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**REGULATION OF PENDRIN BY pH: DEPENDENCE ON GLYCOSYLATION**

Running title: Mathematical Modeling of Pendrin Activity

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## SYNOPSIS

Mutations of the anion exchanger pendrin are responsible for Pendred syndrome, an autosomal recessive disease characterized by deafness and goiter. Pendrin is highly expressed in kidney collecting ducts, where it acts as a chloride/bicarbonate exchanger and thereby contributes to the regulation of acid-base homeostasis and blood pressure. This study aimed to characterize the intrinsic properties of pendrin. Mouse pendrin was transfected in HEK293 and OKP cells; its activity was determined by monitoring changes in intracellular pH induced by variations of trans-membrane anion gradients. Combining measurements of pendrin activity with mathematical modeling, we found that its affinities for  $\text{Cl}^-$ ,  $\text{HCO}_3^-$  and  $\text{OH}^-$  vary with intracellular pH, resulting in increased activity at low intracellular pH. Pendrin maximal activity was also stimulated at low extracellular pH, suggesting the presence of intra- and extracellular proton regulatory sites. We identified 5 putative pendrin glycosylation sites, only two of which are used. Mutagenesis-induced disruption of pendrin glycosylation did not alter its cell surface expression, polarized targeting to the apical membrane and basal activity, but fully abrogated its sensitivity to extracellular pH. The hereto unknown regulation of pendrin by external pH may constitute a key mechanism in controlling ionic exchanges across the collecting duct and inner ear.

**Word count:** 200

**Keywords:** pendrin; anion exchanger; mathematical modeling; transport kinetics; glycosylation; pH.

**Abbreviations:** B-IC, type-B intercalated cells; CCD, cortical collecting duct; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonate.

## INTRODUCTION

Pendred syndrome is an autosomal recessive disease characterized by deafness and goiter [1]. It is associated with mutations of the *PDS* gene which encodes pendrin, an anion exchanger that is a member of SLC26 family (Slc26a4). Pendrin exchanges extracellular chloride against either intracellular bicarbonate or iodide or formate [2-4]. The  $\text{Cl}^-/\text{I}^-$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchange functions of pendrin account for its role in iodide organification in the thyroid and in pH homeostasis of endolymph respectively, which are altered in Pendred syndrome [5, 6]. Even though patients with Pendred syndrome display no clinical signs beyond the thyroid and inner ear, pendrin is expressed in many tissues including mammary glands [7], uterus [8], testis [9], placenta [9], and kidneys [10]. Kidneys even display a higher expression level of pendrin mRNAs than the thyroid gland and inner ear.

In the kidney, pendrin is expressed at the apical border of the type-B intercalated cells (B-IC) present in the cortical collecting duct (CCD). B-ICs play a major role in the regulation of acid-base balance by mediating bicarbonate secretion through an apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger functioning in series with a basolateral plasma membrane  $\text{H}^+$ -ATPase. Recent studies have shown that  $\text{HCO}_3^-$  secretion in CCDs is abolished in pendrin knockout mice, which indicates that apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange in B-ICs is fully mediated by pendrin [10, 11]. Accordingly, both the expression and the activity of the apical anion exchanger are upregulated in CCD after induction of metabolic alkalosis by either administration of aldosterone or  $\text{NaHCO}_3$  loading, and aldosterone-induced metabolic alkalosis is more severe in pendrin knockout mice than in controls [12].

In addition, B-ICs are sites of chloride reabsorption through the apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger in series with basolateral chloride channels *ClCK*. Chloride reabsorption in CCDs is fully abolished in pendrin knockout mice [11], indicating that pendrin-mediated transcellular transport in B-ICs is the only pathway for chloride reabsorption in CCDs. Moreover, the expression of pendrin in the kidney of rats fed different amounts of chloride is inversely correlated with the urinary excretion of chloride, independently of the accompanying cation and acid-base status [13, 14]. This suggests a major role for pendrin in the regulation of chloride balance.

Finally, it was reported recently that coupling of pendrin with the  $\text{Na}^+$ -driven  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (NDCBE/Slc4a8) mediates electroneutral reabsorption of  $\text{NaCl}$  in the CCD [15]. The presence of this thiazide-sensitive pathway for  $\text{NaCl}$  reabsorption, which operates in parallel with the amiloride-sensitive epithelial sodium channel, likely accounts for the fact that isolated alteration of  $\text{Cl}^-$  transport in the distal nephron can impact blood pressure [16-19] and that pendrin knockout mice are protected against mineralocorticoid/salt-induced hypertension [12].

The importance of pendrin in mediating  $\text{Na}^+$  and  $\text{Cl}^-$  reabsorption and  $\text{HCO}_3^-$  secretion in the CCD, and in regulating acid-base balance, extracellular volume and blood pressure, underlies the need to characterize its intrinsic properties, which have remained poorly documented until now. The purpose of this study was to functionally characterize pendrin using in vitro expression models. Our major findings are that pendrin activity is modulated by pH and that this regulation depends on pendrin glycosylation.

## EXPERIMENTAL

**Cloning of mouse pendrin cDNA.** Mouse cDNA was generated from total RNA using Superscript III RT (Invitrogen). The coding sequence of pendrin was amplified by PCR using the platinum *pf*x polymerase (Invitrogen) and the following primers: sense, 5'GTCATCCC-TCGTCGCATC3' and reverse 5'TCTCAGGAAGCAAGTCTACGC3'. After a subsequent A-tailing with Taq polymerase, the mouse pendrin coding sequence was subcloned into a pTarget mammalian expression vector following the manufacturer's protocol (Promega, Madison WI).

**Site-directed mutagenesis.** Asparagines at positions 167, 172, 241, 475 and 567, predicted by the NetNGlyc 1.0 Server to be putative N-glycosylation sites, were mutated to alanines using site-directed mutagenesis on the wild-type pendrin-pTarget construct. Site-specific mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, USA), and mutations were confirmed by sequencing the plasmid DNA (GATC Biotech AG, Deutschland). All mutants were then transiently transfected into Human Embryonic Kidney 293 (HEK293) cells for protein expression assays and stably transfected in Opossum Kidney (OKP) cells for transport activity assays.

**Cell culture and transfection.** OKP and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen) supplemented with 2mM glutamine and 10% fetal bovine serum (Gibco Invitrogen), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a 5% CO<sub>2</sub>/95% O<sub>2</sub> mix. Cells grown in 60 mm dishes were transfected with 1µg of pendrin cDNA construct using Lipofectamine Plus kit (Invitrogen) according to the manufacturer's protocol. Transiently transfected cells were studied 48 h after transfection for protein expression and activity. For stable transfections, cells were selected by their resistance to G418 (500 µg/ml) (Invitrogen). Single clones were isolated and placed into the separate wells of a 24 well plate. Cells were grown to confluence sufficient for detection of pendrin by western blotting and immunostaining. For experiments, one clone was extensively analysed. Non-transfected cells were used as control.

**Cell surface biotinylation.** Cells were placed on ice and rinsed twice with a cold rinsing solution at pH 8 containing PBS, 1 mM MgCl<sub>2</sub>, and 0.1 mM CaCl<sub>2</sub> (PBS-Ca-Mg). Cells were then incubated at 4°C for 1 h in PBS-Ca-Mg pH8, containing 1 mg/ml NHS-biotin. Biotinylation was stopped by washing 3 times with ice-cold PBS-Ca-Mg supplemented with 100 mM glycine. Cells were then washed 3 times in PBS-Ca-Mg. Washed cells were lysed for 20 min at 4 °C in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris/Hepes, pH 7.5; 1% (v/v) Triton X-100) containing protease inhibitors (Complete 1697498, Roche Diagnostics). To quantify protein amounts, 10µg of total cell extract from each sample was used to provide a measure of total pendrin expression, and 50µg of cell lysates were incubated with avidin beads (Sigma) overnight at 4°C. After overnight incubation, samples were centrifuged at 16,000 rpm for 5 min, and the supernatant (the intracellular fraction) was removed. Avidin beads were then washed 3 times with lysis buffer, twice with high salt buffer (500mM NaCl, 5mM EDTA, 50mM Tris, 0.1% triton X100, pH7.5) and once with no salt buffer (10mM Tris pH 7.5) and then denaturated for 20 minutes at 65°C with Laemmli denaturing buffer and subjected to SDS-PAGE and immunoblotting.

**Immunoblotting.** Western blotting was performed on whole cell lysates. HEK293 or OKP cells were solubilised in high-salt lysis buffer (HSB) containing 500 mM NaCl, 50 mM Tris (pH 8), 1% Triton X100, 0.1% SDS, with the complete protease inhibitor cocktail (Roche Diagnostics). For enzymatic deglycosylation 20 µg of proteins were treated with either N glycosidase F or endoglycosidase H following the manufacturer's protocols. Proteins were resolved for 3 h at 300 V in 10% SDS-PAGE (Bio-Rad) and transferred to nitrocellulose membrane (GE Healthcare) for 2 hours at 600 mA. Blots were blocked using 5% non-fat milk in TBS

0.1% Tween 20 for 1 h and then incubated overnight with primary anti-pendrin antibody diluted 1:5000 in TBS 0.1% Tween 20. Blots were then washed 3 times with TBS 0.1% Tween 20, and incubated with horseradish peroxidase-conjugated goat anti rabbit IgG (Promega, Madison, WI, USA) 1:10000 in TBS 0.1% Tween 20. Blots were washed as described above and assayed with ECL using SuperSignal Substrate (Pierce, Rockford, IL, USA) and captured on a light sensitive imaging film (Kodak).

The anti-pendrin antibody (gift from Dr D Eladari), a rabbit polyclonal antibody directed against a peptide corresponding to the C-terminal domain of mouse pendrin (amino acids 630 to 643), has been characterized previously [20, 21].

