

SARA and Hgs attenuate susceptibility to TGF- β 1-mediated T cell suppression

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ABSTRACT Transforming growth factor- β 1 (TGF- β 1) is a pluripotent cytokine that controls peripheral T cell tolerance mainly in mucosal immunity. It is secreted by regulatory T cells (Tr/Th3) but also by other immunologically active cells. Smad anchor for receptor activation (SARA) and hepatic growth factor-regulated tyrosine kinase substrate (Hgs) are involved in TGF- β 1 signaling. Both molecules are known to present Smad2 and Smad3 to the TGF- β receptor complex. The role of SARA and Hgs in TGF- β 1 susceptibility of human CD4⁺ T cells is unclear. We demonstrate here that TGF- β 1 up-regulates SARA mRNA expression in CD4⁺ T cells similar to that of Smad7. However, the increase in SARA expression was lower (6.1 $\pm$ 0.3-fold vs. 25 $\pm$ 4.1-fold) compared with Smad7 and delayed, with a maximum at 12 h compared with 2 h. Th1 and Th2 cell subsets expressed the same levels of SARA and Hgs. Compared with resting cells, significantly lower levels of the two molecules were found in antigen/allergen- or anti-CD3/CD28-stimulated cells. Down-regulation of SARA and Hgs mRNA in preactivated CD4⁺ T cells was accompanied by a twofold increase in a TGF- β 1 responsive reporter gene assay. Overexpression of SARA and Hgs in T cells yielded a dose-dependent decrease in cotransfected reporter gene expression, indicating an inhibitory function of both molecules. Thus, SARA and Hgs are regulators of TGF- β 1 susceptibility in T cells and integrate regulatory signals into the influence of TGF- β 1-mediated suppression of human T cells.—Kunzmann, S., Wohlfahrt, J. G., Itoh, S., Asao, H., Komada, M., Akdis, C. A., Blaser, K., Schmidt-Weber, C. B. SARA and Hgs attenuate susceptibility to TGF- β 1-mediated T cell suppression. *FASEB J.* 17, 194–202 (2003)

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TGF- β 1 is a crucial regulatory cytokine that maintains balanced antigen reactivity in the immune system. T regulatory (Tr) cells are major elements that maintain a regulated stage (reviewed in ref 1). TGF- β 1 abolishes T cell activation without promoting activation-induced

cell death. However, TGF- β 1-mediated control of T cell activity depends not only on the presence of TGF- β 1, but also on the ability of a T cell to transmit and convert intracellularly the TGF- β 1 signal. Differences in TGF- β 1 susceptibility of T cells therefore affect peripheral tolerance and, in turn, dysregulated immunity and development of autoimmune diseases, cancer, and allergies (reviewed in refs 2–5). Induction of peripheral tolerance represents a key step in specific immunotherapy (6).

TGF- β 1 binds to the TGF- β receptor (R)-II, which is a constitutively active kinase. Subsequently, the TGF- β RI is recruited to TGF- β /TGF- β RII complex and is phosphorylated by the TGF- β RII. The activated TGF- β RI phosphorylates Smad2 and Smad3, which form heterodimeric complexes with the common partner Smad4, allowing the complex to translocate into the nucleus. In the nucleus, this complex associates with promoter elements and regulates the transcriptional response of the target genes. Smad7 inhibits phosphorylation of Smad2 and Smad3 by strong association with the TGF- β RI complex, thus blocking TGF- β 1 signaling. TGF- β 1 itself induces the expression of Smad7, creating negative feedback regulation of the TGF- β 1 signal pathway (7). There is strong evidence that enhanced TGF- β 1 production or disruption of downstream signaling cascades in T cells can either prevent or lead to pathogenic situations such as allergic diseases and autoimmune diseases. This was demonstrated in murine asthma (8, 9) and arthritis models (10) and human inflammatory bowels disease (11).

Recent studies revealed proteins interacting in different combinations with Smad molecules and thereby affecting TGF- β 1 signaling pathways. Smad anchor for receptor activation (SARA) and HGF (hepatocyte growth factor)-regulated tyrosine kinase substrate (Hgs) are Smad-interacting proteins that are assumed to facilitate transport of unphosphorylated Smad2 and

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Smad3 from the microtubules to activated TGF- β Rs (12–14).

SARA is a large protein of 1323 amino acids containing a Smad binding domain (SBD) that binds Smad2 or Smad3 and a central FYVE motif (12, 15). The FYVE motif is a zinc finger-like structure that binds phosphatidylinositol-3-phosphate on the cytoplasmic surface of endosomal vesicles (16) and is required for efficient TGF- β /Smad signaling (17). Overexpression of SARA causes clustering of Smad2/3 into a punctate pattern consistent with an association with endosomal vesicles. The carboxy-terminal domain of SARA associates with TGF- β RI, bridging the receptor with Smad2. The resulting phosphorylation of Smad2 causes its release from SARA, which allows movement of Smad2 to the nucleus (12). It was suggested that SARA masks the nuclear import signal of Smad2 and prevents its inappropriate nuclear import before activation (18).

Hgs is characterized by a FYVE domain. It is phosphorylated after stimulation by cytokines and growth factors, including IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), HGF, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF; 19). Overexpression of Hgs inhibited IL-2 or GM-CSF-mediated proliferation (20). Hgs is localized to the cytoplasmic surface of early endosomes regulating vesicular transport (21). In cooperation with SARA, Hgs is assumed to recruit Smad2 and Smad3 to the TGF- β /activin receptor and modulate TGF- β /activin receptor-mediated signaling (13).

SARA and Hgs represent potential regulatory molecules for TGF- β signaling mediated by Smad activation. The aim of the present study was to investigate whether SARA and Hgs participate in signal transduction of TGF- β 1 in human CD4⁺ T cells. Understanding the pathways contributing to TGF- β 1-mediated signaling between regulatory T cells and their targets will improve concepts of peripheral tolerance.

