THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 53, Issue of December 31, pp. 55060–55072, 2004
Printed in U.S.A.

The TRK1 Potassium Transporter Is the Critical Effector for Killing of Candida albicans by the Cationic Protein, Histatin 5*

Received for publication, September 24, 2004, and in revised form, October 12, 2004 Published, JBC Papers in Press, October 13, 2004, DOI 10.1074/jbc.M411031200

Didi Baev‡, Alberto Rivetta‡, Slavena Vylkova‡, Jianing N. Sun‡, Ge-Fei Zeng§,
Clifford L. Slayman¶, and Mira Edgerton¶¶

From the Departments of *Oral Biology and ¶¶Restorative Dentistry, School of Dental Medicine, State University of New York, Buffalo, New York 14214 and the §Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, Connecticut 06520

Salivary histatins (Hsts)1 are structurally related histidine-rich cationic proteins produced by acinar cells in human salivary glands and are key components of the nonimmune host defense system of the oral cavity (1, 2). In vitro, Hst 5 (24 amino acids) kills pathogenic Candida albicans (3–5) and possesses both fungistic and fungicidal activities against a spectrum of other fungi including Candida glabrata, Candida krusei, Saccharomyces cerevisiae, Cryptococcus neoformans (6), and Neurospora crassa.2 Hst 5 is also effective against azole- and amphotericin-resistant strains of these fungi (7), which is of considerable practical importance and is of basic significance as well, suggesting a fundamental difference in their mechanisms of action.

Hst 5 has been shown not to act like conventional pore-forming cationic peptides (8, 9). Instead, it acts via a multistep process that includes initial binding of extracellular Hst 5 to a Candida surface protein Ssa1/2p (10), followed by subsequent entry of Hst 5 into the cytoplasm (11, 12). That the primary target for Hst 5 is indeed intracellular has been verified by the observation that expression of a chromosomally encoded human-salivary histatin 5 gene, under the control of a regulated promoter, yields all of the customary toxic effects (13).

Whatever the primary target for Hst 5, its actions quickly become apparent at the plasma membrane and include noncytolytic efflux of cellular ATP and of potassium and magnesium ions (8, 11) and elevated permeability to small cationic dyes such as propidium iodide (14) but not to larger anionic dyes such as calcein (15). These obvious membrane effects are accompanied by a rapid and irreversible loss of cell volume and ultimately by cell cycle arrest (16). However, all such toxic effects, including cell death, can be attenuated or blocked by a curious range of agents: by protonophores such as CCCP and DNP (8, 17, 18), by elevated extracellular calcium (19, 20), by elevated external potassium (9, 11), and (very surprisingly) by well known anion channel inhibitors such as niflumic acid, 5-nitro-2-(3-phenylpropylamino)benzoic acid, and DIDS (16).

Potassium ions appear to be especially important in these protective phenomena. In addition to their biochemical role as enzyme cofactors (especially in protein synthesis; see Ref. 21), they have several major physiological functions. Under normal circumstances, K+ accounts for ~40% of the cytosolic osmoticum and therefore establishes background conditions against which control of cell turgor must operate. Also, as the major cytoplasmic cation, K+ controls the ionic strength of cytoplasm, a property closely related to its function as a catalytic cofactor. Cytoplasmic K+ can also buffer the electric potential difference across the plasma membrane (V_m, membrane voltage), which in turn supplies energy for the majority of “active” transport processes in fungal membranes. This role normally becomes important only when the membrane itself or its primary voltage generator, the H+-ATPase (22, 23), is damaged or down-regulated.

Tok1p, a cloned potassium channel which is the only certified
K⁺ channel in *Candida* plasma membranes, may be the principal diffusion pathway for voltage stabilization (24–26), but NSC1 (a protein whose encoding gene is not yet known) certainly contributes when extracellular divalent ions fall too low (especially Ca²⁺, ~10 μM or below; see Refs. 27 and 28). Another protein involved in these potassium-related phenomena is the major K⁺ uptake system in *Candida*, Trk1p. This transporter, initially cloned in *Saccharomyces* by Gaber et al. (29), is believed to operate as a proton-coupled system, having similar properties to the high affinity K⁺ uptake system in *Neurospora* (30, 31), and has also been reckoned as a voltage regulator on the basis of dye distribution measurements (32). Finally, it has recently been shown to mediate chloride currents through *Saccharomyces* plasma membrane, which are made easily visible by raising intracellular Cl⁻ above ~10 mM (33).

The possible involvement of Tok1p in histatin toxicity was investigated by creating TOK1-disrupted mutants of *C. albicans* (34). Knockout of TOK1 (tok1Δ/tok1Δ) completely abolished the characteristic currents of Tok1p and also reduced the extent of formation of Hst 5-induced ATP loss, after knock-out of both wild-type TOK1 alleles, clearly demonstrated that Tok1p channels could not be the primary site of Hst 5 action, although they do play a modulating role. NSC1, the putative nonselective cation channel that is responsible for very low affinity K⁺ uptake in *Saccharomyces* (28, 35), is not yet accessible to mutational analysis. We therefore turned to the “active” K⁺ transporter, Trk1p, for further examination of potassium involvement in Hst 5-mediated effects on *Candida*.

The initial experimental objective was the same as that used with Tok1p (34), which was to assess the effects of Hst 5 on killing and ATP loss in *Candida* strains deleted of one and deleted of both TRK1 alleles. However, it proved impossible to delete both TRK1 alleles, and all such attempts resulted in retention of a wild-type allele, a phenomenon associated with essential candidal genes. We therefore decided to overexpress TRK1 protein by introducing an additional TRK1 gene, into the *RP10 locus* of wild-type (CaTK2(wt)) cells, which one copy of TRK1 has been placed at the *RP10* locus; and CaTK3, constructed from CaTK2(wt), has a third copy of TRK1, also inserted at the *RP10* locus.

**DNA Manipulations**—All synthetic oligonucleotides used in strain constructions are listed in Table I and were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Molecular cloning, DNA hybridizations, and electrophoresis were carried out as previously described (30, 31), and has also been reckoned as a voltage regulator on the basis of dye distribution measurements (32). Finally, it has recently been shown to mediate chloride currents through *Saccharomyces* plasma membrane, which are made easily visible by raising intracellular Cl⁻ above ~10 mM (33).

