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The yeast CLC chloride channel functions in cation homeostasis

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ABSTRACT **A defect in the yeast *GEF1* gene, a CLC chloride channel homolog leads to an iron requirement and cation sensitivity. The iron requirement is due to a failure to load Cu^{2+} onto a component of the iron uptake system, Fet3. This process, which requires both Gef1 and the Menkes disease Cu^{2+}-ATPase yeast homolog Ccc2, occurs in late- or post-Golgi vesicles, where Gef1 and Ccc2 are localized. The defects of *gef1* mutants can be suppressed by the introduction of *Torpedo marmorata CLC-0* or *Arabidopsis thaliana CLC-c* and *-d* chloride channel genes. The functions of Gef1 in cation homeostasis provide clues to the understanding of diseases caused by chloride channel mutations in humans and cation toxicity in plants.**

The yeast genome encodes only one ORF, *GEF1*, that has amino acid homology to the CLC voltage-gated chloride channel superfamily (1). The prototype of this superfamily is the *Torpedo marmorata CLC-0*, which has been characterized by electrochemical measurements to be a *bona fide* chloride channel (2–4). Surprisingly, deletion of the *GEF1* gene leads to an iron requirement for growth on medium containing nonfermentable carbon sources (5). The phenotype of the *gef1* mutant was puzzling: Why should strains lacking a putative chloride channel have an iron requirement?

Yeast has both low-affinity and high-affinity iron uptake systems (6, 7). A key component of the high-affinity uptake system Fet3 oxidase requires copper for its activity. The loading of copper onto Fet3 takes place in post-Golgi vesicles and requires the copper-transporting ATPase Ccc2 (8, 9). Numerous intracellular organelles, including clathrin-coated vesicles, endosomes, lysosomes, synaptic vesicles, and Golgi membranes have acidic interiors. This acidification is mediated by a proton translocating electrogenic ATPase. There is evidence for a requirement of parallel movement of chloride to maintain net electroneutrality (10, 11). The homology of Gef1 to the CLC chloride channel superfamily led us to explore the possibility that Gef1 played a role with Ccc2 in the loading of copper into the Fet3 oxidase.

Herein, we describe two roles of Gef1 in yeast—its participation in the high-affinity iron transport system and its function in cation detoxification. Our results suggest that Gef1 is required as an anion channel to provide the counterbalancing charge that will permit cation compartmentalization into organelles or vesicles with acidic interiors.

MATERIALS AND METHODS

Yeast Strains and Plasmids. All strains used are isogenic to W303 (*ura3–1 can1–100 leu2–3, 112 trp1–1 his3–11, 15*). The following plasmids were used to construct the deletions of *GEF1*, *CCC2*, *FET3*, and *TFP1* genes in both mating types. pRG52 (Δ*gef1*::*HIS3*), pRG53 (Δ*gef1*::*LEU2*), pDY118(5) (Δ*ccc2*::*LEU2*), pD*FET3*(5) (Δ*fet3*::*URA3*), and pRG86 (Δ*tfp1*::*LEU2*). Double mutants *gef1 ccc2*, *gef1 fet3*, and *gef1 tfp1* were obtained by crossing the desired single mutant strains and identifying the double mutant among the meiotic progeny by the appropriate combination of disruption markers. The *gef1/gef1* homozygous diploid was generated by crossing haploid *gef1* mutants of opposite mating types. The strain RGY250 used for histochemical analysis was generated via crossing strains RGY84(Δ*gef1*::*LEU2*) with RGY145 (Δ*ccc2*::*LEU2*) and transformed with plasmids pRG151 and pDY219 (5). The plasmid pRG151 was constructed by introducing in-frame a 0.7-kb *Bam*HI fragment containing the *GFP* ORF into the carboxyl terminus of the *GEF1* ORF (the last 27 amino acids were deleted). This construct contains the complete promoter region of *GEF1*. The backbone plasmid is pRS426 (12). *CLCs* constructs were made as follows. Plasmid pRG120 contains a 2.3-kb *Xho*I fragment with the *CLC-0* ORF lacking the last 4 amino acids (provided by Chris Miller, Brandeis University, Waltham, MA). It is cloned into pRS1024 (13), a *PMA1*-based expression vector. PCR products were generated from *At CLC a, c*, and *d* cDNAs (provided by Klaus Steinmeyer via Mirko Hechenberger, Hamburg University, Germany) with the added restriction sites *Pst*I and *Sal*I. Forward primers were 5′-CCCTGCAGATGGATGAAGATGGAAACTTGCAG-3′, 5′-GGCTGCAGATGGATGATCGGCACGAAGGA-3′, and 5′-GGCTGCAGATGTTATCGAATCATCTCCAG-3′. Reverse primers were 5′-CCGTCGACTCTAGCTTTTCCA-CTTTTGTG-3′, 5′-GGGTCGACCCTTATTCACTTGAG-GGGATC-3′, and 5′-GGGTCGACACCTAAAAGATCGT-CTAGAAGCGG-3′. The PCR products digested with *Pst*I and *Sal*I were cloned into pAD4 (14), an *ADH1*-based expression vector, generating plasmids pRG175, pRG176, and pRG178.

