Defective T-Cell Activation Is Associated with Augmented Transforming Growth Factor β Sensitivity in Mice with Mutations in the *Sno* Gene

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The proto-oncogene *Sno* has been shown to be a negative regulator of transforming growth factor beta (TGF-β) signaling in vitro, using overexpression and artificial reporter systems. To examine *Sno* function in vivo, we made two targeted deletions at the *Sno* locus: a 5′ deletion, with reduced Sno protein (hypomorph), and an exon 1 deletion removing half the protein coding sequence, in which Sno protein is undetectable in homozygotes (null). Homozygous *Sno* hypomorph and null mutant mice are viable without gross developmental defects. We found that *Sno* mRNA is constitutively expressed in normal thymocytes and splenic T cells, with increased expression 1 h following T-cell receptor ligation. Although thymocyte and splenic T-cell populations appeared normal in mutant mice, T-cell proliferation in response to activating stimuli was defective in both mutant strains. This defect could be reversed by incubation with either anti-TGF-β antibodies or exogenous interleukin-2 (IL-2). Together, these findings suggest that *Sno*-dependent suppression of TGF-β signaling is required for upregulation of growth factor production and normal T-cell proliferation following receptor ligation. Indeed, both IL-2 and IL-4 levels are reduced in response to anti-CD3 stimulation of mutant T cells, and transfected *Sno* activated an IL-2 reporter system in non-T cells. Mutant mouse embryo fibroblasts also exhibited a reduced cell proliferation rate that could be reversed by administration of anti-TGF-β. Our data provide strong evidence that *Sno* is a significant negative regulator of antiproliferative TGF-β signaling in both T cells and other cell types in vivo.

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initial steps of T-cell activation during a productive immune response. To characterize Sno function in lymphoid cells and other cells in vivo, we used homologous recombination gene targeting in mice to make two deletion mutations. A 5' deletion, removing 1.7 kb of regulatory sequences at the 5' end of the Sno gene, leaves coding sequences intact. This mutation decreased Sno mRNA and protein expression to low basal levels but appeared to leave activation-induced increases intact. A second construct deleted exon 1, removing the coding sequences for the first half of the protein and effectively eliminating Sno expression in homozygous mice. Mice homozygous for either deletion are viable and show no deviant phenotype on gross inspection in either a C57BL/6, 129/Sv, or mixed background. However, both mutations caused a reproducible defect in T-cell activation, accompanied by signs of increased sensitivity to TGF-β. The mutant defect is rescued by the addition of exogenous interleukin-2 (IL-2) to the cultures. This suggests a direct relationship between Sno activity and the production of proliferative cytokines. Indeed, we found that Sno-null splenocytes express reduced levels of IL-2 as well as IL-4 and that Sno overexpression can trans-activate the IL-2 promoter in transfection assays. These results suggest that Sno functions early in the T-cell activation pathway(s) to inhibit tonic TGF-β modulation of the pathway in response to strong activating signals and that early response of the Sno gene to T-cell receptor triggering is required for optimal IL-2 production and subsequent T-cell proliferation.

MATERIALS AND METHODS

RNA isolation and RT-PCR. RNA was isolated as described previously (10) and reverse transcribed using random hexamer primers. Reverse transcription (RT)-PCR was performed with specific primers and [32P]dCTP in the reaction. Pilot studies were used to determine the conditions for linear incorporation of 32P with the amount of input RNA (27 cycles with 0.7 µg of total RNA for Sno and 0.05 µg for GAPDH [glyceraldehyde-3-phosphate dehydrogenase] [Table 1]) (17, 49). One-fifth of the [32P]dCTP-labeled reaction mixture was loaded on a 5% acrylamide gel, electrophoresed, exposed to a phosphor screen, and scanned for image analysis. The band identities were confirmed by the band sizes and by Southern blot hybridization of cold RT-PCR products to radiolabeled Sno probes (not shown). Real-time PCR was performed, using SYBR Green dye and primer concentration conditions that we optimized in pilot experiments, in a Prism 7700 sequence detector (Applied Biosystems, Inc., Foster City, Calif.). Oligonucleotide primer sequences and template amounts are presented in Table 1. Real-time PCR for IL-2 and IL-4 was performed using predeveloped TaqMan primers (Applied Biosystems, Inc.).

Splenoctye stimulation. Splenocytes were isolated from unprimed C57BL/6 mice (Fig. 1) or B6:129/Sv mice of the different genotypes and plated on 1-cm2 diameter dishes precoated with 100 µg of anti-CD3e (aCD3) antibody/ml (catalog no. 145-2C11; BD Biosciences Pharmingen, San Diego, Calif.). Adherent cells were harvested after 1 to 48 h for RNA isolation. Both adherent and nonadherent cells were collected for immunophenotyping; some splenocytes were harvested prior to plating (zero hour).

Targeting vector and gene targeting. A 20-kb 129/Sv strain mouse genomic DNA clone in lambda FIX II (Stratagene) phage was used to generate the targeting vectors. A pGK-neo gene expression cassette (50) was substituted for the 5' untranslated region and the context for the initiation of translation. Fill-in of the lacZ mutant, we generated another allele deleting all of exon 1 (designated ex1) led us to expect that this deletion would remove promoter elements controlling Sno expression. The Sno5a construct also deleted the 500-nucleotide (nt) 5' untranslated region and the context for the initiation of translation. Fill-in of the Neo 5' overhang to make a blunt end disrupted the Neo recognition site but regenerated the ATG codon (boldface); the Kozak consensus context was altered from GTG to GAATTCATGATC (GTG to GaATTcATGAtc). Since the coding region remained intact in the 5a mutant, we generated another allele deleting all of exon 1 (designated ex1), thus deleting all of the biological activities so far described for Sno (Fig. 2A). A lacZ gene with a nuclear localization signal was substituted for the exon 1 coding sequences that were deleted. The targeting constructs (Fig. 2A and 3A) were electroporated into R1 ES cells (42) and selected in 200 µg of G418/ml of irradiated G418-resistant mouse embryoblast fibroblast (MEF) feeder cells isolated from our TgN(pGKneo)066Spw transgenic mice. ES clones exhibiting homologous integration of the targeting vector (n = 2 for 5a and n = 4 out of the 12 clones isolated for ex1) were thawed, expanded briefly, injected into C57BL/6 blastocysts, and implanted into pseudopregnant recipient mice, which were allowed to deliver litters (7). We obtained one or more germ line-transmitting male chimeras for each line. One line of Sno5a was chosen for further analysis. Two lines of Sno5a were studied and behaved identically; the results reported here were from one of these lines. Animal use complied fully with federal guidelines, with prior approval of the animal protocol by the Institutional Animal Care and Use Committee.

