Journal of Molecular Biology Research; Vol. 3, No. 1; 2013
ISSN 1925-430X E-ISSN 1925-4318
Published by Canadian Center of Science and Education

Expression of Human Chloride Channels ClC1 or ClC2 Revert the Petite Phenotype of a *Saccharomyces cerevisiae* GEF1 Mutant

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Received: December 28, 2012 Accepted: June 13, 2013 Online Published: July 2, 2013

doi:10.5539/jmbr.v3n1p68 URL: http://dx.doi.org/10.5539/jmbr.v3n1p68

Abstract

The mechanism of activation of the yeast ClC chloride channel/transporter *GEF1* is unknown, and in this study we tested the ability of human ClC1 and ClC2, two channels with different activation kinetics, to revert the *petite* phenotype of a strain whose *GEF1* gene was deleted. We found that when the human channels are expressed in a low-copy plasmid, the reversion of the phenotype does not occur; in contrast, when the channels are over expressed by means of a strong transcriptional promoter in a multiple-copy plasmid, the cells reach the normal size, and show a normal membrane surface and oxygen consumption. To determine the size variationsof individual cells, we employed flow-cytometry as a quantitative tool to evaluate the *petite* phenotype.

These results suggest that the human ClC channels, when abundantly present in the cells, can support the metabolism disrupted in the knock-out strain. We also observed that the fluorescence emitted by GFP-tagged channels was found mostly towards the periphery of the *wt* yeast, whereas in the *GEF1* knock-out it was detected in intracellular clusters. GFP-tagged channels expressed in *X. laevis* oocytes produced robust currents and did not show any evident difference with respect to the normal ClCs, whereas Gep1p did not show voltage-dependent activation.

Keywords: chloride channel, functional complementation, voltage-clamp, *Xenopus laevis* oocytes

1. Introduction

Chloride channels/transporters (ClCs) are members of a large family present in a wide variety of organisms from bacteria to higher eukaryotes. ClCs carry out multiple physiological roles, from plasma membrane and cell volume modulation to the control of vesicular pH (Fahlke, 2001; Jentsch, Stein, Winreich & Zdebik, 2002; Sardini et al., 2003; Soleimani & Xu, 2006; Jentsch, 2008). A clear example of this functional diversification is illustrated by comparing the properties of mammalian ClC1 and ClC2. They are both located in the plasma membrane; however, whereas ClC1 is activated by plasma membrane depolarization and thus is responsible for the repolarization current in muscle fibers, ClC2 is activated by hyperpolarization, as well as by other mechanisms such as changes in pH and cell volume (Conte, De Luca, Mamrini, & Vrbovà, 1989; Steinmeyer, Ortland, & Jentsch, 1991; Klocke, Steinmeyer, Jentsch, & Jockusch, 1994; Jordt & Jentsch, 1997).

The mechanism of activation of the *Saccharomyces cerevisiae* Gef1p, the sole ClC found in this species of yeast, is still not clearly understood. Gef1p plays a critical role in yeast iron metabolism and is found mainly in the *trans*-Golgi (Greene, Brown, DiDomenico, Kaplan & Eide, 1993; Schwappach, Stobrawa, Hechenberger, Steinmeyer & Jentsch, 1998). Mutations of the GEF1 gene lead to an iron requirement for growth on non-fermentable carbon sources due to a failure to load copper onto the iron uptake system; thus, knocking down the expression of GEF1 leads to *petite* (*pet*) colonies when grown in these conditions (Gaxiola et al., 1998). Gef1p forms a Cl^- transporter/channel in the plasma membrane of the yeast that does not show

voltage-dependent activation when expressed in heterologous systems (López-Rodríguez et al., 2007). Interestingly, several ClC genes from plants, fungi, and vertebrates functionally complement the *pet* phenotype of yeast whose gene *GEF1* had been deleted, whereas others such as the mammalian ClC7 gene, which codes for a protein of the lysosomal membrane, does not revert the mutation (Hechenberger et al., 1996; Gaxiola, Yuan, Klausner & Fink, 1998; Miyazaki et al., 1999; Kida, Uchida, Miyazaki, Sasaki, & Mauro, 2001; Marmagne et al., 2007).

