Identification of a chloride-formate exchanger expressed on the brush border membrane of renal proximal tubule cells

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1. Introduction

1.1. Physiology of the body fluids and the kidney

1.1.1. Composition of the body fluids
The most abundant component of the human body is water. It is distributed among three compartments: an intracellular, an extracellular, and a transcellular compartment (1,2).
Osmotically active substances determine the distribution of water between the intracellular and extracellular compartments.
Sodium is largely an extracellular ion whereas potassium is mainly an intracellular ion. The major anions of the extracellular fluid are chloride, bicarbonate and protein. The major anions of the intracellular fluid are proteins and organic phosphates (3).
Na⁺ and Cl⁻ contribute to about 80 % of extracellular osmolarity and thus the NaCl balance influences the plasma volume which is a determinant of the arterial blood pressure. The arterial pressure or the filling of the cardiac ventricles, respectively, determine the cardiac output. Thus, the maintenance of the volume of body fluids is necessary for normal function of the cardiovascular system.
The kidneys are the main regulator of NaCl and water balance. Working in an integrated fashion with components of the cardiovascular and central nervous system, the kidneys accomplish the balance of water and NaCl by regulating the excretion of both.
Excess intake of electrolytes or water is followed by their excretion from the body, yet their amount in the body may increase. Therefore, excess intake of water leads to an increase in water excretion (diuresis), while excess intake of Na⁺ leads to an increase in Na⁺ excretion (natriuresis). Vice versa, if the excretion exceeds intake,
the amount decreases. Thus, the kidneys have to match the daily intake with the excretion in order to maintain homeostasis of the body fluids.

### 1.1.2. Structure and function of the kidneys

As the structure and the function of the kidneys are closely related with each other the gross anatomic and histologic features of the kidneys are of important value for the understanding of their function.

A frontal section through the human kidney shows a cortex surrounding a central region called the medulla. The medulla consists of papillae that empty into pouches, the calyces (4).

With regards to the vasculature the kidney possesses a portal system with two capillary beds in series. The first system supplies the glomeruli, the second accompanies and surrounds the tubules. The venous blood is drained into interlobular, arcuate, and interlobar veins (5).

In human, each kidney consists of 1 – 1 ¼ million units called the nephron (6). They are all basically similar in structure and function. Each unit consists of a glomerulus, a proximal convoluted tubule, a loop of Henle and a distal convoluted tubule. The distal convoluted tubules turns into collecting ducts; these in turn empty into the renal calyces mentioned above.

The normal filtration rate of human kidneys is around 120 ml/min or 180 l/day. As the average daily urine volume is around 1,5 l, 99 % of the 180 l/day ultrafiltration is reabsorbed. This enormous transport work required for the reabsorption is fulfilled by highly specialized and economically working transport systems. About two-thirds to three fourths of the glomerular filtrate are reabsorbed within the proximal tubule (7,8).
Introduction

1.2. Reabsorption of chloride in the proximal tubule

1.2.1. Fluid reabsorption in the proximal tubule

By definition, the filtered load to the proximal tubule is the product of the single nephron glomerular filtration rate (SNGFR). As mentioned under 1.1.2, the major fraction of the ultrafiltrate is reabsorbed along the proximal tubule. This means primarily the reabsorption of Na\(^+\), Cl\(^-\), HCO\(_3\)\(^-\) and in smaller quantities potassium, phosphate and various filtered organic compounds such as glucose and amino acids. In 1941 Walker et al. (9) made several major discoveries using micropuncture studies: along the length of the proximal tubule fluid reabsorption proceeds and osmolarity and Na\(^+\)-concentration remain approximately the same as in the plasma (Figure 1). Although Cl\(^-\) is reabsorbed throughout the length of the proximal tubule, Cl\(^-\) concentration rises to exceed that in the glomerular ultrafiltrate. They concluded that there must be preferential absorption of Na\(^+\) with a non-Cl\(^-\) anion, most likely bicarbonate in the proximal tubule.

![Profile of ratio of solute concentrations in tubule fluid to those in plasma (TF/P) along the proximal tubule. Modified from Rector et al. (12).](image)

Figure 1:
Profile of ratio of solute concentrations in tubule fluid to those in plasma (TF/P) along the proximal tubule. Modified from Rector et al. (12).
For an anion like Cl\(^{-}\), there are two general driving forces for transepithelial transport. First, the transtubular potential difference must be taken into account. Due to electrogenic cotransport of Na\(^{+}\) with organic substrates like glucose or amino acids, there is a lumen-negative transtubular potential difference in the early proximal tubule (10,11) quantitatively most important. As the rate of electrogenic Na\(^{+}\) - cotransport decreases in the late proximal tubule, the transtubular potential reverses and becomes positive (12).

The question whether Cl\(^{-}\) - reabsorption is primarily paracellular (passive) or transcellular (active) or both has been epivocal for a long time. Neumann and Rector (13) found that Cl\(^{-}\) - absorption in the proximal tubule depends on a high luminal Cl\(^{-}\) - concentration and on a favorable electrochemical gradient. Similarly, Frömter et al. (14) concluded using the split droplet technique that Cl\(^{-}\) - absorption is totally passive with one-half due to diffusion and one-half due to solvent drag. Other studies reported opposite findings: fractions of Cl\(^{-}\) - transport were inhibitable by luminal SITS, luminal furosemide and luminal and peritubular potassium removal (15,16).

In addition, Alpern et al. (17) demonstrated that there is also a significant component of chloride absorption occurring independently of the electrochemical driving force. They concluded that this Cl\(^{-}\) absorbed in the absence of an electrochemical driving force cannot be attributed to passive paracellular chloride diffusion and must therefore be due to either active transcellular chloride transport or solvent drag. As Green et al. (18) found that addition of cyanide to the luminal fluid eliminated chloride absorption when tubules were perfused with a chloride concentration similar to that of plasma ultrafiltrate the idea of active Cl\(^{-}\) - absorption was supported.

Thirdly, even in the presence of an outwardly directed driving force for passive Cl\(^{-}\) - transport, a significant fraction of Cl\(^{-}\) - absorption was inhibited by manipulations that block active Na\(^{+}\)- transport, such as removal of peritubular K\(^{+}\) (16) or addition of ouabain (19).
1.2.2. Identification of active chloride transport in the proximal tubule

As the intracellular Cl⁻ activity in the proximal tubule is higher than would be the case if Cl⁻ movements would simply follow the membrane potential (20), transcellular Cl⁻ fluxes would depend on an energetically uphill accumulation.

Active transport of chloride across the plasma membrane has been described in several epithelia: Secondary active transport of Cl⁻ with Na⁺ and K⁺ had been described already 20 years ago in the shark rectal gland (21), the flounder intestine (22), the Amphiuma early distal renal tubule (23), the rabbit renal medullary thick ascending limb (24) and rabbit medullary collecting duct (25).

K⁺ independent secondary active cotransport of Cl⁻ with Na⁺ had been observed originally in the Necturus gallbladder (26) and flounder urinary bladder (27).

However, neither Na⁺/Cl⁻/K⁺ - cotransport nor Na⁺/Cl⁻ – cotransport could be detected in the proximal tubule (28).

As an approach to determine whether anion exchange pathways for Cl⁻ were present in the proximal tubule, Karniski and Aronson (29) used brush border membrane vesicles: They were able to demonstrate the presence of Cl⁻ / Cl⁻ - exchange as an anion exchange system in the proximal tubule for which Cl⁻ is a substrate (Figure 2).

When they screened for other substrates that might exchange with Cl⁻, an outward formate gradient markedly stimulated Cl⁻ - uptake (Figure 2).
In contrast, acetate (the next larger monocarboxylate) did only minimally induce Cl⁻ transport. Similar results as demonstrated for acetate were obtained when outward gradients for propionate, butyrate, HCO₃⁻, p-aminohippurate, lactate, succinate or sulfate were imposed (29). An enhancement of Cl⁻ uptake into brush-border vesicles other than by formate was only observed with oxalate (30). In order to demonstrate a reversible Cl⁻ / formate exchange process they imposed an outward Cl⁻ gradient driving uphill formate transport. An outward Cl⁻ gradient was able to stimulate the rate of formate influx and caused an overshoot of formate uptake above equilibrium.

Therefore, uphill Cl⁻ transport across the apical membrane of the proximal tubule cells can occur by exchange for intracellular formate. In order to sustain this transport mechanism, exit mechanisms to maintain appropriate outward gradients have to be functional. As the concentration for formate is very low in biological fluids (31,32) formate has to recycle across the membrane to maintain the intracellular concentration.
Due to the primary active extrusion of Na\(^+\) across the basolateral membrane via the Na\(^+\)/K\(^+\) - ATPase (33-36) an inwardly directed electrochemical gradient for Na\(^+\) is present across the luminal membrane. As the major mechanism for H\(^+\) -secretion into the tubular lumen is the Na\(^+\)/H\(^+\) - exchanger (37-43) this in turn provides a favorable driving force for secondary active H\(^+\) - secretion. Consequently, an inwardly directed H\(^+\) - gradient serves as a driving force for translocation of formate into the cell by nonionic diffusion of formic acid (Figure 3). As the pKa of formate is 3.75 (44) the permeability coefficient for diffusion of formic acid across lipid bilayers is 10 \(^{-2}\) cm \(*\) s \(^{-1}\) (45,50). Assuming an intracellular pH of 7.2 and a pH of 6.8 as measured in the tubular fluid the calculated lumen-to-cell unidirectional flux of formic acid is \(\approx 30\) pmol \(*\) min\(^{-1}\) \(*\) mm\(^{-1}\) assuming a concentration of formate in the lumen of approximately 500 \(\mu\)M (50). Formic acid accumulated would then dissociate into H\(^+\) and formate.

