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Structure, Expression, and Chromosome Mapping of LATS2, a Mammalian Homologue of the Drosophila Tumor Suppressor Gene lats/warts

Norikazu Yabuta,* Takayuki Fujii,* Neal G. Copeland,† Debra J. Gilbert,† Nancy A. Jenkins,† Hiroaki Nishiguchi,† Yuichi Endo,§ Shingo Toji,‡ Hiromitsu Tanaka,‖ Yoshitake Nishimune,‖ and Hiroshi Nojima*†

*Department of Molecular Genetics, †Department of Science for Laboratory Animal Experimentation, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan; ‡Mammalian Genetics Laboratory, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702; §Tsurgua Institute of Biotechnology, Toyobo Co. Ltd., 10-24 Toyo-cho, Tsurgua 914-0047, Japan; ‖Department of Biochemistry, Fukushima Medical College, 1-Hikarigaoka, Fukushima 960-1247, Japan; and *Ina Laboratories, MBL Co. Ltd., Ina, Nagano 396-0002, Japan

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We have cloned and characterized LATS2, a novel mammalian homologue of the Drosophila tumor suppressor gene lats/warts. Northern blot analysis showed ubiquitous expression of mouse LATS2 (MmLATS2) mRNA, whereas expression of human LATS2 (HsLATS2) mRNA was enhanced in skeletal muscle and heart. Immunoblotting analysis of fractionated cell lysates showed HsLats2 to be a nuclear protein. We mapped the MmLATS2 gene to mouse chromosome 14 by interspecific backcross analysis. We also mapped the HsLATS2 gene (by fluorescence in situ hybridization) to the 13q11–q12 region, in which a loss of heterozygosity has been frequently observed in many primary cancers and to which the tumor suppressor genes RB and BRCA2 have also been mapped. © 2000 Academic Press

INTRODUCTION

Tumorigenesis in human cancers is a complex process involving a series of genetic changes such as activation of oncogenes or inactivation or loss of tumor suppressor genes. The Drosophila tumor suppressor gene warts was first identified in a mutant that showed a superficial wart-like phenotype due to homozygous loss of this gene (Justice et al., 1995). The same gene, named lats (large tumor suppressor), was also isolated as a recessive overproliferation mutant by screening genetic mosaics (Xu et al., 1995). Its transcript is expressed ubiquitously in many tissues, including the brain, muscle and heart. Immunoblotting analysis of fractionated cell lysates showed HsLats2 to be a nuclear protein. We mapped the MmLATS2 gene to mouse chromosome 14 by interspecific backcross analysis. We also mapped the HsLATS2 gene (by fluorescence in situ hybridization) to the 13q11–q12 region, in which a loss of heterozygosity has been frequently observed in many primary cancers and to which the tumor suppressor genes RB and BRCA2 have also been mapped.

MATERIALS AND METHODS

Isolation of LATS2 cDNA. A subtracted cDNA library was prepared from mouse testis as described previously (Fujii et al., 1999). From this library, we have isolated more than 150 genes whose transcription is up-regulated in sperm-producing testis. One of these cDNA clones encoded a protein similar to the Drosophila tumor suppressor lats, which we named mouse LATS2. The identity of the MmLATS2 gene was confirmed by sequencing the inserts of five independent clones, and a clone matching the consensus sequence was selected for further analysis. The cDNA insert from the mouse LATS2 clone, we isolated a human LATS2 cDNA from a human testis cDNA library by colony hybridization. DNA sequences of the cDNA inserts were determined by the dideoxy chain termination method using T7 promoter-specific fluorescent dye-labeled primers and a SequiTHERM Cycle Sequencing Kit (Epicenter Technologies, Madison, WI). The reaction products were analyzed on a LI-COR Model 4000L DNA sequencer (LI-COR Inc., Lincoln, NE). DNA database searches were carried out against all entries of the DDBJ/
with mice. Mice were immunized subcutaneously by standard protocol fusion protein, and the purified fusion protein was used to immunize 3 produces in N-terminal portion of human Lats2 (amino acids 78–256) was produced in Escherichia coli as a GST (glutathione S-transferase)-fusion protein, and the purified fusion protein was used to immunize mice. Mice were immunized subcutaneously by standard protocol with ~60 μg of fusion protein in Freund's complete adjuvant five times at biweekly intervals and boosted with fusion proteins in Freund's incomplete adjuvant. Preparation of hybridomas producing monoclonal antibody and antibody purification were carried out according to standard procedures (Harlow and Lane, 1988). Anti-14-3-3 polyclonal antibody and anti-HsOrc2 polyclonal antibody were obtained from MBL Co. Ltd. (Nagoya, Japan).