Oxidase Activity and Sensitivity of Fet3 to Proteolytic Digestion. Fet3 oxidase activity was detected in undenaturated samples, by using 7.5% polyacrylamide gels and *o*-dianisidine dihydrochloride as substrate as described (15). Gels for oxidase assays were developed in humid atmosphere at 30°C for 3 days. Sodium azide (10 mM) was included in the oxidase buffer to diminish background signal. Fet3 protease-accessibility was determined as described (16).

Iron Uptake. High-affinity iron uptake was measured with ^{55}Fe as described (17).

Immunofluorescence. Strain RGY250 was grown in YPD added with 1 mM ferrozine to midlogarithmic phase and fixed with 3.7% formaldehyde for 1 h. Spheroplast formation, permeabilization, wash, and incubation with antibodies was done as described by Pringle *et al.* (18). HA.11 mouse mAb (Berkeley Antibody, Richmond, CA) was used as first antibody. Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch) was used as secondary antibody. 4′,6-Diamidino-2-phenylindole (Sigma) was added to mounting medium to stain mitochondrial and nuclear DNA.

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Abbreviation: HA, hemagglutinin.

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RESULTS AND DISCUSSION

The *gef1* Mutant Has a Defect in Copper Metabolism. Yeast strains containing the *gef1* mutation are unable to grow on nonfermentable carbon sources in the absence of high iron concentrations (5). Although there is considerable variation in this phenotype among various strain backgrounds, the addition of ferrozine, an iron chelator, to the medium prevents the growth of *gef1* mutant strains in all genetic backgrounds tested. We have found that the requirement for iron in the *gef1* mutant can be overcome by the addition of copper to the growth medium. This phenotype is similar to that of the *ccc2* mutant, which is defective in a copper transporting ATPase. The explanation for this iron–copper connection for Ccc2 is that copper is required for the activity of Fet3 iron oxidase, which is necessary for the high-affinity iron transport (8). The connection between iron and copper suggests that the iron defect of *gef1* like that of *ccc2* results from a defect in copper metabolism (Fig. 1*A*). Interestingly, strains lacking Tfp1, the 69-kDa subunit of the vacuolar ATPase, are also incapable of growing on low-iron medium containing nonfermentable carbon sources and are rescued by addition of copper (Fig. 1*A*).

The g*ef1*, *ccc2*, and *fet3* mutants also fail to grow in minimal medium buffered at pH 7, a condition where wild-type strains grow well. The addition of copper to the medium at pH 7 permits the growth of *gef1* and *ccc2* mutants but not of *fet3* mutants (Fig. 1*B*). Thus, the iron requirement on nonfermentable carbon sources and the inability to grow at pH 7 appear to result from the same cause—a deficiency in loading copper onto Fet3 with the resulting loss of the high-affinity iron uptake system. The Fet3 oxidase would be required for growth at high pH and not at low pH because iron solubility decreases dramatically with increasing pH (20). As expected by this interpretation, the growth defects of *fet3* mutants is suppressed by higher concentrations of iron, which could now be taken up by the Fet3-independent low-affinity iron transport system (6, 7). As predicted, the addition of iron to the medium at pH 7 restores the growth of *gef1* mutants with either glucose or glycerol/ethanol as carbon sources.