As H\(^+\) - gradient-stimulated formate uptake is sensitive to high concentrations of disulfonic stilbenes (46), H\(^+\) - formate cotransport plays an additional important role in formic acid recycling across the apical membrane (Figure 3).

Hence, the net result is electroneutral Na\(^+\) - coupled Cl\(^-\) - entry via an outwardly directed electrochemical gradient for formate that provides a driving force for tertiary active Cl\(^-\) - absorption via Cl\(^-\)/formate - exchange as illustrated in Figure 3.
Introduction

Figure 3:
Two alternative mechanisms of formate recycling to sustain Na\(^+\) - coupled Cl\(^-\) reabsorption by Cl\(^-\) / formate exchange across the apical membrane have been suggested. The molecular identity of the transporters involved in this process is not known:
Left: H\(^+\) - formate cotransport in parallel with Na\(^+\) / H\(^+\) exchange (modified from Saleh et al (46)).
Right: nonionic diffusion of formic acid in parallel with Na\(^+\) / H\(^+\) exchange (modified from Aronson et al. (47)). In both models, formate is then used to be exchanged for Cl\(^-\) to drive Cl\(^-\) - reabsorption.

As mentioned above oxalate was also able to stimulate Cl\(^-\) - uptake into brush-border vesicles. Similar to formate, oxalate accumulation was markedly stimulated by an outward Cl\(^-\) - gradient, confirming the presence of a Cl\(^-\) / oxalate - exchange process (29). Recycling of oxalate to sustain Na\(^+\) - coupled Cl\(^-\) - reabsorption was shown to occur via oxalate / carbonate exchange and oxalate / sulfate exchange in
parallel with Na\(^+\) / H\(^+\) - exchange (48,49). Neither the molecular identity of the oxalate / carbonate exchanger nor of the oxalate / sulfate exchanger is known.

1.2.3. Chloride – formate exchange: studies in vitro and in vivo

The first observations of active Cl\(^-\) - transport via Cl\(^-\) / formate - exchange across the luminal membrane were made using rabbit renal microvillus membrane vesicles (29,30). The important question whether this transport mechanism contributes significantly to transepithelial chloride reabsorption in the proximal tubule remains to be defined.

Using microperfused tubules in vitro Schild et al. (50,51) were able to demonstrate that physiological concentrations of formate between 0.1 to 1.4 mM (52) reversibly stimulated volume reabsorption without changes in transepithelial potential. The formate-stimulated volume reabsorption could be inhibited both by 4,4’-diisothiocyanostilbene – 2,2’- disulphonate (DIDS), as observed in renal microvillus membrane vesicles, and by ouabain, an inhibitor of the Na\(^+\) / K\(^+\) - ATPase.

In addition, the stimulation of volume reabsorption by formate occurred in the absence of any passive driving force for Cl\(^-\) supporting the idea of an active transport mechanism.

Studies that followed by other groups were able to demonstrate that formate concentrations as low as 10 µM significantly stimulate fluid transport in the isolated perfused proximal tubule from rabbit (53,54).

Wang et al. (54) were able to demonstrate in vivo that formate-stimulated Cl\(^-\) transport is blocked by the apical Na\(^+\) / H\(^+\) - exchanger inhibitor EIPA, indicating functional coupling to the activity of the apical Na\(^+\) / H\(^+\) - exchanger. Stimulation of proximal Cl\(^-\) - transport and fluid reabsorption was also observed with concentrations of oxalate in the physiological range (1 µM). Contrary to the functional coupling to Na\(^+\) / H\(^+\) exchanger observed for formate, oxalate-stimulated Cl\(^-\) transport was insensitive to EIPA but dependent on the presence of sulfate as originally observed in vesicle studies (56,57). The findings that Cl\(^-\) - formate
exchange and Cl⁻ - oxalate exchange depend on different secondary transport processes suggested two separate transport systems as summarized in Table 1.

<table>
<thead>
<tr>
<th>Exchange</th>
<th>EIPA</th>
<th>Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻ - formate exchange</td>
<td>Sensitive</td>
<td>insensitive</td>
</tr>
<tr>
<td>Cl⁻ - oxalate exchange</td>
<td>Insensitive</td>
<td>sensitive</td>
</tr>
</tbody>
</table>

Table 1:
Cl⁻ - formate exchange and Cl⁻ - oxalate exchange represent two separate transport systems, as they can be distinguished with respect to their sensitivities to EIPA and sulfate.

1.2.4. Pathways for chloride exit across the basolateral membrane of the proximal tubule

In order to match the solute entry across the apical membrane of the proximal tubule different mechanisms of Cl⁻ exit across the basolateral membrane have been identified. As mentioned under 1.2.2. the intracellular Cl⁻ concentration is above the electrochemical equilibrium. Patch-Clamp technique has demonstrated the presence of Cl⁻ channels favoring Cl⁻ exit via a passive driving force (58,59). Increased cell volume stimulated by NaCl entry has been shown to activate these channels (60,61). In addition, electroneutral K⁺ – Cl⁻ cotransport has been functionally identified as a mechanism to mediate Cl⁻ efflux (62,63). The molecular identity of this transporter remains to be defined. Third, as intracellular Cl⁻ responded to changes in peritubular Na⁺ and HCO₃⁻ the model of a Na⁺ - dependent Cl⁻ / HCO₃⁻ - exchanger has been developed as a model of Cl⁻ extrusion (64-66). Recently, an electroneutral Na⁺ - driven Cl⁻ / HCO₃⁻ - exchanger has been cloned with heavy expression in brain and
testis and a weaker signal in kidney and ovary (67,68). It is unclear whether this transporter is also expressed on the basolateral membrane of proximal tubule cells. The mechanisms of Cl⁻ exit across the basolateral membrane of the proximal tubule are summarized in Figure 4.

**Figure 4:**
Pathways identified for Cl⁻ exit across the basolateral membrane of proximal tubule cells. Taken from Giebisch et al. (69).
1.3. Identifying anion exchangers responsible for chloride reabsorption

1.3.1. The SLC26A family of mammalian anion transporters and their involvement in human genetic diseases

Molecular genetics and the ongoing Human Genome Project have revealed the presence of many different transporter gene families (70,71). A classical family well described consists of anion exchangers of the SLC4 family. They have been shown to mediate the electroneutral exchange of Cl\(^-\) for HCO\(_3^-\) across the plasma membrane of mammalian cells and thereby contributing to regulation of intracellular pH and cell volume. SLC4A1 is also known as erythroid band 3 protein or AE1 and was the first anion exchanger identified followed by its homologs SLC4A2 and SLC4A3 (72-74).

A distinct gene family is SLC26 including anion transporters that are structurally different from the SLC4 family (75,76). The first functionally characterized member of the family was SLC26A1, also known as Sat-1 (77,78). Sat-1 has been expression cloned from rat liver and functionally characterized as a bicarbonate / sulfate anion exchanger (78). Shortly after, SLC26A2, SLC26A3, SLC26A4 and SLC26A5 were cloned.

The SLC26A2 gene has been recognized as the disease gene mutated in diastrophic dysplasia. Diastrophic dysplasia (DTD) is a rare autosomal recessive chondrodysplasia. The clinical manifestations of DTD are short-limbed short stature, kyphoscoliosis, contractures and multiple dislocations of joints. The consequences of the severe joint dysplasia are mobility impairment in these patients. Thus, they have to undergo repeated corrective orthopaedic surgery (79-81).

SLC26A3 has been identified as the disease gene mutated in congenital chloride diarrhoea, another rare autosomal recessive disease (82,83). The disease is characterized by life-long, high volume diarrhoea with markedly elevated chloride concentrations (82,84). Linkage disequilibrium studies and haplotype analysis
identified a gene known as DRA (down-regulated in colonic adenoma) that it was indeed the gene mutated in CLD patients and functions as a Cl⁻/HCO₃⁻ exchanger mediating colonic Cl⁻ reabsorption (85,86).