To determine if human ClC1 and ClC2 complement the *pet* phenotype of Gef1p$^-$ yeast, we expressed these two genes in a *GEF1* knock-out strain. This paper describes the results of complementation assays and some details of the yeast phenotype revealed by scanning electron microscopy (SEM). To quantify the reversion of the *pet* phenotype, the colony size assay was supported with an analysis of cell volume by flow cytometry, which allowed us to measure the size and estimate the cell surface complexity of up to 5,000 individual cells per second. The results suggest that overexpression of ClC1 or ClC2 rescue the *pet* phenotype of a Gef1p$^-$ strain, whereas expression of the same channels in a single-copy plasmid and under a constitutive promoter do not rescue the mutant phenotype.

2. Methods

2.1 Yeast Strains and Media

The *S. cerevisiae* strains used in this study were RGY30 (*wt*) MATa ura3-1; leu2-3,112 trp1-1; his3-11, 15, and RGY 192 (Gef1p$^-$) MATa*gef1: HIS3*, leu2-3,112; trp1-1; his15. Both strains were donated by Dr. R. Gaxiola (Gaxiola et al., 1998). The cells were propagated on standard YPD media that contained 1% bacto-yeast extract, 2% peptone, and 2% dextrose; they were made competent for transformation by the LiAc method (Greene et al., 1993; Geitz, Schiestls, Willems & Woods, 1995). For auxotroph selection we used a medium without uracil (SC-U), containing 0.67% yeast nitrogen base without amino acids. Induction and analysis of the *pet* phenotype for cells transformed with pYES was made in SC-U supplemented with 0.01% leucine, 0.01% tryptophan, 0.005% histidine, 2% galactose, 2% ethanol, 2% glycerol, and 1mM ferrozine. For the cells transformed with pUG35, we also included 2% galactose to eliminate the possibility that this carbohydrate couldrevert the phenotype, as observed previously (Greene et al., 1993).

2.2 Plasmid Construction

Plasmids carrying either hClC1 (pRc/CMV_hClC1) or hClC2 (pBK/RSV_hClC2) were donated by Dr. Al George and Dr. Gary Cutting, respectively. The ClCs were amplified with Platinum®Taq DNA polymerase using the following conditions: for hClC-1 (35 cycles): 94ºC/30s, 55ºC/30s, and 68ºC/3min, and for hClC-2 (35 cycles): 94ºC/30s, 63ºC/30s, and 68ºC/3min. The translation stop codon was eliminated in both cases.

Two expression plasmids were used for complementation experiments: 1) the multicopy vector pYES2.1/V5-His-TOPO (pYES2.1 TOPO®, Invitrogen) and 2) the centromeric pUG35 (Donated by Dr. J.H.Hegemann), which permitted tagging the hClCs with the green fluorescent protein (GFP) at the carboxy-terminus. RNA was isolated from transformed yeast using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). In addition, RNA was isolated from yeast induced to overexpress *GEF1* with the plasmid pYEX-BX-GEF1 (López-Rodríguez et al., 2007).

2.3 Yeast Complementation Assays

Strains RGY30 (*wt*) and RGY192 (Gef1p$^-$) were transformed with plasmids derived from pYES (pYES-hClC1 or pYES-hClC2) and plated on YPD; after selection in restrictive media, positive colonies were transferred to SC-U supplemented with 2% galactose to induce the GAL promoter. The size of the transformed yeast was visualized and measured under the light microscope. Cell diameters were measured from ten different ocular fields (100X), and statistical analysis was performed with one-way ANOVA and Tukey *post hoc* tests; in order to have a more accurate measure of the cell diameter and membrane complexity, flow cytometry was used, as indicated below.

Plasmids derived from pUG35 (pUG-hClC1 and pUG-hClC2) were also introduced into RGY30 and RGY192; galactose was added to as above this carbohydrate to revert the phenotype, eventhough the MET promoter allowed the constitutive expression of the transgenes (Mumberg, Müller & Funk, 1994).

2.4 Flow Cytometry

Cells grown in liquid YNB were collected from samples of three independent transformations, optic density measured in a spectrophotometer (λ 480 nm) after 4 h of induction with galactose, and sorted using a

Fluorescence Activated Cell Sorter apparatus (FACS calibur; Becton Dickinson). Acquisition and analysis of the FACS data were performed using CELLQUEST software (Becton Dickinson) and SUMMIT V4.3 (DAKO Colorado, Inc.). Frontal light dispersion was a direct indication of cell volume whereas lateral dispersion suggests the complexity of the cell surface. Data analysis was performed with Windows Multiple Document Interface Flow Cytometry Application, Version 2.9 (WinMDI V2.9).

