SDT1/SSM1, a Multicopy Suppressor of S-II Null Mutant, Encodes a Novel Pyrimidine 5’-Nucleotidase*  

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SDT1 (suppressor of disruption of TFIIS 1, YGL224c, also known as SSM1, suppressor of S-II null mutant 1) is Saccharomyces cerevisiae gene identified as a multicopy suppressor of 6-azauracil sensitivity in a null mutant of the transcription elongation factor S-II. We found that overproduction of SDT1 caused hyposensitivity to not only 6-azauracil but also 5-fluorouracil and 5-fluorocytosine. This hyposensitivity was limited to pyrimidine derivatives, and no effect was observed for non-pyrimidine drugs including such clinically used anti-fungal drugs as amphoterin B and fluconazole. Purified recombinant SDT1 protein specifically dephosphorylated 5’-UMP and 5’-CMP. These results suggested that SDT1 conferred pyrimidine-specific hyposensitivity by dephosphorylating active metabolites of 6- or 5-modified pyrimidines, i.e. 6- or 5-modified UMP. This is the first description of a highly specific pyrimidine 5’-nucleotidase in S. cerevisiae.  

A Saccharomyces cerevisiae null mutant of the transcription elongation factor S-II is hypersensitive to 6-AU (1) and MPA, and now a number of transcription elongation-related factors and RNA polymerase II mutants are also known to be 6-AU-hypersensitive (2–6). In yeast cells, 6-AU is transformed to 6-azauracil, which inhibits both IMP dehydrogenase and orotidylate decarboxylase, key enzymes of the purine and pyrimidine nucleotide synthesis pathways, respectively, thereby lowering intracellular GTP and UTP levels and inhibiting transcription elongation (7). MPA specifically inhibits IMP dehydrogenase and lowers the intracellular GTP level (7). The transcriptional stimulation and arrest relief activity of S-II are necessary to support cell proliferation in the presence of 6-AU (8). We have identified SDT1 (9) (also known as SSM1 (10)) as a multicopy suppressor of the 6-AU hypersensitivity of an S-II null mutant (10). The SDT1 null mutant is hypersensitive to 6-AU but not to MPA, and SDT1 overexpression confers hyposensitivity to 6-AU but not to MPA (this study and Ref. 10). Thus, although SDT1 was identified by the screening for a functional substitute for S-II, it can support growth in the absence of S-II only partially if at all. The deduced amino acid sequence of SDT1 has 30–50% identity with S. cerevisiae YER037w, Schizosaccharomyces pombe SPAC24B11, and a putative sugar starvation-induced protein of Arabidopsis thaliana (GenBank™ accession number AC006223). These genes share a haloacid dehalogenase-like hydrolase consensus sequence, but their biological functions are unknown. To elucidate the mechanism of 6-AU hypersensitivity suppression by SDT1 overexpression, we first tested the drug sensitivity of a SDT1 overproducer. The result suggested that drug resistance by SDT1 overexpression is specific to pyrimidine derivatives. Because SDT1 has a hydrolase consensus sequence, we assumed that SDT1 is a metabolic enzyme of pyrimidines and found that the UMPase activity in a yeast cell extract was proportional to the SDT1 gene dosage. We then expressed recombinant SDT1 protein in Escherichia coli, purified it, and showed that SDT1 protein specifically dephosphorylates 5’-UMP and 5’-CMP. These results suggested that SDT1 detoxifies 6-azaUMP by dephosphorylating it to 6-azauridine in 6-AU-challenged yeast cells, and by this means SDT1 overexpression confers suppression of 6-AU hypersensitivity of S-II null mutant.  

