Function and Expression of a Novel Rat Salt-Tolerant Protein: Evidence of a Role in Cellular Sodium Metabolism

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Abstract. Higher dietary salt intake in humans is associated with higher BP, but the BP response to NaCl, so-called salt sensitivity, is heterogeneous among individuals. It has been postulated that modifications in cellular cation metabolism may be related to salt sensitivity in mammalian hypertension. The authors have isolated a novel rat complementary DNA, called salt-tolerant protein (STP), that can functionally complement Saccharomyces cerevisiae HAL1, which improves salt tolerance by modulating the cation transport system. On high-salt (8% NaCl) diets, both Dahl salt-sensitive and salt-resistant rats displayed an elevated BP and increased STP mRNA expression. Immunohistochemistry using an anti-rat STP antibody demonstrated the presence of STP immunoreactivity in the proximal tubules. In cells that transiently expressed STP, the intracellular [Na\(^+\)]/[K\(^+\)] ratio was higher than that in control cells. STP contains predicted coiled-coil and Src homology 3 domains, and shows a partially high degree of nucleotide identity to human thyroid-hormone receptor interacting protein. These results suggest that STP may play an important role in salt sensitivity through cellular sodium metabolism by mediating signal transduction and a hormone-dependent transcription mechanism.

Hypertension is a multifactorial disease that is complicated by genetic and environmental factors. Excessive dietary sodium intake is an important environmental factor in the development and/or maintenance of an elevated BP in some but not all people (1). Although the underlying mechanisms of this salt sensitivity are not yet fully understood, several biochemical alterations and genetic markers have been documented in hypertension in both humans and experimental animals (2). The intracellular sodium concentration is reported to be elevated in various cells of patients with essential hypertension (3–6). Several investigators have described increased serum and urine levels of Na\(^+\), K\(^+\)-ATPase inhibitors in essential hypertension. Several investigators have described increased serum and urine levels of Na\(^+\), K\(^+\)-ATPase inhibitors in essential hypertension.

In 1992, Gaxiola et al. (13) isolated a novel gene, HAL1, in the yeast Saccharomyces cerevisiae, which improves salt tolerance by modulating the cation transport system. To elucidate the genetic factors in rat salt sensitivity, we isolated six rat complementary DNA (cDNA), which encode proteins that can complement HAL1 (14). This approach may make it possible to identify critical processes for salt tolerance. One of these genes showed no significant similarity to any known proteins, with regard to its amino acid sequence, and was registered as salt-tolerant protein (STP). STP improved the growth of a HAL1-deficient yeast strain in media with a high concentration of NaCl, but had no effect in normal media. STP contains 1946-bp nucleotides and an open reading frame that encodes 547 amino acid residues. In a Northern blot analysis, STP was shown to be expressed in several different rat tissues as a single transcript of 2.1 kb (14).

In this study, to assess the physiologic function of STP, we examined the regulation of STP gene expression and the intracellular cation contents in STP-transfected cells. Furthermore, we determined the localization and predicted the structure of STP protein.

Materials and Methods

Materials

Hybond\textsuperscript{TM}-N nylon membrane was obtained from Amersham (Buckinghamshire, United Kingdom), and Immobilon\textsuperscript{TM} transfer membrane was purchased from Millipore (Bedford, MA). Prokaryotic gene fusion vectors pGEX-3X and pGEX-2T, and glutathione-Sepharose 4B were purchased from Pharmacia Biotech (Uppsala, Sweden). Alkaline phosphatase-conjugated goat anti-rabbit IgG was obtained from BioRad Laboratories (Hercules, CA). Isopropyl β-D-thiogalactopyranoside, nitroblue tetrazolium, and 5-bromo-chloro-3-indolyl phosphate p-toluidine salt were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Blocking reagent was purchased from Boehringer Mannheim (Mannheim, Germany). OCT compound was obtained from Miles, Inc. (Elkhart, IN). Alkaline phosphatase-conjugated avidin for immunohistochemistry was purchased from DAKO (Glostrup, Denmark). Biotinylated goat anti-rabbit IgG was obtained from Vector Laboratories (Burlingame, CA). Naphthol-AS-BI-phosphoric acid and levamisole were obtained from Sigma Chemical Co. (St. Louis, MO). New fuchsin powder was from Merck (Darmstadt, Germany). pBluescript ISK(−) phagemid vector and pSG5 eukary-
otic expression vector were purchased from Stratagene (La Jolla, CA). Cell culture media were obtained from Life Technologies (Gaithersburg, MD). Various DNA-modifying enzymes and endonucleases were purchased from Takara Shuzo (Kyoto, Japan) and Nippon Gene (Toyama, Japan).