Immunoprecipitation of Sno protein. To examine Sno protein, 13.5-day postcoitum (dpc) embryos were eviscerated and homogenized in 10 ml of RIPA buffer per g of tissue (56) with fresh protease inhibitor cocktail (Complete, Roche Applied Science, Indianapolis, Ind.). Eviscerated embryos were used because LacZ staining of embryos (not shown) revealed them to have the highest

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**TABLE 1. Oligonucleotide primer sequences and conditions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide (5')</th>
<th>Oligonucleotide (3')</th>
<th>Annealing temp (°C)</th>
<th>Size (nt)</th>
<th>Amt of template (µg in 50 µl)</th>
<th>5'-to-3' sequence</th>
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</thead>
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<tr>
<td>SnoN, SnoN2</td>
<td>oM094</td>
<td>oM065</td>
<td>62</td>
<td>427,289</td>
<td>0.7</td>
<td>CTGCTGCCTCCAGTCTA</td>
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<td></td>
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<td>gM257</td>
<td>60</td>
<td>413</td>
<td>0.125</td>
<td>TGAACAGTCACATCTG</td>
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<td></td>
<td>oM246</td>
<td>oM257</td>
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<td>700</td>
<td>0.125</td>
<td>AGTGGGACCGCTGAGT</td>
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<tr>
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<td>mO247</td>
<td>mO248</td>
<td>60</td>
<td>357</td>
<td>0.065</td>
<td>CACCTGATGTTGTCGCG</td>
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<tr>
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<td>0.05</td>
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<tr>
<td></td>
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<td>mO278</td>
<td>60</td>
<td>134</td>
<td>0.6</td>
<td>CGTTCTGGCTCTTATGT</td>
</tr>
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</table>

a Sequences originally from Clontech.
expression of Sno protein. Precleared lysates were immunoprecipitated overnight with anti-Sno polyclonal antibody (H317; Santa Cruz Biotechnology, Santa Cruz, Calif.) or preimmune serum, electrophoresed, Western blotted using a mixed anti-Sno monoclonal antibody probe generously provided by Shunsuke Ishii (52) and goat anti-mouse horseradish peroxidase-coupled secondary anti-

body (Sigma, St. Louis, Mo.), and developed by Western Lightning chemiluminescent detection (New England Nuclear, Boston, Mass.).

T-cell proliferation assay for function of unprimed T cells. Spleen cells from littermates of each genotype were seeded into microtirs. The following stimulation conditions were employed: 10 ng of 145-2C11 (eCD3) wells bound in 96-well plates, 50 ng of phorbol myristate acetate (PMA)/ml with 1 µg of ionomycin/ml, and 20 U of recombinant human IL-2/ml. Recombinant human TGF-β1 (catalog no. 240-B; R&D Systems, Minneapolis, Minn.) was used at 100 pM. Anti-TGF-β antibody (MAB1835, clone 1D11 anti-TGF-β1, -β2, -β3; R&D Systems) was used at 2 µg/ml. Cells were incubated for 66 h at 37°C with a stimulator in the medium or plate bound and then for an additional 6 h with [3H]thymidine and were then harvested onto glass fiber filters (Tomtec harvester and Wallac 1450 MicroBeta scintillation counter; Perkin-Elmer, Boston, Mass.) and counted in scintillation fluid to determine the [3H]thymidine incorporation.

Transient transfections and luciferase assays. 10T1/2 cells (3 x 10^6 per 60-mm-diameter plate) were transfected with 1 µg of reporter with or without 2 µg of Sno expression construct in a total DNA mass of 3 µg using lipofectAMINE (Gibco-Invitrogen, Grand Island, N.Y.). Transfections and assays were done in triplicate. Cells were harvested 48 h after transfection in groups of six or fewer to minimize variations in activity (8). The washed cell monolayers were scraped into reporter lysis buffer (Promega, Madison, Wis.) for harvest. The lysates were assayed for protein concentration (6) (Bio-Rad [Hercules, Calif.] protein assay), and 50 µg of protein from each sample was measured into tubes in duplicate for luciferase assays in a Pharmingen luminometer with Promega dual-injection reagents. An internal transfection control, such as pSV βgal, was not included with each experimental sample, since it had been demonstrated that Sno activates the simian virus 40 and several other enhancers and distorts the control (K. Jessen and S. Pearson-White, unpublished data), as does Ski (32). pSV βgal transfection controls were done in parallel and stained for LacZ activity (Roche Applied Science) to compare the transfection efficiencies of MEFs of different genotypes.

Cell proliferation assay. MEFs were isolated by trypsinizing and mechanically dissociating 13.5-dpc embryo carcasses as described previously (2). Multiple separate cell preparations were tested for each genotype. MEFs were plated at equal densities in quadruplicate in 96-well plates at the appropriate concentrations of TGF-β1; cultured for 24 h, with the final 3 h with [3H]thymidine and then harvested onto glass fiber filters and counted as described above to determine the [3H]thymidine incorporation. Anti-TGF-β antibody (MAB1835; R&D Systems) was used at 2 µg/ml.

Statistics. Statistical significance was calculated using the paired one-tailed Student’s t test. The probabilities given are the calculated likelihood that the two sets of values are from the same population. All experiments were performed completely at least three times; representative data from one typical experiment are shown.