**Immunofluorescence.** OKP cells plated at confluent density on 12 mm transwell porous cell culture inserts (0.4  $\mu\text{m}$  pore, Costar) for 7 days and HEK293 cells grown on glass coverslips were washed twice with an ice-cold PBS solution at pH 8 containing 1 mM  $\text{MgCl}_2$  and 0.1 mM  $\text{CaCl}_2$ , and were then placed at 4°C for 1h in PBS solution containing 1 mg/ml EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce). After 3 washes with PBS, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 30 min at room temperature, then washed 3 times with PBS and permeabilized with 0.1% Triton X100 for 15 min. Cells were blocked for 30 min with 3% normal goat serum (Vector Laboratories) and incubated with anti-pendrin antibody diluted 1:400 in 3% normal goat serum for 1h. After 3 washes with PBS, cells were incubated for 1h in the dark with FITC-conjugated goat anti-rabbit IgG diluted 1:100 and Cy5-conjugated streptavidin (Sigma Aldrich) at 1:100. Filters were excised from the filter cup with a razor blade and mounted with Vectashield H1200 (Vector Laboratories) mounting media containing 4,6-diamidino 2-phenylindole dihydrochloride for nuclei staining. Slides were visualised with a Zeiss LSM 510 laser-scanning microscope using appropriate filters (Carl Zeiss, Germany).

**Intracellular pH measurement.** Activity of pendrin in transfected cells was assessed based on the rate of variation of intracellular pH ( $\text{pH}_i$ ) in response to the generation of transmembrane gradients of  $\text{Cl}^-$ ,  $\text{HCO}_3^-$  or  $\text{OH}^-$  concentration. The activity of pendrin was calculated as the difference between the rate of pH variation in transfected cells and that in non-transfected cells in the same experimental series. Changes in transmembrane ion gradients were obtained by varying the composition of the extracellular medium, as indicated in the *Results* section. For  $\text{pH}_i$  measurements, HEK293 cells grown to confluence on glass coverslips coated with 0.5 mg/ml poly D-lysine (Sigma) were incubated for 30 min at 37°C in the presence of 5  $\mu\text{M}$  pH sensitive dye 2',7'-bis(2-carboxyl)-5(6)-carboxy-fluorescein (BCECF) (Invitrogen) in isotonic solutions. Changes in intracellular pH were monitored at excitation wavelengths of 500 and 450 nm, at 37°C, in a FluoroMax3 spectrofluorometer (Jobin Yvon, Horiba). Three-point calibration curves (pH 6.0, 7.0 and 8.0) were established using KCl/nigericine.  $\text{Cl}^-$ -containing solutions consisted of NaCl at concentrations varying from 0.5 mM to 120 mM, Na-gluconate, KCl, and K-gluconate. All solutions contained 0.2 mM  $\text{KH}_2\text{PO}_4$ , 0.8 mM  $\text{K}_2\text{HPO}_4$ , 1 mM Ca-gluconate, 1 mM Mg-gluconate and 10 mM HEPES. For  $\text{Cl}^-$ -free solutions, all  $\text{Cl}^-$  containing salts were replaced with gluconate salts. For  $\text{HCO}_3^-$ -containing solutions Na-gluconate was replaced isosmotically with  $\text{NaHCO}_3$ . Bicarbonate containing solutions were prepared temporarily to intracellular pH measurements. The actual concentration of bicarbonate was calculated as explained in **Appendix A**.

The intrinsic buffering power ( $\beta_i$ ; mM  $\text{H}^+$ /pH unit) was measured in transfected and non-transfected HEK293 cells using the  $\text{NH}_4^+$  pulse method. To eliminate all pendrin activity the measurements were done in the presence of 0.5 mM DIDS. Cells were initially loaded with BCECF in  $\text{Na}^+$ - and bicarbonate-free isotonic solutions. NaCl was iso-osmotically replaced with NMDGCl. The  $\Delta\text{pH}_i$  of cell alkalisation was measured after the addition of 20 mM  $\text{NH}_4\text{Cl}$  by iso-osmolar replacement of NMDGCl. The base equivalent transport flux was cal-

culated by multiplying the buffering power ( $\text{mM H}^+/\text{pH unit}$ ) by the transport rate ( $\text{pH unit/min}$ ) and expressed in  $\text{mM/min}$ .

When tested, pharmacological agents (Sigma Aldrich) were added simultaneously with BCECF. They were dissolved in DMSO (Sigma, 1% final concentration), and the same concentration of DMSO was added to controls.

**Measurement of intracellular chloride concentration.** Pendrin-transfected HEK293 cells grown to confluence on glass coverslips were labelled for 24 hours with 5  $\text{mM}$  SPQ (6-methoxy-N-(3-sulfopropyl)-quinolinium, inner salt) (Molecular Probes) in sterile DMEM medium supplemented with 10% bovine serum. Afterwards, cells were washed extensively in isotonic buffer at pH 7.4, prior to 30 min incubation in the same buffer at  $37^\circ\text{C}$ . The isotonic buffers were either  $\text{Cl}^-$ -free solutions or  $\text{Cl}^-$ -containing solutions consisting of NaCl at concentrations varying between 4  $\text{mM}$  and 120  $\text{mM}$ . After incubation cells were washed again 5 times and SPQ fluorescence was measured in a FluoroMax3 spectrofluorometer at excitation and emission wavelengths of 365 nm and 443 nm respectively. Calibration curves were established using KCl/nigericine (5  $\mu\text{M}$ )/tributyltine (10  $\mu\text{M}$ ). For calibration  $\text{Cl}^-$ -containing solutions consisted of KCl at concentrations varying from 4  $\text{mM}$  to 120  $\text{mM}$ , Na-gluconate, Mg-gluconate, Ca-gluconate, K-gluconate and Hepes.

**Statistics.** Values are expressed as means  $\pm$  S.E. The significance of differences between mean values was evaluated using paired or unpaired  $t$  test or analysis of variance as appropriate.  $p < 0.05$  was considered statistically significant.

## RESULTS

**Expression of pendrin protein in HEK293 cells.** We first determined the expression of pendrin protein and its location in HEK293 cells stably transfected with mouse pendrin cDNA. **Figure 1** shows that all the cells derived from a single clone displayed pendrin-positive staining. Pendrin immunoreactivity was mainly present at the plasma membrane since it colocalized with biotin. In contrast, non-transfected or mock-transfected cells did not show any positive stain for pendrin (data not shown).

**Measurements of pendrin activity.** We next characterized the functional expression of pendrin by determining the rate of ion transport in either  $\text{Cl}^-/\text{OH}^-$  or  $\text{Cl}^-/\text{HCO}_3^-$  exchange mode in pendrin cDNA-transfected cells. Previous studies demonstrated that pendrin functions in  $\text{Cl}^-/\text{OH}^-$ ,  $\text{Cl}^-/\text{HCO}_3^-$ , or  $\text{Cl}^-/\text{formate}$  electroneutral exchange mode [22, 23].

*$\text{Cl}^-/\text{OH}^-$  exchange.* Cells were first incubated in a  $\text{Cl}^-$ -free medium for 30 min at 37°C and then monitored for changes in intracellular pH ( $\text{pH}_i$ ) after addition of extracellular  $\text{Cl}^-$ . **Figure 2A** shows that the addition of extracellular  $\text{Cl}^-$  (120 mM) caused a more rapid cellular acidification in pendrin-transfected cells than in non-transfected cells. This phenomenon was reversed after the cells were switched back to a  $\text{Cl}^-$ -free medium (**Figure 2B**). The initial rate of cell acidification, corresponding to the forward rate of  $\text{Cl}^-/\text{OH}^-$  exchange, was  $\sim 0.3$  and  $\sim 0.04$   $\text{pH unit}\cdot\text{min}^{-1}$  in transfected and non-transfected cells respectively. The backward rate of  $\text{Cl}^-/\text{OH}^-$  exchange was  $\sim 0.2$  in transfected cells and  $\sim 0.03$   $\text{pH unit}\cdot\text{min}^{-1}$  in non-transfected cells.

*$\text{Cl}^-/\text{HCO}_3^-$  exchange.* In these series of experiments, cells were first incubated in a bicarbonate-free and  $\text{Cl}^-$ -containing medium for 30 min at 37°C, and then monitored for  $\text{pH}_i$  changes after switching to a bicarbonate-containing and  $\text{Cl}^-$ -free medium. As shown in **Figure 2C**, the concomitant addition of bicarbonate (25 mM) and removal of chloride from the medium caused a transient acidification of both pendrin-transfected and non-transfected cells that is likely accounted for by  $\text{CO}_2$  entry. Afterwards, a more rapid intracellular alkalinisation was observed in pendrin-transfected cells than in non-transfected cells. The initial rate of cell alkalinisation, corresponding to the rate of  $\text{Cl}^-/\text{HCO}_3^-$  exchange, was  $\sim 0.8$  and  $\sim 0.2$   $\text{pH unit}\cdot\text{min}^{-1}$  in transfected and non-transfected cells respectively.

We also confirmed that pendrin is inhibited by 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), as previously described [22]. Pendrin activity determined under forward  $\text{Cl}^-/\text{HCO}_3^-$  exchange mode was fully inhibited by 0.5 mM DIDS: (in  $\text{mM}\cdot\text{min}^{-1} \pm \text{SE}$ ) Control,  $20.1 \pm 1.0$ ; DIDS,  $1.4 \pm 1.4$ ;  $n = 6$ ,  $p < 0.001$ .

**Effect of pH on pendrin activity.** We examined whether pendrin is regulated by pH by measuring its activity while either the extracellular ( $\text{pH}_e$ ) or the intracellular ( $\text{pH}_i$ ) pH was varied. In the first series of experiments, the cells were incubated in a  $\text{Cl}^-$ -free medium at  $\text{pH}_e = 7.4$ ; their  $\text{pH}_i$  rose to an equilibrium value of 7.85. At  $t = 0$ , the cells were suddenly exposed to a 120 mM  $\text{Cl}^-$  medium of variable  $\text{pH}_e$ . The bath switch resulted in  $\text{Cl}^-$  influx and  $\text{OH}^-$  efflux. As shown in **Figure 3A**, the initial flux of  $\text{OH}^-$  out of the cell was found to increase with decreasing  $\text{pH}_e$ .