Preparation of antibody and immunoblotting. To establish a mouse hybridoma producing anti-human Lats2 antibody (3D10), the N-terminal portion of human Lats2 (amino acids 78–256) was produced in Escherichia coli as a GST (glutathione S-transferase)-fusion protein, and the purified fusion protein was used to immunize mice. Mice were immunized subcutaneously by standard protocol with ~60 μg of fusion protein in Freund's complete adjuvant five times at biweekly intervals and boosted with fusion proteins in Freund's incomplete adjuvant. Preparation of hybridomas producing monoclonal antibody and antibody purification were carried out according to standard procedures (Harlow and Lane, 1988). Anti-14-3-3 polyclonal antibody and anti-HsOrc2 polyclonal antibody were obtained from MBL Co. Ltd. (Nagoya, Japan).

For immunoblotting analysis, fractionated samples were mixed with an equal volume of 2x Laemmli's sample buffer and then heated at 100°C for 5 min. For preparation of whole-cell extracts, cells were directly lysed in 1x Laemmli's sample buffer and subsequently heated at 100°C for 5 min. Proteins were resolved by SDS-PAGE on 7.5% gels and then transferred to PVDF membranes (Millipore). Membranes were probed with monoclonal antibody against HsLats2. After being washed, blots were incubated with HRP-conjugated anti-mouse IgG (MBL Co. Ltd., Nagoya, Japan). Immuno-reactive bands were detected by enhanced chemiluminescence (Amersham).

Cell culture and subcellular fractionation. All cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Hyclone Laboratory, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Nuclear and cytoplasmic fractions were prepared by the method of Schreiber et al. (1989). Asynchronously growing HuLa-S3 cells were washed twice with ice-cold phosphate-buffered saline, harvested, and collected in Tris-buffered saline. After centrifugation, the pellet was resuspended in buffer A [10 mM Hapes (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF] and allowed to swell on ice for 15 min, after which 0.6% (final concentration) Nonidet-P40 was added, and the tube was vortexed for 10 s. The nuclear pellet was resuspended in buffer B [10 mM Hapes (pH 7.9) 400 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF], and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract were clarified by centrifugation at 15,000 g for 5 min.

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating C57BL/6J × Mus spretusF1 females and C57BL/6J males as described by Copeland and Jenkins (1996). A tail-snip was collected in Tris-buffered saline. After centrifugation, the pellet was resuspended in buffer A [10 mM Hapes (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF] and allowed to swell on ice for 15 min, after which 0.6% (final concentration) Nonidet-P40 was added, and the tube was vortexed for 10 s. The homogenate was centrifuged (1500g, 30 s) and the supernatant collected as the cytoplasmic fraction. The nuclear pellet was resuspended in buffer C [20 mM Hapes (pH 7.9) 400 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF], and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract were clarified by centrifugation at 15,000 g for 5 min.

Fluorescence in situ hybridization. FISH (fluorescence in situ hybridization) analysis of R-banded human chromosomes was performed as described previously (Endo et al., 1996) using human LATS2 cDNA (5-kb insert in pAPneo vector; Kobori et al., 1999) as a probe. Briefly, 150 ng of biotin-labeled cDNA probe was hybridized to metaphase chromosome spreads, which were prepared as described by Takahashi et al. (1990). For fluorochrome detection, the slides were incubated with fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories, Burlingame, CA) and then washed. The FITC signals were amplified by incubation with biotin-conjugated goat anti-avidin Ab (Vector Laboratories) followed by incubation with FITC-conjugated avidin. Preparations were counterstained with propidium iodide and examined with a laser scanning microscope (Zeiss LSM).

RESULTS

Isolation of Mouse and Human LATS2 cDNAs

To isolate systematically mouse genes whose transcription is specifically induced during spermiogenesis, we prepared an adult mouse testis cDNA library subtracted against biotin-labeled mRNA of prepubertal mouse testis (Fuji et al., 1999) using an improved subtraction method (Kobori et al., 1998). Since mice produce no sperm prior to puberty, this strategy can be expected to yield sperm- or haploid cell-specific cDNA species. In addition to 84 species whose expression was dramatically induced during spermiogenesis (as judged by Northern analysis), we identified 56 species whose transcription was moderately induced. Nucleotide sequence analysis revealed that one of the latter group of cDNAs encoded a predicted protein of 1043 amino acids that was homologous to the Drosophila warts/lats kinase (J ustice et al., 1995; Xu et al., 1995). We named this gene LATS2, since another mammalian homologue of the warts/lats gene was recently isolated and named LATS1 (Tao et al., 1999). By screening a human testis cDNA library by colony hybridization with mouse LATS2 cDNA as a probe, we also isolated human LATS2 cDNA.