RESULTS

Sno mRNA expression in the immune system. Two isoforms of Sno mRNA have been described (46, 47). SnoN is the product of the full-length mRNA. The SnoN2 isoform (also called sno-deE3) is generated by use of an internal alternative splice donor site within exon 3 (46, 47). The SnoN2 transcript is 138 nt shorter than SnoN, predicting the translation of a protein product missing a 46-amino-acid segment (46, 47). Mouse Sno expression contrasts with that of the human gene, which produces SnoN2 as a minor percentage of total Sno mRNA in the tissues examined (46). Mouse SnoN and SnoN2 mRNAs are expressed throughout thymocyte and splenocyte development, appearing first in thymic progenitor cells at 13.5 dpc (Fig. 1A and B), as determined by semiquantitative 32P RT-PCR. The ratio between SnoN and SnoN2 is 1:4 in spleen and in thymus tissue at most ages tested (Fig. 1A and B) but is closer to 1:1 in embryonic 13.5-dpc thymic cells. These expression levels and ratios were confirmed using isoform-specific primers in real-time PCRs (data not shown). It has been shown that the SnoN/SnoN2 ratio is also 1:4 in many nonhematopoietic tissues (e.g., in liver, kidney, and skeletal muscle) but is consistently 1:1 in the brain (46). The functional significance of
the two isoforms is unknown, but tissue-specific differences in their relative expression levels suggest important functional differences between them.

**Sno mRNA is induced upon T-cell receptor stimulation of wild-type splenocytes.** We had previously shown that serum stimulation of fibroblasts upregulates SnoN2 expression by 1 h, resulting in peak expression at 3 h (46). To examine whether Sno was inducible by antigen receptor triggering in T cells, splenocytes from unprimed mice were isolated and cultured for 0, 1, or 3 h with plate-bound antibody specific for the receptor-associated signaling molecule CD3ε, a strong proliferative stimulus for normal T cells. Increased expression of both SnoN and SnoN2 was detectable by 1 h after T-cell receptor stimulation (Fig. 1C). The level of increase in Sno expression was modest but was independently confirmed using isoform-specific primers in real-time PCR experiments (data not shown). SnoN2 mRNA remained the dominant isoform in stimulated T cells, increasing by twofold in splenocytes within 3 h. As expected, autocrine growth factor signaling pathways, represented by IL-2, IL-2 receptor α-chain (IL-2Rα), and IL-4 mRNAs, were induced with slightly delayed kinetics relative to Sno but were also detected by 3 h (Fig. 1C). Ski was also upregulated 2.5-fold upon T-cell receptor stimulation (Fig. 1C).

**Gene targeting produces altered expression of Sno in vivo.** To examine Sno function in vivo, we performed gene targeting in mice. A pGK-neo gene expression cassette (50) was substituted for 1.7 kb of Sno promoter immediately upstream of the NcoI site at the ATG translation start site to a BatEll site 1.7 kb upstream was replaced with the pGKneo cassette. This mutation is called 5Δ due to the removal of sequence 5' of the coding region. The deletion removes a HindIII site, and the resulting larger HindIII fragment (6.5 kb) hybridizes with a probe outside the targeting construct. With the wild-type gene, the probe hybridizes with a 3.5-kb HindIII band. The symbols for the pGKneo cassette, Bgl2 site, and HindII site are defined to the right of the figure. (B) Autoradiogram of Southern blot of 10 μg of DNA from ES cells and two targeted clones digested with HindIII, electrophoresed on a 1.2% agarose gel, alkaline blotted to Hybond N+ (Amersham Biosciences, Piscataway, N.J.), and probed with the radiolabeled 3' probe shown in panel A. Clone 13 appears in duplicate at two concentrations, wt, wild type. (C) The NcoI site at the ATG codon is destroyed, but another is introduced in pGKneo that is closer to the probe, making a 7.4-kb band with the 5' probe; the wild-type band is 8.1 kb. The autoradiogram shows a Southern blot of an NcoI digest from a wild type (+/+) and an Sno5Δ/+ heterozygote probed with the 5' probe shown in panel A. (D) The pGKneo insert introduces a Bgl2 site so that an exon 1 probe hybridizes with a 3.5- instead of a 5.3-kb band when digested with Bgl2. The autoradiogram shows a Southern blot of a Bgl2 digest from a weanling Sno5Δ/+ heterozygote intercross litter probed with exon 1; three mice are homozygous mutant at the Sno locus.
out of 200 clones (ex1) had undergone homologous recombination at one allele of the Sno locus (Fig. 2B and C and 3B) and were successfully passed through the mouse germ line.

When bred from heterozygotes, mice homozygous for either mutant Sno allele are viable at weaning (Fig. 2D and 3C) in the expected 1:2:1 ratio in mice carrying a mixed 129/Sv-C57BL/6 genetic background, as well as in mice backcrossed through several generations onto either 129/Sv or C57BL/6. No gross morphological defects have been noted in homozygotes carrying either mutation, and the mice are not subject to early mortality on a mixed genetic background or after backcrossing. Maintenance of the lines on a 129/Sv background was difficult, probably because of reduced fertility of this strain. However, both mutant alleles were successfully propagated by backcrossing them to C57BL/6. Heterozygote intercrosses were used to generate animals for testing but not to propagate the line. Mice used in these studies resulted from intercrosses using the 11th generation of backcrossing for Sno5α and the 3rd and 4th generations for Snoex1. Two independent lines initially established for the Snoex1 mutant gave identical results in phenotypic screening.

