Isolation and Characterization of a Rat Homologue of the Human Tuberous Sclerosis 1 Gene (Tsc1) and Analysis of Its Mutations in Rat Renal Carcinomas

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ABSTRACT

In the Eker rat, a germ-line mutation in the homologue of the human tuberous sclerosis gene (Tsc2) causes renal cell carcinomas (RCs) with a complete penetrance in all heterozygotes. Tsc2 mutations have also been found in a subset of chemically induced non-Eker rat RCs. Because tuberous sclerosis patients with alteration of either of the two predisposing genes (Tsc1 and Tsc2) show identical symptoms, the products of these two genes are thought to be involved in a common biological pathway. In this study, to analyze the possible overlap between the functions of Tsc2 and Tsc1 gene products, we isolated and characterized a rat homologue of the TSC1 gene (Tsc1). The rat Tsc1 gene, which has an identical exo-intron structure to that of human TSC1 and is localized on rat chromosome 3, has been shown to encode a protein (hamartin) that is highly homologous to the human counterpart with an ~86% amino acid sequence identity. Using PCR-single-strand conformational polymorphism analysis, we identified two splicing donor site mutations in one chemically induced rat RC (1 of 15). This suggests that alterations of the Tsc1 gene may be involved in the development of a subset of rat RCs.

INTRODUCTION

Renal carcinogenesis in the Eker rat is an excellent animal model of dominantly inherited cancer (1). We and others have identified a germ-line mutation in the rat homologue of the human TSC2 gene as a tumor-predisposing factor in the Eker rat (2–4). All rats heterozygous for Tsc2 mutation develop RCs by the age of 1 year (5). LOH of intragenic Tsc2 gene somatic mutations are characteristic of Eker rat RCs, indicating that a second hit of the Tsc2 gene is a cause of RC development (6, 7). The introduction of the Tsc2 gene into Tsc2-deficient cell lines suppresses their growth and tumorigenicity (8, 9), clearly supporting a tumor suppressor function. We have constructed transgenic Eker rats with a wild-type Tsc2 gene and ascertained that germ-line suppression of the Eker phenotype is responsible for both embryonic lethality in homozygotes and tumor predisposition in heterozygotes, and we have finally confirmed that tumors in the Eker rat are caused by the Tsc2 germ-line mutations (10). We have also identified Tsc2 mutations in chemically induced rat RCs, suggesting a more general involvement of Tsc2 alterations in RC development in rats (11).

Tsc2 encodes tuberin, a ~200-kDa protein that contains a Rap1-GAP homology region near its COOH terminus (12). Recently, Rap1-GAP, Rab5-GAP, and transcriptional activator activities, as well as other possible functions of tuberin, were reported (13–16). However, the precise in vivo function of tuberin and the molecular mechanisms of tumor development associated with Tsc2 mutations have not been fully elucidated.

TSC is an autosomal dominantly inherited disease characterized by hamartomatic benign tumors in multiple organs such as the brain, kidney, and heart (17). Approximately half of TSC patients are sporadic cases without any familial history (18). In addition to the TSC2 gene localized on chromosome 16p13.3, another TSC-predisposing gene (Tsc1) was recently identified on chromosome 9q34 (19, 20). TSC1 encodes hamartin, which contains a predicted coiled-coil region and one potential transmembrane region, with a calculated molecular mass of ~130 kDa (20). The symptomatic similarity among human TSC patients associated with either TSC1 or TSC2 mutation suggests that hamartin and tuberin may be involved in a common biochemical pathway in vivo (19, 21). Indeed, direct interactions between tuberin and hamartin have been reported (21).

Because of the highly conserved nature of the Tsc2 gene structure in vertebrates, the function and possible interaction of tuberin with hamartin may be conserved. Thus, we anticipated that studies of rat hamartin might provide some clues for elucidation of the molecular mechanisms of renal carcinogenesis in the Eker rat model. Therefore, we cloned and structurally characterized the rat Tsc1 gene. Moreover, we identified somatic Tsc1 mutations in one chemically induced rat RC.

MATERIALS AND METHODS

Tumor and Normal Tissue Samples. Three RCs induced by EHEN in F344 rats, four RCs induced by DEN in the F2 progeny of a LEC/F344 cross, and one RC induced by DEN in the F2 progeny of a LEC/WKAH cross were selected as described in our previous report (11). Seven RCs induced by EHEN in the F2 progeny of a LEC/F344 cross were also analyzed. For this induction, 500 ppm of EHEN in drinking water was given for 2 weeks from 4 weeks of age, for 1 week from 9 weeks of age, and for 1 week from 12 weeks of age. The animals were sacrificed between 36 and 44 weeks of age, and tumor and normal tissue samples were taken and stored at ~80°C until analysis.

