Coordination of K⁺ Transporters in Neurospora: TRK1 Is Scarce and Constitutive, while HAK1 Is Abundant and Highy Regulated

Alberto Rivetta,⁎ Kenneth E. Allen,⁎ Carolyn W. Slayman,⁎ Clifford L. Slayman⁎

Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, Connecticut, USA; Department of Genetics, Yale School of Medicine, New Haven, Connecticut, USA.

Fungi, plants, and bacteria accumulate potassium via two distinct molecular machines not directly coupled to ATP hydrolysis. The first, designated TRK, HKT, or KTR, has eight transmembrane helices and is folded like known potassium channels, while the second, designated HAK, KT, or KUP, has 12 transmembrane helices and resembles MFS class proteins. One of each type functions in the model organism Neurospora crassa, where both are readily accessible for biochemical, genetic, and electrophysiological characterization. We have now determined the operating balance between Trk1p and Hak1p under several important conditions, including potassium limitation and carbon starvation. Growth measurements, epitope tagging, and quantitative Western blotting have shown the gene HAK1 to be much more highly regulated than is TRK1. This conclusion follows from three experimental results: (i) Trk1p is expressed constitutively but at low levels, and it is barely sensitive to extracellular [K⁺] and/or the coexpression of HAK1; (ii) Hak1p is abundant but is markedly depressed by elevated extracellular concentrations of K⁺ and by coexpression of TRK1; and (iii) Carbon starvation slowly enhances Hak1p expression and depresses Trk1p expression, yielding steady-state Hak1p:Trk1p ratios of ~500:1, viz., 10- to 50-fold larger than that in K⁺- and carbon-replete cells. Additionally, it appears that both potassium transporters can adjust kinetically to sustained low-K⁺ stress by means of progressively increasing transporter affinity for extracellular K⁺. The underlying observations are (iv) that K⁺ influx via Trk1p remains nearly constant at ~9 mM/h when extracellular K⁺ is progressively depleted below 0.05 mM and (v) that K⁺ influx via Hak1p remains at ~3 mM/h when extracellular K⁺ is depleted below 0.1 mM.

Essentially all living cells are metabolically dependent on high (~150 mM) intracellular concentrations of potassium ions, but two fundamentally different strategies are used to maintain high internal [K⁺] in the face of generally low external concentrations (<10 mM). In animal cells, concentative uptake of K⁺ is chemically coupled to the ATP-driven extrusion of sodium ions, and the diffusional efflux of K⁺ ions into extracellular concentrations ranging from nearly 1 to 10 mM determines ordinary resting membrane voltages (Vₘ) of ~70 to ~90 mV. In contrast, most plant cells, fungi, and bacteria possess a strongly electrogenic proton extrusion pump, and concentrative K⁺ influxes are driven by the membrane voltage, which can range beyond ~300 mV (1, 2).

At least four modes of K⁺ uptake occur in these cells: (i) diffusive, fast influx via K⁺-specific channels (3, 4); (ii) simple facilitation, a protein-mediated slow flux formerly termed facilitated diffusion; (iii) H⁺- or Na⁺-coupled symport of potassium ions, formerly termed secondary active transport (5–9); and (iv) in some species, ATP-coupled K⁺ influx pumping (10, 11).

Modes ii and iii have both been attributed to two distinct and large classes of transport proteins, generally dubbed Trk and Kup in bacteria and Trk (or HKT) and HAK in plants and fungi. For the Kup-HAK proteins, no crystal structures have yet been reported, but available sequences define 12 transmembrane helices. This indicates general similarity to the MFS class of membrane transporters and suggests a mechanism of rocker-switched access for transfer of K⁺ ions (12–15). For the Trk-HKT proteins, early sequence data revealed strong similarity to K⁺ channels and led to a theoretical model (16, 17) based on the bacterial potassium channel, KcsA (18). That model, which varied slightly among the bacterial, fungal, and plant homologues, has been substantiated by a variety of point mutation and sidedness studies (19–22). More recently, VpTrkH, a bacterial homologue from Vibrio parahaemo-
protein, not the TRK1 protein, was responsible for K\(^+\)-H\(^+\) symport in the earlier experiments (24).

We have now undertaken a thorough test of the situation directly in Neurospora, making particular use of single-gene deletion strains recently provided by the Neurospora genome project (25). In normal K\(^+\)-containing media, TRK1 protein (Trk1p) is expressed constitutively at a low level and is rather insensitive to growth conditions. HAK1 (Hak1p) is also expressed constitutively but is strongly suppressed by high extracellular potassium and also by the simultaneous presence of Trk1p. Other metabolic conditions impinge on both. For example, carbon starvation of Neurospora, superimposed on K\(^+\) starvation, slowly diminishes Trk1p while simultaneously enhancing Hak1p. One net result of these changes is that the expression ratio Hak1p:Trk1p, which is in the neighborhood of 10:1 under normal laboratory conditions, rises to more than 500:1 after 3 h of starvation for K\(^+\) and carbon.

**MATERIALS AND METHODS**

Strains of *N. crassa* used in this study are listed in Table 1. Knockout strains for single genes encoding the critical potassium transporters TRK1 and HAK1, for the apparently silent homologue TRK2, and for the K\(^+\) channel TOK1 were generated within the *Neurospora* functional genomics project (25) by homologous replacement with the hygromycin B resistance gene (*hph*). These four strains, along with two knockout strains for the DNA repair enzyme *MUS51* and wild-type strains *ORSA* and 74-OR23-1VA, were obtained from the Fungal Genetics Stock Center (Kansas City, KS). Knockout of the *MUS51* gene greatly enhances the frequency of homologous recombination in *Neurospora* (26).

**Genetic loci numbers for TRK1, TRK2, HAK1, and MUS51 are NCU06449, NCU02456, NCU00790, and NCU08290, respectively. All other strains in this study were constructed using standard genetic and molecular biological techniques (27). Strain names, detailed genotypes, and sources/constructs for all strains used in these experiments are listed in Table 1. In order to make the genetic construct(s) in each experiment as clear and direct as possible while minimizing the need for crosses going back to the FGSC strains.

Strains used in this study are listed in Table 1. Knockout strains for single genes encoding the critical potassium transporters TRK1 and HAK1, for the apparently silent homologue TRK2, and for the K\(^+\) channel TOK1 were generated within the *Neurospora* functional genomics project (25) by homologous replacement with the hygromycin B resistance gene (*hph*). These four strains, along with two knockout strains for the DNA repair enzyme *MUS51* and wild-type strains *ORSA* and 74-OR23-1VA, were obtained from the Fungal Genetics Stock Center (Kansas City, KS). Knockout of the *MUS51* gene greatly enhances the frequency of homologous recombination in *Neurospora* (26).

**Genetic loci numbers for TRK1, TRK2, HAK1, and MUS51 are NCU06449, NCU02456, NCU00790, and NCU08290, respectively. All other strains in this study were constructed using standard genetic and molecular biological techniques (27). Strain names, detailed genotypes, and sources/constructs for all strains used in these experiments are listed in Table 1. In order to make the genetic construct(s) in each experiment as clear and direct as possible while minimizing the need for crosses going back to the FGSC strains.
made up in distilled water plus 2% sucrose. In K⁺-free Vogel’s medium, potassium phosphate was replaced by NaH₂PO₄, thus elevating extracellular concentrations of Na⁺ ([Na⁺]ₑ) to 63.5 mM. For all experiments, harvested conidia were washed and resuspended in glass-distilled water prior to inoculation.

Drop tests were devised to compare strains bearing seven combinations of single, double, and triple deletions of TRK₁, TRK₂, and HAK₁ genes (see Fig. 2), as well as two combinations with TOL₁ deleted. Seven-μl aliquots of conidial suspensions (at 10⁷ conidia/ml) were pipetted onto 2% agar plates containing K⁺-free Vogel’s salts, 0.2% glucose, 3% sorbose (to enforce colonial growth), and KCl at 0.03, 0.1, 1, 3, or 10 mM. Plates were incubated at 30°C for 3 days and scanned on a CanoScan 8400F digital scanner (Canon U.S.A., Inc., Lake Success, NY) at 600 dpi.