Expression of Sno is highest in embryonic mouse skin; after birth, Sno expression is highest in brain tissue. In the Snoex1−/− tissues, Sno poly(A)+ mRNA was reduced below the limit of detection with a probe specific for the exon 1 sequence that was deleted (Fig. 4A, top, exon 1 probe). Using this exon 1 probe, a minor amount of Sno mRNA with retarded mobility was detectable in the brains of mice homozygous for the Sno5α mutation, which retains this sequence (Fig. 4A, exon 1 probe). Rehybridization with a separate probe for exons 2 to 5 showed that although exon 1 was deleted from Snoex1−/− mice, downstream initiation produced a 6.4-kb 3′-end RNA species in the brain, but there was much less produced in embryos (Fig. 4A, panels with the probe from exons 2 to 5, lanes 1 and 4). This
FIG. 4. Sno mRNA and protein are expressed in wild-type but not mutant tissue. (A) Five micrograms of poly(A)⁺ RNA from brain or eviscerated 13.5-dpc embryo tissue of mice of the indicated genotypes was electrophoresed and subjected to Northern blotting. The blot was hybridized with a probe for exon 1 and then stripped and rehybridized with a probe for exons 2 to 5 and then with a GAPDH probe to show that the lanes were equally loaded with RNA. The probe used for each autoradiogram is shown to the right of each blot. (B) To examine Sno protein expression, lysates from wild-type or mutant eviscerated 13.5-dpc embryo tissue were electrophoresed, blotted, and probed with a probe for exons 2 to 5. Total protein lysates (140 mg of tissue per lane) were immunoprecipitated with anti-Sno polyclonal antiserum (α-Sno) or preimmune serum (pre). The immunoprecipitated products were electroblotted on a 7.5% polyacrylamide Tris-glycine gel, blotted to polyvinylidene difluoride membranes, and probed with a mixture of four anti-Sno monoclonal antibodies (αSno MAb) (52). X-Ray film exposed by the enhanced chemiluminescence signal was laser scanned to make the image. 293T cells were transfected separately with Sno expression vectors, immunoprecipitated with anti-FLAG-agarose beads, and pooled to provide positive controls (tf’d SnoN/N2 at 80 and 76 kDa; tf’d trunc N/N2 at 40 and 34 kDa). Aliquots of each mouse embryo lysate were electrophoresed, blotted, and probed with anti-Smad4 antibody (Santa Cruz) to show that equal amounts were loaded. Molecular mass markers (not shown) were used to measure the approximate molecular masses of the bands.

Despite the presence of RNA derived from downstream coding sequence in Snoex1−/− mice, no mature full-length protein is detectable in these mice on immunoblots (Fig. 4B). To control for the possibility that truncated mutant proteins are produced, we made Sno exon 1 deletion mutant cDNA constructs containing only cDNA from exons 2 to 6 for SnoN and SnoN2 and used them to transfect 293T cells (Fig. 4B, lane tf’d trunc N/N2). Transfected protein lysates were then electrophoresed in parallel with the lysates from embryo fibroblasts. Although a minor band of the appropriate size for truncated SnoN2 (Fig. 4B, tf’d N2Δex1; 34 kDa) was observed in the Snoex1−/−-immunoprecipitated lane, no truncated SnoN band is detected. The truncated SnoN2 band is also present in the wild-type lane at a similar level. It is therefore unlikely to be responsible for the phenotypes observed in Snoex1−/− cells. Since the SnoΔ⁵ deletion leaves exon 1 coding sequence intact, we expected to see Sno protein in embryo lysates from Snoex1−/− mice but at decreased levels based on the small amounts of Sno mRNA detected by Northern blot analysis. As predicted, Sno protein was detected in mice homozygous for SnoΔ⁵ at reduced levels. We measured ~40% as much Sno⁵ protein as seen in wild-type mice, using densitometric measurement of the laser-scanned autoradiogram (Fig. 4B). The polyclonal antibodies used for the immunoprecipitation and the monoclonal antibody mixture used for the blot hybridization are both capable of binding to any remaining carboxy-terminal peptide that the 6.4-kb Snoex1−/− mRNA might encode. Thus, the homozygous Snoex1 mutation appears to eliminate Sno expression, as expected, while the SnoΔ⁵ mutation significantly decreases Sno expression. An anti-Smad4 probe detected comparable levels of Smad4 in mice of each genotype, suggesting no feedback alteration of Smad4 expression in either line (Fig. 4B).

Sno mutations produce no gross phenotypic changes in homozygous mice. At 3 months of age, littermates of all three genotypes (SnoΔ⁵−/−, Snoex1−/−, and their Sno⁺/+ siblings) displayed no differences in the gross morphology of any organ system. Tissue sections from testis, salivary gland, skeletal muscles, heart, lung, and liver, as well as smears taken from bone marrow and blood, were also unremarkable. No significantly increased incidence of somatic tumors has been observed up to 2 years of age. Splenomegaly (enlarged spleen) was noted among a few SnoΔ⁵−/− and SnoΔ⁵−/+ mice >6 months old in a mixed 129/Sv-C57BL/6 genetic background. However, weights and cell counts of spleens harvested from mice at younger ages...
FIG. 5. T-cell proliferation assays examining functions of unprimed T cells show that Sno\textsuperscript{5\Delta/-} and Sno\textsuperscript{ex1/-} mutant T cells have a T-cell activation defect that is largely compensated for by addition of excess IL-2 or incubation with anti-TGF-β antibody. (A) Spleen cells from litters of each genotype were seeded at a density of 500 × 10\textsuperscript{3} responder cells per microwell in 96-well plates. The cells were incubated for 66 h at 37°C and then for a final 6 h with 1 μCi of [\textsuperscript{3}H]thymidine and then harvested onto glass fiber filters to determine the [\textsuperscript{3}H]thymidine incorporation. The numbers presented are kilocounts of [\textsuperscript{3}H]thymidine per minute (background samples without stimulator were subtracted) in the average of triplicate wells from a representative experiment. The error bars indicate the calculated standard deviations. For T-cell receptor stimulation of splenocytes, 10 ng of 145-2C11 αCD3 allogeneic major histocompatibility complex anti-T-cell receptor (T-cell receptor) monoclonal antibody was preincubated in each well as indicated (aCD3). Additional antibody or cytokines were added as indicated (TGFβ, TGF-β). Control wells had no αCD3 stimulator or other additions to the media and had very low proliferation. The genotypes were wild type (Sno\textsuperscript{+/+}), Sno\textsuperscript{5\Delta/-}, and Sno\textsuperscript{ex1-}.

Analysis on days 2 to 5 of culture showed that the peak proliferative response was on day 3 for both wild-type and mutant cultures; day 3 data are shown. Mutant splenocytes also showed similar decreases in proliferative response after stimulation by the bacterial superantigen staphylococcal enterotoxin B and by mitomycin C-treated allogeneic spleen cells (data not shown), demonstrating that the proliferative defect seen in these mice is not restricted by the type of external stimulus applied through the T-cell receptor or its signaling partners.