DNA and RNA Isolation and Southern Blot and Northern Blot Analyses. DNAs were isolated from tumor samples by proteinase K digestion as described previously (22). Total RNAs were prepared with Isogen reagent (Nippon gene) according to the manufacturer’s instructions. Southern and Northern blot analyses were performed as described previously (3). The membrane filters for hybrid cell panel analysis were kindly provided by Dr. G. Levan (University of Goteborg, Goteborg, Sweden; Refs. 23–25).

RT-PCR. For the initial isolation of partial rat Tsc1 cDNA, first-strand cDNA was synthesized from 5 μg of rat (Long Evans strain) testis total RNA by RT using Superscript II (Life Technologies) and random primers in 20 μl of reaction mixture. A 1-μl aliquot of RT solution was used for PCR in 25 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1 mM MgCl2, 200 mM deoxynucleotide triphosphates, 2 units of Taq DNA polymerase (Toyobo), and 100 pmol each of forward primer HTSF2 (5'-CTGGATCCCACAGAAGCCT-3') and reverse primer H7RS2 (5'-CAGTCGACAGACTTGTGCGG-3'). Temperature conditions were 92°C for 3
min for initial denaturation; 92°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min for the amplification (35 cycles); and 72°C for 3 min for the final extension (protocol I). Similarly, the 5′ end of the human TSC1 cDNA was obtained by RT-PCR using forward primer HTSF1 (5′-GACCATGCGACACACAAACGAAA-3′), reverse primer HTR1 (5′-GGTGACACAGACAAATGCA-3′), and total RNA from the G401 cell line. To obtain the full coding region of rat Tsc1 cDNA, first-strand cDNA was synthesized from rat (Long Evans) kidney total RNA using random primers, and PCR was performed using an Expand High Fidelity PCR kit (Boehringer Mannheim) according to the manufacturer’s instructions. Temperature conditions for PCR were 92°C for 3 min, 55°C for 1 min, and 72°C for 1.5 min for the initial polymerization; 92°C for 1 min, 62°C for 1 min, and 72°C for 1.5 min for the amplification (35 cycles); and 72°C for 3 min for the final extension (protocol II). The primers used were as follows: primers TSRT1 (reverse primer; 5′-CTGGCGCTTC-3′) and TSRT4 (forward primer; 5′-GCGAATTCGGAGAG-3′) for sequence analysis on October 18, 2017. © 1999 American Association for Cancer Research.

### Table 1: The oligonucleotide primer sets, MgCl₂ concentration, and annealing temperature for rat Tsc1 PCR-SSCP analysis

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<th>Annealing temperature (°C)</th>
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a Exon 9 was examined with two primer sets, exon 15 was examined with three primer sets, and exon 23 was examined with three primer sets.

b The product size of exon 9 latter half is for LEK, WKAH, BN, and F344 rats.

**The 5′-RACE.** The 5′-RACE based on in vitro PCR was performed according to the method described by Maruyama et al. (27) with some modifications. Rat brain total RNA (3 µg) was used as a template for the RT reaction using the antisense primer TSRt6 (5′-AGTGCTGCTTATCATGTTGGCT-3′). After the hydrolysis of RNA with 0.5 N NaOH, cDNAs were precipitated and circularized in 40 µl reaction mixture containing 50 mM Tris·HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 20% polyethylene glycol 6000, and 50 units of T4 RNA ligase (Takara) at 16°C. Ligation mixture (0.3 µl) was subjected to PCR using primers TSRT1 (reverse primer; 5′-GGAGACCTTCTCTTCAAGAATG-3′) and TSRT3 (forward primer; 5′-GTGGGCTCATGCTGCTGAAAC-3′). Temperature conditions for PCR were the same as those for protocol I. A 1-µl aliquot of the first PCR solution was used for the second PCR using primers TSRT4 (reverse primer; 5′-GGGACTCTTTCCACATGCTGAAAC-3′) and TSRT5 (forward primer; 5′-ATCTCGAGGTTGGTTGTTAATATCATCCTGGA-3′). Amplified products were subcloned into pBluescript II SK(+). DNA sequences were subjected to DNA sequence analysis after digestion with EcoRI and XhoI for sequence analysis.