To examine the effect of intracellular pH on the activity of the exchanger, the cells were incubated in a 120 mM  $\text{Cl}^-$  medium at variable  $\text{pH}_e$  (6.0, 7.4, and 8.0); their equilibrium  $\text{pH}_i$  then reached varying values (6.2, 7.0, and 7.5). At  $t = 0$ , the cells were exposed to a  $\text{Cl}^-$ -free medium at a fixed  $\text{pH}_e$  of 8.0, resulting in  $\text{Cl}^-$  efflux and  $\text{OH}^-$  influx into the cells. The initial rate of cell alkalinization was seen to increase with decreasing  $\text{pH}_i$ , as displayed in **Figure 3B**.

A decrease in extracellular or intracellular pH enhances the gradient for  $\text{OH}^-$  efflux or influx, respectively, and therefore might account for the observed increase in pendrin activity.



To evaluate whether the pH dependence reflects a true regulation of pendrin or a concentration gradient effect, we then sought to determine the kinetics parameters of pendrin.

**Kinetic parameters of pendrin.** To calculate the affinities of pendrin to  $\text{Cl}^-$ ,  $\text{OH}^-$  and  $\text{HCO}_3^-$ , we first determined pendrin activity by measuring  $\text{pH}_i$  variations in the presence of different anion concentrations, in several exchange modes. In all experiments, the activity of pendrin was calculated as the difference in the initial rate of  $\text{pH}_i$  variation between pendrin-transfected and non-transfected cells. Next, the affinities were calculated based on a theoretical model of pendrin-mediated ion transport.

*Activity of pendrin as a function of external chloride.* Cells were pre-incubated in a  $\text{Cl}^-$ -free medium, then switched to a medium containing different concentrations of  $\text{Cl}^-$  (0.5 to 100 mM), and the initial rate of acidification was measured. As expected, increasing extracellular  $\text{Cl}^-$  concentration increased pendrin activity in a saturable fashion. Linearizing the data according to the Lineweaver-Burk equation yielded an apparent external  $\text{Cl}^-$  affinity of 1.35 mM and a  $V_{\text{max}}$  of 0.3 pH unit. $\text{min}^{-1}$  (**Figure 4A**).

*Activity of pendrin as a function of internal chloride.* To determine the apparent intracellular affinity of pendrin for  $\text{Cl}^-$ , we measured the initial rate of alkalisation during backward  $\text{Cl}^-/\text{OH}^-$  exchange. Thus, after pre-incubation in  $\text{Cl}^-$ -free medium, the cells were switched to a medium containing various concentrations of  $\text{Cl}^-$  and the change in  $\text{pH}_i$  was monitored. When  $\text{pH}_i$  equilibrium was achieved, the cells were switched back to a  $\text{Cl}^-$ -free medium and the initial rate of alkalisation was determined. The internal concentration of  $\text{Cl}^-$  at the onset of the alkalisation phase was determined by SPQ fluorescence in separate experiments. Using this protocol we were able to vary the intracellular concentration of  $\text{Cl}^-$  from 0.5 to 50 mM. This was accompanied by a saturable activation of pendrin, and an apparent internal  $\text{Cl}^-$  affinity of 24 mM and a  $V_{\text{max}}$  of 0.2 pH unit. $\text{min}^{-1}$  were derived from the Lineweaver-Burk plot (**Figure 4B**).

*Activity of pendrin as a function of external bicarbonate.* Following pre-incubation in a bicarbonate-free and  $\text{Cl}^-$ -containing medium, the cells were switched to a  $\text{Cl}^-$ -free medium containing various concentrations of  $\text{HCO}_3^-$  and the initial rate of alkalisation was measured. Bicarbonate was added at concentrations ranging from 1 to 25 mM, and the corresponding actual concentrations of  $\text{HCO}_3^-$  in the medium were calculated as described in **Appendix A**. A Lineweaver-Burk plot of the data gave an apparent external  $\text{HCO}_3^-$  affinity of 2.6 mM and a  $V_{\text{max}}$  of 0.8 pH unit. $\text{min}^{-1}$  (**Figure 4C**).

The curves representing the initial flux across pendrin as a function of external or internal anion concentrations (**Figure 4A-C**) were then used to determine the kinetic parameters of the exchanger. We examined different models (such as the bi bi cyclic reaction scheme, the six-state ping-pong model, an eight-state exchanger scheme) and obtained the best fit to the data using the ping-pong mechanism depicted in **Figure 5**. According to this scheme, after  $\text{Cl}^-$  binds to the empty carrier on one side, it is translocated to the other side; only after it is released can the other ion ( $\text{HCO}_3^-$  or  $\text{OH}^-$ ) bind to the carrier and be translocated back in the opposite direction. Note that this kinetic scheme is identical to that assumed for the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger AE1 [24]. To fully characterize dynamic transport across pendrin, 4 kinetic parameters per ionic species must be determined: the external and internal binding affinities, and the forward and backward translocation rates.

The flux equations are given in **Appendix B**. As discussed therein, the experimental curves in this study can only yield 6 independent parameters, out of a total of 10 (6 affinities and 4 independent translocation rates). We therefore assumed that the internal and external binding affinities of each ion are equal, and we fixed the forward (external)  $\text{Cl}^-$  translocation rate (denoted  $P_{\text{Cl}}^e$ ). The remaining 6 independent parameters (the affinities to  $\text{Cl}^-$ ,  $\text{HCO}_3^-$ , and

$\text{OH}^-$ ; the forward rates for  $\text{HCO}_3^-$  and  $\text{OH}^-$ ; and the backward rate for  $\text{Cl}^-$ ) were then fitted to the experimental data.

Analysis of the experimental results revealed that it was not possible to fit the data shown in **Figures 4A and 4B** simultaneously without accounting for the modulating effect of internal pH ( $\sim 8.0$  in the  $\text{Cl}^-$  influx experiments, vs.  $\sim 7.0$  in the  $\text{Cl}^-$  efflux experiments) on pendrin activity. Moreover, assuming that the kinetic parameters of pendrin are independent of  $\text{pH}_i$ , the model predicts that the driving force in the experiment displayed in **Figure 3B** remains constant, that is, the flux should remain approximately the same as  $\text{pH}_i$  varies between 6.2 and 7.5. Thus, these two sets of results strongly suggest that  $\text{pH}_i$  variations intrinsically modify the kinetics of the transporter, independently of transmembrane concentration gradients. We accounted for this effect by assuming that the binding affinities of  $\text{Cl}^-$  and  $\text{OH}^-$  vary with internal pH.

Fitted parameter values are given in **Table 1**. As described in **Appendix B**, the fitted values of the affinities, and of the translocation rates relative to  $P_{\text{Cl}^-}^c$ , depend very little on the value of  $P_{\text{Cl}^-}^c$ . The affinity of pendrin for  $\text{Cl}^-$  and  $\text{OH}^-$  was respectively calculated as 3 mM and  $1.4 \times 10^{-6}$  M at  $\text{pH}_i = 8.0$ , and as 75 mM and  $2.6 \times 10^{-7}$  M at  $\text{pH}_i = 7.0$ . Predicted flux values based upon these parameters are in good agreement with measured values, as displayed in **Figure 4**.

We then sought to interpret the effect of external pH depicted on **Figure 3A**. If  $\text{pH}_e$  had no intrinsic effect on the kinetic parameters of pendrin, the mathematical model would predict a constant flux in these experiments. Indeed, the ‘effective’ concentration of  $\text{Cl}^-$  (i.e., its concentration divided by its affinity) in the bath was much greater than that of  $\text{OH}^-$  both in the bath and within the cell, so that the intracellular concentration of  $\text{OH}^-$  was the rate-limiting factor that determines the magnitude of the flux. Given that the initial  $\text{pH}_i$  was constant in these experiments, the flux at  $t = 0$  should have remained constant in the absence of an intrinsic effect of  $\text{pH}_e$ . The fact that the flux decreased with increasing  $\text{pH}_e$  strongly suggests that lowering  $\text{pH}_e$  stimulates pendrin activity.

**Glycosylation of pendrin.** Immunodetection of mouse pendrin transiently expressed in HEK293 cells reveals two bands at  $\sim 130$  kDa and  $\sim 90$  kDa (**Figure 6A**). Treatment with endoglycosidase H, which hydrolyzes only the high mannose sugars, did not alter the 130 kDa band and slightly reduced to  $\sim 85$  kDa the apparent molecular weight of the lower band, consistent with core deglycosylation of pendrin. Conversely, upon treatment with N glycosidase F, which removes all glycosides, the  $\sim 130$  kDa band disappeared and the  $\sim 90$  kDa band moved to the same lower molecular weight seen after endoglycosidase H treatment, which corresponds to the expected molecular weight of the non-glycosylated protein (**Figure 6B**). These results confirm that the higher molecular weight band is the complex glycoprotein and the lower band is the core glycosylated protein.

*In silico* analysis of mouse pendrin (NetNGlyc 1.0 Server) predicted five putative N-linked glycosylation sites at asparagines 167, 172, 241, 475 and 567. Site-directed mutagenesis, in which asparagine was substituted for alanine, showed that only the first two sites, N167 and N172, account for the full glycosylation of pendrin (**Figure 6B**).

We next evaluated the role of glycosylation on the membrane targeting, the polarization, and the activity of pendrin. These experiments were performed in OKP cells stably transfected with either wild type or N167A/N172A double mutant pendrin, because OKP cells exhibit a much higher transfection rate (up to 80%) compared to HEK293 cells, and they are polarized when grown on filters. **Figure 7A-C** shows that in polarized OKP cells (as in HEK293 cells; see **Figure 1**), wild type pendrin colocalized with biotin at the plasma membrane. Moreover, pendrin was preferentially expressed at the apical cell border (**Figure 7D**), as in native collect-

ing duct B-ICs. A similar pattern of apical membrane expression was observed with the N167A/N172A double mutant pendrin (**Figure 7E-H**).

