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Identification of a novel mammalian member of the NSF/CDC48p/Pas1p/TBP-1 family through heterologous expression in yeast

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Abstract Two suppressors of the growth deficiency of a potassium transport mutant of *Saccharomyces cerevisiae* were isolated from a mouse cDNA expression library. These suppressors, *SKD1* and *SKD2* (suppressor of K^+ transport growth defect), were cDNAs encoding members of a family of ATPases involved in membrane fusion (*N*-ethylmaleimide-sensitive fusion protein, NSF), cell division cycle regulation (CDC48p), peroxisome assembly (Pas1p), and transcriptional regulation (TBP-1). The SKD1 protein constitutes a novel member of this family with 49–58% amino acid sequence similarity with other family members, and contains a single ATP binding site. The SKD2 polypeptide is the mouse homolog of NSF.

Key words: ATPase; Membrane fusion; Yeast; Suppressor; Potassium transport; TRK

1. Introduction

Potassium homeostasis in *Saccharomyces cerevisiae* involves at least two distinct potassium transport systems [1]. *TRK1* encodes a transmembrane polypeptide thought to constitute the high affinity transporter [2,3], and the *TRK2* gene product is required for lower affinity transport [4]. Mutants lacking either functional *TRK1* or both functional *TRK1* and *TRK2* alleles exhibit a Trk^- phenotype: they are viable, but are unable to grow on minimal media containing low potassium concentrations [5].

Extragenic suppressors of the increased potassium requirement of *trk* mutants have been identified by selecting for growth on potassium-limiting media and include regulators of the TRK2 potassium uptake system [6] and mutations in nutrient transporters ([7] and R.F.G., unpublished results). Heterologous suppressors of the Trk^- phenotype have been isolated from *Arabidopsis thaliana* cDNA libraries [8,9], and at least one of these encodes a voltage-gated inwardly rectifying potassium channel [10].

We screened a mouse macrophage cDNA library constructed in a yeast expression vector for suppression of the Trk^- phenotype, and identified cDNAs conferring growth to the *trk* mutant on low potassium-containing media. We report here the isolation of two types of suppressor cDNAs termed *SKD1* and *SKD2* (suppressor of K^+ transport growth defect) whose full-length versions code for two proteins belonging to the NSF/CDC48p/Pas1p/TBP-1 family of ATPases. This family encompasses proteins involved in a broad spectrum of cellular functions and includes NSF, the *N*-ethylmaleimide sensitive fusion protein and its yeast counterpart Sec18p, key factors for membrane fusion processes including protein trafficking and secretion [11,16], VCP, a clathrin-binding protein involved in membrane transport [23], CDC48p, thought to regulate the cell division cycle by participating in the budding process of yeast cells [12], Pas1p, involved in peroxisome assembly [13], and TBP-1, the human immunodeficiency virus HIV-1 Tat-binding protein, a modulator of transcription [14]. SKD1 is a new member of this family and SKD2 is the mouse homologue of the *N*-ethylmaleimide-sensitive fusion protein NSF.

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The nucleotide sequences of *SKD1* and *SKD2* have been deposited in the GenBank data base under accession numbers U10119 and U10120, respectively.

2. Materials and methods

2.1. Yeast strains and media

Strain CY162 (*MATα* ura3-52 trk1*Δ* *his3Δ200* *his4 15* *trk2Δ1*::pCK64) has been described previously [8]. Yeast media were prepared essentially as described [24]. CY162 cells were grown in YPD supplemented with 100 mM KCl (YPD-100K). All minimal media contained (per liter) 6.7 g yeast nitrogen base without amino acids (Difco, Detroit, USA), and amino acids (AA) as described [24]. When indicated (+ ura), uracil was added at a concentration of 20 mg/l. SD + AA media contained 20 g/l D(+)-glucose and SG + AA media 20 g/l D(+)-galactose. High potassium (100K) media was supplemented with 100 mM KCl. The K^+ concentration in SD + AA and SG + AA media was estimated to be 7 mM [8]. SD + AA and SG + AA solid media contained 20 g/l purified agar (#A7921, Sigma). Solid SG + AA medium used for restreaking candidates for single colonies (Fig. 1) was supplemented with 5 mM KCl.

