Conversion of the 2 Cl⁻/1 H⁺ antiporter ClC-5 in a NO₃⁻/H⁺ antiporter by a single point mutation

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Several members of the CLC family are secondary active anion/proton exchangers, and not passive chloride channels. Among the exchangers, the endosomal ClC-5 protein that is mutated in Dent’s disease shows an extreme outward rectification that precludes a precise determination of its transport stoichiometry from measurements of the reversal potential. We developed a novel imaging method to determine the absolute proton flux in Xenopus oocytes from the extracellular proton gradient. We determined a transport stoichiometry of 2 Cl⁻/1 H⁺. Nitrate uncoupled proton transport but mutating the highly conserved serine 168 to proline, as found in the plant NO₃⁻/H⁺ antiporter atClCa, led to coupled NO₃⁻/H⁺ exchange. Among several amino acids tested at position 168, S168P was unique in mediating highly coupled NO₃⁻/H⁺ exchange. We further found that ClC-5 is strongly stimulated by intracellular citrate, leading to coupled NO₃⁻/H⁺ exchange. Serine 168 has an important function in determining anionic specificity of the exchange mechanism.

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Introduction

The CLC protein family is of paramount physiological importance as underscored by the presence of its members in all phyla (Maduke et al, 2000) and their involvement in human genetic diseases (Zifarelli and Pusch, 2007; Jentsch, 2008). As the first members to be identified and to be extensively studied were ClC-1 channels, it was thought that this would be a common feature of all CLC proteins. This was the case also for ClC-5 when its first electrophysiological characterization was reported (Steinmeyer et al, 1995). However, more recent studies triggered by the finding that the bacterial ClC-ec1 is actually a Cl⁻/H⁺ antiporter (Accardi and Miller, 2004) led to the conclusion that similar to ClC-ec1, also ClC-4 and ClC-5 are Cl⁻/H⁺ antiporters in which the movement of Cl⁻ is thermodynamically coupled to the transport of protons in the opposite direction (Picollo and Pusch, 2005; Scheel et al, 2005).

Despite this functional diversity, the overall protein architecture is conserved across the whole family. CLCs are composed of two subunits, each harbouring an ion translocation pathway (Ludewig et al, 1996; Middleton et al, 1996; Weinreich and Jentsch, 2001; Dutzler et al, 2002). ClC-5 is part of a family branch comprising also ClC-3 and ClC-4 (Steinmeyer et al, 1995). It is mostly expressed in the proximal tubule, a segment of the nephron responsible for the reuptake of small molecular weight proteins (Günther et al, 1998). It is mutated in Dent’s disease, a disorder characterized by defective endocytosis leading to kidney stones and renal failure (Lloyd et al, 1996). It has been proven that ClC-5 colocalizes with an endosomal H⁺-ATPase and with endosomal markers and endocytosed proteins, suggesting a function in the acidification of early endosomes (Günther et al, 1998, 2003; Piwon et al, 2000) (for review, refer Jentsch, 2005; Zifarelli and Pusch, 2007). In support of this view, ClC-5 knockout mice showed an impaired endocytosis (Piwon et al, 2000) and a reduced ability to accumulate H⁺ and Cl⁻ in the endosomes (Piwon et al, 2000; Hara-Chikuma et al, 2005).

In spite of the considerable insights into the function of ClC-5, our knowledge of its molecular function and biophysical properties remains very limited. We have recently proposed a model in which transport activity in ClC-5 occurs in bursts. Within each burst, transport is very fast, in the order of 10⁶ ions/s (Zdebik et al, 2008). According to this model, the unitary transport properties of ClC-5 can be estimated using non-stationary noise analysis, a method that is classically applied to ion channels but not transporters. The apparent single transporter conductance (~0.5 pS; Zdebik et al, 2008) reflects the mean transporter current once it is in the active state (Hilgemann, 1996).

However, several aspects of the ClC-5 function remain to be investigated; one of the most prominent is the transport stoichiometry of the exchanger, that is, the number of Cl⁻ ions transported per each proton. The bacterial ClC-ec1, for which a simple estimate of the transport stoichiometry can be obtained from reversal potential measurements (Accardi and Miller, 2004), shows a 2 Cl⁻/1 H⁺ stoichiometry with almost no slippage (Accardi and Miller, 2004; Accardi et al, 2006; Nguiragool and Miller, 2006; Walden et al, 2007). Unfortunately, this approach is not feasible for ClC-5 because currents mediated by the transporter are extremely outwardly rectifying (Steinmeyer et al, 1995; Friedrich et al, 1999). Initial rough estimates for the Cl⁻/H⁺ stoichiometry ranged from 1 to 5 (Picollo and Pusch, 2005; Scheel et al, 2005). These measurements were, however, associated with a large error. We have now developed a new imaging method to determine the absolute proton flux in Xenopus oocytes from the extracellular proton gradient, and determined a 2:1 Cl⁻/H⁺ stoichiometry, similar to the bacterial ClC-ec1. A 2:1 stoichiometry was also described for the plant vacuolar atClCα NO₃⁻/H⁺ transporter (De Angeli et al, 2006). From a...
mechanistic viewpoint, the different anion specificity is particularly interesting because NO$_3^-$ and other polyatomic anions lead to a partial uncoupling of H$^+$ transport in ClC-ec1 and ClC-5 (Nguitragool and Miller, 2006; Zdebik et al., 2008). However, the molecular mechanism underlying the different anion specificity of ClC-ec1 and ClC-5 compared with the plant atClCa is unknown.