Membrane expression of pendrin with disrupted N-linked glycosylation sites in stably transfected, non-polarized OKP cells was confirmed by cell surface biotinylation followed by avidin bead precipitation and immunoblotting. Results show that pendrin was expressed at a lesser level in cells expressing the N167A/N172A double mutant pendrin than in cells expressing the wild type pendrin, but the fraction of pendrin expressed at the cell surface was similar in the two cell lines (**Figure 8A**). Furthermore, the functional pendrin activity in these two OKP cell lines, as determined under the forward  $\text{Cl}^-/\text{OH}^-$  exchange mode at  $\text{pH}_e$  7.4, was proportional to their expression level of pendrin at the cell surface (**Figure 8B**). However, in contrast to the wild type pendrin, the activity of which increases at low  $\text{pH}_e$  in OKP cells as in HEK293 cells, the activity of the N167A/N172A double mutant pendrin did not increase at  $\text{pH}_e$  6.0 (**Figure 8B**).

Altogether these results indicate that glycosylation is not required for the expression of pendrin at the apical cell surface and its activity, but that it is a determinant of its sensitivity to external pH.

## DISCUSSION

Pendrin has been shown to play a key role in maintaining the homeostasis of the inner ear [25] and in regulating the renal chloride balance [13, 14]; hence the importance of fully characterizing its structure and function. Pendrin expression in *Xenopus laevis* oocytes or in HEK293 cells previously demonstrated that it can function in several distinct modes including  $\text{Cl}^-/\text{I}^-$ ,  $\text{Cl}^-/\text{OH}^-$ ,  $\text{Cl}^-/\text{HCO}_3^-$  and  $\text{Cl}^-/\text{formate}$  exchange [22, 26], and that it is sensitive to DIDS [22]. In this study, we sought to characterize the glycosylation properties of pendrin, its kinetic behaviour, and its regulation by pH. Stable expression of mouse full-length pendrin in HEK293 cells enabled us to examine its function as a  $\text{Cl}^-/\text{OH}^-$  and  $\text{Cl}^-/\text{HCO}_3^-$  reversible exchanger.

We found that the activity of pendrin was significantly higher in the  $\text{Cl}^-/\text{HCO}_3^-$  exchange mode, that is, its predominant transport mode in the kidney collecting duct [27]. In rabbit CCD, Schuster [28] estimated the  $K_m$  for luminal chloride as 4-11 mM based upon his flux measurements, and as 1.7 mM based upon the fluxes reported in another study [29]. However, it is important to note that the  $K_m$  values measured in these previous studies are apparent trans-epithelial kinetic parameters and cannot be considered as true affinity constants in the enzymatic sense; the measured fluxes included a contribution by other transporters, such as basolateral anion exchangers. In contrast, our values were obtained in heterologous expression systems and reflected only the activity of pendrin. Moreover, they were corrected to account for variations in gradients and driving forces as external and/or internal concentrations were themselves varied. The apparent  $\text{Cl}^-$  affinity values that we measured (1.35 mM at  $\text{pH}_i = 8.0$ , 24 mM at  $\text{pH}_i = 7.0$ ) are significantly lower than the true affinity values that we calculated (3 and 75 mM, respectively). From a physiological standpoint, the relatively high affinity of pendrin for external  $\text{Cl}^-$  at high pH values could play an important role in the kidney collecting duct: it would allow the exchanger to function at very low  $\text{Cl}^-$  concentrations and secrete bicarbonate in states of alkalosis.

The pendrin affinity values obtained in this study may also be compared to those that have been reported for the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger AE1. Based upon previously published experimental data [30, 31], Weinstein [24] calculated a  $K_m$  of 50 mM for  $\text{Cl}^-$  and 198 mM for  $\text{HCO}_3^-$ , with the assumption that internal and external affinities were equal. In a later study, Knauf et al. [32] estimated the true  $K_m$  of AE1 as 20 mM for external  $\text{Cl}^-$  (vs. 48 mM for internal  $\text{Cl}^-$ ), and as 2 mM for external  $\text{HCO}_3^-$  (vs. 340 mM for internal  $\text{HCO}_3^-$ ). These AE1 affinity values are comparable to those that we determined for pendrin at physiological pH values (i.e.,  $\text{pH}_i \sim 7.0$ ).

Importantly, we characterized the effect of intracellular ( $\text{pH}_i$ ) and extracellular ( $\text{pH}_e$ ) pH on the kinetics of the transporter. We measured pendrin activity at pH values varying from 6 to 8; note that during ischemic episodes, intracellular pH levels may drop as low as 6.2 [33]. Pendrin as a  $\text{Cl}^-/\text{HCO}_3^-$  exchanger is known to contribute to the regulation of intracellular and extracellular pH [27]. Conversely, we established for the first time that pendrin is regulated by pH. More specifically, we found that pendrin activity *in vitro* is significantly higher at acid  $\text{pH}_e$ . Mathematical analysis of the fluxes showed that the effects of  $\text{pH}_e$  can be accounted for assuming that a low  $\text{pH}_e$  accelerates ion translocation, possibly via allosteric modifications. The effects of  $\text{pH}_i$  on pendrin kinetics could be explained by a  $\text{pH}_i$ -induced modification of binding affinities. The affinity of pendrin for  $\text{Cl}^-$  was predicted to increase by a factor of 25 as  $\text{pH}_i$  decreased from 8.0 to 7.0, whereas that for  $\text{OH}^-$  was reduced by a factor of 5.

The regulation of AE isoforms by pH was previously established by several investigators [34-36]. In particular, the study of Sterling and Casey [34] demonstrated that AE1 and AE3 isoforms are essentially insensitive to pH, whereas AE2 appears to be slightly inhibited by acid pH and more significantly activated by high pH. Although the latter findings would sug-

gest that pH exerts opposite effects on AE2 and pendrin, it should be noted that in contrast to our present measurements of pendrin activity,  $pH_i$  and  $pH_e$  were varied simultaneously in the study of Sterling and Casey; thus, we can not rule out the possibility that  $pH_i$  and  $pH_e$  exert inverse regulatory effects on AE2. Interpreting flux measurements for an acid-base transporter is complicated by the combined effect of ionic concentrations on the driving force and potential pH regulation sites. Our mathematical model enabled us to show that pH-mediated changes in pendrin activity were not due to variations in transmembrane gradients but to an intrinsic pH effect. In the kidney, the stimulating effect of low extracellular pH on pendrin activity suggests that the exchanger is constitutively activated, since urine is known to be acid. This may also be the case in the endolymphatic sac of the inner ear, where pendrin has been shown to contribute to pH buffering by secreting bicarbonate [37]. The endolymph is known to be acidic (pH ~ 6.6-7.1) [38-40], and endolymphatic pH homeostasis is necessary for the prevention of hearing loss. Thus, the constitutive stimulation of pendrin in the endolymphatic sac may represent an important regulation pathway in the inner ear.

Our results indicate that pendrin is glycosylated exclusively at asparagines 167 and 172, which are located in the second putative extracellular loop of pendrin. N-glycosylation of membrane proteins plays a role in their correct folding and their targeting to the membrane [41, 42]. Some studies also suggest that glycosylation may have a role in maintaining the transporter in an active form within the membrane [43, 44]. Hence, the role of N-glycosylation sites varies among different proteins. In contrast to the rat  $Na^+Cl^-$  thiazide-sensitive co-transporter (Slc12a3) which displays diminished surface expression and activity when its N-glycosylation sites are disrupted [43], N-glycosylation of pendrin does not appear to be a pre-requisite for its targeting to the cell surface and polarized expression at the apical cell border. Although N-glycosylation is not required for the basal transport activity of pendrin, mutation of the two N-glycosylated asparagines (Asn) to alanines (Ala) abrogates the external pH dependence of pendrin activity. Several hypotheses can be put forward to account for this effect: 1) the sugar residues are the proton sensor; however, to our knowledge, this has never been reported before; 2) the loss of the sugar residues modifies the conformation of the protein and masks the proton sensor; 3) the replacement of Asn by Ala modifies the conformation of the protein and masks the proton sensor, independently of the loss of the sugar residues; 4) mutated Asn are the proton sensor responsible for the modulation of pendrin activity. Further experiments are needed to address these hypotheses.

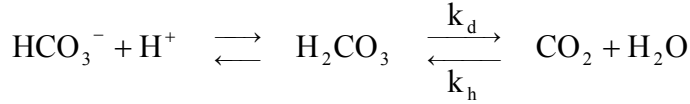
In summary, we characterized both experimentally and theoretically the kinetic behaviour of pendrin. We showed for the first time that pendrin activity is modulated by both internal and external pH, and that the dependence on external pH is lost by mutagenesis-induced disruption of N-glycosylation sites. The sensitivity of pendrin to pH may serve as a key mechanism in controlling ionic exchanges across the plasma membrane of type-B intercalated cells in the kidney collecting duct.