MATERIALS AND METHODS

Reagents

Recombinant (r) human TGF- β 1 was obtained from R&D Systems (Abingdon, UK), rIL-2, rIFN- γ , rIL-4, and neutralizing anti-IL-4 (8F12 and 3H4) were gifts from Dr. C. Heusser, Novartis (Basel, Switzerland). Human rIL-12 and neutralizing anti-IL-12 mAb were from PharMingen (San Diego, CA, USA) and phytohemagglutinin (PHA) was from Sigma Chem. Co (Saint Louis, MS, USA). The mouse anti-human CD28 mAb (clone 15E8) was purchased from the Netherlands Red Cross blood transfusion service (Amsterdam, Netherlands); the mouse anti-human CD3 mAb (clone CRL 8001) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Generation of rabbit anti-Hrs antiserum was described previously (19, 20); rabbit anti-SARA antiserum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rat anti-HA mAb (clone 3F10) was purchased from Roche (Mannheim, Germany), anti-Flag M2 mAb from Sigma. The amino-terminal enhanced fluorescent protein vector pEGFP-N1 and pDsRed2-N1 vectors were from Clon-

tech (Palo Alto, CA, USA). The pGL3ti (CAGA)₁₂ vector and the expression vectors pKU-Hrs-HA and pCMV5B-Flag-SARA (gift of J. L. Wrana) were described previously (12, 20, 22). rBet v 1 was a gift from Dr. R. Valenta (Vienna, Austria) and Der p 1 from Dr. H. Fiebig (Allergopharma, Reinbek, Germany).

Isolation of CD4⁺ T cells

Peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy volunteers by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation. The interphase cells were washed three times and CD4⁺ T cells were purified using anti-CD4-Dynal magnetic beads and Detach-a-Bead antibodies (both Dynal, Hamburg, Germany). The purity of CD4⁺ T cells was initially tested by flow cytometry and was > 95%.

Cell cultures

The human keratinocyte cell line (HaCaT) was provided by Dr. N. Fusenig (Institute of Biochemistry and Molecular Biology, Heidelberg, Germany). Jurkat cells (acute human T cell leukemia; clone E6–1) were obtained from ATCC. All cultures with human CD4⁺ T cells and Jurkat cells were performed in serum-free AIM-V medium (Life Technologies, Basel, Switzerland) in a humidified atmosphere containing 5% CO₂ at 37°C. HaCaT cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M 2 β -mercaptoethanol (all from Life Technologies), and 10% fetal bovine serum (Sera Laboratory, Sussex, UK).

In vitro differentiation of CD4⁺ naive T cells into Th1 and Th2 type cells

CD45RA⁺ cells were negatively isolated from purified CD4⁺ T cells by using the MACS[®] system (Milteny) as described (23). To remove memory T cells, CD4⁺ T cells were incubated with MACS microbead-conjugated anti-CD45RO. Freshly isolated CD4⁺CD45RA⁺ T cells were resuspended in AIM-V medium. The cells were cultured in 24-well plates at a cell density of 3×10^6 /well. They were stimulated with a combination of anti-CD2 mAb (4B2 and 6G4 each 0.5 μ g/mL), anti-CD3 (1 μ g/mL), and anti-CD28 (1 μ g/mL) as well as IL-2 (20 ng/mL). For Th1 differentiation, human IL-12 (10 ng/mL) and neutralizing anti-IL-4 mAb (10 μ g/mL) were added to individual wells. For Th2 differentiation, IL-4 (25 ng/mL) and neutralizing anti-IL-12 (10 μ g/mL) were used. The growing cell cultures were expanded with fresh culture medium containing 25 ng/mL human IL-2. After 12 days the cells were harvested, washed, and restimulated using the same procedure. After two cycles of stimulation, cytokine patterns of differentiated cells were verified by ELISA.

T cell proliferation assay

CD4⁺ T cells (3×10^6) were stimulated on plastic dishes, coated with anti-CD3 (1 μ g/mL) and/or anti-CD28 (2 μ g/mL), or T cells were activated with PHA (2 μ g/mL) and IL-2 (20 ng/mL). Viability of transfected T cells was analyzed by ethidium bromide staining (50 μ M) as described (24). The cultures were set up in 200 μ l AIM-V medium in 96-well flat-bottom microtiter plates (Costar, Corning, NY, USA). Samples in triplicate, containing 10^5 T cells and an equal number of 600 rad irradiated autologous PBMC as antigen-

presenting cells were incubated for 5 days in the presence of 0.3 μ M Der p 1 or Bet v 1 allergen. Cells were pulsed for 16 h with 1 μ Ci [^{3} H]-thymidine (Hartmann, Braunschweig, Germany) and harvested on glass fiber filters using an automated multisample harvester (LKB, Pharmacia-Wallac, Turku, Finland). Filters were transferred in sample bags with liquid scintillation fluid and analyzed using a β -scintillation counter (Pharmacia-Wallac).

