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NOTES

TRK2 Is Not a Low-Affinity Potassium Transporter in *Saccharomyces cerevisiae*

JOSE RAMOS,[1] RAFAEL ALIJO,[1] ROSARIO HARO,[2] AND ALONSO RODRIGUEZ-NAVARRO[2]*

Departamento de Microbiología, Escuela Técnica Superior de Ingenieros Agrónomos, 14071 Córdoba,[1] and Departamento de Microbiología, Escuela Técnica Superior de Ingenieros Agrónomos, 28040 Madrid,[2] Spain

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***TRK1* and *TRK2* encode proteins involved in K^+ uptake in *Saccharomyces cerevisiae*. A kinetic study of Rb^+ influx in *trk1 TRK2*, *trk1 TRK2^D*, and *trk1 trk2* mutants reveals that TRK2 shows moderate affinity for Rb^+. K^+-starved *trk1Δ TRK2* cells show a low-affinity component accounting for almost the total V_{max} of the influx and a moderate-affinity component exhibiting a very low V_{max}. Overexpression of TRK2 in *trk1Δ TRK2^D* cells increases the V_{max} of the moderate-affinity component, and this component disappears in *trk1Δ trk2Δ* cells. In contrast, the low-affinity component of Rb^+ influx in *trk1Δ TRK2* cells is not affected by mutations in *TRK2*. Consistent with the different levels of activity of the moderate-affinity Rb^+ influx, *trk1Δ TRK2* cells grow slowly in micromolar K^+, *trk1Δ TRK2^D* cells grow rapidly, and *trk1Δ trk2Δ* cells fail to grow. The existence of a unique K^+ uptake system composed of several proteins is also discussed.**

K^+ uptake in fungi and plants is mediated by transport systems different from those mediating equivalent processes in animal cells or in prokaryotic organisms. But despite the work devoted to these systems, a comprehensive model of K^+ transport in cell-walled eukaryotic cells is still elusive, with various models proposed. In part, this reflects the diversity of the organisms studied, but it is also due to the lack of basic genetic studies. However, the molecular genetics of K^+ transport in *Saccharomyces cerevisiae* has developed rapidly during the last years (5).

S. cerevisiae cells can grow in media with K^+ concentrations extending from 2 to 4 μM K^+ (11, 17) to 2.1 M K^+ (16). Growth rates and the K^+ contents are almost constant in this wide range of K^+ concentrations (11, 14, 17), and consequently, the K^+ uptake system must adapt to provide almost constant net K^+ uptake. Furthermore, the system also provides rapid K^+ replenishment in K^+-depleted cells (14). To meet these requirements, the K_m of K^+ influx can take any value from approximately 15 μM in K^+-starved cells to 5 mM in cells growing at low-millimolar K^+ concentrations (11, 14, 17), and it may reach values exceeding 50 mM K^+ in cells grown at 0.5 M K^+ (15). This broad capacity of adaptation is affected by mutations in two genes, *TRK1* and *TRK2*. *TRK1* is clearly involved in high-affinity K^+ uptake because *trk1Δ* cells do not grow at micromolar K^+ concentrations and do not exhibit K^+ influx K_ms in the micromolar range (6, 11). In *trk1Δ* cells, disruption of *TRK2* increases notably the K^+ requirement, and its overexpression restores a wild-type K^+ requirement (20–22). In contrast, disruption of *TRK2* does not have an appreciable effect on *TRK1* cells. To account for these observations, it has been proposed that *TRK2* may encode a low-affinity K^+ transporter, dispensable when TRK1 is functional (8, 9). Both TRK1 and TRK2 are putative membrane proteins (55% identical), but their exact function has not been demonstrated and cannot be deduced from their putative amino acid sequences (6, 9).

