

Ectopic Potassium Uptake in *trk1 trk2* Mutants of *Saccharomyces cerevisiae* Correlates with a Highly Hyperpolarized Membrane Potential*

(Received for publication, February 5, 1998, and in revised form, March 31, 1998)

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Null *trk1 trk2* mutants of *Saccharomyces cerevisiae* exhibit a low-affinity uptake of K^+ and Rb^+ . We show that this low-affinity Rb^+ uptake is mediated by several independent transporters, and that *trk1Δ* cells and especially *trk1Δ trk2Δ* cells are highly hyperpolarized. Differences in the membrane potentials were assessed for sensitivity to hygromycin B and by flow cytometric analyses of cellular DiOC₆(3) fluorescence. On the basis of the latter analyses, it is proposed that Trk1p and Trk2p are involved in the control of the membrane potential, preventing excessive hyperpolarizations. K^+ starvation and nitrogen starvation hyperpolarize both *TRK1 TRK2* and *trk1Δ trk2Δ* cells, thus suggesting that other proteins, in addition to Trk1p and Trk2p, participate in the control of the membrane potential. The HAK1 K^+ transporter from *Schwanniomyces occidentalis* suppresses the K^+ -defective transport of *trk1Δ trk2Δ* cells but not the high hyperpolarization, and the HKT1 K^+ transporter from wheat suppresses both defects, in the presence of Na^+ . We discuss the mechanism involved in the control of the membrane potential by Trk1p and Trk2p and the causal relationship between the high membrane potential (negative inside) of *trk1Δ trk2Δ* cells and its ectopic transport of alkali cations.

Potassium is an indispensable element, which is accumulated against large transmembrane concentration gradients in cells living in diluted environments. Probably because of the central role of this element in all forms of life, different mechanisms mediating K^+ uptake have evolved in different types of cells. In plants and fungi, the cellular uptake of K^+ is probably always an electrophoretic process, which takes place in response to the membrane potential created by the H^+ -pump ATPase (1, 2), as described for *Neurospora crassa* (3).

Among all eucaryotic non-animal cells, the maximum information about potassium transport has been obtained in *Saccharomyces cerevisiae*. In this fungus, the biochemistry (4–7) and the genetics of K^+ transport (8–11) and H^+ -pump ATPase (12) have been extensively studied. The *TRK1* gene of *S. cerevisiae* encodes a notable K^+ transporter, which is adapted to

provide the required amount of K^+ in many different nutritional conditions. To perform this function, this transporter changes its K_m , which shows values in the millimolar range in cells growing at millimolar concentrations of K^+ , and as low as 20–30 μM in K^+ -starved cells (5, 8, 13). The V_{max} is also variable, depending on the cellular pH and K^+ content of the cells (5, 6). A second gene, *TRK2* (10, 11), encodes a second K^+ transporter structurally related to Trk1p (11). However, unlike Trk1p, Trk2p shows a very low Rb^+ influx V_{max} in *trk1Δ* cells with a low K^+ content, and the influx is undetectable in *TRK1* cells or in *trk1Δ* cells with a normal K^+ content (7). Using a *trk1Δ* strain overexpressing Trk2p, it has been found that the affinity of the TRK2 transporter for K^+ and Rb^+ is also regulated by the K^+ content of the cell, and that the K_m in K^+ -starved cells is only slightly higher than that of TRK1 (7). Because the V_{max} of TRK2 is very low in comparison to that of TRK1 and the K_m is higher than the K_m of TRK1, the function of Trk2p seems to be superfluous as an independent K^+ transporter.

Deletion of the *TRK1* and *TRK2* genes results in cells that grow normally at high concentrations of K^+ , and slowly at relatively low concentrations, exhibiting a Rb^+ influx kinetics with a K_m of 60 mM and a V_{max} of 9 and 16 $nmol\ mg^{-1}\ min^{-1}$, in the two strains studied so far (7). This low-affinity Rb^+ uptake of *trk1Δ trk2Δ* cells also constitutes the major pathway for Rb^+ uptake in *trk1Δ TRK2* cells, in which Trk2p mediates a minor pathway, as already mentioned. However, in contrast with the relevance of the low-affinity K^+ uptake in *trk1Δ TRK2* and *trk1Δ trk2Δ* cells, this uptake has never been detected in wild strains (4, 5). Because the K_m of the low-affinity Rb^+ influx is much higher than the K_m of TRK1 and the V_{max} not insignificant in comparison to the V_{max} of TRK1, it can be concluded that the low-affinity Rb^+ uptake has not been detected in *TRK1* cells because it does not exist in these cells.

