The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action *in vivo*

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Suppressor of Cytokine Signaling-1 (SOCS-1) is an essential physiological inhibitor of IFN-γ signaling. Mice lacking this gene die in the early postnatal period from a disease characterized by hyperresponsiveness to endogenous IFN-γ. The SOCS box is a C-terminal domain shared with over 30 other proteins that links SOCS proteins to an E3 ubiquitin ligase activity and the proteasome, but whether it contributes to inhibition of cytokine signaling is currently disputed. We have deleted only the SOCS box of the SOCS-1 gene in mice and show that such mice have an increased responsiveness to IFN-γ and slowly develop a fatal inflammatory disease. These results demonstrate that deletion of the SOCS box leads to a partial loss of function of SOCS-1.

Suppressor of Cytokine Signaling-1 (SOCS-1) was discovered independently by three groups and shown to be an important negative-feedback inhibitor of cytokine-activated Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways (1–3). SOCS-1 contains an SH2 domain that recognizes activated JAKs and a kinase-inhibitory region that is proposed to inhibit JAK Tyr kinase activity (4–6). Mice lacking the SOCS-1 gene die with fatty degeneration and necrosis of the liver before weaning and a T-cell-dependent multiorgan inflammatory disease. These mice display hypersensitivity to IFN-γ, and mice lacking both SOCS-1 and IFN-γ fail to develop the postnatal disease seen in SOCS-$1^{-/-}$ mice (7–9).

SOCS-1 also contains a C-terminal domain with homology to the previously defined cytokine-inducible SH2-containing protein (CIS). We termed this homology domain the SOCS box (1). Subsequently, we and others have defined a large family of proteins that contain a C-terminal SOCS box and either an SH2 domain (CIS and SOCS-1 to SOCS-7), ankyrin repeats (ASB-1 to ASB-18), WD40 repeats (WSB-1 and -2 as well as Tubby-like protein), a SPRY domain (SSB-1 to SSB-4), a small GTPase (RAB) domain (4 RAR-like proteins), or a neuralized domain (1 protein) (10–13). The SOCS box from several family members was shown to act as an independent binding domain for the elongin B/C complex (14, 15) that, in turn, is part of an E3 ubiquitin ligase complex (16). The same complex also has been shown to bind to the Von Hippel–Lindau tumor-suppressor protein (that also contains a SOCS box-like sequence) and to induce proteasomal degradation of bound hypoxia-inducible transcription factors (17). Recent *in vitro* studies have demonstrated SOCS-1-dependent ubiquitination and proteasomal degradation of vav and the TEL/JAK2 fusion protein; in the latter case, at least, this ubiquitination depends on an intact SOCS box (18–20). Despite this observation, functional studies of SOCS proteins have generally failed to demonstrate a requirement of the SOCS box for inhibition of cytokine signaling *in vitro* (4–6), and two studies have suggested that the SOCS box instead stabilizes SOCS proteins by inhibiting their proteasomal degradation (5, 14). We have addressed the physiological role of the SOCS box by deleting only this element from the SOCS-1 gene in mice and comparing their phenotype to that of mice lacking the entire SOCS-1 protein.

Materials and Methods

Generation of SOCS-1 Δ/Δ Mice. The murine SOCS-1 gene is shown in Fig. 2. A nested PCR protocol was used to amplify the 2.9-kb *Bam*HI exon-bearing fragment from the SOCS-1 gene that lacked the sequence encoding the C-terminal 41 aa before the translation-termination codon. A 3.2-kb *Bam*HI–*Eco*RV fragment situated immediately 3′ also was isolated, and these two fragments were inserted upstream and downstream of a loxP-flanked PGKneo cassette to produce the targeting vector. This vector was linearized and electroporated into C57BL/6 embryonic stem (ES) cells, and after selection in 175 μg/ml G418, resistant clones were picked and screened. *Hin*dIII-digested genomic DNA isolated from individual clones was hybridized with a 0.7-kb *Bam*HI–*Nhe*I fragment located upstream of sequences in the targeting vector to identify clones in which homologous recombination had occurred. This strategy provided distinction between the endogenous (≈20-kb) and targeted (4.5-kb) SOCS-1 alleles. An ES-cell clone, in which an endogenous SOCS-1 allele had undergone homologous recombination as confirmed by *H*indIII restriction endonuclease analysis and DNA sequencing, was expanded and injected into BALB/c blastocysts to generate chimeric mice. Male chimeras were mated with C57BL/6 females to yield heterozygotes for the targeted SOCS-1 allele, which were interbred to produce wild-type (+/+), heterozygous (Δ/+) and homozygous SOCS box-deleted mutant (Δ/Δ) mice on a pure C57BL/6 genetic background. SOCS-$1^{+/+}$IFN-$\gamma^{-/-}$, SOCS-$1^{\Delta/\Delta}$IFN-$\gamma^{-/-}$, and SOCS-$1^{+/\Delta}$IFN-$\gamma^{-/-}$ mice were generated by crossing SOCS-$1^{\Delta/+}$ mice with C57BL/6 IFN-$\gamma^{-/-}$ mice and then intercrossing SOCS-$1^{+/\Delta}$IFN-$\gamma^{+/-}$ offspring.

