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Tuberous Sclerosis 2 Gene Is Expressed at High Levels in Specific Types of Neurons in the Mouse Brain

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Tuberous sclerosis (TSC) is an autosomal dominant disorder characterized by mental retardation, epilepsy and hamartomatous growth in many tissues. The gene (TSC2) encoding a tumor suppressor protein whose mutations cause TSC, has been demonstrated to be expressed at high levels in the adult and developing brain, raising the question of whether or not the TSC2 gene product has unique roles in differentiation related to cytoskeletal interactions within the central nervous system, in addition to a tumor suppressor function. To determine the expression of TSC2 in functionally distinct neuron types of the mouse brain, we carried out in situ hybridization with digoxigenin-labeled riboprobes for the detection of TSC2 mRNA. High levels of the TSC2 gene were in neurons of the pyramidal and dentate granular layer in the hippocampus, cerebellar Purkinje cells, neurons of the piriform cortex, motor neurons in the medulla and interneurons in the striatum, while intermediate levels were in cortical neurons, striatal neurons, septal neurons, thalamic neurons and neurons in the substantia nigra compacta. Thus, the high expression of the TSC2 gene has restricted distribution in specific neuronal types which are characterized by well-developed dendrites and rich in use-dependent long-term changes in synaptic efficacy. These results suggest that the function of the TSC2 gene product may be involved on a cellular basis in neuronal plasticity and relevant to mental retardation observed in TSC patients.

Key words: tuberous sclerosis gene; cytoskeletal interaction; neuronal plasticity; in situ hybridization; mouse brain

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by mental retardation, epilepsy and development of tumors termed hamartomas such as angiofibroma of the face and on the skin, cardiac rhabdomyoma, ungal fibroma, phakoma of the retina and angiomyolipoma of the kidney (Gomez, 1988). In the central nervous system, cortical tubers in the cerebrum and cerebellum, periventricular nodules and giant cell astrocytoma are characteristic lesions. The frequency of TSC among the general population is about 1 in 10,000 to 30,000 and it occurs in all racial groups (Gomez, 1988; Ohno et al, 1982). Linkage studies have shown two loci for TSC: one on chromosome 9q34.3 (TSC1) and another on chromosome 16p13.3 (TSC2) (Fryer et al., 1987; Kandts et al., 1992; Povey et al., 1994). Recently, the TSC2 gene has been isolated and characterized (The European Chromosome Tuberous Sclerosis Consortium, 1993). The gene for the TSC1 locus on chromosome 9q34 has not yet been identified. Several mutations in the TSC2 gene of TSC patients have been

Abbreviations: bp, base pair; cDNA, complementary DNA; dCTP, deoxy-CTP; DEPC, diethyl pyrocarbonate; kb, kilobase; mRNA, messenger RNA; LTD, long-term depression; LTP, long-term potentiation; NBT, nitroblue tetrazolium salt; RT-PCR, reverse transcription-coupled polymerase chain reaction; tRNA, transfer RNA; TSC, tuberous sclerosis complex; TSC1, locus on chromosome 9q34.3 for TSC; TSC2, locus on chromosome 16q13.3 for TSC

found (The European Chromosome Tuberous Sclerosis Consortium, 1993; Kuma et al., 1995a, 1995b).

Loss of heterozygosity on chromosome 16p13.3 has been observed in the hamartomas from TSC2 patients, suggesting the role of tumor suppressor function of the TSC2 gene product (Green et al., 1994). The Eker rat, one type of animal model for dominantly inherited cancer, which bears a gene mutation in the TSC2 gene and develops tumors in kidneys (renal carcinoma) and other tissues has also been found to exhibit a loss of heterozygosity in tumors (Hino et al., 1994; Kobayashi et al., 1995; Kubo et al., 1995).

The TSC2 gene encodes a 1784-amino acid protein, tuberin, which shares a region of homology with the GTPase-activating protein for rap1 (rap1-GAP) (Wienecke et al., 1995). It has been predicted that this region of homology interacts with rap1 (p21-ras-related protein) which functions to regulate cytoskeletal interactions both in mammalian platelets and in the budding pathway of yeast cells (McCabe et al., 1992; White et al., 1992). The expression of rap1-GAP especially suppresses the random budding due to the expression of rap1, suggesting that rap1-GAP may be required for nonrandom selective budding in yeast cells (McCabe et al., 1992). Recently, it has been demonstrated by Northern blot analysis and in situ hybridization for TSC2 mRNA that the TSC2 gene is expressed at high levels in specific regions of the adult and developing brain (Geist and Gutmann, 1995). Together with the putative function of rap1-GAP as a regulatory factor in budding, finding on the high expression of the TSC2 gene in the mammalian brain raises a new question of whether or not the TSC2 gene product tuberin has unique roles in the development and cellular basis of synaptic plasticity of the central nervous system which are both closely related to cytoskeletal interactions, in addition to the tumor suppressor function.

In this study, using the in situ hybridization technique, we have described the neuronal pattern of the TSC2 gene expression in the mouse brain in order to examine which type of neurons express the most abundant TSC2 gene and have discussed the possible function of the TSC2 in the central nervous system.