Batch cultures were used to quantitate growth and the specific potassium uptake of strains {H9004/H9004/H9004} to 63.5 mM. For all experiments, the ligation mixtures were transformed into E. coli electrocompetent cells (DH10B; 18290-015; Life Technologies/Invitrogen Corp., Grand Island, NY), and the amplified plasmids, sequenced to ensure fidelity, were linearized by incubation with the appropriate restriction enzymes, MluI for TRK₁-HA and KpnI for HA-HAK₁. A homemade high-fidelity DNA polymerase (generous gift of Efim Golub, Department of Genetics, Yale University) was used to carry out all of the PCRs; T4 DNA ligase and restriction endonucleases were purchased from New England Biolabs Inc., Beverly, MA.

**Transformation and genotyping.** The TRK₁-HA and HA-HAK₁ genes were transformed into strains [ΔΔΔ mus] and [ΔΔΔ mus his-3] by electroporation. Stock cultures of the recipient strains were grown on Vogel’s medium plus 2% sucrose with or without 0.5 mg/ml histidine. Conidia from 5- to 7-day-old slants were harvested in sterile distilled water, washed, transferred to fresh medium, and germinated for 2 h at 30°C (50 ml of medium containing 5 × 10⁷ conidia/ml on an orbital shaker at 250 rpm). The germinated conidia were harvested by centrifugation (3,000 × g for 5 min), washed twice with 10 ml 1 M sorbitol, and resuspended at a density of 10⁶ conidia/ml. In each transformation, a 10-μl aliquot was mixed with 1 to 3 μg of linearized DNA, transferred into an ice-cold 0.2-cm electroporation cuvette, and electropulsed via a Bio-Rad Gene Pulser at 1.5 kV (600 Ω and 25 μF; Bio-Rad Laboratories, Hercules, CA).

**Construction of transformation cassettes.** Since deletion of all three K⁺ transporter genes (strains [ΔΔΔ] and [ΔΔΔ mus]) essentially abolishes growth of Neurospora on media containing ≤1 mM KCl (see Fig. 2), growth complementation at low extracellular concentrations of potassium ([K⁺]ₑ) could be used to select for transformation by TRK₁, HAK₁, or their tagged variants in the ΔΔΔ background. Transformation cassettes were designed for exact homologous replacement of the native open reading frames (ORFs). The 5' and 3' untranslated regions (UTRs) of ∼1 kb for each gene were incorporated into the transformation cassettes, such that the introduced tagged genes would be flanked by the intact native 5' and 3' UTRs and thus would be under the control of the native promoters and termination sequences. Two different tags, the HA epitope (YPYDVPDYA) and the FLAG epitope (DYKDDDDK), were tested at a variety of insertion sites. The HA epitope yielded much clearer Western blots than the FLAG epitope for both transporter genes, and the C terminus of TRK₁ and N terminus of HAK₁ proved the best insert sites, so the tagged genes are designated TRK₁-HA and HA-HAK₁, respectively.

In order to assemble the TRK₁-HA cassette, a segment including the 5' UTR, the TRK₁ ORF, and HA was constructed by PCR with wild-type genomic DNA as a template; likewise, a segment of the 3' UTR was amplified by PCR. These segments, bearing an MluI site at the 5' end and also at the 3' end, were spliced, cut with MluI restriction endonuclease, and ligated into the pBl25 vector. The list and explanation of primers (K1-f to K4-r) used in these constructs is given in Table 2. The HA-HAK₁ cassette was assembled similarly, using genomic DNA and the primers (K5-f to K8-r) also shown in Table 2. For both completed constructs, the ligation mixtures were transformed into E. coli electrocompetent cells (DH10B; 18290-015; Life Technologies/Invitrogen Corp., Grand Island, NY), and the amplified plasmids, sequenced to ensure fidelity, were linearized by incubation with the appropriate restriction enzymes, MluI for TRK₁-HA and KpnI for HA-HAK₁. A homemade high-fidelity DNA polymerase (generous gift of Efim Golub, Department of Genetics, Yale University) was used to carry out all of the PCRs; T4 DNA ligase and restriction endonucleases were purchased from New England Biolabs Inc., Beverly, MA.

**TABLE 2 Primers used to construct the transformation cassettes**

| Name* | Sequencea (5'→3') | Region |
|-------|-------------------|--------|
| K1-f  | cacgtCAACGGGTtccagtcaaatgaaagagtgaaagtg | TRK1 5' UTR + TRK1 ORF (from −1694 to 2978) |
| K2-r  | cacgttGAGCCGGTCGctgtggttccgtagaaggtag | TRK1 3' UTR (from 2984 to 4261) |
| K3-f  | cacgttGACGGTGCtcaagccccagctaa | HAK1 5' UTR (from −1325 to 0) |
| K4-r  | cacgttGACGGTTCGctcaagccccagctaa | HAK1 ORF + HAK1 3' UTR (from 1 to 4199) |
| K5-f  | cacgttGACGGTTCGctcaagccccagctaa | TRK1-HA 3' UTR (from −394 to 0) |
| K6-r  | cacgttGACGGTTCGctcaagccccagctaa | PMA1 5' UTR (from −394 to 0) |
| K7-f  | cacgttGACGGTTCGctcaagccccagctaa | PMA1 5' UTR (from −394 to 0) |
| K8-r  | cacgttGACGGTTCGctcaagccccagctaa | PMA1 5' UTR (from −394 to 0) |

a f, forward primer; r, reverse primer.

b Uppercase letters designate the restriction sites used for cloning. Italicics designate start and stop codons. Coordinates for the DNA fragments are defined from 1 = A of the start codons. Underlining designates the coding sequence for the HA epitope.
HA-tagged variants into the *his3* locus, electroporated conidia were spread on histidine-free Vogel’s medium. Because these inserts were designed to reconstitute the *HIS3* wild-type gene, transformants were identified by growth on histidine-free plates. After 3 to 5 days (30°C in the dark), colonies were transferred to slants of the appropriate selective medium (lacking sorbose to allow normal filamentous growth). Homokaryons were obtained by backcrossing, as described by Davis and de Serres (27).

Genotypes of the homokaryotic progeny were verified by PCR via one primer from inside the transformation cassette and one primer outside and using template genomic DNA extracted by the method of Rose et al. (30). Mycelium from an overnight standing culture (1 ml Vogel’s minimal medium at 30°C, ~10 mg wet weight) was removed with a sterile hook, suspended in lysis mix, and broken by a 3-min shock in an SI-D236 cell disruptor (Scientific Industries, Bohemia, NY). The complete lysis mix contained 0.1 M acid-washed glass beads (G-8772; Sigma-Aldrich Inc., St. Louis MO), 0.1 ml lysing buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 1 mM EDTA, 10 mM Tris base, pH 8.0, and NaOH), and 0.1 ml phenol-chloroform (1:1). After brief mixing with 0.1 M Tris–EDTA (TE; pH 8, 1 mM EDTA), the slurry was spun down in a microcentrifuge (16,300 rpm, 5 min), and 0.2 ml of the aqueous phase was mixed with 0.5 ml 100% ethanol. The resulting precipitated DNA was collected by centrifugation, washed in 70% ethanol, and vacuum dried. Dry genomic DNA was resuspended in 50 μl TE, and ~0.2 μg of DNA (volume, ~0.5 μl) was used as the template for PCR (PicoMaxx PCR system 600422; Stratagene, La Jolla, CA).