SLC26A4 or Pendrin has been identified as the disease gene mutated in Pendred syndrome (PDS). Pendred syndrome is an autosomal-recessive disorder characterized by congenital hearing loss and goiter (87,88). The incidence of this disease is approximately 7.5 – 10 in 10.000 (89). Highly homologous proteins have been identified and found conserved in bacteria, yeast, plants and animals, including human. Despite Pendrin’s sequence homology to sat-1 respectively SLC26A1, it does not transport sulfate but functions as a transporter of chloride, iodide and bicarbonate (90). Most interestingly, Pendrin has been found capable of mediating Cl⁻ - formate exchange next to Cl⁻ - OH⁻ / HCO₃⁻ (91,92).

Table 2 summarizes SLC26 family members 1 – 4 and their involvement in human genetic diseases:

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Chromosome location</th>
<th>Disease</th>
</tr>
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<tbody>
<tr>
<td>SLC26A1 (SAT1)</td>
<td>4p16</td>
<td>unknown</td>
</tr>
<tr>
<td>SLC26A2 (DTDST)</td>
<td>5q32</td>
<td>Diastrophic dysplasia (DTD)</td>
</tr>
<tr>
<td>SLC26A3 (CLD, DRA)</td>
<td>7q22–q31</td>
<td>Congenital chloride diarrhea (CLD)</td>
</tr>
<tr>
<td>SLC26A4 (PDS)</td>
<td>7q22-q31.1</td>
<td>Pendred syndrome (PDS)</td>
</tr>
</tbody>
</table>

Table 2: SLC26 family members and their involvement in human diseases.
1.4. Aim of the present study

Whereas several lines of evidence indicate that the transporter primarily responsible for apical Na\(^+\) - H\(^+\) exchange is NHE3 (37-43), the transporter responsible for apical Cl\(^-\) - formate and Cl\(^-\) - oxalate exchange has yet to be identified. Several candidate genes are known that could be either responsible for this activity or could be closely related both functionally and on a molecular level.

Although mRNA encoding pendrin was detected in rat proximal tubules by RT-PCR (91), this work has been challenged by results from other groups. Pendrin has been immunolocalized to the apical membrane of non-type A intercalated cells in the cortical collecting tubule rather than the proximal tubule by several groups (93-95). Studies in knockout mice indicate pendrin plays an essential role in base secretion by the collecting tubule (93) but there is no effect on Cl\(^-\) -formate or Cl\(^-\) - oxalate exchange in proximal tubules (96). Thus, the molecular identity of proximal tubule chloride – formate exchanger is yet to be identified.

1. As a strategy to identify one or more anion exchangers responsible for mediating Cl\(^-\) reabsorption in the proximal tubule EST database screens for homologs of Pendrin with expression in mouse kidney might allow cDNA cloning of Pendrin homologs.

2. Once the full length sequence of a novel Pendrin homolog has been obtained:
   a) Northern analysis will allow to determine the expression profile of this gene
   b) Functional studies in Xenopus oocytes will be necessary to characterize this protein in order to determine its transport properties
   c) Immunocytochemical studies can be used to detect the protein in cells or tissues with regards to its presence and subcellular localization.
2. Material and Methods

2.1. Buffers and Solutions

2.1.1. Buffers used for Northern analysis

2.1.1.1. SSC (20x)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Molarity (in M)</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>3</td>
</tr>
<tr>
<td>NaCitrate</td>
<td>0.3</td>
</tr>
</tbody>
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2.1.1.2. Church and Gilbert buffer

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount:</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>1%</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.001 M</td>
</tr>
<tr>
<td>Phosphate buffer*</td>
<td>0.5 M</td>
</tr>
<tr>
<td>SDS</td>
<td>7%</td>
</tr>
</tbody>
</table>

* Phosphate buffer (2 M) (pH 7.2) is made up by dissolving 142 g Na₂HPO₄·7 H₂O in 999 ml H₂O and adding 1 ml of 85 % H₃PO₄
2.1.2. Buffers used for a 7.5 % polyacrylamide gel

2.1.2.1. Stacking gel

<table>
<thead>
<tr>
<th>Substance:</th>
<th>Amount:</th>
</tr>
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<tbody>
<tr>
<td>30 % Acrylamide, 0.8 %bis</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>1 M Tris-HCl, pH 6.8</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.24 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>120 µl</td>
</tr>
<tr>
<td>Ammonium Persulfate (0.1g in 1 ml)</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

2.1.2.2. Separating gel

<table>
<thead>
<tr>
<th>Substance:</th>
<th>Amount:</th>
</tr>
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<tbody>
<tr>
<td>30 % Acrylamide, 0.8 %bis</td>
<td>7.37 ml</td>
</tr>
<tr>
<td>1 M Tris-HCl, pH 8.8</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>14.98 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>300 µl</td>
</tr>
<tr>
<td>Ammonium Persulfate (0.1g in 1ml)</td>
<td>150 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
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</table>
Material and Methods

2.1.2.3. Sample buffer

<table>
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<tr>
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<tr>
<td>Glycerol</td>
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</tr>
<tr>
<td>β-Mercaptoethanol</td>
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</tr>
<tr>
<td>5 mM Tris base pH 6.8</td>
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2.1.2.4. Transfer buffer

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<tr>
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<tr>
<td>Methanol</td>
<td>800 ml</td>
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<tr>
<td>H₂O</td>
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2.1.3. Buffers used for oocyte preparation and flux-studies

2.1.3.1 Ca²⁺ - free hypotonic oocyte medium

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<td>NaCl</td>
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</tr>
<tr>
<td>KCl</td>
<td>2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
</tr>
<tr>
<td>Tris-HEPES**</td>
<td>5</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
</tbody>
</table>
**Tris-HEPES** is Tris-(Hydroxymethyl)-aminomethan-N-hydroxyethylpiperazin-N’-2-ethan-sulfonat (pKs 7.4) and serves as a buffer

### 2.1.3.2. Ca$^{2+}$ isotonic oocyte medium

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<td>5</td>
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<tr>
<td>pH</td>
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### 2.1.3.3. Chloride - free uptake buffer

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<td>Hemi-calcium-gluconate</td>
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<tr>
<td>HEPES</td>
<td>5</td>
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<tr>
<td>pH</td>
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</tr>
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</table>
2.2. Vector maps

2.2.1. pCR 2.1. TOPO

Figure 5:
PCR 2.1-TOPO vector map. The vector was used for the full-length cloning procedure of a mouse EST. Taken from www.invitrogen.com (97)
2.2.2. pcDNA 3.1

Figure 6: pcDNA 3.1 vector map. The vector was used for the transient expression of a putative anion transporter in COS 7 cells. Taken from www.invitrogen.com (98)
2.2.3. pGH 19

Figure 7:
pGH 19 vector map (99). The Xenopus expression plasmid pGH19 was used for expression studies of the putative anion transporter in Xenopus laevis oocytes. Picture by Knauf / Kraus.

2.3. cDNA cloning of a putative chloride – base exchanger

2.3.1. The GenBank expressed sequence tag (EST) database
The accumulation and analysis of partial, “single-pass” cDNA sequences (“expressed sequence tags”) has become an important component of modern genome research (100-103).
Expressed sequence tags (ESTs) are short DNA sequences generated from the 3’ and 5’ ends of random cDNA clones. The average length of 200 – 500 bp is a function of
current automated sequence technology. Usually, several hundred to several thousand clones are isolated at random from a given cDNA library (104,105). These clones are only sequenced a single time, using universal primers complementary to the vector at the multiple cloning site(s). Thus, ESTs are qualitatively and quantitatively different from traditional database entries in terms of accuracy and completeness. Automated “single pass” sequencing results in a 3% error or base ambiguity rate. Contaminations with vector and other spurious sequences are frequent problems. In addition, it is not generally known in advance if these fragments originate from the coding sequence or non-coding regions from mRNA. Due to the fast sequencing process the number of ESTs is increasing rapidly. Every day a thousand new entries are submitted to publicly available databases, such as dbEST at the NCBI (106,107). The so-called BLAST allows one to submit sequence data to search for sequence similarity and retrieve all records that match to a given sequence. Therefore, EST databases have an immediate practical value of interest to a broad range of researchers as it allows the accelerated “in-silico” cloning of genes from different species for which homologs in other organisms have already been functionally characterized.

2.3.2. Full length cloning of a mouse EST with expression in the kidney
The reported amino acid sequence for human pendrin was used to perform a tblastn search of the GenBank EST database. Four unique mouse EST clones were identified that had neither overlap with each other nor close identity to cDNAs encoding known proteins. Primers were synthesized based on the reported sequences and used to screen a mouse renal cDNA library (Clontech) by PCR to identify those that were expressed in kidney. Products of the expected size were obtained using primers based on two of the EST sequences (GenBank AA871419 and AI747461). These two EST clones were then obtained (Research Genetics, Inc.) and sequenced. The 0.7 kb of AA747461 sequence was found to be entirely contained within the 1.2 kb sequence of AA87141. Analysis of the sequence of
AA871419 indicated the presence of an open reading frame encoding the C-terminus of a predicted protein with approximately 40% identity to pendrin. 5'-RACE was then performed using Marathon-Ready mouse kidney cDNA (Clontech) as template for nested PCR. A 1.8 kb PCR product was successfully amplified, and subcloned into pCR2.1-TOPO (as shown in Figure 5). Sequencing indicated that this PCR product contained 144 bp of 5' UTR and the first ATG of a single open reading frame that was continuous with the reading frame in AA871419. The presence of upstream stop codons in the same reading frame confirmed that the start codon had been identified.