2.5 O_2 Consumption Rates

Yeast strains were grown at 30ºC in SC-U medium with 2% dextrose to an OD_{600} of 3 and then arrested in M phase by adding 1.5 mg/mL of nocodazole in 1% DMSO. After 4 h, yeast were collected by centrifugation for 5 min at 1000 *g* at 4ºC and resuspended in SC-U supplemented with 2% galactose. After 4 h of incubation, cells were counted in a Nuebauer chamber (Optyk Labor), and the culture was diluted in 3 mL of fresh SC-U set at 30ºC. The rate of O_2 consumption was determined using a Clark-type oxygen electrode and YSI Benchtop Biological Oxygen Monitor (5300 model) as reported (López-Rodríguez et al., 2007; Hernandez-Muñoz, Díaz-Muñoz, & Chagoya de Sanchez, 1992).

2.6 Scanning Electron Microscopy (SEM)

Yeast were transformed and fixed in 3% glutaraldehyde in H_2O for 2 h. Then the cells were covered with a thin coat of gold using an Ion Sputter FC 1100 (Jeol) operating at 1200kV and 5 mA for 10 min. Samples were observed and photographed under an electron microscope (Jeol, JSM-54110LV) at a 10,000 X.

2.7 Electrophysiology

Isolation of oocytes and recordings were as described previously (Miledi, 1982). Briefly, *X. leavis* frogs were anesthetized with 0.17% 3-aminobenzoic acid methylester (MS-222) for 20-30 min. The oocytes were manually removed, enzymatically defolliculated with 0.5mg/ml collagenase type I (Sigma) at room temperature for 45 min, and then kept at 16ºC in Barth´s medium (in mM): 88, NaCl; 1, KCl; 0.33, $Ca(NO_3)_2$; 0.41, CaCl; 0.82, $MgSO_4$; 2.4, $NaHCO_3$; 5, HEPES; pH 7.4, containing 0.1 mg/mL gentamycin sulfate. The next day 50 nL of RNA (1 μg/μl) from yeast that were induced to express hClC1, hClC1-GFP, hClC2, hClC2-GFP, or *GEF1* were injected into the vegetal hemisphere, and electrophysiological recordings were obtained 4-5 days later.

To obtain the membrane currents generated by the hClCs we used the two-microelectrode voltage-clamp technique (Miledi, 1982). The oocytes were placed in a 100-μL chamber, impaled with two microelectrodes filled with 3 M KCl (0.5-1.5 MΩ), and clamped at 0 mV. For induction of voltage-activated currents, the oocyte´s membrane potential was stepped from 0 to -120 to +40 in 20-mV steps. All recordings were made at room temperature (20-23ºC) in a chamber continually perfused with Ringer´s solution (in mM): 115, NaCl; 2, KCl; 1.8, $CaCl_2$; 5, HEPES; pH 7.4.

3. Results

3.1 The Gef1p$^-$ Yeast Phenotype is Reverted by Overexpression of hClC1 and hClC2

The first indication showing the complementation of the *pet* phenotype by expressing hClC1 or hClC2 was provided by a simple drop assay. Figure 1A illustrates that the mammalian genes revert the size of spotted cells when expressed in a Gef1p$^-$ strain growing in low-iron and non-fermentable carbon sources. This complementation was found when the ClCs were introduced and induced to express under the GAL1 promoter contained in the plasmid pYES. Flis et al. (2005) reported that expression of the mouse ClC2 was not capable of complementing a Gef1p$^-$ strain when using a single-copy plasmid and thus, we decided to see if this was also true with our strains and the human ClCs.

The ClCs clones derived from pUG35 were grown in restrictive media supplemented with galactose to discard any ability of this carbohydrate to revert the phenotype. Consistent with Flis' findings, neither hClC1 nor hClC2 reverted the *pet* phenotype (Figure 1B). The results above suggested that the reversion of the *pet* phenotype observed in the first series of experiments was due to a dose effect, since the expression derived from pYES is expected to be higher than that driven by the MET25 promoter of the pUG35 vector.