RT-PCR

Total RNA was isolated from human CD4⁺ T lymphocytes using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was eluted in 40 μ L water and subjected to reverse transcription. Approximately 5 μ g total RNA (12 μ L) was reverse transcribed by addition of 500 μ g/mL oligo (dT)₁₂ primer (Roche, Basel, Switzerland), RNase Inhibitor (Roche) (10 U/ μ L), dNTP (5mM each dNTP; Qiagen) and Omniscript transcriptase (Qiagen) (0.2 U/ μ L) for 1 h at 37°C. cDNA was denatured at 90°C for 5 min and used for PCR amplification. For full-length RT-PCR for SARA and Hgs the "expand high fidelity PCR system" (Roche) was used. All other PCR reactions were performed with Taq-polymerase (Qiagen). RT-PCR were performed using the following primers: GAPDH forward 5' CTT CGC TCT CTG CTC CTC CT 3', GAPDH reverse 5' GCT GAT GAT CTT GAG GCT GTT G 3', SARA forward 5' AAA ATG CTG TTG CAG AAG ACC A 3', SARA reverse 5' TTT AAT TGT GAA GGG GAA CAG ACA 3', Hgs forward 5' GCG TCT CCT AGA CAA GGC GA 3', Hgs reverse 5' GTC ATC ACC CCG AAC TGC AC 3', TGF- β RI forward 5' TAT CAC CAA CAG CAT GTG TAT AGC TG 3', TGF- β RI reverse 5' AGC CAG AAC CTG ACG TTG TCA TAT CAT 3', TGF- β RII forward 5' TGA CCC CAA GCT CCC CTA CCA TGA 3', TGF- β RII reverse 5' TGA TGT CAG AGC GGT CAT CTT CCA 3', IL-10 forward 5' ATG CCC CAA GCT GAG AAC CAA GAC 3', IL-10 reverse 5' CCC AGA GCC CCA GAT CCG ATT TTG 3', Smad7 forward 5' AAG TCA AGA GGC TGT GTT GC 3', Smad7 reverse 5' TCT CGT AGT CGA AAG CCT TGA TGG AGA AA 3'. The primer for IL-10, TGF- β RI and TGF- β RII was used as described before (25, 26). Primers for the entire length of the coding sequence of SARA and Hgs: SARA el forward 5' ATG TGG ATT GAT GAA AAT GCT GTT GCA GAA 3', SARA el reverse 5' TTA TAC GAT GTT TTC CAG AAT ATA AAA GAT GAG T 3', Hgs el forward 5' ATG GGG CGA GGC AGC GGC ACC TTC GA 3', Hgs el reverse 5' TCA GTC GAA TGA AAT GAG CTG GGC CTC GCT 3'. PCR products were loaded next to a standard (1 kb plus, Life Technologies) and analyzed on 0.5% or 1% agarose gels. Image analysis was performed using a fluorescence imager analyzer FLA 3000 (Fuji, Dielsdorf, Switzerland) and quantified using the AIDA software (Raytest, Urdorf, Switzerland).

Real-time PCR

Based on the sequence reported in GenBank the PCR primers and TaqMan probes to amplify and detect SARA and Hgs, mRNA were designed using the Primer Express software version 1.2 (Perkin Elmer/Applied Biosystems, Foster City, USA) as follows: SARA forward primer 5' GCT ATC AAG CAG GGA GCA ATG 3', SARA reverse primer 5' CCA AGG CGC TAT CCA GAT CA 3', SARA probe FAM 5' CCA GCC CCT TCC CTC GCA GTA CAT 3' TAMRA, Hgs forward primer 5' TTC CAC AAT GGC GAG TCT GA 3', Hgs reverse primer 5' AAG GTG GTG ACG GCG TTC T 3', Hgs probe FAM 5' CCA CGA GCA GTT CCT GAA GGC GC 3' TAMRA. The TaqMan probes were labeled with 6-carboxy-fluorescein (FAM) as the reporter dye and 6-carboxy-tetramethyl-Rhodamine (TAMRA)

as the fluorescent quencher. The PCR primers and the TaqMan probes were purchased from Microsynth (Balgach, Switzerland). A TaqMan-GAPDH control was used to amplify and detect GAPDH as control recommended by the manufacturer (Perkin Elmer/Applied Biosystems): GAPDH forward primer 5' GCA CCG TCA AGG CTG AGA AC 3'; GAPDH reverse primer 5' GAG GGA TCT CGC TCC TGG A 3'; GAPDH probe FAM 5'CTT GTC ATC AAT GGA AAT CCC ATC ACC ATC 3' TAMRA.

The prepared cDNA was amplified using an UNG-containing PCR mastermix (Perkin Elmer/Applied Biosystems) according to recommendations of the manufacturer. Accumulation of the PCR products was detected in real time by monitoring the probe cleavage-induced mobilization of the reporter dye. Relative quantification was performed using the comparative C_T method as described (27). Before using the $\Delta\Delta C_T$ method for quantitation, a validation experiment was performed to verify that efficiencies of target and control were approximately equal. In some experiments, quantitative analysis of SARA, Hgs, TGF- β RI, and Smad7 expression was performed in a similar manner using the SYBR Green Universal PCR Master Mix (Applied Biosystems).

Immunoprecipitation and immunoblotting

For immunoprecipitations, 6 $\times$ 10⁶ CD4⁺ T or Jurkat cells were lysed in RIPA buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1%NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with 1 mM sodium orthovanadate, 10 μ g/mL aprotinin, and 10 μ g/mL leupeptin. The lysates were incubated overnight with 7 μ L rabbit anti-Hrs or 2 μ g/mL anti-SARA. Protein-G agarose beads (Sigma) were used for precipitation. After 4 h the beads were washed four times with RIPA buffer, boiled, and loaded next to a protein mass ladder (Benchmark, Invitrogen) on a NuPAGE 4–12% bis-tris gel (Invitrogen). The proteins were electroblotted onto a nitrocellulose membrane (Amersham Life Science, Dübendorf, Switzerland) and SARA or Hgs peptides were detected with anti-SARA mAb or anti-HGS serum. The blot was visualized with a LAS 1000 camera (Fuji). For estimation of protein quantity photographs were taken with incremental exposure times. Accumulated signals were analyzed using AIDA software (Raytest).