2.2. RNA isolation and cDNA library construction

Poly(A)$^+$ RNA was isolated from cultured J774.1 cells and the 3–5 kb-size fraction was selected by sucrose gradient centrifugation as previously described [25]. The cDNA library was constructed by ligating cDNAs synthesized using a *Not*I-poly(dT) primer-adaptor (BRL, Gaithersburg, USA), Superscript reverse transcriptase (BRL), and *Eco*RI adaptors (Stratagene, La Jolla, USA) to *Eco*RI/*Not*I digested pYES2 (Invitrogen, San Diego, USA) DNA. This directional library contained approximately 1×10^6 independent recombinants. Library plasmid DNA was prepared using a standard alkaline lysis technique followed by CsCl density gradient centrifugation.

2.3. cDNA library screening

CY162 cells were transformed with total library plasmid DNA by a lithium acetate method [26], grown on SD + AA (100K) plates at a

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density of 300–1000 Ura$^+$ transformants per plate until colonies reached a diameter of ~1 mm, replica-plated to SG + AA plates, and incubated for 4–10 days at 30°C to identify Trk$^+$ clones capable of growth on 7 mM K$^+$. Galactose dependence of Trk$^+$ candidates was assessed by restreaking colonies plates onto both SD + AA and SG + AA media. The Trk$^+$ phenotype was determined to be plasmid dependent when only Ura$^+$ colonies derived from a specific candidate were capable of growth on SG + AA + ura plates and when that plasmid suppressed the Trk$^-$ phenotype when re-introduced into CY162 recipients.

2.4. DNA sequencing and Northern analysis

Full-length *SKD1* and *SKD2* cDNAs were sequenced using the dideoxy sequencing technique with a Sequenase kit (USB, Cleveland, USA). Both strands of the coding region of *SKD1* cDNA were sequenced. A mouse multiple tissue Northern blot (Clontech, Palo Alto, USA) was probed with the full-length ^{32}P-labeled 2.6-kb *SKD1* cDNA and processed according to the manufacturer's instructions.

2.5. Plasmid constructions

The 1.9-kb *Xho*I KAT1 fragment of pKAT1 [2] was subcloned into the polylinker of pYES2, and the resulting construct (pFP113) contained KAT1 fused to a *GAL1* promoter. Truncation 5′-Δ-1 of full-length *SKD1* cDNA was constructed by digesting the pYES2-based plasmid with *Kpn*I and *Sma*I, blunting with T4 polymerase, and circularizing the dropout by ligation. Truncations 5′-Δ-2 and 5′-Δ-3 were constructed by ligating *Nco*I/*Not*I and *Cla*I/*Not*I fragments of the full-length *SKD1* cDNA in pYES2 to *Bam*HI/*Not*I digested pYES2, after blunting the *Nco*I, *Cla*I, and *Bam*HI overhangs with T4 DNA polymerase.

3. Results

3.1. Isolation of SKD cDNAs

Approximately 300,000 CY162 Ura$^+$ colonies were screened for suppression of the growth defect of the *trk1 trk2* double mutant on medium containing 7 mM K$^+$. Out of several hundred isolates displaying growth under selective conditions, the Trk$^+$ phenotype of several candidates was both galactose-inducible and plasmid-dependent, indicating that the transforming plasmid contained a cDNA insert whose expression led to suppression of the growth defect. (Fig. 1).