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**Experimental Procedures**

**Strains and Media—** *Escherichia coli* strain DH5α (Invitrogen) was used as host for plasmids and was grown in LB medium (16). *Saccharomyces cerevisiae* and of *pys* mutants in *E. coli* (37, 38), but our experience using the 1.37-kb DNA fragment yielded very low rates of transformation in *C. albicans*, irrespective of the targeted locus. It seemed likely that the low transformation frequencies might result from too short flanking untranslated regions in the commonly used transforming fragment (424 bp upstream, 129 bp downstream), so we tested transformation with a much longer fragment, 2.37 kb, in which *URA3* gene was flanked by 1.04 kb upstream and 0.52 kb downstream. This length was selected to assure inclusion of the complete *URA3* promoter, along with additional potential regulatory sequences. Experiments quickly found transformation frequencies with this larger fragment to range from 30- to 600-fold higher than with the standard 1.37-kb fragment (37, 38), but we have used this long fragment of *CaURA3* DNA for plasmid constructs in the *CaTRK1* disruption cassette.

**Design of the CaTRK1 Disruption Cassette**—The design principle for the *CaTRK1* disruption cassette is shown in Fig. 1. Fig. 1A depicts the *TRK1* locus, including the ORF (shaded bar, arrowhead), three target regions (5' TR, 3' TR, and 3'-R), of which the two 3' regions are within the ORF; and two short flanking sequences (● and □) are used for control PCR. Fig. 1B depicts the plasmid bearing the disruption cassette (pDBT1U3), which consists, in order of 5'-TR from the *TRK1* locus, cloned as a Sall-BamHI fragment; 3'-R, cloned as a BamHI-KpnI fragment; the wild-type *URA3* gene; and 3' TR, cloned as a SacI-Apal fragment. Since 3' R naturally occupies a region downstream of 3' TR in the *TRK1* locus (these fragments are described below), integration of the disruption cassette into the genome via homologous recombination (1C) results in a pair of directed upstream and downstream of the selectable marker. This construct affords a simple way, via intrachromosomal recombination and upon FOA screening (39, 40), to delete the *URA3* gene and 3' TR (Fig. 1D), leaving only the remnants 5' TR, 3' R, plus (in this case) 221 terminal bp of the *TRK1* ORF. The principal advantages of this design are (a) that the resulting disrupted allele is no longer a target for the same disruption cassette, leaving only the surviving intact allele as target; (b) that no foreign sequences are left in the recipient locus or genome; and (c) that the selectable marker cannot be recycled if, by chance, the cassette integrates ectopically.

**Construction of the CaTRK1 Disruption Cassette**—The three gene-specific fragments 5' TR, 3' TR, and 3'-R of the *CaTRK1* disruption cassette constructs were amplified by PCR using as a template genomic DNA from strain CA14 and the (3'-TRK5'-5'/TRK5'-r, (Sc2)1TRK3'-f/ (A2)1TRK3'-r, and (B2)2TRK3'-f/K22TRK3'-r primer pairs, respectively (see Table I). PCR cycling conditions were as follows: initial denaturation for 3 min at 94 °C, followed by 30 three-step cycles consisting of 15-s denaturation at 94 °C, 15-s annealing at 55 °C, and 30-s elongation at 72 °C. This was followed by a 1-min final elongation at 72 °C.

The 5'TR is the 247-bp fragment spanning positions −287 to −41 upstream of the translational start codon of the *CaTRK1*; 3'TR is the 297-bp fragment spanning positions 2291–2587 within the *CaTRK1* ORF; and 3'-R is the 304-bp fragment spanning positions 2592–2962.
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TABLE I

| Purpose and primer a | Sequence b | Restriction site c |
|----------------------|------------|-------------------|
| Construction of pUC18A | AATgggcccGTAATCATGGTCATAGCTGTTTC | ApaI |
| Construction of pDBU3 | AATgggcccTTCGAGCTCGGTACCCGGG | ApaI |
| Construction of pDBT1U3 bearing the CaTRK1 disruption cassette | AAGggtaccGTATGACCGATACAACAAG | KpnI |
| Construction of pMP2–5′trk1-U3 | AAGgtcgacCAGCAACACATAACAGAAAG | SalI |
| Construction of pDBK1L | AAGggatccCTATATTTGATATACTAATATCTGC | BamHI |
| Control primers | AAGgggcccCTGCATTCTCCTTGGCATATAC | ApaI |

a Lowercase letters f and r indicate that the respective primer is forward or reverse.

b All sequences are given in the conventional 5′-to-3′ direction.

c Restriction sites introduced into primer sequences for cloning purposes are in lowercase letters.
allelic replacement was confirmed by PCR of four randomly selected samples. One of them was grown overnight (at 30 °C) and (K)URA3-f/2TRK1ctrl-r and by Southern analysis (data not shown). A gous strain CaTK1 was obtained.

The LTR1 tract contains the first TRK1 allele (Fig. 1, D). The nucleotide sequence of the fragment was confirmed by analysis of the resulting PCR product. Primers were custom synthesized by Integrated DNA Technologies, Inc. Reporters dyed and quencher dyed for the probes were 6-carboxyfluorescein and Black Hole Quencher, respectively. The Ct value for primers was 54.4–57.7 °C, and the Tm for probes was 66.5 °C for EF51 and 64.0 °C for TRK1, respectively. TaqMan PCR conditions were as follows: 2 min at 50 °C plus 2 min at 95 °C and then 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. Amplification and detection were carried out in 96-well plates on an iCycler iQ real time detection system (Bio-Rad). Each reaction mix contained duplicates of the test cDNA templates, negative RNA controls, no-template controls, and positive genomic DNA controls. All samples contained 1× IQ Supermix (Bio-Rad), 50 nM TaqMan probe, and 50× concentration of both the forward and the reverse primer. Fluorescent data were collected and analyzed with iCycler iQ software. The threshold cycle (CT) value for each sample was calculated by determining the point at which the base line. Each sample was tested in duplicate, and the results were averaged to obtain the final Ct (i.e., the point at which sample fluorescence rises above the background level). Sensitivity was determined by plotting 2-ΔΔCT values with various template dilutions (slope of <0.10). The ΔCT value was calculated by obtaining the difference (ΔCT) between Ct values of the target (TRK1) and the normalizer (EF51). ΔCT for CaTK2(α) was used as a reference (base line) for comparison of each strain. ΔΔCT values were then calculated as the difference between each sample’s ΔCT and the base line’s ΔCT and were transformed to absolute values (2-ΔΔCT) to calculate comparative expression levels.

