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Ras-Guanine Nucleotide Exchange Factor Sos2 Is Dispensable for Mouse Growth and Development

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The mammalian *sos1* and *sos2* genes encode highly homologous members of the Son-of-sevenless family of guanine nucleotide exchange factors. They are ubiquitously expressed and play key roles in transmission of signals initiated by surface protein tyrosine kinases that are transduced into the cell through the action of membrane-associated Ras proteins. Recent reports showed that targeted disruption of the *sos1* locus results in embryonic lethality. To gain insight into the in vivo function of *sos2*, we disrupted its catalytic CDC25-H domain by means of gene targeting techniques. Mating among heterozygous sos2$^{+/-}$ mice produced viable *sos2*$^{-/-}$ offspring with a normal Mendelian pattern of inheritance, indicating that the loss of *sos2* does not interfere with embryo viability in the uterus. Adult homozygous mutant sos2$^{-/-}$ mice reached sexual maturity at the same age as their wild-type littermates, and both male and female null mutants were fertile. Histopathological analysis showed no observable differences between mutant and wild-type mice. Our results show that unlike the case for *sos1*, *sos2* gene function is dispensable for normal mouse development, growth, and fertility.

Ras protein activation in eukaryotes is mediated through the action of Ras-specific guanine nucleotide exchange factors (GEFs) (1, 17) linking the activation of surface receptors by upstream signals to the accumulation of a Ras-GTP complex able to deliver signals to the nucleus. GEFs are highly conserved in evolution, having been initially identified in lower organisms such as *Saccharomyces cerevisiae* (CDC25 and SCD25) (4, 5), *Schizosaccharomyces pombe* (Ste6) (15), and *Drosophila* (Son of sevenless [Sos]) (2). Three types of Ras-specific GEFs have been described in mammals: the highly homologous GRF1 and GRF2 (6, 10, 11, 21), the closely related Sos1 and Sos2 (3, 7, 14), and GRP (9). *sos* and *GRF2* genes are widely expressed in adult tissues and cell lines, while expression of *GRF1* and *GRP* genes is restricted primarily to the brain. All Ras-specific GEFs share a region of homology with the C-terminal 450 amino acids of CDC25 (CDC25-H domain) constituting the catalytic domain of all these proteins (1).

The GEF protein Sos plays a crucial role in the process of coupling protein tyrosine kinases, via the adapter protein Grb2, to Ras activation, facilitating GDP-GTP exchange. Sos1 and Sos2 proteins are constitutively bound to the SH3 domain of Grb2 through the proline-rich region present in their C. termini. The Grb2-Sos complex binds directly to the activated receptors or to a second adapter protein, such as Shc, through the SH2 domain of Grb2 (8, 12, 20).

Alignment of the murine or human Sos1 and Sos2 proteins uncovers a high overall (65% amino acid identity) degree of similarity. Similarity between Sos1 and Sos2 is highest (up to 75% amino acid identity) at their N-terminal regions. In contrast, the homology between the C-terminal regions of Sos1 and Sos2 is more restricted and scattered (overall similarity of 40%), with conserved regions mostly reduced to the short proline-rich motifs responsible for interaction with the SH3 domain of Grb2. These differences between their C-terminal regions are likely to account for the distinct signaling and functional properties of the two Sos proteins. It has been reported that human Sos2 has a higher affinity for Grb2 than Sos1 (25) and that mouse Sos1 is more stable than Sos2, which appears to be degraded by a ubiquitin-dependent process (18). Other differences between Sos1 and Sos2 are related to their protein tyrosine kinase signaling properties: Sos1 participates in short- and long-term signaling, whereas Sos2-dependent signals are predominantly short term (19).

sos1 is essential for embryonic development, with homozygous null *sos1*$^{-/-}$ embryos dying at midgestation in utero (19, 24). To evaluate the functional significance of the highly homologous *sos2* gene, we created a targeted disruption of this locus in mice. In this report we show that *sos2* is completely dispensable for mouse development, since its null mutation resulted in viable mice with no apparent phenotypic effect due to this deficiency.