For expression studies, the coding sequence was amplified by PCR using primers designed to incorporate upstream and downstream EcoR1 and Xba1 restriction sites, respectively (5' primer: CCAAGAATTCCACCATGGGGCTGCAGAGGAGAGACTA; 3' primer: CCGGTCTAGATCAGAGTTTGGTGGGCCAAAACA). This PCR product was subcloned into the pCR2.1-TOPO vector (as shown in Figure 5) and fully sequenced (Yale University Sequencing Facility). The EcoR1 and Xba1 restriction sites were then used to excise and ligate the putative anion-exchanger coding region either into the Xenopus expression plasmid pGH19 (as shown in Figure 7) between the 5’ and 3’-untranslated regions (UTRs) of the Xenopus β-globin gene (99), or into the mammalian expression vector pcDNA3.1(+) (as shown in Figure 6).
2.4. **Northern analysis**

2.4.1. **Detecting mRNA via Northern analysis**

Northern blotting is a technique used for the detection and quantification of RNA. Northern analysis is a variation of the Southern blotting technique developed by Southern et al. in 1975 (108). Whereas the overall procedure of the two techniques is similar, they differ in that Northern blotting refers to RNA-DNA hybridization, whereas Southern blotting employs DNA-DNA hybridization. Due to the similarity of the two techniques, the term Northern blotting was created, as a simple play on words of Southern blotting (109).

Total cellular RNA, or poly (A) RNA, consists of three types of RNA: tRNA (transfer RNA – active in assembly of polypeptide chains), rRNA (ribosomal RNA – part of the structure of ribosomes) and mRNA (messenger RNA – the product of DNA transcription used for translation of a gene into a protein). Northern blotting focuses on detecting mRNA.

Thus, this technique is useful as an adjunct to cDNA cloning (as described under 2.3.2.) because this procedure can indicate which tissues or cell types express a particular gene. Furthermore, the size of a specific mRNA can be compared with the size of a cloned cDNA in order to reveal whether the cloned cDNA is full length. There are several basic steps to Northern hybridization:
Material and Methods

Step 1:
Total RNA that has been isolated and purified from a particular cell is loaded on a denaturing electrophoretic gel. A commercially available RNA-standard is also loaded to determine the size of a specific RNA fragment.

Step 2:
The next step is to pass an electric current through the gel and the RNA moves away from the negative electrode. The distance moved depends on the size of the RNA fragment. As genes are of different sizes the size of mRNA varies also.

Step 3:
Next, the RNA is transferred onto a nitrocellulose or nylon membrane. The membrane is placed over the RNA gel and a highly concentrated salt solution, such as 20X SSC, is then used to transfer the RNA to the membrane by capillary action.

Step 4:
After 48 hours the transfer of the RNA from the gel to the membrane is complete. The RNA can be covalently cross-linked onto the membrane by exposing it to a short pulse of UV-light (not shown).

Step 5:
The blot is placed into a glass tube and a radionlabelled DNA-probe specific for the RNA fragment in question is incubated with the blot overnight. This is usually performed in a hybridization oven, which carefully regulates the temperature and allows the blot to turn constantly.

Step 6:
Unbound probes are washed away from the blot by using high temperature and high salt washing solution. Consequently, only those sequences that have perfectly matched form stable hybrids.

Step 7:
The radioactive probe that has bound to the blot can be detected by placing the membrane next to a X-ray film.
Material and Methods

Figure 8:
Basic steps to Northern hybridization (Knauf/Kraus)

2.4.2. Northern analysis of a novel cDNA with homology to pendrin

A mouse multiple tissue Northern blot (Clontech; Palo Alto, CA) was probed with a 32P-labelled probe. The probe was a PCR-generated cDNA clone spanning nucleotides 1556-1992 of the putative anion-exchanger coding sequence (primers 5’ –AAGTCTTCCGTTCCTCAGCCAC–3’ and 5’GCTCTTAATGCACACAGTGTCCAC–3’). This region has low sequence similarity to pendrin and was specifically chosen to minimize the potential for cross-hybridization to the pendrin transcript.

Sterile redistilled water was added to 25 ng template DNA to a final volume of 8 µl. The DNA was denatured by heating in a boiling water bath for 10 min and chilled quickly on ice. The denatured DNA was mixed with 4 µl of High Prime reaction mixture (Roche Molecular Biochemicals), 3 µl dATP, dGTP, dTTP and 5 µl of [α32P]dCTP. After incubation for 10 minutes at 37°C the reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0) and by heating to 65°C for 10 min. The radiolabelled DNA was separated from unincorporated nucleotides with Sephadex G-50 Quick Spin Columns (Boehringer Mannheim). The column matrix was resuspended by inversion and drained by gravity. The radiolabelled sample was applied to the centre of the column bed and centrifuged in a swinging bucket rotor und eluted from unincorporated nucleotides bound in the column.

The Northern blot was prehybridized in Church-Gilbert solution containing 50 % formamide and 100 µg of denatured salmon sperm DNA/ml for 24 hr at 42°C. The blot was hybridized for 24 hr at 42°C in the same solution containing 10⁶ cpm/ml of either the 32P random prime-labelled for the putative anion exchanger probe or a similarly labelled β-actin probe as a RNA loading control. After hybridization, the blot was washed in 2X SSC containing 0.5 % SDS for 20 min at room temperature and then 1X SSC containing 0.5 % SDS for 20 min at 55°C.
2.5. Immunocytochemical studies

2.5.1. Antibodies as a “research tool”

The use of synthetic peptides as immunogens is a frequently used technique. Most sequences can be used to induce an antibody specific for the peptide itself (110,111). As the carboxy-terminal sequence is often exposed on the surface of the native protein these regions are preferentially used in order to obtain antibodies that will recognize the native protein.

Some antigens of interest might not be immunogenic because they are too small in size or because of the lack of appropriate class II proteins. This lack can be overcome by coupling to carriers such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or purified protein derivative of tuberculin (PPD).

2.5.2. Raising CFEX and Pendrin specific antibodies

Peptides corresponding to the C-terminal 29 amino acids of rat pendrin (CKDPLDLMEAEMNAEELDVQDEAMRLAS) and of the mouse putative anion exchanger (CFEX) (CVFASVHDAVTALSHRKSVPVLATKL), the latter including an additional N-terminal cysteine, were synthesized by the HHMI Biopolymer Laboratory & W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Each peptide was coupled to keyhole limpet hemocyanin (KLH) with the Inject Maleimide Activated Immunogen Conjugation Kit (Pierce Chemical Co.), and used to immunize rabbits to generate polyclonal antisera (Pocono Rabbit Farm). Anti-sera were purified with affinity columns prepared by immobilizing each peptide using SulfoLink Coupling Gel (Pierce Chemical Co.).
2.5.3. Transient expression of CFEX in COS-7 cells

To verify antibody specificity, immunoblotting was performed using COS-7 cells transfected with CFEX cDNA that had been subcloned in pcDNA3.1 (as shown in Figure 6). COS-7 cells were grown in DMEM media with 10% fetal calf serum, 50 U/ml penicillin, and 50 mg/ml streptomycin at 37°C in 5% CO2-95% air. Cells were plated on 6-well tissue culture dishes for 24 hr and used at 80-90% confluence. Cells were transfected with 2 µg plasmid and 10 µl Superfect Reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. After transfection, cells were incubated for 72 hr and then solubilized in SDS sample buffer for immunoblotting experiments.

2.5.4. Immunoblotting experiments on CFEX-transfected COS-7 cells and mouse renal microsomes

COS-7 cells or renal microsomes isolated from mouse kidney cortex (112) were solubilized in sample buffer and proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels according to Laemmlli (113). For immunoblotting, proteins were transferred to polyvinylidene difluoride (PVDF, Immobilon-P, Millipore, Bedford, MA) with a Transphor™ transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) and stained with Ponceau S in 0.5% trichloroacetic acid. For immunoblotting studies, PVDF strips were incubated first in Blotto (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 hr to block nonspecific binding, followed by overnight incubation in primary antibody diluted 1:100 in Blotto. The strips were then washed in Blotto and incubated for 1 hr with a 1:2000 dilution of the horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Zymed, San Francisco, CA). Bound antibody was

31
detected with the ECL enhanced chemiluminescence system (Amersham Corp) according to the manufacturer's protocol.

2.5.5. Tissue preparation and immunofluorescence staining

Adult female C57BL/6J mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Animals were perfusion-fixed with PLP (2% paraformaldehyde, 750 mM lysine, and 10 mM sodium periodate in phosphate buffer, pH 7.4, 22°C) as described by McLean (114) and Larsson (115). The kidneys were removed, cut into 2-4 mm blocks, and postfixed in the same fixative for an additional 4 hr at room temperature. The tissue was cryoprotected by incubation in a phosphate buffer (pH 7.2) containing 2.3 M sucrose and 50% polyvinylpyrrolidone (116), mounted on aluminum nails, frozen, and stored in liquid nitrogen.