Constructions of Strains CaTK2 and CaTK3—Complementation of the CaTK1 heterozygous strain was accomplished by introducing the wild type TRK1 gene into the RPlO region (13) of CaTK1. This strain, possessing two wild-type alleles and the disrupted trk1α site, was named CaTK2. Another strain (CaTK3) expressing three wild-type TRK1 alleles, was made by introducing the extra copy of TRK1 into the wild-type strain CaTK2(α), again at the RPlO locus. To construct both transforms, a 4,636-bp fragment containing TRK1 was PCR-amplified by primers (H/Trk1L-f and IF/Trk1L-r (Table 1). The resulting DNA was digested with HindIII and BamHI and ligated into the large HindIII-BamHI fragment of pDBT3 (34) to generate plasmid pDBT11, which bears both the URA3 selectable marker and the target RPlO locus sequence. Following linearization with NotI (which cuts within the plasmid-borne RPlO target sequence), pDBT11 was transformed into CaTK2(α) transformants, and transformants were selected as URA3 colonies on the selective media. The correct integration of the constructs into the RPlO locus was verified by PCR analysis and Southern blotting of the respective genomic DNA preparations (data not shown).

RNA Isolation and cDNA Synthesis for TaqMan RT-PCR—Total RNA from each strain was isolated as previously described (13) with the following modifications. Cultures of Ca. albicans strains were grown under the same conditions as for functional assays (34) in 7 ml of YNB medium, 2% glucose with the addition of uridine when required. Total RNA was isolated using RNeasy Mini Kit from Qiagen. The samples were both in-coom-treatment with DNase I using the RNase-Free DNase set from Qiagen, and off-column-treated using the TURBO-DNA-Free set from Ambion. The absence of genomic DNA contamination was confirmed by PCR using the following primer pair: EFB1-5′ /H11032 and EFB1-3′ for elongation factor gene EF-1α (CaEFB1) and TRK1–5′/H11032–TRK1 for CaTK1. Following DNase I treatment, 2 μg of total RNA was used per reaction for the first strand synthesis (cDNA) via the RETROscript kit from Ambion as previously described (13). TaqMan RT-PCR Assay—Quantitative real time RT-PCR experiments were carried out as described previously for yeast (40). Primers and TaqMan probes (forward primer EF51 5′-AGG AAG CCA CAG CCA GGA AAG-3′; reverse primer EF51 5′-TGA CTC TCT TCA TCA TCA TCA-3′; forward primer TRK1 5′-CGT CTT CAT CTC CTC AGT CAT-3′; reverse primer TRK1 5′-GGC TCA CTA TGG TGC TCT ATC-3′; probe EF51 5′-CCG CTG CTG CTG CTC CTG CCG-3′; probe TRK1 5′-AAC AAG CCA GCC GGC TCT GAC AGC-3′) were designed using the Primer 3 Input program (available on the World Wide Web at freud.wol.mtu.edu/cgi-bin/primer/3/primer3 www.cgi) and confirmed via Amplify 1.2 Software for PCR. Primers were custom synthesized by Integrated DNA Technologies, Inc. Reporter dyes and quencher dyes for the probes were 6-carboxyfluorescein and Black Hole Quencher, respectively. The Tm of primers was 54.4–57.7 °C, and the Tm for probes was 66.5 °C for EF51 and 64.0 °C for TRK1, respectively. TaqMan PCR conditions were as follows: 2 min at 50 °C plus 2 min at 95 °C and then 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. Amplification and detection were carried out in 96-well plates on an iCycler iQ real time detection system (Bio-Rad). Each reaction mix contained duplicates of the test cDNA templates, negative RNA controls, no-template controls, and positive genomic DNA controls. All samples contained 1× IQ Supermix (Bio-Rad), 50 nM TaqMan probe, and 50× concentration of both the forward and the reverse primer. Fluorescent data were collected and analyzed with iCycler iQ software. The threshold cycle (CT) value for each sample was calculated by determining the point at which the base line. Each sample was tested in duplicate, and the results were averaged to obtain the final Ct (i.e., the point at which sample fluorescence rises above the background level). Sensitivity was determined by plotting 2-ΔΔCT values with various template dilutions (slope of <0.10). The ΔCT value was calculated by obtaining the difference (ΔCT) between Ct values of the target (TRK1) and the normalizer (EF51). ΔCT for CaTK2(α) was used as a reference (base line) for comparison of each strain. ΔΔCT values were then calculated as the difference between each sample’s ΔCT and the base line’s ΔCT and were transformed to absolute values (2-ΔΔCT) to calculate comparative expression levels.
Western Blotting—Anti-Trk1p antibody was prepared to the synthetic oligopeptide (c)SIRRTNVYEEQS found in Trk1p and Trk2p from S. cerevisiae. This corresponds to the sequence (44)SVRTNYYEQS of C. albicans, predicted to span half of the intracellular loop M2c-M1d in the Durell and Guy (44) structural model of the fungal TRK proteins. Trk1p peptide was synthesized in the Durell and Guy (44) structural model of the fungal TRK proteins. Trk1p peptide was synthesized in the Keck Biotechnology Resource Center at Yale Medical School, verified by mass spectrometry, and used in the maleimide-activated conjugation kit (catalog no. 77607) from Pierce. Rabbit anti-Trk1p serum was produced by the animal immunization service at Yale Medical School, after six tri-weekly injections with 100 μg of the mCKLH-conjugated peptide. Trk1p antibody was purified on a peptide affinity column by means of the SulfoLink Kit (catalog no. 44895 from Pierce) and subsequently used at a 100-fold dilution for Western blots. Membrane and cytosolic fractions from each C. albicans strain were prepared as we have previously described (10, 15). Briefly, cells were glass bead-disrupted in the presence of protease inhibitors and centrifuged at 3,200 × g for 5 min to remove unbroken cells and organelles. The supernatant was then centrifuged at 100,000 × g for 1 h at 4 °C to separate cytosolic and membrane fractions. The cytosolic fraction was collected in the supernatant, and the total crude membrane fraction was collected in the protein pellet. Soluble membrane proteins were extracted from the membrane fraction with 10 mM sodium phosphate buffer containing 2.5% Triton X (with protease inhibitors at 4 °C) and were recovered in the supernatant following centrifugation at 100,000 × g for 1 h at 4 °C. Equal amounts of protein (100 μg) from each fraction were separated using 7.5% SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes, and probed with anti-Trk1 antibody, followed by goat-anti-rabbit peroxidase-conjugated secondary antibody (diluted 1:2,500). Fractions were also probed with yeast anti-Pma1 (kindly provided by Dr. Amy Chang, Albert Einstein College of Medicine) and yeast anti-Gas1 (kindly provided by Dr. Randy Schekman, University of California at Berkeley) to verify identity and purity of membrane fractions. Reactive proteins were visualized with one of the 1-Step TMDS detection system (Pierce) according to the manufacturer’s protocol.