Here, we found that the serine residue S168 of ClC-5 is critical for anion selectivity and coupling efficiency. Mutating this residue into a proline is sufficient to convert ClC-5 into a NO$_3^-/H^+$ antiporter. This serine in conserved in most Cl$^-$ channels and transporters; in ClC-ec1, it is involved in the coordination of Cl$^-$ ions (Dutzler et al., 2002, 2003), and in ClC-0 it has been shown to have a critical function for ion selectivity, single-channel conductance, and gating.

In addition, in this study, we directly investigate for the first time the regulation of ClC-5 by intracellular pH. Intracellular pH changes in response to a variety of stimuli and influences a number of cellular functions (Roos and Boron, 1981). Furthermore, there is increasing evidence that such pH changes are not uniform but, instead, subcellular domains might experience specific pH shifts (Pastoriza-Munoz et al., 1987; Aw and Jones, 1989; Swietach et al., 2005).

Results

Validation of the fluorescence-based proton flux assay using a sucrose/proton co-transporter

We developed an imaging system to quantify the proton flux across the plasma membrane of *Xenopus* oocytes. The setup is schematically shown in Figure 1A. Changes in the extracellular proton concentration close to the oocyte surface were imaged using the pH-sensitive dye BCECF. Each measurement was accompanied by a calibration of the BCECF solution (without oocyte) before and after the experiments. An example of calibration is shown in Figure 1B. It can be seen that the ratio change of the BCECF fluorescence is linearly related to the total amount of protons added (see the straight line in Figure 1B). This linearity is very convenient because it allows working on differential signals (e.g., ‘baseline’ subtraction). The analysis of the extracellular BCECF fluorescence is described in detail in the Supplementary data section.

To validate the method, we applied it on the maize sucrose/proton co-transporter ZmSut1 (Carpaneto et al., 2005). The only charged substrate carried by this transporter are protons such that, ideally, the electrically recorded sucrose-induced transport current should coincide with the proton current determined by the fluorescent assay. A typical experiment is illustrated in Figure 1C. The proton gradient $\Delta$H$_{\text{tot}}$ is plotted as a function of the radius for various time points (see legend for symbols) together with the theoretical fit (lines). In this particular experiment, the proton current from the fluorescence data was estimated as 159 nA, whereas for the electrical current, we measured a value of 160 nA, being thus in excellent agreement. Figure 1D shows average values for the ratio of the sucrose induced electrical current, $I_{\text{ele}}$, and the proton current, $I_{\text{H}}$, estimated from the fluorescence assay at different membrane voltages (error bars indicate s.e.m., $n \geq 6$).

Transport stoichiometry of ClC-5

Next, we applied the same method to ClC-5. Results for a typical experiment (at 80 mV) are shown in Figure 2A. In this case, the proton current, $I_{\text{H}}$, was estimated at 0.56 μA, whereas the electrical current, $I_{\text{ele}}$, was measured at 1.51 μA. Assuming a stoichiometric coupling of $N$ chloride ions for each transported proton, the electrical current, being the sum of the proton current and the chloride current, is given by

$$I_{\text{ele}} = I_{\text{Cl}} + I_{\text{H}} = (N+1)I_{\text{H}}$$

and thus

$$N = \frac{I_{\text{ele}}}{I_{\text{H}}}. $$

This ratio is plotted in Figure 2B as a function of the membrane voltage. It can be seen that the stoichiometric coupling ratio is close to 2 and it is independent of the applied voltage. The overall average including all voltages is 1.95 ± 0.11 (s.e.m., $n = 29$).

Previous qualitative experiments have shown that H$^+$ coupling to anion transported is significantly weakened if NO$_3^-$ or SCN$^-$ are substituted for Cl$^-$ (Zdebik et al., 2008). In fact, applying our fluorescence-based H$^+$ transport assay, we determined an approximately six- to ten-fold reduction of the stoichiometric coupling ratio for NO$_3^-$/H$^+$ antiport (Figure 2C). Under such conditions of weak coupling and

![Image](image-url)
large turnover, the classical distinction between a coupled transporter and a passive channel becomes fuzzy, highlighting the positioning of CLC proteins at an ambiguous interface between these two classes of membrane proteins (Gadsby, 2004; Miller, 2006).