Therefore, two important questions remain unanswered about K^+ transport in *S. cerevisiae*, the function of Trk2p and the identity, and consequently the function, of the transporter mediating the low-affinity uptake of K^+ and Rb^+ observed in *trk1Δ* cells. For the latter question two hypotheses have been put forward, that this transport takes place mediated by several non- K^+ transporters, which harbor intrinsic K^+ transport capabilities (14–16), and that it is mediated by a part of the normal K^+ uptake system, which is multimeric and loses its normal properties in the absence of Trk1p (7). The former hypothesis is supported by well documented studies with strains carrying mutations in sugar and amino acid transporters, and with strains overexpressing amino acid permeases (14–16). The latter has been advanced to explain why the low-affinity Rb^+ uptake does not exist in wild strains, and to explain why glucose activates the low-affinity Rb^+ influx of

* This work was supported by European Community Grants BI02-CT-0400 and BI04-CT96-0775 (to A. R.-N.) and BIO4-CT97-2210 (to J. R.), and Ministerio de Educación y Ciencia of Spain Grants PB92-0907 (to A. R.-N.) and PB95-0976 (to J. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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TABLE I
S. cerevisiae strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Reference or source
<i>S. cerevisiae</i>		
W303.1A	<i>Mat a his3 leu2 trp1 ade2 ura3</i>	
W59	W303.1A <i>trk1::LEU2</i>	R. Haro (this laboratory)
WΔ2	W303.1A <i>trk2::HIS3</i>	R. Haro (this laboratory)
WΔ3	W303.1A <i>trk1::LEU2 trk2::HIS3</i>	R. Haro (this laboratory)
UCPA9	W303 expressing the mutant version UCPA9 of the <i>UCPI</i> cDNA	Ref. 18
Y806	<i>Mat a leu2 ade2 ade3 ura3 lys2 ρ^o</i>	Ref. 19
Plasmids		
pYPGE15	Yeast expression vector. <i>PGK1</i> gene promoter and <i>CYC1</i> terminator. 2 μm, <i>URA3</i> yeast marker and <i>Amp^R</i>	Ref. 20
pYES2	Yeast expression vector. <i>GAL1</i> gene promoter and <i>HIS3</i> terminator. 2 μm, <i>URA3</i> yeast marker and <i>Amp^R</i>	Invitrogen
YEp352	Yeast episomal vector. 2 μm, <i>URA3</i> yeast marker and <i>Amp^R</i>	Ref. 21
pRS404	Yeast integrative plasmid; <i>TRP1</i> yeast marker and <i>Amp^R</i>	Ref. 22
pPGKHAK1	pYPGE15 derivative, expressing the 2.3-kb coding region of the gene <i>HAK1</i> of <i>S. occidentalis</i>	M. A. Bañuelos (this laboratory)
pAG8	pYES2 derivative, expressing the 2.3-kb coding region of the gene <i>HAK1</i> of <i>S. occidentalis</i>	M. A. Bañuelos (this laboratory)
pRSHAK	pRS404 derivative containing the 5-kb <i>SnaBI/MluI</i> fragment of pAG8 including the <i>HAK1</i> gene of <i>S. occidentalis</i> the <i>GAL1</i> gene promoter and the <i>HIS3</i> terminator	This study
pPMAHKT1	pDR195 derivative expressing the <i>HKT1 cDNA</i>	F. Rubio
YEpHNM1	YEp352 derivative containing the <i>HNM1</i> gene	This study
YEpHXT3	YEp352 derivative containing the <i>HXT3</i> gene	This study

trk1Δ mutants in the same fashion as it does in wild strains (7, 17).

In this paper we report the characteristics of Rb⁺ uptake, tolerance to hygromycin B, and cellular DiOC₆(3) fluorescence, as determined by flow cytometry, in strains carrying different combinations of wild and null alleles of the *TRK1* and *TRK2* genes. The results suggest that Trk1p and Trk2p are involved in the regulation of the electrical membrane potential. Deletion of these proteins brings the cells to a very high membrane potential, negative inside, which could drive an ectopic uptake of alkali cations.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, Growth Conditions, and Methods—The *S. cerevisiae* strains used in this study are all isogenic to W303.1A (*Mata ura3 his3 leu2 ade2 trp1*) (Table I).

Cells were grown in complex YPD or YPG (1% yeast extract, 2% peptone, 2% glucose or galactose) media, and in mineral media containing either ammonium or arginine as nitrogen sources, as described previously (5). The mineral media contained 5 μM K⁺ and 200 μM Na⁺, and KCl and NaCl were added in each case to obtain the required amounts of K⁺ and Na⁺. Cells with a normal K⁺ content were routinely grown in the arginine medium supplemented with 0.5 mM K⁺ for the *TRK1 TRK2* and *TRK1trk2Δ* strains, 2.5 mM K⁺ for the *trk1Δ TRK2* strain, and 10 mM K⁺ for the *trk1Δ trk2Δ* strain. K⁺-starved cells were obtained by suspending cells with a normal K⁺ content in K⁺-free arginine medium for 3 h (5). The mineral media for nitrogen or phosphate starvation were prepared substituting K⁺ for arginine or sulfuric acid for phosphoric acid, respectively; for nitrogen starvation, amino acid supplementation, if required, was reduced 10-fold. Starvation was achieved by incubating the cells for 5 h in these media. *trk1Δ trk2Δ*-pGALHAK1 cells were grown in YPG and transferred to YPD as indicated in each case. Hygromycin B resistance was tested in YPD.

N. crassa was grown as described elsewhere (23). Cells with a normal K⁺ content were grown at 3 mM K⁺. To obtain low-K⁺ cells, the conidia were grown at 50 μM K⁺ for 6 h and then transferred to 10 μM K⁺ for 1 h. All procedures for manipulating DNA were standard ones (24).

