Chloride Homeostasis in *Saccharomyces cerevisiae*: High Affinity Influx, V-ATPase-dependent Sequestration, and Identification of a Candidate Cl^- Sensor

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Chloride homeostasis in *Saccharomyces cerevisiae* has been characterized with the goal of identifying new Cl^- transport and regulatory pathways. Steady-state cellular Cl^- contents (~0.2 mEq/liter cell water) differ by less than threefold in yeast grown in media containing 0.003–5 mM Cl^-. Therefore, yeast have a potent mechanism for maintaining constant cellular Cl^- over a wide range of extracellular Cl^-. The cell water:medium $[Cl^-]$ ratio is >20 in media containing 0.01 mM Cl^- and results in part from sequestration of Cl^- in organelles, as shown by the effect of deleting genes involved in vacuolar acidification. Organellar sequestration cannot account entirely for the Cl^- accumulation, however, because the cell water:medium $[Cl^-]$ ratio in low Cl^- medium is ~10 at extracellular pH 4.0 even in *vma1* yeast, which lack the vacuolar H^+-ATPase. Cellular Cl^- accumulation is ATP dependent in both wild type and *vma1* strains. The initial $^{36}Cl^-$ influx is a saturable function of extracellular $[^{36}Cl^-]$ with $K_{1/2}$ of 0.02 mM at pH 4.0 and >0.2 mM at pH 7, indicating the presence of a high affinity Cl^- transporter in the plasma membrane. The transporter can exchange $^{36}Cl^-$ for either Cl^- or Br^- far more rapidly than $SO_4^=$, phosphate, formate, HCO_3^-, or NO_3^-. High affinity Cl^- influx is not affected by deletion of any of several genes for possible Cl^- transporters. The high affinity Cl^- transporter is activated over a period of ~45 min after shifting cells from high-Cl^- to low-Cl^- media. Deletion of ORF YHL008c (formate-nitrite transporter family) strongly reduces the rate of activation of the flux. Therefore, Yhl008cp may be part of a Cl^--sensing mechanism that activates the high affinity transporter in a low Cl^- medium. This is the first example of a biological system that can regulate cellular Cl^- at concentrations far below 1 mM.

INTRODUCTION

The regulation of the cytosolic concentrations of inorganic ions is one of the fundamental functions of the plasma membranes of all cells. Many ion homeostatic mechanisms are reasonably well understood, especially with regard to Na^+, K^+, and Ca^{2+}, in which the primary generators of ion gradients are ATP-driven pumps. The general principle of regulation of these cations is that the steady-state level of an ion depends on a balance between uphill and downhill transport processes (pump-leak paradigm) (Tosteson and Hoffman, 1960; Lew et al., 1982).

Regulation of cellular Cl^- is less well understood. In general, the steady-state cytosolic Cl^- concentration is determined by a combination of activities of cation-Cl^- cotransporters (Haas and Forbush, 1998; Lu et al., 1999; Rivera et al., 1999), Cl^--HCO_3^- (or other base) exchangers (Alper et al., 2002; Soleimani and Xu, 2006), and Cl^- channels (Jentsch et al., 2002). In order for cells to regulate cytosolic Cl^-, there must be mechanisms for sensing the Cl^- concentration. One known mechanism is the inhibition of the $Na^+/K^+/2Cl^-$ cotransporter by increases in cytosolic $[Cl^-]$ (Lytle and Forbush, 1996; Gillen and Forbush, 1999; Russell, 2000; Lytle and McManus, 2002), which limits net influx. Possible Cl^--sensing functions may also be associated with CFTR (Jiang et al., 1998) and other airway epithelial Cl^- channels (Tarran et al., 2000), although the molecular identities of the actual Cl^- sensors are not known.

The purpose of the work described here is to determine whether *Saccharomyces cerevisiae* is a useful system for the study of mechanisms of regulation of cellular Cl^-. Very little is known about Cl^- transport and regulation in yeast. Instead, attention has been focused on the wealth of K^+, Na^+, and H^+ transporters and channels available for study in this organism (Gustin et al., 1986; Nass and Rao, 1998, 1999; Bihler et al., 1999; Morsomme et al., 2000; Kuroda et al., 2004). In the early literature on ion transport in yeast, Cl^- was considered an impermeant ion (Conway and Downey, 1950; Rothstein, 1974), because Cl^- transport is far slower than that of many other ions. Most recently Coury et al. (1999) showed that

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Abbreviations used in this paper: APG, arginine/phosphate/glucose medium; 2-DG, 2-deoxy-D-glucose; DNP, 2,4-dinitrophenol; LCAPG, low Cl^- arginine/phosphate/glucose medium; LCYNB, low-Cl^- yeast nitrogen base medium; V-ATPase, vacuolar H^+-ATPase; YNB, yeast nitrogen base medium; YPGE, medium containing 1% yeast extract, 2% peptone, 2% glycerol, 2% ethanol.

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the influx of $^{36}Cl^-$ into *S. cerevisiae* is almost negligible compared with that of $^{86}Rb^+$ or phosphate.

Although the fluxes are small, there is evidence that Cl^- transport has physiological importance in *S. cerevisiae*. Gef1p is the sole member in *S. cerevisiae* of the CLC family (Jentsch et al., 1999), which includes mammalian Cl^- channels and as well as a bacterial protein of known structure (Dutzler et al., 2002; Dutzler et al., 2003) that acts as coupled exchanger of H^+ for Cl^- (Accardi and Miller, 2004). The phenotype of *gef1* strains is slow growth at low Fe^{3+} concentrations, indicating a role for Gef1p in iron metabolism (Greene et al., 1993). Gef1p is expressed in Golgi or post-Golgi, prevacuolar vesicles (Schwappach et al., 1998; Gaxiola et al., 1999). The Cl^- ions transported into these vesicles by Gef1p have two functions. First, the Cl^- flux neutralizes the H^+ pumped into the vesicle by the V-type H^+-ATPase (Wada and Ohsumi, 1992; Gaxiola et al., 1998). The second role of Cl^- is to act as a direct cofactor for Cu^{2+} loading on Fet3p (Davis-Kaplan et al., 1998). In addition to the role of Gef1p as an organellar Cl^- transport pathway, there is recent evidence that Gef1p is also expressed on the plasma membrane (López-Rodríguez et al., 2007).

Little is known about the distribution of Cl^- across the plasma membrane of *S. cerevisiae*. Yeast is expected to have a relatively low intracellular Cl^- concentration, because 100 mM Cl^- inhibits transcription in extracts of *S. cerevisiae* (Lue and Kornberg, 1987). From the steady-state accumulation of $^{36}Cl^-$, Coury et al. (1999) calculated that the intracellular $[Cl^-]$ is ~0.15 mM at extracellular pH 4.0 and extracellular $[Cl^-]$ of 5 mM. This value of cellular $[Cl^-]$ is of course an average over the whole cell, including cytosol, endoplasmic reticulum, Golgi, nucleus, mitochondria, endosomes, and vacuole(s). Although the most prominent ion channels in the vacuolar membrane of *S. cerevisiae* are cation selective (Bertl and Slayman, 1990), the membrane is permeable to Cl^- (Wada and Ohsumi, 1992), as expected given the role of Cl^- as a counterion for vacuolar acidification. Nothing is known about the steady-state distribution of Cl^- across the yeast vacuolar membrane.

The plasma membrane potential of *S. cerevisiae* is not known as accurately as that of *Neurospora crassa* (Ballarin-Denti et al., 1994), but it is probably in the range of −50 to −120 mV, with the magnitude depending on yeast strain and incubation conditions (Vacata et al., 1981). If the membrane potential is in this range, then a cytosolic $[Cl^-]$ of 0.15 mM (Coury et al., 1999) in a medium containing 5 mM Cl^- is not far from that expected from a passive distribution. There are no published data on the cells:medium Cl^- distribution at either higher or lower $[Cl^-]_o$, except those of Conway and Downey (1950), in which intracellular Cl^- was difficult to distinguish from that trapped in the intercellular space.

There have been three published measurements of $^{36}Cl^-$ fluxes in yeast. Groves et al. (1996) showed that expression of the membrane domain of the erythrocyte anion exchanger AE1 (band 3) in yeast causes a large increase in the $^{36}Cl^-$ influx, but the basal flux, though low, was measurable. Coury et al. (1999) measured the $^{36}Cl^-$ influx (5 mM Cl^-) in *S. cerevisiae* and found that it is dependent on extracellular pH (largest at pH 3) and is larger in the absence than in the presence of glucose, consistent with the idea that membrane depolarization increases the influx. The other $^{36}Cl^-$ influx measurement was from this laboratory (Jennings et al., 2007) and indicated that there is a high affinity Cl^- transport pathway in yeast that does not involve the anion exchanger homologue Bor1p.