**Conversion of ClC-5 into a NO3/H+ exchanger**

NO3- uncouples H+ transport in ClC-5 and ClC-ec1 (Nguitragool and Miller, 2006; Zdebik et al., 2008), whereas the plant atClCa shows coupled NO3/H+ antiport (De Angeli et al., 2006). Inspecting the sequence of these three transporters in the highly conserved ion-binding regions reveals that atClCa carries a proline at position 168 (numbering of ClC-5), whereas ClC-ec1, ClC-5, as well as all other known CLC Cl− channels and Cl−/H+ antiporters have a serine at the equivalent position (Figure 3A).

Therefore, we introduced the mutation S168P in ClC-5 and investigated its properties. Currents mediated by this mutant in the standard Cl− solution were outwardly rectifying as WT, but their magnitude was barely above background (Figure 3C). Exchanging extracellular Cl− with NO3- led to a dramatic increase of outward currents (corresponding to inward anion flow) (Figure 3C), suggesting a significant change of anion selectivity (compare with WT traces, Figure 3B). In addition, for the mutant, the activation kinetics of the currents were significantly slower and more pronounced (compare Figure 3B and C).

Comparing the currents in the positive voltage range in the presence of various extracellular anions, we established that S168P has a conductivity sequence (NO3− > I− > Br− – H+)(Figure 3D), which differs from that of WT ClC-5 (NO3− > Cl− > Br− > I−) (Steinmeyer et al., 1995) (see Table I) but is very similar to that of atClCa (NO3− ≈ I− > Br− > Cl−) (De Angeli et al., 2006).

We next investigated whether NO3− transport by this mutant is coupled to H+ movement using an extracellular pH-sensitive microelectrode (Picollo and Pusch, 2005). Figure 3E shows that NO3− transport, activated by positive potentials, produces a significant acidification of the extracellular solution, which readily reverses upon switching off of the clamp, suggesting a stronger coupling of NO3− to H+ transport in the mutant compared with WT (Zdebik et al., 2008). Furthermore,

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**Table I** Conductivity sequences among halide ions and nitrate for WT ClC-5 and various mutants of residue 168

<table>
<thead>
<tr>
<th>Residue</th>
<th>Conductivity sequence</th>
</tr>
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<tbody>
<tr>
<td>S (WT)</td>
<td>NO3− &gt; Cl− &gt; Br− &gt; I−</td>
</tr>
<tr>
<td>A</td>
<td>NO3− &gt; Br− &gt; I− &gt; Cl−</td>
</tr>
<tr>
<td>G</td>
<td>NO3− &gt; Cl− &gt; Br− &gt; I−</td>
</tr>
<tr>
<td>P</td>
<td>NO3− &gt; I− &gt; Br− &gt; Cl−</td>
</tr>
<tr>
<td>T</td>
<td>NO3− &gt; I− &gt; Br− &gt; Cl−</td>
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measurements performed with an inwardly directed pH gradient show that the outward movement of protons is produced by an active transport mechanism, proving a thermodynamic coupling between NO₃⁻ and H⁺ movement through S168P (Supplementary Figure 2).

We quantitatively investigated the NO₃⁻/H⁺ transport stoichiometry of S168P using our fluorescence assay. We found that, at moderately positive voltages (40 and 60 mV), the NO₃⁻/H⁺ stoichiometry was close to 2, whereas H⁺ transport became less coupled to anion transport at more positive voltages (Figure 3F), but still much strongly coupled than WT NO₃⁻/H⁺ exchange (Figure 2C).

