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The *STK2* Gene, Which Encodes a Putative Ser/Thr Protein Kinase, Is Required for High-Affinity Spermidine Transport in *Saccharomyces cerevisiae*

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Eukaryotic polyamine transport systems have not yet been characterized at the molecular level. We have used transposon mutagenesis to identify genes controlling polyamine transport in *Saccharomyces cerevisiae*. A haploid yeast strain was transformed with a genomic minitransposon- and *lacZ*-tagged library, and positive clones were selected for growth resistance to methylglyoxal bis(guanylhydrazone) (MGBG), a toxic polyamine analog. A 747-bp DNA fragment adjacent to the *lacZ* fusion gene rescued from one MGBG-resistant clone mapped to chromosome X within the coding region of a putative Ser/Thr protein kinase gene of previously unknown function (YJR059w, or *STK2*). A 304-amino-acid stretch comprising 11 of the 12 catalytic subdomains of Stk2p is ≈83% homologous to the putative Pot1p/Kkt8p (Stk1p) protein kinase, a recently described activator of low-affinity spermine uptake in yeast. Saturable spermidine transport in *stk2::lacZ* mutants had an approximately fivefold-lower affinity and twofold-lower V_{max} than in the parental strain. Transformation of *stk2::lacZ* cells with the *STK2* gene cloned into a single-copy expression vector restored spermidine transport to wild-type levels. Single mutants lacking the catalytic kinase subdomains of *STK1* exhibited normal parameters for the initial rate of spermidine transport but showed a time-dependent decrease in total polyamine accumulation and a low-level resistance to toxic polyamine analogs. Spermidine transport was repressed by prior incubation with exogenous spermidine. Exogenous polyamine deprivation also derepressed residual spermidine transport in *stk2::lacZ* mutants, but simultaneous disruption of *STK1* and *STK2* virtually abolished high-affinity spermidine transport under both repressed and derepressed conditions. On the other hand, putrescine uptake was also deficient in *stk2::lacZ* mutants but was not repressed by exogenous spermidine. Interestingly, *stk2::lacZ* mutants showed increased growth resistance to Li^+ and Na^+, suggesting a regulatory relationship between polyamine and monovalent inorganic cation transport. These results indicate that the putative *STK2* Ser/Thr kinase gene is an essential determinant of high-affinity polyamine transport in yeast whereas its close homolog *STK1* mostly affects a lower-affinity, low-capacity polyamine transport activity.

Putrescine and the polyamines spermidine and spermine are ubiquitous compounds which play essential roles in the control of several growth processes in eukaryotes (40, 55). Although most cells display a capacity for endogenous polyamine biosynthesis, most, if not all, prokaryotic and eukaryotic cells can accumulate polyamines from their environment (19, 41, 50). Polyamine uptake is an energy-requiring process and relies on high-affinity membrane transport systems which have thus far been molecularly characterized only for bacteria (19). The importance of the contribution of inward transport to the homeostasis of polyamine pools is shown by the fact that genetic defects in polyamine transport or starvation from extraneous polyamine sources markedly sensitizes tumor cells to the antiproliferative effect of drugs that specifically target polyamine biosynthesis, such as α-difluoromethylornithine (1, 18, 43, 51). Polyamine transport in mammalian cells is hormonally regulated (22, 25, 31, 50) and is tightly regulated by negative feedback mechanisms which depend on intracellular polyamine levels (31, 36, 54). An important negative regulator of mammalian polyamine uptake induced by polyamine accumulation is the ornithine decarboxylase antizyme (17, 36, 54), although its mechanism of action on polyamine transport is still unclear.

In the yeast *Saccharomyces cerevisiae*, exogenous polyamines are accumulated by saturable uptake mechanisms (32, 56) and by a vacuolar carrier apparently coupled with H^+/ATPase activity (24). However, little information is available on the molecular identity and regulation of the polyamine carrier(s) in yeast. Recently, the *POT1*/*KKT8* gene, which encodes a putative Ser/Thr protein kinase, has been cloned and shown to be a positive regulator of spermine transport in *S. cerevisiae* (23). *POT1*/*KKT8* could restore low-affinity spermine transport in mutants defective in spermine uptake that were isolated according to their growth resistance to spermine at low Mg^{2+} concentrations (32).

To gain insight into other components of the polyamine transport system in yeast, we have used transposon mutagenesis (5) to disrupt genes involved in polyamine uptake. Selection of disruption mutants for growth resistance to a toxic polyamine analog led to the identification of *STK2*, a novel gene with a Ser/Thr protein kinase signature homologous to *POT1*/*KKT8* (*STK1*). Unlike *STK1*, which affects a low-affinity, low-capacity uptake system and is expressed at very low levels, *STK2* behaves as a major activator of high-affinity spermidine transport and also regulates putrescine and spermine uptake. We also show that spermidine transport is subject to negative

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feedback regulation in yeast as in mammalian cells but that *STK2* is not likely to be involved in the derepression of polyamine transport, since putrescine uptake is also defective in *STK2* disruption mutants but is not subject to repression by spermidine. Evidence showing that a defective *STK2* gene leads to increased growth tolerance of Li^+ and Na^+ in nutrient-rich medium is also presented, suggesting a regulatory link between polyamine and monovalent inorganic cation transport.

MATERIALS AND METHODS

Strains and media. The haploid *S. cerevisiae* FY86 (*MAT*α *his3-*Δ*200 ura3-52 leu2-*Δ*1*) used in this study was obtained from Fred Winston (Harvard School of Public Health, Cambridge, Mass.) and was routinely grown on YPD medium (1% yeast extract, 2% peptone, 2% D-glucose). The minimal medium used for transformant selection was YNB [2% D-glucose, 0.17% amino acid-free yeast nitrogen base, 0.5% $(NH_4)_2SO_4$] supplemented with the appropriate essential amino acids. Polyamine-free YNB medium (H medium) and agar plates were prepared as described previously (2). Yeast transformations were performed by the lithium acetate method (20).

Selection strategy for polyamine transport-deficient mutants. A yeast genomic library of mini-Tn*3::lacZ::LEU2* insertions kindly provided by Michael Snyder (Yale University, New Haven, Conn.) was used for transposon mutagenesis (5). In addition to a *LEU2* selection marker gene, the inserted transposon sequence includes a *lacZ* adjacent to a 38-bp terminal repeat and lacking both a promoter and an initiator ATG codon. β-Galactosidase production in yeast transformed with this library thus requires an in-frame insertion into the coding region of an expressed gene (5). The mini-Tn*3*-mutagenized yeast genomic sequences were released from vector DNA by digestion with *Not*I and transformed into FY86 cells. Approximately 3×10^3 individual Leu^+ transformants were then plated on YPD agar plates containing 20 mM methylglyoxal bis(guanylhydrazone) (MGBG; Sigma, St. Louis, Mo.), a cytotoxic polyamine analog that is known to share the polyamine transport system in mammalian cells (6, 40).

Identification of disrupted genes in polyamine transport mutants. One MGBG-resistant Leu^+ clone selected by the above-described strategy (MGY1) was transformed with the *Hpa*I-cleaved pRSQ-1 rescuing plasmid, which is a modified version of YIp5 (5) (a generous gift of Michael Snyder), and selected on uracil-free YNB plates. Yeast genomic DNA was then isolated from spheroplasts prepared from Ura^+ transformants essentially as described previously (5), except that the spheroplasts were lysed for 30 min at 65°C in 0.5 ml of a buffer containing 0.3% sodium dodecyl sulfate, 200 μg of proteinase K per ml, and 50 mM EDTA. Purified yeast DNA was digested with *Eco*RI, ligated with 400 U of T4 DNA ligase in 250 μl of ligation buffer, and electroporated into *Escherichia coli* (14). Plasmid DNA purified from positive transformants was then digested with *Bam*HI and *Eco*RI to confirm the presence of vector and insert DNA. The size of the *Bam*HI-*Eco*RI yeast genomic fragments thus rescued from MGY1 cells was 0.8 kb. Flanking yeast DNA was then sequenced by the dideoxy chain termination method with the T7Sequencing kit (Pharmacia Biotech). DNA sequences were analyzed with the BLAST and FASTA programs (39). PEST sequences (48) were identified with the PEST algorithm described by Rogers et al. (48). The chromosome localization of the disrupted genes was determined with the no. 77284 hybridization membrane kit for *S. cerevisiae* AB972 (American Type Culture Collection, Rockville, Md.) by Southern blotting with the ^{32}P-labeled *Eco*RI-*Bam*HI fragment rescued from MGY1 cells as specified by the supplier.