Candidacidal Assay—Killing of Candida TRK mutant strains by Hst 5 was measured by the microdilution plate assay, as previously described (34). Assays were performed in triplicate for each strain and each test condition, after cells had been preincubated, at a density of 2.5 × 10^6 cells/ml in 10 mM sodium phosphate buffer (pH 7.2), with the test concentration of Hst 5 for 60 min at 37 °C. For DIDS experiments, cells were preincubated with 1 mM DIDS for 1 h before use in the candidacidal assay. Cell death was calculated as (1 – (number of colonies recovered from Hst 5-treated cells/number of colonies from control cells)) × 100.

ATP Bioluminescence Assay—Histatin 5-induced release of ATP from C. albicans cells was measured as described (34) with the following slight modifications. For each assay, cells were mixed with 31 μM Hst 5 at a density of 10^6 cells/ml and incubated at 37 °C, with shaking. At intervals of 5, 10, 20, 30, 45, and 60 min after exposure to Hst 5, the suspension was centrifuged (6,000 × g, 3 min), and 25 μL of supernatant was assayed for ATP by luminometry, using the FL-AA ATP assay kit (Sigma). Results are expressed as nmol of ATP released/10^6 cells.

Rubidium-86 Influx Measurements—The general design for assessing K+ transporter function described for Saccharomyces (32) was used with the following modifications for Candida. Cells were grown at 37 °C to A_600nm = 0.8–1.0, in 15 ml of YNB medium (Qiobogene) supplemented with 150 mM KCl and with 200 μM uridine (when required). The resulting suspensions were spun down, and the cells were washed twice with glass-distilled water and then resuspended at the same density (2.5 × 10^7 cells/ml) in 1 mM sorbitol containing 2.5% glucose. Cells were potassium- and stearic acid in this solution for 5 h on a rotary shaker (250 rpm) at room temperature. For uptake measurements, the starved cells were centrifuged, washed, and resuspended at 5 × 10^6 cells/ml in transport buffer (50 mM Tris-succinate, 2.5% glucose, pH 5.9). Uptake of ^86Rb^+ was initiated by mixing 225 μL of this suspension with 25 μL of transport buffer containing ^86Rb^+ and cold RbCl at final concentrations of 0.1 μCi/ml and 1.07 mM, respectively. At intervals of 30 s, 1 min, 2 min, and 3 min, 200-μL aliquotes (10^6 cells) were harvested by filtration on 0.45-μm Durapore membranes (Millipore Corp., Bedford, MA), rinsed three times with 2 mM MgCl2, immersed in Ecocscint fluid (Research Products, Inc.), and assayed on a Beckman Coulter LS6500 scintillation counter.

Electrophysiological Methods—Measurements of electric currents through the Candida plasma membrane were made on spheroplasts, using the whole-cell recording configuration of the patch clamp technique, as described previously for Saccharomyces (34), with slight modifications for Candida (34). All C. albicans strains were grown in shaking (250 rpm) liquid YPD medium, to A_600nm = 0.8–1.2, at 30 °C. All media used for preparing and recording from Candida spheroplasts are listed in Table II. To make spheroplasts, ~4 OD units of cells were harvested by centrifugation (500 × g for 5 min), washed twice with 3 ml of Buffer A, resuspended in the same buffer plus 0.2% β-mercaptoethanol, and incubated for 30 °C on a slow orbital shaker (6 rpm), for 30 min. The suspension was then recentrifuged, and the pellet was resuspended in 6 ml of Buffer B (spheroplasting buffer) plus 0.6 units/ml zymolyase 20T (catalog no. 320921; ICN Biomedicals Inc., Irvine, CA) and incubated for 45 min at 30 °C. The resulting spheroplasts were spun down (500 × g for 5 min), gently resuspended in Buffer C (osmotic stabilizing buffer), and incubated stationary, at room temperature (~25 °C), until use. Spheroplasts were preincubated 200 μM uridine-containing buffer, and cells were incubated for 3 min, 200-μL aliquotes (10^6 cells) were harvested by filtration on 0.45-μm Durapore membranes (Millipore Corp., Bedford, MA), rinsed three times with 2 mM MgCl2, immersed in Ecocscint fluid (Research Products, Inc.), and assayed on a Beckman Coulter LS6500 scintillation counter.

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RESULTS

The C. albicans TRK1 Gene—A 4,783-bp nucleotide sequence from C. albicans (strain ATCC 10261) was cloned by pheno-
typic complementation of a trk1Δ trk2Δ strain of S. cerevisiae. The enclosed ORF would translate into a 1,059-amino acid
protein with ~36% sequence identity to each of the Saccharo-
ymces proteins, Trk1p and Trk2p, and ~50% similarity allow-
ning conservative substitutions. The corresponding comparisons
between the two Saccharomyces proteins are ~53% sequence
identity, and ~65% similarity with conservative substitutions
(46). Accordingly, this ORF was assigned the gene name Ca-
TRK1 and registered with NCB! under accession number
AF267125. Details of cloning this gene and characterization of
the expressed protein will be published elsewhere. Sequencing
of the Candida genome (strain SC5314) has now been com-
pleted, and contig 19 (available on the World Wide Web at
www.sequence.stanford.edu:8080/btcontigs19super.html) lists
two alleles, 19-10057 and 19-20057, which differ slightly from
each other and somewhat more from the sequence at AF267125
but clearly represent small variations on the same gene. All of
our BLAST searches from AF267125 or its translation identi-
fied sequences in contig 19-10057 or 19-20057 or in the trans-
lations thereof but failed to identify any other related Candida
sequences. Therefore, C. albicans appears to harbor only a
single protein with significant identity to the Saccharomyces
TRK1 and TRK2 proteins. However, our searches did reveal an
open reading frame, in overlapping contigs 6-2069 and 6-1944,
that potentially encodes an 808-amino acid potassium trans-
porter in the HAK/KUP family.

In order to examine the possible role of the TRK1 protein
(Trk1p) in killing of C. albicans by the cationic salivary protein
Hst 5, we constructed a series of isogenic C. albicans TRK1
mutants from wild-type strain CAI4, here designated CaTK2(wt), which is itself a ura- derivative of SC5314. Single-
allele disruption of the TRK1 locus produced the heterozygous
trk1Δ/TRK1 strain CaTK1; however, two independent strategies
failed to produce a trk1-null strain, suggesting that disruption
of both TRK1 alleles is lethal in C. albicans. Gene-dose modu-
lation for Hst 5 effects was therefore devised by transforming
an additional copy of the wild-type gene into the Candida RP10
locus to produce strain CaTK3 (TRK1/ΔTRK1/ΔTRK1/URA3) that has three functional copies of TRK1. Complementa-
tion of the hemideleted strain CaTK1 yielded strain CaTK2
(trk1Δ/ΔTRK1/ΔTRK1/URA3) that carries two functional cop-
ies of TRK1. All four strains (CaTK2(wt), CaTK1, CaTK2, and
CaTK3) had similar growth characteristics in both YPD and
YNB medium, and none required supplementation with extra
K+ for normal growth. Also, all grew robustly on glycerol as
sole carbon source, demonstrating the presence of normal ox-
idative metabolism.

