Connexin Distribution in the Rabbit and Rat Ciliary Body
A Case for Heterotypic Epithelial Gap Junctions

J. Mario Wolosin,*† Michael Schütte,* and Shuhua Chen*

Purpose. To evaluate the distribution of different α- and β-type connexins (Cx) present in the dual layered ciliary body epithelia (CBE) of both rabbit and rat.

Methods. Immunocytochemical detection of Cx26, Cx32, Cx43, and Cx50 was performed on frozen sections of rabbit and rat ciliary body using indirect immunofluorescent methods. The identity of the antigens recognized by the monoclonal primary antibodies was further confirmed by Western immunoblots. Double labeling experiments based on either conventional or confocal microscopy were carried out to establish the exact spatial relationship between different connexins.

Results. Connexin 50 was found only in the nonpigmented epithelium (NPE) at apical and basolateral membranes, whereas Cx43 was observed exclusively and at a very high concentration in the pigmented epithelium (PE), primarily in the apical cell membrane, with minimal extension to the proximal lateral zone. The correct antigenicity of the antibodies was confirmed by Western blots of rabbit ciliary body membranes. In rabbit, the Cx26 antibody detected an antigen that was abundant in the NPE and was weakly expressed in the PE. In rat, however, the Cx26 staining was confined to capillary wall endothelia. Western blots of ciliary body and liver membranes and liver immunohistology indicated that the Cx26 antibody used does not recognize rabbit Cx26. Cx32 did not yield any substantial epithelial labeling in either species.

Conclusions. The distribution of Cx50 around the entire NPE cell perimeter suggests its involvement in NPE–NPE cell homotypic gap junctions. The concentration of Cx43 and Cx50 at the apical membranes of the PE and NPE cells, respectively, and their complete absence from the opposite cell suggest that these connexins may participate in the formation of heterotypic gap junctions, either with each other or with other yet unidentified connexins. Invest Ophthalmol Vis Sci. 1997;38:341–348.

The dual-layered ciliary body epithelium (CBE) is the site of production of aqueous humor, which is essential for the nutrition of avascular ocular structures and the maintenance of correct intraocular pressure. This active secretory process involves uptake of ions at the serosal side by the pigmented layer (PE), diffusion of these ions through the heterocellular junctional path to the nonpigmented layer (NPE), and transport to the posterior eye chamber. The resultant sustained osmotic gradient is presumed to draw water and, thus, to generate the inflow of aqueous humor. The physical connection of the two distinct CBE layers through apically located, heterocellular gap junctions (GJs) has been demonstrated on the ultrastructural level. Additionally, intracellular dye-injection and subsequent transepithelial dye-transport, as well as whole tissue transepithelial studies, have confirmed the involvement of the heterocellular junctional path in the communication between the two distinct layers. The permeability of the heterocellular path can be modified by pharmacologic agents such as adrenaline and acetylcholine, suggesting that autonomic control of the heterocellular junctional path may set the limit for the overall transepithelial transport capacity.
Gap junctions are formed by an extended family of related amphipathic polypeptides called connexins (Cx) that consist of four transmembrane segments connected by one intracellular and two extracellular loops; the carboxyl and amino termini are located on the cytoplasmic side. A hexamer of such connexins forms a hemichannel, or connexon, in a cell’s membrane.

Theoretically, gap junctions that are formed by docking of two connexons can be built according to four different blueprints: homomeric homotypic (all six subunits of a connexon are identical and both connexons are identical); heteromeric homotypic (connexons are identical but composed of different subunits); homomeric heterotypic (the channel consists of two different homomeric connexons); and heteromeric heterotypic. Heterotypic interactions are limited by the ability of different connexins to dock onto each other. This head-to-head docking appears to be highly dependent on the structural matching of the second extracellular domain. This and other sequence similarities have led to the classification of all known connexins into two subfamilies, α and β. Connexons formed by members of one family are believed to be unable to form GJs with connexons made of members of the other. Within the respective subfamilies, several heterotypic GJs have been generated in vitro using oocytes or HeLa cells transfected to express connexins. In addition, different connexins have recently been identified within the same hemichannel, indicating the existence of heteromeric (homotypic) GJs in vivo. Evidence for the existence of heterotypic GJs in vivo, however, remains inconclusive.

There are strong anatomical, functional, and biochemical differences between the two distinct layers of the CBE. These differences hint at the possibility that the gap junctions connecting the NPE and the PE may be heterotypic. Interestingly, rather than behaving as nonspecific channels, gap junctions display distinct degrees of ionic selectivity, caused by the charge cloud at the cytoplasmic port of the central pore; thus, heterotypic gap junctions generating asymmetric pores between the NPE and the PE cells could form the molecular basis for complex communication patterns between the two epithelial layers. In this context, it is noteworthy that we have recently shown rectification of Ca²⁺ signal transfer; that is, a signal originating in the NPE is able to spread rapidly into the PE whereas the reverse, PE to NPE signal transfer, does not occur.