**Analysis of transport activity of other mutants at position 168**

To systematically study the effect of amino-acid substitutions at position 168 on transport properties, we additionally mutated S168 into A, C, G, H, L, N, Q, T, and Y. Among these mutants, only S168A, S168G, and S168T yielded currents above background in Cl⁻, Br⁻, I⁻, or NO₃⁻ containing solutions. All mutants showed a reduced current expression compared with WT (data not shown). Preliminary results indicated that all mutants (including S168P) exhibited coupled Cl⁻/H⁺ exchange with a stoichiometry of roughly 2:1 at voltages ≤80 mV (G. Zifarelli and M. Pusch, unpublished data). S168A and S168T, but not S168G, changed the conductivity sequence compared with WT as documented in Table I and Supplementary Figure 3. In further experiments, we concentrated on the ability of the mutants to select for NO₃⁻ compared with Cl⁻ in coupled transport. We first performed a detailed analysis of the relative current magnitude in NO₃⁻ compared with Cl⁻ for the various mutants (Figure 4A). As for WT, all mutants show a preference for NO₃⁻ transport compared with Cl⁻ transport. However, for the two mutants S168T and S168P, this preference is much more pronounced. S168A shows an intermediate phenotype, whereas for S168G, Cl⁻ currents are only slightly smaller than NO₃⁻ currents, thus being similar to WT (Figure 4A). But is NO₃⁻ transport coupled to H⁺ transport as in S168P or uncoupled as in WT? To address this question, we determined the relative coupling efficiency, e_{rel}, as described in Materials and methods, comparing the acidification measured with a pH-sensitive microelectrode in Cl⁻ and NO₃⁻ on the same oocytes (see Supplementary Figure 4 for representative recordings). A value of e_{rel} = 1 means that NO₃⁻ transport and Cl⁻ transport are equally well coupled to H⁺ counter-transport. A small value of e_{rel} indicates inefficient NO₃⁻/H⁺ exchange (see Equation (2)). Figure 4B shows the values of e_{rel} for WT and the four functional S168 mutants at 60, 80, and 100 mV. In agreement with the fluorescence data, WT exhibits inefficient and voltage-dependent NO₃⁻/H⁺ transport coupling. S168P is the only mutant that shows a similar coupling efficiency of NO₃⁻/H⁺ exchange and Cl⁻/H⁺ exchange. S168A, S168G, and S168T show an intermediate phenotype, with NO₃⁻ transport being less coupled than Cl⁻ transport. Thus, among all mutants tested, S168P is unique in that it strongly selects for NO₃⁻ against Cl⁻, maintaining stoichiometrically coupled H⁺ antiport.

**Intracellular pH dependence of CIC-5**

To gain more insight in the regulation of CIC-5 by pH, we studied its dependence on the intracellular H⁺ concentration using inside-out patch clamp recordings. Current traces for a typical experiment are shown in Figure 5A. Compared with the physiological pH 7.3, CIC-5 currents are dramatically

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**Figure 4** Nitrate over chloride preference of WT and mutants at position 168. (A) Ratio between current values measured at 80 mV in extracellular solutions containing NO₃⁻ and Cl⁻, respectively, for WT and the indicated mutants of S168. Currents were leak-subtracted. Error bars indicate s.e.m. (n ≥ 15). (B) For each potential (symbols are indicated in the figure), we calculated the relative coupling efficiency, e_{rel} (as described in Materials and methods), comparing acidification (measured with a pH-sensitive microelectrode) and currents in Cl⁻ and NO₃⁻ containing extracellular solutions. Error bars indicate s.e.m. (n ≥ 5).

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**Figure 5** Dependence of CIC-5 on the intracellular proton concentration. (A) Representative recordings from one inside-out patch perfused with intracellular solutions at different pH values as indicated. Voltages range from +160 to -60 mV in 20 mV steps. (B) Normalized current–voltage relationships at pH 5.3 (squares) (n = 9), 6.3 (circles) (n = 8), 7.3 (triangles up) (n = 18), 8.3 (triangles down) (n = 5), 9.3 (diamonds) (n = 3). Currents were normalized to the current measured at pH 7.3 at 160 mV. (C) Plot of normalized currents at 160 mV as a function of pH. The solid line is a Hill fit as described in the main text with I_{max,5.3} = 1.9, I_{max,6.3} = 0.3, K = 6.2 10^{-4} M (pK = 7.21). (D) Plot of the mean values of the pK obtained as in C, versus voltage. (E) Average values of the apparent single channel conductance measured by noise analysis performed on patches perfused with solutions at pH 7.3 (n = 8), pH 6.3 (n = 5), pH 5.3 (n = 4). Error bars indicate s.e.m.
activated by acidic pH, and almost completely suppressed at alkaline pH (Figure 5A and B). We quantified the pH dependence at the most positive voltages (which allowed a reliable determination of the current amplitudes). An example of the analysis performed at 160 mV is shown in Figure 5C. The activation can be well described by a simple 1:1 binding curve of the form

\[ I_{\text{norm}} = I_{\text{min}} + (I_{\text{max}} - I_{\text{min}}) \frac{H/K}{1 + H/K} \]

where \( H \) is the free proton concentration, \( K \) the apparent binding constant (\( pK = -\log(K) \)), \( I_{\text{min}} \) and \( I_{\text{max}} \) are the minimal and the maximal values, respectively. The apparent \( pK \) is \( \sim 7.2 \), very close to the physiological intracellular pH and is voltage independent (in the range 130–160 mV) (Figure 5D).

The increase of currents at acidic pH could simply reflect a larger turnover of the transporter, enabled by the larger H\(^+\) concentration. Alternatively, protons could regulate the probability of the transporter to be in an active state.

To discern between these possibilities, we applied nonstationary noise analysis. This method is classically applied to ion channels but not to transporters. However, we have shown previously that the spectral noise properties of ClC-5 are consistent with the idea that the transporter is ‘gated’, that is, fluctuates between active and inactive states (Zdebik et al, 2008). Because, as for ion channels, individual transport events are not resolved, the noise is dominated by the gating noise (Hilgemann, 1996). Thus, non-stationary noise analysis can be used to obtain estimates of the transporter turnover during the bursts of activity. We will call this quantity the ‘single transporter current’.