Materials and Methods

Production and subcloning of the TSC2 cDNA fragment

Total cellular RNA from a human normal skin fibroblast cell line was purified by the acid-guanidium thiocyanate-phenolchloroform extraction method followed by the elimination of genomic DNA using RNase-free DNase I. The TSC2 cDNA fragment (810 bp of human TSC2 cDNA) was generated from the total RNA by the reverse transcription-coupled polymerase chain reaction (RT-PCR) method using forward primer (F: 5'-AGAAGGCTTCTCCAG-AACT-3') and reverse primer (R: 5'-GGAGGTGGA CCT-CGAGTGCCTT-3'). The RT-PCR primers were designed to amplify a region of 810 bases corresponding to bases 2280–3090 of the human TSC2 sequence (The European Chromosome Tuberus Sclerosis Consortium, 1993). The TSC2 cDNA fragment thus produced was subcloned into pGEM-5Zf(+) plasmid (Promega).

Northern blot analysis

Tissues: RNA was extracted from brain regions and other peripheral tissues of normal C57 BL/KsJ mice (10–12 weeks old) using the cesium chloride ultra centrifugation method. For each sample, 45–135 μg of total RNA was denatured by heating at 65˚C for 10 min with 50% formamide, separated electrophoretically on a 1% denaturing formaldehyde agarose gel and transferred to nitrocellulose (Hybond-N^+, Amersham, Bucks, United Kingdom) by Northern transfer.

Probes: For all mouse tissues, the TSC2 cDNA probes used for Northern blot analysis were the inserts from the cloned human TSC2 cDNA fragment. The TSC2 cDNA probe and γ actin cDNA probe were labeled with [α-^{32}P]CTP using the random primer reaction.

Hybridization conditions: The blots were prehybridized with a buffer containing 50% formamide, 5 × SSC, 5 × Denhardt, 50 mM NaH_2PO_4, 0.5% SDS, 0.25 mg/mL denatured salmon sperm at 42˚C for 2 h and then hybridized at 42˚C overnight in the same buffer containing labeled cDNA probes (4×10^8 cpm/mL). Blots were first washed 3 times at room temperature in 2 × SSC for 15 min and then, for higher stringency, 3 times at 62–65˚C in 0.2 × SSC, 1.0% SDS for 30–60 min. Finally, blots were rinsed with 2 × SSC at room temperature for 10 min. The blots were apposed to a Bio-rad molecular imaging plate for 4 days to visualize the intensity and localization of the probe. To normalize the quality of the RNA, a ^{32}P-labeled actin cDNA probe was used for each blot at 2×10^8 cpm/mL with the same hybridization conditions.

In situ hybridization

Tissues: Normal C57BL/KsJ mice (12–15 weeks old) were deeply anesthetized with sodium pentobarbital and perfused transcardially with ice-cold 20 mL heparinized saline followed by ice-cold 50 mL phosphate buffered 4% paraformaldehyde. The brains were removed, postfixed in the fixative for 1 h, saturated in 30% sucrose prepared by diethyl pyrocarbonate (DEPC) treated water overnight in the cold (4˚C). The brains were cut into the serial coronal sections (40 µm thickness) on a freezing microtome and free-floated in 0.1 M phosphate buffer prepared by DEPC water.

Probes: For in situ hybridization, digoxigenin-labeled antisense and sense riboprobes to TSC2 mRNA were synthetized as follows: the digoxigenin-labeled TSC2 antisense riboprobes were synthesized by the transcription of a human TSC2 cDNA fragment (810 bp) (amplified by RT-PCR and inserted into the pGEM-5Zf(+) plasmid as described above) with SP6 polymerase after linearization of the template DNA by restriction enzyme SphI, while the digoxigenin-labeled TSC2 sense riboprobes were synthesized with T7 polymerase after linearization of the template DNA by restriction enzyme *Eco*RI, using digoxigenin-labeled UTP as substrates (RNA labeling Kit, Boehringer Mannheim, Germany).

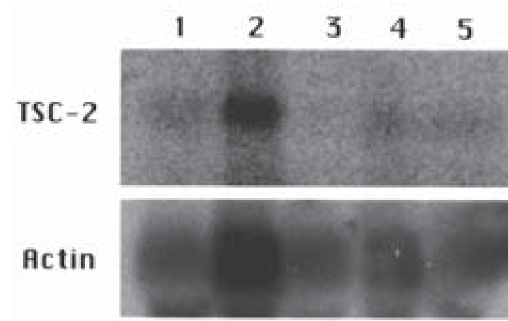

Fig. 1. Expression of TSC2 mRNA in mouse tissues. A poly(A)$^+$RNA Northern blot obtained from various mouse tissues was probed with the TSC2 cDNA probe. The blot was reprobed with an actin cDNA probe as a control for quantity of RNA. Each lane indicates a blot from: 1) the liver, 2) the brain, 3) the kidney, 4) the spleen and 5) the heart, respectively. There is wide spread TSC2 mRNA expression in many tissues with high levels of expression (4.8–3.4% of actin) in the liver, brain and spleen and lower levels (1.2% of actin) in kidney, when corrected for amount of RNA by expression of actin mRNA.