Isolation of plasma membranes. *Neurospora* plasma membranes were obtained by the method of Bowman et al. (31), with slight modifications. The filtered and rinsed cell pad from each batch culture (see above) was lifted, resuspended in 1.5 ml of digesting buffer (0.59 M sucrose, 5 mM EDTA, 50 mM NaHPO₄ at pH 5.8, 1 mM β-mercaptoethanol [β-ME] plus 4,600 U of snail gut enzyme [G3060-04; U.S. Biological], and incubated for 1 h at 30°C, with occasional mixing. Digestion was terminated by addition of 6 ml of cold buffer, the mixture was spun at 5,000 × g for 5 min, and the pellet was resuspended in 7 ml of stabilizing buffer (0.33 M sucrose, 1 mM EDTA, 0.3% bovine serum albumin fraction V, titrated to pH 7.1 with NaOH; see reference 28) plus protease inhibitor cocktail (PIC, 10 μg/ml each of aprotonin, leupeptin, pepstatin, and chymostatin). The suspension was then homogenized in a glass-and-Teflon tissue grinder and spun at 5,000 × g for 5 min. This pellet extraction cycle was repeated three more times, and the resulting supernatants were pooled and then respun twice, first (at 10,000 × g for 10 min) to remove mitochondria and then (18,500 × g for 20 min) to collect the plasma membranes (PMs). The PM pellet was rinsed in 15 ml of washing buffer (1 mM EDTA-Tris at pH 7.5) plus PIC, by resuspension with the glass-Teflon homogenizer, and was spun down again (18,500 × g, 20 min). The final pellet was resuspended in 100 μl of washing buffer, sampled for quality testing, frozen, and stored at ~80°C. (Our standard assay for quality of *N. crassa* membrane preparations is hydrolytic activity of the plasma-membrane ATPase, NcPma1p [31].)

**Quantitative Western blot analysis.** Samples were first assayed for total protein content by the method of Lowery et al. (32) and then separated by SDS-PAGE (33). Electrophoresis was carried out on precast polyacrylamide gradients (4–15% TGX precast gel 456–1086; Bio-Rad) run for 1.5 h at 100 V, and the resulting gels were electroblotted to polyvinylidene fluoride (PVDF) membranes (IPVH 15150; Millipore). These were blocked by 1 h of incubation with 5% fat-free milk in TS buffer (10 mM Trizma base titrated to pH 7.4 with HCl, 0.9% NaCl, and 0.1% Tween 20) and stained for 1 h (25°C) with primary anti-HA monoclonal antibody (MMS-101P; Covance Corp., Berkeley, CA) and for a second hour (25°C) with secondary anti-mouse antibody coupled to horseradish peroxidase (HRP; 4021; Promega Corp., Madison, WI). (MMS-101P proved to be the only non-cross-reacting antibody commercially available. Control tests on wild-type [nontagged] membrane samples found several other commercial preparations to cross-react with an abundant protein running near the mass of NcTrk1p or NcHak1p (~100 kDa).) Blots were visualized using Denville HyGlo ECL reagent (E2400; Denville Scientific, Metuchen NJ) exposed to Kodak XAR autoradiograph film and were imaged on the CanoScan 8400 at 600 dpi. Each row image was analyzed via ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA), measuring band intensities with respect to background levels.

The actual membrane content of HA epitope, whether attached to Trk1p or to Hak1p, was quantitated via reference samples of HA-tagged Pma1p (the plasma membrane ATPase proton pump) from *Saccharomyces cerevisiae*. A membrane-protein suspension in which HA-tagged *S. cerevisiae* Pma1p (ScPma1p) had been established as 20% of the total protein (Coomassie staining; A. B. Mason and K. E. Allen, unpublished data) was loaded onto four lanes of each gel. For quantitation, one was run with four lanes containing ScPma1p N terminally tagged with the HA epitope (red circles) and 2, 3, 4, and 5 μg of membrane protein at 20% purity (A. B. Mason and K.E. Allen, unpublished data), representing 4, 6, 8, and 10 fmol of HA, respectively, attached to Pma1p. The test Neurospora membrane preparations contained 2.5 to 15 μg of membrane protein (black circles), including HA-tagged Hak1p. (A) Blots showing the four calibrating loads of *Saccharomyces* membrane protein and several test loads of *Neurospora* membrane protein. Samples of *Neurospora* were starved of K⁺ for 0.5 to 5 h and then refed 10 mM K⁺ for 1 to 5 h. (B) Plotted points indicate digitization of the bands shown in panel A. The smooth curve indicates the least-square fit of a simple logarithm function to the calibrating points (red). Test points (black) are plotted as per-band intensity (ordinate) and are measured in fmol (abscissa). (Inset, lower right) Single blot comparing *Saccharomyces* HA-Pma1p to the two Neurospora proteins of interest here.

![Figure 1](image_url)
Neurospora and were carried out on strains deleted of the gene (cf. (34, 36)), is useful as a strong, constitutive promoter (37, 38). A plasmid bearing the PMA1 promoter was constructed from plasmid pMF272 (originally designed to target the his3 locus (39)) by modification as follows. A segment containing the ccg-1 promoter, the multiple cloning site (MCS), and SGFP (encoding s-green fluorescent protein) was excised via NotI/EcoRI restriction and replaced with a short fragment containing the same MCS flanked by NotI and EcoRI sites, thus making plasmid pAR282. Separately, a 1-kb segment of DNA located upstream of the NcPMA1 start codon, and designated here Ncpp, was amplified by PCR using genomic DNA from wild-type strain 2489A as the template, along with the PPI-1/PP2-1 primer pair (Table 2). After restriction with NotI/XbaI, this was cloned into pAR282 to give plasmid pAR283. Untagged and HA-tagged versions of both TRK1 and HAK1 were cloned into pAR 283 following XbaI/Xmal cuts for TRK1 and following XbaI/EcoRI cuts for HAK1. The resulting four plasmids were linearized with Drai endonuclease before transformation of the strain (Table 1).

RESULTS

Growth experiments. Expression cloning of Neurospora DNA in a K⁺-deficient strain of Saccharomyces cerevisiae (24, 40) and homology searching in the completed N. crassa genome sequence (25, 41–43) have together revealed three K⁺ transport genes in Neurospora: TRK1, TRK2, and HAK1. To gain an initial idea of the physiological roles of the corresponding transport proteins, combination knockout strains were constructed and tested in drop colony growth experiments over a range of extracellular K⁺ concentrations (0.03 to 10 mM) (Fig. 2A). TRK1, expressed in strains with Δ(TRK1)+ and Δ(TRK2)+, supported robust growth down to 0.1 mM [K⁺]o, while HAK1, expressed in strains Δ(TRK1)+ and Δ(TRK2)+, was significantly less effective, yielding robust growth only down to 0.3 mM [K⁺]o. Deletion of TRK1 from HAK1 strains reduced both colony size (diameter) and mycelial density (reflectance) compared to the control colonies (cf. Δ(TRK1)+ with Δ(TRK1)+ or Δ(TRK2)+ with Δ(TRK1)+ for [K⁺]o of 0.3 mM). The reciprocal deletions of HAK1 had no such effects on TRK1+ colonies. TRK2, expressed alone in strain Δ(TRK1)+, was unable to support growth on low K⁺ and also failed to augment growth due to either TRK1 or HAK1 (cf. Δ(TRK1)+ with Δ(TRK1)+ or Δ(TRK2)+ with Δ(TRK1)+) for this reason, all further experiments (see Fig. 4 to 10) focused on TRK1 and HAK1 and were carried out on strains deleted of the TRK2 gene.

Two other K⁺ transport mechanisms did not play a significant role under the conditions used below and were not considered further: (i) a separate low-affinity system for potassium uptake, indicated by the sharp increase of colony sizes and densities at high [K⁺]o (≥3 mM), especially in strains Δ(TRK1)+ and Δ(TRK2)+, and presumed to be analogous to the Nsc pathway previously described in patch-clamp studies of S. cerevisiae (44, 45), and also (ii) a Neurospora Tok1 K⁺ channel, expected to be a strong outward rectifier and not to allow significant K⁺ influx, again analogously to its homologue in yeast (46–48). Confirmation of the lack of effect of these channels on K⁺-dependent growth was obtained from experiments on several strains deleted of TOK1, as illustrated, for example, in Fig. 2B.

