letters to nature

paramount for *B. fragilis* to maintain a long-term commensal relationship in the human colon. As *B. fragilis* is the anaerobic species most frequently isolated from clinical infections, and the capsular polysaccharides are instrumental in the disease process, phase variation may also contribute to the pathogenic potential of this organism.

## Methods

### Construction of insertion mutants

Sequences of primers used in this study are listed in Supplementary Information Table 2. DNA used for homologous recombination for each of the four mutants was amplified by PCR from *NCTC9343* using the following primers: PSE: U1-F, UpE-R; PSF: U2-F, UpR-R; PSG: U3-F, UpR-R; PSH: U4-F, UpR-R. These 2.1–3.3-kb products were digested with BamHI and cloned into pLS654. Plasmids were introduced into *B. fragilis* NCTC9343 by mobilization from E. coli and cotransfected were selected using erythromycin.

### Demonstration of inversion and PCR/digestion technique

Primers used to demonstrate inversion — in the order of upstream primer, primer between the inverted repeats and downstream primer — are as follows: PSE: UpE-1, UpE-2, UpE-3; PSF: UpF-1, UpF-2, UpF-3; PSG: UpG-1, UpG-2, UpG-3; PSH: UpH-1, UpH-2, UpH-3.

For the PCR/digestion technique in Fig. 4b, c, a PCR is performed, using chromosomal DNA from a phenotypically selected population, which amplifies the PSA or PSB invertible region and some flanking DNA. The PCR product is digested with a restriction enzyme that cleaves asymmetrically between the inverted repeat elements. When the promoter is in one orientation, the two fragments resulting from the digested PCR product differ in size from those fragments that result when the promoter is in the opposite orientation.

### PSF and PSG inversion junctions

Inversion junctions were determined by sequencing PCR products resulting from amplification using the following primers that only amplify DNA in one orientation: PSF: UpF-2, UpF-3; PSG: UpG-2, UpG-3.

### Creation of xy/E reporter constructs

The 420-bp region upstream of *upaY* was amplified by PCR using primers PMA-1 and PMA-3, and was cloned into the *E. coli* BamHI site of the xy/E reporter plasmid pLEC23. The following primer pairs were used for PCR amplification of the invertible regions lacking 7–9 bp of each inverted repeat: PSA: IRA-F, IRA-R; PSB: IRB-F, IRB-R; PSG: IRG-F, IRG-R; PSH: IRH-F, IRH-R. Each PCR product was cloned into pLEC23 in both orientations. Xy/E assay was performed as described (C.M.K., D. Lipsett and L.E.C., unpublished work).

Supplementary Information accompanies the paper on Nature’s website (http://www.nature.com).

Acknowledgements

We thank W. Kalka-Moll for electron microscope photography and J. Daley and S. Lazo-Kallian for assistance with FACS analysis. This work was supported by grants from the National Institutes of Health.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to L.E.C.
(e-mail: lcomstock@channing.harvard.edu).

© 2001 Macmillan Magazines Ltd

Barttin is a Cl− channel β-subunit crucial for renal Cl− reabsorption and inner ear K+ secretion

Raud Estévez†, Thomas Boettger†, Valentín Stein†, Ralf Birkenhöger†, Edgar Otto†, Friedrich Hildebrandt* & Thomas J. Jentsch†

* Zentrum für Molekulare Neurobiologie (ZMNH), Universität Hamburg, Falkenried 94, D-20246 Hamburg, Germany
† Universitäts-Kinderklinik, Universität Freiburg, Mathildenstraße 1, D-79106 Freiburg, Germany

Renal salt loss in Bartter’s syndrome is caused by impaired transepithelial transport in the loop of Henle. Sodium chloride is taken up apically by the combined activity of NKCC2 (Na+-K+-2Cl− cotransporters) and ROMK potassium channels. Chloride ions exit from the cell through basolateral ClC-Kb chloride channels. Mutations in the three corresponding genes have been identified that correspond to Bartter’s syndrome types 1–3. The gene encoding the integral membrane protein barttin is mutated in a form of Bartter’s syndrome that is associated with congenital