Dominance test and tetrad analysis of *STK2/stk2* diploids. The phenotypic dominance of insertion mutants was determined by constructing diploids between FY86 or MGY1 and the haploid strain DRY370 (*MAT***a** *gal2 leu2-3,112 his3-*Δ*1 trp1-289a ura3-52*) (46). Zygotes were identified microscopically and selected for the His^- Leu^- phenotype on YNB agar plates. Growth on YPD plates containing 20 mM MGBG was then assayed for the diploids thus selected. For tetrad analysis, diploids grown in YPD medium were transferred to sporulation medium (1% sodium acetate, 0.1% yeast extract, 0.05% D-glucose) for 5 days at 30°C, and after a 15-min incubation at 37°C with 0.5 mg of Zymolyase per ml in 0.8 M D-sorbitol, tetrads were dissected under the microscope with a micromanipulator. Spores from five asci were then inoculated onto YPD plates with or without L-leucine, and daughter cells from each colony were restreaked on YPD plates with or without 5 mM norspermine to determine their resistance to toxic polyamine analogs.

Cloning of *STK2*. The full sequence of *STK2* (YJR059w [12]), including 693 and 262 bp adjacent to the 5′ and 3′ ends of the coding region, respectively, was isolated as a *Bam*HI fragment (3.4 kb) (Fig. 1A) from a lambda PM-1436 *S. cerevisiae* genomic clone from chromosome X (ATCC 70345) and was inserted into the unique *Bam*HI site of the YCp50 single-copy expression vector. MGY1 cells were transformed with this construct (YCp-STK2), and after selection on plates containing 5.7 mM 5-fluoroorotic acid to remove the *URA3* marker (4), transformants were then plated on YPD medium containing 5 mM norspermine, a cytotoxic spermine homolog (15), and assayed for [^3H]spermidine uptake activity as described below.

Disruption and cloning of *STK1*. The full sequence of the *STK1* (*KKT8*/*POT1*) gene, including 265 and 168 bp adjacent to the 5′ and 3′ ends, respectively, of the *STK1* open reading frame (ORF) (Fig. 1A), was amplified by PCR and cloned into the *Hin*dIII and *Sac*I restriction sites of pBluescript KS(+) (Stratagene), resulting in the pB-STK1 plasmid. The *STK1* gene was disrupted by introducing a *URA3* marker gene between the *Eco*RI and *Bam*HI sites (Fig. 1A), thus deleting catalytic subdomains I to VII of the putative kinase (see Fig. 2). The FY86 and MGY1 strains were then transformed with the resulting 4,978-bp *Hin*dIII-*Sac*I fragment, and after removal of the *URA3* marker by selection on 5-fluoroorotic acid, deletion within *STK1* was confirmed by PCR and Southern blot analysis (data not shown). This selection produced the Ura^- *stk1*Δ and *stk2::lacZ stk1*Δ deletion mutants used in the present study. The pYES-STK1 construct was generated by subcloning the *Sac*I-*Hin*dIII *STK1* fragment from pB-STK1 into the corresponding sites of the pYES2.0 multicopy expression vector (Invitrogen).

RNA isolation and analysis. Total cellular RNA was isolated (49), and 20 μg of total RNA was then electrophoretically resolved on 1.2% agarose–formaldehyde gels and transferred to a nylon membrane. The RNA blots were hybridized with the 2.1-kb *Sac*I-*Hin*dIII *STK1* DNA insert from pB-STK1 or the PCR-amplified 1.5-kb lambda PM-1436 fragment encompassing the full *STK2* ORF as well as 258 nucleotides adjacent to the 3′ end of the STK2 coding region. The *STK1* and *STK2* probes were labeled with [^{32}P]dCTP by using the T7 Quick Prime random primer kit (Pharmacia Biotech) by standard procedures, and the blots were exposed to autoradiographic film at −80°C for 5 to 24 h.

Growth assays. Growth of yeast strains was determined by diluting cultures growing exponentially in YPD medium to an optical density at 600 nm (OD_{600}) of 0.01 in fresh YPD medium containing the indicated addition of norspermine, LiCl, NaCl, KCl, or D-sorbitol and measuring the cell density by turbidimetry at 600 nm.

Polyamine and leucine transport analysis. [*terminal methylene* (N)^3H] spermidine trihydrochloride (2.65×10^4 Ci/mol) and [2,3-^3H]putrescine dihydrochloride (3.65×10^4 Ci/mol) were obtained from Dupont-New England Nuclear (Lachine, Quebec, Canada). [^{14}C]spermine tetrahydrochloride (108 Ci/mol) and [4,5-^3H]leucine (1.08×10^5 Ci/mol) were purchased from Amersham (Arlington Heights, Ill.). Prior to polyamine transport assays, cells were grown to the mid-logarithmic phase (OD_{600} = 1.1 to 1.3), washed three times in glucose-citrate buffer (50 mM sodium citrate [pH 5.5], 2% D-glucose), and resuspended in 80 μl of the same buffer at 6.3×10^7 cells/ml. When the cells were preincubated in spermidine-supplemented medium, the sodium citrate concentration used for the washing step was 100 mM (with the omission of D-glucose) to ensure a more efficient removal of traces of contaminating polyamines. Transport was initiated by the addition of [^3H]spermidine, [^3H]putrescine (each at 50 Ci/mol), or [^{14}C]spermine (10 Ci/mol) at 10 to 200 μM, as indicated, to a final volume of 100 μl and transfer of yeast cells to a water bath at 30°C with mild shaking. The reaction was stopped at predetermined intervals by adding 1 ml of ice-cold stop buffer {glucose-citrate buffer containing 5 mM *sym*-norspermidine [*N*,*N*′-bis(aminopropyl)-1,3-propanediamine]; Sigma}. Cell suspensions were layered on cellulose acetate filters (pore size, 0.45 μm) held on a manifold filter vacuum apparatus prewashed twice with ice-cold stop buffer, and a mild vacuum was applied. The filters were washed three times with stop buffer, and the radioactivity on the filters was then measured by liquid scintillation spectrometry. In time course experiments, nonspecific binding was determined by adding radioactive substrate to the yeast suspension and immediately washing the cells on the filters. Kinetic parameters were determined by adding increasing concentrations of nonradioactive substrate to a fixed concentration of [^3H]spermidine or [^3H]putrescine. Uptake data were best fitted to a pump-and-leak model including a saturable and a diffusional component of the form

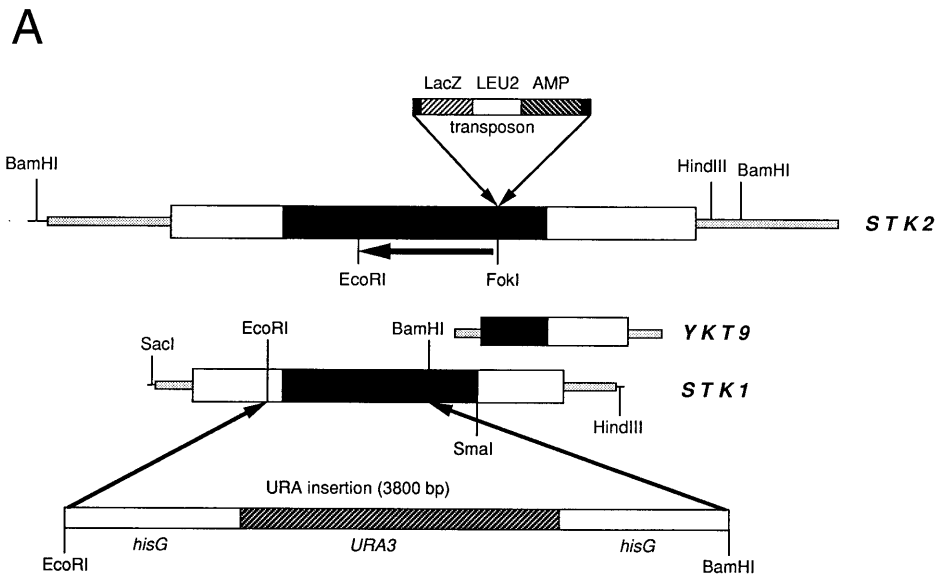
$$V = \frac{V_{max}S}{K_m + S} + PS$$

where V is the initial uptake velocity, S is the substrate concentration, and P is a permeability constant (29). Nonspecific uptake as measured by parallel incubation of yeast cells at 4°C yielded values of P comparable to those derived by the above equation.

