours at 4°C and analyzed by SDS-PAGE.

Delipidation

Lipids were extracted from plasma membranes isolated from fetal, calf and adult lenses (0.2 mg membrane protein) and from 0.2 mg of purified cell-to-cell channels (0.3 nmoles) according to the method of Bligh and Dyer (1959). The chloroform phases were applied on HPLTC silica 60 gel plates. The plates were developed with $CHCl_3$/MeOH/H_2O (20:6.7:1 parts) and the lipids were visualized with phosphomolybdic acid.

Negative staining

Aliquots (~1 μl) of purified proteins at a concentration of ~0.5 mg/ml were spread on glow-discharged carbon-coated grids. Excess solution was blotted by touching the side of the grid on a filter paper. The grids were washed by touching the surface of 5-10 drops of distilled water and stained with a 2% uranyl acetate. The grids were examined in a Philips EM301 or CM12 electron microscope. Measurement of particle diameter and length were performed directly on the negatives in a Nikon comparator.

RESULTS

Purification of lens fiber cell-to-cell channels

Cell-to-cell channels were purified from plasma membranes isolated from fetal and calf lenses. SDS-PAGE shows that the plasma membranes were composed of numerous protein bands, the principal of which migrated at ~26 kDa and is called MIP (Fig. 1B, lane 1 and Fig. 4A). MIP was present in similar amounts in membranes isolated from fetal, calf and adult bovine lenses (Fig. 4A). On the other hand, the cell-to-cell channels are composed of MP70, a protein that appears as a diffuse 55-75 kDa band in SDS-PAGE (Fig. 1B, lane 1 and Fig. 4A).

The first step in the purification procedure transferred the plasma membrane proteins into nonionic detergents micelles ('solubilization'). The aim was to produce stable, monodisperse channel/detergent complexes suitable for subsequent purification by column chromatography. Treatment with octyl-β-D-glucopyranoside (OG) in Buffer 1 solubilized >60% of the total plasma membrane proteins (Ehring et al., 1991). Comparison of SDS-PAGE gels of the plasma membranes and the OG-solubilized proteins showed that these conditions effectively solubilized most types of intrinsic proteins, including MIP and the cell-to-cell channels (Fig. 1B, lanes 1 and 2).

Purification of the channel/detergent complexes required chromatography of the OG-solubilized proteins in three different columns. Chromatography in a cation exchange column (MonoS) eluted cell-to-cell channels in the 'flow-through' fractions (area shaded in the first panel of Fig. 1A). SDS-PAGE showed a dramatic decrease in the amount of MIP and a substantial increase in the amount of the diffuse 55-75 kDa band in the fractions (MP70) (Fig. 1B, lane 3).

Further purification required exchanging OG for decyl-β-maltopyranoside (DeM). OG was effective in transferring the intrinsic proteins from lipid bilayers into micelles. Probably because of its short acyl chain, however, OG produced unstable complexes which remained in solution for only short periods of time (~10 hours). Reduced stability induced unspecific protein-protein aggregation which handicapped further purification. Replacement of OG with DeM formed complexes that remained stable in solution for over extended periods of time (months) and allowed for the purification of the channels.

The DeM/channel complex binds to the anion exchange column MonoQ. This permitted removal of most of the intrinsic lipids from the channel/DeM complex by extensive washes with Buffer 3. The channels were eluted from the MonoQ in a peak at 0.35-0.45 M NaCl with a continuous NaCl gradient formed by mixing Buffer 3 and 4 (area shaded in Fig. 1A, second panel). SDS-PAGE showed that the principal protein of the MonoQ peak was the diffuse 55-75 kDa band (MP70). Contaminants appear as weak bands at ~35 kDa and 26 kDa (Fig. 1B, lane 4).

Chromatography of the channel fractions in a Superose 6 gel filtration column removed the contaminants seen after the cation exchange purification step. Proteins eluted in the void volume (6.5 ml) and in a shoulder that extended up to 12 ml into the internal volume of the Superose 6 column (Fig. 1A, third panel). SDS-PAGE showed that the diffuse 55-75 kDa band eluted from 6.5 ml to 10.5 ml which indicated heterogeneity in the size of the channel/DeM complex. Therefore, all fractions containing the diffuse 55-75 kDa band were studied in the electron microscope.