**Figure 1** Functional characterization of ClC-K/barttin in Xenopus oocytes.

a–f, Measurements of current (I). Barttin (b), ClC-Ka (c) and ClC-Kb (d) alone gave no significant currents. ClC-Ka/barttin co-expression gave large currents (e), and ClC-Kb/barttin moderate currents (f). ClC-Kb/barttin(Y98A) currents. g, h, Steady-state current—voltage relationships for ClC-Ka/barttin and ClC-Kb/barttin(Y98A) respectively, in the presence of: Cl− (diamonds); Br− (squares); NO3− (triangles); N2O3 (circles); , , . I. Permeability ratios (P/P) from reversal potentials for ClC-Ka/barttin (left) and ClC-Kb/barttin(Y98A) (right). Averages from at least two batches of oocytes, five oocytes per batch. j. Effect of extracellular Ca2+ on ClC-Ka/barttin (left) and ClC-Kb/barttin(Y98A) (right) oocytes. k, Effect of extracellular Ca2+ on ClC-Ka/barttin (left) and ClC-Kb/barttin(Y98A) (right) oocytes. l, j and k, conductances at −20 mV were normalized to values at pH 7.4 or 1.8 mM Ca2+, respectively (two or three batches of oocytes, 5–9 oocytes per batch). j. Surface expression of epitope-tagged ClC-Ka/Barttin and ClC-Kb/barttin(Y98A) at 7–13 days post injection. Voltage (V) was clamped between +80 and −100 mV for 0.8 s in 20-mV steps throughout. Error bars, S.E.M.
deafness and renal failure. Here we show that barttin acts as an essential β-subunit for ClC-Ka and ClC-Kb chloride channels, with which it colocalizes in basolateral membranes of renal tubules and of potassium-secreting epithelia of the inner ear. Disease-causing mutations in either ClC-Kb or barttin compromise currents through heteromeric channels. Currents can be stimulated further by mutating a proline-tyrosine (PY) motif on barttin. This work describes the first known β-subunit for CLC chloride channels and reveals that heteromers formed by ClC-K and barttin are crucial for renal salt reabsorption and potassium recycling in the inner ear.

ClC-Ka and ClC-Kb are highly homologous Cl channels that are nearly exclusively expressed in kidney. CLCNKB mutations in Bartter’s syndrome together with immunohistochemical results suggest that human ClC-Kb (the orthologue of rodent ClC-K2) mediates basolateral Cl efflux in the thick ascending limb of Henle’s loop and in more distal nephron segments. Similarly, immunolocalization and the diabetes insipidus observed in Clcnk1 mice indicate that rodent ClC-K (the orthologue of human ClC-Ka) is crucial for transepithelial transport in the thin ascending limb. Whereas ClC-K1 yields Cl currents on heterologous expression, no currents are observed with human ClC-Ka or ClC-Kb, suggesting that ClC-K channels may need β-subunits.

Positional cloning of the gene BSND, which underlies Bartter’s syndrome type 4 (also named BSND; OMIM accession number 602522), identified barttin. When barttin was co-expressed with ClC-Ka in Xenopus oocytes, large Cl currents were observed (Fig. 1c). No currents were seen with barttin, ClC-Ka or ClC-Kb alone (Fig. 1a, b, d). In oocytes, barttin/ClC-Kb co-expression gave small but detectable currents (Fig. 1e). More pronounced effects on ClC-Kb were seen in transfected tsA201 cells (see Supplementary Information A), and in oocytes injected with an activating mutant of barttin (Y98A; see below) (Fig. 1f). In oocytes, voltage activation differed between ClC-Ka/barttin and ClC-Kb/barttin (Fig. 1c, e, f). No such difference was found in mammalian cells (see Supplementary Information A). Barttin also markedly increased currents of rat ClC-K1, which yields small currents by itself (Fig. 2b). The effect seemed to be specific for ClC-K because currents of ClC-1, ClC-2 and ClC-5 were not changed by barttin (data not shown).