EXPERIMENTAL PROCEDURES  
Plasmids and Strains—Kpn1-NcoI 1.8-kilobase pair fragment of the SDT1 gene was inserted into the SmaI site of the multicopy shuttle vector pY0323 (11) to obtain the SDT1-overproducing plasmid pH1275. pY0323 and pRS416 (12) were introduced to BY4742 (Mata his3 leu2 lys2 ura3), which was purchased from Invitrogen, and used as wild type. pH1275 and pRS416 were introduced to BY4742 and used as an SDT1 overproducer.  
Drug Sensitivity Assay—Drug sensitivity was determined by the microbroth dilution method. Approximately 2000 yeast cells/well were inoculated into 200 μl of synthetic medium (0.67% yeast nitrogen base without amino acids, 2% glucose) containing 100 μg/ml lysine, 250 μg/ml leucine, drugs, and 1% Me2SO in 96-well plates. After 40 h of incubation at 30 °C, cell growth was monitored by measuring the optical density at 595 nm.  
Nucleotidase/Phosphatase Activity—Nucleotidase/phosphatase activity was determined as described previously (13) with some modifications. The protein sample was incubated in a 0.1 M Tris-HCl buffer, pH 8.5, containing 10 mM MgCl2, 1 mM dithiothreitol, and substrate nucleotide phosphate in a final volume of 60 μl at 30 °C for 10 min. 150 μl of developer (1.4% ascorbic acid, 0.36% ammonium molybdate, 0.86 mM H2SO4) was then added to the reaction mixture and incubated at 45 °C for 20 min. Absorbance at 820 nm was measured, and the amount of released phosphate was calculated. KH2PO4 solution was used as a standard. One unit of UMPase activity is defined as 1 μmol of phosphate released/min from 5’-UMP.  
Recombinant SDT1 Expression and Purification—The SDT1 gene was amplified by the polymerase chain reaction and inserted into pET21c(+) (Novagen) to obtain a His tag-fused recombinant protein. The resulting plasmid pH1171 was introduced to BL21-CodonPlus(DE3)-RIL (Strategene). The transformant was incubated in LB medium, and recombinant SDT1 was induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside at 18 °C for 18 h. E. coli cells were lysed in extraction buffer (0.1 M Tris-Cl, pH 7.6, 1 mM UMP, 1 mM MgCl2, 0.8 mM phenylmethylsulfonyl fluoride, 17 mM spermidine, 0.21 mM lysozyme, 0.5% sodium deoxycholate) and then centrifuged at 100,000 × g for 1 h to obtain a clear supernatant. Recombinant SDT1 in the resulting cell lysate was absorbed to a nickel-charged resin (His-Bind
Yrimidine 5'-nucleotidase (molecular weight 12,400) were mixed and applied to a Superose 12 HR gel filtration column (Amersham Biosciences) and then loaded onto a nickel-charged HiTrap chelating column (Amersham Biosciences) equilibrated with buffer 1 (0.1 M Tris-HCl, pH 7.6, 1 mM MgCl₂, 1 mM UMP). Recombinant SDT1 was eluted by a 50–300 mM imidazole linear gradient in buffer 1. Active fractions were pooled and then concentrated with a Centricon YM-30 (Amicon).

**Other Methods**—Protein concentration was determined by the method of Bradford (14). Imidazole concentration was determined by absorbance at 320 nm.

**RESULTS**

**Drug Sensitivity of a SDT1 Overproducer**—SDT1 was isolated as a multicopy suppressor of the 6-AU sensitivity of an S-II null mutant (10). To characterize the suppression, we first tested whether the SDT1 overproducer was resistant to other anti-fungal pyrimidine derivatives. A representative result is shown in Fig. 1 and summarized in Table I. The SDT1 overproducer showed hyperresistance to all pyrimidine derivatives tested. The SDT1 overproducer and wild-type cells were equally sensitive to non-pyrimidine drugs such as mycophenolic acid, amphotericin B, and fluconazole. These results suggested that SDT1 confers resistance specifically to pyrimidine derivatives.

**Purification of Recombinant SDT1 in E. coli**—Because SDT1 has a halolacid dehalogenase-like hydrolase consensus sequence, we assumed that SDT1 is a metabolic enzyme of pyrimidines. 6-AU is metabolized to 6-azaUMP by FUR1 protein (15), which inhibits IMP dehydrogenase and orotidic acid decarboxylase (7). Because 6-azauridine has no inhibitory activity to orotidic acid decarboxylase (16), the cellular toxicity of 6-azaUMP could be inactivated by dephosphorylation to 6-azauridine. Phosphatases are included among the members of the hydrolase family. Therefore, we prepared cell extracts from the SDT1 overproducer, the SDT1 null mutant, and wild-type yeast cells and measured their UMPase activity. The SDT1 overproducer cell extract contained approximately twice as much UMPase activity as did the wild-type extract, whereas the SDT1 null mutant extract contained less than half of the amount of the wild-type extract (data not shown). Considering that SDT1 is a member of the hydrolase family, this result suggested that SDT1 itself was UMPase.