Negative staining showed that fractions eluting from 7.5 ml to 9.5 ml (shaded in Fig. 1A, third panel) contained annuli of

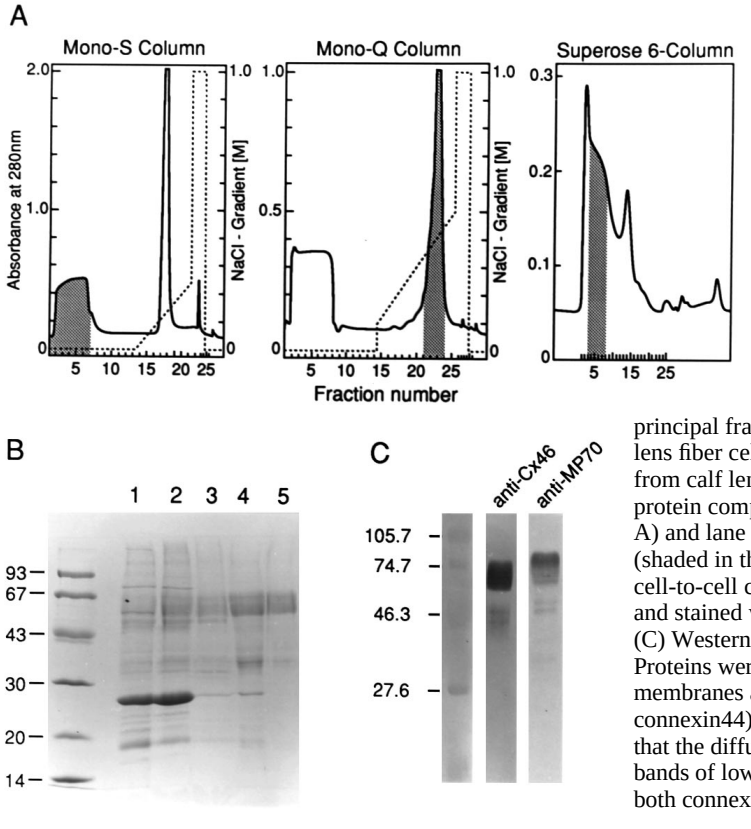

Fig. 1. (A) Chart of the steps used in the purification of cell-to-cell channels starting from the octyl-β-D-glucopyranoside (OG) solubilized proteins of fetal and calf lenses. OG-solubilized proteins were injected in a MonoS cation exchange column. The cell-to-cell channels eluted in the 'flow-through' fractions (area shaded in first panel). After replacing OG with decyl-β-D-glucopyranoside (DeM), the 'flow-through' fractions were injected in a MonoQ anion exchange column and eluted with a NaCl gradient (dotted line in second panel). The channel/DeM complexes eluted in a sharp peak at 0.35-0.45 M NaCl (shaded in second panel). Chromatography in a Superose 6 gel filtration column removed contaminants and aggregated channels. Fractions from 7.5 ml to 9.5 ml (shaded in third panel) contained the 'purified cell-to-cell channels'. (B) SDS-PAGE of the principal fractions of the procedure used to purify cell-to-cell channels from lens fiber cells. Lane 1 shows the protein composition of plasma membranes from calf lenses and lane 2 the OG-solubilized proteins. Lane 3 shows the protein composition of the 'flow-through' fractions (shaded in first panel of A) and lane 4 the proteins in the 0.35-0.45 M NaCl peak from the MonoQ (shaded in the second panel of A). Lane 5 shows the proteins of the 'purified cell-to-cell channel' (shaded in third panel of A). Gels were 12% acrylamide and stained with Coomassie Blue. Molecular mass markers are in kDa. (C) Western blot analysis of 'purified cell-to-cell channels' (lane 5 in B). Proteins were transferred from 10% SDS-PAGE onto nitrocellulose membranes and blotted with either a polyclonal anti-connexin46 (for connexin44) or a monoclonal anti-MP70 (for connexin50). The blots show that the diffuse 55-75 kDa protein band contains both connexins. Weaker bands of lower molecular masses, probably containing 'unmodified' forms of both connexins, are also seen. Methylated molecular mass markers are in kDa.