For a quantitative study of cellular dependence on the two main K⁺ transporters, strains carrying hemagglutinin (HA)-tagged versions of the TRK1 and HAK1 genes were constructed (Table 1) and tested for the ability to grow exponentially in liquid suspension cultures. In the experiment shown in Fig. 3, freshly harvested conidia of N. crassa were rinsed and resuspended in minimal medium at 25°C, where they displayed a typical 3- to 4-h lag before germinating. Cells expressing only TRK1 then grew exponentially at the same specific rate (α) of 0.274 h⁻¹ (doubling time of 2.53 h) at starting [K⁺]o as low as 0.3 mM (blue circles), falling slightly to 0.259 h⁻¹ at 0.1 mM [K⁺]o (green squares). In contrast, cells expressing only HAK1 slowed to a specific growth rate of 0.182 h⁻¹ at 0.3 mM [K⁺]o (red circles) and 0.144 h⁻¹ at 0.1 mM [K⁺]o (data not shown). For cells deleted of both TRK1 and HAK1 and inoculated into [K⁺]o at or below 1 mM (grey triangles), growth was barely detectable and could be described as either quasieponential, α < 0.08 h⁻¹, or apparently linear, ~0.6 mg dry weight/h. These results quantitatively confirm the advantage of Trk1p over Hak1p at low [K⁺]o. Further implications of the growth results, including the time courses of intracellular K⁺ concentration ([K⁺]i) and net fluxes, are considered later (see Fig. 8).

Regulation of HAK1 and TRK1 expression. Most of the Neurospora strains listed in Table 1, including those used for the liquid growth experiments shown in Fig. 3, were constructed explicitly to
allow expression analysis via Western blotting, making use of the HA epitope. Four clear results emerged, which are qualitatively demonstrated in the blots of Fig. 4: (i) expression of Trk1p was only weakly affected by \([K^+]_0\) in the growing cell suspension, whereas (ii) expression of Hak1p was strongly inhibited by 10 mM \([K^+]_0\) and almost completely suppressed by 100 mM \([K^+]_0\). Furthermore, (iii) coexpression of HAK1 had no significant effect on the cellular level of Trk1p, but (iv) coexpression of TRK1 clearly accentuated the inhibitory effect on Hak1p by elevated \([K^+]_0\). In other words, regulation of these two \(K^+\) transport proteins in Neurospora is in no way reciprocal; when the TRK1 gene is present behind its native promoter, it is dominant and sufficient down to the lowest tolerable \(K^+\) levels (Fig. 3). The HAK1 system is more closely tied to Neurospora’s metabolic regulatory program, but it becomes useful mainly when Trk1p is absent or blocked or when other metabolic limitations are imposed, e.g., carbon starvation (see below).

Quantitative analysis of these results required careful calibration of the Western blots, and for this purpose, HA-tagged proton pump protein (Pma1p) from Saccharomyces cerevisiae was used as the standard (see Materials and Methods). Data averaged from three sets of gels similar to those of Fig. 4 yielded the plots of Fig. 5, showing not only that Trk1p is dominant and insensitive to the presence of the HAK1 gene or protein but also that it is scarce, being present at only 1 to 2 fmol per \(\mu\)g of membrane protein (Fig. 5A), at any value of \([K^+]_0\), compared to more than 10-fold that level of Hak1p (Fig. 5B) for low values of \([K^+]_0\). However, as expected from the results shown in Fig. 4, Hak1p was suppressed to very low levels by either elevated \([K^+]_0\), or expressed TRK1 gene/protein.

These results were further emphasized by several experiments with Ncpp, the promoter that normally controls expression of the gene encoding Neurospora’s plasma membrane proton pump (NcPMA1). Ncpp itself is not sensitive to \([K^+]_0\), whether intracellular or extracellular, but it is a routine strong promoter (37, 38). Western blot analysis of HA-tagged Trk1p and Hak1p, expressed behind Ncpp rather than their native promoters, found Ncpp to increase Trk1p approximately 10-fold in isolated plasma membranes, regardless of the potassium level (Fig. 6A). But, Ncpp not only failed to increase Hak1p in membrane preparations but actually decreased expression (Fig. 6B) at all \([K^+]_0\) values below 100 mM; evidently, the native Hak1 promoter is more effective than Ncpp, especially with low extracellular potassium. Cross-expression experiments were fully consistent with these findings. That is,
FIG 6 A strong promoter (Ncpp) enhances expression of TRK1 protein but depresses expression of HAK1 protein. (A) Western blot analysis of HA-tagged Trk1p produced via the native NcTRK1 promoter (strain {TRK1-HA}) or via the Ncpp promoter (Ncpp; strain {NcppTRK1-HA}). Steady-state enhancement by Ncpp was ~5-fold at low \([K^+]_o\), and ~10-fold at high \([K^+]_o\). For the inset, the load is 20 \(\mu\)g membrane protein in each lane. (B) Western blot analysis of HA-tagged Hak1p produced via the native NcHAK1 promoter (strain {HA-HAK1}, NcPMA1; strain {NcppHA-HAK1}; \(\bullet\)). Quantitative gels from two separate experiments were scaled (to values at \([K^+]_o\), of 1 mM) and averaged. Note that steady-state depression by Ncpp, about 2.5-fold at low \([K^+]_o\), was released at high \([K^+]_o\). For the inset, the load is 1.5 \(\mu\)g for each lane.

FIG 7 Rapid shifts of potassium-ion concentration affect Hak1p expression only slowly and do not affect Trk1p expression at all. Time courses of Trk1p-HA (A, \(\bullet\)) and HA-Hak1p (B, \(\bullet\)) and of intracellular \([K^+]_i\) during potassium starvation (0 to 5 h), followed by potassium repletion (5 to 10 h). Removal of \([K^+]_o\) (harvest, rinse, and resuspend) required less than 1 min, which was very fast compared to the decline of \([K^+]_i\). For strains {TRK1-HA} and {HA-HAK1}, the preparative growth medium was \(K^+\)-free Vogel’s salts plus 100 mM KCl and 2% sucrose. For the insets, the load was 5 \(\mu\)g membrane protein in each lane. High-sensitivity ECL reagent was used for panel A.

Modulation of cellular \(K^+\) content, fluxes, and affinities. Apart from questions about molecular details, there are two principal modes for regulating protein function: (i) raising or lowering the amount of the specified protein present, and (ii) modulating the kinetic properties of that protein. Mode-i changes for Trk1p and Hak1p are described in Fig. 4 to 7, especially in response to different potassium regimens. Mode-ii changes can be extracted, in part, from additional properties of exponentially growing cells, e.g., from the time courses of intracellular potassium and of the fluxes implied by the measured cell mass and \([K^+]_i\). Cells sustaining the maximal growth rate (\(a = 0.274 \text{ h}^{-1}\)) had stabilized \([K^+]_i\) within the control range, 165 to 190 mM, by 90 min after the onset of exponential growth (Fig. 8A, triangles and blue circles) and were taking up net \(K^+\) at ~50 mM/h (Fig. 8B), or at approximately 1 mM/min, the previously established requirement for optimal growth of Neurospora (51, 52). However, potassium-limited cells (green squares, red circles, and grey triangles) were characterized by lowered \([K^+]_i\), values at the onset of exponential growth (viz., by extra leakage of potassium from the conidia during the lag phase), by progressive decline of \([K^+]_i\), as growth continued, and by falling net influx (Fig. 8B) as extracellular \([K^+]\) declined due to elevating the level of Trk1p ~10-fold by means of expression behind Ncpp further depressed the expression levels of Hak1p (data not shown).

