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12. The A98R ORF was cloned after amplification of genomic viral DNA with 22 cycles of polymerase chain reaction (PCR) with Taq polymerase [F. M. Ausubel *et al.*, in *Short Protocols in Molecular Biology* (Wiley, New York, ed. 3, 1995)]. The oligonucleotide primers [sense 5′-gagagctcc**ATG**GGTAAAAA-CATTATCATAATGG-3′; antisense 5′-gcatgtcgacT-CACACAGACTGAGCATTGGTAG-3′; Great American Gene Company] contained Nco I or Sal I restriction sites (underlined, respectively) flanking the ORF encoding 568 amino acids (uppercase letters). Codons 4 and 5 were altered to optimize bacterial expression. The PCR product was purified, digested with Nco I and Sal I, and ligated into a modified version of the plasmid pET-8C [B. A. Moffatt and F. W. Studier, *J. Mol. Biol.* **189**, 113 (1986)] (it has an extra Sal I site in the polylinker) cleaved with Nco I and partially digested with Sal I. This construct placed the A98R ORF under the control of a T7 phage promoter. The resulting plasmid, pCVHAS, was transformed into the expression host, *E. coli* BL21(DE3). The A98R protein was expressed by induction with 1 mM isopropylthiogalactoside. After 3 to 5 hours of further growth, the membrane fraction was isolated (*13*). Control membrane preparations were made from cultures with the same vector containing an irrelevant gene (a protein kinase). Total protein was measured according to M. M. Bradford [*Anal. Biochem.* **72**, 248 (1976)]. The paper chromatography method was used to assay for HAS activity (*13*).
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15. Assays with *E. coli*–derived A98R were incubated either with 120 μM UDP-[^{14}C]GlcA or with 150 μM UDP-[^{3}H]GlcNAc. The reactions also contained one unlabeled sugar nucleotide (an authentic precursor or UDP-Glc, UDP-GalA, or UDP-GalNAc) at 300 μM. Less than 5% of the maximal incorporation (assay with UDP-GlcA and UDP-GlcNAc present) was detected if an unnatural UDP-sugar was substituted for UDP-GlcA or UDP-GlcNAc, or if only a single precursor was present.
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27. Membranes (860 μg of protein) were incubated with 120 μM UDP-[^{14}C]GlcA (0.36 μCi) and 840 μM UDP-[^{3}H]GlcNAc (2.6 μCi) in 300 μl of 50 mM Hepes, pH 7.2, with 15 mM $MnCl_2$ for 3 hours at 30°C. EDTA (18 mM final concentration) was then added to stop the HAS activity. Half of the reaction was deproteinized by treatment with 0.5% SDS (w/v) and Pronase (final concentration of 200 μg/ml, 5 hours at 37°C; Boehringer Mannheim). Unincorporated precursors and other small molecules were removed by ultrafiltration (Microcon10, 10^4-daltons cutoff; Amicon). Half of this semipurified sample was injected onto a Sephacryl S-500HR column (1 cm by 50 cm; Pharmacia) equilibrated in 0.2 M NaCl, 5 mM tris, pH 8 (0.5 ml/min, 1-ml fractions). To verify that the identity of the labeled polysaccharide was HA, we treated the other half of the original reaction with HA lyase (30 units at 37°C overnight; Sigma) before the deproteinization step. This treatment degraded the radioactive polymer to small oligosaccharides (tetramers and hexamers) that were removed by ultrafiltration before gel filtration chromatography.
28. Two cultures of NC64A cells (0.9 liter; 1.9×10^{10} cells) were infected with PBCV-1 (multiplicity of infection of 5) and incubated for 50 or 90 min after infection. Another culture served as an uninfected control. The cells were harvested, and the membrane fraction (yield ~3 mg of protein) was prepared as described [P. L. DeAngelis and A. M. Achyuthan, *J. Biol. Chem.* **271**, 23657 (1996)], except that 1 mM mercaptoethanol was substituted for dithiothreitol. The paper chromatography method was used to assay for HAS activity (*13*).
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Specific Inhibition of Stat3 Signal Transduction by PIAS3

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The signal transducer and activator of transcription–3 (Stat3) protein is activated by the interleukin 6 (IL-6) family of cytokines, epidermal growth factor, and leptin. A protein named PIAS3 (protein inhibitor of activated STAT) that binds to Stat3 was isolated and characterized. The association of PIAS3 with Stat3 in vivo was only observed in cells stimulated with ligands that cause the activation of Stat3. PIAS3 blocked the DNA-binding activity of Stat3 and inhibited Stat3-mediated gene activation. Although Stat1 is also phosphorylated in response to IL-6, PIAS3 did not interact with Stat1 or affect its DNA-binding or transcriptional activity. The results indicate that PIAS3 is a specific inhibitor of Stat3.