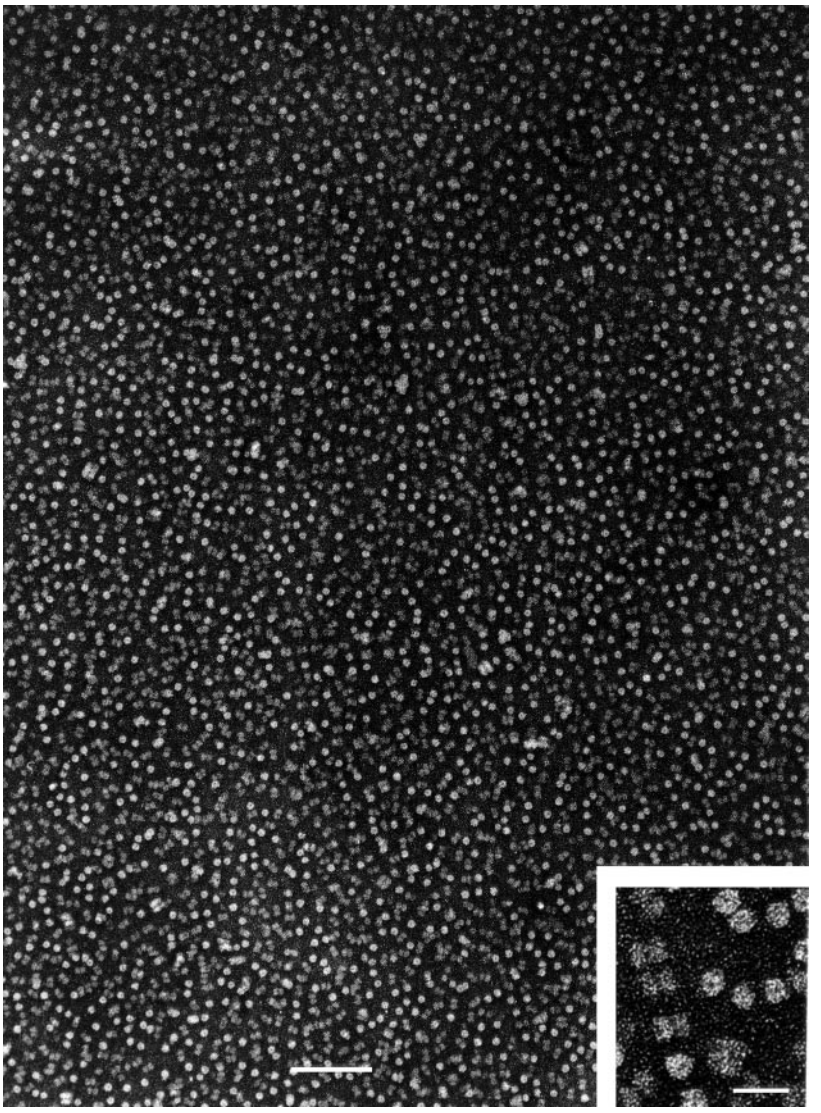

Fig. 2. Negative staining of 'purified cell-to-cell channel' eluting from 7.5 ml to 9.5 ml in the Superose 6 gel filtration column. The channel/DeM complexes appear as annuli ~11 nm in diameter and as ~16 nm long particles (inset). These correspond to top and side views of 'complete' cell-to-cell channels. Bars: 110 nm; 21 nm in inset.

about 11±0.6 nm (s.d., *n*=105) in diameter and particles 16±0.8 nm in length (s.d., *n*=96) and ~11 nm in width (Fig. 2). The ~16 nm long particles were composed of two domains joined through a 3 nm wide, lightly stained region (Fig. 2 inset). SDS-PAGE showed that these fractions contained principally the 55-75 kDa band and only a small amount of weaker bands at ~35 kDa (Fig. 1B, lane 5). These fractions are referred to as 'purified cell-to-cell channels'.

Fractions eluting from 6.5 ml to 7.5 ml also contained principally the diffuse 55-75 kDa protein band, however, negative staining showed aggregates composed of 3-6 channels (not shown, but see Kistler et al., 1993). Such an aggregate can explain why those channels eluted in the column void volume. They were discarded. Negative staining showed that fractions eluting from 9.5 ml to 10.5 ml contained single channels of similar shape and dimensions to those in the 'purified cell-to-cell channel' fractions. However, SDS-PAGE showed a substantial increase in the amount of the protein bands migrating at ~35 kDa that most likely represent endogenous proteolytic fragments of the channels (Kistler et al., 1990). They were also discarded.

Therefore, SDS-PAGE and negative staining showed that fractions eluting from 7.5 ml to 9.5 ml from the Superose 6 column contained 'complete', monodisperse cell-to-cell channels composed principally of a diffuse 55-75 kDa protein band.

Connexins composing the purified lens fiber cell-to-cell channels

Immunoblots showed that the upper part of the diffuse band was recognized by anti-MP70, a monoclonal that crossreacts with sheep and mouse connexin50 (Fig. 1C, second lane) (Kistler et al., 1985; White et al., 1992). The lower part was recognized by an anti-rat connexin46, a polyclonal that also crossreacts with bovine connexin44 (Fig. 1C, third lane) (Gupta et al., 1994). Both antibodies also recognized lower molecular mass bands migrating at about 50 kDa and 40 kDa, respectively (Fig. 1C, lanes 2 and 3). Whereas the weaker bands correlate well with the molecular masses of connexins 44 and 50, the stronger diffuse bands were 15-20 kDa larger than the molecular mass predicted from the amino acid sequence. We refer to the strong bands as the 'modified' and to the weaker bands as the 'unmodified' forms of connexins 44 and 50.