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### Appendix A. Bicarbonate Concentration

Bicarbonate and carbonic acid form an acid/base pair. In water, carbonic acid and carbon dioxide are inter-converted:



where  $k_d$  and  $k_h$  are the  $\text{H}_2\text{CO}_3$  dehydration and hydration rates, respectively taken as 18 and  $0.037 \text{ s}^{-1}$  [45]. We assume that the dissociation of  $\text{H}_2\text{CO}_3$  is rapid and at equilibrium. Thus, the acid-base concentration ratio is given by:

$$\text{pH} = \text{pKa} + \log\left(\frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]}\right) \quad (1)$$

$$\text{That is, } [\text{H}_2\text{CO}_3] = [\text{HCO}_3^-] \times 10^{\text{pKa} - \text{pH}} \quad (2)$$

The temporal concentration variations can be written as:

$$\frac{d[\text{HCO}_3^-]}{dt} + \frac{d[\text{H}_2\text{CO}_3]}{dt} = -k_d[\text{HCO}_3^-] + k_h[\text{CO}_2] \quad (3)$$

$$\frac{d[\text{CO}_2]}{dt} = +k_d[\text{HCO}_3^-] - k_h[\text{CO}_2] \quad (4)$$

Adding Eqs. (3-4) yields:

$$\frac{d}{dt}([\text{HCO}_3^-] + [\text{H}_2\text{CO}_3] + [\text{CO}_2]) = 0 \quad (5)$$

$$[\text{HCO}_3^-] + [\text{H}_2\text{CO}_3] + [\text{CO}_2] = C_{\text{tot}} \quad (6)$$

where the constant  $C_{\text{tot}}$  is the total concentration of all bicarbonate species (i.e.,  $\text{HCO}_3^- + \text{H}_2\text{CO}_3 + \text{CO}_2$ ). Combining Eqs. (2) and (6), we obtain:

$$[\text{CO}_2] = C_{\text{tot}} - [\text{HCO}_3^-](1 + 10^{\text{pKa} - \text{pH}}) \quad (7)$$

Substituting Eqs. (2) and (7) into Eq. (3) and rearranging yields an equation for the time derivative of  $[\text{HCO}_3^-]$  only:

$$\frac{d[\text{HCO}_3^-]}{dt} = -\left(\frac{k_d(10^{\text{pKa} - \text{pH}}) + k_h(1 + 10^{\text{pKa} - \text{pH}})}{(1 + 10^{\text{pKa} - \text{pH}})}\right)[\text{HCO}_3^-] + \left(\frac{k_h}{1 + 10^{\text{pKa} - \text{pH}}}\right)C_{\text{tot}} \quad (8)$$

$$\text{Let } k_1 \equiv \frac{k_d(10^{\text{pKa} - \text{pH}}) + k_h(1 + 10^{\text{pKa} - \text{pH}})}{(1 + 10^{\text{pKa} - \text{pH}})} \quad (9)$$

$$k_2 \equiv \frac{k_h}{k_d(10^{\text{pKa} - \text{pH}}) + k_h(1 + 10^{\text{pKa} - \text{pH}})}$$

Integration of Eq. (8) yields the concentration of bicarbonate as a function of time:

$$[\text{HCO}_3^-] = C_{\text{tot}}(k_2 + (1 - k_2)\exp(-k_1 t)) \quad (10)$$

In our experiments, the total concentration of bicarbonate species is equal to the initial concentration of  $\text{HCO}_3^-$  (denoted  $[\text{HCO}_3^-]_0$ ). Equation (10) can therefore be expressed as:

$$\frac{[\text{HCO}_3^-]_t}{[\text{HCO}_3^-]_0} = k_2 + (1 - k_2)\exp(-k_1 t) \quad (11)$$

where the subscript “t” denotes any time t, and “o” the starting point of the experiment. As demonstrated by Eq. (11), the equilibrium (“eq”) concentration of  $\text{HCO}_3^-$  is:

$$\frac{[\text{HCO}_3^-]_{\text{eq}}}{[\text{HCO}_3^-]_0} = k_2 = \frac{0.037}{18(10^{3.57-7.40}) + 0.037(1 + 10^{3.57-7.40})} = 0.9327 \quad (12)$$

That is,  $\text{HCO}_3^-$  concentration at equilibrium is 6.73% lower than that at the starting point. Given that  $\exp(-3) = 0.05$ , we can also estimate the time needed to reach 95% of the equilibrium concentration ( $t_{95}$ ) as:

$$t_{95} = \frac{3}{k_1} = \frac{3(1 + 10^{3.57-7.40})}{18(10^{3.57-7.40}) + 0.037(1 + 10^{3.57-7.40})} = 75.64 \text{ s} \quad (13)$$

## Appendix B. Kinetic Parameter Evaluation

As depicted on **Figure 5**, the transport of a given species (e.g.,  $\text{Cl}^-$ ,  $\text{HCO}_3^-$ , or  $\text{OH}^-$ ) across pendrin is characterized by 4 parameters: the external and internal binding constants ( $K^e, K^i$ ) and the forward and backward translocation rates ( $P^e, P^i$ ), where the superscripts “e” and “i” respectively denote the extracellular and intracellular compartments. The non-dimensional external and internal concentrations of chloride ( $\gamma$ ), bicarbonate ( $\beta$ ), and  $\text{OH}^-$  ( $\alpha$ ) are defined as:

$$\gamma^e = \frac{[\text{Cl}^-]^e}{K_{\text{Cl}}^e}, \gamma^i = \frac{[\text{Cl}^-]^i}{K_{\text{Cl}}^i} \quad (14a)$$

$$\beta^e = \frac{[\text{HCO}_3^-]^e}{K_{\text{HCO}_3}^e}, \beta^i = \frac{[\text{HCO}_3^-]^i}{K_{\text{HCO}_3}^i} \quad (14b)$$

$$\alpha^e = \frac{[\text{OH}^-]^e}{K_{\text{OH}}^e}, \alpha^i = \frac{[\text{OH}^-]^i}{K_{\text{OH}}^i} \quad (14c)$$

### General Equations

Following the approach of Weinstein [46], we assume that anion binding occurs much faster than translocation, so that the concentration of bound carrier on each side is calculated based on the equilibrium binding constants. Thus, if  $x^e$  and  $x^i$  respectively denote the concentration of unloaded carrier on the external and internal sides, the concentration of carrier bound to  $\text{Cl}^-$  on the external and internal sides is calculated as  $\gamma^e x^e$  and  $\gamma^i x^i$ , respectively. Similarly, the concentration of carrier bound to  $\text{HCO}_3^-$  and  $\text{OH}^-$  is given by  $\beta^e x^e$  and  $\alpha^e x^e$  on the external side, and by  $\beta^i x^i$  and  $\alpha^i x^i$  on the internal side. If  $x_{\text{total}}$  represents the density of total carrier, conservation of total carrier can then be written as:

$$(1 + \gamma^e + \beta^e + \alpha^e)x^e + (1 + \gamma^i + \beta^i + \alpha^i)x^i = x_{\text{total}} \quad (15)$$

That is,  $\delta^e x^e + \delta^i x^i = x_{\text{total}}$  (16)

where:  $\delta^e \equiv (1 + \gamma^e + \beta^e + \alpha^e)$   
 $\delta^i \equiv (1 + \gamma^i + \beta^i + \alpha^i)$

In addition, there is no net flux of loaded and unloaded carrier across the membrane, so that:

$$(P_{\text{Cl}}^e \gamma^e + P_{\text{HCO}_3}^e \beta^e + P_{\text{OH}}^e \alpha^e)x^e = (P_{\text{Cl}}^i \gamma^i + P_{\text{HCO}_3}^i \beta^i + P_{\text{OH}}^i \alpha^i)x^i \quad (17)$$

That is,  $\eta^e x^e = \eta^i x^i$  (18)

where:  $\eta^e \equiv (P_{\text{Cl}}^e \gamma^e + P_{\text{HCO}_3}^e \beta^e + P_{\text{OH}}^e \alpha^e)$   
 $\eta^i \equiv (P_{\text{Cl}}^i \gamma^i + P_{\text{HCO}_3}^i \beta^i + P_{\text{OH}}^i \alpha^i)$

Substituting Eq. (18) into (16) yields:

$$x^e = x_{\text{total}} \eta^i / \sigma \quad (19a)$$

$$x^i = x_{\text{total}} \eta^e / \sigma \quad (19b)$$



where:  $\sigma \equiv \delta^e \eta^i + \delta^i \eta^e$

The net fluxes of  $\text{Cl}^-$ ,  $\text{HCO}_3^-$  and  $\text{OH}^-$  into the cell can then be calculated as:

$$J_{\text{Cl}}^{\text{net}} = J_{\text{Cl}}^{\text{influx}} - J_{\text{Cl}}^{\text{efflux}} = P_{\text{Cl}}^e \gamma^e x^e - P_{\text{Cl}}^i \gamma^i x^i = x_{\text{total}} \left( \frac{P_{\text{Cl}}^e \gamma^e \eta^i - P_{\text{Cl}}^i \gamma^i \eta^e}{\sigma} \right) \quad (20a)$$

$$J_{\text{HCO}_3}^{\text{net}} = J_{\text{HCO}_3}^{\text{influx}} - J_{\text{HCO}_3}^{\text{efflux}} = P_{\text{HCO}_3}^e \beta^e x^e - P_{\text{HCO}_3}^i \beta^i x^i = x_{\text{total}} \left( \frac{P_{\text{HCO}_3}^e \beta^e \eta^i - P_{\text{HCO}_3}^i \beta^i \eta^e}{\sigma} \right) \quad (20b)$$

$$J_{\text{OH}}^{\text{net}} = J_{\text{OH}}^{\text{influx}} - J_{\text{OH}}^{\text{efflux}} = P_{\text{OH}}^e \alpha^e x^e - P_{\text{OH}}^i \alpha^i x^i = x_{\text{total}} \left( \frac{P_{\text{OH}}^e \alpha^e \eta^i - P_{\text{OH}}^i \alpha^i \eta^e}{\sigma} \right) \quad (20c)$$

**Cl/OH exchange: Cl influx**

In the first set of Cl/OH<sup>-</sup> exchange experiments, the dpH/dt variations are proportional to the efflux of OH<sup>-</sup>. Before addition of Cl<sup>-</sup> to the bath at t = 0, the intracellular concentration of Cl<sup>-</sup> is negligible. Thus, we assume that  $\gamma^i \sim 0$  immediately after t = 0. In addition, there is no bicarbonate (i.e.,  $\beta^i = \beta^e = 0$ ). Under these conditions, Eq. (20c) yields the OH<sup>-</sup> efflux as:

$$J_{\text{OH}}^{\text{efflux}} = \frac{x_{\text{total}} P_{\text{OH}}^i \alpha^i P_{\text{Cl}}^e \gamma^e}{\gamma^e [P_{\text{OH}}^i \alpha^i + P_{\text{Cl}}^e (1 + \alpha^i)] + P_{\text{OH}}^i \alpha^i (1 + \alpha^e) + P_{\text{OH}}^e \alpha^e (1 + \alpha^i)} \quad (21)$$