MATERIALS AND METHODS

Sos2 targeting vector and chimeric mouse production. Four mouse genomic *sos2* clones were identified and isolated from a 129SvJ mouse-derived library (Stratagene, La Jolla, Calif.), using a 690-bp probe derived from the sequence immediately downstream of the CDC25-H domain (bp 3093 to 3783) of murine *sos2* cDNA (3) (GenBank accession no. Z11664). Since there is 72.8% homology between *sos1* and *sos2* at the CDC25-H domain, we used sequences downstream of—instead of within-this domain to avoid cross-hybridization with *sos1*.

DNA fragments were isolated from two of these clones and subcloned into pBluescript II (Stratagene). Plasmids pPNT (23), containing *pgk-neo*, and pMC1-TkpA (13), containing thymidine kinase (*tk*) selectable markers, were used to construct the *sos2* targeting vector pLM146 (Fig. 1A). A 2.4-kb *Nde*I-*Nde*I fragment containing sequence of the intron between exons B and C was used as the 5′ arm of the construct, and an *Xba*I/*Xba*I fragment of 6.0 kb containing exon D was used as the 3′ arm of homology. The *neo* cassette from pPNT (*Xho*I/*Bam*HI) was used as a positive marker and replaced a fragment of 0.22 kb that contained exon C (which codes for amino acids 855 to 893, inside the CDC25-H

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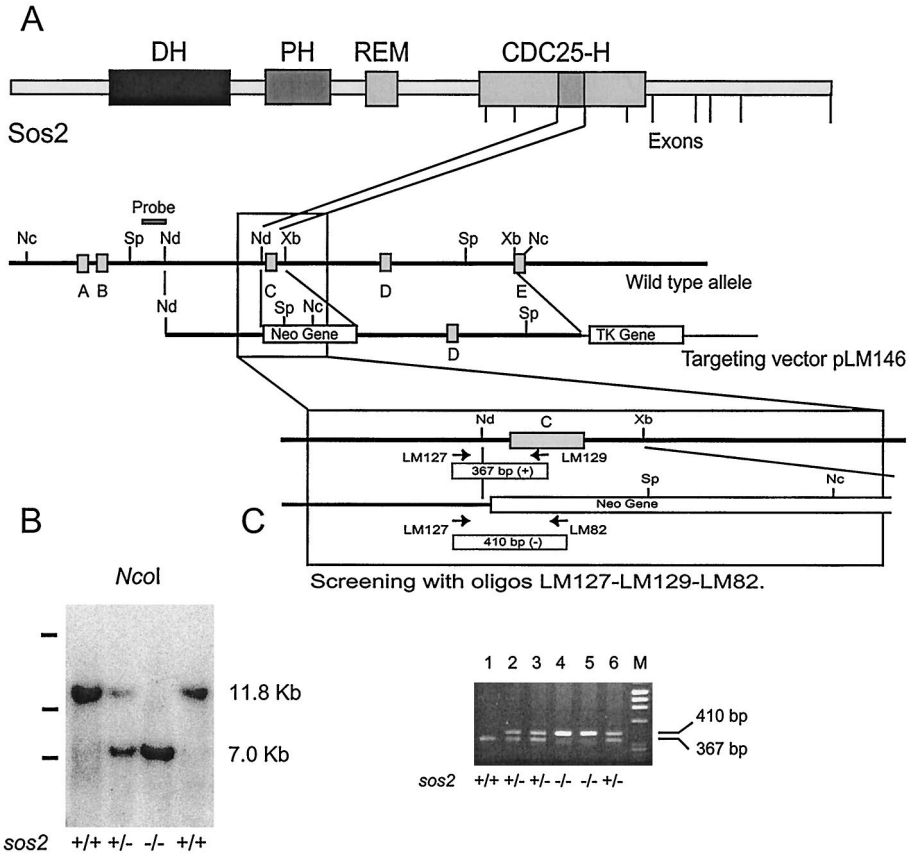