Structure of Mouse and Human Lats2

The predicted mouse Lats2 gene product contained 1043 amino acid residues and was highly homologous to mouse Lats1 at the primary sequence level (Fig. 1A). Sequence similarity was highest in the putative Ser/Thr kinase domain (80% identity) at the C-terminal end of the protein. Mouse Lats2 and fruit-fly Lats/Warts shared 75% sequence identity in the kinase domain (Fig. 1B). Sequence similarity between mouse Lats2 and fruit-fly Lats extended to the N-terminus, although the similarity in this region was much lower than in the kinase domain. Structural comparisons between mouse Lats1 and Lats2 revealed a similar pattern of high similarity in the C-terminal half of the molecule including the kinase domain and low similarity in the N-terminal half. Mouse and human Lats1 and fruit-fly and Caenorhabditis elegans Lats all con-
tain four or five stretches of several proline residues in the N-terminal region, one of which matches the consensus sequence (PXXPXR) for a potential SH3 (Src homology 3) binding site (Feng et al., 1994). However, no such sequences were found in Lats2, suggesting a functional distinction between the mammalian Lats homologues. Human Lats2, but neither mouse Lats2 nor any other Lats homologue, possesses seven repeats of alternating proline–alanine residues (PAPA repeat). Such a sequence was also found in human p57KIP2 (Matsuoka et al., 1995) and may be involved in protein–protein interactions. The polyglutamine repeat found in fruit-fly Lats and yeast Lats-like protein is not found in Lats proteins of other species.

Further analysis of kinase domain amino acid se-

FIG. 1. (A) Amino acid sequence comparisons between mouse Lats2, mouse Lats1, and Drosophila Lats. Identical amino acids are indicated by shaded backgrounds. The Ser/Thr kinase domain is indicated by thick horizontal bars drawn above the amino acid sequence. (B) Schematic drawings of Lats2 proteins of mouse (MmLats2) and human (HsLats2) together with Lats1 proteins of mouse (MmLats1) and human (HsLats1), fruit-fly Lats (DmLats), nematode Lats1 (CeLats1), and budding yeast Lats1-like protein (Cbk1; P53894). The locations of the alternating proline–alanine sequence (PAPA repeat), polyproline sequence (P stretch), and polyglutamine repeat (Q repeat) are shown. Asterisks indicate the locations of consensus sequences (PXXPXR) for potential SH3-binding sites. (C) Dendrogram of the kinase domains of Lats proteins and other Ser/Thr kinases clustered by the PileUp program. The dendrogram indicates similarity on the horizontal axis, proportional to the length of the branch line before bifurcation. Hs, Homo sapiens; Mm, Mus musculus; Dm, Drosophila melanogaster; Nc, Neurospora crassa; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe.
quences was performed to determine the relationship between Lats1, Lats2, and fruit-fly Lats. We conducted BLAST searches using the Lats2 kinase domain against all entries of the DDBJ/GenBank/EMBL Data-Libraries, collected the amino acid sequences of the kinase domains of 20 Ser/Thr kinases, and constructed a dendrogram showing the sequence relationships among these kinase domains using the “PileUp” program of the GCG software package (Genetics Computer Group, Madison, WI). As shown in Fig. 1C, the result clearly indicates that Lats2 is most closely related to Lats1, followed by fruit-fly and nematode Lats.

Expression of the LATS2 Gene

To characterize the LATS2 gene further, we examined its transcription patterns in various mouse tissues. Northern analysis of RNA extracted from various adult mouse tissues using LATS2 cDNA as a probe indicated that expression of the LATS2 gene was ubiquitous, although mRNA levels were comparatively high in ovary and testis (Fig. 2A). We therefore examined the expression pattern of LATS2 in the testis in more detail. Total RNA extracted from testis of mice of varying ages was subjected to Northern blot analysis (Fig. 2B). LATS2 mRNA could be detected in even the earliest prepubertal testis (2–5, 8, and 17 days old); LATS2 transcription increased in postpubertal testis (23 days old) and declined in adult mouse testis (29 and 35 days old). We also examined the detailed localization of LATS2 mRNA using total RNA prepared from fractionated mouse testicular cells, i.e., germ, Leydig, and Sertoli cells (Fig. 2C); the quality of this RNA was verified in a previous report (Koga et al., 1998). Most of the LATS2 mRNA was detected in the germ cell fraction.