Immunoprecipitation and Western Blotting. For analysis of SOCS-1 proteins in mouse organs, tissue extracts were prepared from whole thymus, 80 mg of spleen, 200 mg of liver, and 130 mg of lung tissue 4 h after injection of mice with 5 μg of IFN-γ. SOCS-1 and SOCS-1/ΔSB proteins from each tissue extract were immunoprecipitated with 3 μg of anti-SOCS-1 monoclonal antibody and Western blotted with 1 μg/ml of an independent biotinylated anti-SOCS-1 antibody (4H1).

For signal-transduction studies, adult mice weighing an average of 29 g were given a single i.p. injection of either 0.2 ml of saline or 2 μg of rmIFN-γ (PeproTech, Rocky Hill, NJ) in 0.2 ml of saline. Livers were dissected at the indicated times after injection, immediately frozen on dry ice, and stored at −70°C. Frozen livers were homogenized in RIPA buffer [1% (vol/vol) Triton X-100/1% (wt/vol) sodium deoxycholate/0.1% (wt/vol) SDS/150 mM NaCl/10 mM Tris, pH 7.5] containing 10 μg/ml

Abbreviations: SOCS-1, suppressor of cytokine signaling-1; JAK, Janus kinase; STAT, signal transducer and activator of transcription; CIS, cytokine-inducible SH2-containing protein.

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leupeptin (Auspep, Parkville, Australia), 1 mM iodoacetic acid, 50 μg/ml soybean trypsin inhibitor, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EGTA (Sigma), and 1 mM pervanadate. Pervanadate was prepared by adding hydrogen peroxide (20 mM final) to 1 mM sodium orthovanadate (Sigma) and by incubating at room temperature for 30 min. Lysates were cleared by centrifugation, and protein content was quantitated by using a Coomassie Protein Assay Reagent (Pierce). For immunoprecipitations, liver lysates were incubated with 4 μg of anti-STAT1 antibody (Transduction Laboratories, Lexington, KY) for 30 min on ice and then with 30 μl of 50% protein G Sepharose slurry for 2 h or overnight. After repeated washing in RIPA buffer, bound proteins were eluted from the protein G Sepharose in 40 μl of 2× SDS loading buffer [125 mM Tris·HCl, pH 6.8/2% (wt/vol) SDS/20% (vol/vol) glycerol/100 mM DTT/0.01% bromophenol blue] at 95°C for 2 min, centrifuged at 14,800 × *g* for 5 min, and the supernatant then was loaded on Bio-Rad Criterion SDS/7.5% PAGE gels. Proteins were fractionated by SDS/PAGE under reducing conditions and then electrophoretically transferred to PVDF-Plus membranes (Micron Separations, Westborough, MA). Membranes were blocked with 2% (vol/vol) BSA, 0.1% Tween 20 in phosphate-buffered saline for a minimum of 1 h, and then incubated overnight with anti-phospho-STAT1 antibody (1:1,000, New England Biolabs). Antibody binding was visualized with horseradish peroxidase-conjugated anti-rabbit antibody (Silenus, Paris) and the SuperSignal West Pico chemiluminescent substrate (Pierce).