FIG. 1. Targeted disruption of the murine *sos2* gene in ES cells and mice. (A) Schematic representation of the *sos2* locus and targeting vector. Boxes in the wild-type allele schematics represent the exons of the *sos2* CDC25-H domain. The open boxes in the targeting vector schematics represent the *pgk-neo* and *pgk-tk* selectable marker genes. Position of boundaries of individual exons coding for the C-terminal portion of the protein are indicated by vertical marks. The position of the 5′ flanking probe used in Southern blotting is indicated. DH, Dbl homology; PH, pleckstrin homology; REM, Ras exchange motif. (B) Homologous recombination of the targeting vector in mice was verified by Southern blotting, digesting genomic DNA with *Nco*I, and hybridizing with a 5′-flanking probe. The wild-type allele produced a 11.8-kb band, whereas the mutant allele yielded a 7.0-kb band due the introduction of a new *Nco*I site in the targeting vector. (C) Routine genotyping of mice was performed by PCR using the oligonucleotides indicated (see Materials and Methods for sequences). The LM127 and LM129 primers are specific for the *sos2* gene and amplify a fragment of 367 bp. The LM82 primer is specific for the promoter of the *neo* gene and amplifies a fragment of 410 bp with LM127. Nc, *Nco*I; Sp, *Spe*I; Nd, *Nde*I; Xb, *Xba*I.

domain) (Fig. 1A). The negative marker (herpes-virus *tk*) was placed 3′ of the regions of *sos2* homology. The targeting vector, pLM146, was linearized with *Sal*I and electroporated into CJ7 embryonic stem (ES) cells (22), and homologous recombinants were screened by using a diagnostic *Spe*I restriction enzyme digestion with a 5′ probe external to the targeting vector. Colonies resistant to double G418-fialuridine selection were isolated and expanded. Southern blotting analysis showed that 7 out of 80 double-resistant clones tested had targeted disruption of one *sos2* locus by homologous recombination.

Several of the recombinant ES cell lines were expanded and subsequently injected into 3.5-day C57BL/6 blastocysts to generate chimeras transmitting the mutated *sos2* allele to the progeny. The offspring was analyzed for *sos2* disruption by Southern blotting and PCR analysis. Breeding of mice heterozygous for *sos2* ($sos^{+/-}$ mice) gave rise to homozygous mutant $sos^{-/-}$ mice.

Genotyping of targeted ES cells, mice, and embryos. Genomic DNA was extracted from cultured ES cells, mouse tail biopsies, and embryo yolk sacs as described by Laird et al. (16). ES cells were incubated at 37°C, and tail biopsies and embryo yolk sacs were incubated at 55°C, in lysis buffer (100 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], 200 mM NaCl, 100 μg of proteinase K per ml 4 to 5 h or overnight. DNA was isopropanol precipitated and recovered by lifting the aggregated precipitate from the solution. DNA was washed in 70% ethanol and resuspended in 200 μl of Tris-EDTA (pH 8.0) buffer. For Southern analysis, 20 μl of DNA was digested with *Spe*I, electrophoresed on 0.6% agarose gels, and blotted to GeneScreen Plus membranes (Dupont, Boston, Mass.). A probe flanking the 5′ end of the targeting vector sequence was labeled with a random primer labeling kit (Stratagene) and used in hybridizations. Wild-type and mutated alleles were identified by predicted restriction fragment size differences. Clones displaying homologous recombination were reconfirmed with *Nco*I digestion and the same 5′ probe (Fig. 1B). Digestion of ES cell DNA with enzymes that did not cut within the targeting vector and Southern blotting and hybridization with a *neo* probe showed only a single band, indicating the there was only a single site of vector insertion in the targeted ES cell clones (not shown).

PCR was also used for routine genotyping of DNA isolated from mouse tail biopsies or embryo yolk sacs. Three primers, LM127 (5′-CTTTCTGCCCCTGT AATTTACACCAGATGA-3′), LM129 (5′-GTGGTCCTGACTTAGTTCCAC AGCGTCA-3′), and LM82 (5′-CTACCGGTGGATGTGGAATGTGTGCGA-3′), were used in a 50-μl reaction with 1 to 2 μl of DNA and 1.25 U of *Taq* polymerase (Boehringer Mannheim) under the conditions indicated by the company. The LM127 and LM129 primers are specific for *sos2* and amplify a 367-bp fragment (LM127 bp 14 to 43 and LM129 bp 353 to 380 of GenBank entry AF094681). LM82 primer, specific for the *neo-pgk* promoter (on bp 517 to 543 of GenBank entry M18735), amplifies a 410-bp fragment with LM127. Cycling conditions were 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final cycle at 72°C for 10 min. Amplified products were analyzed directly in 2.5% agarose gels (NuSieve 3:1).