A visual inspection of the cells transformed with pYES under the light microscope revealed that the cell diameter correlated well with the size of the colony (Figure 1A and 2A). The cell diameter of Gef1p$^-$ (5.91μ ± 0.06) and the strain transformed with the multicopy vector (5.95 μ ± 0.03) differed from that of the *wt* strain (6.88μ ± 0.03). Cells expressing hClC1 were clearly larger (6.77 μ ± 0.01) than the knock-out mutant but did not reach the full size of the *wt*, whereas those expressing hClC2 (6.82 μ ± 0.03) were undistinguishable from the *wt* yeast (Figure 2A). The yeast surface was analyzed in samples of these cells under the SEM (Figure 2B and 3B), but other than changes in cells diameter we did not observe any major difference among the samples.

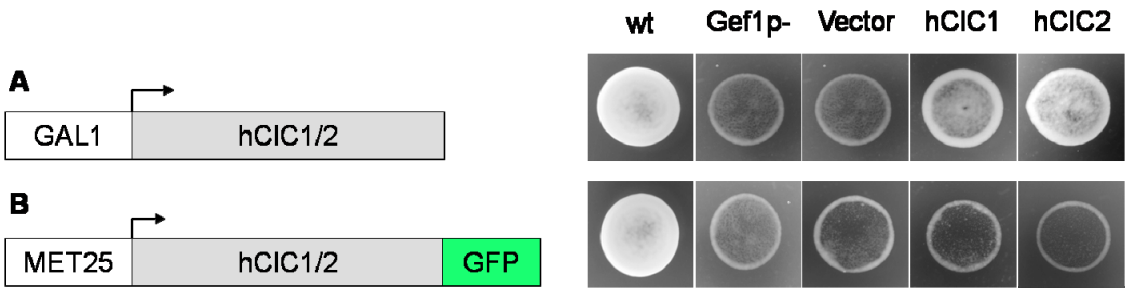

Figure 1. Overexpression of hClCs revert the *pet* phenotype

A. The human ClCs expressed under the GAL1 promoter revert the *pet* phenotype of a Gef1p$^-$ strain. B. In contrast, a centomeric plasmid where the ClCs were expressed under the direction of MET25 did not revert the phenotype of the strain.