On the basis of the K^+ requirements and the K^+ and Na^+ uptake kinetics of the K^+ uptake mutants, two hypotheses have been proposed to explain the organization of the K^+ uptake system of *S. cerevisiae*: (i) a complex system with different binding sites for K^+ or Rb^+ and for Na^+ (11) and (ii) two independent transporters, a high-affinity system encoded by *TRK1* and a low-affinity system encoded by *TRK2* (5, 8, 9). Here we report a kinetic study in different mutants of the K^+ uptake system. The results indicate that the product of *TRK2* does not mediate low-affinity K^+ uptake and suggest that TRK1 and TRK2 are two components of a complex system composed of several proteins.

S. cerevisiae DBY746 (*MATα ura3 leu2 his3 trp1*) was used as the wild type. Strain RH2.2, containing a deletion within the *TRK1* gene in DBY746, was constructed by the one-step gene disruption method (18). Plasmid pRH22 containing the *TRK1* gene was isolated from a yeast genomic library constructed in the shuttle vector YCp50. The bacterial clone harboring this plasmid was identified by in situ hybridization with a 23-mer oligonucleotide reproducing the sequence of a DNA fragment of *TRK1* (starting at position 1000 of the sequence published in reference 6). The 2.3-kb *Xba*I fragment in pRH22 was removed and replaced by the 3.0-kb *Bgl*II fragment of plasmid YEp13 containing the *LEU2* gene, and then the 4.2-kb *Sac*I fragment of the new plasmid was used for transforming DBY746. Disruption of *TRK1* in several Leu^+ transformants was confirmed by Southern blot analysis. Strains R1155 (*MATα ura3 lys9 his4 trk1Δ*), M469 (*MATα ura3 leu2 his3 trp1 trk1Δ trk2*::pCK64), and A105 (*MATα ura3 his4 lys9 trk1Δ TRK2^D-1*), with different levels of expression of TRK2 (6, 9, 20), were also used. Standard protocols (19) and manufacturers' recommendations were followed for routine DNA manipulations. Ammonium phosphate medium, free of K^+ and Na^+, and arginine phosphate medium, free of ammonium, K^+, and Na^+, were prepared as described previously (17). These media contained approximately 2 μM K^+ and were supplemented

* Corresponding author. Escuela Técnica Superior de Ingenieros Agrónomos, 28040 Madrid, Spain.

with KCl to obtain the required K^+ concentration. Cells were normally grown in the medium with arginine because ammonium greatly increases the K^+ requirement (11, 12), with 3 mM K^+ for *TRK1* strains and with 30 mM K^+ for *trk1*Δ strains. These K^+ concentrations saturated the growth rate response of the strains to K^+, and the cells in these media (normal-K^+ cells) contained approximately 500 nmol of K^+ mg^{-1}. K^+-starved cells, containing 250 nmol of K^+ mg^{-1} and a maximal capacity for K^+ uptake, were prepared by incubating normal-K^+ cells in the medium with arginine and without K^+ for 5 h (14). Azide-treated cells with different K^+ contents were prepared as described previously (14). Dry weights are used in all references to weights of cells.

Uptake experiments were carried out in 10 mM morpholinoethanesulfonic acid brought to pH 6.0 with $Ca(OH)_2$, containing 0.1 mM $MgCl_2$ and 2% glucose (incubation buffer). The methods for the cell analyses have been described previously (14, 17). Briefly, normal-K^+ cells or K^+-starved cells were harvested by centrifugation, washed with water, and suspended in incubation buffer. Rb^+ or Li^+ was added to the suspension of cells at time zero, samples were removed by filtration at intervals, and the cation contents were determined in acid extracts by atomic absorption or by atomic emission spectrophotometry. The initial rates of uptake were determined from the time courses of the cellular cation contents. Low rates were constant for long periods of time (17), but sampling time was kept as short as possible and was never longer than 15 min. Technical reasons made it difficult to carry out experiments at Rb^+ concentrations exceeding 100 mM, and K_ms in the 50 to 100 mM Rb^+ range calculated in different experiments showed appreciable variability. Reported figures are from representative experiments, and reported data are means from at least five experiments. The standard errors of these means were lower than 20% of the corresponding mean. K_ms and V_{max}s of two-component kinetics were calculated by a computer-assisted nonlinear regression program, assuming that the actual rate resulted from the addition of two Michaelis-Menten equations. The legitimate use of Rb^+ as a K^+ analog has been demonstrated (17).