Semithin cryosections (0.5 µm) were cut with a Reichert Ultracut E ultramicrotome fitted with an FC-4E cryoattachment and then mounted on gelatin-coated slides. The sections were washed in TBS and then incubated with 1% BSA and 0.5 M ammonium chloride in TBS for 15 min at 20°C in a humidified chamber. The ammonium chloride quenching wash was removed and the sections were incubated with 1% SDS in TBS for 5 min. The sections were washed twice with TBS and then blocked with 10% goat serum and 0.1% BSA in TBS for 15 min at 20°C. The sections were then incubated with anti-CFEX or anti-pendrin primary antibodies diluted in TBS containing 10% goat serum and 0.1% BSA for 16 hr at 4°C. The primary antibodies were removed and the sections were washed 3 times (10 min each wash) with high salt TBS (3.4% NaCl) containing 0.1% BSA and then
incubated for 1 h with a fluorescein-conjugated secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Molecular Probes, Eugene, OR) diluted 1:200 with the TBS/BSA/goat serum blocking buffer. The slides were washed for 10 min with high salt TBS, 2X with standard TBS (0.9% NaCl; 10 min each wash) and mounted in VectaShield (Vector Corp, Burlingame, CA) to prevent fading of the immunofluorescent signal. The slides were then visualized on a Zeiss Axioskop phase-contrast microscope.

To test for specificity of antibodies, peptide preabsorption was performed. Prior to use for immunofluorescence, 10 µl aliquots of the affinity purified primary antibodies were incubated with 30 µg of either the CFEX or pendrin peptide described above in 500 µl of TBS for 16 hr at 4°C. The samples were centrifuged at 18,000xg for 15 min at 4°C. The supernatants were then removed and stored at 4°C until needed. The anti-CFEX/peptide and anti-pendrin/peptide mixtures were used at dilutions of 1:4 and 1:6 respectively.

2.6. The use of Xenopus laevis oocytes for CFEX transport studies

2.6.1. The use of Xenopus laevis oocytes for the functional expression of membrane proteins

Amphibian eggs as a system to determine the function of components injected into them has a long history. In the pioneering studies of Briggs and King in 1952 (117) eggs from the leopard frog *Rana pipiens* were used for studies on nuclear
transplantation. In the following years the South African clawed frog *Xenopus laevis* became popular (118,119) because its oocytes proved to be easier for biochemical experiments such as extraction of nucleic acids (120). Their work established the conclusion that fully grown oocytes contain large reserves of the components required for protein synthesis (121). Gurdon et al. (122) were the first to report that mRNA could be translated into the specific protein when microinjected into *Xenopus* oocytes. Ever since, they have become a popular research tool in particular for studying membrane proteins such as ion channels and transporters. A detailed description of the use of Xenopus oocytes for the study of heterologously expressed membrane proteins is given by Wagner et al. (123).

Next to electrophysiological studies such as patch-clamp technique (124) and two-electrode voltage clamp (123), studies using labelled compounds have become a widely used method for the study of transporters expressed in oocytes. With regards to electroneutral transport mechanisms they are indispensable, but also for investigations of electrogenic transport flux experiments provide an important technique.

### 2.6.2. Oocyte isolation and preparation

Oocytes were isolated from female *Xenopus laevis* frogs by partial ovarectomy under tricane (3-aminobenzoic acid ethyl ester) anesthesia (0.2 % for 5-10 min). A small incision was made in the abdomen and a lobe of ovary was removed. The removed tissue was manually dissected into small clumps of 5-10 oocytes using small forceps and washed for 20 min in Ca²⁺-free hypotonic medium (as described under 2.1.3.1.) to remove blood and damaged tissue. Oocytes were then
defolliculated by treatment with 2 mg/ml collagenase (Worthington type I or Sigma type I) in Ca\(^{2+}\)-free hypotonic solution for 45-90 min with gentle agitation at room temperature. The oocytes were periodically checked and the digestion terminated when the oocyte follicle had been removed with minimal damage to the oocytes. Following the incubation oocytes were washed three times in Ca\(^{2+}\)-free hypotonic media, then washed three times in isotonic solution (as described under 2.1.3.2.). Stage V-VI (Dumont) oocytes were selected and maintained at 18° C in the same isotonic solution supplemented with 50 µg/ml gentamicin and 2.5 mM sodium pyruvate.

pGH19 plasmid DNA was linearized by XhoI digestion and cRNA was transcribed using T7 RNA polymerase (mMESSAGE mMACHINE, Ambion, Austin, TX). Precipitated cRNA was dissolved in sterile H2O and yield and quality were assessed by spectroscopy and agarose gel electrophoresis. On the day of their isolation, oocytes were microinjected with 50 nl of sterile H2O or 50 nl of a cRNA solution containing 25 ng of CFEX cRNA by use of a pneumatic injector. The injected oocytes were then incubated at 18°C for ~48 hr to allow for expression of the protein.

2.6.3. Functional studies on oocytes expressing CFEX

For uptake experiments, oocytes were twice washed at room temperature in 1 ml chloride-free solution before incubation in 500 µl of the same solution containing 3.4 mM \(^{36}\text{Cl}\) or 38 µM \(^{14}\text{C}\) formate. After 30 min incubation, external isotope was removed by washing the oocytes three times with 1 ml of ice-cold chloride-free buffer. For chloride efflux measurements, oocytes were incubated for 60 minutes in a chloride-free buffer containing \(^{36}\text{Cl}\). After three washes with ice-cold chloride-
free buffer, the $^{36}$Cl content of oocytes was measured both initially and 30 min after suspension in solutions containing test anions. Net efflux was calculated as the difference between the initial oocyte $^{36}$Cl content and that remaining after 30 min re-incubation. For experiments in which 10 mM anions (Cl-, formate, HCO$_3^-$, acetate) were tested for effects on $[^{14}$C]formate uptake or $^{36}$Cl efflux (Figs. results), gluconate was isosmotically replaced by each test anion and media were buffered with 5 mM Tris titrated with HEPES to pH 8.5. An external pH of 8.5 was used because it allowed the pH of the HCO$_3^-$ solution to remain stable due to the ampholyte effect (125). Radioisotope content of each individual oocyte was measured by scintillation spectroscopy after solubilization in 0.2 ml 10 % SDS and addition of 3 ml scintillation fluid (Opti-Fluor, Packard). Figure 5 summarizes the important steps.
Material and Methods

1. Step:

cRNA transcribed from a vector containing the sequence of CFEX is injected into the oocyte cytoplasm. The injected cRNA is translated into the protein, modified and finally sorted to the plasma membrane.

2. Step:

In order to study CFEX transport activity, radiolabelled [14C]formate is added to the outside solution.

3. Step:

[14C]formate gets transported into the cell for exchange of Cl⁻. Radioisotope content of each individual oocyte can be counted representing the amount of transport activity.
Figure 9:
Single steps to the expression of CFEX in Xenopus oocytes (by Knauf/Kraus)

2.6.4. Converting counts into molarity
In order to determine the amount of radiolabelled substrate in moles transported by every single oocyte, 5 µl of the uptake solution was counted separately as a standard in all experiments. The standard in cpm was multiplied with the concentration of isotope in the uptake solution in moles. The counts of a single oocyte were put in ratio with the standard in order to convert the counts into the moles transported.

2.7. Statistical analysis
Results shown in the bar graphs represent means ± S.E.M. (Standard error of the mean) for the number of oocytes indicated. In some cases the S.E. values are too small to be visible in the figures. The results were analyzed using paired or non-paired Student’s t-test. P values < 0.05 were considered statistically significant.
3. Results

3.1. Identification of a chloride – base exchanger

In an attempt to identify an anion exchanger capable of mediating Cl⁻- reabsorption in the proximal tubule, the EST database was screened for homologs of pendrin. A novel mouse EST was identified and 5'-RACE was used to obtain the full coding sequence of the corresponding transcript. The sequence (GenBank accession number AY032863) encodes a predicted protein of 735 amino acids.

A database search shown in Figure 1 indicates very high similarity to members of the SLC26A family of mammalian anion transporters described under 1.3.2.. Closest homology was observed to the human SLC26A6 (79% amino acid identity) (126,127) and lower but significant similarity (30-40% identity) to pendrin (89) and other members of the family such as “down-regulated in adenoma” (DRA) (87), diastrophic dysplasia sulfate transporter (DTDST) (128), the sulfate transporter SAT1 (78), and prestin (129). SLC26A6 was previously identified as encoding a putative anion exchanger expressed in renal tubules and pancreatic ducts (126,127), although functional expression studies failed to detect anion transport activity (127).