Message and Protein Expression Levels of C. albicans TRK1
Mutants—Quantitative real time RT-PCR (TaqManTM) was
performed to measure TRK1 transcript levels in all four Can-
dida strains. For relative quantification of gene expression,
transcript levels were normalized to those of the EFβ1 gene by

4 M. Miranda, E. Bashi, and C. L. Slayman, unpublished data.
at the start of measurement, which averaged 3.5 nmol/10^8 cells. This was almost certainly an artifact, probably representing residual Rb^+ left in the cell wall, despite the rinsing of harvested pellets with MgCl_2. The jump was ignored in calculating the TRK-dependent fluxes.

Rubidium influx in CaTK1 cells occurred at only 2.7 nmol/10^8 cells/min, compared with 13.3 nmol/10^8 cells/min in wild-type CaTK2(wt), a functional ratio that is not far from the corresponding transcript expression ratio (1:5; cf. 1:7). No significant difference was found on a point-to-point basis between the strain CaTK2(wt) and that of wild-type cells, and the computed uptake rate (11.9 nmol/10^8 cells/min) was compatible with the Western blots, confirming that placement of the TRK1 gene at the RP10 locus, together with the single remaining normal allele, can restore wild-type protein expression levels (Fig. 2B). Furthermore, the addition of a third TRK1 gene at the RP10 locus to wild-type Candida, in strain CaTK3, augmented rubidium influx ~1.5-fold to 19.5 nmol/10^8 cells/min, as if in direct proportion to gene dosage, compared with the wild-type. Although this coincidence of gene dosage and function may be post-translationally regulated, it is probably best regarded as fortuitous, in view of the much elevated (10-fold) transcript levels in CaTK3. Evidently, the majority of Trk1p resulting from hyperproduction of TRK1 message is nonfunctional and sequestered into a cytosolic compartment. Nevertheless, overexpression of TRK1 did appreciably enhance potassium (rubidium) transport in CaTK3 cells, in agreement with elevation of Trk1p detected in the membrane fraction. These results were supported further by assessment of the novel chloride transport properties of Trk1 proteins in these cells.

A Second Functional Assay: The Chloride Channel through Trk1p—A completely unexpected property of TRK proteins was recently identified by patch clamp experiments on whole protoplast membranes of S. cerevisiae; they conduct large inward currents when the cytoplasm is loaded with chloride ions (33, 52). The molecular mechanism underlying such implied chloride effluxes is unknown, but it apparently serves as a voltage-driven escape for excess cytoplasmic chloride ions. Although the fluxes themselves can be very large, an order of magnitude larger than maximal potassium fluxes, the TRK-mediated Cl^-permeability is fixed and independent of chloride concentration. Similar TRK-related chloride currents have recently been observed in Candida, and typical current records from spheroplasts of all four Candida strains are compared in Fig. 4. These sets of superimposed traces display the amplitude and time courses of membrane current required to step the membrane voltage from ~40 mV (holding voltage) to ~100 mV, +80 mV, etc., to ~180 mV and hold it there for 2.5 s. Positive currents (so-called outward currents; upward traces) reflect the noisy opening and closing of Tok1p channels, which let K^+ out of the cells and which were previously shown (34) not to be critical to
only slightly modulates the killing of *C. albicans* by Hst 5. Very different results emerged with Trk1p, the primary “active” K+ transporter. Averaged results from a homogeneous set of experiments are shown in Fig. 6. The wild-type strain CaTK2(wt) showed normal sensitivity to Hst 5, with ~95% of the cells killed at 125 μM Hst 5, ~81% at 62 μM, and an estimated half-killing concentration of ~20 μM. Significantly, strain CaTK1, having a 7-fold reduction of *TRK1* transcripts and a 5-fold reduction of Trk1p function, was quite insensitive to Hst 5, which produced only ~11% killing at 125 μM (data not shown), with an apparent half-killing concentration of >600 μM (n = 6). This result was not due to reduced cellular uptake of Hst 5, since we found that CaTK2(wt) and CaTK1 had equivalent total cellular levels of Hst 5 following 30- and 60-min incubation with fluorescein isothiocyanate-Hst 5, as assessed by quantitative FACScan analysis (data not shown). Thus, the CaTK1 strain proved profoundly resistant to histatin 5 killing despite normal uptake of peptide.

Complementation of CaTK1 by insertion of a second wild-type *TRK1* gene at the RP10 locus fully restored sensitivity to Hst 5 in CaTK2 with no statistically significant difference in Hst 5 killing between CaTK2(wt) and CaTK2 at any dose. Thus, deletion of the *TRK1* gene itself, not some unrelated or incidental change in the CaTK1 strain, was responsible for the diminished histatin sensitivity. However, a second and very surprising result emerged from killing tests on the *TRK1* overexpression strain, CaTK3. This strain proved significantly less susceptible to Hst 5 killing than the wild type, as judged by a fitted half-killing concentration of 31 μM Hst 5, again computed on the basis of multiple (from three to seven) independent determinations. The extrapolated maximal killing at high Hst 5 doses could be taken as 100% for both CaTK2(wt) and CaTK3. Although the Western blots (Fig. 2B) showed total 120-kDa membrane-bound Trk1p in CaTK3 to be only slightly elevated from the wild-type level, cytosolic Trk1p was greatly elevated. This suggests that a large cytosolic reservoir of Trk1p could sequester Hst 5 and diminish its free concentration, that is accessible to plasma membrane sites. Experiments are in progress to investigate this possibility.

**Correlated Loss of ATP by Hst 5-treated Candida**—The fact that Hst 5 kills *Candida* without overt cell lysis or without its own forming pores in the plasma membrane has been well understood for more than 5 years (8, 9). However, a cardinal feature of the process was shown to be the net loss of cytoplasmic ions and small molecules, especially ATP (8, 11, 18), at a rate (for ATP) that correlated well with the killing effectiveness of Hst 5 (53). It was therefore important to determine whether the same relationships between killing effectiveness and *TRK1* transcript and protein levels also held for ATP loss in the four *C. albicans* strains.