Growth rate constants were calculated from the semilogarithmic plots of the absorbance of cultures versus time. Tubes were inoculated with less than 10^4 cells ml^{-1}, starting the recording of the absorbance when the culture reached approximately 0.04 absorbance units.

Rb^+ influx kinetics in *trk1*Δ cells. *trk1* mutants grow well at high K^+, although both growth rates and K^+ uptake are considerably lower than in the wild type, when the external K^+ is low. This finding suggests that these mutants have only a low-affinity K^+ transporter (6, 11). However, careful study of the reported data shows that the reduction of the K^+ uptake capacity is, surprisingly, more severe than the reduction in the ability to grow at low K^+. A mutant in which K^+-starved cells exhibited a K^+ influx K_m of 11 mM and V_{max} of 11 nmol mg^{-1} min^{-1} grew in 100 μM K^+ with a growth rate constant of 0.05 h^{-1} (11). Even if the cells growing at 100 μM K^+ were K^+ starved, with minimal K^+ content and maximum K^+ uptake capacity, they would need a net K^+ uptake of 14 nmol mg^{-1} h^{-1} to keep a K^+ content of 280 nmol mg^{-1} (11), but they could take only 6 nmol mg^{-1} h^{-1} if the kinetic data are correct. New experiments with this *trk1* mutant revealed that a minor component (V_{max} of 0.6 nmol of K^+ mg^{-1} min^{-1}) of apparent high affinity was still present in K^+-starved cells. This finding suggested that either the mutant retained a low TRK1 activity or K^+ uptake in *trk1* mutants could not be explained simply by a low-affinity system.

To exclude the possibility of a residual function of TRK1, we constructed a mutant strain (RH2.2) carrying a *trk1*Δ null allele and studied the K^+ requirement and the Rb^+ influx kinetics of the mutant. Like the *trk1-1* mutant previously reported (11), RH2.2 required a higher K^+ concentration than the wild type for maximum growth rate, still grew in low K^+ (growth rate constant of 0.09 h^{-1} at 50 μM K^+), and showed complex kinetics of Rb^+ influx: in normal-K^+ cells, Rb^+ influx exhibited a typical Michaelis-Menten kinetics of low affinity (K_m of 65 mM and V_{max} of 16 nmol mg^{-1} min^{-1}), but in K^+-starved cells, it exhibited two kinetic components, a low-affinity component of the same K_m and V_{max} as in normal-K^+ cells and a minor component of moderate affinity (K_m of 0.4 mM and V_{max} of 1.2 nmol mg^{-1} min^{-1}), compared with the K_ms of the high-affinity (80 μM) and low-affinity (6 mM) modes of Rb^+ uptake in wild-type cells (11, 14, 17). The existence of the moderate-affinity component could explain the growth of the *trk1*Δ strain at low K^+ concentrations (Rb^+ uptake was strongly inhibited by K^+, but an exact calculation of the K_i was technically impossible because of the low V_{max} of the system).