For \( \text{pH} \leq 7.3 \), we found that the single transporter current was independent of \( \text{pH} \) (Figure 5E). This result suggests that the current increase is not caused by an increased turnover, but reflects an increased probability of the gated transporter to be in an active state. Unfortunately, for \( \text{pH} > 7.3 \), currents were too small to allow a reliable estimate of the single transporter current. Thus, we cannot exclude that the reduction of currents at alkaline pH is caused, at least partially, by a reduced turnover.

To further test the hypothesis that the current increase at acidic pH reflects a regulation of the probability of ClC-5 to be in an active state, we exploited the fact that substitution of extracellular Cl\(^-\) with NO\(_3^-\) leads to a large uncoupling of anion and proton transport, H\(^+\) transport thus being of relatively minor importance for the overall transport activity. Thus, at difference with the situation in the presence of Cl\(^-\), in NO\(_3^-\) a smaller pH effect may be expected if pH directly affects the turnover. On the other hand, a similar pH effect is expected in NO\(_3^-\), if protons modulate the probability of the transporter to be in an active state. Furthermore, ClC-5 shows a more pronounced activation time-course in NO\(_3^-\), rendering non-stationary noise analysis more reliable. As shown in Figure 6A, pH strongly affects ClC-5 currents when NO\(_3^-\) is the transported anion. In particular, the pH regulation (Figure 6B) is very similar in comparison with the measurements in the presence of Cl\(^-\) (see Figure 5C). Furthermore, the single transporter current is independent from \( pH_{\text{int}} \) in the range 5.8 to 7.3 (Figure 6C). These measurements confirm the conclusion that \( pH_{\text{int}} \) mostly regulates the probability of the transporter to be in an active state with little influence on the turnover rate.

**Discussion**

There is solid evidence that the major physiological function of ClC-5 is related to the acidification of endosomes, consistent with its localization in segments of the nephron with a high endocytotic activity and with its involvement in Dent’s disease, caused by a defective endocytosis. It was assumed previously that ClC-5 was a Cl\(^-\) channel, and this would naturally propose its molecular function: to provide a Cl\(^-\) conductance serving as an electrical shunt to allow efficient endosomal accumulation of protons by the V-type ATPase. The finding that ClC-5 is not a Cl\(^-\) channel but a Cl\(^-\)/H\(^+\) antiporter (Picollo and Pusch, 2005; Scheel et al, 2005) called for a thorough revaluation of this picture. However, a better understanding of the physiological function of ClC-5 is inextricably intertwined with a broader knowledge of its basic, biophysical properties. Among the biophysical features with direct physiological implications, in this work, we focused our attention on three problems. The first, and most important one, is the stoichiometry of transport, which determines the degree of coupling between Cl\(^-\) and H\(^+\). Secondly, we identified a residue that determines the anion selectivity and coupling efficiency of the transporter. Lastly, we investigated the dependence of the transport activity on the cytoplasmic proton concentration. To tackle these questions, we used different approaches.

To study the stoichiometry of transport in ClC-5, we developed a new imaging method that allowed direct quantification of the outward proton flux. Taking advantage of this method, we found a voltage-independent stoichiometry of 2 Cl\(^-\)/1 H\(^+\). The same stoichiometry has been found for the bacterial ClC-ec1 (Accardi and Miller, 2004), for the plant atClC (De Angelis et al, 2006), and for the lysosomal ClC-7 (Graves et al, 2008). Thus, a 2:1 stoichiometry seems to represent a general feature of the Cl\(^-\)/H\(^+\) antiport activity in the CLC family, possibly implying a similar coupling mechanism. This tight coupling suggests that the main
physiological function of CIC-5 is not only to provide a Cl\(^-\) shunt conductance to assist the efficient accumulation of H\(^+\) by the V-type ATPase, but also to actively contribute to the endosomal acidification loading directly protons into the endosomes using the Cl\(^-\) gradient. Alternatively, it is conceivable that the tight coupling between Cl\(^-\) and H\(^+\) movement can provide a means to regulate endosomal chloride concentration (Zifarelli and Pusch, 2007; Jentsch, 2008). Further studies are, however, required to clarify this point.