We also analyzed the expression of human LATS2 using multiple-tissue Northern blots obtained from Clontech (Palo Alto, CA). As shown in Fig. 2D, the expression pattern is distinct from that of the mouse homologue. Human LATS2 is most strongly expressed in skeletal muscle and heart, whereas LATS2 expression in these tissues is moderate in mouse. Of further note is a smaller (3.8 kb) transcript specific to human testis. Comparison of the DNA sequences of several cDNA clones isolated from the human cDNA library suggested that this 3.8-kb transcript has the same open reading frame as the 5.8-kb transcript, but a shorter 3’ untranslated region (data not shown).

Preparation of Antibody and Subcellular Distribution of Lats2

To examine the subcellular distribution of endogenous Lats2 protein, we raised a monoclonal mouse antibody (3D10) against a recombinant GST–fusion protein containing the N-terminal portion (amino acids 78–256) of HsLats2. As shown in Fig. 3A, the anti-HsLats2 monoclonal antibody (3D10) specifically recognized a band of approximately the same size as the 6Myc-tagged HsLats2 protein expressed in 293T human embryonic kidney cells (2 μg/lane of the whole extract). The 3D10 antibody also recognized a C-terminally truncated form of 6Myc-tagged HsLats2, which contained the epitope for 3D10, but not the C-terminal portion of Myc-tagged HsLats2 protein from which the epitope was absent. Although the endogenous HsLats2 cannot be detected by the 3D10 antibody in the whole

FIG. 2. Northern blot analysis of mouse and human LATS2. RNA was prepared from various tissues of an adult mouse (A), from the testis at various developmental stages (B), and from fractionated testicular cells (C). Northern blots of RNA from various human tissues were obtained from Clontech (D). Mouse or human LATS2 cDNA inserts were radiolabeled and used as hybridization probes. After autoradiography, the first probe was stripped from the filter, which was rehybridized with β-actin cDNA as a loading control.
extract of 293T cells, it can be recognized as a single band of 125 kDa (lane 8), which is consistent with the predicted size of HsLats2, when the sample was concentrated by separation of the nucleus from the cytoplasm and subsequent extraction by 300 mM NaCl (5 mg/lane of the concentrated nuclear extract). Thus, immunoblotting with anti-Myc monoclonal antibody (PL14) indicated that the bands detected by 3D10 represented bona fide HsLats2 protein. Together with Lats2 in human (MRC5) cells, 3D10 antibody also recognized the Lats2 proteins of other mammalian species including monkey (COS7) and Chinese hamster (CHO), but not those of rat (Rat1) or mouse (NIH3T3), as shown in Fig. 3B. One of the two major bands that were detected at around 125 kDa in MRC5 and COS7 cells may represent a phosphorylated form of Lats2. The lower band at 116 kDa in COS7 cell extracts may be due to protein degradation, since we often observed many bands of lower molecular mass in cell extracts that were not prepared with rigorous care for the integrity of the proteins (data not shown).

We next examined subcellular fractions for the presence of HsLats2 by immunoblotting. Immunoblot analysis of separated cytoplasmic and nuclear fractions of HeLa-S3 cells showed that almost all of the endogenous HsLats2 protein was localized in the nuclear fraction (N) (Fig. 3C, top). We confirmed that another nuclear protein, HsOrc2, was also localized in the nuclear fraction (Fig. 3C, middle). In contrast, a typical cytoplasmic protein, 14-3-3β, which was also used as a control for successful fractionation, was found exclusively in the cytoplasmic fraction (Fig. 3C, bottom). The result indicates that HsLats2 is a nuclear protein.