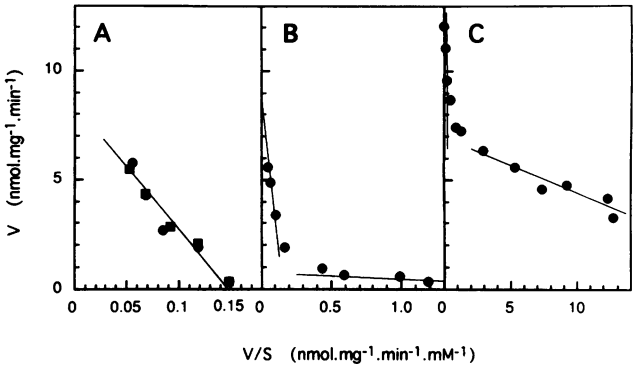

FIG. 1. Eadie-Hofstee plots of the initial rates of Rb^+ uptake in different *trk1*Δ mutants. (A) *trk1*Δ *trk2*Δ normal-K^+ cells (■) and K^+-starved cells (●) (strain M469); (B) *trk1*Δ *TRK2* K^+-starved cells (strain R1155); (C) *trk1*Δ *TRK2*^{D} K^+-starved cells (strain A105). Experimental data in panels B and C can be explained by the addition of two independent processes following Michaelis-Menten equations. The low-affinity processes show the same K_ms in all three panels.

TRK2 exhibits moderate affinity for K^+. Disruption of *TRK2* increases the K^+ requirement of *trk1*Δ cells (8, 9). This finding indicated that either (i) TRK2 somehow governs both the low- and moderate-affinity components of Rb^+ influx in *trk1*Δ cells or (ii) TRK2 governs only a low-rate system of moderate affinity for Rb^+ and there is a separate major system of high rate and low affinity. To test these two possibilities, the Rb^+ influx kinetics were compared in *trk1*Δ *trk2*Δ, *trk1*Δ *TRK2*, and *trk1*Δ *TRK2*^{D} strains (M469, R1155, and A105; null, normal, and overexpressed TRK2, respectively [8, 9, 20]). In normal-K^+ cells of the three strains, Rb^+ influx followed a Michaelis-Menten kinetics of low affinity (K_m of 65 mM and V_{max} of 8.5 nmol mg^{-1} min^{-1}), and an identical kinetics (i.e., no moderate-affinity component) was observed in K^+-starved cells of the *trk1*Δ *trk2*Δ double mutant. In contrast, in K^+-starved cells of the other two strains, *trk1*Δ *TRK2* and *trk1*Δ *TRK2*^{D}, Rb^+ influx had two kinetic components, one of low affinity, with K_m of 65 mM as in normal-K^+ cells, and a moderate-affinity component with K_m of 0.3 mM. This component had V_{max}s of 0.7 nmol mg^{-1} min^{-1} in the *TRK2* strain and 7 nmol mg^{-1} min^{-1} in the *TRK2*^{D} strain (Fig. 1). Thus, the moderate-affinity influx of Rb^+ varied with the expected

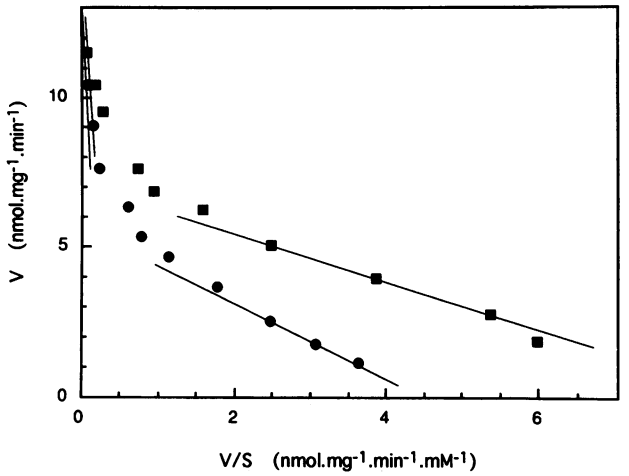

FIG. 2. Eadie-Hofstee plots of the initial rates of Rb^+ uptake in *trk1*Δ *TRK2*^{D} cells (strain A105) treated with 2 mM azide for 5 min (●) or with 10 mM azide for 15 min (■). The K^+ contents were 350 and 250 nmol mg^{-1}, respectively. Experimental data can be explained by the addition of two independent processes following Michaelis-Menten equations.

level of the *TRK2* product, indicating that TRK2 was required for moderate-affinity but not for low-affinity Rb^+ uptake.