Hepatocyte Cultures. Hepatocytes were harvested from 10-week-old mice as described (21). Briefly, livers were perfused retrogradely through the inferior vena cava with Hanks' balanced salt solution (HBSS) without calcium and magnesium and then with the same medium containing 0.5 mM EDTA. EDTA then was removed by flushing the liver with HBSS without calcium and magnesium. Hepatocytes were released by perfusing with HBSS without magnesium but with 5 mM $CaCl_2$ and 0.05% collagenase IV (Sigma). Viable hepatocytes were purified by using a Percoll density gradient (Amersham Pharmacia), and the resulting cell pellet was washed three times by 50 × *g* centrifugation in RPMI 1640 medium containing 10% (vol/vol) FCS and 50 μM 2-mercaptoethanol (RF10 medium). Cells were plated in 175-cm^2 culture flasks (Corning) in the same medium at a density of 5×10^6 cells per flask and were allowed to adhere for 2 h at 37°C.

Results and Discussion

In previous studies, we demonstrated that deletion of the SOCS box abolished the capacity of SOCS-1 to interact with elongins B and C (15). To investigate the capacity of the SOCS box-deleted form of SOCS-1 (SOCS-1/ΔSB) to interact with JAK kinases, we stably expressed FLAG epitope-tagged SOCS-1 or SOCS-1ΔSB in M1 leukemic cells. The SOCS-1 proteins were purified from cellular extracts by using anti-FLAG antibody chromatography and were analyzed by Western blotting with anti-JAK1 and anti-FLAG antibodies. These studies revealed copurification of JAK1 with both SOCS-1 and SOCS-1/ΔSB (Fig. 1), indicating that the deletion of the SOCS box did not disrupt the capacity of SOCS-1 to bind JAK1.

Mice that contained a homozygous deletion of the SOCS box in the SOCS-1 gene (Δ/Δ mice expressing SOCS-1/ΔSB protein) were generated as indicated in Fig. 2. The induction and expression levels of SOCS-1 or SOCS-1/ΔSB proteins were assessed in the thymus, spleen, liver, and lung of wild-type, Δ/+, and Δ/Δ mice 4 h after injection of IFN-γ (Fig. 2*c*). The data showed that Δ/Δ mice produced a truncated SOCS-1 protein of the expected molecular weight for a molecule lacking the SOCS box. The levels of SOCS-1/ΔSB protein in different tissues from Δ/Δ mice were similar to those of SOCS-1 protein in wild-type

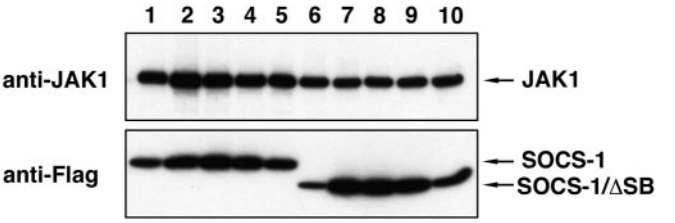

Fig. 1. Interaction of SOCS-1ΔSB with JAK1. Cellular extracts from M1 cells stably expressing either N-terminally Flag-tagged SOCS-1 or SOCS-1 lacking the SOCS box (SOCS-1/ΔSB) were incubated with anti-Flag antibody M2 resin and bound proteins eluted with Flag peptide. (Lanes 1–5) Column eluates from M1 cells expressing full-length SOCS-1. (Lanes 6–10) Column eluates from M1 cells expressing SOCS-1/ΔSB.

mice. Curiously, in Δ/+ heterozygotes only, SOCS-1/ΔSB protein was present at significantly lower levels than the wild-type protein (Fig. 2*c*).