Transfections and reporter gene assays

CD4⁺ T cells were purified as described above and rested in serum-free AIM-V medium (LifeTechnologies) overnight. pCMV5B-Flag-SARA, pKU-Hrs-HA, empty control-vectors (pCMV5B, pKU) and the TGF- β -sensitive pGL3ti (CAGA)₁₂-Luciferase reporter gene were added to 3 $\times$ 10⁶ CD4⁺ T cells, which were previously washed in PBS and resuspended in 100 μ L of NucleofectorTM solution for T cells (Amaxa Biosystems, Cologne, Germany), electroporated using the U-15 program of the NucleofectorTM (Amaxa) and immediately transferred into prewarmed AIM-V medium. Transfected cells were seeded into 24-well plates and TGF- β 1 (1 ng/mL) was added to the cells. Twenty-four h after transfection, luciferase activity in cell lysates was measured by the dual luciferase assay system (Promega Biotech Inc., Madison, WI) according to the manufacturer's instruction in a Berthold Lumat LB 9507 luminometer (Bad Wildbach, Germany). Data were normalized by the activity of Renilla luciferase under the control of thymidine kinase promoter of pRL-TK. All values were obtained from experiments performed in triplicate and repeated at least three times.

Human HaCaT cells were seeded at 4.75 $\times$ 10⁵ cells/well in 6-well culture dishes 24 h before transfection. HaCaT cells

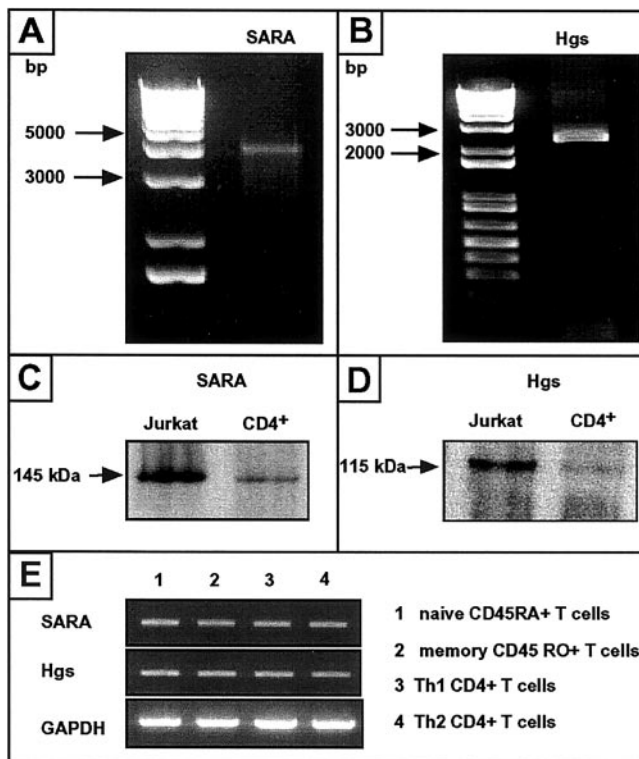


Figure 1. RT-PCR amplicons and protein expression of SARA and Hgs in human CD4⁺ T cells and Jurkat cells. RT-PCR products of SARA (A) and Hgs (B) are resolved on a 0.5% agarose gel next to a DNA standard. Arrows indicate the molecular weight in base pairs (bp). Immunoprecipitates of SARA (C) and Hgs (D) of the human T-leukemic cell line Jurkat (lane 1) and of resting human CD4⁺ T lymphocytes (lane 2) resolved on a 6–12% PAA gel. Arrows indicate the molecular mass in 10³ daltons (kDa). E) mRNA expression of SARA and Hgs in different CD4⁺ T cell subsets. Representative of 3 independent experiments.

were then transiently transfected with the indicated constructs using LIPOFECTAMINE-PLUS reagent in Optimen 1 medium (both LifeTechnologies) and after 2 h maintained in 0.2% FCS culture medium. Twenty-four hours after transfection, cells were incubated for 16 h with or without TGF- β 1 (1ng/mL) in serum-free culture medium and the luciferase activity was measured.

RESULTS

Expression of SARA and Hgs in human CD4⁺ T cells

To determine whether SARA and Hgs are expressed in resting human CD4⁺ T lymphocytes, the expression of mRNA and protein for SARA and Hgs was analyzed in resting human CD4⁺ T lymphocytes using RT-PCR and Western blots of immunoprecipitated proteins. RT-PCR was designed for the entire length of the coding sequence and revealed a specific amplicon of SARA with a distinct, expected size of 3972 bp (Fig. 1A). Similarly, the amplicon of Hgs shows a PCR product of 2331 bp (Fig. 1B). The coding sequence spanning PCR amplicons of both molecules showed no evidence of

splice variants, since only single bands were amplified. Immunoprecipitated SARA and Hgs could be detected with a molecular size of 145 kDa (Fig. 1C, lane 2) and 115 kDa, respectively (Fig. 1D, lane 2). Expression of SARA and Hgs in Jurkat cells was stronger than that of freshly isolated peripheral T cells, in which only a weak signal could be detected (Fig. 1C, D, lane 1). Equal SARA and Hgs mRNA expression was found in human CD45RA⁺ (naive), CD45RO⁺ (memory), Th1 and Th2 cells (Fig. 1E).

TGF- β 1 up-regulates SARA mRNA in resting human CD4⁺ T lymphocytes

In their role as Smad adaptor molecules, alterations in SARA and Hgs expression may provide a means of effective cross-regulation of TGF- β 1-mediated responses with other signaling pathways. Therefore, SARA and Hgs expression may depend on the presence of TGF- β 1 or IFN- γ , as known for Smad7 (7, 28). Thus, resting human CD4⁺ T cells were stimulated with TGF- β 1, IFN- γ , or IL-2 for 12 h. Quantitative real-time RT-PCR demonstrated increased SARA expression to a ddC_T value of 6.1 ± 0.3 , whereas Hgs remained unchanged (ddC_T: 1.1 ± 0.3). This is shown in Fig. 2A, B. TGF- β 1 did not affect housekeeping gene expression. IFN- γ and IL-2 affected neither SARA (Fig. 2A) nor Hgs