Stat3 participates in signal transduction pathways activated by the IL-6 family of cytokines and by epidermal growth factor (*1*, *2*). Stat3 is also activated in cells treated with leptin, a growth hormone that functions in regulating food intake and energy expenditure (*3*). Targeted disruption of the mouse gene encoding Stat3 leads to early embryonic lethality (*4*). Like other members of the STAT family, Stat3 becomes tyrosine phosphorylated by Janus kinases (JAKs). Phosphorylated Stat3 then forms a dimer and translocates into the nucleus to activate specific genes (*5*).

We cloned a protein named PIAS1, which can specifically interact with Stat1 (another member of the STAT family), by the yeast two-hybrid assays (*6*). We searched the expressed sequence tag (EST) database for other PIAS family members and identified a human EST clone encoding a polypeptide related to the COOH-terminal portion of PIAS1 (*7*). We obtained a full-length cDNA containing an open reading frame of 583 amino acids by screening a mouse thymus library with the human EST clone (*8*). The corresponding protein, named PIAS3, contains a putative zinc-binding motif [C_2-$(X)_{21}$-C_2] (*9*), a feature conserved in the PIAS family (Fig. 1A). Northern (RNA) blot analysis indicated that PIAS3 is widely expressed in various human tissues (Fig. 1B).

To study the function of PIAS3, we prepared a specific antiserum (anti-PIAS3c) to a recombinant fusion protein of glutathione-S-transferase (GST) with the 79 COOH-terminal amino acid residues of PIAS3. This antibody detected a protein with a molecular mass of about 68 kD, the predicted size of PIAS3, in both cytoplasmic and nuclear extracts of a number of human and murine cell lines (*10*). To identify which STAT protein interacts with PIAS3, we prepared protein

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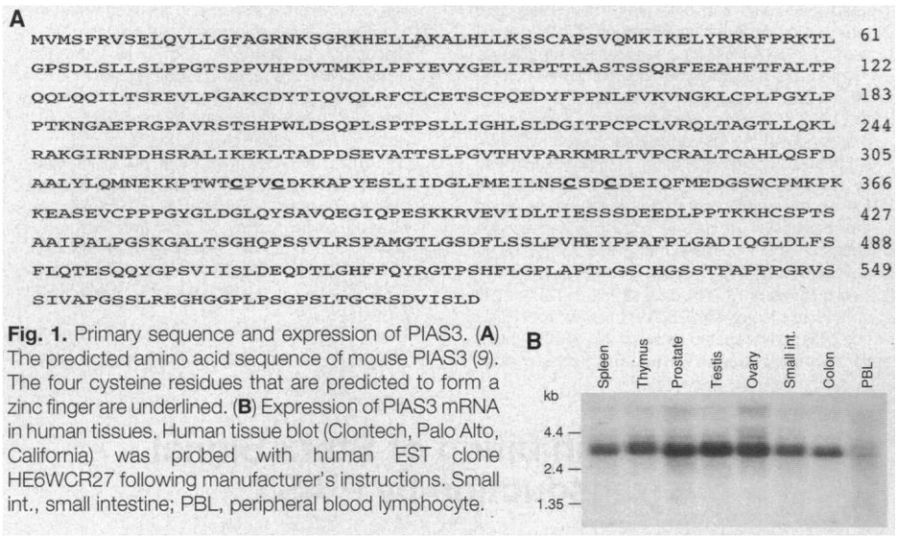

Fig. 1. Primary sequence and expression of PIAS3. (**A**) The predicted amino acid sequence of mouse PIAS3 (*9*). The four cysteine residues that are predicted to form a zinc finger are underlined. (**B**) Expression of PIAS3 mRNA in human tissues. Human tissue blot (Clontech, Palo Alto, California) was probed with human EST clone HE6WCR27 following manufacturer's instructions. Small int., small intestine; PBL, peripheral blood lymphocyte.