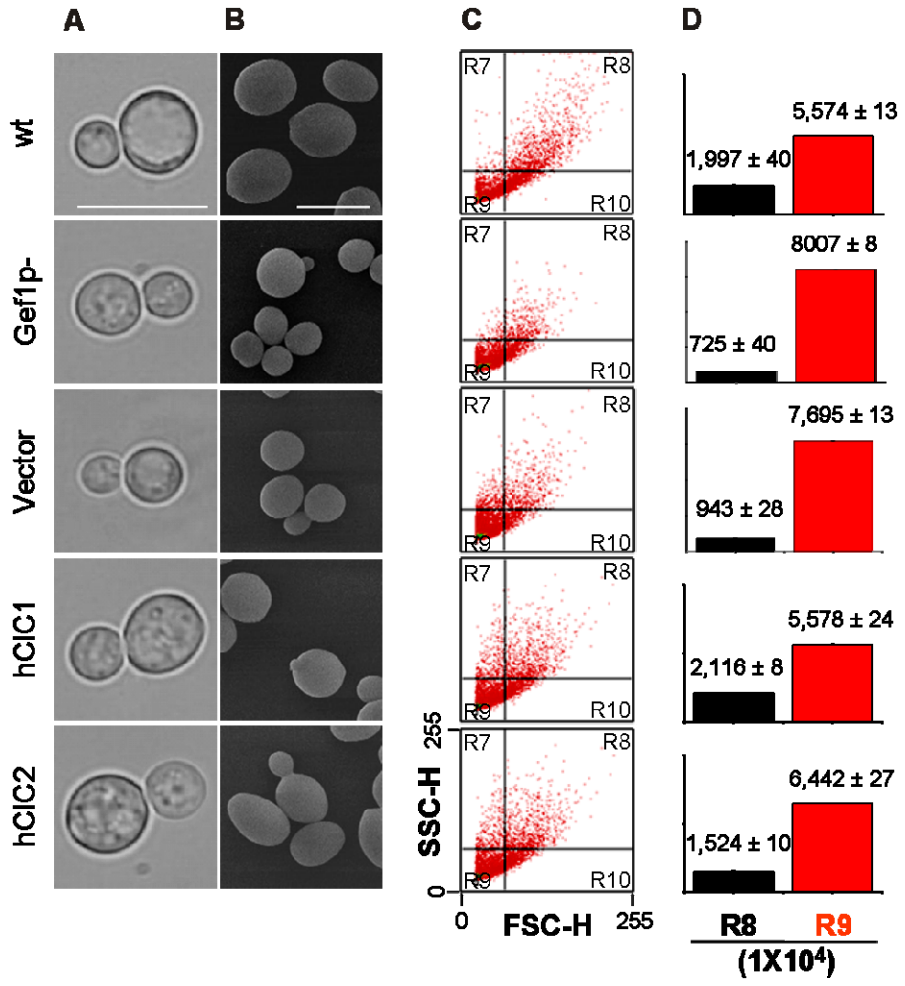

Figure 2. Phenotype of reverted cells

A. Sample images of *wt* and reverted cells seen under the light microscope. Bar = 10 μm. B. Sample images of the yeast under the SEM; notice the diversity of cell sizes within and among samples. Bar = 5 μm. C. Distribution of the cells resulting from the flow cytometry; FSC-H (cell size) and SSC-H (surface complexity), comparative data was subtracted from quadrant R8. D. Distribution of cells (events) in R8 and R9 in three independent experiments (means ± SE).

Observation of the cells under the light microscope showed a wide diversity of cell diameters among samples; thus flow cytometry, a standard easy and quick methodology, was used to analyze a large population of yeast samples to have a better idea of the variants within and among the samples.

The results of flow cytometry were plotted in Figure 2C, which shows the wide variability of cell size regardless of the cell sample, and this was consistent with the diversity of cell diameters observed under the light microscope. Nevertheless, comparing the distribution of cells in quadrants R8 and R9 revealed some difference in volume and complexity of the cell surface between the *wt* and the *GEF1* knock-out; these parameters allowed us to establish a clear *quantitative* reference to determine whether the reversion of the *pet* phenotype occurred or not. The number of events recorded in quadrant R8 for the *GEF1* mutant and for cells transformed with the core vector (pYES) were slightly different (725 ± 4 and 943 ± 28, respectively), whereas the number of cells expressing ClC1 (2116 ± 8) in R8 approached that of the *wt* (1998 ± 14), and the number of ClC2-expressing cells in R8 was intermediate (1524 ± 10), suggesting a partial reversion (Figure 2C-D).

Cells transformed with pUG35 (472 ± 1) showed an unexpected distribution that was different from the *GEF1* mutant (725 ± 4); in addition, the number of small cells was minimally changed by expressing either hClC1 (938 ± 4) or hClC2 (767 ± 8) (Figure 3C-D).

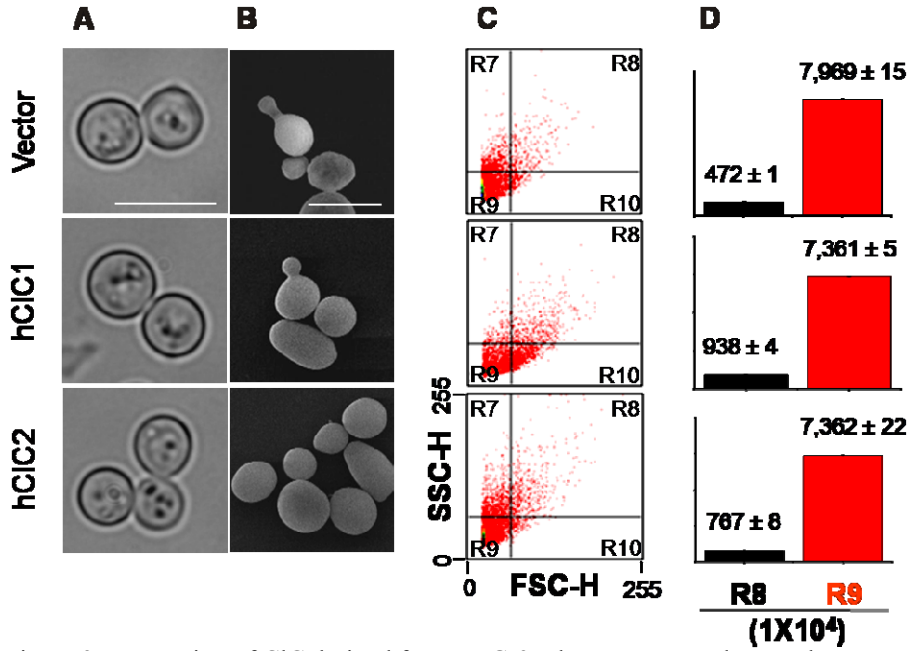

Figure 3. Expression of ClC derived from pUG-35 does not revert the pet phenotype

A. Images of cells under the light microscope. Bar = 10µm. B. Cells seen under the SEM Bar = 5µm. C. Distribution of cells in the flow cytometry assay. D. Distribution of the cells in R8 and R9 in three independent experiments (means ± SE).

