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Brain Research 898 (2001) 364–367

BRAIN RESEARCH

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Short communication

Expression of connexin 30 in the developing mouse cochlea

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Accepted 30 January 2001

Abstract

Mutations in the *GJB6* gene encoding connexin 30 (Cx30) can cause dominant forms of nonsyndromic deafness. By studying immunohistochemical localization of Cx30 in the mouse cochlea at different ages from 0 to 30 days after birth, we found that the expression of Cx30 is nearly the same as that of Cx26. These findings suggest that as well as Cx26, Cx30 may also contribute to the generation and maturation of endocochlear potential.

Theme: Sensory systems

Topic: Auditory, vestibular, and lateral line: periphery

Keywords: Connexin 30; Endocochlear potential; Fibrocytes; Development; RT-PCR

It has been reported that the gap junction consists of at least 13 protein subunits, and four kinds of connexins have been identified to be associated with human hereditary deafness. Mutations in the *GJB2* gene encoding connexin 26 (Cx26) [3,7] and mutations in the *GJB3* gene encoding connexin 31 (Cx31) [10,17] can cause both recessive and dominant forms of nonsyndromic deafness. Mutations in the *GJB1* gene encoding connexin 32 (Cx32) can result in X-linked Charcot-Marie-Tooth syndrome associated with progressive hearing loss [1]. Mutations in the *GJB6* gene encoding Cx30, another gap junction protein, can lead to dominant forms of nonsyndromic deafness [4].

The human Cx30 amino acid sequence is 95% identical to that of the mouse and shares 77% identity with human Cx26 [6]. Cx30 and Cx26 were expressed in the same regions of the adult rat cochlea [9]. Previously, we reported that the generation and maturation of the endocochlear potential (EP) may depend upon the postnatal development of gap junctional communication among the fibrocytes in the cochlear lateral wall and the Na,K-ATPase activity of the type II and suprastrial fibrocytes [15]. By studying the expression and distribution of Cx30 in the developing mouse cochlea, we investigated the gap junction function of Cx30 in the present experiment.

For RT-PCR, the adult CBA/N mice were anesthetized with urethane and the temporal bones were rapidly removed. The cochlea was picked away in sterile phosphate buffer (PB) under a stereomicroscope and prepared for RNA extraction. Brain and liver were removed in parallel. The extraction of polyadenylated RNA and the synthesis of cDNA were performed according to a procedure described elsewhere [5].

To amplify Cx30 cDNA fragments, polymerase chain reaction (PCR) was performed with sense (GAAGTGTGGGGTGATGAGCAGGAG) and antisense (GGCCTCGAAATGAAGCAGTCCACG) primers [2] and cDNA as a template. Incubation was performed at 94°C for 5 min and the PCR reaction proceeded for 35 cycles: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min in a programmable thermal cycler (Model 2400, Perkin Elmer, Norwalk, USA) using a thermostable Taq DNA polymerase (TaKaRa ExTaq, TaKaRa Shuzo, Otsu, Japan). The

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0006-8993/01/$ – see front matter
PII: S0006-8993(01)02216-8

PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Temporal bones were obtained from pigmented CBA/N mice at different development stages between 0 and 30 days after birth (DAB) and at 1 year 2 months. Ears were harvested from at least three pups at each developmental period. Under anesthesia with urethane (1.5–4.5 g/kg, i.p.), the animals were perfused endocardially with 0.01 M phosphate-buffered saline (PBS, pH 7.2), followed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Cochleae were collected and immersed in the fixative for 2 h at room temperature. Subsequent steps to make paraffin sections of the cochlea were carried out as described elsewhere [15].

For the immunostaining of Cx30, the sections were deparaffinized, rehydrated and exposed to 5% normal goat serum in PBS for 1 h, then incubated at room temperature overnight in a rabbit polyclonal antibody against Cx30 (Zymed Laboratories, San Francisco, USA) diluted to 1:200 with 1% bovine serum albumin (BSA) in PBS. After washing with PBS, the sections were flooded for 1 h with a 1:300 dilution of biotinylated goat anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) in 1% BSA–PBS and washed with PBS again, then flooded for 1 h with Vectastain ABC reagent (Vector Laboratories, Burlingame, USA) and thoroughly rinsed in PBS. The sites of the bound primary antibodies were visualized by development for 10 min in 3,3′-diaminobenzidine (DAB)–H_2O_2 substrate medium prior to dehydration and cover slipping. Control sections were processed in parallel in each protocol and included substitution for rabbit antiserum against Cx30 with a similar dilution of non-immune rabbit serum.

As shown in Fig. 1, a single band was detected in both the cochlea and brain after 35 cycles of PCR, while no band was observed in the liver. The nucleotide sequences of the fragments derived from both the cochlea and brain were sequenced, indicating these sequences completely corresponded with that of Cx30 (data not shown). These findings showed the presence of the reverse transcripts for *GJB6* in the cochlea and brain.