**Genomic PCR Analysis for the Determination of Exon-Intron Structures.** Introns lengths were partially determined by PCR using genomic DNA as templates and the Expand Long Template PCR System (Boehringer Mannheim) according to the manufacturer’s instructions. For the amplification of introns between coding exons, primers for SSCP probes [RT-PCR fragments and human TSC1 cDNA clone HA4782 (kindly provided by Dr. T. Nagase, Kazusa DNA Research Institute, Kisarazu, Japan; Ref. 26)].
Tsc1 GENE IN CHEMICALLY INDUCED RENAL CARCINOGENESIS

RESULTS

Structural Analysis of the Rat Tsc1 Gene. To clone rat Tsc1 cDNA and genomic DNA, we initially performed RT-PCR analysis using primers designed from the human TSC1 cDNA sequence (20). A ~1.2-kb fragment was successfully amplified with a primer set (HTSF2 and HTSR2) from rat testis mRNA (data not shown). Sequence analysis of the amplified portion of this fragment revealed a derivation from rat Tsc1 mRNA, showing homology with nt 1030–2229 of the human TSC1 cDNA sequence (data not shown). Subsequently, cosmids and phage clones containing rat Tsc1 genomic DNA regions were isolated by library screening, and their exonic sequences were determined (Fig. 1A). Also, a partial rat Tsc1 cDNA fragment corresponding to nt 739–1722 of human TSC1 cDNA was isolated from a kidney cDNA library. After the identification of sequences corresponding to exons 3 and 23 of the human TSC1 gene in a cosmids clone, the full coding region of rat Tsc1 cDNA was obtained by RT-PCR (Figs. 1A and 2A; GenBank/EMBL/DDBJ accession number AB011821). The 5’-RACE was also performed to analyze the 5’-untranslated region of Tsc1 mRNA (Figs. 1A and 2B; GenBank accession number AB016165).

The exon-intron organization of the rat Tsc1 gene demonstrated essential similarity with the human TSC1 gene, except for the junction

sequence cycle reaction using a dye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems), and sequences were analyzed with an ABI PRISM Model 310 genetic analyzer (Perkin-Elmer Applied Biosystems). For sequencing of PCR products from the SSCP analysis, a SequiTherm cycle sequence kit (Epitritech Sciences) was used with [α-32P]dCTP.

PCR-SSCP Analysis. Details of the oligonucleotide primer sets, sizes of the PCR products, MgCl2 concentrations, and PCR annealing temperatures for the Tsc1 gene are summarized in Table 1. The primer sets for the Tsc2 and Vhl genes were described previously (28, 29). PCR was carried out in a 10-μl volume including 50 ng of template genomic DNA, 1 mM each of the primers, 100 mM MgCl2 and 10 μl of distilled water, and fragments were amplified by PCR with the same primers used for PCR-SSCP.

LOH Analysis. For detection of LOH in the Tsc1 gene region in RCs from LEC/F344 F1 progeny, a polymorphism in the Tsc1 gene was used (851). The 5’-end of a termination codon (TGA) was defined as nt number 1. The 15-mer sequence deleted in a cDNA clone and a termination codon is indicated. Both ends of exonic sequences (except for the 3’ end of exon 23) were also shown in parentheses at both sides of each exonic sequence. In exon 23, the nt number for the 3’ end of a termination codon (TGA) is indicated. The adenine of the first translational initiation codon (ATG) was defined as nt number 1.

Sequence Analysis. For sequencing of the exon-intron boundaries and cDNAs, plasmid DNAs and genomic PCR fragments were subjected to the cycle sequence reaction using a dye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems), and sequences were analyzed with an ABI PRISM Model 310 genetic analyzer (Perkin-Elmer Applied Biosystems). For sequencing of PCR products from the SSCP analysis, a SequiTherm cycle sequence kit (Epitritech Sciences) was used with [α-32P]dCTP.