Despite the defective proliferation seen in response to triggering through the T-cell receptor, T cells from Sno\textsuperscript{ex1/-} mice could proliferate well in culture after stimulation with the combination of PMA and ionomycin. PMA and calcium ionophores together bypass the early steps in T-cell activation that require the T-cell receptor complex by direct activation of downstream intracellular signaling cascades (48). Interestingly, differences were noted in the ability of PMA-ionomycin to induce proliferation of cells carrying the two Sno mutations: Sno\textsuperscript{5\Delta/-} cells proliferated only 75% as well as wild-type cells in response to PMA-ionomycin, whereas Sno\textsuperscript{ex1/-} cells proliferated as well as the wild type under these conditions (Fig. 5B). This may reflect differences in compensation by other genes regulating cell growth that are invoked in Sno-null cells but not in the hypomorph.

Exogenous IL-2 also reverses the Sno mutant defect. T cells require the support of growth factors in order to proliferate after stimulation through the T-cell receptor complex. IL-2 is

(2 to 6 months) or mice with a higher percentage of C57BL/6 background have yielded significant differences in the numbers of splenocytes from wild-type and mutant Sno\textsuperscript{ex1/-} or Sno\textsuperscript{5\Delta/-} spleens. Similarly, age- and sex-matched mutant mice and their sibling controls maintain similar numbers of thymocytes.

T-cell subpopulations from thymus and spleen were analyzed by flow cytometry. There were no significant differences between homozygous mutants of either line and their sibling controls in thymocyte or splenocyte expression of the CD4, CD8, CD25, or CD62L differentiation antigens (data not shown). Sno is not expressed in B cells, and as expected, no differences in the percentages of B220\textsuperscript{+} splenocytes were observed (data not shown). These results have remained consistent through 11 generations of backcrossing to C57BL/6 for the Sno\textsuperscript{5\Delta/-} mutation.

Sno\textsuperscript{5\Delta/-} and Sno\textsuperscript{ex1/-} splenocytes exhibit reduced T-cell activation in response to T-cell receptor stimulation. The T-cell receptor complex consists of an antigen-specific heterodimer, structurally related to immunoglobulin molecules, and an associated multimeric signaling complex designated CD3. Plate-bound αCD3 antibody is a potent T-cell receptor stimulatory signal. Sno\textsuperscript{5\Delta/-} and Sno\textsuperscript{ex1/-} splenocytes have a T-cell proliferation response to αCD3 stimulation that is 15 to 60% of wild type (Fig. 5A; 26 and 36% in the example shown). Heterozygote values were somewhat variable, ranging from fully wild type to intermediate between wild type and homozygous mutant in many experimental replicates (data not shown).
the major proproliferative cytokine produced to support clonal expansion of antigen-activated T cells. It is primarily, if not exclusively, produced by T cells in vivo and functions in an autocrine, as well as a paracrine, fashion. Addition of IL-2 to cultures of Sno\textsuperscript{ext-/-} cells effectively reversed the proliferation defect in response to αCD3 stimulation (Fig. 5A). Although IL-2 completely restored the deficit in proliferative response in T cells from Sno\textsuperscript{ext-/-} mice, it only partially restored proliferation of Sno\textsuperscript{5a/-} cells (66% of wild type). This difference was observed consistently in all the repetitions of this experiment and mirrors the incomplete recovery of proliferative capacity seen with PMA-ionomycin stimulation.

The Sno mutant defect may be due to reduced antagonism of TGF-β signaling. TGF-β is known to inhibit T-cell activation through suppression of IL-2 production (5, 22, 31). Since Sno has been shown to suppress the response to signaling through TGF-β receptors in other cell types (55, 56, 58), we tested whether the Sno mutant defect in T-cell proliferation could be reversed by addition of anspecific anti-TGF-β antibody. Blocking endogenous TGF-β signaling with anti-TGF-β antibody completely rescued the proliferation of the Sno\textsuperscript{ext-/-} mutant T cells in response to αCD3 stimulation. Although rescue of Sno\textsuperscript{5a/-} T cells by anti-TGF-β antibody consistently appeared to be incomplete (averaging 75% of Sno\textsuperscript{ext-/-} recovery), it was not statistically significantly different from the wild type (P < 0.092) (Fig. 5A). Both wild-type and mutant cells were sensitive to the addition of 100 pM exogenous recombinant TGF-β, showing severely reduced levels of proliferation under these conditions (Fig. 5A). The addition of TGF-β reduced cell proliferation of Sno mutant cells to the level of unstimulated control cultures, whereas wild-type cells were not as completely inhibited from proliferating (Fig. 5A), consistent with increased TGF-β sensitivity in mutant cells. High doses of exogenous IL-2 could not completely reverse the suppressive effect of added TGF-β in either wild-type or mutant cells (Fig. 5A), suggesting that inhibition of IL-2 production alone does not completely account for the inhibitory capacity of TGF-β at this concentration.

IL-2 and IL-4 mRNAs are reduced in Sno-null splenocytes and are induced to lower levels after T-cell receptor stimulation. IL-2 and IL-4 mRNA levels were found to be lower in unstimulated Sno-null than in wild type splenic T-cell populations (Fig. 6A), suggesting that steady-state levels of T-cell activation in vivo are decreased in mutant mice. Although IL-2 mRNA levels were higher 1 h after αCD3 stimulation in mutant cells than in wild-type controls, by 3 h (and at all subsequent time points) mutant cells expressed significantly less IL-2 mRNA than control cells (Fig. 6B). Uprogulation of a second cytokine, IL-4, after CD3 ligation was less in Sno\textsuperscript{ext-/-} splenic T cells than in the wild type at all time points tested (Fig. 6A and B).

Sno trans-activates the IL-2 promoter in non-T cells. Since Sno deletion reduces the amount of IL-2 mRNA produced in response to αCD3 stimulation, we tested whether Sno could regulate IL-2 gene expression. 10T1/2 fibroblasts were transiently cotransfected with an IL-2 promoter element (632 bp [28, 54]) driving a luciferase reporter (IL-2-lux) and expression constructs driving FLAG-tagged SnoN or SnoN2. Control transfections were performed with IL-2-lux alone. IL-2-lux activity, in the absence of Sno constructs, was very low, as expected. Cotransfection with either SnoN or SnoN2 increased luciferase activity >60-fold (Fig. 6C). Anti-FLAG immunoprecipitates were used to confirm expression of the transfected Sno constructs (Fig. 6D). 32P RT-PCR demonstrated low-level expression of endogenous SnoN and SnoN2 in untransfected 10T1/2 cells, while the levels were >50-fold higher in transfected cells (Fig. 6D). No additional T-cell-specific factors were cotransfected, suggesting that Sno expression is sufficient to induce activation of IL-2 expression even in a non-T cell.