3.2 Oxymetry

Respiratory metabolism is significantly diminished in strains that lack the *GEF1* gene (Gaxiola et al., 1998); thus, we determined if the level of oxygen consumption was normal in the reverted strains that expressed the hClCs. In three independent experiments the *wt* strain presented a higher respiratory rate (3.65 ± 0.68 nAO_2/min per 10^6 cells) when compared to Gef1p$^-$ transformed with the core plasmid (3.04 ± 0.10 nAO_2/min per 10^6 cells, Table 1). However, when the plasmid expressed either hClC1 or hClC2, the strain exhibited the normal level of oxygen consumption: 3.62 ± 0.05 or 3.59 ± 0.05, respectively. (In three independent experiments, the expression of hClC2 showed a slightly lower rate of O_2 consumption; however, it was not statistically significant. Expression of the hClCs in the *wt* strain did not increase the O_2 consumption (Table 1, RGY30 strain), size of the colony, or microscopic characteristics (not shown).

Table 1. O_2 consumption rates in *wt* (RGY30) and mutant (RGY192) yeast strains

Plasmid/Strain	(nAO_2/min/10^6 cells)
pYES-RGY30	3.658 ± 0.068
pYES-hClC1-RGY30	3.609 ± 0.086
pYES-hClC2-RGY30	3.626 ± 0.063
pYES-RGY192	3.048 ± 0.101
pYES-hClC1-RGY192	3.625 ± 0.052
pYES-hClC2-RGY192	3.596 ± 0.058

3.3 Expression of hClCs Tagged with GFP

In sharp contrast to the results described above, expression of hClC1 and hClC2 fused to GFP using a centromeric plasmid (pUG35) did not completely revert the *pet* phenotype. The spot assay correlated well with the images taken under the light microscope (Figure 1A and 3A), whereas flow cytometry revealed that the characteristics of the mutant were not totally reverted by expressing the ClCs (Figure 3C and D). This was also evidenced when observing the cell structure under the SEM, which showed that cells with the *pet* phenotype remained in the population of yeast transformed with either hClC1 or hClC2, and only a few cells among the population appeared to have reverted to the *wt* phenotype (Figure 3B).

A previous report describing similar results suggested that for the proper expression of ClC2, several codons have to be switched to those more frequently found in yeast. In addition, the overexpression of the Kah1 transporter is also needed to suppress the *pet* phenotype (Flis et al., 2005). In our results, fluorescence derived from the hClCs tagged with GFP and expressed in the *wt* strain was observed in intracellular compartments but mainly distributed around the periphery of the cells (Figure 4); thus,it is not necessary to introduce the yeast preferred codons for proper expression of the hClCs. When those plasmids were used to transform the Gef1p$^-$ strain, roughly 20-25% of the cells expressed the gene, and the fluorescence was found in intracellular compartments (Figure 4); however, as indicated above, this level of expression did not suffice to rescue the *pet* phenotype. A remaining question was if the GFP-tagged channels were functional; to probe that, we used *X. laevis* oocytes to test the electrophysiological properties of these channels.