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of exons 11 and 12 (intron 12; Fig. 1, A and B). The rat exon 11 sequence (115 nt) was found to be 3 nt longer than that of the human sequence at its 3' end. Complementarily, the rat exon 12 sequence (119 nt) was 3 nt shorter than that of the human sequence at its 5' end. The GT/AG rule was kept at all intronic splicing donor and acceptor sites (Fig. 1B). The deduced aa sequence of the rat Tsc1 product consisted of 1163 residues and showed 86% identity with that of human hamartin (Fig. 2A). A putative transmembrane region (aa 127–144) and a potential coiled-coil region (aa 719–998) were conserved between rat and human (Fig. 2A). In the COOH-terminal region encoded by exon 23, a 3-aa deletion and two single aa insertions were found in rat hamartin as compared with human hamartin (Fig. 2A). During RT-PCR analysis, we identified a cDNA fragment lacking nt 2042–2056 (15 bp) at the exon 16-exon 17 junction (data not shown). This 15-bp sequence was pyrimidine rich and ended with an AG dinucleotide (Fig. 1B). These results indicate that the second splicing acceptor site exists in the 5' part of exon 17. The use of this second acceptor site causes a 5-aa (Gly-Ser-Pro-Pro-Ser) deletion (Fig. 2A).

In man, TSCI mRNA has a long 3'-untranslated region (~4.5 kb; Ref. 19). Although we have not yet fully accomplished sequence analysis of the 3'-untranslated region of rat Tsc1 cDNA, substantial homology of the sequenced region of exon 23 with the 3'-untranslated region of human TSCI cDNA was identified (data not shown). In addition, the mRNA size (~8 kb) of rat Tsc1 was similar to that of human TSCI mRNA detected by Northern blot analysis (see below). These results indicate that rat Tsc1 mRNA also has a long 3'-untranslated region. The longest 5'-untranslated region, which was obtained by 5'-RACE, was 184 bp in length and showed an 84.5% sequence identity with that of human TSCI cDNA (Fig. 2B). Thus, the 5'-untranslated region of Tsc1/TSCI mRNA was also conserved.

Fig. 2. Structural conservation between rat Tsc1 and human TSCI genes. A, as sequence alignment of rat and human hamartins. The aa sequence of rat hamartin (Rat) using single-letter codes is aligned with that of human hamartin (Human). The number of the first aa residue of each line is indicated on the left. An asterisk between two sequences indicates the identity of each residue between rat and human. Putative transmembrane and coiled-coil regions are denoted by a box and underlined, respectively. A GSPPS sequence deleted by an alternative splicing event is indicated by dots above the sequence.

B

Fig. 3. Northern blot analysis of Tsc1 expression. Lanes contain 10 μg of total RNA from rat organs. Probes are 5'2.4-kb region of rat Tsc1 cDNA. An ~8-kb band similar in size to that of human TSCI mRNA was detected. The bottom panels show the results with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes using the same blot. a, brain 1; b, liver; c, spleen; d, kidney; e, heart; f, brain 2.
Chromosomal Localization and Expression of the Rat Tsc1 Gene. The chromosomal localization of the rat Tsc1 gene was determined by Southern blot analysis of a human/rat somatic cell hybrid panel. The appearance of rat-specific bands coincided with the presence of rat chromosome 3 in each cell clone (data not shown). The expression of Tsc1 was examined in several rat tissues by Northern blot analysis. A ~8-kb band similar in size to that of human TSC1 mRNA was detected in the brain, liver, spleen, kidney, and heart (Fig. 3).

Mutational Analysis of the Tsc1 Gene in Rat RCs. To investigate the possible involvement of Tsc1 gene alteration in chemically induced renal carcinogenesis in rats, we searched for mutations in 21 coding exons (exons 3–23) of Tsc1 by PCR-SSCP analysis in 15 RCs induced by EHEN or DEN. Of these RCs, eight were used in our previous PCR-SSCP analysis of the Tsc2 and Vhl genes (11). The remaining seven RCs were newly established RCs. In this study, an analysis of Tsc2 and Vhl as well as Tsc1 gene mutations was carried out. With regard to the Tsc1 gene, we identified two mutations in 1 EHEN-induced RC (sample 9) as well as nine polymorphisms in 15 RCs (Fig. 4; Table 2). The two mutations were in the splicing donor site of intron 16, giving rise to a GT to GA transition and a GT to GC transversion. In exon 16, a polymorphic sequence at nt 2020 (G versus C) was found (Table 2), and the rat in which sample 9 RC developed was a heterozygote for this polymorphism. Autoradiography of SSCP showed several discrete shifted bands in sample 9 that were not seen in the normal tissue sample from the same rat or other RC samples (Fig. 3). When these shifted bands were sequenced, both mutations were found with C at nt 2020, whereas the normal band contains either C or G. Thus, the two mutations were generated on the same allele. In sample 9, no mutations were detected in either the Vhl or Tsc2 gene (see below). In intron 9, a polymorphic 20-bp deletion was identified in the LEC allele used here (Table 2). We used this polymorphism as a marker for LOH detection in applicable cases of RC. PCR-SSCP analysis in the seven newly established RCs (data not shown). In contrast, five Tsc2 mutations were found in three samples (Table 3). These results regarding the absence of mutations in Vhl and the presence of mutations in the Tsc2 gene in chemically induced RCs were in line with those from our previous report (11).