Histopathological analysis. More than 30 tissues taken from $sos2^{-/-}$ adult mice (three males and one female, 16 weeks old) after necropsy were fixed in formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Lesions were not found in any tissues. As controls, we examined the same tissues from two 16-week-old wild-type $sos2^{+/+}$ males and three 16-week-old wild-type $sos2^{+/+}$ females. Two 16-week-old $sos2^{+/-}$ males were also examined.

Western blot analysis. Protein extracts were obtained from snap-frozen mouse tissues. Tissues were homogenized in radioimmunoprecipitation buffer (50 mM Tri-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and centrifuged in a Sorvall S1256 apparatus at 30,000 rpm for 30 min. Supernatant was recovered, and proteins were quantified. Lysates (50 to 70 μg/lane) were loaded onto SDS–7.5% polyacrylamide gels, and the proteins were transferred to polyvinylidene difluoride membranes (Millipore Immobilon-P) by electroblotting. Membranes blocked in TTBS (10 mM Tris-HCl [pH 8.0], 150

mM NaCl, 0.05% Tween 20) plus 1% bovine serum albumin were incubated, as appropriate, with 1:100 dilutions of commercial polyclonal antibodies from Santa Cruz Biotechnology, Santa Cruz, Calif.). Antibodies used were anti-C-terminal Sos1 (C-23, *sc-256*), anti-C-terminal Sos2 (C-19, *sc-258*), and anti-N-terminal Sos1/Sos2 (D-21, *sc-259*; recognizing the identical N termini of both Sos1 and Sos2). Western blots were developed using the ProtoBlot Western blotting alkaline phosphatase system (Promega) following procedures recommended by the supplier.

RESULTS AND DISCUSSION

To investigate the in vivo function of the mammalian Sos2 GEF, we used gene targeting techniques to generate mice deficient in the endogenous *sos2* locus. In an approach similar to that used previously for *sos1* (19), we inactivated *sos2* by targeting its catalytic CDC25-H domain, responsible for the guanine nucleotide exchange activity of the molecule (1).

By means of restriction mapping and partial sequencing, we identified the positions of various exons coding for the CDC25-H domain of *sos2* (Fig. 1A). Based on those genomic mapping data, the targeting vector pLM146 (Fig. 1A) was constructed by replacing genomic sequences containing a highly conserved exon (encoding amino acids 855 to 893) of the central region of the Sos2 CDC25-H domain with a *pgk* promoter-driven *neo* cassette. The herpes simplex virus *tk* gene was included downstream of the long arm of homology in pLM146 to provide negative selection for nonhomologous recombination events (Fig. 1A).

The replacement vector pLM146 was electroporated into CJ7 ES cells, which were then selected in the presence of G418-fialuridine. A relatively high frequency of homologous recombination (7 positives out of 80 clones screened) was observed with our construct. Subsequent microinjection of two of the positive ES cell lines was used to generate chimeras that were then mated to C57BL/6 females to give rise to $sos2^{+/-}$ mice. These *sos2* heterozygotes were further inbred and generated *sos2*-null mice at the expected Mendelian frequency of 1/4. Mutant genotypes were initially confirmed by Southern hybridization (Fig. 1B), and further routine genotyping was carried out by PCR using oligonucleotides hybridizing within both the *sos2* gene and the *pgk-neo* cassette (Fig. 1C).

To confirm that the modification of the *sos2* gene resulted in a null mutation, we examined Sos2 expression by Western immunoblot in tissues from wild-type and mutant mice. Using antibodies specific for the C-terminal region of Sos2 protein (Santa Cruz Biotechnology), we were unable to detect in tissues from −/− mutant mice the presence of the full-length Sos2 protein that is easily observable in their wild-type counterparts (Fig. 2A). In contrast, antibodies specific for Sos1 revealed the presence of similar levels of this protein in both wild-type and mutant *sos2* tissues (Fig. 2B), indicating that there is no overexpression of Sos1 to compensate for the absence of Sos2.