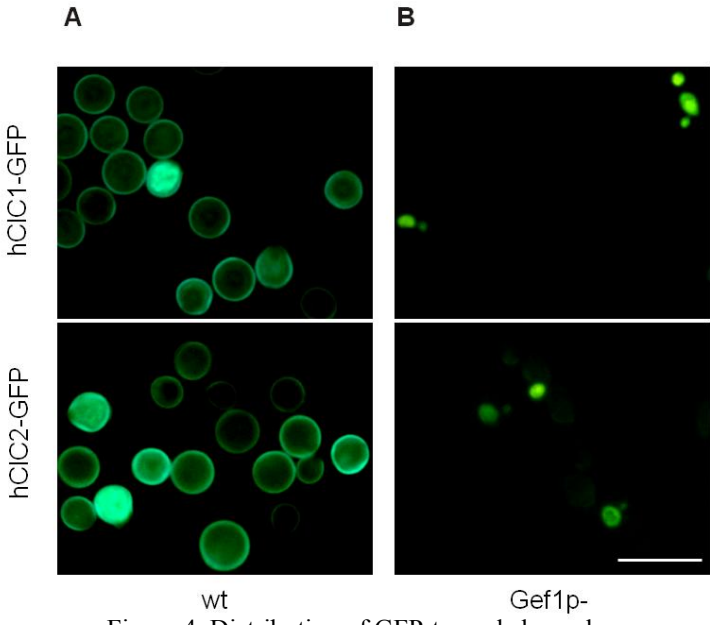

Figure 4. Distribution of GFP-tagged channels

A. fluorescence of ClC1 and ClC2 expressed in the *wt* strain was observed in the periphery of the cells. B. The same plasmids did not revert the *pet*phenotype, and the fluorescence was localized in intracellular compartments. Bar = 10μm.

3.4 Functional Expression of hClC1 and hClC2 Channels in X. laevis Oocytes

Injection of 50 nl of RNA (1 μg/μl) isolated from yeast expressing either hClC1 or hClC2 into frog oocytes induced the expression of functional channels; in contrast, *GEF1* did not present a voltage-dependent current. The resting membrane potential of oocytes injected with the hClC1 was usually around -25 mV, while the uninjected oocytes as well as those expressing hClC2 oscillated between -35 and -40 mV. Voltage stepping the oocytes from 0 to -120 to +40 in 20-mV steps elicited currents derived from the expressed channels. Sample currents generated by hClC1, hClC2, and the GFP-tagged channels are shown in Figure 5. hClC1 and hClC1-GFP showed a fast activation and a pronounced deactivation at voltages more negative than -100 mV, as previously demonstrated (Lorenz, Pusch, & Jentsch, 1996; Pusch, Steinmeyer, & Jentsch, 1994). hClC2 and hClC2-GFP showed a slow activation upon hyperpolarization of the plasma membrane, similar to that previously reported (Gründer, Thiemann, Pusch & Jentsch, 1992; Thiemann, Gründer, Pusch & Jentsch, 1992). This indirect assessment of the channels expressed in yeast gives no indication that the GFP tag alters the properties of the channel.