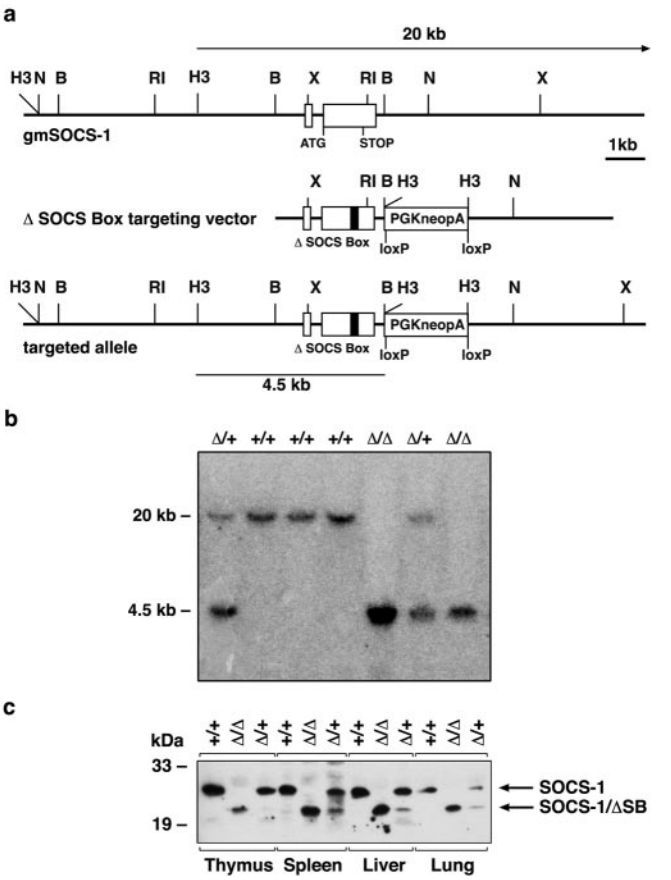

Fig. 2. Generation of Δ/Δ mice through specific deletion of the SOCS box from the SOCS-1 gene by homologous recombination in embryonic stem cells. (*a*) The murine SOCS-1 gene is shown (B, *Bam*HI; N, *Nde*I; X, *Xba*I; RI, *Eco*RI; H3, *Hin*dIII) with the exons represented by open boxes and the deleted SOCS box shown by shaded boxes. (*b*) Southern blot of *Hin*dIII-digested genomic DNA extracted from the tails of mice derived from a cross between heterozygous (Δ/+) mice hybridized with a probe located immediately 5′ to the sequence included in the targeting vector. This distinguishes the 20-kb wild-type genomic fragment from a 4.5-kb targeted allele fragment. (*c*) Western blot showing expression of a smaller SOCS-1 protein consistent with the absence of the SOCS box in various tissues of Δ/Δ mice 4 h after injection with 5 μg of IFN-γ. Extracts were prepared from thymus, spleen, liver, and lung tissue; SOCS-1 and SOCS-1/ΔSB proteins were immunoprecipitated with anti-SOCS-1 monoclonal antibody and then Western blotted with a second biotinylated anti-SOCS-1 antibody. +/+, wild-type mice.

Table 1. Pathology in moribund Δ/Δ mice

Pathology*	Frequency	%
Skeletal muscle–infiltration	18/20	90
Heart–infiltration	18/20	90
Lung–lymphoid foci	12/20	60
–alveolar infiltration	13/20	65
–pneumonia	5/20	25
Pancreas–dispersion and infiltration	11/20	55
Salivary gland–lymphoid foci	11/18	61
Cornea–infiltration	10/19	53
–ulcer	3/19	16
Skin–infiltration of dermis	10/20	50
Marrow–granulocytes > mononuclear cells	10/20	50
Thymus–atrophy or cortical thinning	19/20	95
Liver–trabecular atrophy	8/20	40
–hematopoietic foci	7/20	35
Spleen–no germinal centers	11/20	55
–excess erythropoiesis	11/20	55
Gut–infiltration	3/20	15
Kidney–lymphoid foci	3/20	15
–polycystic disease	1/20	5
–necrosis	1/20	5

*Assessed by histological examination of organs from moribund mice of 40–90 days of age.