Sno-deficient embryo-derived fibroblasts also exhibit reduced antagonism of TGF-β signaling. MEFs derived from embryonic skin express Sno at high levels. Sno\textsuperscript{ext-/-} MEFS grow more slowly than wild-type MEFS in the absence of added TGF-β (P < 0.002) (Fig. 7). Furthermore, Sno\textsuperscript{5a/-} and Sno\textsuperscript{ext-/-} cells show reduced proliferation rates at lower TGF-β concentrations than wild-type cells (0.5 and 1.0 pM TGF-β) (Fig. 7B). Both results indicate increased sensitivity to TGF-β. Sno\textsuperscript{5a/-} proliferation rates were reproducibly intermediate between the wild type and Sno\textsuperscript{ext-/-} at 0.5 pM TGF-β and below, possibly indicating a protein dosage effect (Fig. 7). Culture in the presence of anti-TGF-β boosted the levels of proliferation of both mutant and wild-type MEFS almost twofold over untreated cells (Fig. 7A). The observation that neither Sno\textsuperscript{5a/-} nor Sno\textsuperscript{ext-/-} proliferation was equal to that of the wild type in the presence of anti-TGF-β suggests that additional factors may be suppressing growth in Sno mutant MEFS independently of TGF-β signaling pathways.

Both Sno\textsuperscript{5a} and Sno\textsuperscript{ext} mutations modulate the response of the TGF-β-responsive reporters 3TP-lux and A3-lux. To confirm that Sno levels modulate TGF-β sensitivity, we tested the activity of a TGF-β-responsive promoter in MEFS with the three different genotypes. 3TP-lux is a composite reporter used by other investigators to measure cellular TGF-β responses (70); it contains an element from the human PAI-1 promoter (30) together with a 3× multimerized tetradecanoyl phorbol acetate response element from the human collagenase gene (15) coupled with a luciferase coding sequence. 3TP-lux expression is increased relative to the wild type in Sno\textsuperscript{5a/-} and Sno\textsuperscript{ext-/-} cells in the absence of added TGF-β (P < 0.001) (Fig. 8A, control). Exogenous TGF-β increases 3TP-lux expression in cells of all three genotypes, demonstrating that TGF-β signaling is intact in each case (Fig. 8A). Compilation of data from five separate experiments showed a small but statistically significantly higher activation of 3TP-lux by TGF-β in Sno\textsuperscript{ext-/-} cells than in wild-type cells (Fig. 8B). Luciferase activity in Sno\textsuperscript{5a/-} cells was indistinguishable from that in the wild type in this compilation and is not shown. Cotransfection of the reporter construct with SnoN reduced the level of 3TP-lux expression to below pretransfection levels (Fig. 8A), although the luciferase activity in Sno\textsuperscript{ext-/-} cells remained higher than in wild-type cells. Transfection of FLAG-tagged SnoN reversed the TGF-β-induced increase in activity of the reporter construct (Fig. 8A). In order to confirm that the transfection efficiencies were comparable for all genotypes and preparations of MEFS, we transfected a pSVβgal construct in parallel and stained for beta-galactosidase activity (Fig. 8C). Sno\textsuperscript{ext-/-} cells express LacZ in their nuclei because of the knock-in design. However, cyttoplasmic staining is seen only in cells exposed to the pSVβgal construct during the transfection procedure and appears with comparable frequencies for all
three cell types (Fig. 8C). Similar results were obtained with the A3-lux reporter construct, in which the activin response elements are activated by Smad2/Smad4/FAST-1 or FAST-2 (for forkhead activin signal transducer) (73) (Fig. 8D). Each of these transfections included a FAST-2 expression plasmid. Snoex1/H11002/H11002 cells once again exhibited significantly increased luciferase activity in the presence of TGF-β/H9252. In this instance, neither the control levels without added TGF-β nor the 8- to 10-fold increase after addition of TGF-β was significantly different from the wild type, suggesting that although Sno can repress TGF-β signaling through Smad2/Smad4, it also works through Smad3.

The TGF-β response of an endogenous gene, JunB, confirms differential effects of the Sno5 and Snoext1 mutations on TGF-β signaling. We used quantitative real-time RT-PCR to examine expression of JunB, a major target for induction by TGF-β (35, 38), in MEFs. The elevation of JunB levels in Snoext1/−/− MEFs was significantly (P < 0.019) above that seen in wild-type MEFs 2 h after TGF-β administration (Fig. 9A). L7 ribosomal protein mRNA levels were similar in all samples, showing that equivalent amounts of template were used in all examples (Fig. 9B). In some repetitions of this experiment, JunB was elevated in Snoext1/−/− cells in the absence of added TGF-β, with an additional increase as shown in the presence of TGF-β, but we consistently observed no effect in Sno5/H9004/H11002/H11002 mutant cells. This suggests that JunB expression in MEFs is relatively insensitive to TGF-β signaling, since it appears that the low residual Sno protein levels in Sno5/H9004/H11002/H11002 mutant cells may provide enough TGF-β opposition to keep JunB at “wild-type” levels. The observation that a TGF-β-responsive endogenous gene is expressed at elevated levels in response to TGF-β in Snoext1/−/− cells provides additional evidence that Sno is a significant modulator of TGF-β signals.