After rearrangement, the flux can be rewritten as:

$$J_{\text{OH}}^{\text{efflux}} = \left[ \frac{x_{\text{total}} P_{\text{OH}}^i P_{\text{Cl}}^e}{P_{\text{OH}}^i + P_{\text{Cl}}^e (1 + 1/\alpha^i)} \right] \frac{\gamma^e}{\gamma^e + \frac{P_{\text{OH}}^i (1 + \alpha^e) + P_{\text{OH}}^e \alpha^e (1 + 1/\alpha^i)}{P_{\text{OH}}^i + P_{\text{Cl}}^e (1 + 1/\alpha^i)}} \quad (22)$$

In other words,

$$J_{\text{OH}}^{\text{efflux}} = J_{\text{OH}}^{\text{efflux,max}} \frac{[\text{Cl}^-]^e}{[\text{Cl}^-]^e + K_{\text{Cl}}^{\text{e,app}}}, \quad (23a)$$

where the maximal flux and apparent affinity are given by:

$$J_{\text{OH}}^{\text{efflux,max}} = \left[ \frac{x_{\text{total}} P_{\text{OH}}^i P_{\text{Cl}}^e}{P_{\text{OH}}^i + P_{\text{Cl}}^e (1 + 1/\alpha^i)} \right] \quad (23b)$$

$$K_{\text{Cl}}^{\text{e,app}} = K_{\text{Cl}}^e \left( \frac{P_{\text{OH}}^i (1 + \alpha^e) + P_{\text{OH}}^e \alpha^e (1 + 1/\alpha^i)}{P_{\text{OH}}^i + P_{\text{Cl}}^e (1 + 1/\alpha^i)} \right) \quad (23c)$$

Note that Eq. (23) represents a Michaelis-Menten relationship, in accordance with the kinetic curve shown in **Figure 4A**.

**Cl/OH exchange: Cl efflux**

In the second set of experiments, the the  $dpH_i/dt$  variations are proportional to the influx of  $OH^-$ . By symmetry with the preceding set of experiments, we have:

$$J_{OH}^{inf\ lux} = J_{OH}^{inf\ lux, max} \frac{[Cl^-]^e}{[Cl^-]^e + K_{Cl}^{i, app}}, \quad (24a)$$

where the maximal flux and apparent affinity are given by:

$$J_{OH}^{inf\ lux, max} = \left[ \frac{x_{total} P_{OH}^e P_{Cl}^i}{P_{OH}^e + P_{Cl}^i (1 + 1/\alpha^e)} \right] \quad (24b)$$

$$K_{Cl}^{i, app} = K_{Cl}^i \left( \frac{P_{OH}^e (1 + \alpha^i) + P_{OH}^i \alpha^i (1 + 1/\alpha^e)}{P_{OH}^e + P_{Cl}^i (1 + 1/\alpha^e)} \right) \quad (24c)$$

### *Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange*

In the  $Cl^-/HCO_3^-$  exchange experiments, the  $dpH_i/dt$  variations are proportional to the net flux of  $HCO_3^-$  into the cell; it can be shown that the net flux of  $OH^-$  is negligible in comparison. Before addition of  $HCO_3^-$  to the bath at  $t = 0$ , the intracellular concentration of  $HCO_3^-$  is negligible. Under these conditions, immediately after  $t = 0$ ,  $\beta^i \sim 0$  and there is no  $HCO_3^-$  efflux. The flux of  $HCO_3^-$  into the cell is given by:

$$J_{HCO_3}^{inf\ lux} = \frac{x_{total} P_{HCO_3}^e \beta^e P_{Cl}^i \gamma^i}{\beta^e [P_{Cl}^i \gamma^i + P_{HCO_3}^e (1 + \gamma^i)] + P_{Cl}^i \gamma^i (1 + \gamma^e) + P_{HCO_3}^e \gamma^e (1 + \gamma^i)} \quad (25)$$

Rearranging Eq. 25 yields:

$$J_{HCO_3}^{inf\ lux} = \left[ \frac{x_{total} P_{Cl}^i P_{HCO_3}^e}{[P_{Cl}^i + P_{HCO_3}^e (1 + 1/\gamma^i)]} \right] \frac{\beta^e}{\beta^e + \frac{P_{Cl}^i (1 + \gamma^e) + P_{Cl}^e \gamma^e (1 + 1/\gamma^i)}{[P_{Cl}^i + P_{HCO_3}^e (1 + 1/\gamma^i)]}} \quad (26)$$

Thus, the  $HCO_3^-$  influx can be expressed as:

$$J_{HCO_3}^{inf\ lux} = J_{HCO_3}^{inf\ lux, max} \frac{[HCO_3^-]^e}{[HCO_3^-]^e + K_{HCO_3}^{e, app}} \quad (27a)$$

where the maximal flux and apparent affinity are given by:

$$J_{HCO_3}^{inf\ lux, max} = \left[ \frac{x_{total} P_{Cl}^i P_{HCO_3}^e}{[P_{Cl}^i + P_{HCO_3}^e (1 + 1/\gamma^i)]} \right] \quad (27b)$$

$$K_{HCO_3}^{e, app} = K_{HCO_3}^e \left( \frac{P_{Cl}^i (1 + \gamma^e) + P_{Cl}^e \gamma^e (1 + 1/\gamma^i)}{[P_{Cl}^i + P_{HCO_3}^e (1 + 1/\gamma^i)]} \right) \quad (27c)$$

### **Parameter Fit**

For the 3 species considered here, there is a total of  $4 \times 3 = 12$  parameters (notwithstanding the total carrier density  $x_{\text{total}}$ , which is not an intrinsic property of pendrin). However, these parameters are not all independent; since there is no net flux across the exchanger when external and internal concentrations are equal, we have:

$$\frac{P_{\text{Cl}}^e K_{\text{Cl}}^i}{K_{\text{Cl}}^e P_{\text{Cl}}^i} = \frac{P_{\text{HCO}_3}^e K_{\text{HCO}_3}^i}{K_{\text{HCO}_3}^e P_{\text{HCO}_3}^i} = \frac{P_{\text{OH}}^e K_{\text{OH}}^i}{K_{\text{OH}}^e P_{\text{OH}}^i} \quad (28)$$

Thus, the number of independent parameters (in addition to  $x_{\text{total}}$ ) is 10. As shown in Eqs. (23, 24, 27), the 3 maximum fluxes and the apparent external affinities for  $\text{HCO}_3^-$  and  $\text{Cl}^-$  are constant in each set of experiments, that is, they do not depend on variable concentrations. In contrast, the apparent internal affinity for  $\text{Cl}^-$  (Eq. 24c) is a function of the intracellular pH (i.e.,  $\alpha^i$ ) which is variable. This means that our experiments can yield a total of 6 parameters, and that the others have to be assumed. We therefore assumed that the internal and external binding affinities of a given ion are equal, as is usually done [46], and we fixed the forward  $\text{Cl}^-$  translocation rate ( $P_{\text{Cl}}^e$ ). The remaining 6 parameters were fitted to the data shown in **Figures 4A-C** using the non-linear data fitting optimization procedure `lsqnonlin` on Matlab®.

The forward  $\text{Cl}^-$  translocation rate ( $P_{\text{Cl}}^e$ ) was varied between  $10^{+2} \text{ s}^{-1}$  (i.e., the turnover rate for the  $\text{Na}^+ \text{-K}^+ \text{-ATPase}$ ) and  $10^{+6} \text{ s}^{-1}$  (i.e., that of an ion channel) [46]. For each value of  $P_{\text{Cl}}^e$ , the remaining six parameters were fitted to the experimental data. The error function was the same in all cases, and the affinity values and the ratios of the translocation rates to  $P_{\text{Cl}}^e$  were found to be almost insensitive to the value of  $P_{\text{Cl}}^e$ . The value of  $P_{\text{Cl}}^e$  was then fixed at  $10^{+4} \text{ s}^{-1}$ , a mid-range value. The fitted parameter values corresponding to  $P_{\text{Cl}}^e = 10^{+4} \text{ s}^{-1}$  are shown in **Table 1**.

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## FIGURE LEGENDS

**Fig. 1. Immunolocalization of pendrin in stably transfected HEK293 cells.** Confluent HEK293 cells transfected with pendrin were biotinylated and then stained for surface biotin (**A**, streptavidin-Cy5), nucleus (**B**, 4,6-diamidino 2-phenylindole dihydrochloride), and pendrin (**C**, anti-pendrin antibody followed by FITC-conjugated goat anti-rabbit IgG). The stained slides were visualized by confocal microscopy. **D** shows the merge of the three labelings.

**Fig. 2. Representative traces showing pendrin-mediated changes in intracellular pH.** Changes in  $\text{pH}_i$  in pendrin-transfected (black traces) or non-transfected (grey traces) HEK293 cells were induced by modifying the composition of the extracellular medium, as shown on top of each diagram. The initial rate of  $\text{pH}_i$  variation was determined over the first 20 sec period. The insets illustrate the transport mode of pendrin in each condition. In the absence of bicarbonate, pendrin was studied either under forward (**A**) or backward (**B**)  $\text{Cl}^-/\text{OH}^-$  exchange mode. In the presence of bicarbonate, it was measured under forward  $\text{Cl}^-/\text{HCO}_3^-$  exchange mode (**C**).

**Fig. 3. pH dependence of pendrin activity.** **A.** After pre-incubation in a  $\text{Cl}^-$ -free medium at pH 7.4, cells were exposed to a medium containing 120 mM  $\text{Cl}^-$  at different pH values, and the initial rate of intracellular acidification was monitored. **B.** Cells were first incubated in a medium containing 120 mM  $\text{Cl}^-$  at pH 6.0, 7.4 or 8.0. After equilibration of intracellular pH, at 6.2, 7.0 and 7.5 respectively, cells were exposed to a  $\text{Cl}^-$ -free medium at pH 8.0, and the initial rate of intracellular alkalinisation was measured.