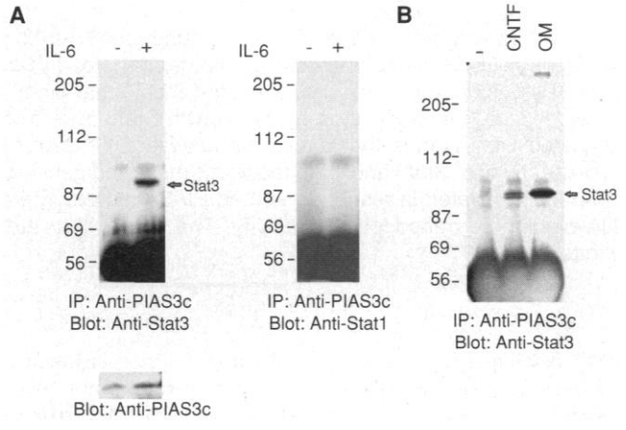

Fig. 2. The in vivo interaction of PIAS3 with Stat3. (**A**) Treatment with IL-6 induced the interaction of PIAS3 with Stat3. Protein extracts from M1 cells, untreated (−) or treated with IL-6 for 10 min (+), were subjected to immunoprecipitation (IP) with anti-PIAS3c. The blot was probed with anti-Stat3 (Santa Cruz Biotechnology, Santa Cruz, California) (top left). The same blot was then reprobed with anti-PIAS3c (bottom left). The filter was washed and reprobed with anti-Stat1 (right). (**B**) Treatment with CNTF or OM induces the interaction of PIAS3 with Stat3. Protein extracts from human HepG2 cells, untreated or treated with CNTF or OM for 10 min, were subjected to immunoprecipitation with anti-PIAS3c. The blot was probed with anti-Stat3. Whole-cell extracts were prepared with lysis buffer containing 1% Brij, 50 mM tris (pH 8), 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), aprotinin (3 μg/ml), pepstatin (1 μg/ml), and 0.1 mM sodium vanadate. The mixture was rotated at 4°C for 30 min and centrifuged at 13,000*g* for 5 min. The supernatant was used for immunoprecipitation with anti-PIAS3c (1:100 dilution). Immunoprecipitation and protein immunoblotting were done as described (*14*).

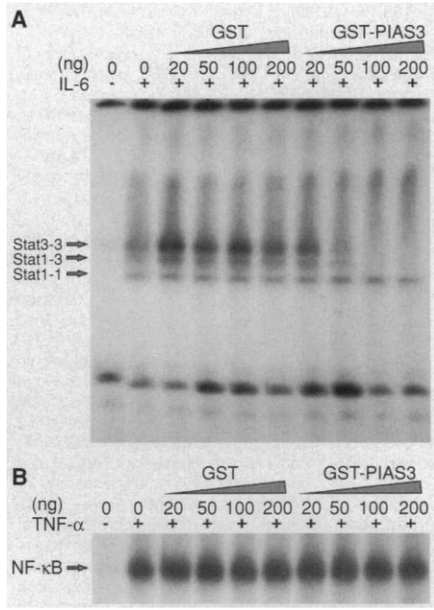

Fig. 3. Inhibition of the DNA-binding activity of Stat3 by PIAS3. (**A**) Electrophoretic mobility shift assays were performed with nuclear extracts prepared from HepG2 cells with (+) or without (−) IL-6 treatment in the absence or presence of various amounts of either GST or GST-PIAS3 proteins (20 to 200 ng) as indicated. Mobility shift assays were done as described (*14*). The probe used is a high-affinity Stat3-binding site to which both Stat1 and Stat3 can bind (*1*, *15*). Stat1-1, Stat1 homodimer; Stat3-3, Stat3 homodimer; Stat1-3, Stat1 and Stat3 heterodimer. (**B**) Same as (A), except that nuclear extracts were prepared from MCF7 cells with (+) or without (−) TNF-α treatment. The probe was derived from the NF-κB-binding site in the promoter of the NF-κB inhibitor I-κB gene (*15*). GST-PIAS3 was constructed by insertion of the cDNA into the Sal I and Not I cloning sites of pGEX4T-1 (Pharmacia). The concentration of GST-PIAS3 was estimated on 7% SDS–polyacrylamide gel electrophoresis with various dilutions of bovine serum albumin as the standard.