The *gef1* Mutant Shows Loss of Fet3-Associated Oxidase Activity and High Affinity Iron Uptake. Our genetic and physiological analyses hinted that the low-iron-sensitive phenotype of the *gef1* mutant results from a defect in the loading of Cu^{2+} onto Fet3. Direct biochemical measurements reveal that both *gef1* and *ccc2* mutants have a defect in Fet3-associated oxidase activity. Addition of copper to the growth medium restores the Fet3 oxidase activity in both mutants (Fig. 2*A*). Moreover, addition of copper to inactive Fet3 apoprotein obtained from *gef1* cells restores Fet3 oxidase activity (Fig. 2*A*). The Fet3-associated oxidase activity of wild type, *gef1*, and *ccc2* is sensitive to proteolysis with proteinase K, indicating that Fet3 apoprotein is present at the cell surface in all three strains (Fig. 2*B*). High-affinity iron uptake is absent when *gef1* or *ccc2* cells are grown without copper, but addition of copper to the growth medium restores high-affinity iron transport in both mutants (Fig. 2*C*), a result consistent with the view that the iron requirement of *gef* mutants results from a defect in copper transport.

***Gef1* and *Ccc2* Colocalize When Grown on Low Iron Medium.** The functional connection between the putative chloride channel and copper transport suggested that they may be localized to similar compartments. Previous results have shown that Ccc2 localizes to late- or post-Golgi vesicles where the copper loading of the Fet3 apoprotein takes place (8). The intracellular localization of Gef1 and Ccc2 was determined by visualization of Gef1 tagged with GFP and Ccc2 tagged with the influenza hemagglutinin (HA) epitope. Epifluorecensce analysis of cells expressing these functional fusions with a CELLscan System (21) shows that Gef1 and Ccc2 indeed colocalize. Colocalization of the same Gef1 and Ccc2 signals upon rotation of the image by 90° provides strong support for the conclusion that the two proteins are in the same compartment (Fig. 3).