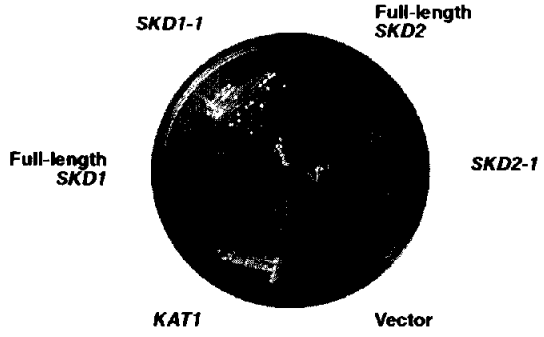

Fig. 1. Growth of CY162 transformants on low potassium medium. CY162 cells transformed with either pYES2 (vector negative control); pFP113 (KAT1 positive control); *SKD1-1* cDNA in pYES2; full-length *SKD1* cDNA in pYES2; *SKD2-1* cDNA in pYES2; and full-length *SKD2* cDNA in pYES2 were restreaked onto SG + AA medium supplemented with 5 mM KCl and grown for 7 days at 30°C.

Restriction site and DNA hybridization analyses of these cDNA inserts indicated that they belonged to different groups. *SKD1* was defined by two identical 2.4 kb *(SKD1-1)* cDNAs and a shorter 2.0 kb insert, *SKD1-2*, lacking the 5′-end of *SKD1-1* cDNA. The *SKD2* group consisted of two identical 3.0-kb cDNAs *(SKD2-1)*. *SKD1*- and *SKD2*-mediated suppression of the Trk$^-$ phenotype was not inhibited by potassium channel blockers TEA and Ba^{2+} (data not shown) and was weaker than that conferred by expression of the plant inwardly rectifying potassium channel KAT1 [8]: cells expressing *SKD1-1* or *SKD2-1* cDNAs formed smaller colonies on low-potassium containing media than cells expressing KAT1 cDNA (Fig. 1).

3.2. Sequence analysis of SKD cDNAs and isolation of full-length clones

The nucleotide sequences of *SKD1-1* and *SKD1-2* cDNAs contained a reading frame extending to the 5′ end of these

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-229          GCCAGAGGCGGGACCAGAGCCCTAAGGGGCTCGAAATTACGGAAGTTTTGTGCGGAGGGTCGGGTGGCTGCTGGAGTTCCTGCTGGATTTACGGCTCCGGAGTTGGGGC  -121
                                                    ⇩                                                          ⇩
-120 TGCTGCTGGGGGGGGTCTCAGAGCCGCCAGCCTCCGCACGGTTTGCGGTGGTGACCCGGGCGCGACTCGACGGCGCTGCTGCGGGCCTTGTCTGTAGTAGGGGACCCCTCGGATCTCCACC  -1

   1 ATGGCGTCCACGAACACCAACCTGCAGAAAGCAATAGATCTTGCAAGCAAAGCAGCCCAGGAAGACAAAGCTGGGAACTATGAGGAAGCTCTTCAACTCTACCAACATGCTGTGCAGTAT  120
   1  M  A  S  T  N  T  N  L  Q  K  A  I  D  L  A  S  K  A  A  Q  E  D  K  A  G  N  Y  E  E  A  L  Q  L  Y  Q  H  A  V  Q  Y   40
                                                    *                                                          *
 121 TTTCTCCATGTTGTTAAATATGAAGCACAAGGTGATAAAGCCAAGCAAAGTATCAGGGCCAAGTGTACAGAATATCTCGATAGAGCAGAAAAACTAAAGGAATATCTGAAGAAAAAGGAG  240
  41  F  L  H  V  V  K  Y  E  A  Q  G  D  K  A  K  Q  S  I  R  A  K  C  T  E  Y  L  D  R  A  E  K  L  K  E  Y  L  K  K  K  E   80

 241 AAGAAACCACAGAAGCCTGTGAAAGAGGCACAGTCAGGCCCAGTTGATGAGAAGGGGAATGACAGTGATGGGGAAGCAGAATCTGATGATCCTGAAAAAAAGAAACTGCAGAATCAACTT  360
  81  K  K  P  Q  K  P  V  K  E  A  Q  S  G  P  V  D  E  K  G  N  D  S  D  G  E  A  E  S  D  D  P  E  K  K  K  L  Q  N  Q  L  120