extracts from murine myeloblast M1 cells, which were untreated or treated with IL-6. Proteins immunoprecipitated with anti-PIAS3c were analyzed by protein immunoblot with anti-Stat3. Stat3 was present in a PIAS3 immunoprecipitate from IL-6–treated M1 cells but not in an immunoprecipitate from untreated M1 cells (Fig. 2A). A reblot of the filter with anti-PIAS3c showed that similar amounts of PIAS3 were present in each lane. IL-6 stimulation can induce tyrosine phosphorylation of Stat1 as well as Stat3 (*10*, *11*). The protein blot was therefore washed and reprobed with antibody to Stat1. Stat1 was not present in PIAS3 immunoprecipitates (Fig. 2A). Furthermore, PIAS3 was not found to be associated with Stat1 in a number of cell lines treated with interferon γ (*10*). These results indicate that PIAS3 specifically interacts with Stat3.

Stat3 can be activated by other cytokines in the IL-6 family, such as ciliary neurotrophic factor (CNTF) and oncostatin M (OM) (*2*). In human HepG2 cells, Stat3 was associated with PIAS3 in cells stimulated with CNTF or OM but not in untreated cells (Fig. 2B).

Tyrosine-phosphorylated Stat3 binds to a specific DNA sequence in its target genes (*1*, *2*). We tested the effect of PIAS3 on the DNA-binding activity of Stat3. Nuclear extracts from HepG2 cells were prepared and analyzed in mobility gel shift assays, with a high-affinity Stat3-binding site as the probe (*1*, *11*). Treatment with IL-6 induced the binding of three distinct gel shift complexes (*1*, *11*) corresponding to a Stat3-Stat3 homodimer, a Stat3-Stat1 heterodimer, and a Stat1-Stat1 homodimer (Fig. 3A). We prepared and purified a recombinant fusion protein of GST with PIAS3 (GST-PIAS3) and added it (in 20- to 200-ng quantities) to IL-6–treated HepG2 nuclear extracts. GST-PIAS3 (100 ng) completely inhibited the DNA-binding activity of the Stat3-Stat3 homodimer and the Stat3-Stat1 heterodimer (Fig. 3A) but had no effect on the DNA-binding ability of the Stat1-Stat1 homodimer. As a control, GST alone did not inhibit the DNA-binding ability of any of the three complexes. A similar inhibitory effect of PIAS3 on the DNA-binding activity of Stat3 was observed in nuclear extracts