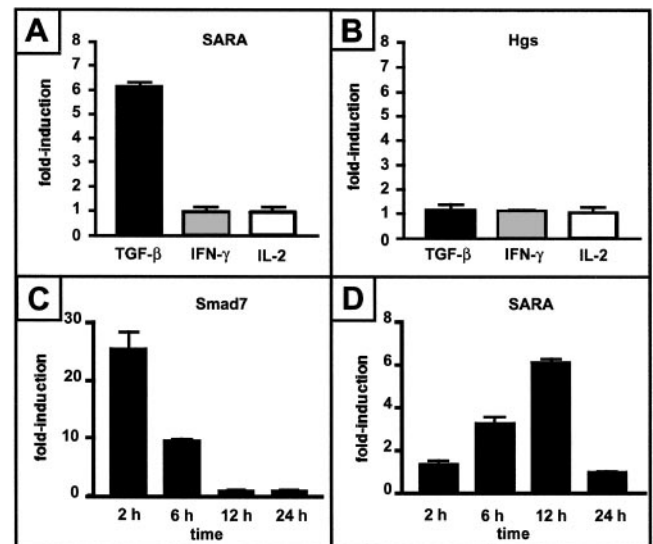


Figure 2. TGF- β 1 up-regulates SARA mRNA in human CD4⁺ T lymphocytes. Human CD4⁺ T cells were cultured for 12 h in medium alone or together with the indicated cytokines (TGF- β 1 1 ng/mL, IL-2 20 ng/mL, INF- γ 50 ng/mL). Relative quantitation of SARA (A) and Hgs (B) gene of human CD4⁺ T cells expression are shown using the comparative C_T method. Kinetic analysis of TGF- β 1-induced expression of Smad7 and SARA is shown in C-D. Resting human CD4⁺ T cell were incubated for 2, 6, 12, and 24 h with TGF- β 1 (1 ng/mL). Smad7 (C) and SARA (D) mRNA were quantified by real-time quantitative PCR and “fold” induction were calculated as ratio to values of cells, which were not incubated with TGF- β 1. Error bars indicate standard error of the mean. Representative of 3 independent experiments.

expression (Fig. 2B). SARA and Smad7 showed different expression kinetics in CD4⁺ T cells. Resting human CD4⁺ T cells were incubated for 2, 6, 12, and 24 h with TGF- β 1. Within 2 h of TGF- β 1 stimulation, Smad7 mRNA was induced 25 ± 4.1 -fold over background levels (Fig. 2C). In contrast, SARA was not increased at that time (1.3 ± 0.2). Whereas the expression of Smad7 mRNA was induced at maximum 2 h after TGF- β 1 stimulation, the maximum expression of SARA mRNA was observed 12 h after treatment of the cells with TGF- β 1 (5.7 ± 0.5), when Smad7 expression had already reached background levels (Fig. 2D). Expression of SARA mRNA reached background levels 24 h after TGF- β 1 stimulation.

TCR engagement regulates SARA and Hgs mRNA expression

To analyze whether SARA and Hgs mRNA expression could be regulated by TCR-mediated signals, expression of resting or 1, 3, and 5 days with allergen or anti-CD3/CD28 mAbs-activated CD4⁺ T cells was analyzed. SARA and Hgs mRNA were detected in resting T cells, but their expression was reduced 1 day after the CD3/CD28 activation. The reduction was even more pronounced on days 3 and 5 of stimulation (Fig. 3A, B). As reported (26), we found a down-regulation of TGF- β RII transcripts after CD3/CD28 activation. TGF- β RI expression was unchanged under these conditions, whereas IL-10 expression used as a positive control was enhanced.

To determine whether allergen-specific T cell activation leads to a down-regulation of SARA and Hgs transcripts as observed for polyclonal activation, T cells from patients allergic to birch pollen or house dust mite were stimulated with the major birch pollen allergen Bet v 1 or the house dust mite allergen Der p 1 for 5 days, using autologous irradiated PBMC as APC. Similar to polyclonal stimulation, the expression of SARA and Hgs was down-regulated within 5 days, whereas TGF- β -RI was not changed relative to the housekeeping gene (Fig. 4).

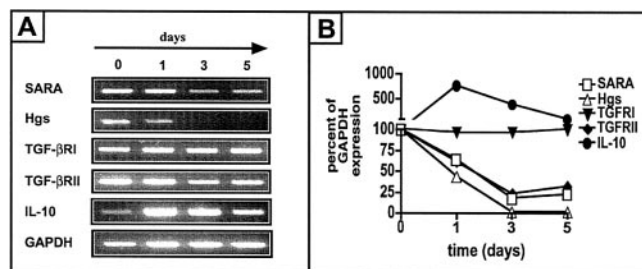


Figure 3. T cell activation regulates SARA and Hgs mRNA expression. Human CD4⁺ T lymphocytes were stimulated with anti-CD3 mAb (1 μ g/mL) and anti-CD28 mAb (2 μ g/mL) for 1, 3, and 5 days in medium (A, B). RT-PCR for SARA, Hgs, TGF- β RI, TGF- β RII, IL-10, and GAPDH was performed. B) Densitometric analysis of PCR results shown in panel A in relation to GAPDH expression. Representative of 3 independent experiments.

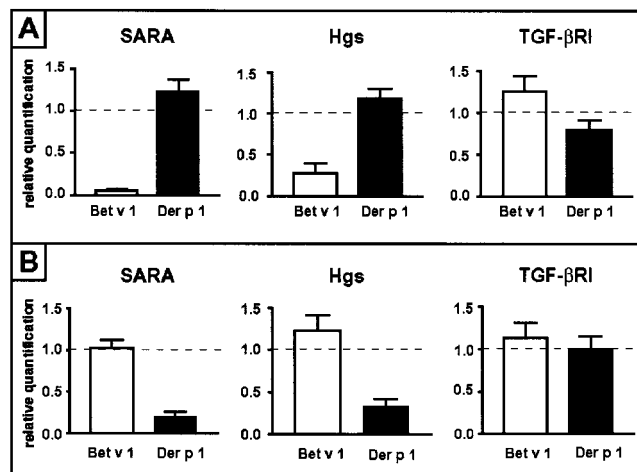


Figure 4. Down-regulation of SARA and Hgs mRNA by antigen/allergen-specific T cell stimulation. Antigen-specific T cell stimulation by Bet v 1 from a birch pollen allergic patient is shown compared with an irrelevant allergen (Der p 1) and in relation to GAPDH expression using real-time quantitative RT-PCR (comparative C_T method; A). Error bars indicate standard error of the mean. Representative of 3 independent experiments. Similar results were obtained from patients allergic to house dust mite vs. an irrelevant allergen (Bet v 1; B).