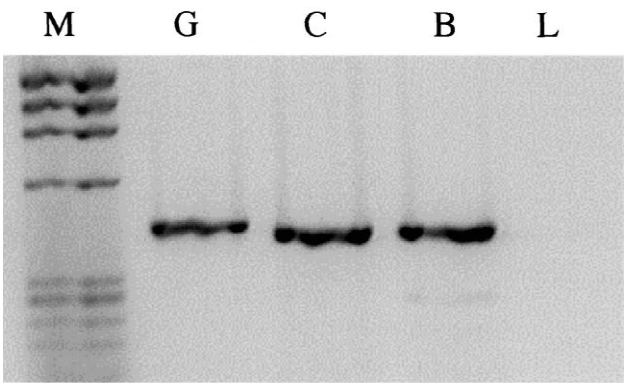

Fig. 1. Electrophoresed gel of RT-PCR analysis of mouse cochlea and brain. Approximately 431 bp bands were detected with Cx30 primers in the cochlea (C) and brain (B), respectively. No band was observed in the liver (L). Glyceraldehyde 3-phosphate dehydrogenase (G) was amplified simultaneously from the cochlear template as a positive control. M, molecular weight marker (ϕX174/HaeIII).

In the basal turn of the 0 DAB cochlear lateral wall, a small amount of reaction product was scattered among the connective tissue cells and among the outer sulcus cells (Fig. 2a). In the apical turn, however, no remarkable immunostaining was observed in the cochlear lateral wall. Very thin connexin 30-like immunoreactivity was maintained in the strial basal cell area from 3 to 6 DAB (Fig. 2b and c), and increased rapidly on 10 DAB in the above area (Fig. 2d). A small amount of reaction product was sparsely distributed among the fibrocytes in the spiral ligament and the suprastrial zone. Immunoreactivity of Cx30 among the spiral ligament fibrocytes was distributed more densely at 12 DAB (Fig. 2e). The distribution of immunoreactivity among the fibrocytes and along the strial basal cell area in the cochlear lateral wall reached the adult pattern at 15 DAB (Fig. 2f). No remarkable difference was observed between 15 DAB and 30 DAB specimens (Fig. 2g). In the mice of 1 year 2 months (Fig. 2h), the dense immunoreactivity was still maintained among the fibrocytes and along the strial basal cells in the cochlear lateral wall. The expression pattern of Cx30 was comparable to the Cx26 in the developing mice cochlea [15]. In contrast to these positive findings, no staining was observed in the control sections.

In the present study, the existence of Cx30 was confirmed by RT-PCR and sequencing in both cochlea and brain. Moreover, the changes in the expression pattern of Cx30 were also investigated by immunohistochemistry in the mouse cochlear lateral wall during the postnatal period. Immunoreactivity of Cx30 was detected as early as 0 DAB among the immature connective tissue cells, which would develop into mature basal cells of the stria vascularis. The immunoreactivity along the strial basal cell area reached nearly the mature pattern on 10 DAB. However, even at this stage, Cx30 was sparsely distributed among the spiral ligament fibrocytes. The distribution of immunoreactivity among the fibrocytes and along the strial basal cell area in the cochlear lateral wall reached the adult pattern at 15 DAB. Comparing these results with those of our previous study of Cx26 [15], it was clear that the expression and distribution of Cx30 and Cx26 were identical in the developing mouse cochlea. However, in our other study of Cx31 [16], no immunoreactivity was observed in the inner ear before 10 DAB. The expression and distribution of Cx31 appeared at 12 DAB and reached the adult pattern at 60 DAB in the fibrocytes of the cochlear lateral wall. It is well known that the EP in the mouse cochlea rapidly increases from 10 DAB and reaches the adult level about 2 weeks after birth [11]. The results of the present study suggest that, as well as Cx26, Cx30 may also contribute to the rapid growth and maturation of the EP.