Polyatomic anions, like NO\(_3^-\), are transported by CIC-5 and the bacterial CIC-ec1 with much weaker coupling to the H\(^+\) movement in comparison with Cl\(^-\) (Nguiatragool and Miller, 2006; Zdebik et al., 2008), probably because these anions induce slippage (Nguiatragool and Miller, 2006), whereas NO\(_3^-\) is transported by the plant atClCa with a coupled 2 NO\(_3^-\)/1 H\(^+\) stoichiometry (De Angeli et al., 2006). An understanding of the molecular determinants responsible for such a different anion selectivity is of relevance in both biophysical and physiological terms. We have found here that serine 168 is critical for the anion selectivity. This residue is conserved in most Cl\(^-\)-channels and Cl\(^-\)/H\(^+\) antiporters of the CLC family. Its mutation to proline, as found in the plant NO\(_3^-\)/H\(^+\) antiporter atClCa, alters the conductivity sequence compared with WT (Steinmeyer et al., 1995) and makes it similar to what is found for atClCa (De Angeli et al., 2006). This finding, by itself, may not seem surprising, as NO\(_3^-\) permeates also the WT. However, there is an important difference: in WT CIC-5, NO\(_3^-\) permeation is almost uncoupled from H\(^+\) transport, whereas for S168P, NO\(_3^-\) transport is much tightly coupled to H\(^+\) movement.

An important function of the serine in the ‘signature sequence’ GSGPE for CLC channel gating and selectivity has been established more than a decade ago (Ludewig et al., 1996). Its function became evident when Dutzler et al. (2002, 2003) identified S107 (corresponding to S168 in CIC-5) as one of the residues that coordinates Cl\(^-\) ions in the crystal structure of CIC-ec1. In CIC-5, the corresponding S168 most likely has the same critical function. To achieve a deeper insight into the mechanism of anion selectivity, we mutated S168 to several other residues. Among all mutations, S168P is unique in that it greatly diminishes transport of Cl\(^-\) and at the same time confers highly coupled NO\(_3^-\)/H\(^+\) antiport activity to CIC-5, the same features observed in the activity of the plant atClCa (De Angeli et al., 2006) and that are critical for plant anion homeostasis. This result shows that S168 is the major determinant of anion selectivity. Interestingly, a proline at the position corresponding to 168 is found only in plant CLCs. Three of the seven Arabidopsis CLCs contain a serine and four carry a proline. On the basis of our results, the latter ones are all predicted to be NO\(_3^-\)/H\(^+\) exchangers, for which the exclusion of Cl\(^-\) as a transported anion is physiologically important. A BLAST search revealed that in eukaryotic CLCs, apart from serine and proline, only alanine and glycine are found at the position corresponding to 168, and only in unicellular organisms. These observations are in agreement with our mutational analysis. Most mutations introduced at position 168 resulted in a non-functional transporter, highlighting the strict constraints that the residue at position 168 must obey to preserve transport (and/or correct membrane targeting). Apart from serine and proline, only the residues alanine, glycine, and threonine were tolerated at position 168. We are in no position to propose a stringent structural interpretation of the mutants’ behaviour, but, due to the peculiar chemical properties of proline, its introduction can be expected to lead to a backbone rearrangement. The fact that S168P is the only mutant that dramatically changes transport properties is compatible with the hypothesis that such a structural change underlies the specific features of S168P, and this would also explain the relatively minor effect of the other mutants. Clearly, structural studies are required to clarify this point.

Surprisingly, mutant S168G is very similar to WT with efficient H\(^+\) pumping and identical ion selectivity. The structure of a construct of the bacterial CIC-ec1 harbouring the S107G mutation (corresponding to S168G of CIC-5) showed no major alterations of secondary structure (Lobet and Dutzler, 2006). However, the S107G mutation in CIC-ec1 is almost completely uncoupled (Jayaram et al., 2008), suggesting significant differences between CIC-5 and CIC-ec1 in the detailed mechanism of anion binding.

Intracellular pH is a fundamental physiological parameter that both reflects and influences many cellular functions, but its effect on CIC-5 has not been investigated yet. Here, we found that intracellular protons activate CIC-5 currents with an apparent pK of 7.2. The simplest explanation of this pH dependence would be that a larger concentration of protons directly increases the outward H\(^+\) transport—because more protons are available. Recently, however, we have proposed a model for the transport activity of CIC-5 (Zdebik et al., 2008), in which transport does not take place continuously, but rather occurs in bursts, and pH could regulate the probability of the transporter to be in the active, transporting conformation. In the framework of this model, the mean current during a burst of activity, the ‘single transporter current’, directly reflects the elementary turnover rate. We found that the single transporter current is pH independent in the range between pH 5.3 and 7.3. This suggests that internal pH does not influence the turnover rate, but rather modulates the duration and/or the frequency of the bursts of transport. In other words, our results indicate that intracellular acidification exerts an effect by increasing the probability for CIC-5 to be in an active, transporting state. Furthermore, this result implies that for pH \(\leq 7.3\), H\(^+\) delivery from the bulk intracellular solution to the transporter is not a rate-limiting step in the transport cycle. These measurements in external Cl\(^-\), the physiologically relevant anion for CIC-5, were corroborated by analogous measurements in extracellular NO\(_3^-\). As NO\(_3^-\) transport is associated with a greatly reduced H\(^+\) transport, a priori, a drastically altered pH dependence might be expected. However, we found that NO\(_3^-\) transport has a very similar pH dependence as Cl\(^-\) transport, supporting our conclusion that pH\(_{int}\) does not directly impinge on the single transporter turnover rate. It is noteworthy that the single transporter current in NO\(_3^-\) is not larger than that in Cl\(^-\) (it is actually slightly smaller), indicating that the tighter coupling in Cl\(^-\) is not associated with a reduced capacity of ion transport. On the contrary, it appears that the slippage occurring in NO\(_3^-\) is associated with a less-efficient overall transport. We therefore interpret the increased macroscopic current seen in NO\(_3^-\) as an increased probability of the transporter to be in the active state in the presence of NO\(_3^-\). Nevertheless, it should be kept in mind that noise analysis is an indirect method and that in the absence of...
direct measurements of a single transporter turnover, these considerations have to be regarded with some caution.