Leucine transport was assayed by incubating yeast cells in citrate-glucose buffer containing 200 μM [^{14}C]leucine (10 Ci/mol) for up to 20 min and determining the radioactive content at predetermined intervals.

RESULTS

Disruption of the YJR059w ORF confers growth resistance to toxic polyamine analogs. To identify genes involved in polyamine transport, the haploid FY86 (wild-type) strain was transformed with a mini-Tn*3::lacZ::LEU2* genomic bank, and the Leu^+ transformants thus obtained were selected on minimal medium plates containing 20 mM MGBG, a toxic polyamine analog (40). That concentration of MGBG completely prevented colony formation by wild-type cells on agar plates (data not shown). The *Eco*RI fragment containing yeast genomic



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STK2    1 MAGNGKDKEVDKSPSVSTLKLLGKRLFNSSSHTDNSSLLLSAEQLGNGRSLRKRPTSPSISGSGSGGNSPSSSAGARQRSASLHRRKNNA 90
                                                                                                    ::| :      |    :   :     :| :
STK1    1                                                                                          MTVSHNHSTKISQQPISSVSAFKFFGKKLLS 31

STK2   91 SVGFSNGSVSSHKSSVALQDLIKHNNNPYLNSPSDILGTGTGIASTRDRDRAVLDREKEKERARNKERNTHHAGLPQRSNSMASHHFPNE 180
          |   :     :              : :     |: :  :    :     :                    :|  :   : |::  :
STK1   32 SSHGNKLKKKASLPPDFHSTSTNDSESSSPKLPNSLKTSRRANSFAHTTNSKRSLSSASTKILPPAGSSTSISRGNRHSSTSRNLSNSKF 121

STK2  181 NIVYNPYGISPNHARPDTAFADTLNTNKENDLSFYMHDGNSKIRMLPLPIANPNDFLPEDMKQYSVHLTDNFVFDTDNKPIGSGGSSEVR 270
          :     |          :  : : :  |: || ||:|||:|||||||:|| :||::||::||: |: |:|||||| :|| || ||| |||
STK1   122 SSERLVYNPYGVSTPSTSLSSVSTSMKKDPDLGFYLHDGDSKIRMLPIPIVDPNEYLPDEMKEASIQLSDNFVFDDENKTIGWGGSCEVR 211

STK2  271 KVKSSYRQKDVYALKKLNMIYHESPEKFYKRCSKEFIIAKHLSHNVHITNTFYLLKVPTTTYTTRGWGFIMELGVKDLFQLMERTGWKNV 360
          |::| ||:|||:|||||||||:|:|||||||||||||||| |||||||||| |:|||| ||||||||:||||::||| :::::||::|
STK1   212 KIRSKYRKKDVFALKKLNMIYNETPEKFYKRCSKEFIIAKQLSHNVHITNTFLLVKVPTTVYTTRGWGFVMELGLRDLFAMIQKSGWRSV 301

STK2  361 PFNEKYCLFKQVAQGIKFCHDNGIAHRDLKPENVLISKEGICKLTDFGISDWYHVIPHDYTSPVKTCQGMIGSPPYTPPEVMYFDAKKHY 450
             ||:|:||||| |:|||| |||||||||||||:| :|:||||||||||||  ||| :|||| | |||||||| |||||::|:||||
STK1   302 ALAEKFCIFKQVACGVKFCHDQGIAHRDLKPENVLLSPDGVCKLTDFGISDWYHTDPHDLSSPVKKCAGMIGSPPYAPPEVMFYDSKKHY 391

YKT9     1                                                                                    MEYHLARNFKNGHASRVA 18
                                                                                                      ↓
                                                                                                      || |||||||  |:|:|
STK2   451 PEKFQKPYNPLAMDSYALGIMLITMINNIIPFIDSCNTDARFREFEVSYDNFINHQNPHFRDNKGCHKPGPGSEYSLARNFKNTDATRIA 540
             : | ||:| |:| | |||:|:|::||::|||::||: |  ||::  :|:|||   :  ||:     |    |:  |     :
STK1   392 DTELQQPYDPRALDCYGLGIILMTLVNNVIPFLESCSFDTGFRDYCDAYENFIRLHDRAFRNRAITARGREWSITWLEISRTDMHLAWHG 481

YKT9    19 WRLADPEAATRYTIDDLFEDPWFQGIETCVDANDKYVCKKPIIKTTTYENPRGFHIATDVAATTP--TSNPFLKNRVPI---RSMVDIAA 108
          ||||||  |||||:|||| ||:|| |||||: ||  : : | :: ::  |                 |||||||        |||::||
STK2   541 WRLADPNPATRYTMDDLFNDPFFQQIETCVEPNDDDLVRVPELRKSTSTNDFSENSLDAPHDQEVIHTSNPFLKKETLTSKPRSMLEIAE 630
             |    | |: :                :   :  :  :      |     | :           |
STK1   482 GSLTQKPPPTKPSTTSSKTHGSKELRLVLMPTTNMCVRNLLSKPLRTRIRGVSISLQMLLQPHPPQTHSLRTESPSGQWstop        531

YKT9   109 HPSPTAVLASSPPPPPPATHVPAEALFTLRETPPPQLATLTLSEEPPATPAPSAPSAPSARVRGHSPHRVGASPSEHCQQLGPstop    187
           ||                  |  |      :            :|             :         :   : ::
STK2   631 SPSLKQKSKVKDSAKTKTHDVGDEGGNESTKPKQQDKKENLKKDEVKNGDKDKVIEEATTTNVDSILEKPTPTSTKVEDNLSEDDSTMKE 720

STK2   721 LKSMLNSTPTTPTHNGPTPLPAKAGTQLDKRMSDLSLKSETPASTKNFSAPNVSSSSNSLRSLGSPSVSSSKKKKVIHHHLDITNSVTNM 810
STK2   811 SSVSAFISRstop 819
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FIG. 1. Restriction map and amino acid sequence alignment of *STK1*, *STK2*, and *YKT9*. (A) Aligned maps of the three genes with restriction sites mentioned in the text. The thick rectangles represent the predicted ORFs with aligned regions of homology. The solid portions of the *STK2*, *YKT9*, and *STK1* ORFs represent the regions of high homology as aligned with the *STK2* ORF. The site of insertion of the mini-Tn*3* transposon into *STK2* determined for the MGY1 clone is shown, together with the *lacZ* and *LEU2* marker genes. The region of *STK1* deleted by the introduction of the *URA3* marker (with *his* repeats) used for construction of the *stk1*Δ cells is indicated at the bottom. (B) Sequence homology between *STK1*, *STK2*, and *YKT9*. The region of Stk2p deduced from the sequence of the *Eco*RI fragment rescued from MGY1 cells is underlined. The alignment with *STK1* (*POT1*/*KKT8*) (23) and *YKT9* (9) was generated by using the FASTA program (39). Identical and conserved residues are indicated by vertical lines and double dots, respectively. An asparaginyl residue (arrow) found in the *STK2* ORF is missing in the chromosome X contig sequence released at the Saccharomyces Genome Database (Stanford University, Stanford, Calif.). The PEST sequences identified in *STK1* and *STK2* are underlined twice.

DNA adjacent to the inserted *lacZ* was isolated from one MGBG-resistant clone (MGY1) from about 500 positive transformants by a plasmid rescue strategy (5). Three other insertion mutants with a lower-level resistance to MGBG were isolated by this procedure, and the disrupted genes in these mutants were different from the lesion found in MGY1 cells (26).