Overexpression of SARA and Hgs in primary CD4⁺ T cells using non-retroviral transfection nucleofection technique

The function of SARA and Hgs in primary T cells was analyzed in a nonviral transfection technique. Transfection or infection of primary and resting human CD4⁺ T cells has so far been technically difficult. Nucleofection technology was applied to transfect CD4⁺ T cells with the green fluorescent protein (GFP) coding plasmid (pEGFP-1) as control or SARA or Hgs. Expression and viability was analyzed 24, 48, and 72 h after transfection. Microscopic analysis of T cells shows uniform expression of GFP in $37 \pm 2.0\%$ of the cells (Fig. 5A) 24 h after transfection. Viability ranged from $80 \pm 1.6\%$ on day 1 to $62 \pm 2.0\%$ on day 3 after transfection. rHgs expression was found as early as 3 h after transfection and was maximal after 12 h (Fig. 5B).

T cell activation influences TGF- β 1 responsiveness

To determine whether there is a difference between the TGF- β 1 responsiveness of resting and activated T cells, the TGF- β 1-sensitive (CAGA)₁₂-Luc reporter was transfected in resting and activated T cells and the difference in TGF- β 1 response was determined by measuring the luciferase activity. Resting T cells transfected only with the (CAGA)₁₂-Luc reporter showed a 2.2 ± 0.14 -fold increase of luciferase activity 24 h after TGF- β 1 addition vs. cells not treated with TGF- β 1 (Fig. 6). When T cells were prestimulated with anti-CD3/28 for 24 or 72 h before the transfection of the reporter construct, a 3.4 ± 0.4 -fold or a 4.7 ± 0.48 -fold increase

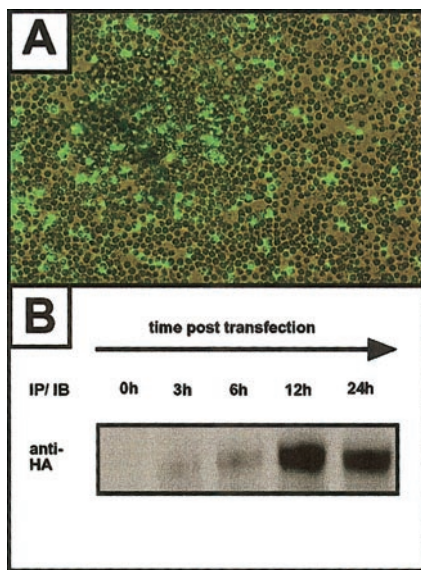


Figure 5. Non-retroviral transfection of molecules in primary CD4⁺ T cells with help of the Nucleofector™ technology. Resting human CD4⁺ T lymphocytes (3×10^6) were transfected with the green fluorescent protein coding plasmid pEGFP-1 (2 μ g) to show recombinant protein expression. A) A phase contrast image with a digitally overlaid fluorescent image of a T cell culture 24 h after transfection. Some cells moved during fluorescent acquisition. Resting human CD4⁺ T lymphocytes (3×10^6) were transfected with the HA tagged Hgs protein coding plasmid pKUHgsHA (2 μ g). To monitor the time course of recombinant Hgs expression, immunoprecipitation and immunoblotting against HA were done 0, 3, 6, 12, and 24 h after transfection (B).

of luciferase activity was observed. When resting T cells were prestimulated with PHA 24 h before the transfection of the luciferase construct, a $5.1 (\pm 0.73)$ increase of luciferase activity was consistently observed. Both results indicate that T cell activation more than doubles their response to TGF- β 1 (Fig. 6).

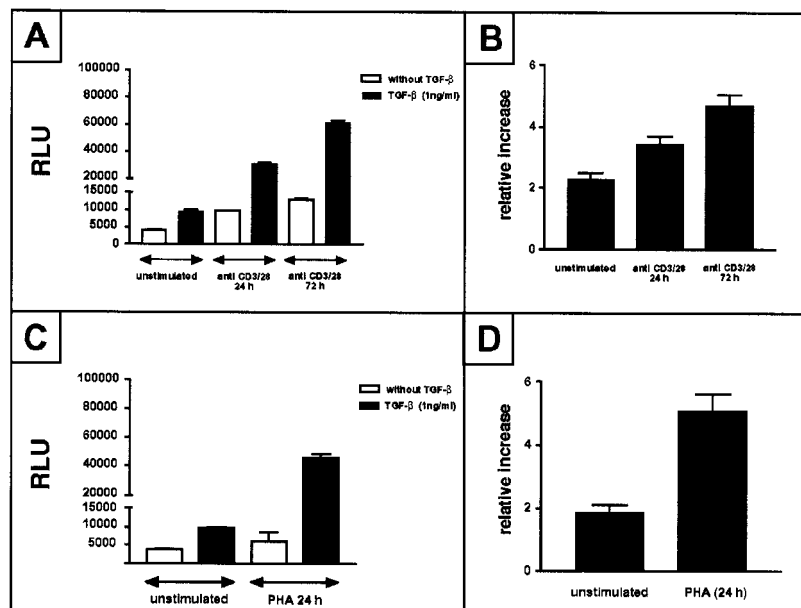


Figure 6. T cell activation enhances TGF- β 1 response of human CD4⁺ T lymphocytes. Luminometric analysis of (CAGA)₁₂-Luc reporter (2 μ g) transfected T cells (3×10^6) prestimulated with anti-CD3/28 mAb (1 μ g/mL, 2 μ g/mL) for 24 or 72 h (A, B) or with PHA (2 μ g/mL; C, D) for 24 h in the presence or absence of TGF- β 1 (1 ng/mL) compared with unstimulated cells. Firefly luciferase activity of (CAGA)₁₂-Luc was normalized by Renilla luciferase activity of pRL-TK vector (5 ng) using dual luciferase reporter system. B, D) Relative increase of luciferase activity without and with prestimulation. All values were obtained from experiments performed in triplicate; error bars indicated standard error of the mean. Representative of 3 independent experiments.