**Antibody**

Polyclonal anti-STP antiserum directed against partial amino acid residues of STP (359 to 417 and 421 to 479 amino acid residues) was produced. Each fragment was generated as glutathione S-transferase (GST) fusion proteins and used to immunize the rabbits. These regions were amplified by PCR with primers that contain either a BamHI or an EcoRI site. The PCR products were digested with BamHI and EcoRI, and subcloned into the prokaryotic gene fusion vector pGEX-2T or pGEX-3X. The constructed plasmids were transformed into JM101, and protein expression was induced with isopropyl β-D-thiogalactopyranoside. The recombinant GST fusion proteins were purified by means of affinity chromatography on immobilized glutathione. Rabbits were injected with 500 μg of total GST-fusion protein in complete Freund’s adjuvant. Rabbits were boosted every 3 wk with an identical regimen, except that incomplete adjuvant was used. The specificity of the STP antiserum was demonstrated by immunoprecipitation of in vitro translated STP. In vitro translated STP was made according to the manufacturer’s directions.

**Western Blot Analysis**

Adult Wistar rat kidney cortex and medulla were separated and homogenized in phosphate-buffered saline with a Teflon homogenizer. The resulting homogenates (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%) and then electrophoretically transferred to an Immobilon™ membrane. The blotted membrane was blocked in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) containing 0.5% blocking reagent and 0.05% Tween 20. The membrane was incubated with anti-STP antiserum (1:2000) for 1 h and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000) for 1 h. Alkaline phosphatase activity was visualized using nitroblue tetrazolium and 5-bromo-chloro-3-indolyl phosphate p-toluidine salt.

**Immunohistochemistry**

Kidneys from adult Wistar rats were fixed in Bouin solution for 3 h and frozen in OCT compound. Serial frozen sections (3.0 mm) of the kidney were cut on a cryostat and thaw-dried on separate slides at room temperature. STP immunostaining was performed by the alkaline phosphatase-labeled streptavidin-biotinylated method (15). Antibody dilution studies (1:500, 1:1000, 1:2000, 1:4000, and 1:10,000) were performed. A 1:1000 dilution was found to be most satisfactory for this antibody in that it provided minimum nonspecific binding and good sensitivity. Sections were incubated with the primary antibody against STP (1:1000) for 30 min and then reacted with the secondary biotinylated anti-rabbit IgG (1:200) for 30 min. They were covered by alkaline phosphatase-conjugated streptavidin diluted 1:200. Sections were colorized with naphthol-AS-BI-phosphoric acid as a substrate and hexazonited new fuchsin as a coupler, and then counterstained with hematoxylin. To exclude any nonspecific staining, preimmune rabbit serum was used instead of primary antibody as a negative control.

**Rats**

Male inbred Dahl salt-sensitive (SS/Jr/Mol referred to as S) and Dahl salt-resistant (SR/Jr/Mol referred to as R) rats were obtained from Møllegaard Breeding Center (Ejby, Denmark) through Seiwa Experimental Animal, Ltd. (Fukuoka, Japan). All of the rats were treated in accordance with the guidelines of our institute. The rats were fed a regular (0.5% NaCl)-, intermediate (2.0% NaCl)-, or high (8.0% NaCl)-salt diet for 2 wk. Body weight, systolic BP (SBP), and pulse rate were measured weekly. To confirm the Dahl salt-sensitive and salt-resistant phenotypes, SBP was determined by tail-cuff plethysmography.

**Northern Blot Analysis**

Total RNA were prepared from rat kidneys by the guanidine thiocyanate method. Twenty micrograms of total RNA were denatured with 50% formamide/6.5% formaldehyde and electrophoresed on 1.2% agarose gels containing 6% formaldehyde. The separated RNA was transferred to a Hybond™-N nylon membrane and hybridized with 32P-labeled full-length STP cDNA and a human G3PDH probe. Signal intensities of mRNA were measured by densitometric analyzer. The expression levels of each mRNA were normalized by the signal intensities of G3PDH mRNA on the same line. Data are expressed as the fold increase relative to the control.