The MGY1 clone expressed strong β-galactosidase activity (data not shown), indicating that an in-frame genomic insertion yielding a fusion protein had occurred. Diploid His^+/Leu^+ cells constructed by mating the His^-/Leu^+ MGY1 clone with the haploid strain DRY370 (His^+/Leu^-) were unable to grow on 20 mM MGBG or 5 mM norspermine, unlike the MGY1 mutants, indicating that the mutation in the latter was recessive. Moreover, meiotic analysis showed that the *LEU2* marker and the locus responsible for MGBG resistance cosegregated in a 2:2 fashion against the Leu^- and the MGBG-sensitive phenotypes (data not shown), as expected from a single insertional event. A single yeast *Eco*RI genomic DNA fragment of 0.8 kb, which mapped to the right arm of chromosome X, could be rescued from the MGY1 clone. Sequencing of that fragment with a *lacZ*-specific primer identified a 747-bp DNA segment that was virtually identical to the portion of the YJR059w ORF encoding residues 305 to 552 (Fig. 1B) (12).

Primary structure of the *STK2* (YJR059w) coding sequence. The sequence of YJR059w includes a 2,460-bp ORF encoding a protein of 819 amino acids with a calculated molecular weight of 87,728 (Fig. 1B). Only one discrepancy was found between the published sequence of YJR059w (12) and our own sequencing data, namely, the presence of an extra asparaginyl residue at position 513. Sequence analysis showed that one segment (residues 208 to 513) of the protein encoded by YJR059w is highly homologous to the region encompassing residues 149 to 453 of the product of the *POT1*/*KKT8* gene, which encodes a putative Ser/Thr protein kinase reported as a positive effector of low-affinity spermine uptake in *S. cerevisiae* (23). In fact, this 304-amino-acid stretch comprises 207 identities and 44 conservative changes, with a resulting 82.6% homology between the corresponding domains of the two ORFs. There is no significant homology between the coding sequences of the two genes outside that stretch. Moreover, the ORF of YJR059w is larger than that of *POT1*/*KKT8*, with the N- and C-terminal portions encoded by the former extending 59 and 200 residues, respectively, beyond the corresponding extremities of the product of the *POT1*/*KKT8* ORF, which comprises 560 amino acids, after alignment with YJR059w (Fig. 1A). Because of the high homology between the two genes and because the name *POT1* is already used for another locus (42), we have proposed the names spermidine/spermine transport kinases 1 and 2 (*STK1* and *STK2*) for the products of the *POT1*/*KKT8* and YJR059w ORFs, respectively.

STK2 most likely encodes a Ser/Thr protein kinase, as shown by the presence of invariant residues characteristic of catalytic subdomains I, II, III, VIb, VII, VIII, and IX of these kinases (16) (Fig. 2). It is noteworthy that the region of homology between the *STK2* and *STK1* coding regions encompasses the sequences encoding catalytic subdomains I to IX and, to a lesser degree, X (Fig. 2), as well as about 50 residues immediately upstream of subdomain I (Fig. 1B). Both Stk1p and Stk2p have potential PEST sequences, which are hydrophilic regions rich in Asp, Glu, Pro, Ser, and Thr residues (often present in rapidly degraded proteins) (48), located at residues 49 to 62 (PEST score = 20.49) and residues 585 to 601 (PEST score = 6.86), respectively, i.e., outside the regions of homology between the two putative kinases (Fig. 1B).

Sequence comparison revealed that the most closely related congeners of *STK1* and *STK2* in *S. cerevisiae* are a group of structurally related ORFs with a Ser/Thr protein kinase signature including, in relative order of homology to *STK1* and *STK2*, *KCR8* (52) > *KKQ8* (57) ≈ *HAL5* (30) ≈ *NPR1* (58, 59) (Fig. 2). Most of the homology among these ORFs and the *STK1* and *STK2* coding sequences is found in the sequences encoding catalytic subdomains I, II, III, VIb, VII, VIII, and IX. Among these related ORFs, only *KCR8* and *NPR1* have thus far been functionally characterized. Whereas Npr1p is required for the posttranslational activation of Gap1p and other amino acid permeases upon transfer to poor nitrogen sources (58, 59), disruption of *KCR8* results in salt hypersensitivity (52).

The regions of Stk2p encompassing residues 523 to 589 and 607 to 631, which lie in a domain sharing no homology with the product of the *STK1* ORF, are highly homologous to segments (residues 2 to 68 and 84 to 106, respectively) of the N-terminal portion of the product of *YKT9* (YKL199c), a 187-residue-encoding ORF whose product overlaps the C-terminal end of the product of the *STK1* ORF (9) (Fig. 1B). These two segments have 75 and 74% homology, respectively, to the corresponding regions encoded by *STK2*, and the reading frame of *YKT9* is shifted by −1 nucleotide relative to that of *STK1* (data not shown).

Expression of *STK1* and *STK2* mRNAs. Northern blot analysis was performed to assess the expression of *STK1* and *STK2* (Fig. 3). The *stk2::lacZ* (MGY1) mutants transformed with the pYES-STK1 overexpression vector exhibited three major transcripts (2.7, 1.5, and 1.1 kb) hybridizing to the *STK1* probe. No *STK1*-specific transcript could be detected in the wild type, in *stk2::lacZ* cells transformed with a YCp50 vector carrying the *STK2* gene, or in *stk1*Δ cells. On the other hand, low levels of two mRNAs (1.5 and 1.1 kb) corresponding to the two shorter *STK1*-specific transcripts were found in *stk2::lacZ* cells. The nature of the major 2.7-kb *STK1*-specific transcript found exclusively in *STK1*-overexpressing cells is unclear but could have resulted from aberrant polyadenylation. On the other hand, hybridization with a *STK2* DNA probe identified a 2.8-kb mRNA species in wild-type and *stk1*Δ cells but not in *stk2::lacZ* cells, in which an approximately 5-kb mRNA corresponding to the *STK2-lacZ* fusion transcript was detected. Transformation of *stk2::lacZ* cells with the YCp-STK2 expression vector restored the expression of the 2.8-kb *STK2*-specific mRNA species. The *STK2* cDNA probe also hybridized to a ca. 1.5-kb transcript present at similar levels in all strains and to an additional, ca. 1.0-kb band that was apparently unique to *stk2::lacZ* cells. These extra RNA species could not be detected under higher-stringency conditions (data not shown).

***STK2* disruption promotes resistance to toxic polyamine analogs by decreasing high-affinity spermidine transport.** To assess the respective roles of *STK1* and *STK2* in the regulation of polyamine transport, we determined the relative growth resistance of *stk2::lacZ* and *stk1*Δ mutants to norspermine, a spermine homolog that is cytotoxic in yeast (15). Norspermine was used in these experiments to circumvent the problem of MGBG precipitation in phosphate-containing media (26) and because of its closer homology to natural polyamines. As shown in Fig. 4A, growth rates of wild-type, *stk2::lacZ*, and *stk1*Δ cells were identical, indicating that disruption of either *STK1* or *STK2* does not affect cell viability or the ability to grow in a nutrient-rich medium. Growth of the wild-type parental strain was strongly inhibited by 1 mM norspermine, whereas *stk2::lacZ* cells were fully resistant to the spermine homolog (Fig. 4B). On the other hand, *stk1*Δ mutants exhibited a much more limited resistance to norspermine (Fig. 4B), with an approximately threefold increase in the half-maximal growth-

