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Sp2 regulates interferon-γ-mediated *socs1* gene expression

Martine Letourneur[1], Lyne Valentino[1], Julia Travagli-Gross, Jacques Bertoglio, Josiane Pierre*

INSERM U749, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry, France

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ABSTRACT

Suppressor of cytokine signalling (SOCS) proteins are inducible feedback inhibitors of Janus kinase (JAK) and signal transducers and activators of transcription signalling (STAT) pathways. Interferon (IFN)-γ induces the expression of the *socs1* gene in several cell types through several cis elements present in its promoter and their binding proteins. *Socs1* expression is induced in the human keratinocytes HaCaT cell line through sequential activation of STAT1 and IRF-1. Comparison of the 5′-upstream sequences of the mouse and human *socs1* genes identified conserved binding sites for IRF-1 regulatory elements. Although this response element is able to bind IRF-1 in human cells, no IFN-γ responsiveness was observed with human *socs1* promoter reporter constructs containing this element. In contrast the mouse *socs1* promoter was fully responsive. The mouse promoter contains two cis-acting elements which modulate its expression and are recognized by IRF-1 and Sp2. Despite the absence of Sp2 in the 5′-upstream sequence of the human promoter, silencing of Sp2 by RNA interference clearly demonstrated that Sp2 is required for IFN-γ-induced regulation of *socs1* mRNA both in human and mouse.

1. Introduction

Interferons (IFN) are multifunctional cytokines that have antiviral, antiproliferative, and immunomodulatory effects (for review see Stark et al., 1998). IFN are used in the clinical management of malignant tumours, multiple sclerosis and chronic viral hepatitis (Baffis et al., 1999; Gresser, 1997). Type II interferon (IFN-γ) is a pleiotropic cytokine produced by activated T cells and NK cells, and is the most potent activator of the proinflammatory functions of keratinocytes. In fact, IFN-γ-activated keratinocytes express a broad array of chemokines, cytokines, and membrane molecules that direct the recruitment, activation, and retention of specific leukocytes in the skin (Albanesi et al., 1999, 2001; Federici et al., 2002). The reciprocal activation of T lymphocytes and keratinocytes has a primary role in the amplification of skin inflammation during immune-mediated skin diseases.

IFN-γ receptors consist of two ligand-binding IFNGR1 chains associated with two signal-transducing IFNGR2 chains. Receptor activation triggers JAK kinases activation which in turn the STAT1 transcription factors. After dimerization, activated STAT1 translocates to the nucleus and induces target gene transcription by binding to γ-activated sequences (GAS) in the promoters of IFN-γ-responsive genes (Darnell, 1997; Darnell et al., 1994). Interferon-regulated factors (IRFs) were also identified as transcriptional regulators of IFN-stimulated genes. Transcription of the *irf-1* gene is inducible by both IFN-α and IFN-γ (Haque and Williams, 1994; Pine et al., 1994; Sims et al., 1993) and this inducible transcription factor binds to an interferon regulatory factor binding element (IRF-E), AANNGAAA which is also a consensus sequence for IRF-2 (Tanaka et al., 1993).

Suppressors of cytokine signalling (SOCS) are a family of proteins that act in a feedback loop to inhibit cytokine responses and activation of the JAK/STAT pathway (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). SOCS1 is one of the eight members of the SOCS gene family which is characterized by a C-terminal SOCS box region and a central phosphotyrosine-binding Src homology 2 domain (Hilton et al., 1998). The SH2 domain of SOCS proteins interacts with phosphorylated tyrosine residues in tyrosine kinases and, in many cases, negatively regulates their activity (Krebs and Hilton, 2001; Yasukawa et al., 2000). The SOCS boxes of SOCS1 and SOCS3 have been found to mediate interaction with elongin B/C complex and to target SOCS1 interacting proteins for proteasomal-mediated degradation via ubiquitinylation; such proteins include JAK2 (Ungureanu et al., 2002), Tel-JAK2 (Frantsve et al., 2001; Kamizono et al., 2001), p65 NF-κB (Ryo et al., 2003), Vav (De Sepulveda et al., 2000), FAK (Liu et al., 2003) and Mal (Mansell et al., 2006). SOCS1 is a critical regulator of IFN-γ signalling since hyper-responsiveness to IFN-γ was found in *socs1* deficient mice (Alexander et al., 1999; Cornish

Abbreviations: GAS, γ-activated sequence; IFN, interferon; IFNGR, IFNγ receptor; IL-4 RE, IL-4 responsive element; IRF, IFN-regulated factor; IRF-E, IRF-binding element; SBE, STAT6-binding element.

* Corresponding author. Tel.: +33 146835483; fax: +33 146835496.
E-mail address: josiane.pierre@u-psud.fr (J. Pierre).

[1] Authors contributed equally to this work.

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et al., 2003). Mice lacking the gene for both *socs1* and *ifn* develop chronic inflammatory lesions in kidney, skin and various other organs (Metcalf et al., 2002). Keratinocytes are primary target of IFN and after IFN exposure they synthesize numerous inflammatory cytokines involved in the initiation of skin diseases (Albanesi et al., 2005). Keratinocytes also express *socs1* to minimize consequences of IFN treatment.

Regulation of *socs1* gene expression occurs at several levels. The silencing of *socs1* gene by hypermethylation has been reported and is associated with the development of hepatocellular carcinoma (Yoshikawa et al., 2001) and was then further found in several other cell lines. Transcription of *socs1* mRNA is rapidly induced by various cytokines. Direct transcriptional repression has been described either for the transcriptional repressor GFI-1B (Jegalian and Wu, 2002) or for the transcription factor, ETS-1 (Travagli et al., 2004). *Socs1* expression is also controlled through translational repression (Gregorieff et al., 2000; Schluter et al., 2000).