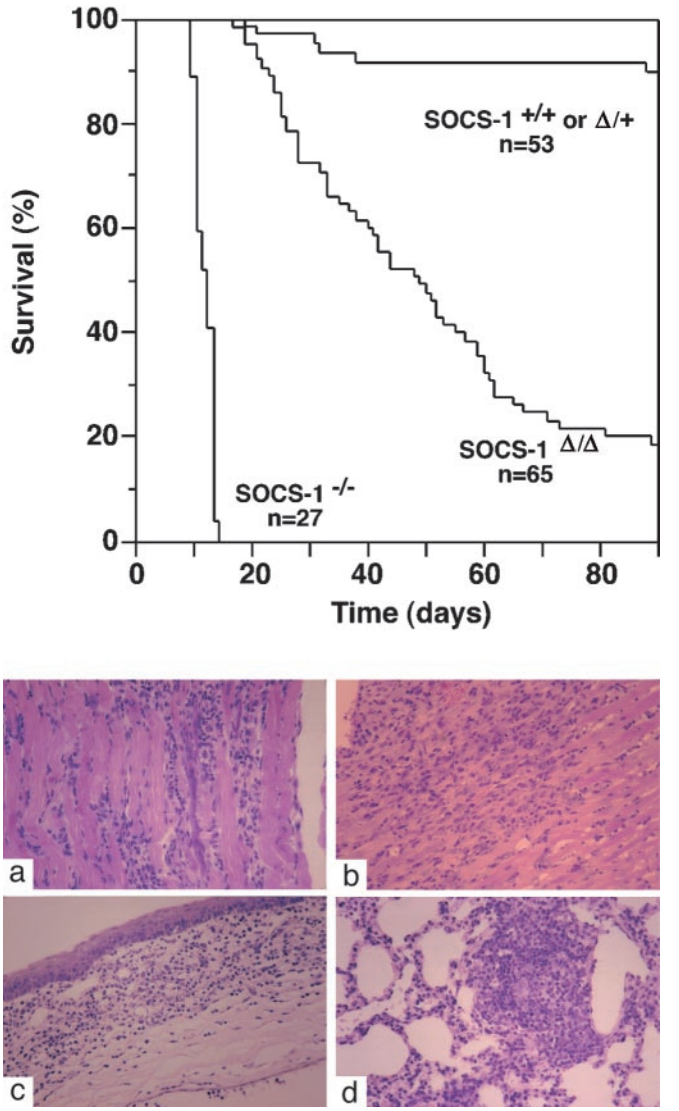

Fig. 3. Disease onset and pathology in mice with deletion of the SOCS box in the SOCS-1 gene. (*Upper*) Survival curves for SOCS-$1^{-/-}$, Δ/Δ, and pooled Δ/+ and +/+ mice on a pure C57BL/6 genetic background (*n* = number of mice). Mice were killed when moribund. (*Lower*) Histology of tissue sections taken from moribund Δ/Δ mice between the ages of 40 and 90 days. (*a*) Skeletal muscle showing extensive cellular infiltration and destruction of muscle cells. (*b*) Heart tissue showing extensive cellular infiltration. (*c*) Cornea with extensive cellular infiltration. (*d*) Lymphoid focus in lung with infiltration of alveolar walls.

Homozygous Δ/Δ mice were healthy at birth but died prematurely compared with Δ/+ or wild-type animals (Fig. 3). However, the onset of disease was prolonged compared with SOCS-$1^{-/-}$ mice, and it most closely matched that seen previously for SOCS-$1^{-/-}$IFN-$\gamma^{+/-}$ mice (22). In fact, the predominant disease states seen in Δ/Δ mice were remarkably similar in frequency and type to those of moribund SOCS-$1^{-/-}$IFN-$\gamma^{+/-}$ mice of the same age (22). Body weight was reduced and extensive inflammatory lesions were seen in skeletal and heart muscle, as well as in the cornea, sometimes with corneal ulceration. The infiltrating cells were predominantly lymphoid cells and macrophages, but the damage to muscle cells was often more severe than that seen in SOCS-$1^{-/-}$IFN-$\gamma^{+/-}$ mice. In addition, lymphoid foci were observed in the salivary glands, lungs, and kidneys of Δ/Δ mice, and the ratio of granulocytes to mononuclear (lymphoid and erythroid) cells was elevated in the bone marrow. Like SOCS-$1^{-/-}$ mice, Δ/Δ mice showed atrophy and dispersion of pancreatic acinar tissue with lymphoid and macrophage infiltration of both the pancreas and dermis and atrophy or thinning of the thymic cortex. Although liver cells did not show fatty degeneration typical of SOCS-$1^{-/-}$ mice, there were areas where the liver trabeculae were thin and widely separated, suggesting previous damage to hepatocytes. The frequencies of these pathologies in Δ/Δ mice are shown in Table 1, and typical sections are shown in Fig. 3. This phenotype suggested that SOCS-1/ΔSB acted as a partial loss of function mutant with significantly reduced capacity to inhibit responses to endogenous IFN-γ.