 361 CAAGGTGCCATTGTTATAGAGCGACCAAATGTGAAGTGGAGTGATGTTGCTGGTCTCGAAGGAGCCAAAGAAGCTCTTAAAGAGGCTGTGATTCTGCCTATTAAGTTTCCTCATCTGTTT  480
 121  Q  G  A  I  V  I  E  R  P  N  V  K  W  S  D  V  A  G  L  E  G  A  K  E  A  L  K  E  A  V  I  L  P  I  K  F  P  H  L  F  160

 481 ACAGGAAAGCGGACACCTTGGAGAGGAATCCTATTATTTGGACCACCAGGAACAGGAAAATCTTATTTGGCTAAAGCTGTTGCAACGGAAGCAAACAACTCAACATTTTTCTCAATATCT  600
 161  T  G  K  R  T  P  W  R  G  I  L  L  F  G  P  P  G  T  G  K  S  Y  L  A  K  A  V  A  T  E  A  N  N  S  T  F  F  S  I  S  200
                                                                                                    ⇩
 601 TCTTCCTCTGACCTTGTGTCTAAGTGGGGGGAAAGCGAAAAACTGGTTAAGAACTTATTCCAGCTTGCCAGAGAGAACAAGCCTTCTATCATCTTCATCGATGAGATTGATTCTCTGTGT  720
 201  S  S  D  L  V  S  K  W  L  G  E  S  E  K  L  V  K  N  L  F  Q  L  A  R  E  N  K  P  S  I  I  F  I  D  E  I  D  S  L  C  240

 721 GGTTCCAGAAGTGAGAATGAGAGCGAGGCTGCACGGAGAATTAAGACGGAGTTCCTGGTTCAAATGCAAGGAGTTGGTGTGGACAATGATGGCATTTTGGTTCTGGGAGCTACAAATATA  840
 241  G  S  R  S  E  N  E  S  E  A  A  R  R  I  K  T  E  F  L  V  Q  M  Q  G  V  G  V  D  N  D  G  I  L  V  L  G  A  T  N  I  280

 841 CCCTGGGTTCTGGATTCTGCAATTAGGCGAAGATTTGAGAAACGTATTTATATTCCTTTGCCCGAAGCCCATGCCCGAGCAGCCATGTTTAGACTGCACTTGGGCAGCACTCAGAACAGC  960
 281  P  W  V  L  D  S  A  I  R  R  R  F  E  K  R  I  Y  I  P  L  P  E  A  H  A  R  A  A  M  F  R  L  H  L  G  S  T  Q  N  S  320

 961 CTCACAGAAGCAGACTTCCAAGAACTTGGGAGGAAGACGGATGGCTATTCAGGAGTAGATATAAGTATCATTGTACGGGATGCACTTATGCAGCCTGTGAGAAAAGTTCAGTCAGCTACT 1080
 321  L  T  E  A  D  F  Q  E  L  G  R  K  T  D  G  Y  S  G  V  D  I  S  I  I  V  R  D  A  L  M  Q  P  V  R  K  V  Q  S  A  T  360

1081 CACTTCAAAAAGGTTCGTGGACCCTCAAGAGCTGATCCTAACTGCATTGTAAATGACCTGCTGACACCCTGCTCTCCGGGAGACCCTGGGGCTATTGAAATGACATGGATGGATGTTCCT 1200
 361  H  F  K  K  V  R  G  P  S  R  A  D  P  N  C  I  V  N  D  L  L  T  P  C  S  P  G  D  P  G  A  I  E  M  T  W  M  D  V  P  400