In the work described here we have determined the steady-state cellular Cl^- contents in *S. cerevisiae* grown in synthetic medium derived from YNB (Sherman, 1991) and APG (Rodriguez-Navarro and Ramos, 1984; Nass et al., 1997) in which $[Cl^-]$ was varied from 0.003 to 100 mM. We find that the cellular Cl^- content varies only slightly over a wide range of extracellular $[Cl^-]$. The effects of deletion of genes involved in vacuolar acidification indicates that a significant fraction of the cellular Cl^- is sequestered in the vacuole or in prevacuolar vesicles. Even in cells having no vacuolar H^+-ATPase, the cellular $[Cl^-]$ in media containing very low $[Cl^-]$ is much higher than expected for electrochemical equilibrium. The elevated cellular $[Cl^-]$ is the consequence of a pH-dependent high affinity Cl^- transporter that is activated rapidly upon exposure of cells to low $[Cl^-]$ media. The molecular identity of the high affinity transporter is not known, but the rapid activation of the transporter is dependent on the gene product of ORF YHL008c, which is homologous to formate-nitrite transporters. YHL008cp therefore may be a component of a Cl^--sensing mechanism.

MATERIALS AND METHODS

Yeast Strains and Media

Salts and buffers were purchased from either Fisher Scientific or Sigma-Aldrich. Some of the $^{36}Cl^-$ distribution studies were performed in *S. cerevisiae* strain FKY 282 (*MAT**a** SRP40 pep4::LEU2 ura3-12 leu2-3,-112 his3-11,-15 trp01-1 ade2-1*, kanamycin resistant) (Groves et al., 1996). Most of the distribution and all the transport studies were performed on haploid strains obtained from the Eusoscarf Yeast Deletion Project via Invitrogen. Strains were all in the background of BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). Deletions used in this study, with Euroscarf identification numbers, are as follows: BOR1 (Y01169), GEF1 (Y06838), NHX1 (Y04290), SUL1 (Y07176), TRK1 (Y01296), TRK2 (Y05121), VMA1 (Y03883), YBR235w (Y03375), YHL008c (Y00955), and YPR003c (Y02830).

Low-Cl YNB medium (LCYNB) was prepared as follows. All concentrations of salts, vitamins, trace minerals, and amino acids are as listed by Sherman (1991) for YNB medium (with 2% glucose and 20 mg/liter uracil), with the following substitutions to remove Cl^-: $Ca(OH)_2$ for $Ca(Cl)_2$; Na_2SO_4 for NaCl; $Fe(NO_3)_3$ for $Fe(Cl)_3$; arginine, lysine, and histidine-free bases instead of hydrochlorides. To make up for the base added with $Ca(OH)_2$, the equivalent amount of H_2SO_4 was added.

Low Cl^- medium based on APG (Rodriguez-Navarro and Ramos, 1984; Nass et al., 1997), designated here as LCAPG, was prepared using 10 mM arginine (free base), 8 mM H_3PO_4, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $Ca(OH)_2$, and the other amino acids, vitamins, trace minerals, glucose, and uracil as in LCYNB described above. For the experiments examining the effect of extracellular $[K^+]$, the KH_2PO_4 concentration was varied from 0.3 to 3 mM.

In most of the experiments the media included Cl^- present as pyridoxine HCl (2 μM) and thiamine HCl (1 μM). In addition, from the stated upper limits on the amounts of Cl^- in the salts used to prepare the media, we estimate that the final Cl^- concentration resulting from Cl^- in these salts is ~2 μM. For measurement of initial influx or steady-state distribution, the $^{36}Cl^-$-specific activity was calculated from the concentration of $^{36}Cl^-$, the specific activity of the $Na^{36}Cl$ stock solution, and the isotope dilution caused by the presence of 5 μM nonradioactive Cl^- (3 μM from pyridoxine and thiamine if present, and 2 μM as contaminants in other salts). In the $^{36}Cl^-$ efflux experiments, in which it is important to minimize exchange of cellular $^{36}Cl^-$ for nonradioactive Cl^-, and in the $^{36}Cl^-$ distribution experiments at the lowest Cl^- concentrations, the Cl^- in the pyridoxine HCl and thiamine HCl stock solutions was removed with 50 mg Dowex 1 (OH^- form) per ml of stock solution. In the experiments with 2,4-dinitrophenol (DNP), possible contaminating Cl^- was removed by extracting DNP (Sigma-Aldrich) into ethyl ether from an aqueous solution that had been acidified to pH 2.0 with H_3PO_4.

Steady-State Cellular Cl^-

Cells from 1 ml of stationary culture were centrifuged, washed once in LCYNB or LCAPG medium, resuspended in 50 ml of the same medium containing 2% glucose and 20 mg/L uracil, and divided into aliquots. $Na^{36}Cl$ (DuPont NEN) was added carrier-free to concentrations of between 1 uM and 1 mM. (The half-life of ^{36}Cl is so long [3×10^5 years] that a 1 mCi/ml stock solution contains ~2 M $Na^{36}Cl$.) At total Cl^- concentrations >0.3 mM, $Na^{36}Cl$ was present at a concentration of 0.3 mM, and nonradioactive Cl^- was added as NH_4Cl, NaCl, or KCl. Cultures were incubated aerobically overnight with rotary shaking at 225 RPM, 30°C. To determine the cellular Cl^- and the Cl^- distribution, cells were centrifuged 2 min at 4,000 RPM, and the $^{36}Cl^-$ in aliquots of extracellular medium was determined by scintillation counting. Cells were immediately washed twice in ice-cold water and cellular $^{36}Cl^-$ activity measured by liquid scintillation counting. Cellular Cl^- contents (mEq/liter cell water) were calculated from the extracellular specific activity, the amount of cell water present, and the total CPM in the cell pellet. The cell water (not including periplasm) was assumed to be 50% of the wet weight of the cell pellet, because 1 g (0.9 ml) of packed cells has total water of 0.78 g (Jennings et al., 2007), with 0.28 g (~31% of the pellet volume) of this water outside the plasma membrane (Conway and Downey, 1950). Cellular K^+ was determined on lysates (following heating with 10 cell volumes of water for 15 min at 100°C and centrifuging cell solids) with a K^+ selective electrode (Fisher Scientific).

In the experiments in Fig. 3, cells were grown overnight in LCAPG medium without $^{36}Cl^-$, centrifuged, and resuspended in fresh LCAPG medium, 2% glucose, plus 8 μM $Na^{36}Cl$. Media were buffered either with 5 mM K-citrate (initial pH 4.5), or with 5 mM K-citrate, 10 mM bistris, 20 mM tris (initial pH 7.6). After 2 h of aerobic incubation at 30°C, 5 ml aliquots of suspension were diluted in 40 ml of ice-cold water, centrifuged, the supernatant aspirated completely, and the cellular $^{36}Cl^-$ determined by scintillation counting.

Unidirectional $^{36}Cl^-$ Influx and Efflux

Cells grown overnight in LCAPG were centrifuged and resuspended in fresh LCAPG, buffered as described in the figure legends. After incubating aerobically for 15 min at 30°C, $Na^{36}Cl$ was added from a 10 mM stock solution to final total concentrations (including the small amount of nonradioactive Cl^- in the medium) of 9–133 μM. Influx was allowed to proceed for various times (60 s for initial influx, longer times for total accumulation) before the suspension was diluted with 10 volumes of ice-cold water, centrifuged, and washed once with cold water. The $^{36}Cl^-$ in the pellet was determined as described above. The efflux of $^{36}Cl^-$ was measured by loading cells with $^{36}Cl^-$ for at least 1 h, 30°C, in LCAPG, 5 mM K-citrate, pH 4.5, with 8 μM $^{36}Cl^-$. Cells were then centrifuged, resuspended in media containing no radioactivity, and incubated further as described in the figure legends. Cellular radioactivity was determined for most of the time points as the total activity minus the supernatant activity (following 1-min spin in a microfuge). At late time points, in which extracellular and total activity are similar, the cellular radioactivity was determined by counting the pellet directly.

Statistical analyses (paired *t* test) were performed using the statistical package in Sigma Plot (Systat).

RESULTS

Steady-State Cellular Cl^- Contents

Fig. 1 A shows the cellular Cl^- contents (mEq/liter cell water) as a function of extracellular $[Cl^-]$ in overnight cultures of haploid yeast strains grown aerobically in media containing 0.003–100 mM Cl^-. At Cl^- concentrations below 4 mM, the Cl^- normally present in YNB or APG was replaced by $SO_4^=$. At higher concentrations, Cl^- was added as NH_4Cl, KCl, or NaCl, with indistinguishable results.