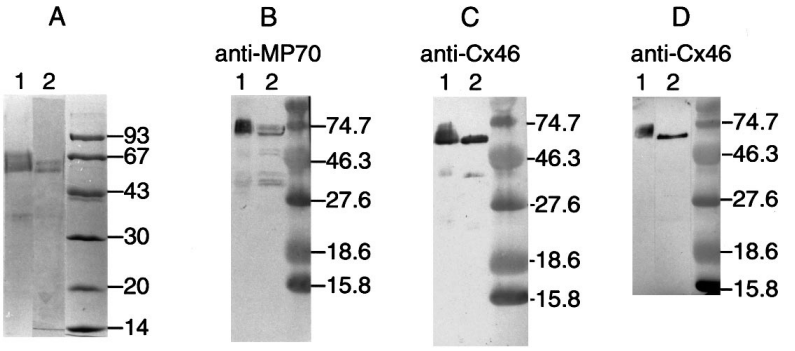

Fig. 3. (A) SDS-PAGE of the purified cell-to-cell channel fractions before (lane 1) and after dephosphorylation with alkaline phosphatase (lane 2) showing the transformation of the diffuse 55-75 kDa band into two sharper bands. In three different preparations, densitometry showed the two bands were present at ratios of 1.2 connexin44 to 1 connexin50. Incubation of the purified channel fractions with buffer, without the enzyme did not transform the diffuse 55-75 kDa band into the two sharp bands (not shown). Gels were 12% acrylamide and stained with Coomassie Blue. Molecular mass markers are in kDa. (B) Immunoblots of purified cell-to-cell channel fractions with anti-MP70 (connexin50) before and after dephosphorylation with alkaline phosphatase (lanes 1 and 2). Before dephosphorylation, the antibody recognized principally the diffuse 55-75 kDa band (lane 1). After dephosphorylation, the antibody recognized sharper, double bands at ~70 kDa (the 'modified form) and sharper double bands at ~50 kDa (the 'unmodified' form). Lower bands at ~35 kDa are also seen but were not identified. (C and D) Immunoblots of purified cell-to-cell channel fractions from fetal (C) and calf (D) lenses with anti-connexin46 (connexin44). (C) A blot showing cell-to-cell channels purified from fetal lenses whereas (D) channels from calf lenses. Before dephosphorylation with alkaline phosphatase, the antibody recognized a strong, diffuse 55-70 kDa band in channels purified from both fetal and calf lenses (lane 1, C and D). After phosphorylation, the antibody recognizes a single, sharper band at 55-60 kDa (the 'modified connexin44) (lane 2, C and D). 'Unmodified' connexin44 appears as a weak band at ~42 kDa only in channels purified from fetal lenses (lanes 1 and 2, C).

To account for the decreased electrophoretic mobility of the modified connexins 44 and 50, we tested for glycosylation and phosphorylation Deglycosylation with endoglycosidase H and neuroaminidase did not alter either the appearance or the position of the 55-75 kDa band (not shown). Dephosphorylation with alkaline phosphatase on the other hand changed the diffuse band into two distinct bands (Fig. 3A). The sharper bands were the 'modified' forms of connexins 44 and 50 because their molecular masses were larger than those predicted from the sequence (Fig. 3B and C). Densitometry determined a ratio of 1.2 connexin44 to 1 connexin50.

Immunoblotting showed that alkaline phosphatase affected both connexins differently. Dephosphorylation of connexin50 showed double bands at the position of both the 'modified' and 'unmodified' forms (Fig. 3B) in channels purified from either fetal or calf lenses. In contrast, dephosphorylation shifted the mobility of the modified form of connexin44 from calf lenses (Fig. 3D) but not from fetal lenses (Fig. 3C). The slightly higher mobility of fetal connexin44 was also seen in plasma membranes (Fig. 4B) suggesting that it is not an artifact of the purification procedure.

We have also determined the relative amounts of 'modified' and 'unmodified' forms of connexins 44 and 50 in fetal, calf and the cortex of adult lenses. 'Modified' connexin44 was present in similar quantities in membranes isolated from fetal and calf lenses but it was greatly decreased in membranes from adult lenses (Fig. 4B). 'Unmodified' connexin44, on the other hand, was present in membranes from fetal lenses only (Figs 3C and D, and 4B, first lane). Previous studies did not detect 'unmodified' connexin44 in fetal lenses probably because of low protein loading (Jarvis et al., 1993). In contrast, 'modified' and 'unmodified' forms of connexin50 were present in equal amounts in plasma membranes from fetal, calf and the cortex of adult lenses. Thus, it appears that the two connexins are processed independently.

Lens plasma membranes have an unusual lipid composition that includes the highest concentration of cholesterol and sphingomyelin in the body (Fig. 5, lanes 1-3). Thin layer