A tblastn search of the NCBI working draft sequence of the human genome indicated no higher match than to exons of SLC26A6. Accordingly, it is very likely that the cDNA cloned is the mouse ortholog of human SLC26A6.
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Results

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DRAmouse  LQVFVFVYLYSLSQLIGTVSTSDTQGTVKFLQPLPAYSDFPSFKKLSVLSPFQI
DDSTmouse  FQGFVFSYVLSDALLSGFVTGSAFTITLSQAKY1LGLSLPIRSHVGVSYTIIH1FRNI
SAT1rat  LRLGFYSTRQQLDQFAGMAGAVTITLSQAKY1LGVR1PRHCQGLMV1HTWSSLQNVK

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CFEXmouse  ETVPGTGTVTAIVGAVLAVLKLNEKLHRLPLPIGELLTLIGATGISYGVKLDNDRFKV
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DRAmouse  KTVNADLTVS1LLVYVVFKE1NQYRSKLV1P1P1EI1V1ATGVSYGCNPEDRFQG
DDSTmouse  TTVNADLTVS1LLVYVVFKE1NQYRSKLV1P1P1EI1V1ATGVSYGCNPEDRFQG
SAT1rat  QANLDCVDTSACLAVLWTAK1LSDRYRHYL1VP1VP1ELTVI1AVTISVF

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CFEXmouse  DTVNADT1G1PL1LFPN1P1DS1VES1FLVYDA1IA1VGFVS1TS1M1TLK1H1G1YQVD1GNQEL
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DRAmouse  KTVNADLTVS1LLVYVVFKE1NQYRSKLV1P1P1EI1V1ATGVSYGCNPEDRFQG
DDSTmouse  TTVNADLTVS1LLVYVVFKE1NQYRSKLV1P1P1EI1V1ATGVSYGCNPEDRFQG
SAT1rat  QANLDCVDTSACLAVLWTAK1LSDRYRHYL1VP1VP1ELTVI1AVTISVF

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SLC26A6human  VALGLS11G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
prestinmouse  VALGLS11G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
pendrinmouse  VALGLS11G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
DRAmouse  VALGLS11G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
DDSTmouse  VALGLS11G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
SAT1rat  VALGLS11G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL

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pendrinmouse  QAVLS11F1G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
DRAmouse  QAVLS11F1G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
DDSTmouse  QAVLS11F1G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
SAT1rat  QAVLS11F1G1FF1QCFPVS1CM1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL

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DDSTmouse  PAVLAA1I1V1N1V1K1G1M1F1Q1V1F1S1C1M1S1VQESTG1T1QVA1VAV1SL1V1LL1V1L1GF1L1FRDL
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<td>SAT1rat</td>
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Results

prenstinmouse: AVLGSQ--------VREAMAEQETASLPQEDMENPATPTPEA------
pendrinmouse: AILHILQNQVKSREGQDSLLETVARIRDPLMLLEAEAEEDVQDEAMRAS
DRAmouse: AILHIL--MKKDYSTSKFNSQEKERKDFITIN-TNGGLRNYRECQVPVEKTF----
DDSTmouse: AVAFAEDSQN----------------------Q--KGVCVNGLSLSGD------
SAT1rat: AVETAEDSQN----------------------Q--KGVCVNGLSLSGD------

*: 

Prim.cons.: AVLHAQ334K222222222233E33R3333TFAL52G62VFN22VL6TE222RLAS

Figure 10:
Sequence alignment of CFEX.

The predicted CFEX protein has a large hydrophobic domain predicted to contain 10-12 membrane-spanning segments (Figure 11) and an intracellular hydrophilic domain containing a consensus site for tyrosine phosphorylation and a possible PDZ interacting domain at the C-terminus, as previously described for SLC26A6 (126,127).

Figure 11:
Hydropathy analysis of CFEX using Kyte-Doolittle (131) with an amino-acid window of 20.
3.2. Expression of CFEX in mouse tissue

The expression of CFEX in mouse tissues was evaluated by Northern analysis as described under 2.4.. The principal transcript of approximately 2.7 kb was most abundant in heart, liver and kidney, with lesser degrees of expression noted in testis, brain, lung and skeletal muscle as shown in Figure 12. The ubiquitous pattern of expression of a 2.7 kb transcript with greatest abundance in heart and kidney is similar to that reported for SLC26A6 in human tissues (127). However, whereas CFEX was well-expressed in liver, SLC26A6 was barely if at all detectable in this organ. Thus, if CFEX and SLC26A6 represent orthologs of the same gene, there are significant species differences in its level of expression in different organs.
Figure 12:
Northern analysis of CFEX expression in mouse tissues and the respective size of about 2.7 kb of the transcript (a). Actin control shown in lower panel (b).
3.3. Immunolocalization of CFEX and Pendrin in mouse kidney

To evaluate whether CFEX is expressed in the brush border membrane of proximal tubule cells, a CFEX-specific anti-peptide antibody was generated for immunohistochemical studies (as described under 2.5.1.). To verify that the antibody is specifically reacting with the intact CFEX protein, immunoblotting was performed using COS-7 cells transiently transfected with CFEX cDNA. As demonstrated in Figure 4, the antibody labeled a polypeptide of approximately 78 kDa in CFEX-transfected cells that was absent in control cells. Antibody staining was blocked by preincubation of the antibody with the immunizing CFEX peptide against which it had been generated (not shown). These findings confirmed the CFEX-specificity of the antibody. In mouse kidney membranes, the antibody labeled a single band of approximately 90 kDa, indicating expression of CFEX in native renal tissue. The small disparity in molecular weight may reflect incomplete glycosylation in the transfected overexpressing COS-7 cells.
Results

Figure 13:
Immunoblot to verify specificity of anti-CFEX antibody. Lane 1: untransfected COS-7 cells; Lane 2: CFEX-transfected COS-7 cells; Lane 3: mouse kidney membranes. 20µg of protein were loaded in each lane.

In order to determine the cellular and subcellular localization of CFEX in mouse kidney, indirect immunofluorescence microscopy was carried out on semithin (0.5 µm) cryosections of PLP-fixed mouse kidney, as illustrated in Figure 14. The anti-CFEX antibody stained the apical surface (brush border membrane) of proximal tubules (Figure 14A). Confirming its specificity, this staining was absent when the antibody was preincubated with the CFEX peptide against which it was generated (Figure 14B). A higher power view confirmed the staining of the brush border membrane of proximal tubule cells (Figure 14C and 14D).
Figure 14:
Immunolocalization of CFEX in mouse kidney. A. Staining of proximal tubules with anti-CFEX antibody in presence of pendrin peptide. B. Absence of staining when antibody was blocked with CFEX peptide. C. Brush border staining. D. Phase image for panel C. Magnifications: A and B * 160; C and D * 420.

In further experiments it was noted that not all proximal tubule segments were stained, indicating heterogeneity with respect to CFEX expression as shown in lower magnification in Figure 15. A more careful examination showed weak or no staining in the early proximal tubule segment (S1) and a strong signal in the late proximal tubule
Results

(straight segment, S3) consistent with the hypothesis that CFEX may mediate proximal tubular Cl− reabsorption.

Figure 15:
Immunolocalization of CFEX in mouse kidney. A Phase image. B. Staining of proximal tubules with anti-CFEX antibody. Note glomerulum and beginning S1 segment in the upper part of the figure showing almost no staining indicating low expression of CFEX in the early proximal tubule. Magnification * 160

Because it has been proposed that pendrin may mediate apical Cl−- base exchange in the proximal tubule (92), we performed additional immunocytochemical studies with an
anti-pendrin antibody. As shown in Figure 16A and 16B, the anti-pendrin antibody failed to stain the apical membrane of proximal tubule cells but labeled the apical membranes of cells in the cortical collecting tubule. This staining was blocked when the anti-pendrin antibody was preincubated with the pendrin peptide against which it had been generated (Figure 16C and 16D).

Figure 16: Immunolocalization of pendrin in mouse kidney. A. Staining of a connecting segment or cortical collecting duct with the anti-pendrin antibody in presence of CFEX peptide. B. Phase image for panel A. C. Absence of staining when anti-pendrin antibody was preincubated with the immunizing pendrin peptide. D. Phase image for panel C. *: cortical collecting duct. Magnification * 160.
The observation of pendrin labeling on the apical membrane of cortical collecting duct cells rather than proximal tubule cells is in agreement with the results of recent reports (94-96). Taken together, the findings in Figure 14 - 16 indicate that the expression of CFEX but not pendrin can be detected on the brush border membrane of proximal tubule cells.