Figures 4 to 6 demonstrate steady-state regulation of Hak1p versus Trk1p over a time scale of hours during exponential growth, but those data say nothing about how rapidly regulatory shifting occurs when the extracellular potassium is suddenly raised or lowered. Figure 7 addresses that question for both transporter proteins. Cells of strain {HA-HAK1}, which had been pregrown in 100 mM \(K^+\) to be optimally loaded with potassium, were quickly rinsed and resuspended in \(K^+\)-free medium (Fig. 7B). Intracellular \([K^+]_i\) had declined by ~65% when the first measurements were made, 30-min postrinse, and rose almost equally fast when \([K^+]_o\) was restored to 100 mM at 5 h. However, the corresponding upregulation and downregulation of HA-tagged Hak1p required several hours, rising with an apparent time constant \(\tau\) of 3.44 h (half-time of 2.37 h) and falling with a \(\tau\) of 1.60 h (half-time of 1.10 h). These numbers can be compared to time constants of ~20 min for activating or deactivating high-affinity transport of either glucose or ammonium ions, induced by the appropriate starvation or replenishment (49, 50). In the obvious control experiment on {TRK1-HA}, HA-tagged Trk1p barely responded to potassium depletion, as is shown in Fig. 7A.
continued uptake. Cells with the most severe potassium limitation, lacking both $K^+$ transporters and started at $[K^+]_0$ of 1 mM or lower (leftmost of Fig. 8B, sink into several hours of actual $K^+$ depletion by growth. In Fig. 9B and C, strains bearing TRK1-HA and/or HA-HAK1 were inoculated into $K^+$-free Vogel's medium plus 0.1 mM KCl. Loss of $K^+$ from the conidia during the lag phase had slightly increased extracellular $K^+$ at time zero, but the onset of exponential growth was accompanied by $K^+$ influxes of $\sim 45$ mM/h in the presence of Trk1p with or without Hak1p (Fig. 9B) and $\sim 18$ mM/h in the presence of Hak1p alone (Fig. 9C). In both cases, the initial plots of flux versus $[K^+]_o$, were hyperbolic but were offset from the origin (0 μM) by 49.7 μM for Trk1p and 103.6 μM for Hak1p, corresponding to projected growth times of $\sim 5.5$ and $\sim 1.9$ h, respectively. However, as $[K^+]_o$ approached those offset values, the fluxes stabilized at $\sim 9$ mM/h with Trk1p, with or without Hak1p, and at $\sim 3.2$ mM/h with only Hak1p. The stabilized fluxes continued for several hours, to the end of exponential growth, despite continually declining extracellular potassium. Progressive, adaptive, increasing affinity of both transporters for substrate ($K^+$) thus is a necessary inference. (HAK protein made no significant contribution either to mass expansion or to $K^+$ net flux in the presence of Trk1p. That is, Trk1p-HA expression was equivalent to that of Trk1p-HA plus HA-Hak1p. It is not known whether Hak1p was deactivated or simply insignificant when present under these conditions.)

Quantitatively, these results reemphasize that growth of $K^+$-limited (or $K^+$-starved) cells of *N. crassa* is better supported via Trk1p than via HAK protein; the effective $K_m$ for $K^+$ transport is lower and the effective $V_{max}$ is higher with Trk1p than with Hak1p when each is tested separately. Finally, it is clear that exponential growth of *Neurospora* is not rigidly dependent upon either the net $K^+$ influx or the actual intracellular concentration.

Cross-pathway effects: carbon starvation. The first demonstration that transport of sugars and other neutral molecules can be unequivocally electroforethoretic (55) solidified the notion of membrane transport within the complex of metabolic reactions then emerging as targets for glucose regulation, especially in *N. crassa* (56–58). Glucose withdrawal leads to the upregulation of high-affinity glucose transport (55, 56), to proliferation of vacuoles (59), to enhancement of cytoplasmic buffer capacity (D. Sanders and C. L. Slayman, unpublished results), and probably, as in *Saccharomyces* (60), to gradual disassembly of the vacuolar ATPase (B. Bowman, unpublished results). In addition, full carbon starvation increases membrane resistivity (49, 50), thereby magnifying voltage changes associated with active transport of potassium ions (5). The latter fact affords a robust approach for quantitative study of the $K^+$ transporters in *Neurospora* (A. Rivetta, K. E. Allen, and C. L. Slayman, unpublished data), but accurate analysis requires knowledge of the separate effects of starvation upon the two proteins Trk1p and Hak1p.

This information was obtained by a simple extension of the experiment shown in Fig. 7, removing extracellular sugar from the mycelial suspension after a suitable period of starvation for $K^+$. As shown in Fig. 10A, tagged Trk1p declined with a time constant of 2.8 h, corresponding to a half-time of 1.9 h. Neither the rate nor the apparent endpoint was influenced by the presence (circles) or absence (triangles) of HAK1/Hak1p. However, the observed decline of Trk1p-HA during pure $K^+$ starvation was unexpectedly fast (Fig. 10A, dashed line) and not distinguishable from that with sugar starvation.

Hak1p itself, on the other hand, barely responded to 1 h of pure $K^+$ starvation (Fig. 10B) but then rose from control values around 15 fmol/μg membrane protein to steady values near 65 fmol/μg membrane protein over the 3 h of carbon starvation (glucose removal). Although the presence (circles) or absence (squares) of

---

**FIG 8** Time courses of intracellular $K^+$ concentration and net flux during exponential growth of *Neurospora* on limiting potassium. The experiment and measurements were the same as those described for Fig. 3. (A) Cell-associated potassium measured and $[K^+]_o$ calculated as described in Materials and Methods. Smoothed curves were drawn by hand. Different starting values of $[K^+]_o$, reflect net loss of cellular potassium during the lag phase (4 to 0 h). Scatter of measured $[K^+]_o$, values (± 1 SD) was $\sim 20\%$ of the mean value. (B) Implied net fluxes were calculated from the increment of total cellular potassium over each successive 16-min interval along the appropriate smooth curve in panel A and then divided by the average cell mass during the same interval along the corresponding fitted curve in Fig. 3. Positive values represent net influx.

---
TRK1/Trk1p did not affect the increase of Hak1p associated with carbon starvation (~50 fmol/µg membrane protein), TRK1-Trk1p did lower the control value of Hak1p at the onset of K+ starvation by approximately 70%. This result is qualitatively consistent with the results shown in Fig. 5.

Overall, the Hak1p:Trk1p ratio, which varied in the range of 10:1 to 30:1 (depending mainly on K+ repletion) in normally grown Neurospora mycelium (Fig. 5, 6, and 8), was increased to ~500:1 by the regimen of serial starvation for K+ and carbon. This emphasizes again that HAK1-Hak1p is fundamentally incorporated into the metabolic program of Neurospora in a manner that TRK1-Trk1p is not.

DISCUSSION

Basic conclusions. Both of the TRK and HAK families of transporter genes are distributed widely in the plant and fungal kingdoms (61–64), and they can be complemented by two families of cation-pumping ATPases (10, 11), although the latter are not present in Neurospora (65). In higher plants, regulation of K+ uptake involves not only several isoforms of the HAK and/or TRK proteins but also many specialized tissues and organs. Regulation in these systems therefore must be very complex. On the other hand, regulation in the best-known simple eukaryotic system, the yeast S. cerevisiae, has evolved mainly around two K+ transporters, both of the TRK family, while completely excluding the HAK family. N. crassa, in possessing K+ transporters from both families while having only moderate cytological specialization, affords intermediate complexity for characterizing and modeling transporter function and regulation. The principal results, presented in Fig. 4 to 7, are simple, clear-cut, and quite distinct from most previous reports on plants or fungi.