Hybridization conditions: The sections were processed by a free-floating method previously described (Saji et al., 1995). Briefly, the free-floating sections were prehybridized at 55˚C for 3 h in 50% formamide, 10% dextran, 2 × Denhart's solution, 2 × SSC (1 × SSC is 0.15 M sodium chloride + 0.015 M sodium citrate), 0.5 mg/mL denatured salmon sperm DNA, 1 mg/mL yeast tRNA and 0.25% SDS. Hybridization with digoxigenin-labeled riboprobes was performed by adding the probes (10 ng/mL) to the prehybridization buffer containing the sections (50–100 µL/section) and incubating at 55˚C overnight. The sections were washed with the incubation in 50% formamide, 2 × SSC at 45˚C for 20 min and in 2 × SSC at 37˚C for 10 min, followed by RNase A treatment (20 mg/mL in 2 × SSC) at 37˚C for 30 min and by rinsing with a dilutional series of SSC solution at room temperature (2 × SSC twice, 1 × SSC twice, 0.5 × SSC for 10 min each). Then the sections were processed for immunohistochemistry using anti-digoxigenin alkaline phosphatase

conjugate (1:500, Boehringer Mannheim). To visualize the hybrid molecules, the sections were incubated in a reaction solution with X-phosphate and nitroblue tetrazolium salt (NBT) for 24 h and mounted on subbed slides.

Results

Expression of TSC2 gene in the various mouse tissues analyzed by Northern blot

Northern blot analysis of poly $(A)^+$RNA from several mouse tissues (liver, brain, kidney, spleen and heart) revealed that the TSC2 mRNA was widely expressed in many tissues including the brain. In all tissues examined, there was one transcript evident as a 5.5 kb RNA signal (Fig. 1). When corrected for amount of RNA by expression of actin mRNA as a control, high levels of TSC2 mRNA were expressed in the liver (4.8% of actin), brain (3.4% of actin), spleen (4.2% of actin), with moderate levels in the heart (2.9% of actin) and lower levels in the kidney (1.2% of actin).

Neuron type specific expression of the TSC2 gene in the brain demonstrated by in situ hybridization

Figure 2 shows coronal sections of the hippocampus hybridized for TSC2 mRNA with antisense TSC2 riboprobes or sense TSC2 riboprobes. As seen in Figs. 2A and F, the section hybridized with sense TSC2 riboprobes indicated the red colored background and neurons which were weakly stained by non-specific binding of labeled riboprobes. In addition to the red colored background staining by non-specific binding of labeled probes shown in the hippocampal section hybridized with sense TSC2 probes (Fig. 2A), the hippocampal section hybridized with antisense TSC2 probes demonstrated neurons with a purple colored cytoplasm in the CA1-CA4 pyramidal layers, dentate granular layer and hilus (Figs. 2B to E) which expressed TSC2 mRNA at a high level. In the dendritic area or area rich in fiber tracts like the striatum radiatum, striatum oriens and dentate molecular layer in the hippocampus (**r**, **o** in Figs. 2C to E, and **m** in Fig. 2E), there were a few TSC2 gene expressing cells.

In the coronal sections hybridized with antisense TSC2 probes, large interneurons in the striatum (arrows in Fig. 3B), large neurons of the Purkinje cell layer in the cerebellum (**p** in Fig. 3E), neurons of the piriform cortex (Fig. 3D) and large neurons of the facial nucleus in the medulla (**f** in Fig. 3F) exhibited expression of TSC mRNA at high levels with the intensely purple-colored cytoplasm. On the other hand, most neurons in the cerebral cortex (Fig. 3A), septum (not shown), thalamus (not shown), striatum (Fig. 3B), substantia nigra compacta (**c** in Fig. 3C), and reticular formation in the medulla (Fig. 3F) expressed moderate or slight levels of TSC2 mRNA, having their cytoplasm weakly stained with purple-colored TSC2 signals (Fig. 3). As seen in Fig. 3E, the cerebellar cortex had a typical laminar distribution of TSC2 mRNA signals which consisted of a moderately signal expressing granular layer (**g** in Fig. 3E), a highly expressing Purkinje cell layer (**p** in Fig. 3E) and a molecular layer (**m** in Fig. 3E) with the slightest expression of TSC2 signals. Besides the cerebellar molecular layer and hippocampal striatal areas, other reticular areas like the substantia nigra reticulata (**r** in Fig. 2E) exhibited only the slightest expression of TSC2 mRNA.

The degree of TSC2 gene expression in various types of neurons is summarized in Table 1.

Discussion

An essential step in unraveling the functions of the TSC2 gene product tuberin in the mature mammalian brain is to determine which neuronal types show the most abundant expression of the TSC2 gene and to establish the common morphological features of the high TSC2 expressing neurons. The present study demonstrates (i) that TSC2 signals are expressed in the pyramidal neurons of the hippocampal CA1-CA4, granule cells of the dentate gyrus, neurons of the piriform cortex, large interneurons of the striatum, Purkinje