Over a range of extracellular $[Cl^-]$ from 0.003 to 5 mM, the cellular Cl^- contents are remarkably constant: 0.2 ± 0.1 mEq/liter cell water, and the cell water:medium Cl^- distribution ratio varies over several orders of magnitude (Fig. 1 B). The dashed line represents the expected distribution if Cl^- were passively distributed across the plasma membrane, with a membrane potential of −120 mV and Cl^- uniformly distributed in all cell water. If the actual membrane potential is closer to −180 mV, as is true for *Neurospora crassa* (Ballarin-Denti et al., 1994), then the Cl^- ratio for a passive distribution would be 10-fold lower. It is clear that the observed Cl^- distribution is reasonably close to a passive distribution only at extracellular $[Cl^-]$ >10 mM. At very low extracellular $[Cl^-]$ (<0.1 mM) the cellular Cl^- contents are orders of magnitude higher than those expected for a passive distribution.

Effect of K^+ Gradient on Cl^- Accumulation

It is known that K^+ starvation hyperpolarizes the *S. cerevisiae* plasma membrane (Madrid et al., 1998); therefore, the membrane potential, though not known precisely, should be more negative in media of very low K^+ concentration. To try to determine whether plasma membrane potential has any effect on the accumulation of cellular Cl^- at very low extracellular Cl^- concentrations, cells were grown in media with low K^+ concentrations. The strain chosen for this experiment has a deletion for a gene, SUL1, that is not expressed at the methionine concentrations used here

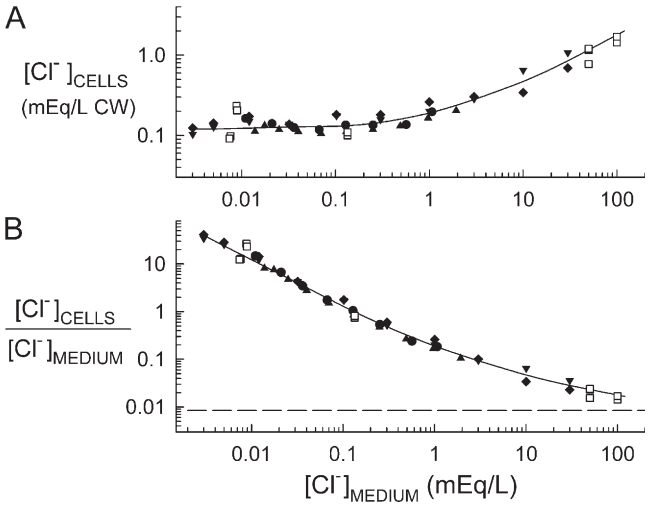

Figure 1. Cl^- contents (A) and distribution ratios (B) for *S. cerevisiae* grown in media containing varying concentrations of Cl^-. Solid symbols: cells were washed and suspended at A_{600} of 0.05 in media in which all but ~5 μM Cl^- had been replaced by $SO_4^=$, and Cl^- was added back as $Na^{36}Cl$ (0.001–0.3 mM), plus, for higher concentrations, 0.7–100 mM NH_4Cl, KCl, or NaCl. Suspensions were incubated aerobically 16–20 h at 30°C, and the cellular $^{36}Cl^-$ contents were determined as described in Materials and methods. Unless otherwise indicated the final extracellular pH was 3.5 ± 0.5. Open symbols: cells were grown in APG, washed, and incubated 2 h at 30°C in APG containing various concentrations of $^{36}Cl^-$ before determining cellular $^{36}Cl^-$ contents. The dashed line in B is the distribution ratio predicted from a Nernst distribution, with plasma membrane potential of −120 mV. The solid curves through the data have no theoretical significance. ◆: strain BY4741, YNB, 25 mM K-citrate buffer, final pH 4.4. ▼, ▲: strain BY4741, YNB. ●: strain FKY282, YNB. □: SUL1 deletant (BY4741 background), 2 h, APG.

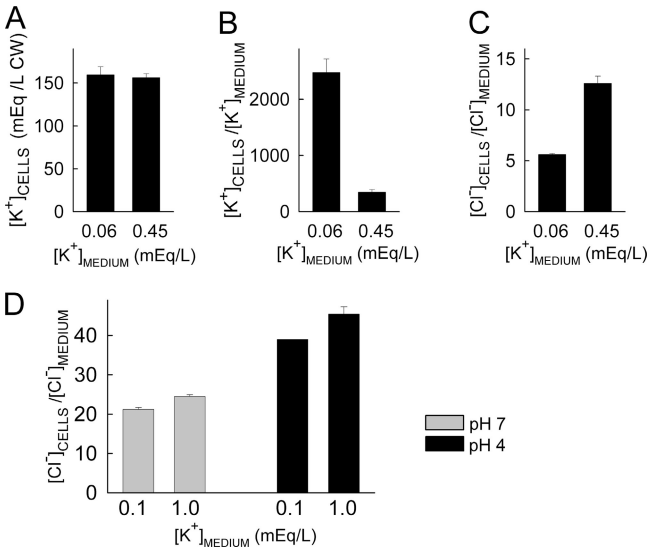

Figure 2. (A–C) Effect of low extracellular K^+ on the cells:medium $^{36}Cl^-$ ratio of cells grown in low Cl^- medium. Cells (*sul1* in BY4741 background) were grown 16 h in LCAPG, 2% glucose, and $^{36}Cl^-$ (total of 8 μM Cl^-). The medium initially contained either 1 mM K^+ (normal APG) or 0.3 mM K^+. After aerobic growth at 30°C for 16 h, cells were centrifuged, washed once in cold water, and the $[K^+]$ (B) and $[^{36}Cl^-]$ (C) distribution ratios were measured. The final extracellular K^+ concentrations were 0.45 mM and 0.06 mM, respectively, for the media that had initially contained 1 mM and 0.3 mM K^+. The total cellular K^+ contents were the same in the two media (A). (D) Effect of low extracellular K^+ on the cells:medium $^{36}Cl^-$ ratio of cells following a 2-h incubation. Cells (*sul1*) were grown 16 h in LCAPG medium containing 2 mM K^+ and then incubated 2 h in fresh LCAPG medium containing 8 μM $^{36}Cl^-$ and either 0.1 mM or 1 mM K^+. The media were buffered either at initial pH 4.5 with 5 mM Na-citrate or initial pH 7.6 with K-citrate, 10 mM bistris, 20 mM tris.

(Cherest et al., 1997); this strain is used for comparisons with other deletants in the same background (BY4741) in further experiments below.

Fig. 2 C shows the cells:medium $[^{36}Cl^-]$ ratio in cultures grown 16 h in LCAPG medium containing 5 μM $^{36}Cl^-$ (plus ~5 μM nonradioactive Cl^-) and (initially) either 0.3 mM or 1 mM K^+. (We found, in agreement with other laboratories [Rodriguez-Navarro and Ramos, 1984; Hess et al., 2006], that similar experiments could not be performed in YNB medium because of the toxicity of NH_4^+ in low K^+ media). In medium initially containing 0.3 mM K^+, the final cell density is only about half that in 1 mM K^+, but the cellular K^+ (~150 mEq/liter cell water) is the same in both media (Fig. 2 A). The cells:medium $^{36}Cl^-$ ratio is lower in cells grown in low K^+ medium. In four measurements on two separate cell preparations, the cells:medium $^{36}Cl^-$ ratio in media with 0.06 mM K^+ is less than half that in the medium with 0.45 mM K^+ (Fig. 2 C).

The effect of the K^+ gradient on the Cl^- distribution is much smaller if the cells are grown in LCAPG medium containing 2 mM K^+ and then incubated for 2 h in LCAPG medium containing 8 μM $^{36}Cl^-$ and either 0.1 mM or 1 mM K^+ (Fig. 2 D). There is slightly less Cl^- in the cells incubated in 0.1 mM K^+, but the difference is smaller than that observed following a 16-h incubation. Therefore, the effect of extracellular K^+ observed following a 16-h incubation does not appear to be a direct effect of the K^+ gradient. Instead, adjustments in gene expression that allow the cells to grow in very low K^+ may have indirect effects on the accumulation of Cl^-. Because there is little acute effect of the K^+ gradient on cellular Cl^-, we can draw no conclusions about the effect of membrane potential on the cells:medium $^{36}Cl^-$ ratio in low Cl^- medium.

Effect of Extracellular pH

To test the effect of extracellular pH on Cl^- distribution, cells were grown overnight in LCAPG medium (~5 μM Cl^-) and then incubated at least 2 h in fresh medium containing 8 μM $Na^{36}Cl^-$, buffered at either pH 4.0 or pH 7. In all strains tested, the steady-state Cl^- contents are substantially higher at extracellular pH 4.0 than at pH 7.0 (Fig. 2 D and Fig. 3 A), suggesting a possible H^+-coupled Cl^- influx.