The activation kinetics of ClC-5 currents upon voltage jumps to positive potentials are much more pronounced in NO3- than in Cl-. This finding by itself provides additional support for the concept of a ‘gated’ transporter: the probability of the transporter to be in an active, transporting state is voltage dependent. This probability depends on the permeating anion, a feature well known from CLC Cl- channels (Pusch et al., 1995).

Independently of the precise biophysical mechanism, our results suggest that modulation of ClC-5 by intracellular pH is physiologically relevant, for example to regulate the degree of endosomal acidification. In this regard, it is interesting to note that transcellular pH gradients have been described in proximal tubule cells (Aw and Jones, 1989), and that it has been reported that intracellular pH progressively declines along the proximal tubule (Pastoriza-Munoz et al., 1987).

Materials and methods

Molecular biology and oocyte expression
Human ClC-5 and ZmSut1 were expressed in Xenopus oocytes as described previously (Carpaneto et al., 2005; Picollo and Pusch, 2005). Oocytes were kept in a solution containing (in mM) 100 NaCl, 10 HEPES, 2 KCl, 1 MgCl2, 1 CaCl2, pH 7.5, for 3–6 days (ClC-5) or for 2–5 days (ZmSut1) at 18°C.

Patch clamp measurements and noise analysis
Inside-out patch clamp measurements were performed as described by Zdebik et al. (2008). Briefly, patch pipettes were pulled from aluminosilicate capillaries (Hilgenberg, Malsfeld, Germany), coated with Sylgard, and fire-polished to a resistance of approximately 0.5–1 MΩ. The extracellular (pipette) solution contained (in mM) 100 NMDGCl, 10 HEPES, 5 MgCl2, pH 7.3. The standard intracellular solution contained 100 mM NMDGCl, 10 HEPES, 2 MgCl2, 1 EGTA, pH 7.3. The pH was adjusted with NaOH or H2SO4 to the desired values. For pH <7.3 MES buffer and for pH 9.3 Bis-Tris-propanol buffer were used instead of HEPES. For patch measurements with external NO3-, the pipette solution contained (in mM) 100 NMDG- NO3-, 10 HEPES, 5 MgCl2, pH 7.3. The 10 mM Cl- arising from the NMDGCl in this solution served to maintain a stable electrode potential. Control whole-oocyte two-electrode voltage-clamp and acidification measurements showed that this low concentration of Cl- had a negligible influence on the current magnitude and H+ transport activity. Solutions were changed by inserting the patch-pipette into the opening of ~0.5-mm-diameter glass perfusion capillaries. Perfusion flow was driven by gravity. Data for non-stationary noise analysis were obtained by repeatedly applying a voltage step to 140 mV. The mean current, I, and the variance, σ², were calculated from these traces as described (Zdebik et al., 2008), and the variance was plotted as a function of the mean current and fitted to the following equation

\[ \sigma^2 = I^2 - I_s^2/N \]  

(1)

where I is the apparent single transporter current and N the number of ‘channels’.

Two-electrode voltage-clamp
Two-electrode voltage-clamp was performed with a Turbotec 03 amplifier (npi, Tamm, Germany) and a custom acquisition program (GePulse) at room temperature (20–25°C) as described earlier (Zdebik et al., 2008). The standard bath solution contained (in mM): 100 NaCl, 4 MgSO4, 10 HEPES, pH 7.3. For measurements of the anion conductance, NaCl was substituted with equimolar amounts of NaNO3, NaBr, or NaF. For the analysis shown in Figure 4, the contribution of leak currents was estimated and subtracted assuming a linear leak conductance and assuming that currents at voltages more negative than ~20 mV were pure leak.