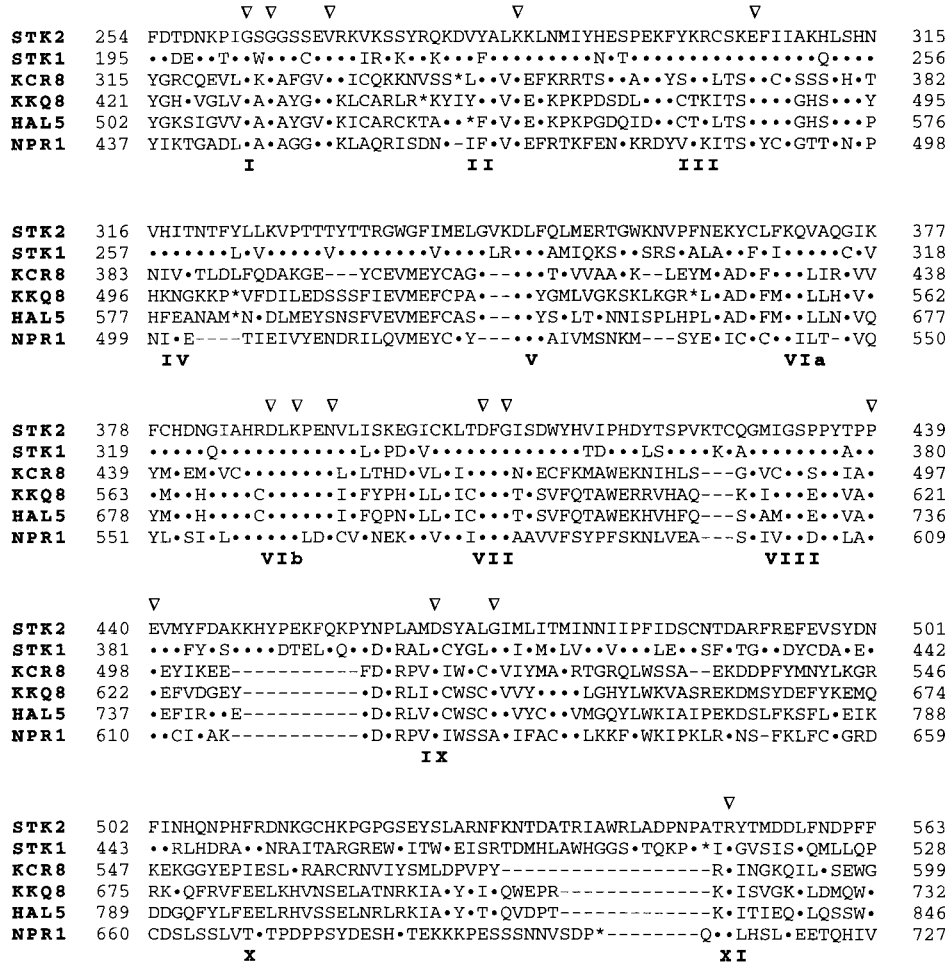

FIG. 2. Coding-sequence homology between Stk2p and related Ser/Thr protein kinases in yeast. Optimal alignments were computer generated with the FASTA program (39). The product of the *STK2* ORF is aligned in order of decreasing homology (from top to bottom) with the products of the ORFs for the enhancer of low-affinity spermine transport *STK1* (23), the *KCR8* (YCR101) halotolerance gene (52), the genes for two putative Ser/Thr protein kinases of unknown function, *KKQ8* (57) and *HAL5* (30), and the nitrogen permease reactivator *NPR1* (58). Gaps introduced for optimal alignment are indicated by hyphens. Small deletions in protein sequences have been introduced to allow direct alignment with *STK2* and are indicated by asterisks. Residues identical to the corresponding position in Stk2p are indicated by solid dots. Roman numerals under the sequence blocks designate the standard catalytic subdomains of Ser/Thr protein kinases (16). Residues conserved in all eukaryotic Ser/Thr protein kinases are indicated by arrowheads above the sequence blocks.

inhibitory concentration of the polyamine analog (data not shown). Thus, *stk2::lacZ* mutants were cross-resistant to MGBG and norspermine, two polycationic compounds with rather dissimilar structures, which are substrates of the polyamine transport system in higher eukaryotes (6, 44).

To determine whether the resistance of *stk2::lacZ* cells to toxic polyamine analogs resulted primarily from a defect in specific polyamine transport, the kinetic parameters of the spermidine uptake by the various yeast strains were then compared. Michaelis-Menten analysis showed that the spermidine affinity for saturable uptake was decreased about fivefold in the *stk2::lacZ* disruption mutants (K_m = 27 ± 6 μM) compared to wild-type cells and *stk1*Δ mutants (K_m = 4.8 ± 0.7 and 5.5 ± 0.7 μM, respectively) (Fig. 5). The K_m value measured for wild-type spermidine transport is in good agreement with that (≈4 μM) previously reported for *S. cerevisiae* (56). There was also a nearly twofold reduction in the V_{max} (39 ± 2 pmol/min/10^7 cells) of saturable spermidine transport in *stk2::lacZ* cells relative to wild-type and *stk1*Δ cells (V_{max} = 67 ± 2 and 75 ± 2 pmol/min/10^7 cells, respectively). Spermidine transport activity (Fig. 6) and sensitivity to MGBG and norspermine (data not shown) were restored to wild-type levels in *stk2::lacZ* cells transformed with a single-copy expression vector encoding the full *STK2* gene, whereas transformation with vector alone had no effect on spermidine uptake. Thus, resistance of *stk2::lacZ* cell growth to toxic polyamine analogs is correlated with a strong reduction in saturable spermidine transport as a result of decreased affinity and V_{max}, and *STK2* disruption was clearly responsible alone for the defect in polyamine transport observed in *stk2::lacZ* mutants.

Since no defect in the initial velocity of spermidine influx could be detected in *stk1*Δ mutants despite their low but consistent resistance to norspermine, the time course of spermidine accumulation was compared for the three strains. Spermidine accumulation was decreased to about 80 and 50% of control levels over a 20-min incubation period in *stk1*Δ and *stk2::lacZ* cells, respectively, at 200 μM substrate (Fig. 7A), whereas *STK1* deletion had only a marginal effect on time-dependent spermidine uptake at 10 μM substrate (see Fig. 9C). Furthermore, low-affinity spermine accumulation was similarly reduced by about 20 to 25% in *stk1*Δ and *stk2::lacZ* cells (Fig. 7B). Thus, *STK2* disruption primarily depresses the

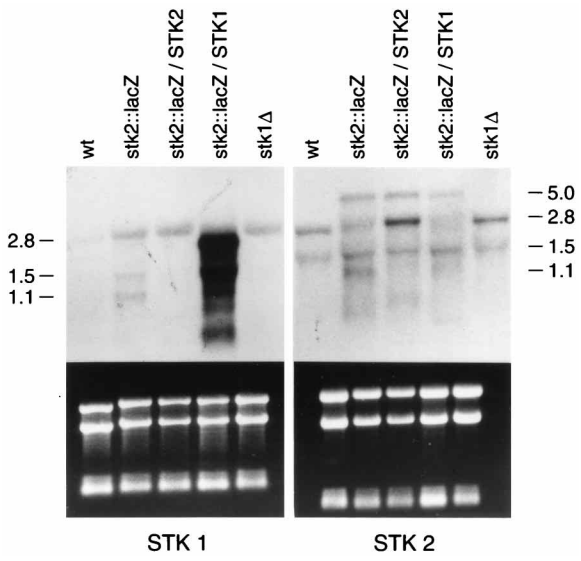

FIG. 3. Northern blot analysis of *STK1* and *STK2* expression. Total RNA isolated from wild-type (wt) cells, *stk2::lacZ* cells, *stk2::lacZ* cells transformed with YCp-STK2 (*stk2::lacZ*/STK2) or pYES-STK1 (*stk2::lacZ*/STK1), and *stk1*Δ cells was probed with *STK1* or *STK2* cDNA. The low level of hybridization to the 26S rRNA detected in all strains in the left panel is due to the lower stringency that was used to demonstrate the upregulation of *STK1*-specific transcripts in *stk2::lacZ* cells. Estimated lengths (in kilobases) of mRNA species are indicated, and ethidium bromide-stained agarose gels are shown below each fluorogram.

initial velocity of a high-affinity spermidine transporter, whereas *STK1* deletion affects a component of spermine and spermidine transport with a lower affinity and a smaller contribution to total uptake activity.

stk2::lacZ cells were also strongly deficient in putrescine uptake (Fig. 8). Putrescine accumulation was approximately time linear and fivefold lower in *stk2::lacZ* cells than in wild-type cells over a 30-min incubation (Fig. 8A), in contrast to spermidine uptake, which reached an apparent plateau after 10 to 20 min (Fig. 7A; also see Fig. 9). As shown in Fig. 8B, putrescine uptake showed no apparent saturation up to 3 mM and had a threefold-lower initial velocity than did spermidine uptake at concentrations below 100 μM. The initial velocity of putrescine uptake was about twofold lower in *stk2::lacZ* cells than in wild-type cells at all substrate concentrations tested (3×10^{-7} to 3×10^{-3} M).