Rb⁺ Uptake—Cells were separated from the culture medium by centrifugation, washed, and suspended in testing buffer composed of 10 mM MES¹ buffer brought to pH 6.0 with Ca(OH)₂, containing 0.1 mM MgCl₂ and 2% glucose. In some experiments with *trk1Δ trk2Δ*-pR-

SHAK1 strain carrying the *pGAL1-HAK1* gene, galactose-substituted glucose, and in the experiments designed to test the effect of divalent cations on Rb⁺ influx, the pH of the testing buffer was adjusted with NaOH, and MgCl₂ was withdrawn. To start the experiments, Rb⁺ was added to the cell suspension and, at short intervals, samples of cells were removed by filtration from the testing buffer. To determine the Rb⁺ content, the cells were treated with HCl and the extracts analyzed by atomic emission spectrophotometry, as described previously (5). In all cases, we determined the initial rate of Rb⁺ uptake from the time course of the net accumulation.

Flow Cytometry—*S. cerevisiae* and *N. crassa* cells were grown as described in each case. *S. cerevisiae* was always grown at low cell density (<0.3 mg dry weight ml⁻¹), and as a consequence the concentration of glucose was very similar in all experiments (close to 2%). Cells were harvested from the culture medium, suspended in testing buffer (2 × 10⁶ cells ml⁻¹), and exposed to DiOC₆(3) cyanine dye (3,3'-dihexyloxycarbocynine iodide, Molecular Probes, Eugene, OR) at 1 nM for 30 min at 28 °C, in the dark. To test yeast cell viability, propidium iodide was added at the moment of the analysis. Flow cytometric analyses were performed in a FACScan (Becton Dickinson) and EPICS XL (Coulter Electronics) flow cytometers, equipped with argon lasers. For the determination of the DiOC₆(3) fluorescence, excitation at 488 nm and a 525-nm dichroic LP filter were used. For propidium iodide, a 590-nm dichroic filter and a 610-nm LP absorbance filter were added. Because the cellular fluorescence was size dependent, we normalized all the results measuring the fluorescence of single small cells (25). In all experiments, a control sample of wild-type cells (W303.1A strain) grown in the arginine medium at 0.5 mM K⁺ was analyzed in parallel with the other samples, and the fluorescence values given by the flow cytometer were always referred to the fluorescence of the control cells and expressed as a percentage. All measurements were repeated at least in three different days.

RESULTS

Characteristics of the Low-affinity K⁺ Transport of trk1Δ trk2Δ Cells—The kinetic study of the influx of the alkali cations in *trk1Δ trk2Δ* cells with different K⁺ contents and the competitive inhibitions exerted between them indicated that these cells took up alkali cations with little differences in affinities (we determined *K_m* values of 60 mM K⁺, 60 mM Rb⁺, 110 mM Na⁺, and 100 mM Li⁺ in the WΔ3 strain), and without regulation by the K⁺ content. Although this uptake shows homogeneous kinetics, experiments with different inhibitors showed that it involves different pathways. Ammonium inhibited 45% of the low-affinity Rb⁺ uptake of *trk1Δ trk2Δ* cells, but showed

¹ The abbreviations used are: MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone.

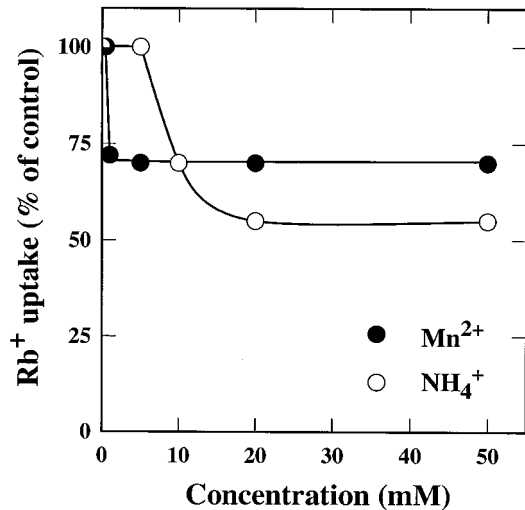


FIG. 1. Inhibition of Rb⁺ influx in *trk1Δ trk2Δ* cells by ammonium or Mn²⁺. Uptake was tested at 100 mM Rb⁺ in K⁺-starved cells.

no effect over the remaining 55%; low concentrations of Mn²⁺, and to a lesser extent other divalent cations (Mg²⁺, Ca²⁺, and Ba²⁺), inhibited 30%, but showed no effect on the remaining 70% (Fig. 1); and ammonium plus Mn²⁺ (20 mM each) inhibited 60% of the uptake. These results suggest the existence of at least three pathways for Rb⁺ uptake in *trk1Δ trk2Δ* cells, one inhibited by Mn²⁺, another inhibited by ammonium and insensitive to Mn²⁺, and a pathway insensitive to both ions. It is worth mentioning that the described inhibition of Rb⁺ uptake by ammonium or Mn²⁺ in *trk1Δ trk2Δ* cells was different from that found in wild-type cells. In the latter, ammonium was a competitive inhibitor of Rb⁺ uptake, and Mn²⁺ and other divalent cations did not show any significant effect (not shown).