Over the last few years, molecular probes specific to individual subtypes of connexin have been developed. Accordingly, we have employed antibodies directed against a variety of connexins (Cx26, Cx32, Cx43, and Cx50) to investigate the nature and distribution of different gap junction proteins in the pigmented and albino rabbit ciliary body. Cx43 has previously been identified in the bovine PE.

**METHODS**

**Immunocytochemistry**

New Zealand rabbits were killed by CO₂ narcosis and asphyxiation; Wistar rats were killed by cervical dislocation. Eyes were enucleated and ciliary bodies were dissected free of other ocular tissues and vitreous humor as described elsewhere. All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After a short rinse in ice-cold Ringer’s solution, individual ciliary body strips were incubated in cryoembedding medium (OCT Tissue Tek) and then frozen in liquid N₂. Frozen sections (15 μm) were cut on a cryostat (International Equipment, Needham Heights, MA) and mounted on sylane-precoated slides. The sections were fixed and permeabilized for 10 minutes in Histocut (Amresco, Solon, OH) containing 0.1% Triton X-100 and subsequently washed three times for 5 minutes in phosphate-buffered saline (PBS). Nonspecific binding sites were blocked by a 45-minute incubation in 5% bovine serum albumin (BSA) (Type V, Sigma, St. Louis, MO; in PBS). Primary incubation was done for 45 minutes using monoclonal IgG antibodies directed against Cx26, Cx32 (Zymed, San Francisco, CA), or Cx43 (Chemicon, Temecula, CA) or a monoclonal IgM antibody that recognizes Cx50 (a generous gift from Dr. D.A. Goodenough, Harvard Medical School). Immunoreactivity was revealed using the indirect method with either tetramethylrhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or IgM (Sigma), as appropriate. After three washes of 10 minutes in PBS, the sections were mounted in Vectashield (Vector Labs, Burlingame, CA) and examined on a fluorescent inverted microscope (Olympus IMT-2, Tokyo, Japan) or analyzed by confocal microscopy (Leitz CLSM, Wetzlar, Germany). Small pieces of rat and rabbit liver also were processed for Cx26-like immunoreactivity as described above. For double labeling experiments on the confocal microscope, the individual frames were scanned alternatingly in single wavelength excitation for FITC and TRITC to minimize nonspecific crossover and the resultant double labeling picture was assembled by merging corresponding image (tiff) files.

**Immunoblotting**

Freshly dissected rabbit ciliary body or liver tissue (approximately 0.5 g of either rabbit or rat tissue) were suspended in 2 ml of homogenization solution (HS;
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FIGURE 1. Immunofluorescence of connexins in the rabbit and rat CBE. (a to d) Rabbit. (a) Cx26. Both nonpigmented epithelium (NPE) and pigmented epithelium (PE) are labeled. The stain is moderate in the NPE and weak in the PE. Note the absence of labeling in the basolateral PE membrane (arrow). (b) Cx32 shows a similar distribution to Cx26 in the NPE. There is no visible PE staining. (c) Cx50. The immunoreactivity is characterized by its sharply punctuated appearance in the NPE. Note the lack of labeling in the PE and the isolated spots on the endothelial cells. (d) Cx43. The immunoreactivity is visible as a band of intense fluorescence in the apical membrane of the PE (arrow). Occasionally, an extension of the immunofluorescence in between the PE flanks is apparent (short arrow). (e to g) Rat. (e) Cx26. The immunoreactivity is confined to the endothelium (arrow) lining the intraprocessal blood vessels (v). The epithelial layers are devoid of staining. (f) Cx50. NPE (N) cell membranes are heavily labeled. Note the fine feathery extensions at the basolateral aspect of the NPE cells (small arrow). Staining is most dense at the apical membrane (arrow). Some stromal endothelial (E) staining is also present. (g) Cx43. The staining occurs only in the PE (P) cells and is concentrated at their apical membranes (long arrow), even though some labeling extends into the proximal lateral flanks (short arrow). Bars = 20 μm (a to d,f,g), 10 μm (e).

125 mM sucrose, 0.5 mM EDTA in 10 mM Tris-HCl, pH 7.7), minced with scissors for 5 minutes, and subsequently diluted to 10 ml in HS. A 15 ml Potter Elvehjem tissue grinder was used for motor-driven (200 rpm) homogenization of minced tissue. The resultant suspensions were filtered through two layers of surgical gauze, further homogenized by 20 passes through a Dounce type A glass homogenizer, and the filtrates were spun at 400 × g for 10 minutes in a fixed angle rotor to obtain nuclear (and debris) pellets. Supernatants were centrifuged two times at 10^4 × g for 10 minutes and the resultant pellets for each tissue were combined to obtain plasma membrane–mitochondrial pellets, which were used for immunoblot analysis of connexins.