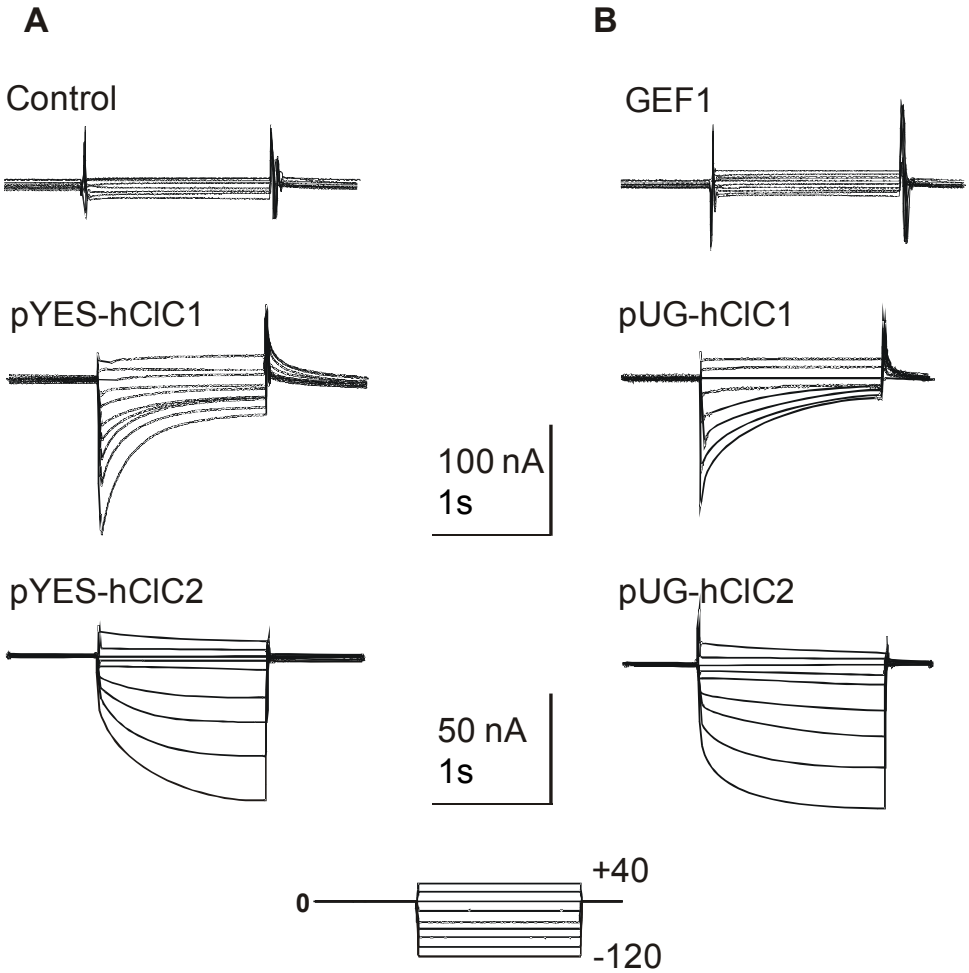

Figure 5. Functional expression in frog oocytes

Neither control nor GEF-1-injected oocytes generated a voltage-activated current, whereas oocytes injected with RNA from yeast induced to express ClC1 and ClC2, whether tagged or not with GFP, generated voltage-gated currents.

4. Discussion

The aim of this study was to determine whether the opposite activation kinetics of hClC1 and hClC2, *i.e.* either slow or fast activation, respectively, as well as other functional differences were related to their ability to revert the *pet* phenotype of a Gef1p$^-$ strain of *S. cerevisiae*. Initially, we observed that both hClCs were able to revert

the *pet* phenotype of the colonies formed by *GEF1* mutant cells; however, a previous report by Flis et al. (2005) contrasted with our observations. Thus, we repeated our experiments using a centromeric expression plasmid as reported by Flis et al. (2005); in this case our results were consistent with those of Flis et al. (2005); that is to say: the expression of hClC1 or hClC2 derived from pUG35 did not rescue the mutant phenotype. Therefore, we can explain our initial results by a dose effect: overexpression of the hClCs under the GAL1 promoter in pYES permits many channels to be properly located in the cell. In contrast, expression of hClCs under the MET25 promoter and in a centromeric plasmid did not induce the expression of sufficient, properly located protein to complement the functions lost in the *GEF1* mutant.

A second possibility to explain the inability of our pUG35-derived plasmids to revert the *pet* phenotype is the presence of GFP at the carboxy-terminus of the receptor. However, the membrane currents generated by oocytes injected with the ClCs showed no evident differences between the channels that were tagged or not with GFP. The fluorescence detected in yeast that were induced to express the GFP-tagged human channels indicates that it is not absolutely necessary to change the codons to those prefered by *S. cerevisiae*, as reported by Flis et al. (2005). This may reflect differences in the nucleotide sequence between the murine cDNAs used in their studies and the human genes that we used in our experiments. Furthermore, the *wt* strain expressing ClC1 or ClC2-GFP presented fluorescence at the cell periphery.

Considering that hClC1 and hClC2 show obvious differences in their activation mechanism and kinetics, we had aimed to correlate their ability to revert the *pet* phenotype with the specific properties of one of the channels; unexpectedly, both human ClCs induced the reversion. *GEF1* does not show voltage dependence either in HEK cells or in *X. laevis* oocytes heterologously expressing the protein (López-Rodríguez, 2007) (Figure 5) for what is considered mainly as an intracellular chloride transporter. There is also some evidence showing the functional role of ClC1 and ClC2 in intracellular compartments as well as their active role in transporting protons and their modulation by pH (Steinmeyer et al., 1991; Bösl et al., 2001). This last functional property may explain the ability of both channels to compensate for the absence of Gef1p in the-knockout yeast.

Acknowledgements

This work was supported by grants from CONACYT (101851) and UNAM-PAPIIT to RM and AM-T. FR-S and AL-R were supported by fellowships from CONACYT-Mexico and Posgrado en Ciencias Biomédicas-UNAM. Dr. R. Gaxiola donated yeast strains. Dr. A. George and Dr. G. Cutting donated plasmids carrying ClCs. We thank Dr. D.D. Pless for editing the final version of this manuscript.

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