Extracellular pH measurements using a pH-sensitive microelectrode
Proton transport was qualitatively measured by monitoring the acidification of the extracellular solution close to the oocyte using a pH-sensitive microelectrode as described previously (Picollo and Pusch, 2005; Zdebik et al., 2008). Briefly, a silanized microelectrode was fire-filled with a proton ionophore (Cocktail B, Fluka), back-filled with a solution containing a phosphate-buffered saline, and connected to a custom high-impedance amplifier. The electrodes were routinely checked and responded consistently with a slope of 57–61 mV/pH unit. The pH-sensitive microelectrode was pushed gently onto the vitelline membrane without rupturing the plasma membrane. The oocyte was simultaneously voltage-clamped and acidification was induced by applying a train of voltage-clamp pulses to a positive test potential. Solutions contained 0.5 mM buffer (HEPES for pH >6.5, otherwise MES).

Determination of the relative coupling efficiency
The microelectrode method cannot be used to determine absolute coupling coefficients. However, comparing measurements in the presence of different anions performed on the same oocyte with the pH-sensitive microelectrode in the same solution, the relative coupling efficiency of anion/proton exchange was estimated in the following way. We applied a train of defined length of activating voltage pulses and measured the final change in extracellular pH, ΔpH, and the net current during the voltage pulses, I_{net}, in the presence of Cl-, or NO3-, from the same oocyte. The current is the sum of the proton current, I_p, and the anion current, I_{anion}, and the latter is equal to I_{anion,*}H+, where I_{anion,*} is the number of anions transported for each proton:

\[ I_{net} = I_p + I_{anion,*} = (1 + r_{anion,*})I_p \]  

As a first approximation, the acidification is proportional to the proton current:

\[ \Delta pH \sim I_p \]  

and thus

\[ I_{net} = \frac{\Delta pH}{I_p} = f(1 + r_{anion,*}) \]  

The proportionality factor, f, depends on the distance of the microelectrode from the oocyte surface, the local density of membrane expression, and other unknown factors. For the same oocyte and microelectrode position, however, f is identical for Cl- and NO3-. Therefore, the ratio of these quantities in Cl- and NO3-, respectively, defining the relative coupling efficiency, \( e_{rel} \),

\[ e_{rel} = \frac{I_{net}(Cl) \Delta pH(NO3)}{I_{net}(HCl) \Delta pH(NO3)} = \frac{1 + r_{Cl}}{1 + r_{NO3}} \]  

(2)

is a measure of the relative efficiency of NO3-/H+ exchange compared with Cl-/H+ exchange. A small value of \( e_{rel} \) indicates that NO3- transport is less coupled than Cl- transport, that is, \( f_{NO3} > f_{Cl} \).

Fluorescence-based measurements of proton transport
For ClC-5, the solution contained (in mM) 120 NaCl and 4 MgCl2, 0.2 BICEF (Sigma, Milan), pH 7.3 (with NaOH). For ZmSut1, the control solution contained (in mM) 150 sorbitol, 30 NaCl, 4 MgCl2, 0.2 MES, pH 6, whereas the measuring solution contained 120 sorbitol, 30 sucrose, 30 NaCl, 0.2 BICEF, pH 6. Thus, BICEF was the only buffer for the fluorescence measurements. Fluorescence was evoked and monitored using a Till monochromator-based imaging system with an Imago cooled CCD camera mounted on an Axiovert Zeiss microscope and controlled by TillVision software (Till photonics, Münch, Germany). Fluorescence was excited alternately at 490 and 440 nm with 1 ms excitation duration, acquiring a pair of images every 100 ms using a 515 nm emission filter. The fluorescence ratio (490/440) was calculated online. A trigger from TillVision synchronously started a voltage-clamp pulse protocol to initiate proton flux and to record the transmembrane current. For ClC-5, the voltage protocol consisted of a 3- to 5-s-long depolarization to a voltage ranging from 20 to 120 mV. For ZmSut1, the voltage was first held at a positive value (from 20 to 40 mV) to minimize proton transport (Carpaneto et al., 2005) and was then switched to a test voltage (from ~100 to ~40 mV) to evoke H+ influx. For ClC-5, leak currents were estimated from current responses to

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negative voltages, and subtracted. For ZmSut1, leak currents were estimated from the current response in the absence of sucrose, and subtracted.

For calibration, 150 or 200 µl test solution were placed in the recording chamber, and fluorescence ratios were measured after successive addition of 2.5 µl of 1 mM NaOH (for ZmSut1 measure-ments) or 1 mM HCl (for CIC-5). The fluorescence ratio changes linearly with the amount of protons (or OH⁻) added (see Figure 1B), and the slope of this relationship was used to convert fluorescence ratios into changes of total proton concentration. Calibration was performed before and after a set of experiments. BCECF solutions were prepared freshly before use.

The experimental fluorescence ratios were analysed as described in detail in the Supplementary data, to extract the net trans-membrane proton current.

Supplementary data
Supplementary data are available at The EMBO Journal (http://www.embojournal.org).

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