Rb⁺ uptake in *trk1Δ trk2Δ* cells was also found to be very sensitive to uncouplers, much more than in *TRK1 TRK2* or *TRK1 trk2Δ* cells. These experiments were carried out in K⁺-starved cells, in order to test cells in identical physiological conditions, finding that at 20 μM CCCP, Rb⁺ influx (initial rate of uptake at 100 mM Rb⁺) in *trk1Δ trk2Δ* cells was inhibited by 80–100%, whereas this concentration showed no effect on *TRK1 TRK2* cells (Fig. 2).

Overexpression of Hxt3p and Hnm1p Partially Suppresses the Phenotype of *trk1Δ trk2Δ* Cells—In a different part of the study of the low-affinity K⁺ uptake of *S. cerevisiae*, we had isolated mutants suppressing the *trk1Δ trk2Δ* phenotype, and found that many of them mapped on the *HXT3* gene, as is consistent with previous reports (14, 15, 26). The sequence of the *HXT3* mutated genes revealed that some of these mutations were located in the 5' non-coding region. This suggested that these mutations produced overexpression of the encoded protein, and hence that the wild HXT3 transporter was able to transport K⁺. To test this possibility, we inserted the *HXT3* gene in a multicopy plasmid and transformed it into the *trk1Δ trk2Δ* strain, finding that the growth at low K⁺ was improved significantly (not shown).

Considering this result, we searched for other genes which, when overexpressed, suppress the *trk1Δ trk2Δ* phenotype. For this purpose, we transformed a *trk1Δ trk2Δ* strain with a library of genomic DNA in a multicopy plasmid and screened for clones suppressing the high K⁺ requirement of *trk1Δ trk2Δ* cells. Using the ammonium medium with 2 mM K⁺, we isolated a clone with a 9-kilobase insert containing the YGL077c open reading frame, which encodes the HNM1 choline transporter (27). By subcloning this gene alone in a multicopy plasmid, we found that *HNM1* partially suppressed the *trk1Δ trk2Δ* pheno-

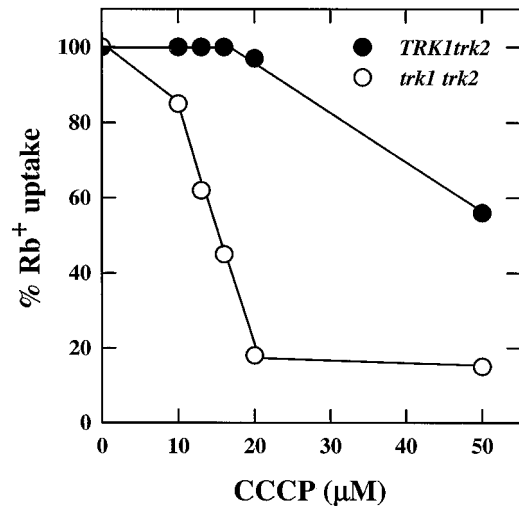


FIG. 2. Inhibition of Rb⁺ influx in *TRK1 trk2Δ* and *trk1Δ trk2Δ* cells by CCCP. Uptake at 100 mM Rb⁺ of K⁺-starved cells.

type (not shown). Although the overexpression of Hxt3p or Hnm1p improved the growth rate of the mutant, it increased less than 10% the rate of Rb⁺ uptake, suggesting that these proteins made a small contribution to the total K⁺ uptake of *trk1Δ trk2Δ* cells.

The finding that the overexpression of the HXT3 and HNM1 transporters partially suppressed the *trk1Δ trk2Δ* phenotype, as it is the case of some amino acids and the inositol transporters (16), gave clear support to the notion that multiple K⁺ pathways, possibly making small individual contributions, composed the low-affinity K⁺ uptake in *trk1Δ* and *trk1Δ trk2Δ* mutants. Because the low-affinity K⁺ uptake does not exist in wild strains, the question was how the lack of one protein, Trk1p, could open all these pathways.

The Lack of Trk1p Triggers the Low-affinity Uptake of Rb⁺—Two different reasons could trigger the general response that furnished several different transporters with the capacity of transporting K⁺, the loss of Trk1p by itself or the K⁺ deficiency generated by its loss. To distinguish between these two possibilities, we used the *trk1Δ trk2Δ* strain transformed with the *HAK1* gene of *Schwanniomyces occidentalis* (28). This gene encodes a K⁺ transporter not related to Trk1p and Trk2p, that restores K⁺ uptake, and consequently a normal growth at low K⁺, in *trk1Δ trk2Δ* cells (28). Therefore, if *trk1Δ trk2Δ* cells expressing Hak1p still kept the low-affinity K⁺ and Rb⁺ uptake, the mediation of the K⁺ deficiency in this uptake could be ruled out.