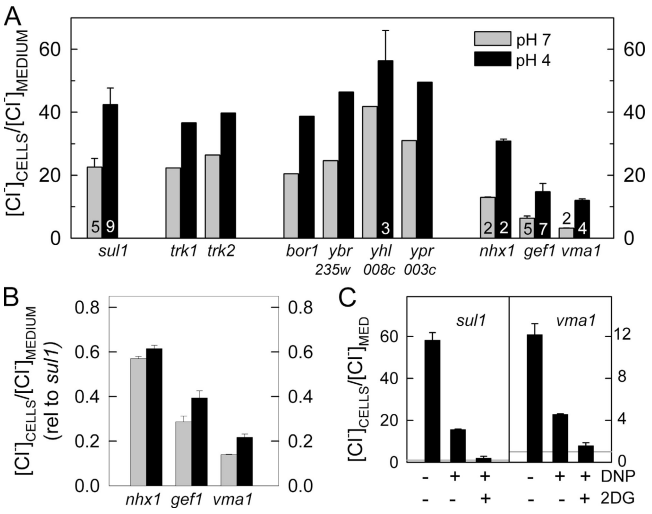

Figure 3. (A) Cell water:medium distribution ratio of $^{36}Cl^-$ in various deletant strains. Cells were grown overnight in LCAPG and then incubated 2 h at 30°C in fresh medium plus 8 μM $Na^{36}Cl$. Cellular $^{36}Cl^-$ contents at the end of the incubation were determined as described in Materials and methods. Media were buffered with 5 mM K-citrate (initial pH 4.5, final pH 4.1; black bars), or 5 mM K-citrate, 10 mM bistris, 20 mM tris (initial pH 7.6, final pH 6.7; gray bars). Strains with deletions of the indicated genes were all in the background of BY4741. Bars labeled *yhl*, *ybr*, and *ypr* refer respectively to strains deleted in YHL008c, YBR235w, and YPR003c. Data represent single determinations except for bars labeled to indicate the number of determinations. Error bars are SEM. (B) Cellular Cl^- contents in low Cl^- media in paired experiments with control strain *sul1*. Cells were incubated in LCAPG media containing 8 μM $^{36}Cl^-$, and cellular Cl contents were determined after sufficient time to reach a steady-state distribution (>1.5 h). Bars represent mean ± SEM of the ratio between cellular $[Cl^-]$ in the test strain (*nhx1*, *gef1*, or *vma1*) and control strain *sul1* at either pH 4.0 (black bars) or pH 7.0 (gray bars). Results of paired Student *t* tests (pH 4 and pH 7.0 combined): *sul1* vs. *nhx1*, $P < 0.02$ ($n = 4$); *sul1* vs. *gef1*, $P < 10^{-5}$, $n = 12$; *sul1* vs. *vma1*, $P < 0.002$, $n = 6$. (C) Cells:medium $^{36}Cl^-$ distribution ratio for *sul1* and *vma1* cells incubated as in A and B but with 0.2 mM DNP or DNP and 20 mM 2-deoxyglucose replacing 2% glucose during the 2 h incubation with $^{36}Cl^-$. The gray horizontal lines indicate a cells:medium ratio of unity.

Genes for Possible Plasma Membrane Cl^- Transporters Have No Effect on Cl^- Distribution

Deletion of any of several genes with homology to plasma membrane Cl^- transporters has no effect on Cl^- accumulation at either pH 4.0 or pH 7.0 (Fig. 3 A). For example, BOR1, the single ORF in the family of bicarbonate transporters (Alper et al., 2002; Romero et al., 2004), is known to catalyze boric acid efflux (Takano et al., 2002; Takano et al., 2007), probably as exchange with H^+ (Jennings et al., 2007). Deletion of BOR1 does not affect $^{36}Cl^-$ accumulation in a low Cl^- medium, in agreement with our earlier conclusion that Bor1p does not transport Cl^- (Jennings et al., 2007). Similarly, deletion of YBR235w, the single *S. cerevisiae* member of the Cl^--coupled cation transporter family (André and Scherens, 1995; Gamba, 2005), also has no effect on $^{36}Cl^-$ distribution. The same is true of YPR003c, a member of the SCL26 family, which in humans includes several Cl^- transporters (Soleimani and Xu, 2006).

The effect of deletion of the genes for the K^+ transporters Trk1p and Trk2p (Ko and Gaber, 1991) was also tested, because, under some conditions, these proteins can mediate a Cl^- current (Kuroda et al., 2004). There is no effect of deletion of either of these genes on Cl^- accumulation in a low Cl^- medium. This finding is consistent with the work of Kuroda et al. (2004), who showed that Trk1p and Trk2p do not mediate significant Cl^- currents when the cytosolic Cl^- concentration is <1 mM. Another yeast Cl^- channel is the voltage-dependent anion channel in the mitochondrial outer membrane (Forte et al., 1987); this channel was not tested because mitochondria, with a matrix-negative inner membrane potential, should not be a major cellular Cl^- compartment.

In one strain, *yhl008c*, the data suggest that there is slightly more Cl^- than in any of the other strains (Fig. 3 A), although the difference between this strain and the control strain *sul1* is small and of questionable statistical significance ($0.1 > P > 0.05$). The YHL008c ORF is homologous to formate-nitrite transporters (Suppmann and Sawers, 1994; Deloménie et al., 2007). This strain is examined further below in reference to regulation of Cl^- transport.

Genes Involved in Organellar Acidification Affect Cl^- Distribution

The only gene deletions that were found to reduce steady-state Cl^- accumulation in low Cl^- medium are those involved in organellar acidification: the genes code for the subcellular Na^+-H^+ exchanger Nhx1p (Nass and Rao, 1998), the CLC family member Gef1p (Greene et al., 1993; Schwappach et al., 1998; Flis et al., 2002), and the vacuolar H^+-ATPase catalytic subunit Vma1p (Kane, 2006). Fig. 3 B shows the results of paired comparisons between each of these deletions and the control deletion *sul1*. In the *nhx1* strain the amount of cellular $^{36}Cl^-$ is ~60% that of the control strain at either pH 4.0 or pH 7. The effect of deletion of GEF1 is larger; cellular Cl^- is ~35% of control. Deletion of VMA1 has a very large effect; cellular Cl^- in *vma1* cells is only ~14% that of the control strain at pH 7, and 20% at pH 4.

The effects of deletion of NHX1, GEF1, or VMA1 are consistent with the idea that Cl^- moves through Gef1p into a subcellular compartment (post-Golgi, prevacuolar vesicles) as a counterion for the H^+ pumped by the ATPase (Davis-Kaplan et al., 1998; Gaxiola et al., 1998), and that Nhx1p facilitates the accumulation of Cl^- by exchanging cations for H^+ in prevacuolar compartments (Nass and Rao, 1998, 1999).

Effect of ATP Depletion

Depletion of ATP has a major effect on the accumulation of $^{36}Cl^-$ in low Cl^- media (Fig. 3 C). Cells from control

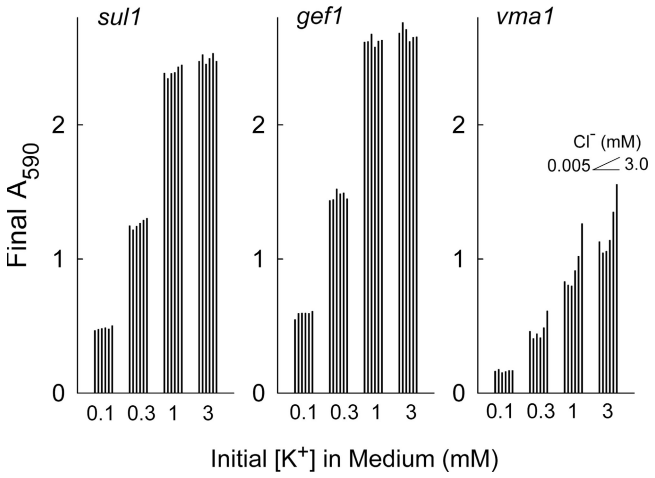

Figure 4. Effect of low K^+ and Cl^- concentrations on growth of *sul1*, *gef1*, and *vma1* strains of *S. cerevisiae*. Cells from each strain were suspended at initial A_{590} of 0.1 and incubated aerobically at 30°C with rotary shaking for 24 h. The medium was derived from LCAPG, with increasing amounts of Cl^- added as arginine Cl^-. The initial concentrations of Cl^- in the media were ~0.005, 0.035, 0.10, 0.30, 1.0, and 3.0 mM (left to right) in each group of six bars. The initial concentrations of K^+ (added as KH_2PO_4) were 0.1, 0.3, 1.0, and 3.0 mM.