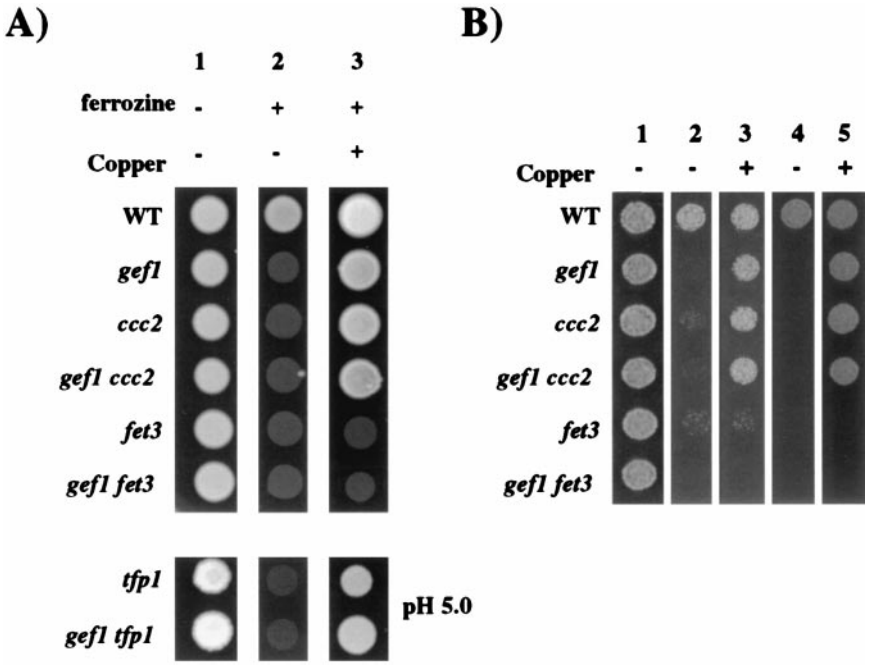

FIG. 1. (*A*) Growth of *gef1*, *ccc2*, *fet3*, and *tfp1* mutants in low-iron-containing medium. Approximately 10^5 cells of the indicated strains were grown for 3 days on YPD (1% yeast extract/2% Peptone/2% dextrose; Difco) (lane 1), YPEG (2% ethanol/glycerol) supplemented with 1 mM ferrozine (Fluka) (lanes 2 and 3) and with 0.1 mM $CuSO_4$ (Sigma) as indicated. For the *tfp1* mutants, the conditions were as above, but the medium was buffered with 50 mM Mes Tris at pH 5 (Sigma). (*B*) Growth of *gef1*, *ccc2*, and *fet3* mutants in minimal medium at pH 7. Approximately 10^4 cells of the indicated strains were grown for 1 day on YPD (Difco) (lane 1), SD (Difco; synthetic medium with 2% dextrose) (19) buffered with 50 mM Mes Tris at pH 7 (Sigma) (lanes 2 and 3), and SGE (synthetic medium with 2% glycerol and ethanol) buffered with 50 mM Mes Tris at pH 7 (Sigma) (lanes 4 and 5). $CuSO_4$ (Sigma) was added to 0.1 mM to lanes 3 and 5.

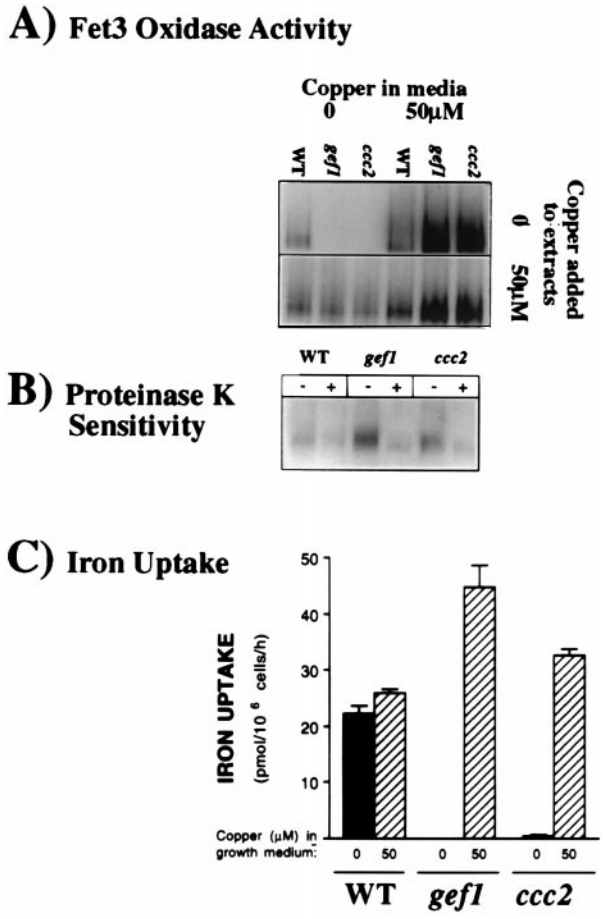

FIG. 2. Analysis of Fet3p function in gef1 cells. The copper status of Fet3p was characterized in congenic wild-type, *gef1*, and *ccc2* cells by using assays of oxidase activity and high-affinity cellular iron uptake, previously shown to correlate with the presence of copper-loaded (holo-) Fet3p (15–17). (*A*) Oxidase assay. Cells were grown in YPD medium alone or with copper added. Duplicate samples were homogenized by using procedures that either preserve Fet3p in its apoprotein state or reconstitute Fet3p as a holoprotein, depending on the copper concentration *in vitro* (16). Solubilized membrane extracts (40 μg per lane) were separated in a nondenaturing gel and analyzed *in situ* for oxidase activity with *o*-dianisidine dihydrochloride as substrate. (*B*) Sensitivity of Fet3p to proteolytic digestion in intact cells. Cells grown in basal YPD medium as in *A* were divided into equal aliquots and either treated (+) with enzymes, zymolyase 100T and proteinase K or not (−). A proteinase inhibitor then was added to both treated and untreated samples, and these samples were chilled. The enzymes were also added to the untreated sample to make a control. This digestion protocol distinguishes cell surface from internal forms of Fet3p (15). Oxidase gels were then prepared as *A*, with copper added to membrane extracts to reconstitute holo-Fet3p. (*C*) High-affinity iron uptake assay. Uptake of radioactive iron was measured in cells grown as in *A* with procedures specific for high-affinity iron uptake (17).