For a high expression of Hak1p in *S. cerevisiae*, we used two different constructs, placing the coding region of the *HAK1* gene under the control of the *PGK1* or *GAL1* gene promoters. Both constructs transformed into the *trk1Δ trk2Δ* strain allowed the transformants to grow at K⁺ concentrations even lower than those needed for *TRK1* strains. In contrast, in these experiments the low-affinity uptake was present or absent depending on the testing conditions. In the *pPGK1-HAK1* transformant, Rb⁺ influx in K⁺-starved cells showed only one kinetic component, corresponding to *HAK1*, whereas in normal K⁺ cells, Rb⁺ influx showed both the *HAK1* and the low-affinity kinetic components. In the *pGAL1-HAK1* transformant, cells grown on galactose (YPG) and tested on galactose showed only the Rb⁺-influx kinetics of *HAK1*, but shortly after the transfer to glucose (YPD) they showed both the *HAK1* and the low-affinity kinetic components. A longer incubation in glucose progressively decreased the former without affecting the latter (not shown).

TABLE II
Sensitivity to hygromycin B in *S. cerevisiae* cells expressing different K^+ transporters

Strains (genotypes)	YPD	YPD + 50 mM KCl
	$\mu\text{g ml}^{-1}$	
TRK1 TRK2	50	100
TRK1 <i>trk2</i>	50	100
<i>trk1</i> TRK2	15	50
<i>trk1 trk2</i> - <i>pYPG15</i>	10	50
<i>trk1 trk2</i> <i>P_{PGK1}</i> -HAK1	20	50
<i>trk1 trk2</i> <i>P_{PMA1}</i> -HKT1	75	100

trk1 Δ *trk2* Δ Strains Are Hypersensitive to Hygromycin B—The results obtained with the HAK1 transporter indicated that Hak1p performed the K^+ transport functions of Trk1p, but that only in very special conditions did it prevent the low-affinity Rb^+ uptake. Among all the possible causes for this behavior, changes in the membrane potential were the most likely. The working hypothesis was that *trk1* Δ *trk2* Δ cells could be hyperpolarized, and that the low-affinity Rb^+ uptake required such hyperpolarization. This hypothesis gave a satisfactory explanation to the high sensitivity of the low-affinity Rb^+ uptake to uncouplers (Fig. 2), and was testable by determining the resistance of the different strains to hygromycin B, an amino glycoside antibiotic for which, as in the case of Dio-9 (29, 30), the resistance of the cells depends on their membrane potential (31, 32). The tests were performed in YPD, which contains sufficient K^+ (15 mM) for normal growth of the *trk1* Δ *trk2* Δ strain, using the different mutants in the TRK1 and TRK2 genes, and the *trk1* Δ *trk2* Δ strain transformed with the *pPGK1-HAK1* gene or the HKT1 cDNA under the control of the PMA1 promoter (Table II). HKT1 encodes a K^+ - Na^+ symporter in wheat (33–35), which is related to the TRK transporters (36). Transformation with HKT1 suppressed the K^+ uptake deficiency of our *trk1* Δ *trk2* Δ strain, consistent with previous results (33–35). The tests of hygromycin B resistance (Table II) showed that the *trk1* Δ strains, either TRK2 or *trk2* Δ , were inhibited by one-fifth of the concentration tolerated by the TRK1 strains, and that HKT1 but not HAK1 suppressed the hypersensitivity of the *trk1* Δ *trk2* Δ strain. In *pma1* mutants the sensitivity to hygromycin B depends on the K^+ content of the medium (31), and we found the same effect in *trk* mutants. However, even after the addition of 50 mM K^+ to the YPD testing medium, the differences were still appreciable (Table II).

The different sensitivity to hygromycin B of the *trk1* Δ and TRK1 cells in a medium which does not impose a K^+ limitation on *trk1* Δ strains indicated that hypersensitivity to hygromycin B could not be a direct consequence of K^+ deficiency. The same conclusion could be reached considering that *trk1* Δ *trk2* Δ cells expressing Hak1p were also sensitive. Although these cells can grow at K^+ concentrations one thousand times lower than those existing in YPD (28) they were sensitive to hygromycin B. Taken together, these results suggested that the *trk1* Δ mutation was associated with a hyperpolarized plasma membrane, and that this defect could be suppressed by the expression of some K^+ transporters but not by all of those able to complement the K^+ uptake deficiency.

Differences in the Membrane Potential Can Be Estimated by DiOC₆(3) Fluorescence and Flow Cytometry—The electrical membrane potential of yeast cells is not known, but comparative assessments of membrane potentials in different conditions may be obtained with fluorescent cyanine dyes (37, 38). The cyanine dye DiOC₆(3) has been used to stain internal membranes in yeast (39) and to detect dysfunctional mitochondria (18, 25, 40). To use this dye as a probe for the membrane