The answer to this question was unequivocally positive, as is shown by the data in Fig. 7. All data plots for ATP release during the first hour could be reasonably fit by straight lines, revealing the following quantitative relationships. Standard Hst 5 treatment (31 μM) released ATP from wild-type CaTK2(wt) cells at 0.20 nmol/10^6 cells/min and from CaTK1 cells at 0.045 nmol/10^6 cells/min (~22%). Although this residual flux was larger than the corresponding residual killing (~10%, Fig. 6), many processes could dispel an exact correspondence, including the metabolically cumulative effects of sustained ATP loss. Complementation of CaTK1 with the *TRK1* gene at the RP10 locus (strain CaTK2) reestablished Hst 5-induced ATP loss at 0.18 nmol/10^6 cells/min, again essentially identical with the wild-type value. Overexpression of *TRK1*, in strain CaTK3, had qualitatively the same effect on ATP efflux as upon Hst 5-induced killing, in that it actually

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**FIG. 3.** Trk1p-mediated rubidium influx rises with the *TRK1* copy number. ○, *C. albicans* strain CaTK2(wt); ■, CaTK1; ●, CaTK2; ▲, CaTK3. For uptake assays, K+—starved cells (10^6/ml; see “Experimental Procedures”) were suspended in transport buffer (50 mM Tris-succinate at pH 5.9 plus 2.5% glucose) and equilibrated for 10 min, after which 1.07 mM RbCl labeled with 0.1 μCi of 86Rb was added. Cells were harvested and washed by vacuum filtration at intervals of 20 s and 1, 2, and 3 min, and 86Rb was assayed by liquid scintillation spectrometry. Each data point represents the mean ± S.D. of at least three independent experiments. The four sets of data were fitted by linear regression, forced to a common ordinate intercept. Calculated slopes (fluxes, in units of nmol/(10^6 cells × min)) were 19.5, 13.3, 11.9, and 2.7, read from top to bottom.

Hst 5 effects. The positive currents varied randomly in amplitude from strain to strain, unrelated to the presence or absence of TRK genes.

The inward, negative currents (downward in the tracing sets of Fig. 4) were dependent upon both *TRK1* and high intracellular chloride, as is made clear by comparison of the top left panel (CaTK2(wt)) with the panels directly to the right (CaTK1) and directly below (0.1 mM Cl–), showing that replacement of intracellular (pipette) chloride with gluconate (Buffer Gl), leaving both intracellular and extracellular K+ unchanged, nearly abolished the negative currents. In *Candida*, these Cl– currents increased with a half-time of about 20 s, stabilizing after ~2 min. Comparison of the records for the four different *Candida* strains (Fig. 4, top row) makes clear that the relative magnitudes of chloride currents, under standard conditions in the different strains, had roughly the same proportion as the rubidium fluxes shown in Fig. 3. This was true when either the time courses of current or the corresponding steadystate currents were compared.

The record sets in Fig. 4 were selected as representative (viz. near average for the data collected from each strain). Security in the above statements about relative amplitudes is reinforced by Fig. 5, plotting the averaged negative currents (last half of each tracing), for all measurements on each strain, against the clamped membrane voltage. Because data at positive membrane voltages are irrelevant (Tok1p), the plots were truncated at ~60 mV. These so-called current-voltage plots (I-V plots) display a characteristic and important parameter for each I-V plot: the limiting slope conductance at large negative voltages, indicated by the dashed line (Fig. 5, top left panel). Comparing the four strains, these limiting conductances defined the same relationships to the number of expressed *TRK1* genes as did the rubidium influxes in Fig. 3. This fact is demonstrated by the phase plot in Fig. 5, inset, showing that Cl– conductance is linearly proportional to Rb+ influx.

**Susceptibility of *C. albicans* TRK1 Mutants to Killing by Histatin 5—Previous experiments (34) had demonstrated that the inwardly rectifying potassium channel, Tok1p, in *Candida*...
reduced the rate of ATP loss, compared with that in wild-type cells, to 0.11 nmol/10^6 cells/min, or ~55% of the control value. Thus, here again, ATP release seemed to be the hallmark of the degree of lethality of Hst 5. These data imply a complex kinetic relationship between expressed functional TRK1 protein and the process of ATP efflux.

\[ \text{CaTK2(wt)} \quad \text{CaTK1} \quad \text{CaTK2} \quad \text{CaTK3} \]

\[ [\text{Cl}]_{\text{in}} \]

183 mM

0.1 mM

\[ 1 \text{s} \]

\[ \pm 100 \text{ mV} \]

\[ -180 \text{ mV} \]

\[ -160 \text{ mV} \]

\[ 50 \text{ pA} \]

\[ 100 \text{ mV}, \quad 80 \text{ mV}, \ldots \text{down to} -180 \text{ mV}, \text{each from a holding voltage of} -40 \text{ mV}. \]

Current traces for each test, as well as the voltage protocol, are shown superimposed. The upward currents (all six panels) represent K⁺ efflux through Tok1p, the K⁺ channel (34). Downward currents (top row) represent (~95%) chloride efflux through Trk1p, the K⁺ transporter (33, 52). Vertical comparison (two left columns) demonstrates that reduction of intracellular chloride to submillimolar range nearly abolished the downward currents. All record sets are representative (near average) for several replicates obtained from independent experiments.

\[ \text{FIG. 4. Large inward currents through Trk1p are carried by chloride ions and vary with TRK1 expression.} \]

Patch clamp records from spheroplasts of C. albicans. Top row, whole-cell current traces, recorded with chloride-filled pipettes, from spheroplasts expressing 1–3 copies of the wild-type TRK1 gene, as indicated. Bottom row, left, whole-cell currents from two strains recorded with low chloride pipettes. Bottom row, right, voltage protocol imposed to produce the current traces in all six panels; a staircase of 2.5-s pulses was applied to the membrane, clamping to +100 mV, +80 mV, ... down to -180 mV, each from a holding voltage of -40 mV. Current traces for each test, as well as the voltage protocol, are shown superimposed. The upward currents (all six panels) represent K⁺ efflux through Tok1p, the K⁺ channel (34). Downward currents (top row) represent (~95%) chloride efflux through Trk1p, the K⁺ transporter (33, 52). Vertical comparison (two left columns) demonstrates that reduction of intracellular chloride to submillimolar range nearly abolished the downward currents. All record sets are representative (near average) for several replicates obtained from independent experiments.

\[ \text{FIG. 5. Chloride currents mimic Rb⁺ fluxes and rise monotonically with the number of TRK1 genes expressed.} \]

Summary of current-voltage (I-V) plots corresponding to the record sets in Fig. 4. Current values, averaged over a 1-s interval (1.25–2.25 s on each trace), ± S.E. for the n experiments with each strain, are plotted against the clamped membrane voltage (n = 7, 23, 7, and 8, respectively, for the four panels read across). Complete plots of Trk1p currents (downward) are shown, whereas the Tok1p currents (upward) are truncated at +60 mV. For each plot, maximal slope conductance was estimated from a straight line drawn through the -160-mV and -180-mV points (parallel dashed line, top left panel). I-V plots were corrected for small leakage currents, as already described (33). Inset, phase plot of Rb⁺ fluxes (from Fig. 3) against maximal slope conductances to demonstrate equivalence of the two methods for measuring functional protein in the four strains. The regression line is forced through the origin.