1201 GGAGATAAACTCTTGGAGCCAGTTGTTTCCATGTGGGATATGCTGCGGTCCCTGTCCAGCACAAAGCCAACAGTCAATGAGCAGGACTTGTTGAAGCTGAAGAAGTTTACAGAAGATTTT 1320
 401  G  D  K  L  L  E  P  V  V  S  M  W  D  M  L  R  S  L  S  S  T  K  P  T  V  N  E  Q  D  L  L  K  L  K  K  F  T  E  D  F  440

1321 GGCCAGGAAGGCTAACACAAAGACAGCAAGAACATGTTTATGCTGCATTTCTTCTCTTTTGTAGGTATTTTGCCTTTCTTGGATGACATTCAGATTATTTCCAGCAAAACTCGTTTA...  2615
 441  G  Q  E  G  END                                                                                                            444
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Fig. 2. Nucleotide and deduced amino acid sequence of the coding region of *SKD1* cDNA. The first nucleotides of suppressing cDNAs *SKD1-1* and *SKD1-2* are indicated by an asterisk above the sequence. Three in-frame stop codons upstream from the putative ATG translation initiation codon are underlined. Arrows indicate the first nucleotide of the three 5′ deletions constructed.

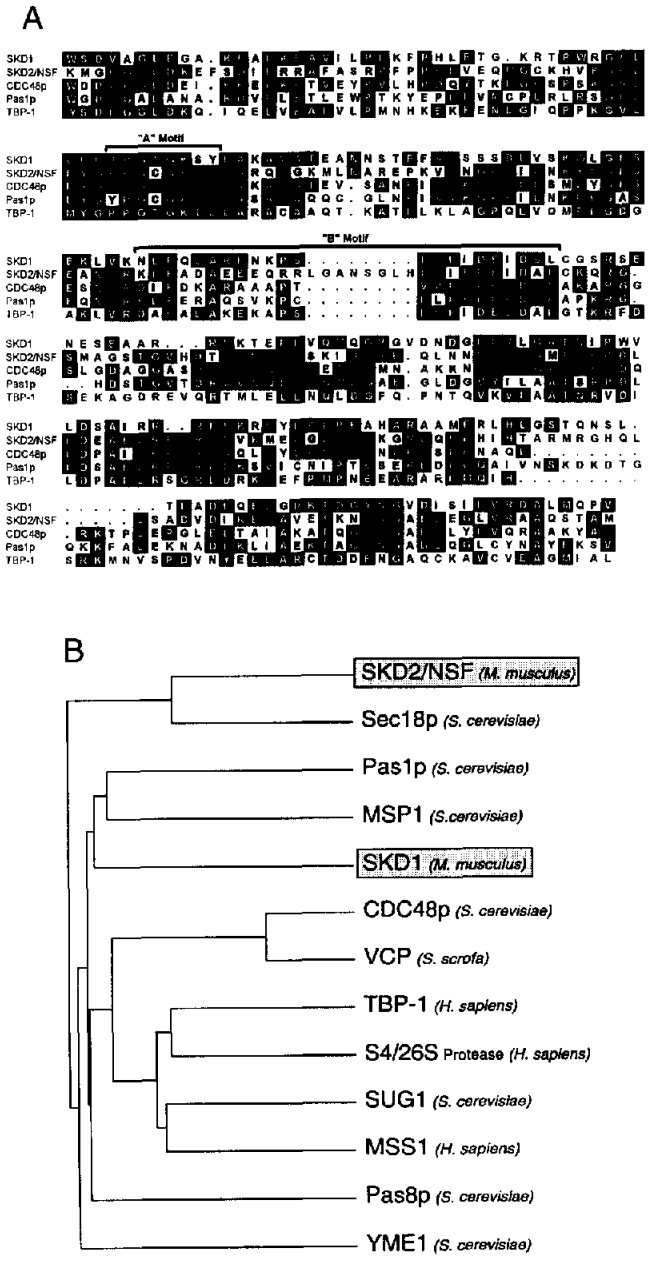

Fig. 3. Sequence comparisons and family relationships. (A) Amino acid sequence comparison between the conserved domain of SKD1 and other members of the NSF/CDC48p/Pas1p/TBP-1 family of ATPases. Alignments were performed with the PileUp program of the GCG software package (Genetics Computer Group, Wisconsin, WI). Black boxes indicate conserved amino acids among the five sequences (score > 1.0 on the Dayhoff PAM-250 matrix). Amino acid residues 133-353 of SKD1 are compared with the corresponding residues of the first repeat of SKD2/mouse NSF (this study), the second repeats of CDC48p [12] and Pas1p [13], and the homologous region of TBP-1 [14]. (B) Dendrogram of *S. cerevisiae* and mammalian members of the NSF/CDC48p/Pas1p/TBP-1 family of putative ATPases including Sec18p [16], Pas1p [13], MSP1 [15], CDC48p [12], VCP [17], TBP-1 [14], the S4 subunit of the 26S protease [18], SUG1 [19], MSS1 [20], PAS8 [21], and YME1 [22]. Distance along the horizontal axis between branch points is proportional to the difference between sequences.

cDNA clones. The full-length *SKD1* cDNA was isolated from the same J774.1 library using *SKD1-1* as a probe and contained an open reading frame coding for a 444-amino acid polypeptide with a molecular mass of 49.4 kDa and a pI of 7.5 (Fig. 2). The putative translation initiation codon is preceded upstream by three in-frame stop codons indicating that the cDNA contained the entire coding region for the SKD1 polypeptide. *SKD1-1* and *SKD1-2* cDNAs lacked 400 and 466 5′-end nucleotides present in the full-length *SKD1* cDNA, respectively. Full-length copies of *SKD1* mRNA (2.6 kb) were present in high abundance in the J774.1 library.