To test whether SDT1 had UMPase activity, we expressed histidine tag-fused recombinant SDT1 in *E. coli* and purified it to near homogeneity (Fig. 2A). As shown in Fig. 2B, recombinant SDT1 protein and UMPase activity were co-eluted from the nickel-charged HiTrap chelating column by an imidazole linear gradient. This result showed that SDT1 has UMPase activity. As shown in Table II, the recovery of UMPase activity was 21%, and the specific activity increased 14-fold. The apparent Kₘ value and the relative Vₘₐₓ values were determined from the Lineweaver-Burk plots (Fig. 3). The average Kₘ values of two independent experiments for 5'-UMP and 5'-CMP were 1.2 and 2.3 mM, respectively. The average Vₘₐₓ values of two independent experiments for 5'-UMP and 5'-CMP were 23 and 20 μmol/min/mg, respectively.

**Substrate Specificity of SDT1**—We next tested which nucleotide phosphates were dephosphorylated by SDT1. As shown in Table III, SDT1 specifically dephosphorylated 5'-UMP and 5'-CMP. Purine nucleotides, 2'(3')-UMP, deoxyribonucleoside monophosphates, nucleoside diphosphates and triphosphates, or p-nitrophenyl phosphate were dephosphorylated at less than one-tenth efficiency compared with 5'-UMP. This is the first description of a highly specific 5'-pyrimidine nucleotidase in *S. cerevisiae*. PN-I (pyrimidine 5'-nucleotidase type I) purified from human erythrocytes is the sole example of a pyrimidine-specific nucleotidase so far (17). PN-I dephosphorylates deoxyribonucleotide monophosphate, which is not dephosphorylated by SDT1. The other known 5'-nucleotidases have broader specificity and dephosphorylate both pyrimidine and purine nucleotides (18, 19). SDT1 shares no sequence similarity with the other nucleotidases reported. A BLAST search of the GenBank database provided no nucleotidase as a similar protein. These results suggested that SDT1 is the sole known member of a novel nucleotidase family.

**Native Molecular Weight Estimation**—Several nucleotidases form oligomers (19). To see whether the SDT1 protein formed an oligomer, purified SDT1 was loaded onto a Superose 12 gel filtration column with molecular weight marker proteins. SDT1 was eluted just after β-macroglobulin (molecular weight 35,000), and its estimated native molecular weight was 33,000 (Fig. 4). Because the deduced molecular weight of SDT1 was 32,000, the result showed that SDT1 existed as a monomer in the solution.

**DISCUSSION**

SDT1 was isolated as a multicopy suppressor of 6-AU sensitivity in an S-II null mutant (10). The SDT1 null mutant is 6-AU-sensitive, and SDT1 expression is regulated by S-II at the...
transcription level (10). To elucidate the 6-AU sensitivity suppression mechanism by SDT1, we first tested whether SDT1 overexpression caused multidrug resistance or whether its effect was limited to pyrimidine derivatives. Because the latter situation was the case as summarized in Table I and SDT1 has a hydrolase consensus sequence, we assumed that SDT1 might be a pyrimidine-metabolizing enzyme. We then found that there was a correlation between the SDT1 gene dosage and UMPase activity in a yeast cell extract. We next purified recombinant SDT1 from E. coli and showed that SDT1 was a 5'-UMP-specific and 5'-CMP-specific nucleotidase (Figs. 2 and 3 and Table III). SDT1 existed as a monomer (Fig. 4), whereas several nucleotidases existed as oligomers (19). Because 6-azaUMP is thought to be an active metabolite of 6-AU, these results suggested that SDT1 detoxifies 6-azaUMP by dephosphorylating it to 6-azauridine in 6-AU-challenged yeast cells, and therefore SDT1 overexpression suppresses 6-AU hypersensitivity.

The reported cellular functions of nucleotidases are nucleotide level regulation, uridine formation for intercellular transport, and phosphate source generation (19). We propose here that nucleotidases can be scavengers of "ill-modified" nucleotide phosphates and be induced to destroy them. This idea is consistent with the report that SDT1 is induced in the presence of 6-AU or the DNA-alkylating agent methyl methanesulfonate (20) and is dispensable in general growth conditions. SDT1 may have higher affinity to such ill-modified nucleotide phosphates.

The table shows the drug sensitivity of SDT1 overproducer.