In cells of the *trk1*Δ *TRK2*^{D} strain growing at 30 mM (high) K^+, the moderate-affinity component of Rb^+ uptake was not detected, although this component presented a high V_{max} in K^+-starved cells. This finding suggested the possibility that the activity of TRK2 might be regulated, e.g., by the internal K^+ and by the internal pH, like TRK1 (13). To test this possibility, we measured Rb^+ influx in normal-K^+ cells treated with azide to produce a rapid decrease of the K^+ content (14). This treatment triggered the appearance of the moderate-affinity Rb^+ influx, whose kinetic characteristics depended on the resulting K^+ content (Fig. 2): cells initially containing 500 nmol of K^+ mg^{-1} and no moderate-affinity component of Rb^+ uptake contained 350 nmol of K^+ mg^{-1} after a 5-min treatment with 2 mM azide and showed a moderate-affinity component of Rb^+ influx of K_m of 1.25 mM and V_{max} of 5.5 nmol mg^{-1} min^{-1}, and cells treated for 15 min with 10 mM azide, containing 250 nmol of K^+ mg^{-1}, showed a K_m of 0.76 mM and a V_{max} of 7.0 nmol mg^{-1} min^{-1}. Although the latter K^+ content is at the lower limit attainable either by azide treatment or K^+ starvation (13, 14), the Rb^+ influx K_m of azide-treated cells was significantly higher than that found in K^+-starved cells (compare Fig. 1C and 2). An analogous difference in K_m for the two types of treatment is also known for TRK1-mediated Rb^+ influx (14), suggesting that the regulation of TRK1 and that of TRK2 are similar. However, for TRK1, Rb^+ uptake is activated by decreasing the internal pH with permeant acids (13, 17), and no such activation occurred in *trk1 TRK2*^{D} cells (experiments like those presented in Fig. 2 performed in the presence of 10 mM butyric acid at pH 5.0).

The notable V_{max} of the TRK2 system in K^+-starved *TRK2*^{D} cells enabled the calculation of the K^+ K_i of this system. Experiments as in Fig. 1C but also including different added external K^+ concentrations showed that K^+ inhibited Rb^+ uptake competitively, with a K_i of 35 μM (the K_i of K^+ in the low-affinity system could not be calculated analogously because experiments of Rb^+ uptake were not performed at concentrations higher than 100 mM Rb^+). Remarkably, in K^+-starved cells, the ratio between the K_m of Rb^+ and the K_i of K^+ in TRK2 was not significantly different from that found in TRK1 (17), i.e., 8.6 and 6.6, respectively.

There are several examples of repeated genes encoding transport systems in *S. cerevisiae* yeast (7, 10), and *TRK2* seems to be a copy of *TRK1*, perhaps a defective copy. However, its unusually large promoter region as well as its transcriptional regulation (9, 21, 22) suggest that TRK2 may also perform physiological functions unknown at this moment.