The putative 444-amino acid SKD1 polypeptide contains Walker-type A and B motifs [27,28] forming a putative ATP binding site (Fig. 3A) and is homologous (49–58% similarity and 25–30% identity overall) to members of the NSF/CDC48p/Pas1p/TBP-1 family of ATPases [11–22]. The highest degree of homology between SKD1 and these proteins (58–67% similarity and 30–39% identity) is found in a consensus domain characteristic of this family of proteins of approximately 230 amino acid residues containing the ATP binding site (Fig. 3A). Unlike NSF, CDC48p, and Pas1p, which contain 2 repeats of this domain, SKD1 contains a single domain as found in TBP-1 [14] and MSP1 [15]. Multiple sequence alignments show that SKD1 is most closely related to Pas1p [13] and MSP1, and may define a new distinct subfamily (Fig. 3B).

We isolated the full-length *SKD2* cDNA from the same J774.1 cDNA library, using as a probe *SKD2-1* cDNA. The *SKD2-1* cDNA sequence lacked the first 587 nucleotides of full-length SKD2 cDNA (Fig. 4). The putative SKD2 polypeptide had over 99% homology with the Chinese hamster *N*-ethylmaleimide-sensitive fusion protein NSF [11]. The mouse NSF polypeptide differs from its Chinese hamster homologue by 10 conservative amino acid substitutions.

3.3. *Trk⁻ phenotype suppression by truncated SKD1 and SKD2 cDNAs*

Expression vectors containing the full-length *SKD1* and *SKD2* cDNAs did not suppress the Trk⁻ phenotype of CY162 cells, indicating that suppression by *SKD1* and *SKD2* was only mediated by truncated versions of these cDNAs (Fig. 4). To test whether the conserved ATP binding domain of the SKD1 protein was required for suppression and whether sequences within the 5′-untranslated region of the full-length cDNAs caused a lack of suppression, 5′-end truncations of the full length *SKD1* cDNA were constructed.

Deletion 5′-Δ-1 removed 166 base pairs of the untranslated region including one in-frame stop codon and deletion 5′-Δ-2 removed 229 base-pairs of the untranslated region including all three in-frame stop codons. Neither of these two truncations resulted in suppression of the Trk⁻ phenotype of CY162 cells (Fig. 4), indicating that the lack of suppression by the full-length *SKD1* cDNA is not due to the presence of inhibitory 5′-untranslated sequences.