Polyamine transport in yeast is under feedback repression by exogenous polyamines: effect of defective *STK1* and *STK2* function. In yeast, sugar (3, 38) and amino acid (13, 21, 53, 63) transport is regulated by various feedback mechanisms that qualitatively and quantitatively depend on substrate availability. For instance, expression of the general amino acid permease encoded by *GAP1* is controlled at transcriptional and posttranslational levels by nitrogen catabolite repression (53, 58, 63). Moreover, polyamine transport in animal cells is known to be under negative feedback control by intracellular polyamine levels by the ornithine decarboxylase antizyme (7, 17, 31, 33, 35, 50, 54), but similar repression mechanisms have not yet been described for yeast.

We therefore assessed the effect of preincubation with exogenous spermidine on subsequent polyamine transport activity. Yeast cells were grown for 20 to 24 h in an amine-free basal medium supplemented or not supplemented with 100 μM spermidine, and the time course of [^{3}H]spermidine uptake was determined. As shown in Fig. 9A, prior incubation with spermidine strongly depressed subsequent spermidine accumulation in wild-type cells. The rate of polyamine accumulation in spermidine-repressed cells was only slightly lower than that measured in cells grown in YPD medium prior to the assay, which is consistent with the fact that complete yeast extract contains substantial amounts of spermidine (26). Spermidine accumulation was extremely low in *stk2::lacZ* cells preincubated with spermidine, and spermidine starvation derepressed

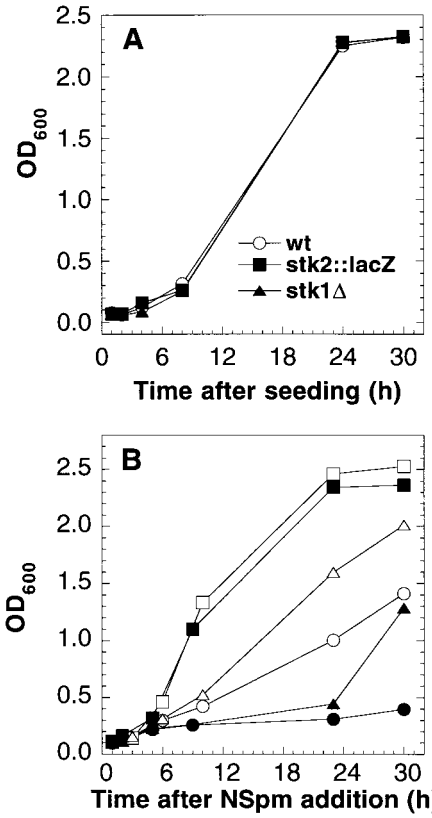

FIG. 4. Effect of *STK1* and *STK2* disruption on growth tolerance of norspermine (NSpm). (A) Basal growth rates of wild-type (wt), *stk2::lacZ*, and *stk1*Δ cells. (B) Effect of 300 μM (open symbols) or 1 mM (solid symbols) norspermine on growth of wild-type, *stk2::lacZ*, and *stk1*Δ cells in YPD medium.

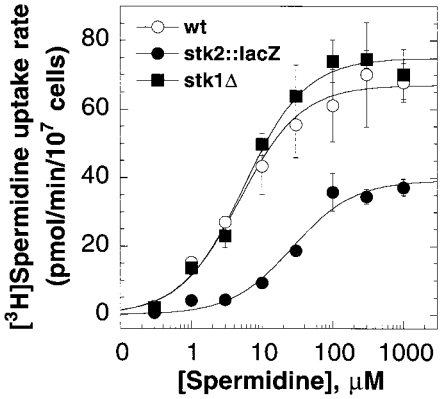

FIG. 5. Initial velocity of [^{3}H]spermidine uptake in wild-type (wt), *stk2::lacZ*, and *stk1*Δ cells. Specific uptake was determined after correction for a diffusional component, as described in Materials and Methods. Data represent the means ± standard deviations for triplicate determinations from three independent experiments.

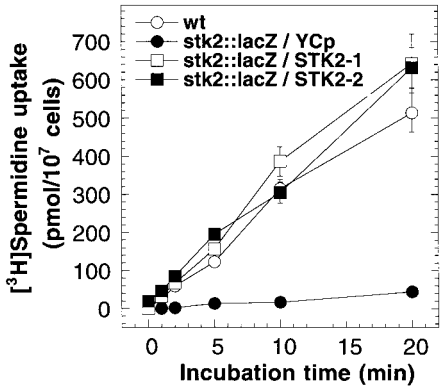

FIG. 6. Complementation of the spermidine transport defect of *stk2::lacZ* cells by the full-length *STK2* gene. The time course of spermidine uptake was measured in wild-type (wt) cells, in *stk2::lacZ* cells transformed with vector alone (*stk2::lacZ*/YCp), and in two independent clones (STK2-1 and STK2-2) of *STK2*-transformed *stk2::lacZ* cells, with 10 μM [^{3}H]spermidine as the substrate. Data are the means ± standard deviations for triplicate determinations from a representative experiment.

spermidine uptake to about 60% of the rate observed in spermidine-repressed wild-type cells (Fig. 9B). Whereas *stk1*Δ mutants underwent derepression of spermidine uptake to the same level as wild-type cells (Fig. 9C), disruption of both *STK1* and *STK2* almost fully abolished high-affinity spermidine transport under either repressing or derepressing conditions (Fig. 9D). These results clearly indicate that (i) residual spermidine transport present in *stk2::lacZ* mutants is due to the activity of the intact *STK1* gene, (ii) expression of either *STK1* or *STK2* is essential for high-affinity spermidine transport, and (iii) the spermidine uptake activities regulated by *STK1* and *STK2* are both repressed by spermidine. However, prior incubation in a polyamine-free medium did not lead to an increase in the rate of putrescine accumulation in either wild-type or *stk2::lacZ* cells (Fig. 10). Thus, spermidine selectively represses spermidine transport, and the low rate of putrescine uptake in *S. cerevisiae* grown in YPD medium cannot result from feedback inhibition by exogenous polyamines present in the yeast extract.

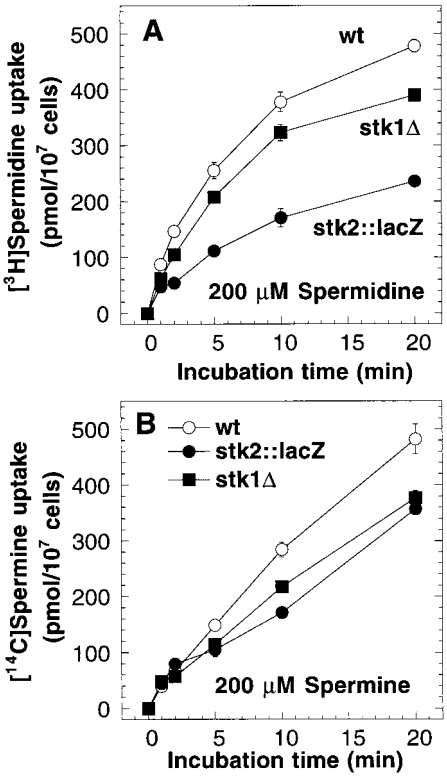

FIG. 7. Effect of *STK1* and *STK2* disruption on the time course of [^{3}H]spermidine (A) and [^{14}C]spermine (B) uptake (each present at 200 μM) in wild-type (wt), *stk2::lacZ*, and *stk1*Δ cells. Data represent the means ± standard deviations for triplicate determinations of total uptake after correction for the initial diffusional component.

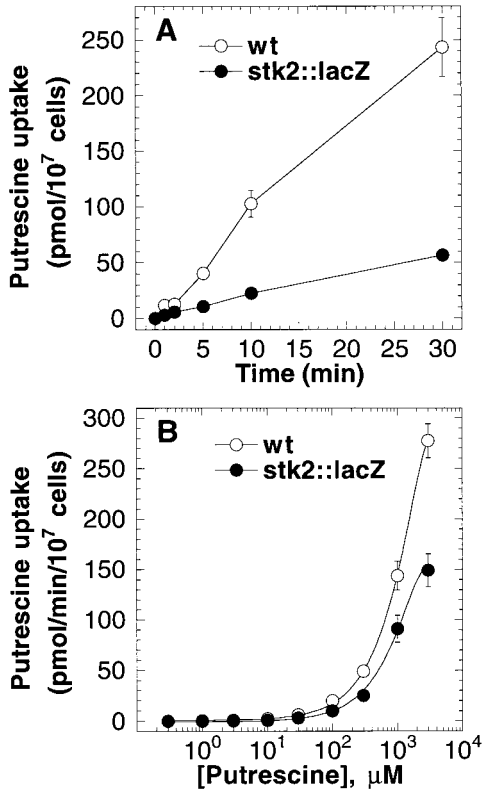

FIG. 8. Characteristics of putrescine transport in wild-type (wt) and *stk2::lacZ* cells. (A) Time course of putrescine uptake, using 50 μM [^{3}H]putrescine. (B) Concentration dependence of the initial velocity of putrescine uptake for a 1-min incubation period.