DISCUSSION

In this report, we isolated and characterized the rat Tsc1 gene and performed a structural comparison with human TSC1 as an initial step toward the analysis of the biochemical pathways in which tuberin and hamartin may be involved in rat renal carcinogenesis. The high degree of structural similarity observed suggests functional conservation of hamartin in these species. The predicted coiled-coil region, in particular, appears to be highly conserved and thus may be functionally important, possibly for multiple protein-protein binding.

As seen in human TSC, Tsc1 and Tsc2 deficiency might cause a similar phenotype in the rat and thus facilitate RC development. As one approach to investigate this possibility, we searched for Tsc1 mutations or deletions in chemically induced RCs; some of these RCs contained somatic Tsc2 mutations that we have identified previously (11). The two Tsc1 splice donor site mutations found in sample 9 appeared to be on the same allele, as revealed by the presence of the same polymorphic sequence in their neighborhood. These splice do-

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4 S. Hasegawa and O. Hino, unpublished observations.
nor site mutations may affect splicing. Tsc2 mutations were not detected in sample 9. Several possibilities could explain the occurrence of these double mutations. One possibility is that two (or more) independently arising RCs were included in sample 9, and Tsc1 mutations were formed separately in different RCs during the early stages of renal carcinogenesis. Advanced carcinomas are known to be monoclonal, but Novelli et al. (31) reported mixed karyotype adenomas in familial adenomatous polyposis and suggested cell to cell cooperation in adenoma formation before monoclonality due to a dominantly growing clone. Merritt et al. (32) also observed that intestinal adenomas in the Min mouse, an experimental model for hamartomas and tuberin and the molecular mechanisms of tumor development. Advanced carcinomas are known to be monoclonal, but Novelli et al. (31) reported mixed karyotype adenomas in familial adenomatous polyposis and suggested cell to cell cooperation in adenoma formation before monoclonality due to a dominantly growing clone. Merritt et al. (32) also observed that intestinal adenomas in the Min mouse, an experimental model for familial adenomatous polyposis, have a polyclonal structure. The other possibility is that the Tsc1 mutations occurred separately within the same tumor after the initiation of RC development. In either case, the finding of Tsc1 mutations suggests the involvement of the Tsc1 alteration in RC development in the rat.

The mutations analyzed in this study and reported previously are summarized in Table 3. The 15 chemically induced RCs showed a chromophoric type histology with a tubular or papillary pattern, not a chromophilic type histology with a tubular or papillary pattern, not a chromophilic type histology. They showed the same histopathological features, comparing the one with Tsc1 mutation versus others with Tsc2 mutations.

The frequency of Tsc2 mutations (8 of 15) was higher than that of Tsc1 mutations (1 of 15) in chemically induced RCs (P < 0.05). We are now trying to ascertain whether chemical renal carcinogenesis is possible in transgenic rats constructed with extra copies of the wild-type Tsc2 gene. This experiment may also provide evidence of the involvement of Tsc1 alterations in RC development in the rat.

In man, loss of the TSC1 locus has been detected in hamartomas or in other RCs. We know that our SSCP analysis may not detect mutations in primer annealing sites. Another possible reason is a reduction of expression of the wild-type Tsc1 allele by some epigenetic mechanism, such as methylation of the promoter region. Detection of two hits of Tsc1 in RCs will strengthen the case for the involvement of Tsc1 alterations in renal carcinogenesis. Phenotypic analysis of Tsc1 knockout mice should also clarify this possibility and the in vivo interactions of hamartin and tuberin.

In summary, we have isolated and characterized the rat homologue of the human TSC1 gene. Structural analysis of rat Tsc1 rendered mutational analysis of RC samples possible, and we identified two mutations in one EHEN-induced RC by PCR-SSCP analysis. Biochemical studies using the Tsc1 cDNA cloned here as well as further mutational analysis of rat RCs should provide clues to the functions of hamartin and tuberin and the molecular mechanisms of tumor development associated with TSC1/Tsc1 or TSC2/Tsc2 mutations.

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