Deletion 5′-Δ-3 removed 928 base pairs from the 5′-end of full-length *SKD1* cDNA and did not suppress the Trk⁻ phenotype (Fig. 4). This latter construct removed the putative ATP binding site of SKD1 but preserved the first ATG codon found in *SKD1-1* and *SKD1-2* cDNAs, indicating that translation of sequences upstream from this ATG were important for suppressor function of SKD1 and included its putative ATP binding site. In addition, this result showed that translation initiation of *SKD1-1* and *SKD1-2* suppressor cDNAs may not utilize a typical ATG start codon, since no ATG sequences are found

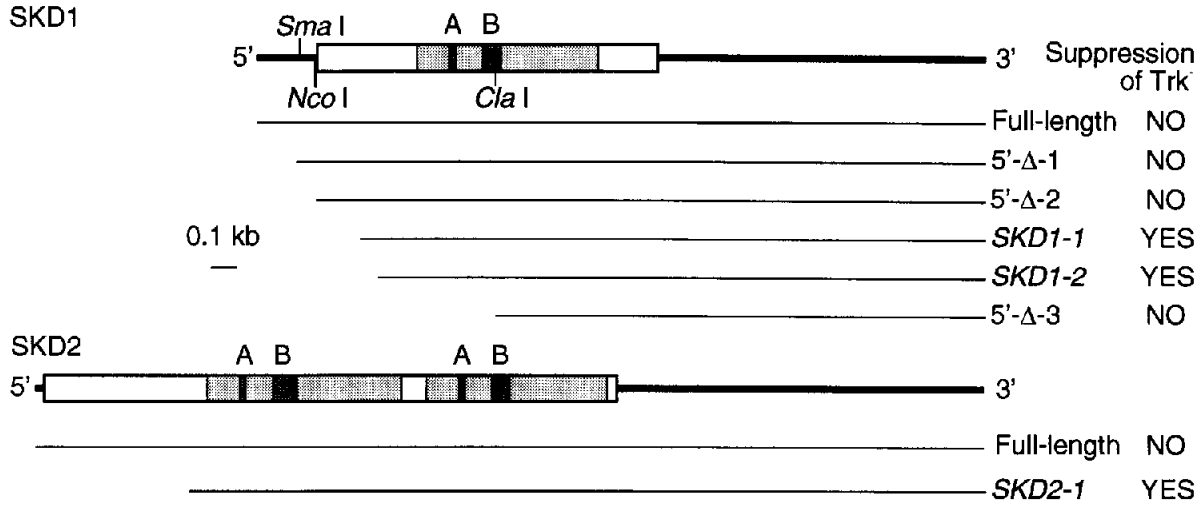

Fig. 4. Effect of 5'-truncations of *SKD* cDNAs on Trk⁻ suppression. The open reading frame of *SKD1* and *SKD2* cDNAs are boxed. Shaded regions indicate the conserved sequences among members of the NSF/CDC48p/Pas1p/TBP-1 family of ATPases, and darker boxes show the position of Walker A and B motifs of the ATP binding site.

between the first nucleotide of these truncated cDNAs and the upstream transcription initiation site of the *GAL1* promoter of the pYES2 vector.

3.4. Tissue distribution of SKD1 mRNA

SKD1 mRNA appears to be ubiquitous. *SKD1* probes hybridized to a mRNA species of approximately 3.0 kb that was detected in all mouse tissues tested, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis, with particularly high expression in kidney (Fig. 5).