The data suggest that the degree of lethality of Hst 5 is correlated with the rate of ATP loss.
Simultaneous Blockade of Hst 5-induced Killing and Trk1p-mediated Chloride Conductance—Discovery of the fact that Hst 5-induced killing of *C. albicans* was accompanied by major leakage of ATP (53), suggested that histatin must produce either greatly enhanced anion permeability or generalized breakdown of the plasma membrane. Attention was further focused on anion permeability by the fact that anion-channel blockers, such as DIDS, protected *C. albicans* cells against Hst 5-induced killing as well as against ATP loss. Bringing those observations together with the Trk1p-mediated chloride conductance prompted us to examine the effects of DIDS on the chloride currents through Trk1p. As is shown in Fig. 8, both the large inward (downward) currents in CaTK2(wt) and the small inward currents in CaTK1 were essentially completely blocked by 0.1 mM intracellular DIDS. Quantitatively, the currents at -180 mV were reduced from (-1175 pA/cell (CaTK2(wt))) or (-125 pA (CaTK1)) to the range of (-3-5 pA, as can be seen by comparing the summary I-V plots in the two left panels of Fig. 8: control versus DIDS. The corresponding limiting conductances at high voltage were 3.3 nS for CaTK2(wt) and 0.6 nS for

**FIG. 6.** Disruption of a single TRK1 allele nearly abolishes killing of *Candida* by Hst 5. Symbols are as in Fig. 3. Test cultures of 2.5 x 10⁶ cells/ml were incubated in 10 mM sodium phosphate buffer plus Hst 5 (15.5–182 μM) for 1 h at 37 °C, pH 7.2. Control cultures were handled similarly, but without Hst 5. Loss of cell viability was calculated as (1 - (number colonies grown from each test culture/number of colonies from the corresponding control)) x 100. Each data point represents the mean ± S.D. of at least three independent experiments. Smooth curves were fitted as saturating (Scatchard) functions with a common maximum. The apparent LD₅₀ values of Hst 5 were as follows (in μM): 21, 24, 31, and >600, respectively, for CaTK2(wt), CaTK2, CaTK3, and CaTK1.

**FIG. 7.** Hst 5-induced ATP loss parallels the killing effect. The symbol key and incubation conditions are as in Fig. 6. Cells were grown overnight in YNB medium, washed, resuspended (10⁶ cells/ml) in buffer with 31 μM Hst 5, and incubated for the indicated times to establish ATP efflux. Extracellular ATP was measured by luminometry of cell supernatants and is expressed as nmol of ATP/10⁶ cells. Each data point represents the mean ± S.D. for at least three independent experiments. Regression lines were fitted through the origin and each set of six test points. Calculated slopes (effluxes, in units of nmol/10⁶ cells x min) were 0.20, 0.18, 0.11, and 0.045, read from top to bottom.
CaTK1 in the control measurements and ∼0.09 nS for both DIDS-inhibited preparations. These numbers, for the particular cells demonstrated in the left and middle panels of Fig. 8 are fully compatible with those computed in Fig. 5 as multicellular averages. The results in Fig. 8 are representative of four different experiments conducted for each panel.

In an attempt to identify other anion channels that might also be affected by DIDS and related inhibitors, we searched the C. albicans genome data base (available on the World Wide Web at www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi) and identified open reading frames for two potential C. albicans chloride channel proteins: one residing on contig 6-2519 and encoding a protein of 879 amino acids. Both CLC4, and CLC5 chloride channels, but the actual expression of these proteins differ greatly from ScGef1p, the chloride channel protein in S. cerevisiae, which is localized in endomembranes (the C. albicans GEF1 locus resides on contig 6-2519).

Testing of C. albicans single allele deletion strains for these two loci showed them to be at most 15–20% more resistant to Hst 5 than the wild-type, observations which argue that, like the potassium channel Tok1p (34), the two identified chloride channels could play a modulating role, but not a primary role, in Hst 5 killing of C. albicans.

Trk1p-mediated chloride conductance modulated by DIDS is a thoroughly remarkable result, which not only confirms that Trk1 protein is the critical effector of Hst 5 toxicity in Candida but also proffers a mechanism: that Hst 5 binding to Trk1p produces a leakage pathway through either that protein itself or a larger complex involving Trk1p.

DISCUSSION

Mediation of Hst 5 Toxicity by Trk1p—We have constructed an isogenic set of C. albicans strains expressing one, two, or three copies of the TRK1 gene, in order to investigate possible functional roles of TRK1 protein in toxic actions of the cationic antimicrobial peptide, Hst 5. Although the quantitative relationships among TRK message, expressed protein, and functional roles of TRK1 protein in toxic actions of the cationic antimicrobial peptide, Hst 5. Although the quantitative relationships among TRK message, expressed protein, and functional roles of TRK1 protein in toxic actions of the cationic antimicrobial peptide, Hst 5.

Further insights into the molecular mechanisms underlying Hst 5 toxicity are provided by two surprising findings: (a) that the 3-TRK1 strain (CaTK3) displays a 50% increase of Trk1p function in the plasma membrane (Figs. 4 and 6), compared with the wild type, but a 40–50% decrease of Hst 5 sensitivity, and (b) that the recently discovered anion permeability (Cl−) of Trk1p and the Hst 5-induced efflux of cytoplasmic ATP are equally inhibited by disulfonic stilbenes.

Anion Channeling through Cation Transporters—Hst 5-induced potassium loss, along with the reduction of Hst 5 sensitivity observed in elevated extracellular K+, initially brought our attention to the K+ channel, Tok1p (34) and then to this work on Trk1p. However, reduction of Hst 5 toxicity by anion channel inhibitors such as DIDS seemed unrelated until the
ability of TRK proteins to conduct anions was recognized (33). In retrospect, chloride conductance through TRK-like proteins almost certainly underlay earlier observations in fungi, especially the chloride-mediated depolarization of *Neurospora* plasma membranes (54), but the general phenomenon of anion channeling through cation transporters only began to emerge a decade ago, in animal systems such as the EAAT proteins, which are responsible for sodium-coupled uptake of excitatory amino acids (55–57).