**Fig. 4. Pendrin activity as a function of anion concentration.** Pendrin activity was measured (black squares) as described in Figure 3 in the presence of different concentrations of  $\text{Cl}^-$ ,  $\text{OH}^-$  and  $\text{HCO}_3^-$ . Also displayed are calculated values (white squares), computed using the mathematical model of pendrin-mediated ion transport. Insets show the Lineweaver-Burk plots of the measured data.

**Fig. 5. Kinetic scheme of pendrin.** Pendrin-mediated ion transport across the cell membrane is represented according to a ping-pong mechanism.  $\text{Pd}^\circ$  and  $\text{Pd}^*$  refer to the free and ion-bound forms of pendrin, and  $P_{\text{Cl}}$  and  $P_{\text{HCO}_3}$  are the translocation rates of chloride and bicarbonate respectively (see Appendix B for details).

**Fig. 6. Glycosylation of pendrin.** **A.** Electrophoretical profile of pendrin in HEK293 cells transiently transfected with pendrin, in the absence of treatment or after treatment with endoglycosidase H, N glycosidase F, or sham treatment. Note that non-transfected cells display a non specific band at  $\sim 130$  kDa. **B.** Electrophoretical profile of HEK293 cells transiently transfected with wild type pendrin (WT), or mutated pendrin with disruption of either only N167 or both N167 and N172 glycosylation sites.

**Fig. 7. Immunolocalization of pendrin in stably transfected OKP cells.** Confluent OKP cells stably transfected with either wild type (**A-D**) or N167A/N172A double mutant pendrin (**E-H**) were biotinylated and then stained for surface biotin (**A & E**, streptavidin-Cy5) and pendrin (**B & F**, anti-pendrin antibody followed by FITC-conjugated goat anti-rabbit IgG).

The stained slides were visualized by confocal microscopy. **C & G** show the merges of the two labellings. **D & H** show *x-z* reconstructions of confocal microscopy merge image stacks.

**Fig. 8. Membrane expression and activity of pendrin with disrupted N-linked glycosylation sites.** **A.** Pendrin immunoblot of biotinylated and whole cell proteins in OKP cells stably transfected with either wild type or N167A/N172A double mutant pendrin. The upper panel shows the blot and the lower panel shows the densitometry measurements of all the bands in each lane. **B.** Pendrin activity, measured under forward  $\text{Cl}^-/\text{OH}^-$  exchange mode at different external pH values (6.0, 7.4, and 8.0) in OKP cells stably transfected with either wild type or N167A/N172A double mutant pendrin. Values are means  $\pm$  SE from 6-9 samples in 2-3 experiments.

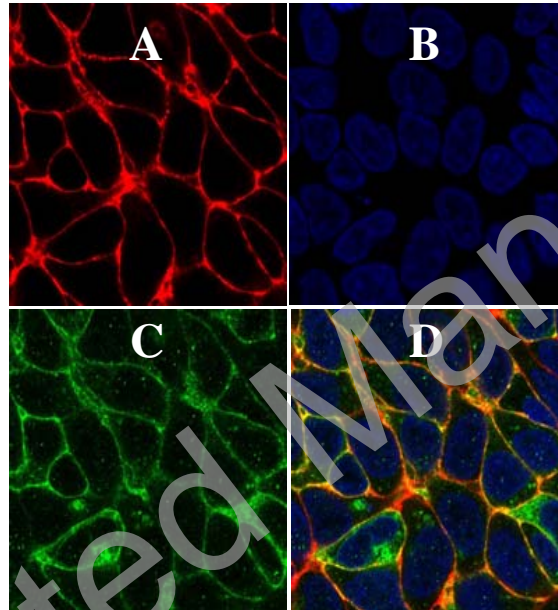
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**Table 1**  
**Pendrin kinetic parameters**

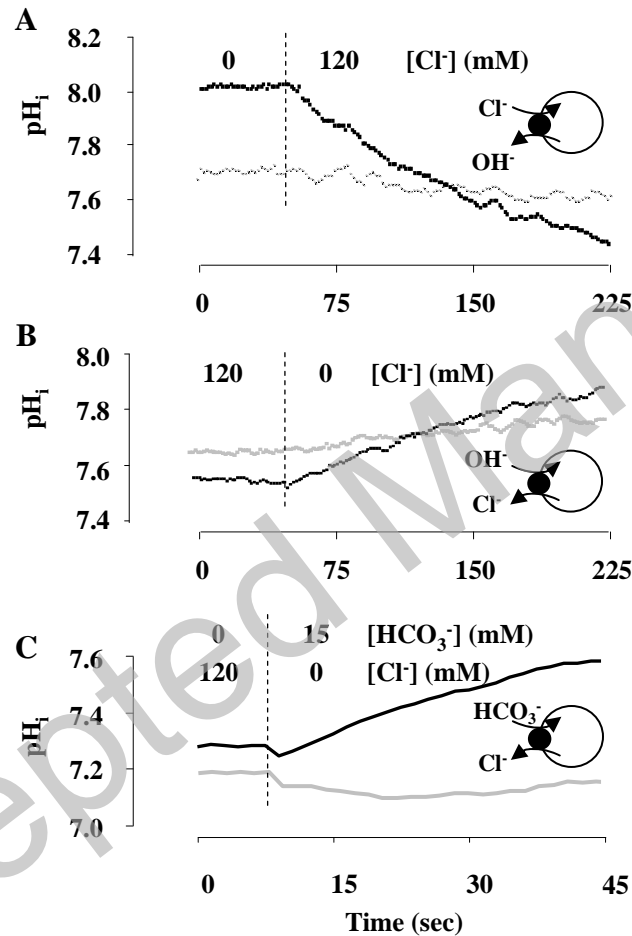
Ratio of $\text{Cl}^-$ backward rate to $\text{Cl}^-$ forward rate, $P_{\text{Cl}}^i/P_{\text{Cl}}^e$	1.239
Ratio of $\text{HCO}_3^-$ forward rate to $\text{Cl}^-$ forward rate $P_{\text{HCO}_3}^e/P_{\text{Cl}}^e$	10.76
Ratio of $\text{OH}^-$ forward rate to $\text{Cl}^-$ forward rate, $P_{\text{OH}}^e/P_{\text{Cl}}^e$	0.262
$\text{Cl}^-$ affinity, $K_{\text{Cl}}$	3.0 mM ( $\text{pH}_i = 8.0$ ) 75 mM ( $\text{pH}_i = 7.0$ )
$\text{HCO}_3^-$ affinity, $K_{\text{HCO}_3}$	5.9 mM ( $\text{pH}_i = 7.2$ )
$\text{OH}^-$ affinity, $K_{\text{OH}}$	$1.38 \times 10^{-6}$ M ( $\text{pH}_i = 8.0$ ) $2.62 \times 10^{-7}$ M ( $\text{pH}_i = 7.0$ )

The parameters were fitted to the data shown in **Figure 4**, and correspond to an external pH of 7.4. The  $\text{Cl}^-$  forward rate ( $P_{\text{Cl}}^e$ ) was fixed at  $10,000 \text{ s}^{-1}$ ; the ratios of the other rates to  $P_{\text{Cl}}^e$  were found to be independent of its value. Note that the  $\text{HCO}_3^-$  and  $\text{OH}^-$  backward rates are not independent parameters; they can be obtained by using Eq. (28).



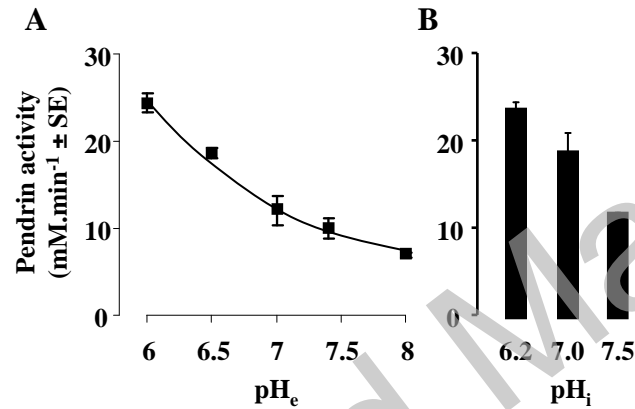
**Figure 1. Immunolocalization of pendrin in stably transfected HEK293 cells.**

Confluent HEK293 cells transfected with pendrin were biotinylated and then stained for surface biotin (**A**, streptavidin-Cy5), nucleus (**B**, 4,6-diamidino 2-phenylindole dihydrochloride), and pendrin (**C**, anti-pendrin antibody followed by FITC-conjugated goat anti-rabbit IgG). The stained slides were visualized by confocal microscopy. **D** shows the merge of the three labellings.



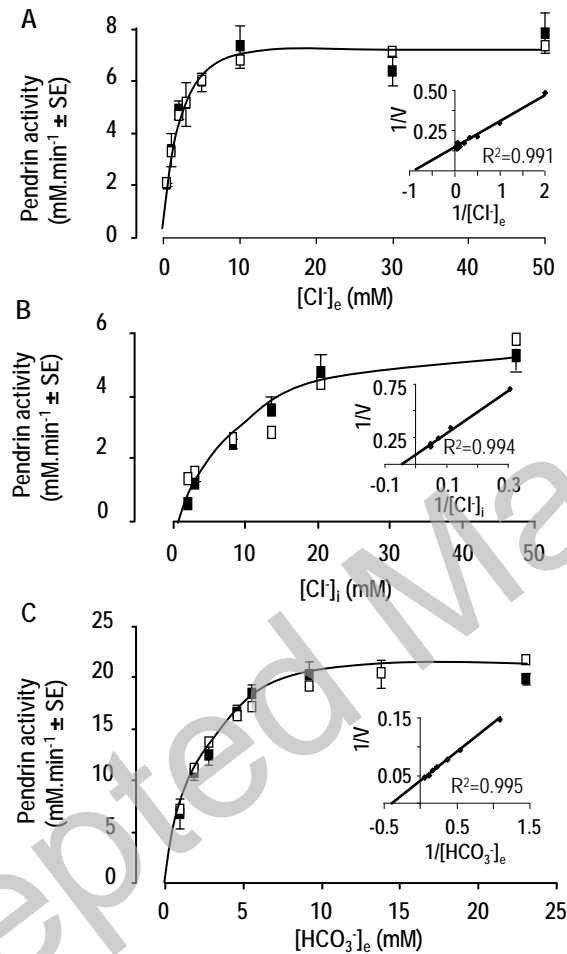
**Figure 2. Representative traces showing pendrin-mediated changes in intracellular pH.**

Changes in  $pH_i$  in pendrin-transfected (black traces) or non-transfected (grey traces) HEK cells were induced by modifying the composition of the extracellular medium, as shown on top of each diagram. The initial rate of  $pH_i$  variation was determined over the first 20 sec period. The insets illustrate the transport mode of pendrin in each condition. In the absence of bicarbonate, pendrin was studied either under forward (A) or backward (B)  $Cl^-/OH^-$  exchange mode. In the presence of bicarbonate, it was measured under forward  $Cl^-/HCO_3^-$  exchange mode (C).