**Construction of Expression Plasmid**

A cDNA insert of STP was prepared by digestion with XhoI and Xhol, and ligated to the Nhel and XhoI site of pBluescript II SK(−), in which the EcoRV site was transformed to the Nhel site. This construct was digested with EcoRI and XhoI, and then inserted into the EcoRI and XhoI site of the modified pSG5 expression vector. The resulting expression plasmid was designated pSST.

**Transfection and Measurement of Cytosolic [Na+] \([K^+]\) Contents**

Twenty micrograms of the control vector pSG5 and pSST were transfected into 1 × 10^6 COS-1 cells using an electroporation apparatus (Gene Pulser, BioRad). The transfected cells were cultured in minimum essential medium containing 10% fetal calf serum in 60-mm dishes for 2 d before the following experiments. The cells were incubated with minimum essential medium containing the indicated concentrations of NaCl for 1 h. After incubation, cells were washed twice with ice-cold 0.1 M potassium phosphate buffer, pH 7.4, and harvested with 0.3 ml of distilled water. The cell suspensions were homogenized with a 26-gauge needle for 30 strokes and then centrifuged at 100,000 × g for 30 min. The resultant supernatants were used to quantify the cytosolic [Na^+] and [K^+] contents by atomic absorption spectrometry. Four separate experiments were conducted.

**Coiled-Coil Prediction**

An algorithm was used to predict the propensity for coiled-coils based on the statistical preference of different amino acids for each position in the heptad repeat. Sequences from the Protein Data Bank were analyzed using a computer program based on this algorithm (16). With a window size of 28 residues, sequences with scores above 1.3 were considered to have a high probability of being coiled-coil structures.

**Statistical Analyses**

BP values were compared using ANOVA followed by the Bonferroni/Dunn test. Differences between the cytosolic free [Na^+] and [K^+] contents in transfected cells and in control cells were evaluated with ANOVA followed by multiple comparisons with Fisher pro-
Results

Western Blot Analysis of Rat Kidney Homogenates

We raised antibodies against the fusion proteins between amino acid residues of STP (from 359 to 417 and from 421 to 479) and GST (Schistosoma japonicum). In a Western blot analysis of rat kidney cortical homogenate, a major protein of 60 kD was recognized by the antiserum (Figure 1A). However, no immunoreactivity was observed in medullary homogenate (Figure 1B). STP contains 1946-bp nucleotides and an open reading frame that encodes 547 amino acid residues. The size of the molecule recognized by the anti-STP antiserum appears to be similar to the calculated value of 57 kD.

Immunologic Localization of STP

We also investigated the localization of STP. Twelve different sections from three animals were studied and gave the same results. Immunohistohemical examination revealed that STP was localized mainly in the proximal tubules of Wistar rat kidney (Figure 2B). There was no staining in the medulla, glomerulus, or distal tubules. These distributions were in agreement with immunoblotting results. No immunoreactivity was observed when preimmune serum was used in place of antiserum against STP (Figure 2A).

Effects of Salt Loading on BP and STP Gene Expression

To assess the effects of salt loading on BP and STP, we examined the changes in SBP and in the expression of STP mRNA in S and R rats under different conditions of dietary salt intake. The rats were fed a regular (0.5% NaCl)-, intermediate (2% NaCl)-, or high (8% NaCl)-salt diet for 2 wk. At the end of the 2-wk period, SBP was measured and the rats were killed to determine the STP mRNA level. In both the S and R rats used for this experiment, SBP significantly increased with a high (8% NaCl)-salt diet compared with a regular (0.5% NaCl)-salt diet (S: 198.0 ± 4.6 mmHg versus 167.7 ± 3.9 mmHg, n = 6, P < 0.05; R: 147.3 ± 3.0 mmHg versus 136.7 ± 2.7 mmHg, n = 6, P < 0.05) (Table 1). SBP remained unchanged in both S and R rats whose diets contained either 2% or 0.5% NaCl. The expression of STP mRNA was higher with a high-salt diet than with a regular-salt diet, but there was no difference in the expression level between S and R rats (Figure 3). The levels of STP mRNA were not increased with an intermediate-salt diet (2% NaCl) (data not shown).