It should be noted that in our targeting strategy, the deletion of exon C from the genomic sequences contained in pLM146 (Fig. 1A) creates an out-of frame disruption which might, at least potentially, give rise to a truncated protein (857 residues, expected molecular size of ca. 105 kDa) in the hypothetical case that the resulting mutated locus could be transcribed into a stable or functional RNA, which in turn would need to be able to be translated into a stable protein. To check for the possible occurrence of a shorter Sos2 protein in mutant *sos2* animals, we performed Western blot analysis of the same tissues using antibodies specific for the amino-terminal region of Sos2. Using a commercial antibody recognizing specifically the common amino-terminal region of Sos1 and Sos2 in mice and humans, we failed to detect the presence of any low-molecular-weight form of Sos2 protein in tissues of the mutant mice (Fig. 2C). These results confirmed the absence of any form of Sos2 protein in our homozygous −/− mutant mice.

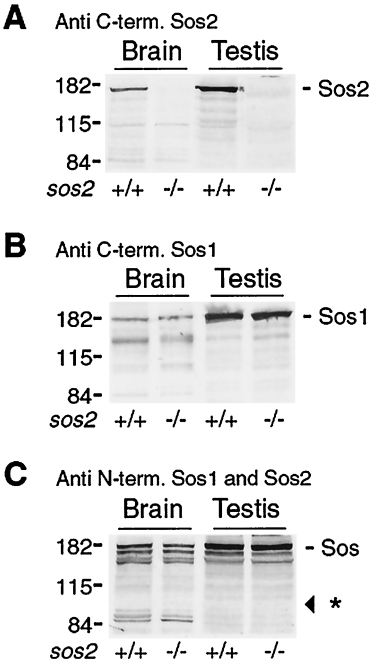

FIG. 2. Western blot to detect Sos proteins in extracts from wild-type and $sos2^{-/-}$ mouse tissues. Fifty- to 70-μg aliquots of total protein from brains and testes of wild-type and $sos2^{-/-}$ mice were electrophoresed on SDS–7.5% polyacrylamide gels. (A) Immunodetection of Sos2 protein. It is present in wild-type and absent in mutant mouse tissues. (B) Immunodetection of Sos1 protein. It is present in all tissues regardless of whether they are deficient for Sos2. There is no apparent increase in the amount of Sos1 protein in tissues that are deficient for Sos2. (C) Immunodetection using antibodies specific for the common N-terminal region of Sos1 and Sos2 proteins. A shortened, truncated Sos2 protein form was not detected in $sos2^{-/-}$ mutant mice at the theoretical position indicated (◀*), confirming the absence of any Sos2 protein form in the mutant *sos2* mice. Sizes are indicated in kilodaltons.

We have kept mutant *sos2* mice in our laboratory for about 1.5 years now. Lesions were not found in any tissues of the $sos2^{-/-}$ mice that could be attributed to the null mutation. Both the heterozygous +/− and homozygous −/− mutant *sos2* mice developed normally, with males and females being fertile. In addition, the mutant mice showed no obvious defects, and their long-term survival rates were indistinguishable from those of their wild-type littermates. Thus, the absence of *sos2* in mice did not compromise development or fertility in mice. Finally, histopathological analysis of more than 30 tissues in four adult $sos2^{-/-}$ animals revealed no gross anatomical defects or histopathological changes (data not shown).

Since the absence of the highly homologous Sos1 protein results in embryonic lethality, probably due to impaired placental development (19), we wished to address the effect of the absence of Sos2 on early development. Histopathological analysis of $sos2^{-/-}$ embryos at day 12 of gestation did not show any difference from their wild-type littermates and development of the placenta appeared also to be normal in −/− animals. This is consistent with the significantly lower levels of *sos2* expression relative to *sos1* expression observed in wild-type placenta (19).

Our results confirm that in sharp contrast to Sos1, Sos2 is dispensable for embryonal and adult mouse development, as well as for normal growth and fertility of the knockout animals.

This very significant functional difference between two otherwise remarkably similar proteins is most likely related to our previously reported observations (19), indicating that Sos1 participates in both short- and long-term signaling through the Ras-mitogen-activated protein kinase pathway, while Sos2-dependent signals are predominantly short term.

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