Chromosomal Location of the Mouse LATS2 Gene

The chromosomal location of mouse LATS2 was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × M. spretus)F1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2700 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and M. spretus DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse cDNA probe. A 3.6-kb PstI M. spretus RFLP was used to follow the segregation of the LATS2 locus in backcross mice. The mapping results indicated that LATS2 is located in the central region of mouse chromosome 14 linked to Gzmb, Gjb2, and Nfl. Although 176 mice were analyzed for every marker in the segregation analysis (Fig. 4), up to 189 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies. The ratios of
nants were detected between LATS2 and Gjb2 in mouse. LATS2 was located on mouse chromosome 14 by interspecific backcross analysis. The segregation patterns of LATS2 and flanking genes in 176 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 176 animals were typed (see text). Each column represents the chromosome, and the positions of loci in human chromosomes, where known, are shown at the right. References for the human map positions of loci cited in this study can be obtained from GDB (Gene DataBase), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

Chromosomal Location of the Human LATS2 Gene

The close linkage between Gjb2 and LATS2 in mouse suggested that the human homologue of LATS2 might also map to 13q. We performed FISH analysis using the cDNA insert of our human LATS2 clone as a probe. As shown in Fig. 5, specific FISH signals generated from biotinylated human LATS2 cDNA were observed at the centromeric region of the long arm of human chromosome 13. No other reproducible signal was observed on metaphase chromosomes. Thus, FISH analysis indicated that the human LATS2 gene maps to the 13q11–q12 region.

**DISCUSSION**

In this study, we report the cloning and characterization of mouse and human cDNAs encoding Lats2. We showed that Lats2 is a bona fide mammalian homologue of the Drosophila tumor suppressor Lats/Warts by amino acid sequence comparison (Fig. 1). C. elegans also has a gene encoding a Lats-like protein. Together, these proteins define a new subfamily of protein kinases that are closely related to but distinct from the Ndr protein kinases and the cyclic AMP-dependent kinases. The C-terminal regions of mammalian Lats1 and Lats2 contain the putative Ser/Thr protein kinase domain and closely resemble each other, whereas sequence similarity in the N-terminal region of the molecule is very low. The absence in mammalian Lats2 of the putative SH3-binding motifs found in mammalian Lats1 and Drosophila Lats suggests that the role of Lats2 in the potential signal transduction pathway may be distinct from those of Lats1 and Drosophila Lats. Homology searches for Lats-like proteins in lower eukaryotes identified fission yeast Orb6 and budding yeast Dbf2, Dbf20, and Cbk1/Knq1 (P53894). However, sequence analysis suggests that Dbf2 and Dbf20 are distant relatives and that Cbk1/Knq1 and Orb6 are more closely related to Ndr protein kinases (Millward et al., 1995). It is noteworthy that disruption of the CBK1 gene showed no alteration of phenotype (http://genome-www.stanford.edu/Saccharomyces/), while Orb6 is required to maintain cell polarity throughout the interphase period of the cell cycle and to promote actin reorganization during morphological transitions (Verde et al., 1998).

The Drosophila lats/warts gene has been identified as a tumor suppressor (Xu et al., 1995; Justice et al., 1995). Mutations in lats cause overgrowth phenotypes and a variety of developmental defects, and mosaic flies harboring lats mutant cells develop large tumors in various organs. Lats1−/− mice develop large soft-tissue sarcomas and ovarian stromal cell tumors with metastasis to the lungs and are susceptible to carcinogenic treatments, which cause them to develop soft-tissue sarcomas. The susceptibility to spontaneous and induced tumors of Lats1−/− mice strongly indicates that Lats1 acts as a tumor suppressor. The phenotypic dif-
ference between fruit-fly lats and mouse Lats1 mutants suggests the possibility of redundancy among lats-like genes in mammals. Our identification of LATS2 as a new member of the LATS family may help to explain the phenotypic difference between fruit-fly lats and mouse lats1/2 mutants, in that Lats2 may partially complement the function of Lats1.

The similarity in structure and mRNA expression patterns between Lats1 and Lats2 suggests that the latter may also function as a tumor suppressor. We mapped in this report the human LATS2 gene to chromosome 13q11–q12, in the vicinity of the well-documented tumor suppressor genes RB (at 13q14) and BRCA2 (at 13q12–q13). Loss of heterozygosity (LOH) in this chromosome region has been frequently observed in primary cancers of the breast (Lee et al., 1988), liver (Wang and Roger, 1988), lung (Weston et al., 1989), ovary (Sato et al., 1991), and bladder (Cairns et al., 1991). The RB gene, mutations in which are responsible for retinoblastoma, is considered to be the most likely candidate gene involved in these tumors. BRCA2, the second gene responsible for familial breast cancer, is also a candidate gene in tumors showing LOH at 13q. However, RB and BRCA2 have been identified as the targets of allelic deletions on chromosome 13q in only a few cases to date. We propose that the LATS2 gene is another candidate tumor suppressor gene that may be responsible for tumors showing LOH at 13q.

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