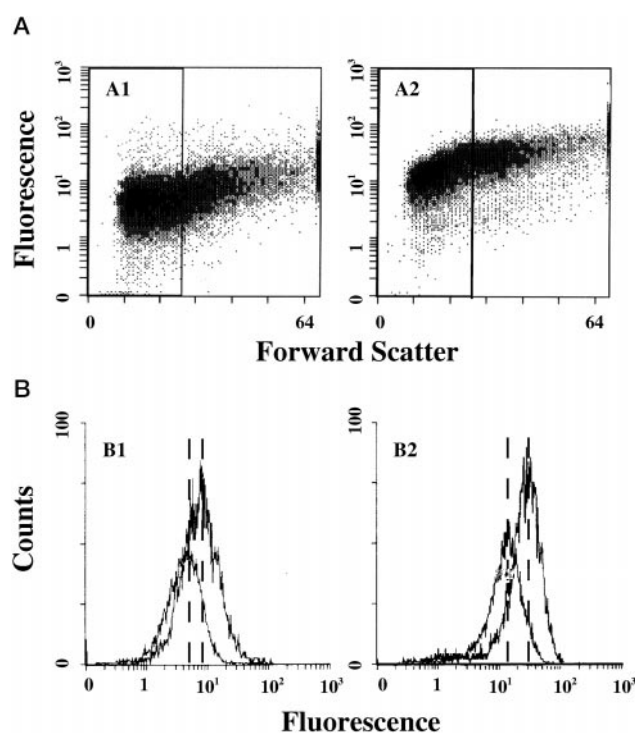


FIG. 3. Flow cytometric analyses of TRK1 TRK2 cells. A, plot of the DiOC₆(3) fluorescence as a function of the forward scatter. The vertical line divides two subpopulations of cells, depending on their size, single cells, or cells with small buds, and large single cells or cells with large buds. B, distribution (counts) of the cells according to their cellular DiOC₆(3) fluorescence. The two subpopulations shown in A have been plotted independently, and higher fluorescence correspond to larger cells. A1 and B1, cells with a normal K^+ content; A2 and B2, K^+ -starved cells.

potential, avoiding the interference of the fluorescence of the internal membranes, we reduced the concentration of the dye in the staining medium (testing buffer) to 1 nM and tested only fermenting cells in which the function of the mitochondria is highly reduced (41). Fermenting cells stained with 1 nM DiOC₆(3) and analyzed by flow cytometry showed size-dependent fluorescence (Fig. 3A). Taking the whole population or dividing it into two subpopulations, roughly corresponding to single cells and cells with small buds, and large cells and cells with large buds, K^+ -starved cells showed a much higher fluorescence than cells with a normal K^+ content (Fig. 3, A and B).

To investigate the contribution of the mitochondria to the overall fluorescence of the cells, we performed some preliminary experiments with ρ^+ and ρ^0 strains, finding that in cells growing at 2% glucose and stained with 1 nM DiOC₆(3) the difference in fluorescence between the ρ^+ and ρ^0 strains was negligible. Then we used a yeast strain expressing a mutant version (UCPA9) of the uncoupling protein of brown adipose tissue, when induced with galactose. The expression of this mutant protein produces a complete uncoupling of the mitochondria, thus abolishing the contribution of the mitochondria to the cell fluorescence (18, 42). In the first experiment, we grew the cells in 2% glucose or 2% galactose, finding no differences in fluorescence (Table III). In the second experiment, the cells were first grown on lactate (respiratory conditions) and then galactose was added at a concentration (1%) that could be fermented when the mitochondria was not functional. The results showed that the fluorescence of the cells on lactate was reduced to a half upon the addition of galactose (Table III). From these results we concluded that, using 1 nM DiOC₆(3), the mitochondria did not make any significant contribution to the cell fluorescence in fermenting conditions, and it contributed

50% in respiratory conditions.

The results presented in Fig. 3, *A* and *B*, indicated that K^+ starvation greatly increased the cellular fluorescence, probably reflecting a hyperpolarization of the membrane potential. This hyperpolarization was consistent with the results obtained in *N. crassa* cells using direct measurements (3), if we assumed that higher fluorescence corresponded to more negative inside membrane potential. To test this directly we measured the cellular DiOC₆(3) fluorescence in *N. crassa*, selecting in the flow cytometer the population of germinated conidia which were only slightly larger than *S. cerevisiae* single cells. Although *N. crassa* does not ferment, the known contribution of the mitochondria to the fluorescence of *S. cerevisiae* respiring cells stained with 1 nM DiOC₆(3) prompted us to perform the test. Giving a 100% value to the cellular DiOC₆(3) fluorescence of *N. crassa* normal- K^+ cells, for which the membrane potential is -200 mV, the fluorescence of low- K^+ cells, for which the membrane potential is -300 mV (3), was 205%. The addition of K^+ to the low- K^+ cells of *N. crassa*, which is known to cause considerable depolarization (3), also produced a remarkable decrease of the cellular DiOC₆(3) fluorescence, to 141% upon addition of 10 mM K^+ . All these results supported that the cellular DiOC₆(3) fluorescence could be used to assess differences in the membrane potential of isogenic strains differing only in genes encoding K^+ transporters, and that the increase in fluorescence can be taken as hyperpolarization of the plasma membrane.

trk1Δ Cells Are Highly Hyperpolarized—

TABLE IV
Cellular DiOC₆(3) fluorescence of *S. cerevisiae* cells expressing different K⁺ transporters

Expressed as percentage of the cellular fluorescence of *TRK1 TRK2* cells growing in 0.5 mM K⁺ arginine medium. The values are mean ± S.D. The number of repetitions were: 8 for *TRK1 TRK2* and *trk1 trk2*, 5 for the other strains in basal conditions, 3 in all other cases.