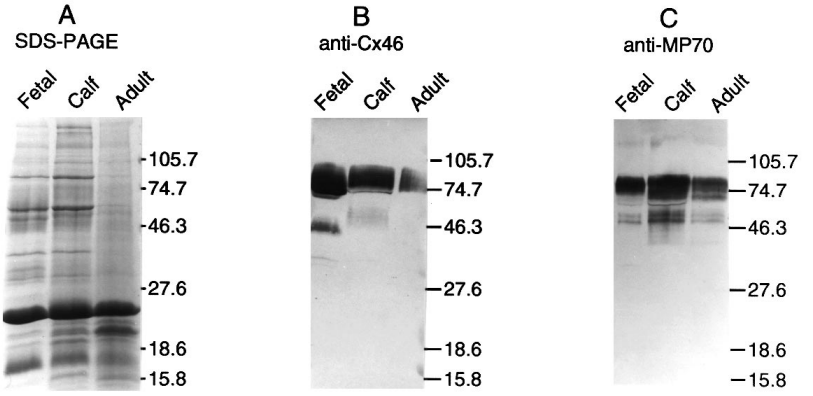

Fig. 4. Plasma membranes from fetal, calf and the cortex of adult bovine lenses were blotted with anti-connexin46 (connexin44) and anti-MP70 (connexin50) to show the 'modified' (band of 55-75 kDa) and 'unmodified' (bands at 44 kDa and 50 kDa) forms of both connexins. (A) Coomassie Blue stained SDS-PAGE gels of plasma membranes from fetal, calf and the cortex of adult bovine lenses. The gels were 12% acrylamide, loaded with 20 μg protein per lane and stained with Coomassie Blue. (B) Immunoblots of plasma membranes from fetal, calf and adult bovine lenses with anti-connexin46 (connexin44). The 'modified' form of connexin44 appeared as a diffuse 55-75 kDa band that decreased progressively with age. 'Unmodified' connexin44 was found only in membranes from fetal lenses. Blots of membranes from calf lenses exhibited a weak and diffuse band of unknown origin migrating between the two forms. Each lane was loaded with 15 μg of membrane protein. (C) Immunoblots of plasma membranes from fetal, calf and the cortex of adult bovine lenses with anti-MP70 (connexin50). 'Modified' connexin50 appears as a diffuse band that remained somewhat constant with age. In contrast to the distribution of connexin44 (B), 'unmodified' connexin50 appeared as a double band at ~50 kDa in membranes from all lenses. The loading was 15 μg of membrane protein per lane.

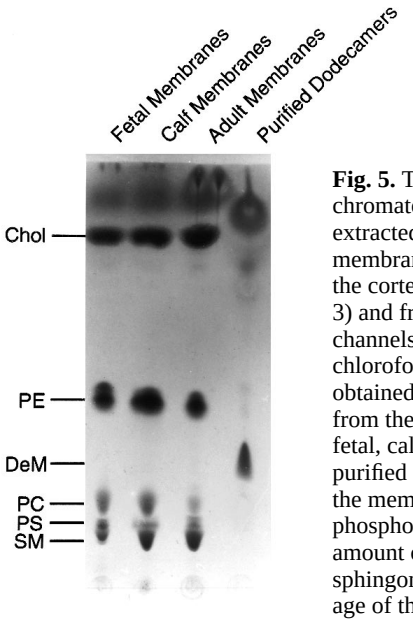

Fig. 5. Thin layer chromatography of lipids extracted from plasma membranes from fetal, calf and the cortex of adult lenses (lanes 1-3) and from purified cell-to-cell channels (lane 4). The lanes show chloroform/methanol extracts obtained from 0.2 mg of protein from the plasma membranes of fetal, calf, and adult lenses, and purified cell-to-cell channels. In the membranes, the amount of phospholipid decreased and the amount of cholesterol and sphingomyelin increased with the age of the animal (Fig. 5 lanes 1-3). In the purified channel fractions, the phospholipids, sphingomyelin and cholesterol were replaced by decyl-β-D-maltopyranoside (DeM). Visualization was with phosphomolybdic acid. Standards are cholesterol (Chol), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and sphingomyelin

chromatography indicates that the purification procedure used in this study removed cholesterol, sphingomyelin and other phospholipids from the purified preparations. This indicates that intrinsic lipids do not participate in the stability of the channels.

DISCUSSION

Purified cell-to-cell channels appeared as annuli ~11 nm diameter and as particles ~16 nm in length and ~11 nm wide. The ~16 nm particles must correspond to side views of 'complete' cell-to-cell channels. They are long enough to span the junctional membranes and the intervening extracellular gap and also exhibit two distinct domains joined by a constriction that collects stain molecules. This view is consistent with a channel composed of two hemichannels joined through the extracellular domains (Unwin and Zampighi, 1980, Unwin and Ennis, 1984). The annulus, on the other hand, exhibits a larger diameter than that of the channels in the junction (~8 nm) (Revel and Karnovsky, 1967). Such a discrepancy is not unexpected since the channels were purified as a complex with DeM molecules, a fact that should increase their diameters, but not their lengths.

While the ~16 nm long particles can only be best explained as the side view of a 'complete' channel (dodecamer), the ~11 nm annulus may correspond to the top view of either a 'complete' channel or a hemichannel (hexamer). Gel filtration chromatography showed that top and side views eluted in the same fractions, indicating that the annulus and the ~16 nm long particle must have very similar, if not identical, molecular masses. Taken together, the data indicate that the purified fractions must contain 'complete' cell-to-cell channels, not a mixture of channels and hemichannels.

The protein composition of lens cell-to-cell channels has been a topic of controversy (for review see Zampighi et al., 1992). Due to its abundance and location in junctions, early work advanced the hypothesis that MIP formed the lens cell-to-cell channels (Peracchia and Peracchia, 1980). This view was challenged by the identification of the 16-18 nm thick junctions (Zampighi et al., 1982) and the demonstration that they were composed of MP70 (Kistler et al., 1985; Kistler and Bullivant, 1987; Gruijters et al., 1987; Zampighi et al., 1989). The observation that purified 'complete' channels are composed of equal amounts of connexins 44 and 50 provides strong support to the view that channels located in the 16-18 nm thick junction mediate lens cell communication.