The important background physiological conditions are ample carbon source, moderated salts (especially 63.5 mM Na+ and 25 mM NH4+), and pHo buffered (citrate/phosphate) in the range of 5.8 to 5.3, well within the broad optimum for K+ transport in N. crassa (66). Under these conditions, Neurospora Trk1p is kinetically a more effective transporter than Hak1p, in having both a higher per-site turnover number and a lower Michaelis constant (K0.5) for substrate K+ (Fig. 2, rows 1 to 3 and columns 2 and 4). It also supports robust growth below 100 µM K+, compared with ~300 µM for Hak1p. Otherwise put, Trk1p provides for stable potassium accumulation in Neurospora while behaving like a classic constitutive enzyme: it is expressed at a nearly constant level over wide-ranging K+ concentrations and can even override sudden shifts of concentration (Fig. 7A), but it is susceptible to approximately 10-fold depletion during energy limitation (i.e., carbon starvation) (Fig. 10A). In absolute terms, Trk1p expression is low in Neurospora, in the range of 1 ± 0.5 fmol/µg membrane protein, which translates to ~4 nmol Trk1p per liter of intracellular water (ICW). Since the net flux of ~50 mmol/h - liter ICW
Scarce TRK1 Dominates Abundant HAK1 in *Neurospora*

Figure 10: Carbon starvation demonstrates further involvement of HAK1-Hak1p in the metabolic program of *N. crassa*. Time courses of TRK1p-HA depression (A) and HA-Hak1p enhancement (B) during 1 h of starvation for K⁺, followed by 3 h of starvation for sugar and K⁺. Note that simultaneous expression of both genes, TRK1 and HAK1, appeared irrelevant to the effect of carbon starvation on the expression time course of either protein, even though the steady-state prestarvation level of Hak1p was significantly reduced by co-expression of TRK1. The general protocol was similar to that described for Fig. 7, starting with cells grown for 16 h in Vogel’s medium plus 2% sucrose. The smooth curve shown in panel A is least-square fitted to all 10 data points; the dashed line represents the expected time course of TRK1p-HA during the hour of starvation for K⁺ alone, based on data from Fig. 7A. Smooth curves shown in panel B were drawn by hand and are identical, except for the vertical separation of 12.6 fmol. For the panel A inset, the load is 15 fmol/μg membrane protein in each lane; for the inset in panel B, the load is 1 μg membrane protein in each lane.

(FIG. 8B, blue circles and triangles) equals 14 μmol/s · liter ICW, a per-site transport number of 3.5 × 10⁷ K⁺ ions/site · s is implied, which is high for conventional ion transporters but very low for conventional ion channels.

Hak1p, on the other hand, behaves like a classic derepressible enzyme in *Neurospora*, present at low levels (1 to 5 fmol/μg membrane protein; Fig. 5B) when its substrate is available at high concentration but elevated 10-fold or more when substrate is scarce and when Trk1p is missing (Fig. 4 and 5B). Its implied per-site turnover number, in sustained support of exponential growth (as in Fig. 8B), lies in the range of 1 × 10⁵ to 3 × 10⁷ K⁺ ions/site · s, appropriate for a conventional ion transport protein. However, carbon starvation, on top of K⁺ limitation, can drive up the expression of Hak1p by another order of magnitude (Fig. 10B), and under that condition, Hak1p becomes the dominant K⁺ uptake protein in *Neurospora*, by a factor of >500, over Trk1p. Since carbon starvation was the practical background condition for discovery of membrane currents due to K⁺-H⁺ cotransport (2, 5), it is clear that Hak1p must have been the responsible protein. This inference was also reached earlier by Haro et al. (24), who compared Northern blot measurements in *Neurospora* to transport measurements on *NcHak1p* heterologously expressed in *Saccharomyces*. A similar relationship between Trk1p and Hak1p has also been described recently for the methylophytic yeast *Hansenula polymorpha* (67). Present data do not rule out the possibility that Trk1p also carries out H⁺-K⁺ cotransport, but they do make that mode unnecessary. Detailed quantitative analysis of the underlying electrical data is needed to clarify the actual functioning of both transport proteins (Rivetta et al., unpublished).

Adaptations. Mechanistically more mysterious is the fact that both TRK1p and Hak1p are able to increase their affinities (or decrease their Michaelis constants) for the transported substrate, as K⁺ limitation becomes increasingly severe. Such a process had been implied before (53, 54, 68, 69); here, it is demonstrated and quantitated (Fig. 9B and C). We have no explanation for why the velocity-versus-concentration plots (flux versus [K⁺]o) are offset from the origin, but before zero is actually reached the proteins appear to change, stabilizing their fluxes at low values that are still sufficient for exponential growth. This condition of TRK1p and Hak1p might be termed the sliding-affinity mode; it occurs without perturbing the effectiveness of TRK1p compared to that of Hak1p. The growth studies (Fig. 3) showed that Trk1p can take up potassium as fast from ~0.1 mM [K⁺]o (10 to 11 mM/H at 5 h; Fig. 3C) as can Hak1p at ~0.3 mM [K⁺]o and in the sliding-affinity mode, Trk1p stabilizes at a 3-fold higher net flux than Hak1p, while it also starts the process at a lower value of [K⁺]o. The origin of these changes, viz., the binding protein(s) presumed to be co-actively involved, remains to be identified.

However, elevation of membrane voltage, such as has been demonstrated to occur during carbon starvation (50, 55), could have exactly that effect on transporter kinetics. This possibility has been described at least twice in theory (70, 71) and has previously been argued to account for flux data in *S. cerevisiae* (72). Elevation of membrane voltage during carbon starvation results from increased membrane resistance, which in turn reflects metabolic downregulation of temporarily unneeded membrane transporters. Such behavior is an energy shift device to enhance the cells’ ability to scavenge the depleted nutrient, namely, sugars in the case of carbon starvation and potassium, we suggest, in the present experiments.

Adaptation to sustained potassium limitation is certainly a property of the whole organism, not solely of the K⁺ transporters. Cells expressing Trk1p and germinated in 0.1 mM K⁺ sustained their starting exponential growth rate (α = 0.259 h⁻¹) (Fig. 3, green squares) for more than 8 h despite the ~50% decline of intracellular [K⁺] that accompanied cellular volume expansion and reduction of net influx (Fig. 8A and B) due to extracellular [K⁺] depletion. Similar adaptation took place in cells expressing only Hak1p (Fig. 3, red circles) but with lower absolute rates, viz., α = 0.188 h⁻¹ started in 0.3 mM K⁺ (Fig. 8) and α = 0.144 h⁻¹ in 0.1 mM K⁺ (data not shown). Even cells missing both defined K⁺ transporters ([ΔΔΔΔ μs]) grew exponentially when constrained on low K⁺ (Fig. 3, triangles), though very slowly, as [K⁺], continued to fall. In this case, residual K⁺ flux necessarily occurred through a low-affinity/nonselective pathway, probably corresponding to that doubled NSC1 in *Saccharomyces* (44, 45). That path would carry current under voltage clamp conditions, but...
probably also would mediate ion exchange: \( K^+ \) for \( H^+ \), \( Na^+ \), amino cations generally, and \( NH_4^+ \) in particular (52, 72). It is likely that ammonium ions (with a crystal radius of 1.48 Å) substituted for potassium ions (crystal radius of 1.33 Å) in those enzymes whose activities normally make use of bound \( K^+ \) (74); \( NH_4^+ \) was abundant (25 mM) in the low-\( K^+ \) media employed in these experiments. However, ionic substitution also could have been more general, since other fungi, especially \( S. \) cerevisiae, have been found to grow when intracellular \( K^+ \) is replaced by a variety of alkali and alkali-metal cations (73, 75, 76).

**Signaling.** The liquid growth measurements (Fig. 3), together with simultaneous potassium determinations (Fig. 8A), show that the growth rate in \( Neurospora \) is not dependent on intracellular \([K^+]_p\) per se; rather, it is dependent on extracellular \([K^+]_o\). An important implication of this finding is that potassium’s primary effect in metabolic regulation, at least in the presence of abundant ammonium ions, should be on signaling rather than upon simple enhancement of cytoplasmic enzyme activities. The same conclusion has also been reached via theoretical analysis of \( K^+ \) transport in \( Saccharomyces \) (77, 78). That seems like a sensible arrangement; given that \( K^+ \) is a bulk constituent of cytoplasm (also partially sequestered in vacuoles), its actual concentration should respond only slowly to threats. However, a sudden drop of \([K^+]_o\) itself would be a direct threat. We have made some effort, thus far unsuccessful, to discover possible signaling vectors. In particular, deletion experiments have failed to show involvement of either \( Neurospora \)’s silent \( K^+ \) transporter (Trk2p) or its plasma membrane \( K^+ \) channel (Tok1p) in modulating growth itself or the particular properties of Trk1p or Hak1p.