Membrane material (5 to 10 μg per slot) was electrophoresed in 1.5 mm thick minigels using the Laemmi buffer system with 10% or 12% polyacrylamide main gels. Prestained standards (Biorad, Richmond, CA) were included in one slot. Electrophoresed protein was transferred to nitrocellulose membrane using a Semiphor semi-dry transfer unit (Hoefer Scientific, San Francisco, CA) with constant current of 0.8 mA/cm^2 for 1 hour. Nitrocellulose membranes were stained
with Ponceau S solution (Sigma) for detection of any transport abnormalities. After a 1-hour incubation in TBS (0.05% Tween 20, 100 mM NaCl, 20 mM Tris-HCl, 5% nonfat dry milk), the membranes were reacted sequentially for 3 hours with the primary antibodies described above and subsequently with horseradish peroxidase-conjugated secondary antisera (goat anti-mouse IgG or IgM, respectively) for 2 hours. Horseradish peroxidase activity was detected by chemiluminescence (ECL detection system, Amersham, UK).

**RESULTS**

We investigated the distribution of four different GJ proteins within the ciliary body epithelium of rabbit and rat. In rabbit, Cx26-like signal was expressed in both epithelial layers (Fig. 1a). In the NPE, intense staining was observed in all aspects of the plasma membrane. In the PE, a weak Cx26-like immunoreactivity was present apically and laterally but not basally. The immunoreaction against Cx32 in rabbit resulted in an extremely faint NPE-labeling, barely detectable above background level (Fig. 1b). Cx50 immunoreactivity, in contrast, was present exclusively in cells of the PE (Fig. 1d). In cross-sections perpendicular to the axis of the ciliary processes, which allows precise optical separation of PE and NPE cells, it was apparent that Cx43 is localized predominantly to the apical PE cell membrane with occasional extensions to the lateral flanks of the PE cells. No differences in the staining pattern for Cx26, Cx43, or Cx50 could be identified when equivalent sections from three different animals were compared. Additionally, the NPE and PE connexin segregation patterns described above were preserved throughout the valleys and ridges of the pars plicata as well as in the pars plana.

In the rat ciliary body, Cx26 (Fig. 1e) and Cx32 (not shown) immunoreactivity was seen only in the endothelial cells of the intraprocessal blood vessels, but not in either epithelial layer. Cx50 staining was confined to the NPE (Fig. 1f) and a few stromal structures. At the basolateral shoulder of the NPE cells, fine, feather-like extensions of the plasma membrane were visible; such a pattern is reminiscent of the lateral membrane interdigitations characteristic for the NPE.8 On the other hand, the extensive corona seen in the rabbit was not present. As found in the rabbit, Cx43 was seen exclusively in the PE, with the highest concentration in the apical membrane (Fig. 1g).

The molecular identity of the polypeptides recognized by the monoclonal antibodies was further assessed by Western immunoblots. The Cx43 immunoblot of rabbit ciliary body membranes revealed a single band of approximately 43 kDa (Fig. 2A). The Cx50 immunoblot showed a predominant band of 50 kDa.
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kDa and a less intense band at 70 kDa, consistent with the known molecular weights for two variants of the Cx50 protein. The Cx26 antibody failed to yield a band at the expected molecular weight with either ciliary body (Fig. 2A) or rabbit liver (Fig. 2B) membranes. The antibody did yield the expected result when used on a rat liver membrane preparation (Fig. 2B) or a mouse liver control (provided by Zymed; not shown). In addition to these bands, Cx26 recognized an approximately 105 kDa polypeptide on the rabbit CBE membranes and a 50 to 60 kDa band on both rabbit and rat liver membranes. Finally, the Cx26 antibody yielded the expected punctate staining pattern in rat liver sections (Fig. 3a) but failed to do so in the identically processed rabbit liver (Fig. 3b). The immunoblots also were probed with Cx32 antibody. There were no positive bands in rabbit ciliary body or liver membranes, but the proper molecular weight band (32 kDa) was present in the rat liver run (not shown).