Gap junctions are extremely resistant to solubilization with mild detergents, a property that greatly facilitated their isolation but has hampered the purification of individual channels and hemichannels. Presently, connexin32 from liver or overproduced in a baculovirus/insect cell expression system (Stauffer et al., 1991), and MP70 from lens fiber cells (Kistler and Bullivant, 1988; Kistler et al., 1993) have been solubilized under mild conditions. Connexin32 was purified by column chromatography and shown to be assembled in hexamers (hemichannels) by single particle rotational averaging (Stauffer et al., 1991). Lens MP70 was only partially purified and shown by negative staining to be assembled in 'complete' channels (Kistler et al., 1993). Our studies extend the work of Kistler and co-workers by showing that 'complete', stable, monodisperse and delipidated channels can be purified in large quantities from bovine lenses. Chemical and structural methods may now be used to study the channels to learn about their high resolution structure and function.

The purified channels were composed principally of phosphorylated connexin44 and connexin50 and of only small quantities of the dephosphorylated forms. This result is in agreement with previous studies showing that mouse connexin50, rat connexin46 and bovine connexin44 are phosphorylated in plasma membrane fractions (White et al., 1992; Paul et al., 1991; Gupta et al., 1994) and connexin46 in cultured fiber cells (Jiang et al., 1993). In crude lens homogenates, dephosphorylation with alkaline phosphatase shifted the mobility of connexin44 to the molecular mass of the unphosphorylated form (Gupta et al., 1994). In the purified channel fraction, however, alkaline phosphatase transformed the diffuse 55-75-kDa band into two sharper bands, but it did not decrease the mobility to that of the dephosphorylated forms. The discrepancy may be due to differences in the preparations (purified channels versus crude lens homogenates), to the experimental conditions used in the enzymatic reaction, or to the possibility that the enzyme dephosphorylated only partially the channel/detergent complexes.

The identification of channels composed of phosphorylated connexins may be important to the lens. Previous studies have shown that phosphorylation may control gap junction assembly (Musil et al., 1990) or can modify channel properties such as gating (Burt and Spray, 1988) and unitary conductance (Saez et al., 1986). The importance of phosphorylation in channel function has been supported by expression in heterologous systems. Injection of cDNA coding for bovine connexin44 and rat connexin46 in *Xenopus* oocytes expressed the unphospho-

rylated form of both connexins. This resulted in single oocyte depolarization and lysis (Gupta et al., 1994; Paul et al., 1991). Although these results can be rationalized by assuming that hemichannels formed of unphosphorylated connexin44 are open, it remains to be explained why the expression of unphosphorylated connexin50 (or of any other connexin) does not lyse single oocytes.

Is the fiber cell-to-cell channel a 'mixed' channel?

Assembly of cell-to-cell channels is a complex process that involves the interaction of connexin within the plane of the membrane to form hemichannels, and across the intercellular space to form the 'complete' channel. Cells expressing a single type of connexin assemble homotypic cell-to-cell channels. Cells expressing two (Kuraoka et al., 1993; Nicholson et al., 1987; Traub et al., 1989; Zhang and Nicholson, 1989) or more types of connexins can assemble either homotypical or 'mixed' cell-to-cell channels. Homotypic channels can be localized in the same gap junction or be segregated into different junctions. Depending on the subunit composition of the hemichannel, 'mixed' cell-to-cell channels can be assembled 'heterotypically' (hemichannels composed of a single connexin) or heteromerically (hemichannels composed of two or more types of connexins).

Heterotypic channels have been assembled by pairing *Xenopus* oocytes expressing different types of connexins (Swenson et al., 1989; Werner et al., 1989). Conclusive evidence for 'mixed' cell-to-cell channels in tissues, however, does not exist. In the lens, connexin46 and connexin50 have been localized in the same fiber cells, and more importantly, in the same junction (Paul et al., 1991; Louis et al., 1993; TenBroek et al., 1992). This paper shows that the purified channels contain equal amounts of connexins 44 and 50. It seems unlikely that the fractions are composed of mixtures of homotypic channels assembled of either connexin44 or connexin50. The channels were purified based on differences in their molecular mass, hydrodynamic radii and charge. This strategy should have separated homotypic channels because the calculated pI of connexins 44 and 50 differs by more than 2 pH units. Therefore, the simplest explanation that can rationalize our results in purified cell-to-cell channels, and those in plasma membranes (Paul et al., 1991; TenBroek et al., 1992) and in fiber cells (TenBroek et al., 1994) is that connexins 44 and 50 are assembled in the same channel ('mixed' or 'hybrid').