Further attempts to investigate the primary signaling mechanisms in potassium homeostasis will attempt to bypass the so-called huggingness feature of most metabolic networks and focus on those circuits that are central to \( K^+ \) homeostasis while avoiding minor paths and side branches. Potassium accumulation in this particular model organism, \( N. \) crassa, is propitious for critical pathway mapping for several reasons: (i) only two effector proteins are involved in \( K^+ \) uptake; (ii) those proteins behave in iden
tifiably different fashions; (iii) a single energizing protein (Pma1p) drives transport through both effector proteins (via membrane voltage) and must be coregulated; and (iv) only the efflux pathway(s) remains uncertain. Changes in the expression of Pma1p still need to be characterized, but the integrated system regulating \( K^+ \) content of \( Neurospora \) inversely adjusts the amount of Hak1p present, while holding Trk1p nearly constant, under varied conditions of potassium starvation or repletion. The same system clearly enhances expression of Hak1p and suppresses expression of Trk1p under carbon starvation. Modulations of Trk1p, Hak1p, and Pma1p under other conditions, e.g., osmotic stress, ammonium starvation, and \( pH \) stress, still need to be characterized, but the main thrust for future experiments is that genes/proteins central to the regulation of \( K^+ \) transport should, when perturbed, displace all three effector proteins in predictable, coordinated fashion. This idea is a kind of protein triangulation.

There are now two main conduits for such investigation. The most direct is to screen for transcription factors which modulate pHAK, the promoter element for the HAK1 gene. This can be done, starting with [HAK1], by inserting a selectable reporting marker behind pHAK in the HAK1 locus and then subjecting the strain to insertional mutagenesis. By varying background \([K^+]_o\) during selection on the reporter agonist, this procedure can be biased to identify mutants that either enhance or suppress the activity of pHAK. Alternatively, \( Neurospora \) homologues of genes that have been implicated in \( K^+ \) regulation elsewhere can be tested in deletion and/or overexpression experiments. Prime candidates at present are two serine/threonine (S/T) protein kinases, Hal4p and Hal5p, described as positive regulators of the TRK proteins in \( Saccharomyces \) (79, 80), and two phosphatase-related peptides, Ppz1p and Ppz2p, plus calcineurin, described as negative regulators (81, 82). Homologues of all of these are present in \( Neurospora \) and can be evaluated by crossing the existing single-gene deletion strains (25) with [HA-HAK1] or [TRK1-HA] or with other constructs described above. A global strategy for characterizing S/T protein kinase genes in \( Neurospora \) has already been described (107 total) (83), and other candidate \( K^+ \) regulatory genes can be extracted from full-genome microarray studies (see, e.g., Tian and collaborators [84, 85]) under different conditions of \( K^+ \) limitation.

**ACKNOWLEDGMENTS**

We are indebted to Brett Mason for calibrated samples of HA-tagged \( Saccharomyces \) PMA1 protein, for use of the CanoScan digital scanner, and for much helpful advice concerning membrane protein assays; to Tong Wang for use of the Instrumentation Laboratory atomic absorption photometer; and to Efim Golub for the high-fidelity DNA polymerase, as well as for advice on bacterial transformations.

The work was supported in part by NIH research grant GM60696.