Suppression of *gef1* Phenotypes by Heterologous CLC Members. Remarkably, the growth defect of the yeast *gef1* mutant at pH 7 is suppressed in *gef1* strains that express the ray *Torpedo marmorata CLC-0* gene (Fig. 4*A*). These results are informative because the function of the Torpedo *CLC-0* as a voltage-gated chloride channel has been established by direct electrochemical measurements (1–4). The yeast Gef1 protein was previously inferred to be a chloride channel because of its homology to the *CLC* superfamily and because another putative member of the *CLCs* (*At CLC-d)* suppressed the respiratory phenotype (22).

The *Torpedo* protein also suppresses other disparate phenotypes of the *gef1* mutant. In addition to their iron requirement, the *gef1* mutant strains manifest sensitivity to a number of salts: NaCl, tetramethylammonium chloride and $MnCl_2$. Two *Arabidopsis thaliana* chloride channel superfamily members, *AT CLC-c* and *-d*, were also able to suppress the pH-induced phenotype (Fig. 4*A*) and the salt sensitivity of *gef1* diploids (Fig. 4*B*). Because 2 mM $MnCl_2$ inhibits the growth of the *gef1* mutant (Fig. 4*B*) but 1 M NaCl does not, the toxicity of these salts in *gef1* strains appears to be related to the nature and concentration of the cation and not to that of the anion. In agreement with this observation, we found that 2 mM $MnSO_4$ was as toxic as 2 mM $MnCl_2$ (Fig. 4*B*).