Peculiarly in *Candida*, but not in *Saccharomyces*, the TRK-dependent chloride conductance is blocked by classic anion channel inhibitors, especially DIDS. However, most importantly, in protecting the organism from Hst 5 toxicity, DIDS reacts with the same protein identified by the expression level experiments as the likely target for primary action of Hst 5. Our current working hypothesis to account for these facts is that the structural path for Cl⁻ through Trk1p is distorted by Hst 5 binding so that it permits the efflux of larger anions, such as ATP, but DIDS “seals” the channel against Hst 5 action. Trk1p-dependent loss of ATP and other large anions initiates cellular shrinkage and eventual cell cycle arrest that are thus blocked by DIDS (16). Moreover, as evidenced in Fig. 6A, the end result produced by DIDS is identical in the two strains (CaTK2/wt) and CaTK1, suggesting that elimination of the small residual Hst 5 sensitivity of CaTK1 by DIDS mimics the likely behavior of true trk1-null strains and re-emphasizes that the TRK1 transporter system qualifies as the critical target/effecter for Hst 5-mediated killing of *C. albicans*.

Overexpression of TRK1—The unexpected conflict between elevated Trk1p function and diminished Hst 5 toxicity observed in the overexpression strain, CaTK3, has at least two possible explanations. Most simply, cytoplasmically localized Trk1p is likely to be sequestered within endomembranes (karmellae or vesicular-tubular clusters) (49), as a device to counter the threat of excessive membrane permeability. Then, if direct interaction between Hst 5 and Trk1p occurs as postulated above, elevated endomembranial Trk1p could also bind significant Hst 5, decreasing the free concentration available to the effector site, which is plasma membrane-localized Trk1p.

An alternative possibility is that plasma membrane-bound Trk1p may have different oligomeric states, only some of which are susceptible to Hst 5. This hypothesis can be formalized in a simple kinetic scheme, for example with three distinct oligomers of the transporter: (a) naked Trk1p (T), which does not transport and does not bind Hst 5; (b) Trk1p bound to an activator (A), to make AT, which does transport and does bind Hst 5; and (c) Trk1p doubly bound to A, to make TAT, which transports (through both Ts) but does not bind Hst 5.

As the membrane concentration of T rises, the concentration of A is fixed and becomes limiting. Formally, this scheme appears as follows,

$$A + T \leftrightarrow AT \quad (Eq. 1)$$

$$AT + T \leftrightarrow TAT \quad (Eq. 2)$$

with the equilibrium constants shown below. The total concentration of A in the membrane is fixed, independently of Trk1p, so that the following is true.

$$A_{\text{tot}} = A + AT + TAT \quad (Eq. 3)$$

Then the concentration of AT in the membrane is given by the following expression,

$$AT = A_{\text{tot}} K_1 T (1 + K_1 T + K_2 K_1 T^2) \quad (Eq. 4)$$

where the concentration of T can vary among the different strains. The 210-kDa protein seen in Western blots (Fig. 2B) is a candidate for at least the AT component of this set.

That Trk1p indeed normally functions in oligomeric associations is suggested by a variety of additional information. Purely on the basis of structural modeling, Durell and Guy (44) postulated that yeast TRK proteins (in *S. cerevisiae* and *Schizosaccharomyces pombe*) should form symmetric dimers or tetramers. Without such clustering, the monomers, which fold like potassium channels (49), would have unusually well conserved surface residues, exposed primarily to membrane lipids. Hetero-oligomerization of TRK proteins in *Saccharomyces* is also a strong possibility because of the presence of three genes/proteins of unknown function but having strong sequence homology with plant potassium-channel β-subunits. Furthermore, bacterial TRK proteins, such TrkH in *E. coli*, are well known to be assisted by accessory subunits: TrkA, a NAD⁺-dependent dehydrogenase (58), and SapD (TrkE), an ATP-binding protein (59). Thus, it seems likely that metabolic regulation of *Candida* Trk1p will involve accessory binding proteins, which should act by modulating the kinetic properties of expressed protein, poised at “normal” with the wild-type ratio of functional Trk1p to accessory protein. Further direct studies on *Candida* TRK protein will be required to test both of these models.

Energetic Factors—The well established ability of protonophores, such as CCCP and DNP, to reduce Hst 5 killing of *C. albicans* cells (8) can also be mediated in part via Trk1p. Although it is generally assumed that these classical uncoupling agents permeabilize plasma membranes as well as mitochondrial membranes, the hard evidence for such effects on plant and fungal cells is sparse, and where it does exist, the window between pure uncoupling effects and gross depletion of ATP is very narrow (60). In other words “uncoupler” effects on intact fungal cells are as likely to result from ATP depletion as from plasma membrane permeabilization, unless ATP levels and membrane conductance, explicitly, are measured. Also, sustained ATP depletion usually triggers down-regulation of membrane transport, by decreased membrane conductance (or leakiness), for cells that are not killed (60). Therefore, CCCP and DNP could depolarize *Candida* plasma membranes, with or without “tightening” them.

Since we have already noted that different doses of wild-type TRK1 have no significant effect on intracellular accumulation of Hst 5, uptake of this polycation, presumably voltage-driven, is certainly mediated by other proteins, such as Nsc1. Thus, two distinctly different routes exist for uncoupler-mediated protection against Hst 5 toxicity: decreased uptake of the peptide because of membrane depolarization and down-regulation of Trk1p function, following from ATP depletion, which would be expected to choke its DIDS-sensitive anionic pathway. As suggested by Gyurko et al. (17), mitochondrial de-energization could play a role in both of these phenomena, via diminished ATP synthesis in the presence of uncouplers. However, differences in energy metabolism cannot account for the reduced susceptibility of CaTK1 to Hst 5, because all four strains were screened to verify the presence of normal oxidative metabolism. The fact that CaTK1 cells have typical respiration and oxidative phosphorylation but are almost completely resistant to Hst 5 killing demonstrates that the mitochondria themselves cannot be the critical locus for Hst 5 cytotoxicity, contrary to previous suggestions (14).

Our findings show that decreased expression of TRK1 and its product results in profound resistance to actions of the anti-fungal cationic protein Hst 5 and identifies the TRK1 potassium uptake system as critical for Hst 5 candidacidal activity. Whether other antimicrobial peptides/proteins may also exert
their activities through similar effectors remains to be discovered. Trk1p and/or other potential cognate activator proteins represent a specific target site by which Hst5 exerts cytotoxic effects against C. albicans, to provide nonimmune protection in the human-host defense system.

Acknowledgment—We thank Dr. Esther Bashi for cataloguing and maintaining the cultures of Candida at Yale, for technical assistance with the electrophysiological experiments, and for helpful criticism of experimental designs.

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