3.4. Functional studies of CFEX expressed in Xenopus oocytes

Given that CFEX is expressed on the apical membrane of proximal tubule cells and is therefore a candidate for mediating brush border Cl\(^-\)-formate exchange, we next examined its ability to mediate Cl\(^-\)-gradient-stimulated formate uptake when heterologously expressed in Xenopus oocytes. Intracellular Cl\(^-\) is approximately 30 mM in Xenopus oocytes in isotonic saline media (131). We measured uptake of 38 µM \(^{14}\text{C}\)formate after washing and reincubation of oocytes in the absence of external Cl\(^-\) imposing an outward Cl\(^-\) gradient. As shown in Figure 17, formate uptake was greatly stimulated into oocytes injected with CFEX cRNA as compared to water-injected controls. The CFEX-mediated formate uptake was almost completely inhibited by the disulfonic stilbene DIDS (0.1 mM), a known inhibitor of renal brush border Cl\(^-\)-formate exchange (29,30,50). These results demonstrate that CFEX can function as a DIDS-sensitive formate transporter.
Results

Figure 17:
CFEX-mediated formate uptake. Uptake of 38 μM [14C]formate was assayed in water- and CFEX cRNA-injected Xenopus oocytes for 30 minutes. 0.1 mM DIDS completely abolished CFEX-induced formate uptake. The number of oocytes (n) for each group is given.

As one method to test the anion specificity of CFEX, we compared the effects of various anions added to the external solution as inhibitors of [14C]formate uptake. As indicated in Figure 18, uptake of 38 μM [14C]formate was strongly inhibited by unlabeled formate (10 mM), as expected for a saturable formate transport process. Formate uptake was very strongly inhibited by external Cl\(^-\) (10 mM) suggesting that Cl\(^-\) may indeed be a substrate for the same transporter. Significant inhibition of formate uptake also resulted from addition of external HCO\(_3^-\), although the effect was smaller than for Cl\(^-\) and formate. In contrast, acetate, a monocarboxylate with the next longer carbon-chain, had no effect on formate uptake mediated by CFEX. Bicarbonate can
lead to an intracellular acidification of oocytes (132), which in turn could inhibit CFEX activity. However also acetate acidifies oocytes (133) and had no effect on formate uptake suggesting that intracellular acidification was not responsible for the effect of bicarbonate on formate uptake. The findings in Figure 18 indicate that CFEX shares several properties with the renal brush border Cl⁻-formate exchanger, including the absence of affinity for acetate (29,30,50). However, the inhibition by HCO₃⁻ raised the possibility that CFEX might function as a more general Cl⁻-base exchanger as described for pendrin (15).

![Figure 18](image)

**Figure 18:**
Effect of external anions on [¹⁴C]formate uptake. Uptake of 38 μM [¹⁴C]formate was assayed in water- and CFEX cRNA-injected Xenopus oocytes in the presence of various anions. Effects of 10 mM of each indicated anion on CFEX-mediated [¹⁴C]formate uptake were assessed by isosmotic replacement of gluconate in the media. Chloride, formate and to a lesser extent bicarbonate abolished formate uptake.
To test whether CFEX mediates Cl\(^-\) transport, as predicted for a Cl\(^-\)-formate or Cl\(^-\)-base exchanger, we next measured \(^{36}\)Cl uptake. As demonstrated in Figure 10, uptake of 3.4 mM \(^{36}\)Cl was greatly stimulated into oocytes injected with CFEX cRNA as compared to water-injected controls. As in the case of CFEX-mediated formate flux, \(^{36}\)Cl uptake was almost completely inhibited by 0.1 mM DIDS. Moreover, as also shown in Figure 19, CFEX-mediated Cl\(^-\) uptake was unaffected by imposition of an outside-acid pH gradient, arguing against significant Cl\(^-\)-OH\(^-\) exchange. In these experiments, \(^{36}\)Cl uptake was measured in the absence of added external Cl\(^-\) so that an outward gradient of unlabeled Cl\(^-\) was present. Under these conditions, CFEX-mediated \(^{36}\)Cl uptake most likely represented Cl\(^-\)-Cl\(^-\) exchange, which is a known mode of the renal brush border Cl\(^-\)-formate exchanger (29,30). Exchange of Cl\(^-\) for endogenous formate might also have contributed to the measured \(^{36}\)Cl uptake.
Results

Figure 19:
CFEX-mediated Cl⁻ uptake. Uptake of 3.4 mM ³⁶Cl was assayed in water- and CFEX cRNA-injected Xenopus oocytes. Media containing 5 mM HEPES were titrated with Tris base to pH 7.5 or 6.5. Effects of 0.1 mM DIDS were also measured.

A final series of transport studies were performed to test directly whether CFEX functions as an anion exchanger. To this end, CFEX cRNA-injected oocytes were preloaded with ³⁶Cl by preincubation for 60 minutes under the same conditions used to measure ³⁶Cl uptake in Figure 10. The oocytes were then washed and resuspended in media containing 10 mM external Cl⁻, formate, HCO₃⁻ or acetate and reincubated for an additional 30 minutes. As shown in Figure 20, external Cl⁻ and formate each markedly stimulated net loss of ³⁶Cl compared to the control medium. In contrast, no appreciable stimulation of ³⁶Cl efflux followed exposure to external acetate or HCO₃⁻. These results are consistent with CFEX-mediated Cl⁻ - Cl⁻ and Cl⁻ - formate exchange,
whereas exchange of Cl\(^-\) for acetate or HCO\(_3^-\) was minimal. These findings are in accordance with the known modes of the renal brush border Cl\(^-\)-formate exchanger (29,30).

**Figure 20:**
Effect of external anions on \(^{36}\text{Cl}\) efflux. Oocyte content of \(^{36}\text{Cl}\) was assayed after the initial 60 min period of incubation with 3.4 mM \(^{36}\text{Cl}\), and after 30 min reincubation in \(^{36}\text{Cl}\)-free K-gluconate media without (control) or with isosmotic replacement of gluconate by 10 mM of each indicated anion.
4. Discussion

4.1. Discussion of sources of error

4.1.1. Demonstration of antibody specificity
The basic principle of immunochemical techniques is that a specific antibody will bind a specific antigen to give an antibody-antigen complex (as described in detail under 2.5.1.). However, very often more than one band is being observed in Western blot when probed with a given antibody or a more diffuse staining or high background is seen in immunolocalization studies. These problems raise the question whether a specific antibody-antigen reaction has taken place or non-specific binding is being observed.

There are two common methods in order to study the antibody specificity. First, it is generally studied by competing with excess of antigen (protein or peptide) where a small volume of antibody is first reacted with excess peptide to neutralize it. The neutralized antibody can no longer bind to another antigen. In case of Western blotting a band of interest will disappear, in the case of immunolocalization studies a staining pattern will vanish. Consequently, the band or staining that is competed by the antigen/peptide is supposed to be specific. If more than one band disappears by peptide/antigen competition then those bands have also the antigenic determinants. They can be considered as fragments of the large antigen or multimer. Second, serum that has been taken from the animal before immunization can be used as a negative control. In order to proof the specificity of an antibody-antigen reaction, the band seen with serum used after immunization should not be present when the serum is used from the same animal taken before immunization. Similarly, staining observed in immunolocalization studies should be absent when preimmunization serum is used.
4.1.2. General aspects of the oocyte expression system
As described in the method section, Xenopus laevis oocytes are a popular tool for the functional expression of membrane proteins. Possible sources of error are endogenous Xenopus proteins that might be similar to the ones that are thought to be studied. Endogenous Xenopus proteins could be activated by substances injected into the oocyte. Similarly, intracellular signalling pathways examined in oocytes do not necessarily correspond to mammalian cell pathways. Proteins missing in the oocyte compared to the native cell could alter the function of the protein expressed.
Nevertheless, Xenopus oocytes allow a clear cut between a real transport process and an artefact: the relatively high amounts of injected cRNA lead to an extremely high expression of the protein to be studied. By routine, the cRNA injected oocytes are compared to water injected oocytes to exclude that an endogenous transporter is being studied. In addition, antibodies can be used in order to detect the presence of a protein. Thus, they allow examining a well-defined question of interest.
One disadvantage is that no quantitative conclusions can be made from observations in oocytes. In vivo studies are indispensable for these kinds of questions.

4.1.3. Oocytes and cRNA
Oocyte and cRNA quality have an enormous influence on the expression rate observed under experimental conditions: the transport rate of a certain substrate can be stimulated compared to the H2O-injected control between 0 and 100 fold. This variation depends on the frog prepared, the collagenase used for the preparation and the age of the oocyte. Similarly, there are differences in the results depending on the quality of the cRNA. With regards to its purity it is important that the cRNA contains no residues from unincorporated nucleotides or the reagents it has been cleaned with such as ethanol or lithium. Storage at -80°C and fresh preparations at every second month help preventing a degradation of the cRNA.
In order to minimize the variables mentioned above, 5 – 10 oocytes were checked prior to each experiment for their protein expression. Oocytes with a transport rate stimulated less
than 40fold were discarded. In addition, a series of experiments was not carried out on a single oocyte preparation but repeated instead with oocytes from different frogs.

4.1.4. Solutions

The pH and the osmolarity of the solutions used (as listed under 2.1.3) were checked on a daily basis. Deviations more than 1% were not tolerated. All the solutions were sterile filtered using a 0.22 µm table top filter system (Beckman Dickensen) and kept at 4°C until the experiment. All the chemicals were kept at the temperature recommended by the vendor and all chemicals protected from light. The radioisotopes were kept at 4°C and a sterile syringe used to take out the amount needed for an experiment.