4. Discussion

Using a mouse macrophage cDNA expression library, we have isolated two different types of suppressors of a yeast potassium transport growth defect, *SKD1* and *SKD2*. Sequence comparison data indicate that the SKD1 protein is a new member of the NSF/CDC48p/Pas1p/TBP-1 family of putative ATPases with diverse cellular functions, including membrane fusion and biogenesis [11–22]. The SKD1 protein has a single consensus domain containing the ATP binding site and is most closely related to the yeast Pas1p and MSP1 polypeptides, involved in peroxisome assembly and mitochondrial protein sorting, respectively [13,15]. *SKD2* encodes the mouse homolog of the human *N*-ethylmaleimide sensitive fusion protein NSF [11].

The inability of full-length *SKD* cDNAs to suppress the Trk⁻ phenotype could be due to either toxicity of efficiently translated products or a lack of functional expression in the yeast host. Full-length cDNAs coding for mammalian inwardly rectifying potassium channels IRK1 [29] and HIR [30] do not suppress the Trk⁻ phenotype of CY162 cells either (F.P. and C.A.V., unpublished data), perhaps because of a similar lack of expression in the heterologous host.

Since both *SKD1* and *SKD2* suppressor cDNAs encode members of the same family of ATPases, we postulate that suppression of the Trk⁻ phenotype by expression of these truncated cDNAs occurs through a common mechanism.

Depletion of intracellular potassium reversibly inhibits coated pit formation and receptor-mediated endocytosis in fibroblasts [31,32], and intracellular transport of secretory proteins between the endoplasmic reticulum and the Golgi complex in cultured hepatocytes [33]. Since the molecular mechanisms governing intracellular membrane-fusion processes are conserved from yeast to man (reviewed in [34]), the Trk⁻ phenotype could involve defects in K^+-dependent membrane assembly or secretory functions. Overexpression of NSF or other key factors involved in these events could lead to partial suppression of the growth defect. Since expression of either SKD1 or SKD2 suppresses the growth defect of *trk* cells, these two proteins could be involved in the same pathway or be part of the same protein complex. Therefore, SKD1 may be a protein involved in membrane fusion and secretory processes like NSF.

Alternatively, ATP depletion caused by overexpression of an

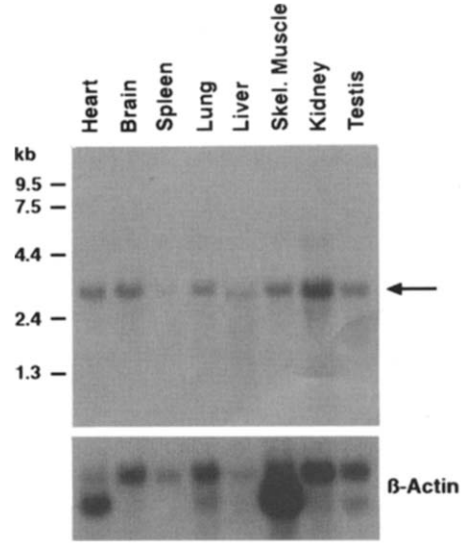

Fig. 5. Tissue distribution of *SKD1* mRNA. (Top) Northern analysis of mouse poly(A)+ RNA from various tissues probed with the full-length ^{32}P-labeled 2.6-kb *SKD1* cDNA. Arrow indicates position of the ~3 kb *SKD1* mRNA. (Bottom) The same blot was probed with ^{32}P-labeled human β-actin cDNA for comparison and shows hybridization to both 2-kb and muscle-specific 1.7-kb messages.

heterologous ATPase could lead to inhibition of an ATPase involved in K^+ efflux. This would prevent excessive potassium efflux from CY162 cells and allow growth of these cells in media with limited potassium content. An energy-dependent potassium efflux is present in *S. cerevisiae* [3,35], and the pH sensitivity of yeast plasma membrane ATPase *pma1* mutants is specifically suppressed by millimolar K^+ concentrations, suggesting that ATPase activity and K^+ homeostasis are coupled phenomena [36].

The finding that putative ATPases SKD1 and NSF are able to suppress the growth defect of Trk^- cells indicates that our expression system could be a powerful method to screen heterologous expression libraries for new members of fusion protein or ATPase protein families in yeast.

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