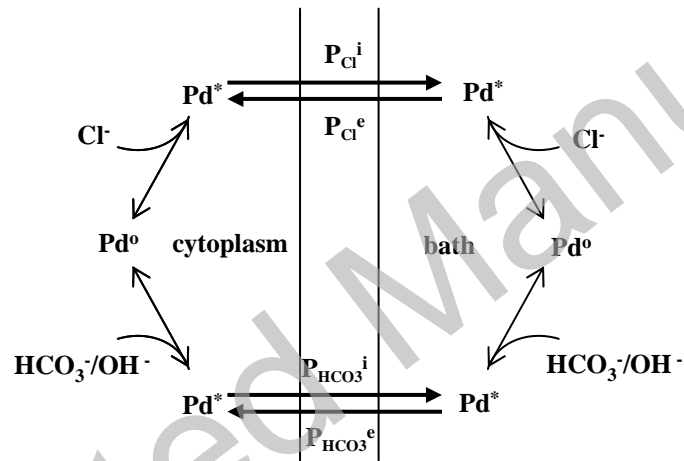
**Figure 3. pH dependence of pendrin activity.**

- A. After pre-incubation in a Cl<sup>-</sup>-free medium at pH 7.4, cells were exposed to a medium containing 120 mM Cl<sup>-</sup> at different pH values, and the initial rate of intracellular acidification was monitored.
- B. Cells were first incubated in a medium containing 120 mM Cl<sup>-</sup> at pH 6.0, 7.4 or 8.0. After equilibration of intracellular pH, at 6.2, 7.0 and 7.5 respectively, cells were exposed to a Cl<sup>-</sup>-free medium at pH 8.0, and the initial rate of intracellular alkalinisation was measured.



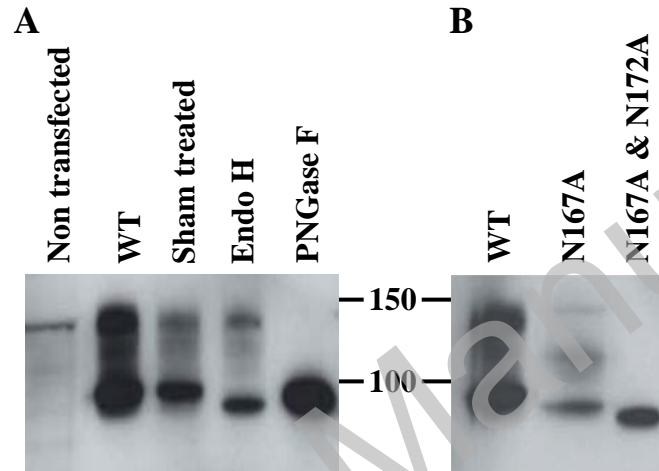
**Figure 4. Pendrin activity as a function of anion concentration.**

Pendrin activity was measured (black squares) as described in Figure 3 in the presence of different concentrations of Cl<sup>-</sup>, OH<sup>-</sup> and HCO<sub>3</sub><sup>-</sup>. Also displayed are calculated values (white squares), computed using the mathematical model of pendrin-mediated ion transport. Insets show the Lineweaver-Burk plots of the measured data.



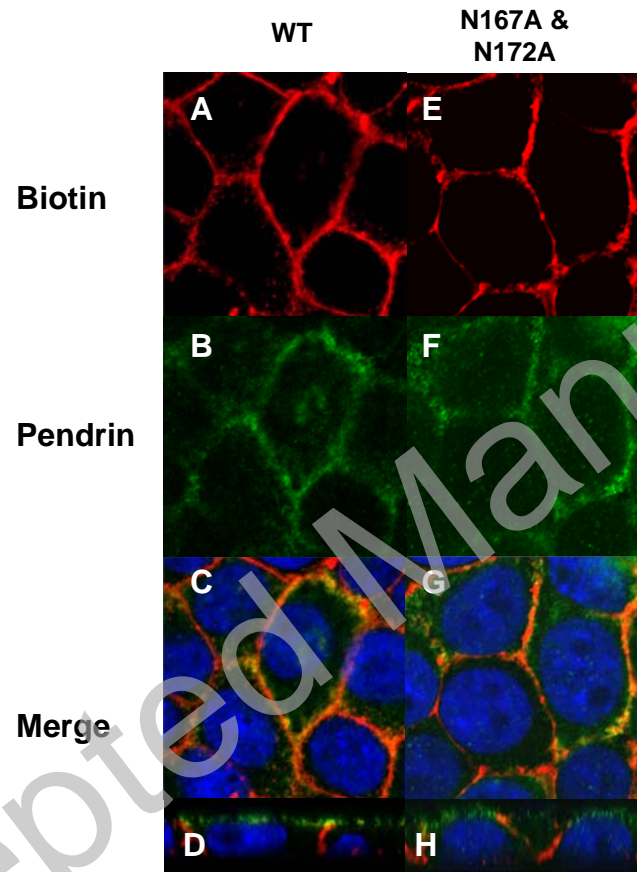
**Figure 5. Kinetic scheme of pendrin.**

Pendrin-mediated ion transport across the cell membrane is represented according to a ping-pong mechanism. Pd° and Pd\* refer to the free and ion-bound forms of pendrin, and  $P_{Cl}$  and  $P_{HCO_3}$  are the translocation rates of chloride and bicarbonate respectively (see Appendix B for details).



**Figure 6. Glycosylation of pendrin.**

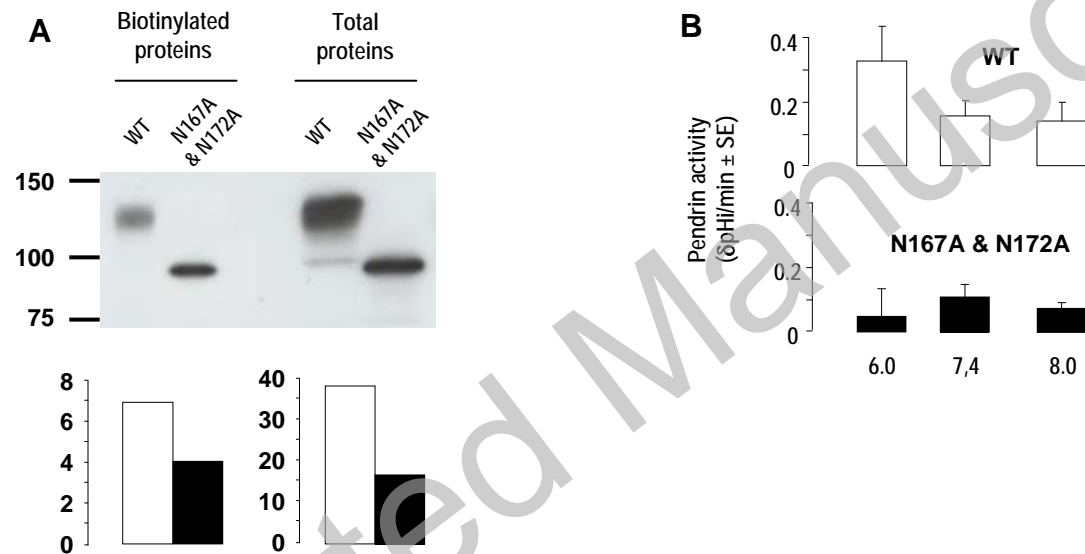
- A. Electrophoretical profile of pendrin in HEK293 cells transiently transfected with pendrin, in the absence of treatment or after treatment with endoglycosidase H, N glycosidase F, or sham treatment. Note that non-transfected cells display a non specific band at ~ 130 kDa.
- B. Electrophoretical profile of HEK293 cells transiently transfected with wild type pendrin (WT), or mutated pendrin with disruption of either only N167 or both N167 and N172 glycosylation sites.



**Figure 7. Immunolocalization of pendrin in stably transfected OKP cells.**

Confluent OKP cells stably transfected with either wild type (A-D) or N167A/N172A double mutant pendrin (E-H) were biotinylated and then stained for surface biotin (A & E, streptavidin-Cy5) and pendrin (B & F, anti-pendrin antibody followed by FITC-conjugated goat anti-rabbit IgG). The stained slides were visualized by confocal microscopy. C & G show the merges of the two labellings. D & H. show *x-z* reconstructions of confocal microscopy merge image stacks.





**Figure 8. Membrane expression and activity of pendrin with disrupted N-linked glycosylation sites.**

- A. Pendrin immunoblot of biotinylated and whole cell proteins in OKP cells stably transfected with either wild type or N167A/N172A double mutant pendrin. The upper panel shows the blot and the lower panel shows the densitometry measurements of all the bands in each lane.
- B. Pendrin activity, measured under forward Cl<sup>-</sup>/OH<sup>-</sup> exchange mode at different external pH values (6.0, 7.4, and 8.0) in OKP cells stably transfected with either wild type or N167A/N172A double mutant pendrin. Values are means ± SE from 6-9 samples in 2-3 experiments.