Double staining techniques in conjunction with confocal microscopy were used to examine the spatial relationship between Cx43 and Cx50, the main connexins identified in either PE or NPE of the two species. Figure 4 shows a double labeling experiment for Cx43 (in red) and Cx50 (in green) carried out in the rabbit. The individually labeled frames are shown for Cx50 (Fig. 4A) and Cx43 (Fig. 4B) as well as the superimposed picture showing both markers (Fig. 4C). A thin, yellow band exactly at the contact zone between both apical membranes suggests spatial overlap between both markers. Equivalent results were obtained in rat ciliary body (Fig. 3d). Additionally, notwithstanding the limited spatial resolution of light microscopy, it was possible to determine that both Cx43 and Cx50 occur in plaques at the same position in opposing apical PE-NPE membranes.

DISCUSSION

Gap junctions are pores in the membranes of adjacent cells, formed by specific connexins that allow the exchange of ions and organic solutes. They occur between homologous and heterologous cells and are an essential part of the communication system of all multicellular organisms. Gap junctional permeability may be regulated or otherwise affected by a variety of modulators and messengers. This regulation allows cells either to act in concert, as in a true syncytium, or to reduce the amount of intercellular communication to permit them to act in isolation.

Our data show that the ciliary body contains connexins organized in punctate patterns, consistent with the observation that GJs occur in plaques consisting of a large number of individual channels. The Western blot analysis provides assurances for the correct identification of Cx43 and Cx50 by the immunohistochemical procedures. The results obtained with the Cx26 antibody, on the other hand, require more careful consideration. In rat, both the staining pattern in the liver and on the Western immunoblot are fully consistent with a positive recognition of a Cx26 epitope by the antibody used. In rabbit, the situation is different, in that the antibody does not yield positive results in either immunoblots or immunohistochemistry of the liver. It appears, therefore, that the Cx26 antibody used is unable to recognize rabbit Cx26; it binds to another, currently unidentified protein present in the CBE membrane. The additional recognition of a second, unexpected 52 kDa polypeptide in both rat and rabbit liver and a third 105 kDa component (in rabbit) suggest that the epitope recognized by the Cx26 monoclonal antibody, or a similar one, may be present in more than one protein. Our findings regarding the CBE distribution of Cx43 are fully consistent with an earlier study by Coca-Prados et al in bovine eyes.

The accumulation of Cx50 and Cx43 at the NPE and PE apical membranes, respectively, suggests the existence of heterotypic junctions at the heterocellular boundary. The simplest interpretation of the data is that Cx43-assembled PE connexons establish whole gap junctions with the available Cx50 connexons present in the NPE apical domain; however, mechanically paired oocytes transfected to express either of the above mentioned connexins were unable to establish viable junctional coupling even though both Cx43 and Cx50 belong to the same α subfamily. Nevertheless, the formation of intercellular junctions may depend on the specific state of the connexins involved, which, in turn, may be influenced by the specific biological environment. For example, despite the predicted incompatibility of connexins consisting of α- and β-subtype connexins and the experimental confirmation of such incompatibility in studies of Cx32 (β) and Cx43 (α)-transfected HeLa cells, Swenson et al have reported the formation of highly viable heterotypic junctions between Cx32 and Cx43-built connexons in the paired oocytes. This apparent discrepancy stresses the relative value of results obtained in vitro transfection systems for in vivo situations. Ultimate support for a Cx43-Cx50 coupling requires demonstration that both polypeptides reside in close proximity within the domain of an ultrastructurally recognizable GJ. At present, though, the required immunoelectronmicroscope study is prevented by the inability of available antibodies to recognize glutaraldehyde-fixed tissue (Wolosin, unpublished).

An alternative option is that the PE Cx43 or the NPE Cx50 interacts with not-yet-identified, compatible connexins expressed in the respective opposite cell rather than with each other. The connexin protein
family has expanded in recent years to include at least 12 distinct members. This report examined only four such proteins. Further studies to determine whether other connexins able to interact with Cx43 or Cx50 are present in these cells will be necessary.

A final possibility is that neither Cx43 nor Cx50 are involved in the formation of the heterocellular apex-to-apex junctions. This, however, seems unlikely; because the spatial distribution of connexons appears to be determined by the availability of compatible docking partners in the opposite membrane, the accumulation of Cx43 in the PE apex provides strong support for the notion that this connexin is part of a heterotypic structure formed between the two layers of the CBE. Furthermore, there are no apicolateral tight junctions in the PE to prevent any unanchored membrane protein from diffusing freely throughout the cell surface. Moreover, if these connexons are not involved in the formation of GJs, intriguing questions arise as to their function as well as to the nature of the entities responsible for the well-established NPE–PE junctional path.

In conclusion, the highly asymmetric distribution of Cx43 and Cx50 at the NPE–PE boundary suggests the existence of heterotypic GJs and, thus, may provide a unique opportunity to identify and further study these structures in vivo.

Key Words

ciliary body, connexin 43, connexin 50, gap junctions, heterotypic

References

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