Intracellular Cation Contents in STP-Transfected Cells

To determine the physiologic function of this gene, we examined whether intracellular cation contents were affected by STP. We constructed the expression plasmid pSST and transiently transfected it into COS-1 cells. Cells were incubated for 1 h in medium containing various NaCl concentrations, and the cytosolic [Na+] and [K+] contents were then quantified by atomic absorption spectrometry. As indicated in Figure 4, the intracellular [Na+]/[K+] ratio in STP-transfected cells was higher than that in control cells at NaCl concentrations of 116 to 216 mM. Statistically significant differences in the [Na+]/[K+] ratio between STP-transfected and control cells were found at 116 mM NaCl (P = 0.038) and 166 mM NaCl (P = 0.026). The intracellular [Na+]/[K+] ratio was not significantly different at 216 mM NaCl (P = 0.597 versus control cells).

Sequence Analysis and Prediction of the Structure of STP

The sequence of STP was examined to evaluate its structural similarity to known protein sequences. Upon close examination of the sequence, we found that it may possess features that are characteristic of an α-helical coiled-coil. The amino acid...
Figure 2. Immunohistochemical localization of STP in rat kidney. The alkaline phosphatase-labeled streptavidin-biotin method was used for immunostaining of STP protein. The reaction product is a highly chromogenic bright red. (A) Normal rabbit serum control did not show staining. Hematoxylin counterstain, ×80. (B) Strong expression of STP (arrows) was found in the proximal tubules. Hematoxylin counterstain, ×80.

Table 1. Systolic blood pressure in Dahl salt-sensitive and Dahl salt-resistant rats

<table>
<thead>
<tr>
<th>Strain/Diet</th>
<th>Systolic Blood Pressure (mmHg)</th>
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<tr>
<td></td>
<td>0.5% NaCl</td>
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<tr>
<td>DS (n = 6)</td>
<td>161.0 ± 6.2</td>
</tr>
<tr>
<td>DR (n = 6)</td>
<td>134.2 ± 2.5</td>
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<sup>a</sup> Values are expressed as means ± SEM (n = 6). BP values were compared using ANOVA followed by the Bonferroni/Dunn test. DS, Dahl salt-sensitive; DR, Dahl salt-resistant. <sup>b</sup> P < 0.05 versus 0.5% NaCl diet.

residues of STP were analyzed using an algorithm designed to detect coiled-coil domains (a VAX Pascal program) (16). The results show that STP has the potential to form coiled-coils (Figure 5). The analysis revealed that a 56-amino acid segment from residue 121 to 176, a 39-amino acid segment from 340 to 378, and a 29-amino acid segment from 390 to 418 could possibly form coiled-coils.

Discussion

Yeasts are being extensively studied at the molecular level to gain fundamental insight into the structure and function of eukaryotic genes. In yeast, gene knockouts can be used to determine whether such regions are important for specific cellular functions that can be detected phenotypically. This technique provides a potentially powerful approach to identifying and studying new genes and their proteins when functional expression is possible in yeast (17–20). To elucidate the genetic basis of salt sensitivity in mammalian hypertension, we isolated six genes that can complement *Saccharomyces cerevisiae* HAL1, which confers salt tolerance by modulating the cation transport system in yeast. These genes were identified as β-globin, α-crystallin, androgen-regulated protein, mitochondrial cytochrome b, a homologue of infant brain cDNA, and a novel gene, called salt-tolerant protein (STP) (14).

The Dahl salt-resistant rat has mutations in the cytochrome P450(11B) (21,22) and P450(AS) (23) genes. In linkage analyses, angiotensin-converting enzyme gene, atrial natriuretic peptide receptor gene, growth hormone promoter region, Na<sup>+</sup>, K<sup>+</sup>-ATPase α1 gene, endothelin-3 gene, nitric oxide synthase
Figure 3. Effects of NaCl on regulation of STP gene expression. Induction of STP mRNA by salt loading. Dahl salt-sensitive and Dahl salt-resistant rats were fed chow containing standard salt (0.5% NaCl) or high salt (8% NaCl) for 2 wk. The animals were then killed and total RNA was prepared from their kidneys. Twenty micrograms of total RNA were subjected to a Northern blot analysis. Signal intensities of mRNA were measured by a densitometric analyzer. The expression levels of each mRNA were normalized by the signal intensities of G3PDH mRNA on the same line. Data are expressed the fold increase relative to the control.