4.2. Discussion of results

4.2.1. Identification of the CFEX cDNA sequence

The identification of new genes using the GenBank expressed sequence tag (EST) database provides a rich source for discovering new putative anion exchangers. The SLC26 gene family was of special interest as SLC26A4 has been demonstrated to mediate Cl⁻-formate exchange next Cl⁻ - OH⁻ / HCO₃⁻ (92,93). However, as Pendrin is not expressed on the brush border membrane of proximal tubule cells but the apical membrane of collecting tubule cells as shown in Figure 14, the molecular identity of the proximal tubule Cl⁻-formate exchanger has been searched for.

In addition, this gene family is of special interest as three members of the family have already been identified to be associated with recessive diseases, namely SLC26A2-A4 (80 - 84, 88,89). Consequently, unidentified genes might also play an important role in acid-base transporter associated diseases so far unknown.
In the present work the cloning and functional characterization of CFEX is being described. Sequence analysis indicates that CFEX is very likely the mouse ortholog of human SLC26A6 as they share 79% amino acid identity. Close homology was also observed to other members of the SLC26A family such as Pendrin (30-40% identity) (89) and “down-regulated in adenoma” (DRA) (87). Similar to SLC26A6, CFEX has 20 coding exons of which most of the exons are similar in size to the corresponding 20 coding exons of SLC26A3 and SLC26A4 (Figure 10). It is interesting to note that the three last amino acids of the COOH-terminus of SLC26A6 (TRL as seen in Figure 10) comprise the same PDZ-interacting domain as CFTR indicating a similar interaction pathway and polarization signal. Thus, in a recent report it could be demonstrated that CFTR markedly stimulates SLC26A6 mediated Cl⁻ and HCO₃⁻ transport (134). It has also been demonstrated very recently (135) that the C-Terminus of SLC26A6 binds the PDZ domains of NHERF (NHE3 regulatory factor) as well as to CAP70 (CFTR associated protein of 70kDa) in vitro. Only the intact C-terminus of SLC26A6 but not a mutation missing the PDZ interaction motif bound full length NHERF confirming the specificity of the PDZ-interaction while the truncation did not affect the chloride transport function.

However, although CFEX is most likely the mouse ortholog of SLC26A6, this important motif is different from SLC26A6 (TKL versus TRL as seen in Figure 10). Therefore, observations made for CFEX might not count for SLC26A6 and vice versa.
4.2.2. Functional characterization of CFEX
In the present work the preliminary functional characterization of CFEX, a Cl\(^-\)-formate exchanger expressed on the brush border membrane of proximal tubule cells is being described. The transport properties of CFEX, including its lack of detectable affinity for acetate and its ability to mediate Cl\(^-\)-formate and Cl\(^-\)·Cl\(^-\) but not Cl\(^-\)·OH\(^-\) exchange (Figures 18-20), closely resemble those described for the renal brush border Cl\(^-\)-formate exchanger in physiological studies utilizing brush border membrane vesicles (29,30) and perfused tubules (50,55). Similarly, HCO\(_3\)\(^-\) was a poor substrate for exchange with Cl\(^-\) in studies of renal brush border vesicles (29,30), and CFEX mediated little if any Cl\(^-\)·HCO\(_3\)\(^-\) exchange under the experimental conditions applied. Although no significant inhibition by HCO\(_3\)\(^-\) of CFEX-mediated formate transport was observed in these studies, raising the possibility that external HCO\(_3\)\(^-\) is a modifier of CFEX activity, measurements of intracellular pH have shown that CFEX can also function as a Cl\(^-\)·HCO\(_3\)\(^-\) exchanger (138). It is interesting to note that formate stimulation of proximal tubule NaCl absorption is abolished when tubules are perfused with 25 mM compared to 5 mM HCO\(_3\)\(^-\) (50). This observation has been attributed to a requirement for luminal acidification to permit the pH-dependent recycling of formic acid to replenish intracellular formate and thereby sustain Cl\(^-\) absorption by apical membrane Cl\(^-\)-formate exchange (50). The present findings raise the additional possibility that luminal HCO\(_3\)\(^-\) has a modifier role to regulate the rate of Cl\(^-\)-formate exchange in the proximal tubule.

In addition to Cl\(^-\)-formate exchange, a significant component of Cl\(^-\) absorption in the proximal tubule can be mediated by Cl\(^-\)·oxalate exchange (29, 32, 137, 138). Studies in membrane vesicles have suggested that Cl\(^-\)-formate exchange is mediated by a separate pathway than that mediating Cl\(^-\)-formate exchange (30, 137). However, recent studies have revealed that CFEX functions in multiple modes with affinity also for oxalate and sulfate (138,139).
In the past it had been postulated that three distinct transporters are responsible for proximal tubule anion transport, namely Cl⁻-formate, Cl⁻-oxalate and oxalate-sulfate exchange (29, 30, 48). As CFEX seems to be capable of mediating all three forms of anion exchange listed above, the results presented in this work seem to oppose the previous findings (29, 30, 48). Multiple arguments can, however, bring the previous findings into accord with the data now presented. First, the kinetic studies of Cl⁻-formate, Cl⁻-oxalate and oxalate-sulfate exchange have been made using brush border membranes of rabbit kidneys. Therefore, species differences have to be taken into account, which open the possibility that CFEX may account for all three anion exchange activities in mouse kidney and additional transporters may be involved in rabbit. Second, it might be possible that CFEX associates with additional subunits in native kidney membranes that are missing in Xenopus oocytes allowing multiple transport modes. Third, additional transporters mediating most of Cl⁻-formate exchange have yet to be identified.

4.2.3. Role of CFEX in tissues other than the kidney

As SLC26A6 is very highly expressed on the apical membrane of pancreatic duct cells (126), CFEX may also play a role in transcellular Cl⁻ transport in epithelia other than the proximal tubule. Moreover, Northern analysis as shown in Figure 12 indicates that CFEX expression is not limited to epithelial cells. For example, CFEX is relatively highly expressed in heart, and Cl⁻-formate exchange has been described in cardiac myocytes as well as vascular smooth muscle cells (140).

The physiological role of Cl⁻-formate exchange in such non-epithelial tissues is presently unknown. It is also possible that CFEX might function as a more general Cl⁻-base exchanger in some native tissues under physiologic conditions.
4.3. Conclusion and unresolved issues

In conclusion, this work shows that CFEX mediates Cl\(^-\)-formate exchange and is expressed on the brush border membrane of proximal tubule cells. Accordingly, CFEX may be responsible for mediating formate-stimulated NaCl absorption in the proximal tubule. It may thereby play an important role in renal NaCl reabsorption. However, recent studies suggest that CFEX might function as a more general Cl\(^-\)-base exchanger. Given its wide tissue distribution, CFEX may also contribute to transcellular Cl\(^-\) transport in additional epithelia such as the pancreas, and contribute to transmembrane Cl\(^-\) transport in non-epithelial tissues such as the heart.

The characterization of CFEX knockout mice will allow to clarify the question whether CFEX is responsible for Cl\(^-\)-formate and Cl\(^-\)-oxalate exchange or if there are separate gene products responsible for formate and oxalate stimulated Cl\(^-\) reabsorption. In addition, it is clearly warranted to identify new tissue specific members as transport proteins are often organized in membrane micro domains through specific interacting proteins.
Summary
A key function of the proximal tubule is retrieval of most of the vast quantities of NaCl and water filtered by the kidney. Physiological studies using brush border vesicles and perfused tubules have indicated that a major fraction of Cl⁻ reabsorption across the apical membrane of proximal tubule cells occurs via Cl⁻-formate exchange. The molecular identity of the transporter responsible for renal brush border Cl⁻-formate exchange has yet to be elucidated. As a strategy to identify one or more anion exchangers responsible for mediating Cl⁻ reabsorption in the proximal tubule, the EST database was screened for homologs of pendrin, a transporter previously shown to mediate Cl⁻-formate exchange. This work reports the cDNA cloning of CFEX, a mouse pendrin homolog with expression in the kidney by Northern analysis. Sequence analysis indicated that CFEX very likely represents the mouse ortholog of human SLC26A6. Immunolocalization studies detected expression of CFEX but not pendrin in the brush border membrane of proximal tubule cells. Functional expression studies in Xenopus oocytes demonstrated that CFEX mediates Cl⁻-formate exchange. Taken together, these observations identify CFEX as a prime candidate to mediate Cl⁻-formate exchange in the proximal tubule and thereby to contribute importantly to renal NaCl reabsorption. However, a more detailed functional characterization of CFEX will be necessary in order to clarify its main transport function under physiological conditions. Given its wide tissue distribution, CFEX may also contribute to transcellular Cl⁻ transport in additional epithelia such as the pancreas, and contribute to transmembrane Cl⁻ transport in non-epithelial tissues such as the heart.
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“Functional characterization and immunolocalization of the transporter encoded by the life-extending gene Indy.”

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“Identification of a chloride-formate exchanger expressed on the brush border membrane of renal proximal tubule cells”

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