Enhanced surface expression of ClC-Ka, but not of the muscle channel ClC-1 (Fig. 1l), and bicarbonate exchange of ClC-Kb3,19 resulted in significant reductions of ClC-Ka or ClC-Kb (Fig. 3a, b). Hence this mutation might cause disease by a mechanism that is not reflected in our expression system. Disease-causing ClC-Kb mutations (G10S) increased ClC-Ka currents over those obtained with wild-type barttin, and did not reduce effects on ClC-Kb when studied in the framework of the activating Y98A mutant (Fig. 3a, b). Hence this mutation might cause disease by a mechanism that is not reflected in our expression system. Disease-causing ClC-Kb mutations (G10S) resulted in significant reductions or the loss of ClC-K/barttin currents (Fig. 3c).

In situ hybridization showed barttin expression in specific nephron segments and in the stria vascularis. If barttin acts as a β-subunit for ClC-Ka and ClC-Kb, these proteins should colocalize in membranes. Immunofluorescence revealed that all nephrin

**Figure 2** Basic structural and functional features of barttin. a, Proposed topology. Described and newly identified mutations are indicated. The PY motif is compared with similar motifs of ENaC and ClC-5 (ref. 14) (inset). The asterisk shows the tyrosine mutated to alanine in barttin(Y98A). Truncations are indicated by slashes and the number of the stop codon. b, Effect of truncated barttin on ClC-K1 in oocytes. Averaged conductances at −20 mV of two batches (11 oocytes). c, Effect of the PY-motif mutation Y98A (‘PY’) on ClC-Ka (left) and ClC-Kb (right). Normalized currents of 15 oocytes from three batches. WT, wild type.

**Figure 3** Functional consequences of disease-associated mutations in Xenopus oocytes. a, b, Effect of barttin (B) missense mutations on ClC-Ka (a) and ClC-Kb (b). c, Effects of CLCNKB mutations (G10S) on ClC-Kb/barttin. In b and c, barttin(Y98A) was used to obtain sufficient currents. Conductances at −20 mV are averages of 7–25 oocytes each. WT, wild type.
Figure 4 barttin and ClC-K proteins in murine kidney. a–d, overviews of renal cortex (a, b) and medulla (c, d) reveal strict co-expression of barttin (red) and ClC-K (green) and vice versa (Fig. 4a–h). As the ClC-K antibody does not distinguish between the highly homologous ClC-K1 and ClC-K2 proteins, this suggests that barttin forms heteromers with ClC-K1 (ClC-Ka) in the thick ascending limb of Henle and more distal segments (Fig. 4e–l). Staining for the basolateral anion exchanger AE1 (green) identifies α-intercalated cells (Fig. 4f, i, j) and barttin (red) in basolateral membranes of which stained for barttin (Fig. 4f, i, j) and ClC-K (Fig. 4e). Barttin was also detected in basolateral membranes of intercalated cells of the collecting duct (Fig. 4h, k, l), which are expressed in apical membranes of the thick ascending limb, the basolateral membranes of which stained for barttin (Fig. 4f, i, j) and ClC-K (Fig. 4e). Barttin was also detected in basolateral membranes of intercalated cells of the collecting duct (Fig. 4h, k, l), which are known to express ClC-K2 (ClC-Kb) as well (Fig. 4g). On the basis of the staining for the basolateral anion exchanger AE1, which identifies α-intercalated cells, both acid-secreting and base-secreting β-intercalated cells express barttin basolaterally (Fig. 4k), but intervening aquaporin-2-expressing (green) principal cells appear devoid of barttin (Fig. 4l).