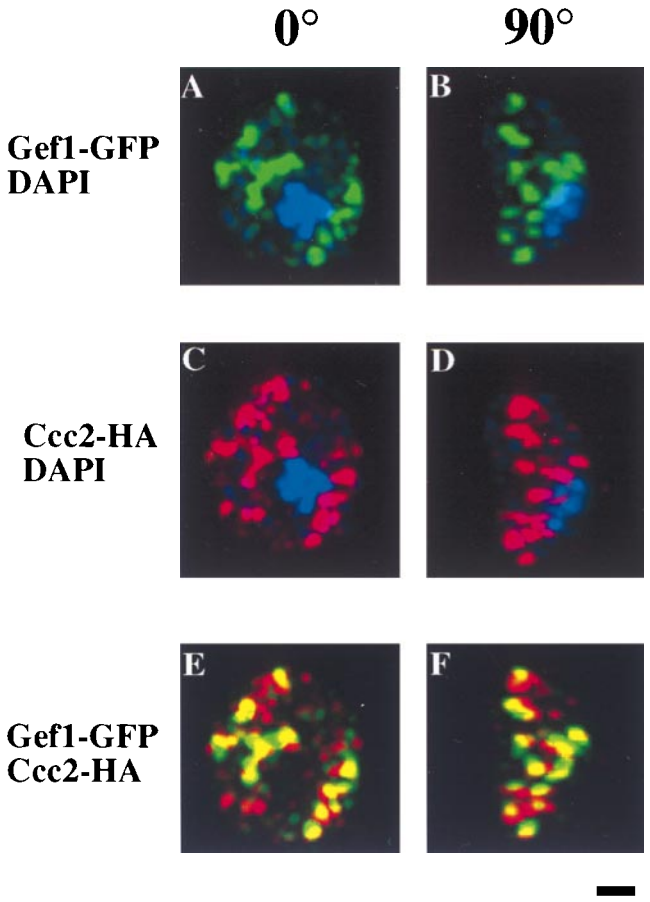

FIG. 3. Distribution of fluorescence in *gef1 ccc2* cells transformed with two constructs: a *GEF1-GFP* fusion and a *CCC2*-$(HA)_3$ tagged fusion. The strain RGY250 (*gef1 ccc2*) was transformed with plasmids pRG151;*GEF1-GFP* and pDY219;*CCC2*-$(HA)_3$. Transformants were grown in iron-depleted medium, fixed, and stained with antibodies to HA epitope and 4′,6-diamidino-2-phenylindole. Cells were viewed by charge-coupled device microscopy and sectioned by using SCANALYTICS (Billerica, MA) (21). (Bar = 1 mm.) (*A*) Image obtained from Gef1-GFP fluorescence. (*B*) The same image rotated 90°. (*C*) Image obtained from inmunodetection of Ccc2-$(HA)_3$. (*D*) The same image rotated 90°. (*E*) Image obtained from the superimposition of *A* and *C*. (*F*) Image obtained from the superimposition of *B* and *D*. 4′,6-Diamidino-2-phenylindole was omitted from images *E* and *F*.

Model for Gef1 Function. High-affinity iron uptake in yeast is mediated by the Fet3–Ftr1 oxidase–permease complex, which requires the Menkes disease Cu^{2+}-ATPase homolog for the loading of Cu^{2+} onto Fet3 oxidase (8). Our studies identify two additional proteins necessary for Fet3 activity: the Tfp1 subunit of the vacuolar H^+-ATPase and the yeast CLC chloride channel Gef1. The requirement for the triad of factors (Ccc2, Tfp1, and Gef1) can be explained by the following model: The loading of Cu^{2+} onto Fet3 apoprotein requires an acidified environment, the lumen of the late- or post-Golgi vesicles. Cu^{2+} transport into the vesicles is accomplished by