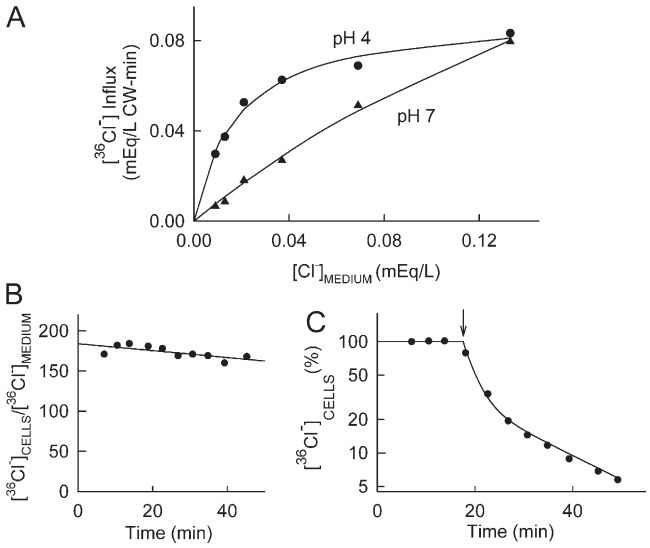

Figure 5. (A) Initial $^{36}Cl^-$ influx as a function of concentration. Cells (*sul1*) were grown overnight in LCAPG and then incubated in fresh medium at either pH 4.0 or pH 7. Media were buffered with 5 mM K-citrate (initial pH 4.5, final pH 4) or 5 mM K-citrate, 10 mM bistris, 10 mM Tris (initial pH 7.6, final pH 6.8). The initial (1 min) influx of $^{36}Cl^-$ was measured at 30°C in LCAPG with the indicated concentration of total Cl^- (0.005 mM nonradioactive Cl^- plus various added amounts of $^{36}Cl^-$). The solid curves through the data are rectangular hyperbolae with $K_{1/2}$ = 0.018 mM, J_{Max} = 0.092 mEq/L cell water-min (pH 4), and $K_{1/2}$ = 0.30 mM, J_{Max} = 0.261 mEq/L cell water-min (pH 7). (B) Very slow efflux of $^{36}Cl^-$ following resuspension of $^{36}Cl^-$-loaded cells (*sul1*) in LCAPG medium. Overnight culture of *sul1* cells in LCAPG was incubated in fresh LCAPG, 5 mM citrate, pH 4.5, and 8 μM $^{36}Cl^-$. After 2 h at 30°C, cells were centrifuged and resuspended in fresh medium containing no $^{36}Cl^-$ and incubated at 30°C. (C) Rapid efflux of $^{36}Cl^-$ following addition of extracellular Cl^-. Overnight culture of *sul1* cells in LCAPG was prepared and incubated as in B, except that 2 mM KCl was added at the arrow. Vertical axis is logarithmic; the curve through the data represents the sum of two exponentials with 70% of the tracer efflux with rate constant 0.41/min and the remaining 30% with rate constant 0.050/min.

(*sul1*) or *vma1* strains were incubated 2 h in LCAPG containing 8 μM $^{36}Cl^-$ as Fig. 3 A. The medium contained either 2% glucose, 0.2 mM 2,4-dinitrophenol (DNP), or DNP plus 20 mM 2-deoxoglucose (2-DG), which is known to strongly deplete ATP in *S. cerevisiae* (Chuang et al., 2005). As in Fig. 3 A, the cells:medium $^{36}Cl^-$ ratio in the *vma1* strain in glucose medium is only ~20% of that in the *sul1* strain. DNP alone causes a decrease in the accumulation of $^{36}Cl^-$, but the cellular $^{36}Cl^-$ in both strains is still higher than that in the medium. DNP plus 2DG reduces cellular $^{36}Cl^-$ to levels that are not distinguishable from that in the medium. The fact that ATP depletion has a strong effect on $^{36}Cl^-$ accumulation even in the *vma1* strain indicates that *S. cerevisiae* cells are capable of concentrating Cl^- by a mechanism that does not require the V-ATPase but that is dependent on cellular energy metabolism. Therefore, the cellular Cl^- in *vma1* cells is not likely to be bound passively to the cell wall or outer surface of the plasma membrane.

Minimal Effect of Extracellular Cl^- on Growth

Despite the fact that *gef1* and *vma1* cells have significantly lower cellular Cl^- in LCAPG than other strains, there are at most very minor effects of low Cl^- on cell growth. Fig. 4 shows the final cell density of *sul1*, *gef1*, and *vma1* strains grown 24 h in APG media (final pH ~3.5) having various concentrations of Cl^- and K^+. Each of the four groups of six bars represents cells grown at a given initial K^+ concentration with 0.005, 0.03, 0.1, 0.3, 1, and 3 mM Cl^-. As in Fig. 2, a low K^+ concentration results in a lower final cell density, and the *vma1* strain, as expected (Kane, 2006), grows to a lower density then the other strains. At a given K^+ concentration, varying the Cl^- has little effect on final cell density. In the *vma1* strain the data suggest an effect of Cl^- on final cell density, but there is no effect of Cl^- on growth rate of exponential cultures (unpublished data). We conclude that there are at most very small effects of Cl^- on growth, even in the *vma1* strain, which has a lower total cellular Cl^- than the other strains.

High Affinity Cl^- Transporter

Fig. 5 shows that cells grown in a low Cl^- medium have a high affinity pathway for initial $^{36}Cl^-$ influx, and that the apparent Cl^- affinity is increased by low extracellular pH. Cells (*sul1*) were grown in LCAPG medium, and the initial $^{36}Cl^-$ influx was measured in LCAPG plus various concentrations of $^{36}Cl^-$. At extracellular pH 4, the influx vs. extracellular Cl^- is described reasonably well by a hyperbola, with $K_{1/2}$ ~0.018 mM, similar to that observed previously in low Cl^- YNB medium (Jennings et al., 2007).

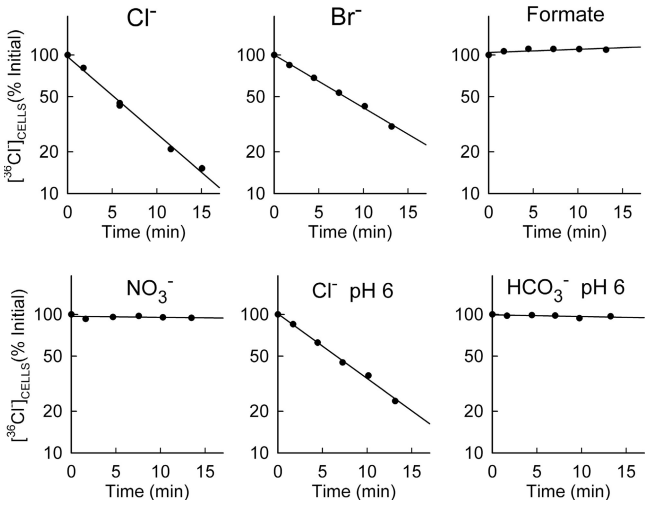

Figure 6. Exchange of cellular $^{36}Cl^-$ for extracellular Cl^- or Br^-, but not for NO_3^-, HCO_3^-, or formate. Cells (*sul1*) were grown in LCAPG and incubated 2 h, 30°C, in fresh LCAPG + 8 μM $Na^{36}Cl$. Cells were centrifuged and resuspended at 30°C in LCAPG plus 2 mM KCl, KBr, K-formate, KNO_3, or $KHCO_3$, as indicated. Cellular $^{36}Cl^-$ as a percent of the value at the first time point is plotted on a logarithmic scale as a function of time. Unless otherwise indicated, the effluxes were performed in media buffered at pH 4.5 with 5 mM K-citrate. The two experiments at pH 6.0 were in media buffered with 5 mM K-citrate and 10 mM bistris. The semi-log plots are fit with straight lines because the time points are too early to reflect the slow component shown in Fig. 5.

At extracellular pH 7, the $K_{1/2}$ is over 10-fold higher than at pH 4.0 (Fig. 5 A). This finding suggests that H^+ is co-transported with Cl^-. However, the V_{max} of the influx is also higher at pH 7.0 than at pH 4, indicating that the effects of pH on the kinetics of Cl^- influx are not simple. Irrespective of the detailed kinetics, the $^{36}Cl^-$ influx at very low extracellular [Cl^-] is lower at pH 7.0 than at pH 4. This is consistent with the fact that the steady-state distribution ratio is also lower at pH 7.0 (Fig. 2 D and Fig. 3 A).