DISCUSSION

In this report, we show that Sno plays an important role in opposing TGF-β signaling in both lymphocytes and fibroblasts.
Sno mRNA is expressed in the thymus as early as embryonic day 13.5 postcoitum, and Sno expression continues through adulthood. Sno mRNA is also expressed in the spleen at all developmental stages from fetal to adult, with upregulation in primary mouse splenocytes within 1 h of T-cell receptor stimulation in vitro. We examined the role of Sno in T-lymphocyte function using two targeted deletions in mice: a hypomorphic mutation with a 1.7-kb deletion upstream of the ATG codon (Sno<sup>5Δ</sup>) and a null mutation with deletion of exon 1 (Sno<sup>ex1</sup>) that removes the coding sequence for the entire domain constitutively to prevent tonic TGF-β signaling in T cells and the demonstration that Sno functions constitutively to prevent tonic TGF-β-mediated suppression of T-cell activation during the early steps in an antigen-specific immune response. We saw no evidence that Sno activity plays a role in opposing the postulated role for TGF-β in maintaining homeostatic control of the T-cell population size in vivo (22), since splenic-T-cell numbers from mutant mice were not different from those of wild-type controls on an inbred (C57BL/6) genetic background.

Analysis of any mutant allele must exclude the possibility that the mutant phenotype is due to the influence of the deletion on a gene closely linked to the target primary, as was noted for Arf/Ink4a (51), or to an unintended second mutation site. The similarity of the phenotypes in T cells for our two independent mutations is good evidence not only that it is a target-specific effect but also that it does not result from a second mutation at an unknown site.

Mice with another null mutation in the Sno gene were reported to die in early embryogenesis (52), whereas our null mice are viable. It is puzzling that our two mutations differ so dramatically from a third mutation at the same locus (52). The results with our mutants are strongly substantiated; two out of two ES clones identified for Sno<sup>ex1</sup> mice and the single line of Sno<sup>5Δ</sup> mice have similar phenotypes. We have also confirmed possible second-site mutations, unless they are tightly linked to Sno, through our backcrossing regimen. The report of an earlier attempt to create Sno-null mice described intercrossing two different ES clone-derived germ line Sno lines to exclude the possibility of second-site mutations (52). However, this does not exclude the possibility that a lethal second-site

FIG. 7. (A and B) Wild-type and mutant MEFs show different DNA synthetic rates (A) and Sno mutant cells are more sensitive to TGF-β (B). MEFs were isolated from litters of embryos derived from intercrossed mice that were either both wild type or both homozygous mutant. The genotype of each MEF preparation was verified by PCR; multiple preparations gave the same results in these experiments. Equal numbers of cells were plated in quadruplicate sets of microwells and untreated or treated with increasing concentrations of TGF-β or panspecific anti-TGF-β antibody at 2 μg/ml for 24 h. The cells were metabolically labeled for the final 3 h with 1 μCi of [3H]thymidine per well and harvested onto glass fiber filters to determine the [3H]thymidine incorporation. The asterisks above the control bars (panel A, control; panel B, 0.0 PM) indicate results that were statistically significantly (P < 0.05) different from the wild-type control. In panel A, the asterisks above the other bars indicate results that were statistically significantly (P < 0.05) different from the corresponding untreated control cells. In panel B, the asterisks at 100 PM TGF-β indicate significant (P < 0.02) difference from the wild type; the other asterisks indicate significant difference (P < 0.009) from the wild type. Incorporation into mutant cells was statistically significantly different from the wild type in the presence of anti-TGF-β antibody (Sno<sup>5Δ</sup>/+, P < 0.013; Sno<sup>ex1</sup>/+, P < 0.001), whereas incorporation in mutants and the wild type was not significantly different in the presence of 5 PM TGF-β. The genotypes were wild type (Sno<sup>+/+</sup>), Sno<sup>5Δ</sup>−/−, and Sno<sup>ex1</sup>−/−. Two independent experiments are shown with different absolute [3H]thymidine incorporation levels in the controls.
FIG. 8. MEFs from Sno\(^{+/-}\) embryos show increased activity of the 3TP-lux and A3-lux TGF-β-responsive promoters, either with or without TGF-β supplementation of the cultures. (A) The activity of a TGF-β-responsive promoter element, 3TP-lux, was tested in transfected MEFs. The genotypes were wild type (Sno\(^+/+\)), Sno\(^{5A+/+}\), and Sno\(^{ex1/}\). “Control” indicates the level of luciferase activity of transfected 3TP-lux reporter alone. +TGF-b, TGF-β (100 pM) was added; +Sno, pCMV-SnoN expression construct was cotransfected. Sixty-millimeter-diameter dishes were transfected in triplicate, and the relative light units (RLU) emitted by the luciferase reporter were measured in duplicate in a luminometer. The error bars indicate the calculated standard deviations from each group of six values measured. The asterisks above the control bars indicate results that were statistically significantly \((P < 0.05)\) different from the wild type. The asterisks above the other bars indicate results that were statistically significantly \((P < 0.05)\) different from the corresponding untreated control cells. The results for the mutants were significantly different from the corresponding wild-type results under each condition (Sno\(^{+/-}\), P < 0.0007; Sno\(^{5A+/+}\), P < 0.034). (B) The increase with added TGF-β was plotted for Sno\(^{-/}\) and Sno\(^{ex1/}\) cells from combined data from five experiments. Sno\(^{5A+/-}\) cells had the same 2.7-fold increase as Sno\(^{+/+}\) cells and were not plotted. The asterisk indicates that the 3.2-fold increase in luciferase in the presence of TGF-β was statistically significantly \((P = 0.016)\) higher in Sno\(^{ex1/}\) cells than the 2.7-fold increase in the wild type. (C) The activity of a different TGF-β-responsive promoter element, A3-lux, was tested in wild-type and Sno\(^{ex1/}\) MEFs cotransfected with a FAST-2 expression vector. The asterisk indicates that the activity in the presence of TGF-β was statistically significantly higher in Sno\(^{ex1/}\) than in Sno\(^{+/+}\) MEFs \((P < 0.005)\). (D) To confirm that MEFs of the three genotypes were transfected with similar efficiencies, a pSV\(\beta\)gal construct was transfected in parallel in the same experiment, and the dishes were stained and photographed. The transfection efficiencies were similar among the three genotypes. The Sno\(^{ex1/}\) cells expressed \(lacZ\) from the knock-in construct, seen in the nuclear staining in the figure. The transfected pSV\(\beta\)gal gave cytoplasmic staining and was thus distinguishable from the nuclear-staining Sno\(^{ex1/}\) background.