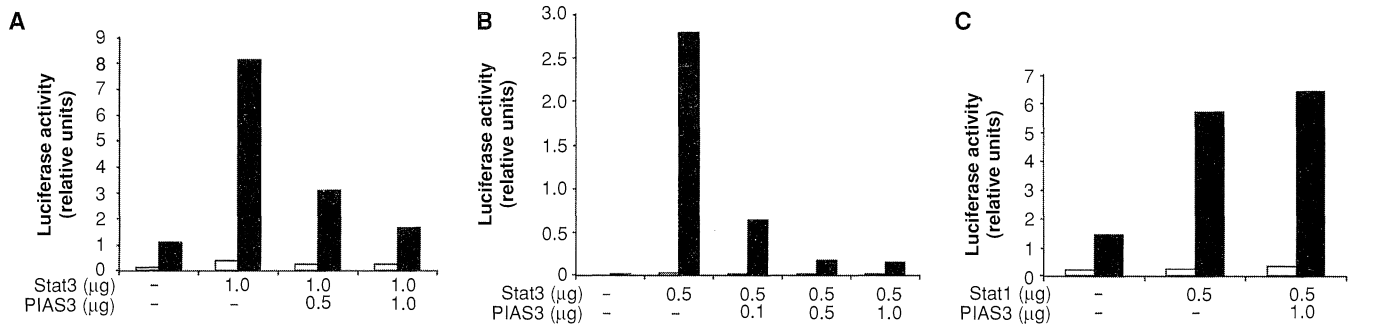

Fig. 4. The effect of PIAS3 on STAT-mediated gene activation. (**A**) Inhibition of Stat3-mediated gene activation in response to IL-6. HepG2 cells were transiently transfected with (4×)IRF-1 luciferase reporter construct together with an empty expression vector, Stat3, or various amounts of FLAG-PIAS3 vectors, alone or in combination as indicated. Twenty-four hours after transfection, cultures were either left untreated (open columns) or treated with IL-6 (10 ng/ml) (R&D Systems, Minneapolis, Minnesota) for 6 hours (solid columns), and cell extracts were prepared and measured for luciferase activity (Promega, Madison, Wisconsin). (**B**) Inhibition of Stat3-mediated gene activation in response to IFN-α. Human 293 cells were transfected with (4×)IRF-1 luciferase reporter construct together with Stat3 or PIAS3 (or both) as indicated. Twenty-four hours after transfection, cells were left untreated (open columns) or treated with IFN-α (5 ng/ml) (Hoffmann–LaRoche, Nutley, New Jersey) for 6 hours (solid columns), and luciferase activity was determined. (**C**) The effect of PIAS3 on Stat1-mediated gene activation. Same as (B), except that Stat3 was replaced with Stat1 in cotransfection assays. FLAG-PIAS3 was constructed by insertion of the cDNA into the Sal I and Hind III sites of pCMV5-FLAG. HepG2 cells were transfected by a modified calcium phosphate method (*16*). Cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 25-hydroxycholesterol (2.5 μg/ml) and were maintained at 35°C and 3% CO_2 for 3.5 hours during transfection. Two hundred ninety-three cells were transfected by the calcium phosphate method (*17*). Data shown are taken from one representative experiment and were repeated at least three times. The relative luciferase units were corrected for relative expression of β-galactosidase.

prepared from IL-6–treated M1 and MCF7 cells (*10*). To further demonstrate the specific inhibitory effect of GST-PIAS3 on Stat3, we tested the effect of GST-PIAS3 on the DNA-binding activity of nuclear factor kappa B (NF-κB). Nuclear extracts prepared from untreated MCF7 cells or MCF7 cells treated with tumor necrosis factor–α (TNF-α) were analyzed by mobility gel shift analysis with an NF-κB–binding site as the probe. TNF-α induced the formation of an NF-κB gel shift complex. The presence of either GST or GST-PIAS3 had no effect on the DNA-binding activity of NF-κB (Fig. 3B). We conclude that PIAS3 can specifically inhibit the DNA-binding activity of Stat3.

To test the effect of PIAS3 on Stat3-mediated gene activation, we transiently transfected HepG2 cells with expression vectors encoding Stat3 and FLAG-tagged PIAS3. Interleukin-6 can induce the association of PIAS3 with Stat3 in HepG2 cells (*10*). A luciferase reporter construct [(4×)IRF-1] containing four copies of the STAT-binding sequence from the interferon regulatory factor–1 (IRF-1) gene was used (*12*). Cotransfection of Stat3 with (4×)IRF-1 resulted in about 20-fold stimulation of luciferase expression when cells were treated with IL-6 (Fig. 4A). In the presence of various amounts of PIAS3 (0.5 μg and 1 μg), Stat3-mediated induction of luciferase expression in response to IL-6 stimulation was inhibited (Fig. 4A). We also performed luciferase assays in human embryonic 293 cells. Interferon α (IFN-α) stimulation can activate Stat3 in 293 cells (*10*, *12*). Cells cotransfected with Stat3 and (4×)IRF-1 reporter construct showed a 150-fold increase of luciferase expression in response to IFN-α (Fig. 4B). In the presence of PIAS3 (1 μg), however, the IFN-α–induced, Stat3-dependent gene activation was almost completely inhibited. PIAS3 (1 μg) had no such inhibitory effect on Stat1-mediated transcription activated in response to IFN-α (Fig. 4C). These results are in accord with our findings that PIAS3 does not interact with Stat1 or inhibit its DNA-binding activity and indicate that PIAS3 is a specific inhibitor of Stat3-mediated gene activation.

Recently, a family of cytokine-inducible inhibitors of STAT signaling has been reported (*13*). Members of this family of proteins, named SOCS, JAB, or SSI, are relatively small protein molecules that contain mainly SRC homology 2 domains. One member of this family, SOCS-1, can bind to JAK1, JAK2, JAK3, and Tyk2. Thus, SOCS-1 may function as a general inhibitor for JAK-STAT signaling pathways through the inhibition of the tyrosine kinase activity of JAKs. The identification of a PIAS protein that can directly inhibit STAT function indicates that JAK-STAT signaling pathways can be suppressed at multiple steps, in a general or specific manner. It seems that the overall strength of STAT signaling for a given cell type may be largely affected by the relative level of STAT and PIAS protein expression.

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7. The database search was done with the Baylor College of Medicine Search Launcher. The name of this EST clone is HE6WCR27 (GenBank accession number H58757).
8. The nucleotide sequence of murine PIAS3 has been submitted to GenBank (accession number AF034080).
9. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr.
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