The response of Δ/Δ mice to IFN-γ was examined by measuring the amount of activated STAT-1 in the liver 4 h after i.v. injection of IFN-γ. STAT-1 Tyr phosphorylation was elevated in Δ/Δ mice compared with wild-type or Δ/+ animals (Fig. 4*a*). The elevated levels of activated STAT-1 in Δ/Δ mice could be caused by the induction of increased amounts or a decreased rate of inactivation of STAT-1. To address this issue and to eliminate the complication of variable levels of endogenous IFN-γ, Δ/Δ mice were generated on an IFN-$\gamma^{-/-}$ genetic background, and the time course of induction and inactivation of activated STAT-1 in the liver in response to injected IFN-γ was measured (Fig. 4*b*). On an IFN-$\gamma^{-/-}$ background, activated STAT-1 accumulated with similar initial kinetics and to a similar level in both Δ/Δ and Δ/+ animals, but the decline of activated STAT-1 levels to baseline was slowed by at least 2 h in the livers of Δ/Δ mice. In contrast, the levels of full-length SOCS-1 in Δ/+ animals and of SOCS-1/ΔSB in Δ/Δ animals were nearly identical throughout the time course (Fig. 4*c*), but, as was seen in Fig. 2, the levels of SOCS-1/ΔSB protein were again reduced in Δ/+ animals. These data are consistent with the current model in which activated STATs contribute to increasing the transcription of the SOCS-1 gene and the accumulation of SOCS-1 protein. Once produced, SOCS-1 inhibits JAKs, and in doing so, the level of phosphorylated STAT-1 declines. Because the levels of SOCS-1/ΔSB in Δ/Δ liver were similar to those of full-length SOCS-1 in wild-type animals at the 2- to 6-h time points, the data also