In the inner ear, barttin colocalized with ClC-K in K+–secreting marginal cells of the stria vasularis (Fig. 5d). The basolateral staining for both proteins contrasts with the apical localization of the KCNQ1 K+ channel (Fig. 5a). Barttin (green) was also found in K+-intercalated cells and base-secreting vestibular dark cells, where it colocalized in basolateral membranes with ClC-K (not shown) below apical membranes that expressed KCNQ1. No balance problems were reported in BSND, but humans can adapt well to vestibular disturbances.

This work has identified a β-subunit for ClC-K Cl− channels. In the kidney, ClC-K/barttin heteromers mediate Cl− reabsorption by facilitating its basolateral efflux (Fig. 6a). In the stria, ClC-K/barttin channels drive K+ secretion by recycling Cl− for the basolateral NKCC1 cotransporter (Fig. 6b). This role is analogous to that of ROMK in Cl−-reabsorbing cells of the thick ascending limb, where it recycles K+ for the apical NKCC2 cotransporter (Fig. 6a).

Because barttin is crucial for ClC-Kb function, its inactivation results in renal salt wasting as do mutations in ClC-Kb. However, because barttin also associates with ClC-Ka, additional symptoms are expected. These may resemble the diabetes insipidus-like phenotype observed on disrupting mouse ClC-K1 (Fig. 6a). Indeed, BSND patients present with more severe renal symptoms than patients having mutations in ClC-Kb. Unlike mutations in barttin, mutations in the ClC-Kbα-subunit do not cause deafness, nor was deafness described in mice disrupted for ClC-K1.

Figure 5 barttin and ClC-K protein in the inner ear. a, overview of a P0 (postnatal day 0) mouse cochlea stained for barttin (green), KCNQ1 (red) and nuclei (blue). RM, Reissner’s membrane (ruptured during sectioning); SM, scala media; ST, scala tympani; SV, scala vestibuli. The box is enlarged in b. Barttin (green) and KCNQ1 (red) reveal complete colocalization (yellow). e, dark cells surrounding the crista ampullaris (CA) express barttin (green) and KCNQ1 (red). Scale bar in a indicates 38 μm in a, 7 μm in b, 22 μm in c and d, and 31 μm in e.
Immunohistochemistry

Anaesthetized adult mice were perfused through the left ventricle with PBS followed by 4% paraformaldehyde (PFA) in PBS. Adult cochleae were dissected from the temporal bone and embedded in paraffin wax. 3-μm sections were stained for Glial fibrillary acidic protein (GFAP) using the primary antibody (Dako, clone G0595) and visualized using the secondary antibody (Alexa Fluor 488 goat anti-rabbit, Invitrogen). Counterstaining was performed with DAPI (Molecular Probes). The localization and quantification of barttin was performed using a confocal microscope (LSM 700; Carl Zeiss, Jena, Germany), with z-stacks being captured to assess the subcellular localization of barttin. The images were processed with Adobe Photoshop CS6 (Adobe Systems). The data were analyzed using ImageJ (NIH) and GraphPad Prism (GraphPad Software).

Acknowledgements

We thank S. Alper for the AE1 antibody, M. Knuper for the aquaporin-2 antibody, and M. Knuper for advice on inner ear immunohistochemistry, and I. Endlicher and M. Kolster for technical assistance. R.E. is a recipient of a Marie Curie Human Potential Fellowship of the European Union, and E.H. is a Heisenberg scholar of the Deutsche Forschungsgemeinschaft (DFG). This work was supported by grants from the DFG, the Fonds der Chemischen Industrie, and the Prix Louis Jeantet de Medicine to T.I.T., and from the Federal State of Baden-Württemberg to E.H.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to T.I.T. (e-mail: jentsch@rmm.uni-hamburg.de).

References


Supplementary Information accompanies the paper on Nature’s website (http://www.nature.com).