Figure 4. Intracellular [Na⁺]/[K⁺] ratios. Intracellular [Na⁺]/[K⁺] ratios in COS-1 cells transfected with control vector (□) or pSST (●) after 1 h of incubation in medium containing the indicated concentrations of NaCl. Cells were washed with ice-cold potassium phosphate buffer, collected with distilled water, and subjected to subcellular fractionation. The cytosolic free [Na⁺] and [K⁺] contents were measured by atomic absorption spectrometry. Intracellular [Na⁺]/[K⁺] ratios were compared using ANOVA followed by multiple comparisons with Fisher’s protected least significant difference methods. Data are means ± SEM from four independent experiments. *P < 0.05 versus control cells.

decrease sodium efflux by modulating Na⁺, K⁺-ATPase, Na⁺, K⁺-cotransport, and other membranous ion transport systems.

Immunohistochemical analysis of STP expression demonstrated that high levels of STP immunoreactivity are localized in the proximal tubules of Wistar rat kidney. An important function of proximal tubules is sodium reabsorption. Approximately 65% of sodium reabsorption occurs in the proximal tubules due to the active transport of sodium by proximal tubule epithelial cells. Because pathologic reabsorption of sodium in the kidney is known to result in hypertension, a genetic change resulting in an increase in renal sodium reabsorption could be the etiologic basis of hypertension. However, the underlying cellular and molecular bases responsible for increased sodium reabsorption leading to high BP are unknown. Salt-sensitive essential hypertension is thought to result from a lowering of the threshold for sodium intake at which sodium homeostasis can be maintained by the kidney without an increase in BP (29). The slope of the renal function (pressure-natriuresis) curve is significantly lower in salt-sensitive than salt-resistant patients, suggesting a disturbance in renal tubular sodium reabsorption (30,31). The STP gene may increase renal sodium reabsorption in proximal tubules, and thus be an important determinant of hypertension associated with excess salt intake.

The amino terminus of proto-oncogene tyrosine-protein kinase FER shows 20 to 30% homology with some regions of
elucidate the physiologic roles of STP transcription mechanism. Additional studies are needed to
regulate genes that increase the intracellular sodium concent-
tration by mediating signal transduction and a hormone-depen-
dent transcription pathway. This finding demonstrates that
STP may be involved in a signal transduction and/or hormone-dependent transcriptional path-
way by mediating protein-protein interactions.

In conclusion, we have isolated a rat eDNA, STP, that encodes a protein that is induced by NaCl loading. STP contains predicted coiled-coil and SH3 domains. The sequence and properties of STP suggest that this protein may contribute to regulate genes that increase the intracellular sodium concentration by mediating signal transduction and a hormone-dependent transcription mechanism. Additional studies are needed to elucidate the physiologic roles of STP.

Figure 5. Probability of forming a coiled-coil. The amino acid se-
quence of STP was analyzed using an algorithm to detect coiled-coil
domains (a VAX Pascal program). The score is derived from a
28-residue sequence using an MTIDK matrix. The P(S) score, which
measures the probability that any residue and its surrounding se-
quence will form coiled-coils, is plotted against the number of amino
acids in the sequence.

STP. A fragment that contained 400 amino acids of the amino
terminus of FER was predicted to form a coiled-coil and may
be able to associate with the catenin-like substrate pp120 (32).
Coiled-coil domains have been shown to be present in myosin,
 laminin, α- and β-tubulins, G-protein β subunit, some bacterial
transfer RNA synthetases, and members of the heat shock
protein family. In several proteins, the predicted coiled-coil
segments may be quite dynamic and play functionally important
roles, such as in protein–protein association, signal trans-
duction, and protein oligomerization (33). STP contains a Src
homology 3 (SH3) domain at its C terminus. Several proteins
involved in the ion transport system contain proline-rich Se-
tant roles, such as in protein-protein association, signal trans-
duction and/or hormone-dependent transcriptional path-
way. Trip 10 may be able to associate with the catenin-like substrate ppl20 (32).

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