Exchange of $^{36}Cl^-$ for Nonradioactive Cl^-

Cells loaded with $^{36}Cl^-$ in media containing 8 μM $^{36}Cl^-$ and then resuspended in LCAPG containing no added Cl^- can maintain a very high cells:medium $^{36}Cl^-$ ratio for many minutes (Fig. 5 B). There is only very slow loss of $^{36}Cl^-$ despite the fact that the ratio of intracellular to extracellular [$^{36}Cl^-$] is over 100. This gradient does not represent a true steady state and cannot be generated except by resuspending preloaded cells in tracer-free medium. The experiment shows, however, that there is little efflux of $^{36}Cl^-$ in a medium containing $SO_4^=$ (2 mM), $H_2PO_4^-$ (8 mM), and traces of iodide (0.6 μM), molybdate (1 μM), and NO_3^- (4 μM). If 2 mM KCl is added (Fig. 5 C), $^{36}Cl^-$ leaves the cells rapidly, indicating that the cells have a mechanism for $^{36}Cl^-$–Cl^- exchange. The first ~70–80% of the $^{36}Cl^-$ efflux has a time course that is close to a single exponential, but the remaining $^{36}Cl^-$ efflux is slower (Fig. 5 C).

Exchange of $^{36}Cl^-$ for Anions other than Cl^-

Several anions other than Cl^- were tested as possible exchange partners for $^{36}Cl^-$ (Fig. 6). Cells were loaded in LCAPG (8 μM $^{36}Cl^-$) as in Fig. 3 and resuspended in LCAPG containing various anions. The efflux of $^{36}Cl^-$ is almost as rapid in the presence of 2 mM Br^- as in Cl^-. In contrast, the presence of 2 mM NO_3^- or formate does not stimulate efflux of $^{36}Cl^-$.

Because of the ubiquitous presence of HCO_3^-, it is of interest to determine whether HCO_3^- is an exchange partner for Cl^-. It is difficult to study HCO_3^- transport at pH 4.5 because HCO_3^- is converted mainly to CO_2 at this pH. Instead, bicarbonate was added as 4 mM $KHCO_3$ in

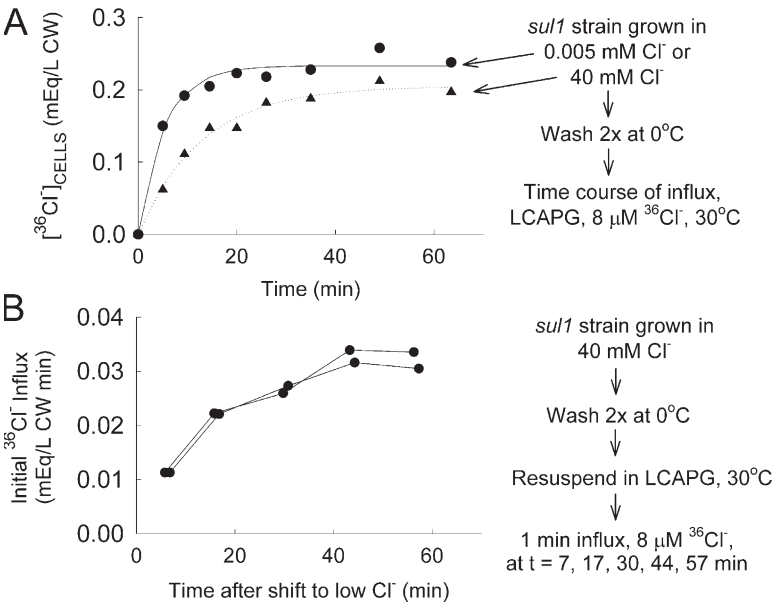

Figure 7. Activation of high affinity Cl^- transport following shift from 40 mM Cl^- to low Cl^- medium. (A) Cells (*sul1*) were grown overnight in LCAPG with no added Cl^- (~0.005 mM; ●) or 40 mM added NaCl (▲), and then washed twice in cold LCAPG, resuspended in LCAPG, pH 4.5, 30°C, with 8 μM $Na^{36}Cl$, and the accumulation of $^{36}Cl^-$ measured over the next 50 min. (B) Cells (*sul1*) were grown in APG + 40 mM NaCl, washed twice in cold LCAPG, and suspended at $t = 0$ in LCAPG, pH 4.5, 30°C. At the indicated times, 8 μM $Na^{36}Cl$ was added and the tracer influx measured for 1 min. Data are from two separate cell preparations.

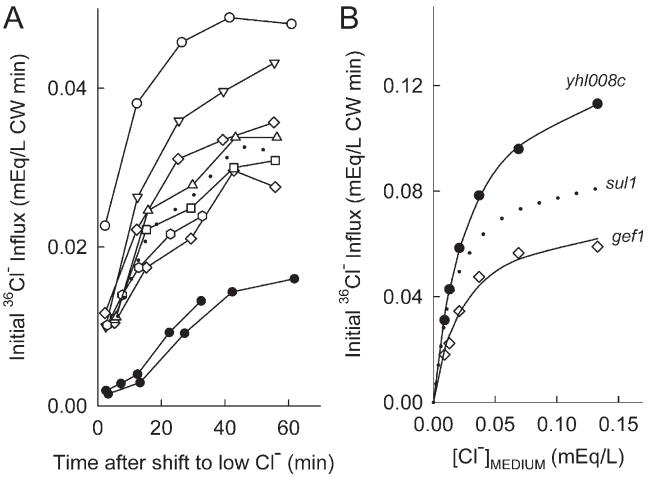

Figure 8. (A) Activation of high affinity Cl^- transport in *yhl008c* strain compared with other deletant strains. Cells were grown in APG containing 40 mM NaCl as in Fig. 5 A, washed in cold LCAPG, and resuspended at $t = 0$ in LCAPG, pH 4.5, 30°C. At the indicated times, 8 μM $Na^{36}Cl$ was added and the tracer influx measured for 1 min. Deletant strains are *trk1* (○), *trk2* (▽), *gef1* (◇), *ypr003c* (△), *bor1* (□), and *yhl008c* (●). The dotted line represents the *sul1* data in Fig. 5 A. (B) High affinity Cl^- flux in strains *yhl008c* (●) and *gef1* (◇) after overnight growth in low Cl^- medium. Cells were prepared and initial influx was measured at pH 4.0 as a function of extracellular Cl^-, as in Fig. 3 A. For comparison, the dotted curve represents the data from Fig. 3 A for control strain *sul1*.

medium buffered at pH 6. At this pH, about half the HCO_3^- is quickly converted to CO_2 (raising the pH slightly in the buffered medium), leaving ~2 mM HCO_3^-. Addition of HCO_3^- does not stimulate efflux of $^{36}Cl^-$ (Fig. 6, bottom right). In this experiment the pCO_2 in the atmosphere above the suspension was not controlled. In separate experiments conducted in a 70% CO_2, 30% O_2 atmosphere, the presence of HCO_3^- again did not stimulate efflux of $^{36}Cl^-$, indicating that there is no evidence for a $^{36}Cl^-$–HCO_3^- exchange process in *S. cerevisiae*. At pH 6.0 the exchange of $^{36}Cl^-$ for extracellular Cl^- is slightly slower than at pH 4.5, but the Cl^-–Cl^- exchange mode of the transporter is clearly functional at extracellular pH 6, and Cl^-–HCO_3^- exchange under the same conditions is undetectable.

Rapid Activation of High Affinity Cl^- Influx following a Shift from High Cl^- to Low Cl^- Medium

In media containing >1 mM Cl^-, *S. cerevisiae* should have no need for a high affinity Cl^- transporter, because the cells exclude rather than accumulate Cl^- (Fig. 1). To determine whether the high affinity Cl^- influx is regulated, cells (*sul1*) were grown in either LCAPG medium or APG containing 40 mM KCl. Cells were then washed in cold LCAPG medium to remove extracellular Cl^-, resuspended in LCAPG plus 8 μM $^{36}Cl^-$ at 30°C, and the accumulation of $^{36}Cl^-$ measured over the next 30 min. As shown in Fig. 7 A, the initial $^{36}Cl^-$ influx is lower in cells grown in 40 mM Cl^-, but the final accumulation of $^{36}Cl^-$ is nearly independent of whether the cells had been grown in high or low Cl^-. This suggests that the high affinity Cl^- influx activates rapidly following exposure of cells to low Cl^- medium.

To examine the time course of activation of the high affinity Cl^- flux in more detail, cells were grown in APG plus 40 mM Cl^-, washed in cold LCAPG, and resuspended at 30°C in LCAPG containing no $^{36}Cl^-$. At various times, 8 μM $^{36}Cl^-$ was added to aliquots of the suspension, and the initial (1 min) flux was measured (Fig. 7 B). The flux is low at early times but activates to a steady-state level with a half time of ~20 min.

The high affinity Cl^- influx activates with roughly the same time course following a shift to LCAPG in all the deletant strains tested except for *yhl008c* (Fig. 8 A). In this strain the 1-min $^{36}Cl^-$ influx is initially much lower than in the other strains and also activates more slowly (Fig. 8 A, solid symbols). In the same strain incubated in low Cl^- medium for several hours, the flux eventually becomes slightly higher than that in the control strain (Fig. 8 B). Therefore, Yhl008cp, an uncharacterized protein with homology to bacterial and *Euglena* formate or nitrite transporters (Suppmann and Sawers, 1994; Deloménie et al., 2007), is definitely not the high affinity Cl^- transporter, but it may have a role in activation of the transporter following a shift to low Cl^- media.