Strains ^a (genotypes)	Cells with normal K ⁺ content			K ⁺ -starved cells			
	Basal	10 mM KCl	10 mM NaCl	Basal	0.5 mM KCl	10 mM KCl	10 mM NaCl
<i>TRK1 TRK2</i>	100	100 ± 17	127 ± 32	232 ± 29	126 ± 10	100 ± 9	254 ± 32
<i>TRK1 trk2</i>	93 ± 11	110 ± 24	128 ± 22	201 ± 39	130 ± 12	100 ± 21	241 ± 23
<i>trk1 TRK2</i>	185 ± 32	180 ± 35	197 ± 13	316 ± 47	327 ± 23	333 ± 33	345 ± 19
<i>trk1 trk2</i>	246 ± 44	250 ± 40	344 ± 50	403 ± 80	553 ± 44	620 ± 81	628 ± 60
<i>trk1 trk2 HAK1</i>	192 ± 33	206 ± 35	189 ± 19	382 ± 67	267 ± 33	292 ± 10	575 ± 30
<i>trk1 trk2 HKT1</i>	235 ± 49	243 ± 40	175 ± 21	421 ± 51	390 ± 21	275 ± 28	230 ± 47
<i>trk1 trk2 HKT1 /Na⁺b</i>	25 ± 5	30 ± 2	25 ± 4	30 ± 5	25 ± 2	25 ± 4	28 ± 4

^a K⁺ contents in the growing media were: 0.5 mM for *TRK1 TRK2*, *TRK1 trk2*, *trk1 trk2 HAK1*, and *trk1 trk2 HKT1*, 2.5 mM for *trk1 TRK2*, and 10 mM for *trk1 trk2* strains.

^b *trk1 trk2 HKT1 /Na⁺* cells were grown in the arginine medium supplemented with 0.5 mM NaCl and 0.5 mM KCl and suspended in testing buffer containing 0.5 mM Na⁺ and 0.5 mM K⁺. K⁺-starved cells were prepared following the standard method, and then suspended in testing buffer supplemented with 0.5 mM NaCl.

TABLE V
Effect of pH changes and CCCP addition on the cellular DiOC₆(3) fluorescence of K⁺-starved cells of *trk1Δ* and *trk2Δ* mutants of *S. cerevisiae*
Expressed as percentage of the cellular fluorescence of *TRK1 TRK2* cells growing in 0.5 mM K⁺ arginine medium.

Strains ^a (genotypes)	pH				CCCP (μM)	
	7	6	5	4	5	10
<i>TRK1 TRK2</i>	223 ± 18	227 ± 28	176 ± 15	156 ± 25	148 ± 30	91 ± 10
<i>TRK1 trk2</i>	261 ± 47	253 ± 38	171 ± 22	158 ± 34	177 ± 36	76 ± 19
<i>trk1 TRK2</i>	333 ± 59	322 ± 66	225 ± 30	171 ± 17	254 ± 42	128 ± 33
<i>trk1 trk2</i>	489 ± 77	457 ± 81	461 ± 63	472 ± 57	397 ± 77	268 ± 37

^a Cells were grown as stated in Table IV and charged with DiOC₆(3) in the testing buffer (pH 6.0), following the standard method; then, tartaric acid, TAPS, or CCCP were added to reach the selected pH or the reported concentration of CCCP. Measurements were made 60 to 90 s after the addition.

cellular DiOC₆(3) fluorescence of nitrogen and phosphate-starved cells, finding that fluorescence increased as a consequence of nitrogen starvation but not after phosphate starvation. We have described that *trk1Δ trk2Δ* cells hyperpolarized in response to K⁺ starvation, increasing their cellular DiOC₆(3) fluorescence over the already high normal value. Unlike this response, nitrogen starvation did not show any effect on the DiOC₆(3) fluorescence of *trk1Δ trk2Δ* cells.

DISCUSSION

The results presented in this report indicate that the growth of *trk1Δ trk2Δ* mutants of *S. cerevisiae* is supported by an ectopic K⁺ uptake mediated by several transporters, which, as a whole, resembles a low-affinity single transporter for K⁺, Rb⁺, Na⁺, and Li⁺. The view is supported by the manner in which this low-affinity uptake is inhibited by ammonium and by divalent cations (Fig. 1), and by the number of different and unrelated transporters (amino acids, sugar, inositol, and choline transporters) (this report and Refs. 14–16) that by over-expression partially suppress the defective growth of *trk1Δ trk2Δ* strains at low K⁺. Our results also indicate that in the absence of Trk1p, or Trk1p and Trk2p, the membrane potential is exceptionally high, negative inside. Taken together, these results pose two questions, whether there is any causal relationship between the ectopic low-affinity K⁺ and Rb⁺ uptake and the high membrane potential, and what the cause of this high membrane potential is? Regarding the former question, it is clear that both the low-affinity Rb⁺ uptake and the high membrane potential are the consequences of the deletion of the *TRK1* gene. Furthermore, the ectopic K⁺ uptake must result from a change in a general property of the membrane, because it is unlikely that the deletion of the *TRK1* gene can modify the specific properties of many different transporters, making all of them permeable to alkali cations. Considering these observations and the high sensitivity of the low-affinity Rb⁺ uptake to CCCP, the most likely cause for the ectopic uptake of K⁺ is the unusually high membrane potential exhibited by *trk1Δ* strains.