SARA and Hgs overexpression attenuates TGF- β 1 responsiveness

To measure TGF- β 1 sensitivity of T cells overexpressing SARA or Hgs, the TGF- β 1-sensitive (CAGA)₁₂-Luc reporter was transfected in primary CD4⁺ T lymphocytes together with an expression plasmid for SARA or Hgs. SARA (Fig. 7A) and Hgs (Fig. 7B) were found to inhibit TGF- β 1-induced luciferase activity of activated T cells in a dose-dependent manner (Fig. 7C, D). SARA overexpression reduced TGF- β 1 sensitivity 2.3 ± 0.4 -fold if cells were transfected with 2 μ g plasmid and Hgs reduced TGF- β 1 responsiveness 2.9 ± 0.3 -fold. SARA and Hgs did not affect the activity of the reporter gene construct in the absence of TGF- β 1. Overexpression of SARA and Hgs at various concentrations had no influence on the viability of the human CD4⁺ T cell compared with cells transfected with the empty vector (data not shown). The inhibitory effect of SARA and Hgs on TGF- β 1 sensitivity was confirmed in the non-lymphoid human keratinocyte cell line (HaCaT) using a lipid-based transfection technique (Fig. 7E, F). Overexpression of SARA and Hgs in this cell line inhibited luciferase activity by 2.9 ± 0.3 -fold and 4.3 ± 0.5 -fold, respectively.

DISCUSSION

The present study demonstrates that the two recently described Smad binding proteins, SARA and Hgs, are expressed, regulated and involved in TGF- β 1 signaling in human CD4⁺ T lymphocytes. Previous studies revealed that mRNA of SARA and Hgs can be detected in virtually all tissues (12, 19, 20). In line with these data, we showed the expression of Hgs and SARA mRNA in human CD4⁺ T cells. Comparable expression levels were detected in memory and naive T cells. The same

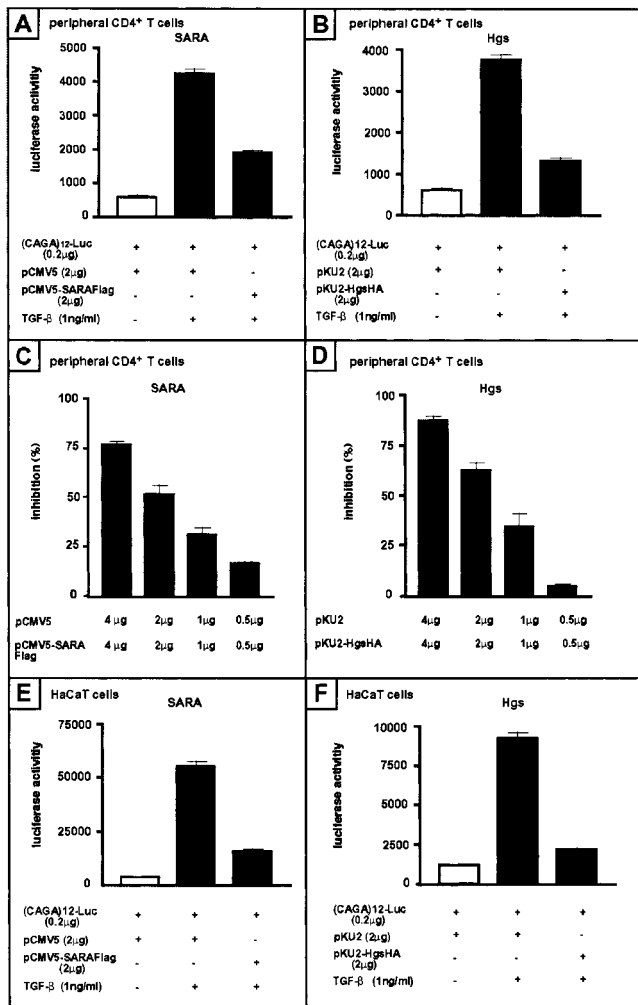


Figure 7. SARA and Hgs overexpression attenuates TGF-β1 responsiveness. Human CD4⁺ T lymphocytes were transfected with the indicated constructs for SARA (A) and Hgs (B). pCMV5 and pKU2 represent empty vectors as control for the expression constructs (pCMV5-SARA, pKU2-Hgs). Titration of SARA and Hgs expression constructs in T cells transfected with the reporter gene in the presence of TGF-β1 (1 ng/mL) (C, D). HaCaT cells were transfected with the same set of plasmids using a cationic lipid reagent (Lipofectamine PlusTM reagent; E, F). Luciferase activity of (CAGA)₁₂-Luc reporter was normalized to pRL-TK activity as described in the legend to Fig. 5. Error bars indicate standard error of the mean. Representative of 3 independent experiments.

levels were observed whether the differentiated T cells were of Th1 or Th2 phenotype. For the first time it is shown that SARA and Hgs protein are expressed in nontransformed, freshly isolated human T cells.

It is known that proteins regulating the TGF-β signal transduction such as Smad7 are controlled via feedback regulation (7, 28). Present results confirm that Smad7 gene expression in T cells is up-regulated by TGF-β1. SARA is up-regulated by TGF-β1. Despite the similarity in feedback regulation, SARA showed a delayed induction by TGF-β1 at significantly lower levels. SARA was not inducible by IFN-γ, as described for Smad7 (28). In contrast, Hgs remained unaffected by TGF-β1 treat-

ment and appeared not to be involved in these feedback regulatory pathways.