**Table I**

| Drug          | IC<sub>50</sub> (µg/ml) |
|---------------|--------------------------|
| 6-AU          | 16                       |
| 5-FU          | 0.20                     |
| 5-FC          | 4.7                      |
| MPA           | 3.6                      |
| AMPB          | 0.59                     |
| FCZ           | 11.0                     |

**Table II**

| Fraction          | Protein (mg) | Activity (units) | Specific activity (units/mg) | Recovery (%) |
|-------------------|--------------|------------------|-------------------------------|--------------|
| Cell extract      | 300          | 370              | 1.2                           | 100          |
| His Bind          | 12           | 130              | 10.5                          | 35           |
| Ni-charged HiTrap | 4.7          | 78               | 16.5                          | 21           |

**Fig. 2.** Purification of recombinant SDT1. A, the nickel-charged HiTrap chelating fraction was subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. Lane 1, precision standard marker (Bio-Rad); lane 2, purified recombinant SDT1 from the nickel-charged HiTrap chelating column. Ten micromolars 5'-UMP was used as a substrate for the UMPase assay. ●, UMPase activity; ○, protein concentration; □, imidazole concentration.

**Fig. 3.** Kinetic parameters estimation. Various amounts of 5'-UMP or 5'-CMP were incubated with 0.2 µg of purified recombinant SDT1, and released phosphate was measured as described under "Experimental Procedures." Representative results are shown. A, substrate/velocity plot. ●, 5'-UMP; ○, 5'-CMP. B, Lineweaver-Burk plot. ●, 5'-UMP; ○, 5'-CMP.
Nucleotidase/phosphatase activity of recombinant SDT1 was determined for a variety of nucleoside phosphates as described under "Experimental Procedures." All nucleoside phosphate was used at 10 mM. The average of two independent experiments was shown as a relative activity compared to 5'-UMP. 5'-monophosphate; PNPP, p-nitrophenyl phosphate.

| Nucleotide | Relative activity (5'-UMP = 100) | Nucleotide | Relative activity (5'-UMP = 100) |
|------------|---------------------------------|------------|---------------------------------|
| 5'-UMP     | 100                             | 5'-UTP     | 5.0                             |
| 5'-CMP     | 87                              | 5'-CTP     | 7.6                             |
| 5'-TMP     | 0.83                            | 5'-ATP     | 4.9                             |
| 5'-AMP     | 0.33                            | 5'-GTP     | 10.3                            |
| 5'-GMP     | 0.15                            | 5'-dUMP    | 0.74                            |
| 5'-IMP     | 0.22                            | 5'-dCMP    | 0.23                            |
| 2'(3')-UMP | 0.08                            | 5'-dAMP    | 0.0                             |
| 2'(3')-AMP | 0.01                            | 5'-dGMP    | 0.17                            |
| 5'-UDP     | 11                              | 5'-OMP     | 5.3                             |
| 5'-ADP     | 1.3                             | PNPP       | 0.0                             |

Hypersensitivity to 6-AU of other transcription elongation factors may be because of the failure of SDT1 up-regulation as well. Probably, S-II suppresses 6-AU sensitivity by up-regulating SDT1 and IMD2 to detoxify 6-AU and to increase the GTP level (21), respectively, and by stimulating transcription elongation of the genes whose transcriptions are arrested because of nucleoside triphosphate shortage. Moreover, the shortages of nucleoside triphosphate would cause nucleotide misincorporation to messenger RNA, and it may be one of the mechanisms whereby 6-AU stops yeast cell proliferation. S-II can help to proofread nucleotide misincorporation (22). S-II seems to play key roles to support yeast cell growth very efficiently under low level nucleotide conditions by stimulating transcription elongation, proofreading, and induction of enzymes such as drug-metabolizing SDT1 and nucleotide-synthesizing IMD2.

This paper suggests that UMP/CMPase SDT1 is involved in pyrimidine-derived anti-fungal drug resistance of yeast. It is possible that some of the pyrimidine-derived anti-fungal drug (e.g. 5-fluorocytosine)-resistant fungi overproduce SDT1. It is also possible that there are pyrimidine nucleotidase-overproducing cancer cells in pyrimidine derivative (e.g. 5-fluouracil)-resistant cancer. If this turns out to be the case, the SDT1 overproducer and recombinant SDT1 protein can be used to develop pyrimidine-nucleotidase-resistant anti-fungal or anti-cancer drugs.

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