Expression of multiple connexins may represent a 'safety' mechanism that ensures formation of cell-to-cell channels when a particular connexin is not expressed, is expressed at low levels, or is vulnerable to endogenous proteolysis. It may also be used to alter channel properties such as conductance, gating, or voltage-dependence. For example, heterotypic channels assembled from liver connexin32 and connexin26 exhibited voltage dependence and asymmetry not present in homotypic channels formed by either connexin (Barrio et al., 1991; Rubin et al., 1992; Versalis et al., 1993). It is not possible to determine which, if any, of these possible functions is occurring in the lens. Perhaps the complexity detected in the architecture of lens cell-to-cell channels is related to the vectorial transport of ions, metabolites, and water between posterior and anterior surfaces, which is crucial in maintaining lens transparency and homeostasis.

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REFERENCES

Barrio, L. C., Suchyna, T., Bargiello, T., Xu, L. X., Roginski, R. S., Bennet, M. V. L. and Nicholson, B. J. (1991). Gap junctions formed by connexins 26 and 32 alone and in combinations are differently affected by applied voltage. *Proc. Nat. Acad. Sci. USA* **88**, 8410-8414.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917.

Bok, D., Dockstader, J. and Horwitz, J. (1982). Immunocytochemical localization of the lens main intrinsic protein (MIP26) in communicating junctions. *J. Cell Biol.* **92**, 213-220.

Burt, J. M. and Spray, D. C. (1988). Inotropic agents modulate gap junction conductance between cardiac myocytes. *Am. J. Physiol.* **254**, H1206-H1210.

Costello, M. J., McIntosh, T. J. and Robertson, J. D. (1985). Membrane specializations in mammalian lens fiber cells: Distribution of square arrays. *Curr. Eye Res.* **4**, 1183-1201.

Costello, M. J., McIntosh, T. J. and Robertson, J. D. (1989). Distribution of gap junctions and square junctions in the mammalian lens. *Invest. Opthalmol. Vis. Sci.* **30**, 975-989.

Ehring, G., Zampighi, G., Horwitz, J., Bok, D. and Hall, J. E. (1990). Properties of channels reconstituted from the major intrinsic protein of lens fiber membranes. *J. Gen. Physiol.* **96**, 631-664.

Ehring, G., Lagos, N., Zampighi, G. and Hall, J. E. (1991). Phosphorylation modulates the voltage dependance of channels reconstituted from the major intrinsic protein of lens fiber membranes. *J. Membr. Biol.* **126**, 75-88.

Gruijters, W. T. M., Kistler, J., Bullivan, S. and Goodenough, D. A. (1987). Immunolocalization of MP70 in lens fiber 16-17 nm intercellular junctions. *J. Cell Biol.* **104**, 562-572.

Gupta, V. K., Berthoud, V. M., Atal, N., Jarillo, J. A., Barrio, L. C. and Beyer E. C. (1994). Bovine connexin44, a lens gap junction protein: molecular cloning, immunologic characterization, and functional expression. *Invest. Ophthalmol. Vis. Sci.* **35**, 3747-3758.

Jarvis, L., Kumar, N. M. and Louis, C. F. (1993). The developmental expression of three mammalian lens fiber cell membrane proteins. *Invest. Ophthalmol. Vis. Sci.* **34**, 613-620.

Jiang, J. X., Paul, D. L. and Goodenough, D. A. (1993). Posttranslational phosphorylation of lens fiber connexin 46: A slow occurrence. *Invest. Ophthalmol. Vis. Sci.* **34**, 3558-3565.

Kistler, J., Kirkeland, B. and Bullivant, S. (1985). Identification of a 70,000-D protein in lens membrane junctional domains. *J. Cell Biol.* **101**, 28-35.

Kistler, J. and Bullivant, S. (1987). Protein processing in lens intercellular junctions: cleavage of MP70 to MP38. *Invest. Opthalmol. Vis. Sci.* **28**, 1687-1691.

Kistler, J. and Bullivant, S. (1988). Dissociation of lens fibre gap junctions releases MP70. *J. Cell Sci.* **91**, 415-421.

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

Kistler, J., Schaller, J. and Sigrist, H. (1990). Mp38 contains the membrane embedded domain of the lens fiber gap junction protein MP70. *J. Biol. Chem.* **265**, 13357-13361.

Kistler, J., Bond, J., Donalson, P. and Engel, A. (1993). Two distict levels of gap junction assembly in vitro. *J. Struct. Biol.* **110**, 28-38.

Kuraoka, A., Iida, H., Hatae, T., Shibata, Y., Itoh, M. and Kurita, T. (1993). Localization of gap junction proteins, connexins 32 and 26, in rat and guinea pig liver as revealed by quick-freeze, deep-etch immunoelectron microscopy. *J. Histochem. Cytochem.* **41**, 971-980

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lo, W. K. and Harding, C. V. (1984). Square arrays and their role in ridge formation in human lens fibers. *J. Ultrastruct. Res.* **86**, 228-254.