In the experiment in Fig. 8 B, the concentration dependence of $^{36}Cl^-$ influx was also measured in the *gef1* strain, demonstrating that these cells have a high affinity Cl^- influx that is very nearly as large as that of the control strain. Therefore, even though the *gef1* strain has 65% lower steady-state Cl^- contents in LCAPG (Fig. 3), the initial $^{36}Cl^-$ influxes are quite similar to those of the control strain, suggesting that the plasma membrane Cl^- influx in the *gef1* strain is normal and that the lower steady-state levels are the result of reduced organellar sequestration of Cl^-.

DISCUSSION

The work described here has shown that *S. cerevisiae* maintains total cellular Cl^- within a narrow range even if extracellular $[Cl^-]$ is varied >10,000 fold. The Cl^- distributions and fluxes indicate the existence of a Cl^- transporter with a very high apparent affinity for Cl^- at acid extracellular pH. The activation of this transporter depends on a Cl^--sensing mechanism, one component of which may be the formate-nitrite transporter homologue Yhl008cp. The experiments also provide evidence that there are steep Cl^- gradients across the membranes of vacuoles or prevacuolar vesicles in *S. cerevisiae* grown in low Cl^- media. These gradients are dependent on the vacuolar H^+-ATPase, the yeast CLC Gef1p, and, to a lesser extent, the cation–H^+ exchanger Nhx1p. A model based on these findings is in Fig. 9.

Two Sites of Concentrative Cl^- Transport: Plasma Membrane and Vacuole/Prevacuole

The Cl^- distribution data in Fig. 3 indicate that, in a very low Cl^- medium, there are two sites of uphill Cl^- influx in *S. cerevisiae*: the plasma membrane and subcellular membranes of compartments that are acidified by the vacuolar H^+-ATPase. There is clearly a very large effect of deleting VMA1 on the cells:medium [Cl^-] ratio, indicating that a substantial fraction of the cellular Cl^- is sequestered in V-ATPase-dependent compartments. However, even in *vma1* cells, the cells:medium Cl^- ratio is far higher than that expected for electrochemical equilibrium (Fig. 3). These experiments were performed in medium containing arginine rather than NH_3/NH_4^+ as the main nitrogen source. Therefore, the NH_3-dependent vacuolar acidification mechanism (Plant et al., 1999) in cells lacking the vacuolar H^+-ATPase should not be operative. Accordingly, in a low Cl^- medium, the plasma membrane itself is capable of maintaining a cytosolic Cl^- concentration that is much higher than that in the medium.

ATP Dependence

ATP depletion strongly reduces the cellular Cl^- contents of both control and *vma1* strains (Fig. 3 C). Therefore, the concentrative transport of Cl^- across vacuolar (or prevacuolar) membranes and across the plasma membrane is ATP dependent. The ATP dependence of Cl^- accumulation in organelles is clearly expected if the transport requires luminal acidification by the V-ATPase. The fact that ATP depletion also prevents Cl^- accumulation in the *vma1* strain is consistent with the idea that plasma membrane Cl^- influx takes place by H^+–Cl^- cotransport driven by the H^+ gradient.

At extracellular pH 7, the cells:medium [Cl^-] ratio is ~2 in the *vma1* strain in low Cl^- media (Fig. 3). A plasma membrane 1:1 H^+/Cl^- cotransporter could account for this accumulation if the cytosolic pH were at least 7.3. Alternatively, if there is a 2:1 H^+/Cl^- cotransporter, the inward electrochemical H^+ gradient could easily drive cytosolic Cl^- far above the extracellular concentration, because the membrane potential would help drive the influx. Yeast have $SO_4^=$ transporters that cotransport at least two H^+ ions with $SO_4^=$ (Roomans et al., 1979), and it is possible that there is a similar transporter for Cl^-. (It is clear that the high affinity Cl^- transporter is not a $SO_4^=$ transporter, because Cl^- does not exchange for $SO_4^=$, and the high $SO_4^=$ concentration in YNB medium does not compete with Cl^- for high affinity transport sites.)

A Regulated Plasma Membrane Cl^- Transport Process with Very High Apparent Affinity

The initial flux of $^{36}Cl^-$ into *S. cerevisiae* that have been incubated in low Cl^- media is a saturable function of the extracellular [Cl^-], with a $K_{1/2}$ of ~0.02 mM (Fig. 5 A). The true Cl^- affinity of the transporter cannot be inferred from flux measurements (e.g., Fröhlich and Gunn, 1986), but the apparent affinity for Cl^- is at least 100-fold higher than those of mammalian anion exchangers (Gunn et al., 1973; Fröhlich and Gunn, 1986) or cotransporters (Dunham et al., 1980; Isenring et al., 1998). The high affinity Cl^- transporter, denoted HACT in Fig. 9, exhibits a robust Cl^-–Cl^- and Cl^-–Br^- exchange activity, but it does not transport formate, bicarbonate, nitrate, phosphate, or sulfate. The fact that the apparent Cl^- affinity (Fig. 5 A) as well as the steady-state Cl^- distribution ratio (Fig. 3 A) depend on pH suggests that the high affinity Cl^- influx is mediated by a H^+–Cl^- cotransporter (or OH^-/Cl^- exchanger). Whatever the nature of the transport process, it must be able to produce steady-state

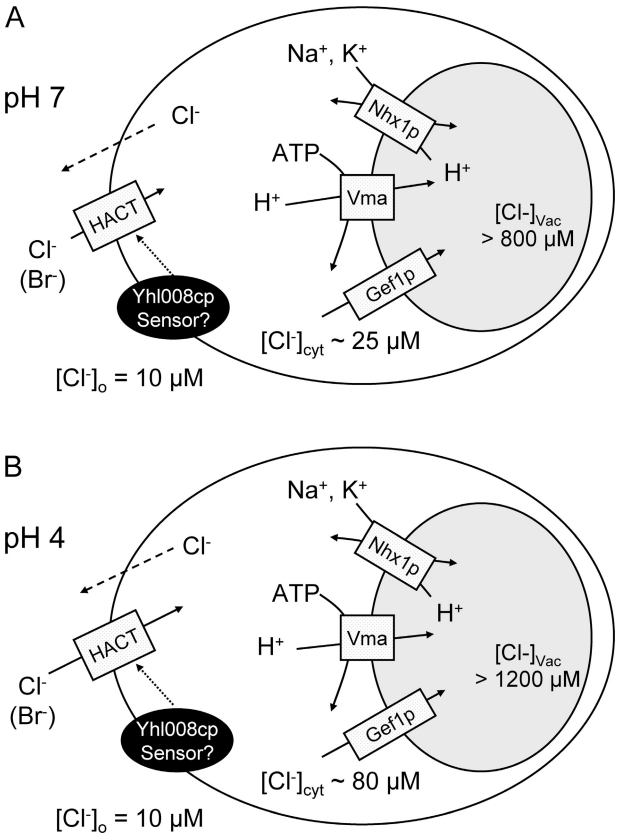

Figure 9. Possible transport mechanisms accounting for the cellular accumulation of Cl^- in pH 7.0 (A) or pH 4.0 (B) media containing 10 µM Cl^-. The elevated cellular Cl^- content is proposed to be a consequence of two processes. (1) Influx across the plasma membrane via a high affinity Cl^- transporter (HACT), which is regulated by a mechanism that includes Yhl008c (depicted here on the plasma membrane, but the actual cellular location is not known). The Cl^- gradient across the plasma membrane is higher at extracellular pH 4.0 than at pH 7, consistent with H^+–Cl^- cotransport across the plasma membrane. The dashed arrow represents downhill efflux of Cl^- through a pathway that is unknown but must be very slow in a low Cl^- medium (Fig. 5 B). (2) Sequestration of Cl^- in the vacuole or prevacuolar compartment by a process that is powered by the V-ATPase (Vma), with Cl^- transport (probably as Cl^-/H^+ exchange; see text) through Gef1p, and the pH gradient modulated by Nhx1p.

cytosolic Cl^- levels that are higher than those in the extracellular medium despite the presence of a very negative membrane potential.

We do not know the molecular identity of the high affinity Cl^- transporter. Several candidate transporters have been ruled out on the basis of the lack of effect of gene deletion on Cl^- accumulation in low Cl media: Bor1p (anion exchanger homologue now known to be a H_3BO_3 transporter [Takano et al., 2002; Jennings et al., 2007]), YBR235w (Cl^--coupled cotransporter homologue), and YPR003c (Pendrin, DRA homologue). We did additional studies on YBR235w, including transport measurements in the deletant strain, which showed that the high affinity flux is present, as well as overexpression of the protein, without an increase in Cl^- transport (unpublished data). The functions of Ybr235wp and Ypr003cp remain unknown.