FIG. 9. Sno\(^{ex1/-}\) MEFs show enhanced activation of endogenous JunB in response to TGF-β. (A) Real-time PCR measured levels of JunB endogenous mRNAs in wild-type and Sno mutant MEFs with (+TGFb) or without (−TGFb) incubation with 100 pM TGF-β for 2 h. The quantified levels calculated for each sample are presented in the histograms. Each sample was measured in quadruplicate and standardized against a dilution curve generated in the same experiment, using the same JunB primers and twofold serial dilutions of template (not shown). The genotypes are indicated below panel B. The asterisks indicate results that were statistically significantly \((P < 0.015)\) different from the corresponding untreated control cells. The difference in the presence of added TGF-β between Sno\(^{ex1/-}\) and the wild type was significant \((P < 0.018)\). (B) \(L7\) ribosomal protein loading control real-time PCR results are presented as a histogram, showing that the samples contained comparable levels of RT-RNA; the profiles were not normalized. The error bars indicate the calculated standard deviations.
but it is expressed at low levels in wild-type as well as mutant cells (Fig. 4B). This carboxyl-terminal fragment of Sno carries the lysine residues that are responsible for ubiquitination and degradation of Sno (55) and thus might turn over rapidly. This domain, including only the carboxyl-terminal half of Sno, would lack SKIP binding (13); N-CoR binding (44); TAF$_2$110 binding (11); Smad 2, 3, or 4 binding (56, 58); transcriptional activation or repression (11); oncogenic transformation; and muscle differentiation-promoting activities (12). However, such a 34-kDa carboxyl-terminal Sno species would be predicted to retain the ability to heterodimerize with Ski (12, 25, 41) and thus could have dominant-negative activity. If truncated Sno protein in these cells has dominant-negative activity, we would expect even heterozygous cells to have phenotypes approaching that of the homozygous mutant. However, we have not observed heterozygote phenotypes that are comparable to homozygous mutant phenotypes, strongly suggesting the absence of such dominant-negative activity. Also, when the Sno$^{5-}$ mutation is combined with the Ski-null mutation, double-homozygous mutant mice die early in embryogenesis, a full 12 days earlier than Ski$^{-/-}$ mice in the absence of the Sno$^{5-}$ mutation (3; S. Pearson-White and C. Colmenares, unpublished data). This shows that Sno and Ski compensate for some of each other’s functions. The Sno$^{5-}$ mutation alone confers a mild phenotype, but this is altered dramatically when Ski is deleted, showing that there is no dominant-negative effect of the Sno$^{5-}$ mutation or, by analogy, of the Sno$^{ext}$ mutation with a similar mild phenotype. The Sno-null mouse described elsewhere also had only exon 1 deleted, and experiments that would have detected a downstream mRNA species or truncated protein were not reported (52). Because of its lethality in the homozygous state, all of the experiments with this mutation were obligatorily performed in heterozygous mice (52). One possible explanation for the effects reported with this mutation might be a dominant-negative effect on Ski function.

Sno is one of many proteins with a negative regulatory influence on TGF-β signaling and is likely, therefore, to have a modulatory rather than a binary controlling influence. Evi-1 (34), oncogenic Ras (33), Ski (1, 36, 57, 71), c-Jun (16), lefty (4), TGFIF1 (68, 69), and TGFIF2 (39) also participate in the inhibition of TGF-β signaling. In the face of so many alternative modulators of TGF-β, it is significant that phenotypic changes in TGF-β effects in lymphocytes are measurable in the absence of Sno. This suggests that Sno is a very important modulator of TGF-β signaling in certain cell types and that Sno may be the critical factor regulating TGF-β activity in T cells.

In primary T cells, TGF-β does more than just inhibit IL-2 production to suppress T-cell activation, since the addition of IL-2 does not rescue T-cell proliferation in the presence of high doses of TGF-β (Fig. 5A). TGF-β also enhances IL-2R expression by IL-2 (19) and plays a significant role in T-cell development and terminal differentiation (22, 31, 66). Sno deletion appears not to affect T-cell-developmental aspects of TGF-β signaling, since immunophenotyping revealed no differences among T-cell subpopulations in Sno mutant mice (data not shown). Furthermore, it appears to play no role in homeostatic regulation of the peripheral T-cell pool.

Sno$^{5-/-}$ and Sno$^{ext/-/-}$ mice show some differences in phenotype. It is only in MEFs, in the regulation of the JunB response to TGF-β, that the two mutants show completely distinct responses: Sno$^{ext/-/-}$ cells hyperactivate JunB in response to TGF-β, whereas Sno$^{5-/-}$ cells do not (Fig. 9). Subtle differences were also seen in other assay systems. Both the activation of the TGF-β-responsive promoter 3TP-lux and the $[^3H]$thymidine incorporation rate are intermediate in Sno$^{5-/-}$ cells between wild-type and Sno$^{ext/-/-}$ cells (Fig. 7 and 8), and reversal of proliferative defects in splenocytes by both IL-2 and anti-TGF-β antibodies was less complete in Sno$^{5-/-}$ than in Sno$^{ext/-/-}$ cells. In these cases, the Sno$^{5-}$ mutation appears to be more resistant to complete reversal than Sno$^{ext}$. While these differences may result from a subtle dose effect for Sno, it is also possible that compensatory developmental changes in interacting pathways not seen in the Sno$^{5-/-}$ hypomorph alter expression in the Sno$^{ext/-/-}$ null mutant.

There is also evidence in data from both mutants that Sno regulates functions other than those induced by TGF-β. Anti-TGF-β antibody was not able to completely rescue proliferation of Sno$^{5-/-}$ or Sno$^{ext/-/-}$ fibroblasts to the level attained by the wild type with added anti-TGF-β antibody (Fig. 7A). It is not known which additional pathway requires Sno function. Sno and Ski are linked to several other signaling pathways through the Smad proteins, which are also targets of the Ras/MAPK, Wnt/β-catenin, and nuclear hormone signaling pathways (27, 37). Ski and Sno can also interact with Gli3, which responds to Sonic hedgehog signaling (14). Our results show that Sno is important in the negative regulation of TGF-β signals in vivo in T-cell activation but also suggest that Sno may modulate signaling through another pathway still to be identified.

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