Louis, C. F., Arneson, M., Jarvis, L. and TenBroek, E. M. (1993). The developmental expression and organization of membrane proteins of the

mammalian lens. In *Gap Junctions. Progress in Cell Research* (ed. J. E. Hall, G. A. Zampighi and R. M. Davies), pp. 177-186. Elsevier, Amsterdam.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurment with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275

Musil, L. S., Cunningham, B. A., Edelman, G. M. and Goodenough, D. A. (1990). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* **111**, 2077-2088

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

Paul, D. L., Ebihara, L., Takemoto, L. J., Swenson, K. I. and Goodenough, D. A. (1991). Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membranes of Xenopus oocytes. *J. Cell Biol.* **115**, 1077-1089.

Peracchia, C. and Peracchia, L. L. (1980). Gap junction dynamics: Reversible effects of divalent cations. *J. Cell Biol.* **87**, 708-718.

Revel, J. P. and Karnovsky, J. J. (1976). Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33**, C7-C12.

Rubin, J. B., Versalis, V. K., Bennett, M. V. L. and Bargiello, T. A. (1992). Molecular analysis of voltage dependence of heterotypic gap junctions formed by connexin 26 and 32. *Biophys. J.* **62**, 183-195.

Rup, D. M., Veenstra, R. D., Wang, H. Z., Brink, P. R. and Beyer, E. C. (1993). Chick connexin56, a novel gap junction protein. *J. Biol. Chem.* **268**, 706-712.

Saez, J. C., Spray, D. C., Nairn, A. C., Hertzberg, E. L., Greengard, P., Bennett, M. V. L. (1986). cAMP increases junctional conductance ans stimulates phosphorylation of the 27-kDa principal gap junction polypeptide. *Proc. Nat. Acad. Sci. USA* **83**, 2473-2477.

Stauffer, K. A., Kumar, N. M., Gilula, N. B. and Unwin, N. (1991). Isolation and purification of gap junction channels. *J. Cell Biol.* **115**, 141-150.

Swenson, K. I., Jordan, J. R., Beyer, E. C. and Paul, D. L. (1989). Formation of gap junctions by expression of connexins in Xenopus oocyte pairs. *Cell* **57**, 145-155.

TenBroek, E., Arneson, M., Jarvis, L. and Louis, C. F. (1992). The distribution of the fiber cell intrinsic membrane proteins MP20 and connexin46 in the bovine lens. *J. Cell Sci.* **103**, 245-257.

TenBroek, E., Johnson, R. and Louis, C. (1994). Cell-to-cell communication in a differentiating ovine lens culture system. *Invest. Ophthalmol. Vis. Sci.* **35**, 215-228.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Traub, O., Look, J., Dertmietzel, R., Bruemmer, F., Huelser, D. and Willecke, K. (1989). Comparative characterization of the 21-kDa and 26-kDa gap junction proteins in murine liver and cultured hepatocytes. *J. Cell Biol.* **108**, 1039-1051.

Unwin, P. N. T. and Zampighi, G. (1980). Structure of the junction between communicating cells. *Nature* **283**, 545-549.

Unwin, P. N. T and Ennis, P. D. (1984). Two configurations of a channel-forming membrane protein. *Nature* **307**, 609-613.

Versalis, V. K., Bargiello, T. A., Rubin, J. R. and Bennett, M. V. L. (1993). Comparison of voltage-dependent properties of gap junctions in hepatocytes and in Xenopus oocytes expressing Cx32 and Cx26. In *Gap Junctions* (ed. J. E. Hall, G. A. Zampighi and R. M. Davies), pp. 105-120. Elsevier, Amsterdam.

Werner, R. E., Levine, E., Rabadan-Diehl, C., Dahl, G. (1989). Formation of hybrid cell-cell channels. *Proc. Nat. Acad. Sci USA* **86**, 5380-5384.

White, T. W., Bruzzone, R., Goodenough, D. A. and Paul, D. L. (1992). Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. *Mol. Biol. Cell* **3**, 711-720.

Zampighi, G., Simon, S. A., Robertson, J. D., McIntosh, T. J. and Costello, M. J. (1982). On the structural organization of isolated bovine lens fiber junctions. *J. Cell Biol.* **93**, 175-189.

Zampighi, G. A., Hall, J. E., Ehring, G. R. and Simon, S. A. (1989). The structural organization and protein composition of lens fiber junctions. *J. Cell Biol.* **108**, 2255-2275.

Zampighi, G. A., Hall, J. E. and Simon, S. A. (1992). The specialized junctions of the lens. *Int. Rev. Cytol.* **136**, 185-225.

Zampighi, G. A., Kreman, M., Boorer, K. J., Loo, D. D. F., Bezanilla, F., Chandy, G., Hall, J. E. and Wright, E. M. (1995). A method for determining the unitary functional capacity of cloned channels and transporters expressed in Xenopus laevis oocytes. *J. Membr. Biol.* (in press).

Zhang, J. T. and Nicholson, B. (1989). Sequence and tissue distribution of a second hepatic gap junction, CX26, as deduced from its cDNA. *J. Cell Biol.* **109**, 3391-3401.

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