**REFERENCES**

1. Slayman CL, Sanders D. 1985. Steady-state kinetic analysis of an electroenzyme. Biochem. Soc. Symp. 50:11–29.
2. Platt MR, Slayman CL. 1987. Role of “active” potassium transport in the regulation of cytoplasmic pH by non-animal cells. Proc. Natl. Acad. Sci. U. S. A. 84:2737–2741.
3. Schroeder JJ. 1988. \( K^+ \) transport properties of \( K^+ \) channels in the plasma membrane of \( Vicia \) faba guard cells. J. Gen. Physiol. 92:667 –683.
4. Lebady A, Very Sentenac A-AH. 2007. \( K^+ \) channel activity in plants: genes, regulations and functions. FEBS Lett. 581:2357–2366.
5. Rodriguez-Navarro A, Platt MR, Slayman CL. 1986. A potassium-potassium symport in \( Neurospora \) crassa. J. Gen. Physiol. 87:649–674.
6. Maathuis FJM, Sanders D. 1994. Mechanism of high-affinity potassium uptake in roots of Arabidopsis thaliana. Proc. Natl. Acad. Sci. U. S. A. 91:9272–9276.
7. Rubio F, Gassmann W, Schroeder JJ. 1995. Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. Science 270:1660–1663.
8. Rodriguez-Navarro A, Rubio F. 2006. High-affinity potassium and sodium transport systems in plants. J. Exp. Bot. 57:1149–1160.
9. Arino J, Ramos J, Sychrová H. 2010. Alkali metal cation transport and homeostasis in yeasts. Microbiol. Mol. Biol. Rev. 74:95–120.
10. de Souza FSJ, Gomes SL. 1998. A P-type ATPase from the aquatic fungus Blastocladiella emersonii similar to animal Na,K-ATPases. Biochim. Biophys. Acta 1383:183–187.
11. Benito B, Garciaedelas B, Schreier P, Rodriguez-Navarro A. 2004. Novel P-type ATPases mediate high-affinity potassium or sodium uptake in fungi. Eurycaryot. Cell 3:359–368.
12. Jardetzky O. 1966. Simple allosteric model for membrane pumps. Nature 211:969–970.
13. Abramson J, Smirnova I, Kasho V, Verner G, Kaback HR, Iwata S. 2003. Structure and mechanism of the lactose permease of \( Esherichia coli \). Science 301:610–615.
14. Law CJ, Maloney PC, Wang D-N. 2008. Ins and outs of major facilitator superfamily antiproters. Annu. Rev. Microbiol. 62:289–305.
15. Smirnova I, Kasho V, Kaback HR. 2011. Lactose permease and the alternating access mechanism. Biochemistry 50:9684–9693.
16. Durell SR, Guy HR. 1999. Structural models of the KtrB, TrkH, and Trk1,2 symporters, based on the crystal structure of the KcsA \( K^+ \) channel. Biophys. J. 77:789–807.
17. Durell SR, Hoo Y, Nakamura T, Bakker EP, Guy HR. 1999. Evolutionary relationship between K+ channels and symporters. Biophys. J. 77:775–788.
18. Doyle DA, Cabral JM, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, MacKinnon R. 1998. The structure of the potassium channel: molecular basis of K+ conduction and selectivity. Science 280:69–76.
19. Tholema N, Bakker EP, Suzuki A, Nakamura T. 1999. Change to alanine of one out of four selectivity filter glycines in KtrB causes a two orders of magnitude decrease in the affinities for both K+ and Na+ of the Na+-dependent K+-upstream system KtrAB from Vibrio alginolyticus. FEBS Lett. 450:217–220.
20. Kato Y, Sakaguchi M, Mori Y, Saito K, Nakamura T, Bakker EP, Sato Y, Goshima S, Uozumi N. 2002. Evidence in support of a four-transmembrane domain topology for the Arabidopsis thaliana Na+/K+ translocating AtHKT1 protein, a member of the superfamily of K+ transporters. Proc. Natl. Acad. Sci. U. S. A. 99:6428–6433.
21. Mäser P, Hosoo Y, Goshima S, Horie T, Eckelman B, Yamada K, Yoshida K, Bakker EP, Shinmyo A, Oiki S, Schroeder JI, Uozumi N. 2002. Glycine residues in potassium channel-like selectivity filters determine potassium selectivity in four-loop-per-subunit KHT transporters from plants. Proc. Natl. Acad. Sci. U. S. A. 99:6428–6433.
22. Zeng G-F, Pypaert M, Slayman CL. 2004. Epitope tagging of the yeast K+–carrier, TRK2, determines fractions which consist with a channel-like structure. J. Biol. Chem. 279:3003–3013.
23. Cao Y, Jin X-S, Huang H, Derege MG, Levin EJ, Kabaleeswaran V, Pan Y-P, Punta M, Love J, Weng J, Quick M, Kloss B, Bruni R, Martinez-Hackett E, Hendrickson WA, Rost B, Javitch JA, Rajashankar RK, Jiang Y, Zhou M. 2011. Crystal structure of a potassium ion transporter TrkH. Nature 470:519–523.
24. Haro R, Sainz I, Rubio F, Rodriguez-Navarro A. 1999. Cloning of two genes encoding potassium transporters in Neurospora crassa and expression of the corresponding cDNAs in Saccharomyces cerevisiae. Mol. Microbiol. 31:511–520.
25. Colot HV, Park G, Turner GE, Ringelberg C, Crew CM, Litvinkova L, Weiss RL, Borkovich KA, Dunlap JC. 2006. A high-throughput gene knock-out procedure for Neurospora reveals functional connections for multiple transport factors. Proc. Natl. Acad. Sci. U. S. A. 103:10353–10357.
26. Nomiyama Y, Suzuki K, Iishi C, Inoue H. 2004. Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining. Proc. Natl. Acad. Sci. U. S. A. 101:12248–12253.
27. Davis RH, de Serres FJ. 1970. Genetic and microbiological research techniques for Neurospora crassa. Methods Enzymol. 17A:79–143.
28. Vogel HJ. 1956. A convenient growth medium for Neurospora (Medium N). Microb. Gen. Bull. 13:42–46.
29. Slayman CW, Tatum EL. 1964. Potassium transport in Neurospora. I. Intracellular sodium and potassium concentrations, and cation requirement for growth. J. Gen. Physiol. 49:424–443.
30. Bowman BJ, Bowman BJ, Slayman CW. 1981. Isolation and characterization of plasma membranes from wild type Neurospora crassa. J. Biol. Chem. 256:12336–12342.
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
32. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
33. Wondrack W, Fieser LF, Fieser ML. 1973. The relationship between ATP and an electrogenic pump in the plasma membrane of Neurospora crassa. J. Membr. Biol. 14:305–338.
34. Bowman BJ, Slayman CW. 1977. Characterization of plasma membrane adenine triphosphatase of Neurospora crassa. J. Biol. Chem. 252:3357–3363.
35. Hager KM, Mandalia SM, Davenport JW, Speicher DW, Benz EJ, Jr, Slayman CW. 1986. Amino acid sequence of the plasma membrane ATPase from Neurospora crassa: deduction from genomic and cDNA sequences. Proc. Natl. Acad. Sci. U. S. A. 83:7693–7697.
36. Capiaux E, Vignais ML, Sentenac A, Goffaux A. 1989. The yeast H+–ATPase gene is controlled by the promoter binding factor TFU. J. Biol. Chem. 264:7437–7446.
37. Figler RA, Omote H, Nakamoto RK, Al-Shawi MK. 2000. Use of chemical chaperones in the yeast Saccharomyces cerevisiae to enhance heterolo-
60. Kane PM. 1995. Disassembly and reassembly of the yeast vacuolar H^+-ATPase in vivo. J. Biol. Chem. 270:17025–17032.
61. Rodríguez-Navarro A. 2000. Potassium transport in fungi and plants. Biochim. Biophys. Acta 1469:1–30.
62. Senn ME, Rubio F, Bañuelos MA, Rodríguez-Navarro A. 2001. Comparative functional features of plant potassium HvHAK1 and HvHAK2 transporters. J. Biol. Chem. 276:44563–44569.
63. Britto DV, Kronzucker HJ. 2008. Cellulolar mechanisms of potassium transport in plants. Physiol. Plant 133:637–650.
64. Gomez-Parras JL, Riaño-Pachón DM, Benito B, Haro R, Sklodowski K, Rodríguez-Navarro A. 2011. A sodium and potassium uptake system in fungi, The transporter diversity of Magnaporthe oryzae. Fungal Genet. Biol. 48:812–822.
65. Slayman CW, Slayman CL. 1970. Potassium transport in Neurospora: evidence for a multi-site carrier at high pH. J. Gen. Physiol. 55:758–786.
66. Cabrera E, Alvarez MC, Martín Y, Siverio J, Ramos J. 2012. K^+ uptake systems in the yeast. Biochim. Biophys. Acta 1814:229–237.
67. Chapman JB, Johnson EA, Kootsey JM. 1983. Electrical and biochemical properties of an enzyme model of the sodium pump. J. Membr. Biol. 74:139–153.
68. Sanders D, Hansen U-P, Gradmann D, Slayman CL. 1984. Generalized kinetic analysis of ion cotransport systems: a unified interpretation of selective ionic effects on Michaelis parameters. J. Membr. Biol. 77:123–152.
69. Madrid R, Gómez MJ, Ramos J, Rodríguez-Navarro A. 1998. Ectopic potassium uptake in trk1 trk2 mutants of Saccharomyces cerevisiae correlates with a highly hyperpolarized membrane potential. J. Biol. Chem. 273:14838–14844.
70. Conway EJ, Gaffney HM. 1966. The further preparation of inorganic cationic yeasts and some of their chief properties. Biochem. J. 101:385–391.
71. Page MJ, Di Cera E. 2006. Role of Na^+ and K^+ in enzyme function. Physiol. Rev. 86:1049–1092.
72. Conway EJ, Breen J. 1945. An "ammonia"-yeast and some of its properties. Biochem. J. 39:368–371.
73. Conway EJ, Moore PT. 1954. A sodium-yeast and some of its properties. Biochem. J. 57:523–528.
74. Kschischo M, Kahm M, Navarrete C, Ramos J. 2011. Actuators of yeast potassium homeostasis, p 10. Abstr. 8th Eur. Conf. Math. Theor. Biol.
75. Kahm M, Navarrete C, Llopis-Torregrosa V, Herrera R, Barreto L, Yenush L, Ariño J, Ramos J, Kschischo M. 2012. Potassium starvation in yeast: mechanisms of homeostasis revealed by mathematical modeling. PLoS Biol. 8:e1002548. doi:10.1371/journal.pcbi.1002548.
76. Yenush L, Mulet J, Ariño J, Serrano R. 2002. The Ppz protein phosphatases are key regulators of K^+ and pH homeostatic implications for salt tolerance, cell wall integrity, and cell cycle progression. EMBO J. 21:920–929.
77. Yenush L, Merchant S, Holmes J, Serrano R. 2005. pH-responsive, posttranslational, posttranslational regulation of the Trk1 potassium transporter by the type 1-related Ppz1 phosphatase. Mol. Cell. Biol. 25:8683–8692.
78. Mulet JM, Leube MP, Kron SJ, Rios G, Fink GR, Serrano R. 1999. A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. Mol. Cell. Biol. 19:3328–3337.
79. Goossens A, de la Fuente N, Formet J, Serrano R, Portillo F. 2000. Regulation of yeast H^+-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters. Mol. Cell. Biol. 20:7654–7661.
80. Park G, Servin JA, Turner GE, Altamirano L, Colot HV, Collopy P, Litvinikova L, Li L, Jones CA, Díala F-G, Dunlap JC, Borkovich KA. 2011. Global analysis of serine-threonine protein kinase genes in Neurospora crassa. Eukaryot. Cell 10:1553–1564.
81. Tian C, Beeson WT, Iavarone AT, Sun J, Marletta MA, Cate JHD, Glass NL. 2009. Systems analysis of plant cell wall degradation by the filamentous fungus Neurospora crassa. Proc. Natl. Acad. Sci. U. S. A. 106: 22157–22162.
82. Tian C, Li J, Glass NL. 2011. Exploring the bZIP transcription factor regulatory network in Neurospora crassa. Microbiology 157:747–759.