Although the molecular bases of SOCS1 activity and regulation by various cytokines have been extensively investigated, limited information exists on its transcriptional regulation by IFN-γ, especially concerning the human promoter. We therefore examined the IFN-γ-activated transcription factors implicated in regulating *socs1* human promoter in comparison with the mouse promoter. Comparative gene analysis is a powerful approach to gain insight into gene regulation and function. To this end, we cloned the *socs1* promoter regions of mouse and human origin upstream a luciferase reporter gene. Functional promoter analyses using reporter assays revealed that the presence of tandem repeats of the core sequences of IRF-E, observed in the mouse promoter lead to a better IFN-γ responsive promoter. In addition we have characterized a putative Sp2 binding site just in front of the IRF-E. Specific inhibition of Sp2 expression by RNA interference inhibits *socs1* mRNA expression. These results demonstrate a decisive role of Sp2 in regulating *socs1* expression.

2. Materials and methods

2.1. Cell culture

The human keratinocyte cell line HaCaT was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics (50 μg/ml penicillin and 50 μg/ml streptomycin), with 1 mM sodium pyruvate and 10% foetal calf serum (FCS).

2.2. Cytokine, antibodies, and reagents

Human recombinant IFN-γ, kindly provided by J. Wietzerbin, was added to the culture media at a final concentration of 10^3 U/ml. Anti-STAT1 (sc-346), anti-IRF-1 (sc-497), anti-IRF-2 (sc-498), anti-Sp2 (sc-643) polyclonal antibodies and Sp2 siRNA (sc-29489) were from Santa-Cruz. Control siRNA (#1027310) was from Qiagen. HA-IRF-1 and HA-Sp2 cDNAs were generous gift of Drs. I. Dusanter-Fourt and J.M. Horowitz, respectively. Plasmid encoding wt socs-1 promoter (−743/+690) luciferase construct was a generous gift of D. Hebenstreit.

2.3. PCR analysis

Total RNA was extracted with Trizol (Life technologies, Gaithersburg, MD, USA) as described by the manufacturer and quantified at OD 260 nm and further treated with DNase from Roche. 2 μg of total cellular RNA was reverse-transcribed using an oligo dT primer and 2 units of AMV reverse transcriptase (Promega, Madison, WI, USA) and then used as template for PCR. The specific primers used and PCR reactions were performed as described (Travagli et al., 2004). 1 μl of RT was amplified with the *socs1*-specific primers and GAPDH-specific primers and analyzed by real time PCR using a Light Cycler (Roche). Results obtained with *socs1*-specific primers were normalized against GAPDH.

2.4. Plasmids constructs

The promoter region of mouse genomic *socs1* DNA (−952/+44) was constructed by PCR from the PCR2-*socs1* plasmid (generous gift of P. De Sepulveda). The proximal 960 bp and 177 bp fragments of the mouse promoter were cloned into the pGL3 basic vector containing the firefly luciferase reporter gene (luc). Each promoter fragment was amplified by polymerase chain reaction (PCR) using unique forward primers and a common reverse primer that ends 45 nucleotides after the start of transcription previously described (Saito et al., 2000), cut by restriction enzymes (listed in Table 1, part A) and then inserted at the same site into the pGL3 basic vector. Sequences of all promoter constructs were verified by sequencing. The numbering of all constructs begins at the start of transcription.

The −590 h*socs1* and −170 h*socs1* were generated from −750 h*socs1* previously described (Travagli et al., 2004) by PCR amplification using forward primers described in Table 1, part A. The PCR fragments were cut by appropriate restriction enzymes and inserted at the same site into pGL3 basic vector.

Site-specific mutations in either the mouse or human *socs1* promoter were performed using the quick change site-directed mutagenesis kit (Stratagene) by using the oligonucleotides described in Table 1, part B.

2.5. Chromatin immunoprecipitation (ChIP) analysis

ChIP experiments were performed as described (Travagli et al., 2004). The primers used for PCR amplification are described in Table 1, part C.

2.6. Immunoprecipitation and Western blotting

After stimulation by IFN-γ (10^3 U/ml), cells were lysed in a buffer containing 50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM NaPP, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin. The cell lysates were clarified by centrifugation. 500 μg of total protein was incubated with appropriate antibodies overnight and with protein G-Sepharose for 1 h at 4 °C. The immunoprecipitates were separated through 8% SDS-PAGE and proceeded for Western blotting as described (Travagli et al., 2004).

2.7. Electrophoretic mobility shift assays (EMSA)

HaCaT cells were stimulated with IFN-γ during 30 min or 2 h, scrapped in PBS supplemented with 1 mM Na_3VO_4, and pelleted. Nuclear extracts, radiolabeled double-strand oligonucleotides were prepared and EMSA experiments performed as previously described (Travagli et al., 2004). The ^{32}P labeled probes used in EMSA are described in Table 1. Supershift experiments were performed using the anti-STAT1, anti-IRF-1, anti-IRF-2 or anti-Sp2-specific antibodies. Oligonucleotides used as probes in EMSA assays contained the same mutated nucleotides as those used to introduce the mutations in reporter plasmids.

2.8. Transfection experiments

For transient transfection, cells were seeded at 4×10^5 cells per well in 6-well dishes 24 h before transfection. The different plasmids DNA were adjusted to 3 μg of DNA and 6 μl of jet-PEI solution (Polyplus transfection, Illkirch, France) in serum-free culture medium. After 16-h incubation, cells were stimulated or not with