The theory of recycling K^+ from the perilymph to

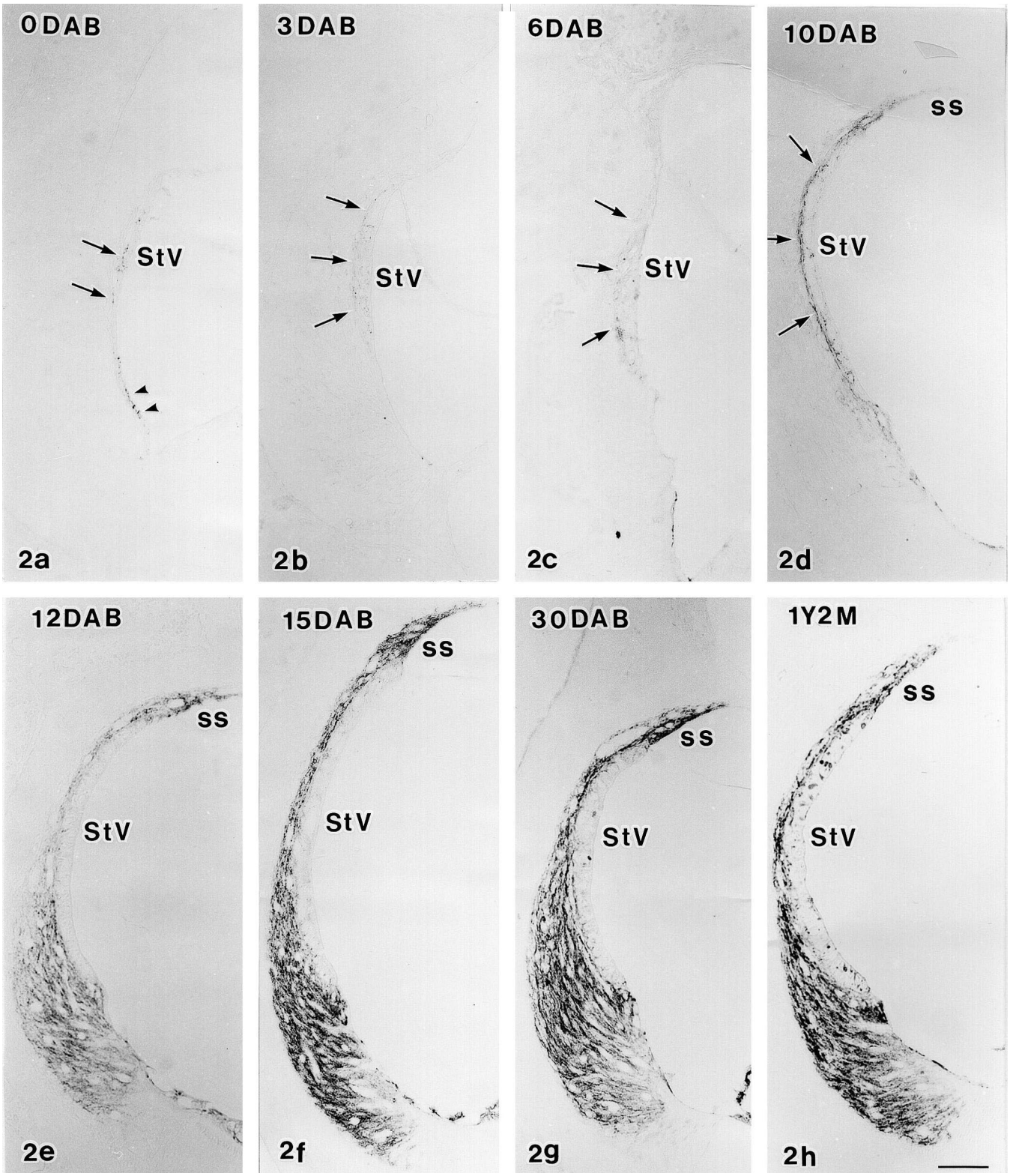

Fig. 2. (a–h) Representative photomicrographs of immunostaining using antibodies against connexin 30 in the developing mice cochlear lateral wall. (a) 0 days after birth (DAB): a small amount of immunoreactivity was scattered among the connective tissue cells which would grow into strial basal cell area in the future (arrows) and among the outer sulcus cells (arrowheads). (b) 3 DAB and (c) 6 DAB: very thin immunostaining of Cx30 was maintained in the strial basal cell area (arrows). (d) 10 DAB: intense immunostaining was observed in the above area (arrows), and small amounts of immunoreactivity were sparsely distributed among the fibrocytes in the spiral ligament and the suprastrial zone. (e) 12 DAB: the immunoreactivity among the fibrocytes and along the strial basal cell area was distributed more densely. (f) 15 DAB: the expression of the immunoreactivity among the fibrocytes and along the strial basal cell area in the cochlear lateral wall reached the adult pattern. (g) 30 DAB. (h) 1Y 2M, 1 year 2 months; StV, stria vascularis; SS, suprastrial zone. Bar=50 μm.

endolymph has been established by previous reports [8,12–14]. When the sensory epithelium is stimulated by mechanical vibration in the cochlea, K^+ ions are expelled basolaterally by sensory hair cells and taken into the supporting cells, then passed through a network of gap junctions from the epithelial supporting cells to the spiral ligament fibrocytes. By the activity of Na,K-ATPase and Na-K-Cl cotransporter within type II fibrocytes, K^+ ions are transported to the intrastrial space, passing through gap junction channels rich in Cx26, Cx30 and Cx31. Finally the K^+ ions are secreted to the endolymph by the marginal cells of the stria vascularis. It remains unclear which portion is damaged in the K^+ transport mechanism, but the dominant forms of nonsyndromic deafness observed in patients with *GJB6* mutations may result from a complete deficit of the K^+ recycling pathway involving the epithelial cell gap junction system and the connective tissue cell gap junction system.

This study suggested that the expression and distribution characteristics of Cx30 in the developing cochlea would not only contribute to the growth and maturation of EP, but also play an important role in the gap junctional communication in ion transport mechanisms.

Acknowledgements

We wish to express our gratitude to Dr. Tomonori Takasaka for his valuable suggestions. This work was supported by a Grant-in-Aid for Science Research (No. 11557122 to K.I and No. 12770943 to Y.K.) from the Ministry of Education, Science and Culture, Japan.

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