Besides feedback regulation, the activation of T cells by the TCR regulates molecules of the TGF-β signaling cascade by up-regulation of the TGF-β binding capacity (29). The effect of TCR activation on the TGF-β signaling cascade is very important for the translation of the external signal into cellular decisions such as inhibited proliferation or reduced cytokine production. SARA and Hgs expression decreased after TCR engagement by antigen-specific or polyclonal stimulation to a degree similar to that observed for TGF-βRII. In contrast, TGF-βRI expression remained constant. It is of particular interest that allergens can trigger this mechanism. SARA and Hgs are similarly down-regulated in another antigen-specific system in which cow milk-specific T cells were stimulated with cow milk antigen (M. Tiemessen, personal communication). Thus, knowledge of such molecular mechanism may provide promising targets for modulation of T cell responses in the TGF-β1-rich environment of allergic inflammation (30, 31). The antigen-driven regulation of TGF-β1 signaling molecules demonstrates that TGF-β1-induced signal transduction is regulated by external factors. Therefore, it is important to understand the functional effect of SARA and Hgs on TGF-β1-induced signal transduction.

The present study introduces non-retroviral, recombinant expression of proteins together with TGF-β1-sensitive reporter gene analysis in primary, nontransformed human T cells. This assay allows reproducible quantification of TGF-β1 signals into the nucleus even in resting T cells. Analysis of TGF-β1 responsiveness by reporter gene constructs allows a more precise analysis of the TGF-β1-induced signaling cascade. Indirect measurements like proliferation and cytokine production always include integration of TGF-β1 unrelated signals. We demonstrated that resting T cells are less TGF-β1 responsive than PHA- or anti-CD3/CD28-stimulated cells. This mechanism is important at the initiation phase of an immune responses, when TGF-β1 is abundant. Moreover, this regulatory pattern is important to limit or terminate T cell activation.

Overexpression of SARA or Hgs drastically reduced TGF-β1 responsiveness of T cells in a dose-dependent manner. Therefore, these molecules represent natural attenuators of TGF-β1 susceptibility. Previous studies showed that Hgs overexpression inhibits Smad3-mediated transcription in the Mv1Lu cell line (32) or even enhances TGF-β1 responsiveness (13). Overexpression of SARA had no effect in Mv1Lu cell line (12). Differences might result from the relative role of phosphatidylinositol phosphates, which play a crucial role in T cell activation. In fact, activation of phosphatidylinositol 3-kinase is a hallmark of costimulation, leading to increased levels of phosphatidylinositol-phosphates (PIPs) at the site of activation (for a review, see ref 33). These lipids can recruit proteins, carrying a pleckstrin- or a FYVE domain as contained in SARA and Hgs. Therefore, PIPs are likely to play a different role in

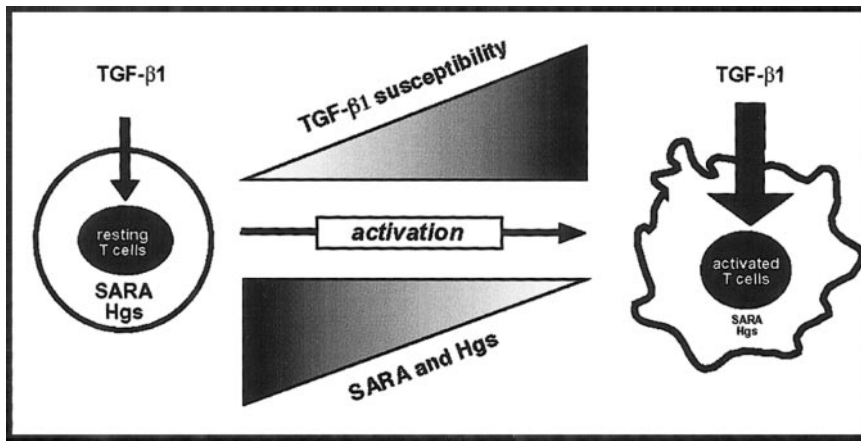


Figure 8. Relationship of the TGF- β 1-attenuators SARA and Hgs in resting and activated T cells. Expression level of SARA and Hgs is higher in resting cells than activated T cells. Activated T cells show an enhanced TGF- β 1 responsiveness.

Mv1Lu cells than in T cells. A possible mechanism of SARA and most likely for Hgs-mediated attenuation of TGF- β 1 responsiveness may be the competition with Smad4 binding sites that are necessary to translocate Smad2 into the nucleus (34). In this scenario overexpression of SARA would block Smad2 against interaction with Smad4.

Our data suggest that SARA and Hgs play an important role in the control of TGF- β 1 responsiveness in T cells. It is tempting to speculate that the negative regulator Smad7 limits the intensity of TGF- β 1-mediated suppression at an early stage, whereas SARA and Hgs integrate signals that limit the duration of suppression. Besides cytokines, the activation level of T cells influences the expression of SARA and Hgs and the response to TGF- β 1. The increased TGF- β 1 susceptibility of activated human CD4⁺ T lymphocytes correlated with activation-dependent down-regulation of the attenuators (Fig. 8). Other members of the TGF- β 1 signaling pathway (TGF- β RI, TGF- β RII) did not show changes of expression pattern, which could explain the increased responsiveness of activated human CD4⁺ T cells to TGF- β 1.

Consequently, SARA and Hgs are interesting signal integrating molecules that influence T cell TGF- β 1 responsiveness under regulatory and activatory conditions. Both molecules act as regulators of immune tolerance, since TGF- β 1 is known to be an important factor for generation of T regulatory cells, which suppress autologous reactions and control the specific immune response. [FJ]

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