Regulation of the High Affinity Cl^- Influx by a Mechanism Involving Yhl008cp

The high affinity Cl^- influx is activated relatively rapidly following a shift from a high Cl^- to a low Cl^- medium (Figs. 7 and 8). It is not clear whether the change in extracellular Cl^- is sensed directly, or whether the shift from high to low Cl^- medium causes a drop in cytosolic Cl^- that then triggers the activation of Cl^- influx. In either case, Yhl008cp may be involved in either sensing low Cl^- or transmitting the signal that eventually activates the transporter. It is not known to what extent transcriptional, translational, and/or posttranslational mechanisms contribute to the activation of the transporter.

The sequence of Yhl008cp is homologous to that of bacterial formate transporters, but the protein has been shown not to be a monocarboxylate transporter (Makuc et al., 2001). The only other experimental information on its properties is that the GFP fusion with Yhl008cp gives a low signal, possibly localized to the vacuole (Huh et al., 2003). Yhl008cp may be another example of a protein that is in a family of transporters but is actually a sensor (Forsberg and Ljungdahl, 2001); however, its true role in Cl^- regulation remains to be determined.

Evidence for Large Subcellular Transmembrane Cl^- Gradients

The fact that deletion of VMA1, GEF1, or NHX1 causes a decrease in cellular Cl^- is strong evidence that, at least in low Cl^- media, a substantial fraction of cellular Cl^- is in a subcellular compartment that is acidified by the V-type H^+-ATPase. Although it is widely accepted that organellar acidification takes place by way of an ATP-driven H^+ influx and a parallel influx of Cl^- (Wada and Ohsumi, 1992; Forgac, 1999; Jentsch et al., 2002), there have been few estimates of the sizes of Cl^- gradients across organellar membranes in intact cells. The cells:medium $^{36}Cl^-$ distribution ratios we have determined in control and *vma1* strains allow a rough estimate of the Cl^- distribution between vacuole and cytosol. At extracellular pH 7.0 in media containing 10 μM Cl^-, the cells:medium Cl^- ratio is 20 in the control strain and ~2 in the *vma1* strain (Fig. 3). The simplest interpretation of this finding is that in the control strain under these conditions, ~90% of the cellular Cl^- at pH 7.0 is in a compartment that is dependent on Vma1p (Fig. 9). If this compartment is the vacuole, which occupies ~25% of cell volume (Wiemken and Dürr, 1974), then the concentration of Cl^- in the vacuole must be nearly 30 times as high as that in the cytosol. If Cl^- is actually concentrated more in prevacuolar vesicles than in the vacuole itself, then the concentration of Cl^- in the vesicles must be even higher, because these vesicles occupy a smaller fraction of the cell volume. A 30-fold Cl^- gradient across the vacuolar membrane is much steeper than has been reported for the alga *Eremosphaera viridis*, where there is an approximately threefold gradient (6.2 to 2.2 mM) of Cl^- between the vacuole and the cytosol (Bethmann et al., 1995). However, high inorganic anion gradients across vacuolar membranes are not unprecedented; there is a 30-fold NO_3^- gradient across the *Arabidopsis thaliana* vacuolar membrane (Cookson et al., 2005). In this context it should be noted that, given the complexity of vacuolar contents, our estimate of total subcellular Cl^- does not allow an estimate of electrochemical activity gradients across subcellular membranes.

Is Gef1p a Conductive Channel or a Stoichiometric Cl^-/H^+ Exchanger?

Gef1p forms conductive channels when reconstituted in lipid bilayers (Flis et al., 2002). However, these channels were observed at Cl^- concentrations roughly 1,000-fold higher than those in yeast cytosol under the present conditions, and it is possible that Gef1p behaves differently in high vs. low Cl^- solutions. The vacuolar membrane potential in higher plants and in *Neurospora crassa* is roughly 30 mV (lumen positive) (Bertl and Slayman, 1990; De Angeli et al., 2006). If the vacuolar membrane potential is of this magnitude in *S. cerevisiae*, then Cl^- transport through a conductive channel can account for only an approximately threefold ratio of vacuolar to cytosolic $[Cl^-]$.

On the basis of the presence of a glutamate residue in the position of Glu203 in bacterial CLC-ec1, Gef1p is expected to be a stoichiometric Cl^-/H^+ exchanger rather than a conductive Cl^- channel (Accardi et al., 2005). The equilibrium condition for Cl^-/H^+ exchange across a membrane with a membrane potential of −30 mV (cytoplasm negative relative to lumen; see Bertl et al., 1992) is

$$[Cl^-]^n_{Cyt} / [Cl^-]^n_{Vac} = ([H^+]_{Cyt} / [H^+]_{Vac}) * e^{[(n+1)*(-30F/RT)]}, \quad (1)$$

where n is the number of Cl^- ions transported per H^+ ion, the subscripts refer to cytosolic and vacuolar

concentrations, and F, R, and T have their usual meanings. If the vacuolar luminal pH is 6.2 (Preston et al., 1989) and the cytosolic pH is 7.2, then a Cl^- gradient of ~20-fold could be generated by a 2:1 Cl^-/H^+ exchanger. A 1:1 exchanger could produce an ~100-fold gradient under these conditions. Our data do not allow a distinction to be made between a 2:1 and 1:1 exchanger, but our data are much more consistent with Gef1p acting as a Cl^-/H^+ exchanger than as a Cl^- channel.

A recent paper (López-Rodríguez et al., 2007) concluded that, in addition to being an organellar Cl^- transporter, Gef1p also mediates Cl^- efflux across the plasma membrane in yeast grown in YPGE medium. The Cl^- concentration in medium containing 1% yeast extract and 2% peptone is 350 mg/liter, or 10 mM, according to Dionex Application Note 123 (http://www1.dionex.com/en-us/webdocs/4082_AN123_07May07_LPN1030_2.pdf). Our data do not address the possibility that Gef1p mediates plasma membrane Cl^- efflux in this medium. However, in the low-Cl^- media in which all the transport experiments described here were performed, if Gef1p (either as a conductive channel or a Cl^-/H^+ exchanger) mediates a Cl^- efflux across the plasma membrane, the efflux is overridden by the high affinity, concentrative Cl^- influx that we have described. Certainly Gef1p is not responsible for the high affinity Cl^- influx.

Nonexponential Time Course of $^{36}Cl^-$–Cl^- Exchange

The time course of $^{36}Cl^-$ -Cl^- exchange in a medium containing 2 mM Cl^- is not distinguishable from a single exponential for the first 70–80% of the efflux (Fig. 5 C and Fig. 6), but the remainder of the efflux is slower (Fig. 5 C). This slower component could represent exchange with a subcellular compartment, or it could be the result of progressive inactivation of the transporter following exposure to 2 mM Cl^-. These possibilities are difficult to distinguish, and a systematic study of the time course was not performed.

Plasma Membrane Conductive Cl^- Permeability

Fig. 2 C shows that, in the long-term presence of very high outward K^+ gradients, the ability of *S. cerevisiae* to accumulate Cl^- in a low Cl^- medium is diminished. However, the K^+ gradient does not have a major effect on Cl^- accumulation during a 2-h incubation (Fig. 2 D). Therefore, in a low Cl^- medium, the conductive Cl^- flux must not be very large; otherwise, the cells would not have a higher Cl^- concentration than the medium. Nonetheless, there must be a finite conductive Cl^- permeability in the plasma membrane, and this conductive permeability may become a major determinant of the cellular Cl^- distribution at high extracellular Cl^-, when there is a large inward gradient (Fig. 1). It is not know whether Gef1p (López-Rodríguez et al., 2007), Trk1p, Trk2p (Rivetta et al., 2005), or some other protein is responsible for the conductive Cl^- flux.

Could Yeast Live and Grow without Any Cl^- At All?

The finding that yeast grown in low Cl^- media retain Cl^- at concentrations much higher than those in the medium suggests that yeast require Cl^- for growth. However, it is possible that the Cl^- regulation that we have observed serves a nonessential purpose (at least under the conditions tested thus far) and that yeast do not have an absolute Cl^- requirement for growth. Even in media that are nominally Cl^- free, there are traces of Cl^-, and we have not tried to lower the Cl^- concentrations of media below ~2 μM. The purpose of the growth studies was to see whether there was an obvious effect of Cl^- on growth in the range of Cl^- concentrations in which we measured the Cl^- distribution, and not to perform a comprehensive study of the effects of extremely low Cl^- on yeast growth. Such a study would require systematic variations in the carbon source, iron, copper, Cl^-, and pH, as well as deletion and overexpression of genes involved in iron and copper metabolism (Davis-Kaplan et al., 1998).

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