K^+ requirements of *trk1*Δ *TRK2* and *trk1*Δ *trk2*Δ cells. If the growth of *trk1*Δ strains at low K^+ was the consequence of the activity of TRK2, a *trk1*Δ *trk2*Δ strain should present very defective growth at low K^+. Growth experiments at 1 mM K^+ showed that the *trk1*Δ *trk2*Δ strain (M469) doubled in 15 h (growth rate constant of 0.05 h^{-1}) and contained approximately 300 nmol of K^+ mg^{-1}, whereas the *trk1*Δ *TRK2* strain (R1155) showed a doubling time of 4.5 h (growth rate constant of 0.15 h^{-1}) and contained 450 nmol of K^+ mg^{-1}. Because K^+ influxes must be sufficient to maintain the K^+ content of the cells, they must have minimal values of 15 nmol mg^{-1} h^{-1} in *trk2*Δ cells and 68 nmol mg^{-1} h^{-1} in *TRK2* cells, and these values can be accounted for by the described kinetic characteristics of the strains. The K^+ uptake capacity at 1 mM K^+ is 17 nmol mg^{-1} h^{-1} in *trk2*Δ cells (K^+ influxes can be calculated from the Rb^+ influx kinetics, assuming the same V_{max}s for K^+ and Rb^+ and that the ratios between the Rb^+ and K^+ K_ms are 2 in the low-affinity mode [11, 17]) and 59 nmol mg^{-1} h^{-1} in *TRK2* cells (17 nmol mg^{-1} h^{-1} through the low-affinity component plus 42 nmol mg^{-1} h^{-1} through the moderate-affinity component; the latter influx is calculated assuming the same V_{max}s for K^+ and Rb^+, 0.7 nmol mg^{-1} min^{-1}, and that the K_i of K^+ for Rb^+ influx, 35 μM, is the K_m of K^+ influx [17]). At 30 μM K^+, *trk1*Δ *trk2*Δ cells did not show appreciable growth, and *trk1*Δ *TRK2* cells doubled in 8 h (growth rate constant of 0.09 h^{-1}) and contained 300 nmol of K^+ mg^{-1}. This growth requires a net K^+ uptake of 26 nmol mg^{-1} h^{-1}, and the difference between this uptake and that expected from the kinetics of TRK2 is within the limits of experimental error (a kinetics with a V_{max} of 0.7 nmol mg^{-1} min^{-1} and a K_m of 35 μM K^+ provides 20 nmol mg^{-1} h^{-1} at 30 μM K^+).

The capacity reported here of the *trk1*Δ *TRK2* and *trk1*Δ *trk2*Δ mutants to grow at low K^+ is higher than previously reported for the same strains (9), but the two sets of data are consistent. The differences may be explained by the different nitrogen sources of the testing media; ammonium medium used in the previous report is known to increase the K^+ requirement by comparison with arginine used in present work (11, 17); in addition, growth rate constants were not reported previously (9).

The number of the K^+ uptake systems. The proposal that TRK1 is a high-affinity K^+ transport system and TRK2 is a low-affinity transporter (8, 9) is not consistent with the actual K_m value of Rb^+ and K_i value of K^+ in TRK2. It could be proposed that yeast cells have three K^+ uptake systems. The real low-affinity transporter would be the system mediating K^+ and Rb^+ uptake in *trk1*Δ *trk2*Δ cells, TRK1, the high-affinity system, and TRK2, a system of moderate affinity. However, in this hypothesis, the physiological function of the low-affinity transporter in wild-type cells is not clear: kinetic evidence of the presence of this transporter has not been found (14, 17), and it would not be related to the low-affinity mode of K^+ uptake (K_ms of Rb^+ influx of 65 and 6 mM [11], respectively). Furthermore, with a multiple K^+ uptake system, it is difficult to explain that K^+, Rb^+, Na^+, and Li^+ are taken up through the same transport system in wild-type strains (2–4, 11) and that a *trk1-1* mutation affects K^+ or Rb^+ uptake much more than

Na^+ uptake (11), a differential effect also observed in *trk1*Δ mutants (the K_m of Li^+ increased only twofold, from 50 to 100 mM, in K^+-starved cells of RH2.2).

These results can be explained if there is a single K^+ uptake system composed of one or several unknown proteins in addition to TRK1 and TRK2, the latter being present in a small number of transporters in normal conditions. The main function of TRK1 would be the regulation of K^+ influx in response to the K^+ content of the cell and to the cell pH (13, 14, 17). Interestingly, glucose activation of the K^+ uptake system (12) would occur in one of the yet unknown proteins because activation is normal in *trk1*Δ *trk2*Δ cells (1).

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