Disruption of *STK2* leads to a salt-resistant phenotype. The *stk2::lacZ* and wild-type cells exhibited similar kinetics of leucine uptake (data not shown), indicating that the loss of *STK2* function does not lead to a general decrease in membrane transport activity. On the other hand, *KCR8*, which encodes the closest Ser/Thr kinase homolog of Stk1p and Stk2p, has been reported to promote halotolerance in yeast (52). We thus assessed the effect of *STK1* or *STK2* disruption on growth tolerance of Na^+, Li^+, and K^+ (Fig. 11). Li^+ is often used as a more toxic substitute for Na^+ and permeates the same influx and efflux pathways in yeast (45). Whereas *stk1*Δ cells had no effect on Li^+ resistance, *stk2::lacZ* cells were clearly more tolerant of this cation (Fig. 11A). Disruption of both *STK1* and *STK2* did not lead to a further increase in Li^+ tolerance (data not shown), suggesting that only *STK2* influences Li^+ tolerance. A comparable increase in growth resistance to Na^+ was observed in *stk2::lacZ* cells, whereas only a marginal difference was noted for K^+-induced growth

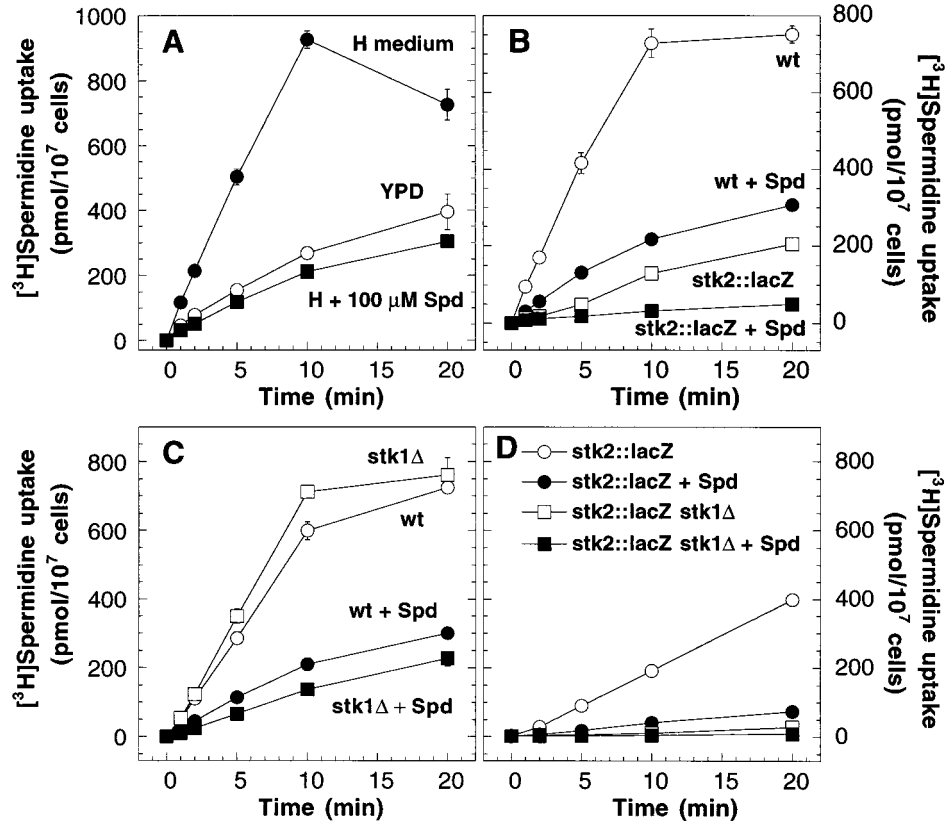

FIG. 9. Downregulation of spermidine transport by exogenous spermidine. Cells were grown for 20 to 24 h in YPD medium or in amine-free basal medium (H medium) supplemented or not supplemented with 100 μM spermidine (Spd), as indicated, after which the time course of [^{3}H]spermidine uptake was measured at 10 μM substrate. (A) Effect of medium composition on spermidine uptake in wild-type cells grown in YPD medium, in H medium containing 100 μM spermidine, or in H medium with no amine. (B and C) Effect of exogenous polyamine deprivation on spermidine uptake in wild-type (wt) cells and either *stk2::lacZ* cells (B) or *stk1*Δ cells (C) grown in the absence (open symbols) or presence (solid symbols) of spermidine. (D) Effect of exogenous polyamine deprivation on spermidine uptake in *stk2::lacZ* cells and *stk2::lacZ stk1*Δ double-deletion mutants grown in the absence (open symbols) or presence (solid symbols) of spermidine. Data are the means ± standard deviations for triplicate determinations from at least two independent experiments.

inhibition (Fig. 11B). Resistance to Na^+ and Li^+ was not due to increased osmotolerance, since *STK2* disruption had no effect on growth with osmotically equivalent concentrations of D-sorbitol (data not shown).

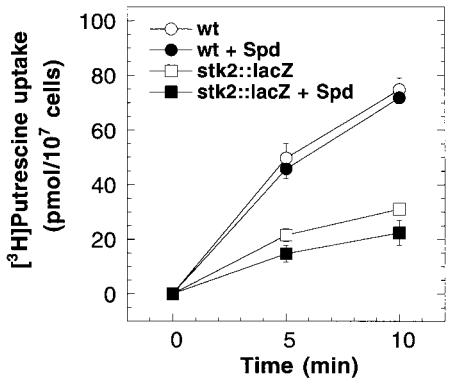

FIG. 10. Spermidine does not repress putrescine transport. Wild-type (wt) or *stk2::lacZ* cells were grown for 20 to 24 h in polyamine-free basal medium (H medium) in the absence (open symbols) or presence (solid symbols) of 100 μM spermidine, before measurement of the time course of [^{3}H]putrescine uptake with 50 μM substrate.

DISCUSSION

Using a transposon mutagenesis approach, we have identified *STK2* as a novel gene involved in the regulation of polyamine transport in yeast. Despite the high degree of homology between the catalytic core region of *STK1* and *STK2*, these two putative protein kinases are differently involved in the regulation of spermidine transport. *STK2* is clearly required for the expression and/or activity of a high-affinity spermidine transporter, whereas *STK1* expression regulates a saturable spermidine uptake system with a fivefold-lower affinity and a twofold-lower V_{max}.