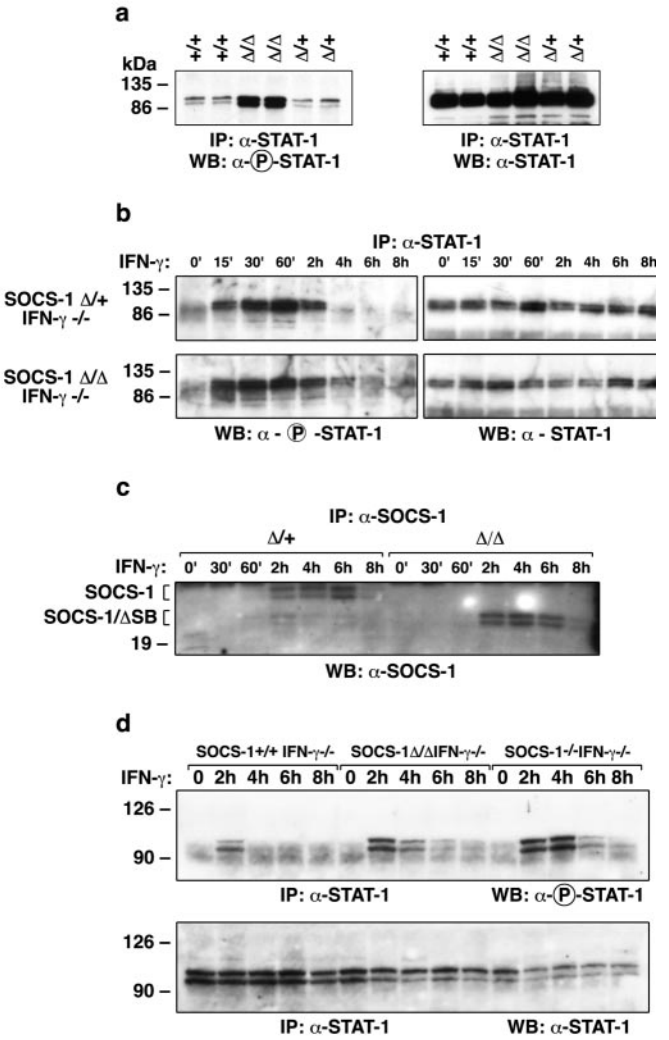

Fig. 4. Response of Δ/Δ mice to IFN-γ. (*a*) Enhanced levels of phosphorylated STAT-1 in livers of Δ/Δ mice 4 h after injection of IFN-γ. Protein was extracted from livers, immunoprecipitated with an anti-STAT-1 monoclonal antibody, and Western blotted either with an antibody specific for Tyr-phosphorylated (activated) STAT-1 (*Left*) or total STAT-1 (*Right*). +/+, wild-type mice; Δ/+, heterozygous mice. (*b*) Time course of STAT-1 activation and deactivation in the liver after a single injection of IFN-γ into Δ/Δ or Δ/+ mice bred onto an IFN-$\gamma^{-/-}$ genetic background. Activated (phosphorylated) STAT-1 (*Left*) and total STAT-1 (*Right*) are shown. Immunoprecipitation and Western blotting were essentially as described for *a*. (c) Time course of SOCS-1 or SOCS-1/ΔSB induction and clearance in the livers of the same animals as described in *b*. Immunoprecipitation and Western blotting were essentially as described for Fig. 2*c*. (*d*) Comparison of STAT-1 response to IFN-γ in primary hepatocyte cultures derived from +/+, Δ/Δ, and −/− mice all bred to an IFN-$\gamma^{-/-}$ background. Cultures were stimulated with IFN-γ for 10 min and then harvested at the indicated time points. Immunoprecipitation and Western blotting of activated STAT-1 were performed as described above.

clearly suggest that SOCS-1/ΔSB is defective in terminating signal transduction and hence in reducing the levels of activated STAT-1.

These data were confirmed in isolated hepatocyte cultures from Δ/Δ and +/+ mice on an IFN-$\gamma^{-/-}$ background (Fig. 4*d*), suggesting that this behavior of full-length and truncated SOCS-1 was independent of more complex cellular interactions. Consistent with the disease incidence profiles in Fig. 3, the prolonged activation of STAT-1 seen in hepatocytes of Δ/Δ mice was intermediate between that seen in SOCS-$1^{+/+}$ and SOCS-$1^{-/-}$ mice (Fig. 4*d*). On an IFN-$\gamma^{-/-}$ background, the levels of activated STAT-1 declined by 4 h in hepatocytes from wild-type mice, by 6 h in Δ/Δ mice, and by ≈8 h in SOCS-$1^{-/-}$ mice. This finding suggests that two separate mechanisms are involved in the termination of signal transduction by SOCS-1, one involving the SOCS box and the other involving the SH2 and N-terminal domains. A role for the SH2 and N-terminal domains has already been proposed in which the SH2 domain binds to a phosphorylated Tyr in the JAK-activation loop, and the N-terminal domain inhibits JAK Tyr kinase activity, thus preventing further activation of STAT-1 (4–6). A role for the SOCS box was suggested by analogy with the Von Hippel–Lindau tumor-suppressor protein where interaction with elongins B/C targets associated substrates (the HIF-1α transcription factor) to proteasomal degradation (17). Paradoxically, however, data have been presented that loss of the SOCS box has no effect on the ability of SOCS-1 to inhibit STAT activation in over-expression systems *in vitro* (4–6), and that instead, it leads to instability of the SOCS-1 protein itself (5, 14). An explanation of these observations is offered by the present results. In over-expression systems, direct inhibition of the JAK/STAT pathway by the SH2 and N-terminal domains is sufficient to completely inhibit this pathway so no further effect is seen through the SOCS box. However, at physiological SOCS-1 levels, only partial inhibition of the pathway may be achieved by the former mechanism, and additional inhibition then is achieved by targeting bound proteins to proteasomal destruction through the SOCS box.

The selective loss of truncated SOCS-1 in heterozygous (Δ/+) animals (Figs. 2*c* and 4*c*) confirms the previously reported relative instability of the truncated compared with the full-length protein in cell lines (5, 14) and suggests that the SOCS box, by recruiting other proteins to the complex, also may have a role in stabilizing SOCS-1. This finding is consistent with the recent observation that binding of the elongin B/C complex stabilizes the von Hippel–Lindau protein against proteasomal degradation (23). The elevated levels of the truncated protein in Δ/Δ compared with Δ/+ mice could be a result not only of biallelic expression but also of the sustained elevation of STAT-1 (which induces SOCS-1 expression; ref. 3) or the inability of the truncated proteins to recruit proteasomal complexes that destroy associated signaling molecules as well as SOCS-1 in Δ/Δ mice. In either case, the present study indicates clearly that the SOCS box is an important element in the SOCS-1 protein that contributes to inhibition of cytokine signaling. It also suggests that the independent ability of this domain to recruit E3 ubiquitin ligase activity will provide an important clue to the roles of the many other proteins that contain a SOCS box.

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