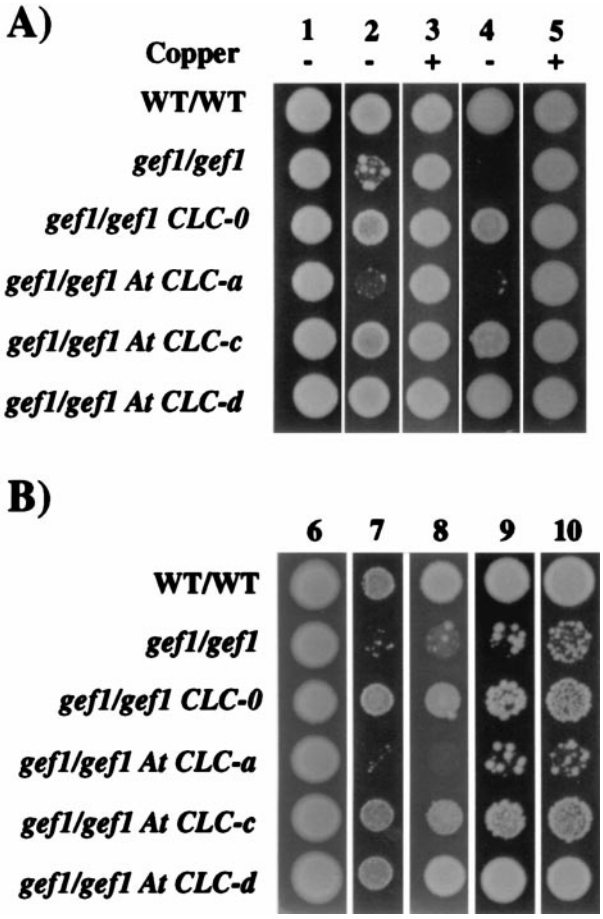

FIG. 4. Expression of *Torpedo* and *Arabidopsis CLC* genes in *gef1/gef1* diploids. (*A*) Vector pRS1024 (13) was introduced into wild-type and *gef1/gef1* diploids. Plasmids pRG120, pRG175, pRG176, and pRG178 were introduced into the *gef1/gef1* diploid. Approximately 10^4 cells of the indicated strains were grown for 4 days in YPD (lane 1), SD buffered with 50 mM Mes Tris (pH 7; Sigma) (lanes 2 and 3), and SGE buffered with 50 mM Mes Tris (pH 7) (lanes4 and 5). $CuSO_4$ (Sigma) at 0.1 mM was added to lanes 3 and 5. Diploids were used because they gave a better discrimination of phenotypes. (*B*) Approximately 10^5 cells of the indicated strains were grown for 3 days in YPD (lane 6), 7 days in YPD supplemented with 1.75 M NaCl (lane 7) (Sigma), 3 days in YPD supplemented with 0.45 M tetramethylammonium chloride (lane 8) (Sigma), 3 days in YPD supplemented with 2 mM $MnCl_2$ (lane 9) (Sigma), and 3 days in YPD supplemented with 2 mM $MnSO_4$ (lane 10) (Sigma).

Ccc2. Acidification is mediated by the vacuolar H^+-ATPase. Both of these transport processes will increase the membrane potential of the vesicles thereby impeding further transport of the cations. The compensatory transport of an anion via Gef1 will promote electroneutrality allowing both the acidification of the lumen and the delivery of Cu^{2+} onto Fet3 (Fig. 5). The increased cation sensitivity of *gef1* mutants (Fig. 4*B*) can as well be explained by this model: Sequestration of toxic cations should also require a counterbalancing anion transport. This detoxification event could take place in the vacuole. *In vitro* studies with purified vacuole vesicles have shown that H^+/ion antiport is the principal mechanism of vacuolar uptake of divalent cations such as Mn^{+2} (23). Nass *et al.* (24) recently reported a Na^+/H^+ exchanger required for intracellular sodium sequestration that appears to be in the yeast vacuole.

The easily scored phenotypes of the yeast *gef1* mutant provide a facile system to study the basic functions of anion transport and its relationship to cation homeostasis. Future work should provide important insights in human diseases that affect ion balance, such as the Menkes disease, the Bartter's

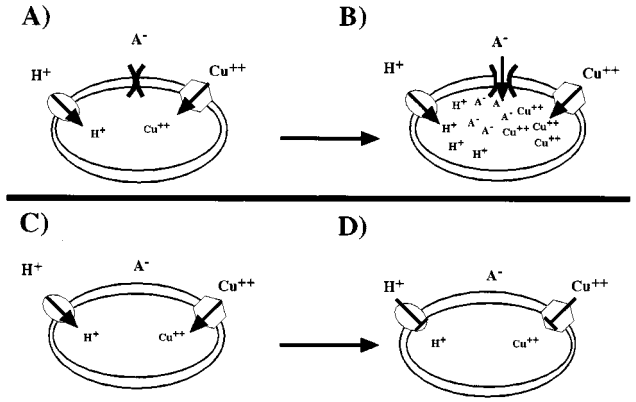

FIG. 5. Model for the role of Gef1 in the regulation of intravesicular acidic pH and transmembrane voltage. (*A* and *B*) Wild-type. (*A*) Protons H^+ and copper Cu^{2+} are actively transported into the lumen of post-Golgi vesicles through the Vma1 and Ccc2 ATPases. Anions A^- line up at the Gef1 anion channel represented at its closed state. (*B*) The Gef1 channel opens when a certain voltage is reached in the lumen of the vesicle and allows the entrance of anions that will reduce the membrane potential and facilitate further H^+ and Cu^{2+} transport. (*C* and *D*) *gef1* mutant scenario. (*C*) Same as in *A* but in the Gef1 anion-channel deficient mutant. (*D*) The intravesicular voltage will reach high values and no further H^+ and Cu^{2+} transport will take place.

syndrome type III, and several inherited kidney stone diseases (25–27). This basic knowledge should also shed light on the important agricultural problem of salt stress. Sodium compartmentalization, a well-documented adaptative response to salt stress in higher plants, depends upon proper anion cotransport (28).

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