Regarding the high membrane potential of *trk1Δ* and *trk2Δ* cells, our results reveal that Trk1p and Trk2p are not only K⁺ transporters but also essential regulators of the membrane potential. To fulfill this function they must either counter the activity of the pump or control the conductance of the plasma membrane. Before going further in the discussion of the function of Trk1p and Trk2p, it is worth mentioning that a pitfall in the use of the cellular fluorescence of the DiOC₆(3) probe to assess differences in membrane potentials is extremely unlikely, considering the conditions for our assessments: (i) we used the cellular DiOC₆(3) fluorescence only for comparative purposes in entirely isogenic cells, except for the genes considered in each case; (ii) the contribution of the mitochondria to these changes in fluorescence has been ruled out; (iii) we did a control using *N. crassa* cells, for which the membrane potential has been measured using intracellular electrodes (3). Furthermore, the hyperpolarized state of the *trk1Δ* and *trk1Δ trk2Δ* cells can be also deduced by contrasting the phenotype of these mutants with the phenotype of *pma1* mutants. The latter are more resistant to hygromycin B than the wild type, whereas *trk1Δ* and *trk1Δ trk2Δ* mutants are strikingly more sensitive (Table II). Assuming that the phenotype of *pma1* mutants is the result of a lower membrane potential (32), it can be concluded that the phenotype of *trk1Δ* and *trk1Δ trk2Δ* mutants is the result of a higher membrane potential.

Nitrogen starvation hyperpolarized *TRK1 TRK2* cells but not *trk1Δ trk2Δ* cells, whereas K⁺ starvation hyperpolarized both types of cells. This suggests that at least two parallel and additive routes control the membrane potential, one dependent on Trk1p and Trk2p, and the other independent of these proteins. Apparently, K⁺ starvation activates both routes, whereas nitrogen starvation activates only that dependent on Trk1p and Trk2p. The mechanisms involved in the control of the membrane potential cannot be established at this moment. However, there are only two ways to achieve this control, either modifying the activity of the pump or triggering a “safety

depolarizing current" when the membrane potential reaches a certain value. In the latter case, because the function can be performed in the absence of K^+ , or any other alkali cation (Table IV), it is likely that the ions moving are either H^+ inward or anions outward. The Trk1p-Trk2p dependence of low-pH depolarization (Table V) indicates that an inward movement of H^+ may be involved in the route of control depending on Trk1p and Trk2p.

The capacity of Trk1p and Trk2p to control the membrane potential is not a general property of K^+ transporters, because Hak1p cannot perform these functions (Tables III and IV). Although the results supporting this conclusion have been obtained using a heterologous expression, the high level of conservation in proteins and functions among fungi, and even among fungi and higher plants, suggests that it is correct. It is very interesting that in *S. occidentalis*,² in *N. crassa*,³ and in *Debaryomyces hanseni*,⁴ HAK1 type K^+ transporters coexist with TRK2 K^+ transporters. Whether the function of these TRK2 transporters in these species is more related with the control of the membrane potential than to K^+ uptake is now under study. Interestingly, in barley plants K^+ uptake is mediated by HAK1 transporters (43–46), and wheat plants have a HKT1 transporter whose function is not clear (47). Hkt1p shows homology with Trk1p and Trk2p (36) and, when expressed in *S. cerevisiae*, it produced strong depolarization in the presence of Na^+ (Table IV). Therefore, the involvement of Hkt1p in the control of the membrane potential in higher plants is an attractive possibility.

The conclusion of this and previous reports (14–16) identifying independent mechanisms for Rb^+ influx in *TRK1 TRK2* and *trk1Δ trk2Δ* cells indicates that the mechanisms involved in the activation of the V_{max} of Rb^+ influx by glucose in both types of cells (17) may be also different. Interestingly, we found that the addition of glucose hyperpolarized both wild type and *trk* mutant cells (not shown), which is consistent with the known activating effect of glucose on the H^+ -pump ATPase (48). The possibility that this hyperpolarization brings about the increase of the V_{max} of Rb^+ influx in all cases is an attractive idea. Unfortunately, this cannot be tested with the techniques used in this report.

Finally, *trk1Δ trk2Δ* mutants have been extensively used for cloning heterologous K^+ -transport genes. Our results indicate that genes expressing low-rate, low-affinity K^+ uptake in these mutants, may encode non- K^+ transporters that support ectopic K^+ uptake.

Acknowledgments—We thank R. Haro for providing WΔ3, WΔ2, and W59 strains; F. Rubio for providing plasmid pPMAHKT1; F. Bouillaud for strain UCPΔ9; A. Alvarez and A. Vázquez (flow cytometry service of the Departamento de Microbiología, Facultad de Farmacia, UCM) and P. Lastres (flow cytometry service of Centro de Investigaciones Científicas, CSIC) for helping with flow cytometry experiments; and E. Rial for permitting the use of his laboratory in preparing many experiments of flow cytometry.

² R. Madrid and A. Rodríguez-Navarro, unpublished results.

³ R. Haro, L. Sainz-Pastor, F. Rubio, and A. Rodríguez-Navarro, unpublished results.

⁴ C. Prista, M. C. Loureiro-Dias, and J. Ramos, unpublished results.

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