STK1 was first identified as a gene restoring low-affinity spermine transport in a chemically mutagenized yeast clone (YTM22-8). However, functional complementation of the spermine transport defect by *STK1* in the YTM22-8 mutants was obtained with a high-copy-level expression vector (23). In fact, *STK1* could not complement the spermine transport defect of these mutants when borne on a single-copy vector (YCp50), and after submission of the present report, Nozaki et al. independently showed that a YCp50-borne *STK2* gene could almost fully restore spermine uptake activity in the same cells (37). These authors proposed that *STK2* affects a low-affinity polyamine transport (37), based on unpublished kinetic data and on the fact that the phenotype selected for complementation was the ability to grow in the simultaneous presence

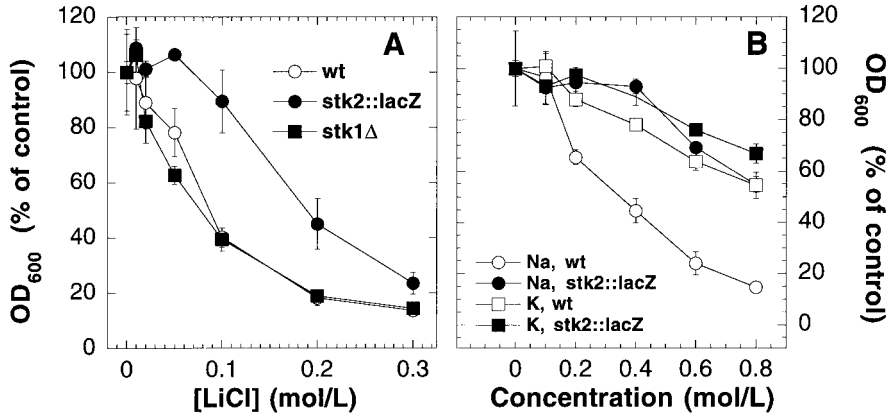

FIG. 11. *STK2* disruption confers growth tolerance of high Li^+ and Na^+ concentrations. Cells were grown overnight in the presence of increasing concentrations of the indicated cation, after which the cell growth was assessed by measuring the OD_{600}. (A) Effect of LiCl on growth of wild-type (wt), *stk2::lacZ*, and *stk1*Δ cells. (B) Effect of NaCl and KCl on growth of the wild-type (wt) (open symbols) and *stk2::lacZ* (solid symbols) cells. Data are the means ± standard deviations for triplicate determinations from a representative experiment.

of spermine and low (50 μM) Mg^{2+} concentrations (23), a combination known to be deleterious for growth (32). This low-affinity spermine uptake system could be a divalent cation transporter which is blocked by spermine and, to a much lesser extent, spermidine and putrescine (32). The current results indicate that *STK1* and *STK2* clearly regulate two modes of high-affinity, saturable spermidine transport with different K_m and V_{max} values. Discrepancies between the roles attributed to *STK2* in specific spermidine (this report) and spermine (37) transport are not clear but may be due to differences between the two transport systems and/or to the use of different conditions (i.e., citrate-based [pH 5.5] and HEPES-based [pH 7.2] buffers, respectively) for polyamine uptake assays.

The high degree of conservation between the catalytic domains of Stk1p and Stk2p strongly suggests that these putative kinases are functionally related. The current results cannot discriminate between the possibilities that differences between the spermidine uptake systems regulated by *STK1* and *STK2* reflect (i) the existence of distinct spermidine permeases with different kinetic properties or (ii) the posttranslational modification(s) of a single carrier species due to the differential activity of these genes, leading to different kinetic modes for this transporter. The fact that *STK1* disruption had only a marginal effect on spermidine uptake could merely reflect the higher velocity and affinity of the spermidine uptake system specifically targeted by *STK2*. Nevertheless, the possibility that a regulatory interplay exists between the two genes cannot be ruled out, since *STK2* disruption apparently increased *STK1* mRNA levels, pointing to a possible repression of *STK1* expression by *STK2*. The relative contribution of *STK1* and *STK2* to spermidine transport is somewhat analogous to the situation described for the *TRK1* and *TRK2* genes, which encode high- and low-affinity K^+ carriers, respectively, in *S. cerevisiae* (10, 28). Whereas *TRK1* deletion confers low K^+ uptake capacity with a lower affinity, *TRK2* disruption leads to a phenotype that is virtually indistinguishable from the wild type because Trk1p is the dominant K^+ carrier expressed under standard conditions (27, 61). Nevertheless, disruption of both *TRK1* and *TRK2* severely impairs K^+ uptake, indicating that the expression of both genes contributes to K^+ homeostasis (28).

It is noteworthy that the *STK1*-specific transcripts detected in *stk2::lacZ* cells were shorter (1.5 ± 0.1 and 1.1 ± 0.1 kb) than those expected for the predicted *STK1* ORF (≈1.7 kb), which raises questions about the actual structure of the *STK1* gene product. The high homology noted between *STK2* and the *STK1*/*YKT9* overlapping sequences most probably arose from a gene duplication event involving chromosomes X and XI, as evidenced by examination of the corresponding nucleotide sequences of extensive chromosomal regions adjacent to the *STK1* and *STK2* genes (12). The striking degree of in-frame sequence conservation between *YKT9* and the sequence of *STK2* encoding catalytic subdomain XI (Fig. 1B and 2) suggests that the actual structure of Stk1p may differ from the ORF product predicted for *STK1* (9, 23), as also noted by Nozaki et al. (37). Therefore, a careful reexamination of the *STK1* gene sequence and the structural characterization of its transcript(s) are required to clarify the nature of the Stk1p product.

A role for protein kinases in the control of membrane transport in yeast has been documented at both the transcriptional (8, 38, 60) and posttranslational (58, 62) levels. The function of the Ser/Thr kinase encoded by *NPR1* in the posttranslational regulation of several yeast amino acid permeases, including Gap1p, has been extensively investigated (13, 53, 58, 59, 63). Gap1p is known to be phosphorylated in vivo and is rapidly dephosphorylated and inactivated upon transfer to repressing conditions (53). The Gap1p inactivation process is thought to be mediated, at least in part, by the constitutive action of the *NPI1* gene, encoding a ubiquitin-protein ligase (11). The identity of the kinase(s) that phosphorylates Gap1p and the phosphorylation sites have not been determined. However, the fact that Npr1p regulates Gap1p posttranslationally makes it a likely candidate as a proximate effector of Gap1p phosphorylation (58, 59). The structural homology between *NPR1* and the *STK1*/*STK2* genes led us to assess the possibility that the latter functions in a manner analogous to *NPR1* in the control of polyamine transport. The present results demonstrate that spermidine downregulates its own transport but not that of putrescine in *S. cerevisiae*. Although feedback repression of putrescine and polyamine uptake by exogenous substrate in mammalian cells is well documented (22, 31, 36, 50, 54), a polyamine-dependent regulation of spermidine transport had not been previously found in yeast. Whereas disruption of both *STK2* and *STK1* strongly prevented the derepression of spermidine transport, *stk2::lacZ* mutants were also strongly deficient in putrescine transport, which is not repressed by exogenous amines. The lack of coordinate changes in spermidine and putrescine uptake under derepressing conditions suggests

that if Stk2p behaves as a polyamine-repressed, Npr1p-like reactivator of spermidine transport, it must also play an additional, permissive role in putrescine uptake. A simpler explanation might be that *STK1* and *STK2* are constitutively required for expression or activity of the putrescine/polyamine permease(s) and that Npr1p or other closely related kinases act as reactivators of the spermidine permease(s) under derepressing conditions. Isolation of the polyamine permease gene(s) should help to resolve the actual role of *STK1* and *STK2*.

Defective Stk2p activity markedly increases tolerance of Li^+ and of high Na^+ concentrations, with little or no effect on growth sensitivity to K^+ or osmotolerance per se. Moreover, *STK1* and *STK2* do not up regulate the rates of polyamine and Na^+ (or Li^+) fluxes in a coordinate fashion, since disruption of both genes did not further increase halotolerance, while suppressing spermidine transport. In fact, neither *STK1* nor *STK2* disruption had any effect on salt tolerance in minimal medium, even under conditions where spermidine transport is fully suppressed (26), indicating a major difference in the general control of polyamine and Na^+ transport by nutrient availability and suggesting that polyamines do not share a major influx pathway for Na^+. Although disruption of various genes confers salt hypersensitivity in yeast, fewer are known to negatively regulate halotolerance (45, 60). The only other known examples are the *PPZ1* and *PPZ2* Ser/Thr phosphatases (45), which are thought to repress the function or expression of the *ENA1* gene, which encodes a P-ATPase that catalyzes Na^+ and Li^+ efflux (47). *STK2* could conceivably repress outward Na^+ transport by antagonizing the same phosphorylation pathway counteracted by the *PPZ* phosphatases. Alternatively, Stk2p kinase activity might oppose the action of calcineurin, a type 2B Ser/Thr phosphatase that is required for the activation of *ENA1* expression (34). Interestingly, disruption of the *KCR8* gene, which encodes a Ser/Thr protein kinase homologous to *STK1* and *STK2*, leads to salt hypersensitivity (52), in marked contrast to *stk2::lacZ* mutants. This suggests that the family of structurally related Ser/Thr protein kinases identified in the present work is involved in both positive and negative control of ion transport.

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