GENOMIC ORGANIZATION AND CYTOKINE-MEDIATED INDUCIBILITY OF THE HUMAN TRIM-8/GERP GENE

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Cytokine signaling is negatively regulated by a set of SH2 domain-containing proteins, the Suppressors of Cytokine Signaling (SOCS) acting as intracellular modulators. Experimental evidence indicates that SOCS gene expression is induced by cytokines and pro-inflammatory stimuli and is highly controlled both at transcription and translation level. Furthermore, SOCS proteins appear rapidly degraded inside the cells, mostly controlling their stability by interacting with specific molecules such as elongin B and C. It has been shown that SOCS-1/JAB, a member of the SOCS family, interacts with TRIM-8/Gerp, a new ring protein specifically binding SOCS-1 recombinant polypeptide in-vitro and in-vivo. Trim-8/Gerp, transcribes a 3.0-kb mRNA, spans 551 AA and is highly conserved during evolution. In addition, it can be induced by IFN-γ in epithelial and lymphoid cells and is expressed mostly ubiquitously in murine and human tissues. Here in this report we present the genomic organization of this new SOCS-1 interactor, and we add new tools for extending investigation of the complex mechanism that undergoes negatively regulation of cytokine signaling.

Cytokines including Interleukins (ILs) and Interferons (IFNs) control a set of different cell functions: proliferation, differentiation and immunomodulatory events (1-3). Moreover, they are actively involved in the pathophysiology during viral infections, play a central role in the development of normal hemopoietic system and in the pathogenesis of autoimmune diseases. (4,5). Cytokines partecipate to a complex network of transduction mechanisms by interacting with their cognate receptors, expressed on the surface of target cells, which usually lack intrinsic protein tyrosine kinase (PTKs) activity (6). Subsequently, cytokine signaling triggers the activation of a set of cytoplasmic proteins, members of the Jak family of PTKs, which associate to the cytokine-bound receptor and activate the family of transcription factors STATs (6).

Being the cytokine effect restricted in intensity and duration, a complex mechanism of negative feedback regulation was proved to prevent the deleterious consequences of excessive cytokine signaling. Principally, the protein tyrosine phosphatases such as SHP-1 and SHP-2 have shown to play a role by dephosphorylating activated Jak kinases complexes and attenuating cytokine response. In the last few years, another mechanism of negatively regulation of cytokine activity has been widely studies and a cohort of new protein members associated to such events have been cloned and characterized. These new polypeptides are termed Suppressor of Cytokine Signaling or SOCS-1 and represent a bunch of adapter molecules featured by a central SH-2 interacting domain and a conserved carboxy-terminal motif, namely the SOCS box.(7-12)

Experimental data have pointed out the followings: (i) SOCS molecules can bind cytokine transducers as Jak tyrosine kinases inhibiting their activity in-vitro and in-vivo (13-15); (ii) deregulated

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expression of SOCS protein perturbs cytokine-related cellular proliferation and hematopoietic differentiation in murine and human organs (16,17). Finally, mice lacking functional SOCS-1, one of the members of the gene family, have monocyte and polymorphonuclear infiltration of different organs and liver ipoinsufficiency with marked signs of fatty degeneration and epatocyte apoptosis. As a hallmark of the network of regulation, SOCS mRNA and proteins are induced by cytokines at early times and are thought to be controlled either at transcription or translation level (18-21). Control of initiation of translation and protein stability is the main features of such regulation. In particular endogenous SOCS protein are barely detected in cytokine-treated cellular extracts (20) and it has been shown that their stability can be much improved by specific interaction with elongin B and C at the SOCS box domain.

Recently a new Ring protein, termed TRIM-8/Gerp, has been cloned and associated as a specific interactor of SOCS-1.

In this paper, we present the genomic organization of TRIM-8/Gerp. The locus spans 12 kb and is organized in 7 exons with a first untranslated leader sequence. Analysis of the 5' of the gene and of putative promoter region give evidence of GAS like consensus elements which might modulate the responsiveness to the IFN-γ. The protein is overall induced by cytokines, in particular IFN-γ, in murine and human epitheloid and lymphoid cells and is highly conserved during evolution.

MATERIALS AND METHODS

Molecular hybridization

Screening of a murine pre-B full-length c-DNA library was performed according to standard procedures. Briefly 10^6 recombinant phages were plated using competent host and grown O/N at 37 C. Plaques were transferred onto nitrocellulose filters (Shoe & Shoeless,), fixed by UV crosslinking and hybridized into 50% formamide, 5X SSC (1X SSC = 0.15 Mol/L sodium chloride, 0.015 Mol/L sodium citrate), 0.02 Mol/L sodium phosphate, 10% dextran sulfate at 42 °C for 16 hrs with a ^{32}P-labeled c-DNA fragment. p50, corresponding to 612 bp of the 5' coding portion. Filters were subsequently washed and exposed at -80 for two days using intensifying screen. Autoradiograms were obtained and used for phage isolation and further purification.

Generation of recombinant TRIM-8 expression vectors

Plasmid p50 was obtained inserting a Bam-Bgl II 612 bp fragment from the original pGAD-NOT containing the Ring domain of TRIM-8/Gerp into the Bam HI site of the pcDNA3.1 (HisC)-Xpress tagged vector (Invitrogen, San Diego, CA).

The Ring- TRIM-8/Gerp construct was obtained amplifying by PCR a 240 bp segment from the TRIM-8/Gerp full length c-DNA template using primers: TRIM-8/Gerp -Bam.consF1 and R3 (derived at the beginning and at +225 from translation initiation site, respectively). Subsequently, amplified products were inserted into the pCR2.1 PCR vector (Invitrogen, San Diego, CA) and checked for proper insertion by gel analysis. *Bona fide* clones were cut with Bam-RI and inserted into the Bam-RI site of pcDNA 3.1-HisC-Bam-RI vector linked in-frame with the Xpress epitope.

For SOCS constructs the pcDNA3.1-Xpress tagged was used as well, but SOCS sequences were inserted with an additional HA epitope.

Northern analysis

Northern blot was performed as previously described (31 - 33). Briefly 20 ug of total RNA were fractionated in a formaldehyde-denaturing agarose gel and transferred onto Hybond nylon filters (Duralon, Stratagene, CA). Hybridization was in 50% formamide, 5X SSC (1X SSC = 0.15 Mol/L sodium chloride, 0.015 Mol/L sodium citrate), 0.02 Mol/L sodium phosphate, 10% dextran sulfate at 42 °C for 16 hrs with a ^{32}P-labeled c-DNA (pC31) fragment corresponding to the 3' unique sequence of p202 gene. Autoradiograms were developed over-night and/or after three days of exposure.

Transfections

For functional studies transfection were performed in the human HeLa cell line, using the lipofectamine plus procedure according to the supplier (GIBCO-BRL, Gaithersburg, MD).

Between 3 – 5 μg of plasmid + 1 μg of XP-

LacZ vector as internal control and a carrier plasmid up to 6 ug maximum were used. A Lipofectamin based transfection was applied to HeLa cells according to supplier's standard procedures. Next day, cells were washed three times with serum-free DMEM and supplemented with fresh medium for additional 8 hrs before cell harvesting.

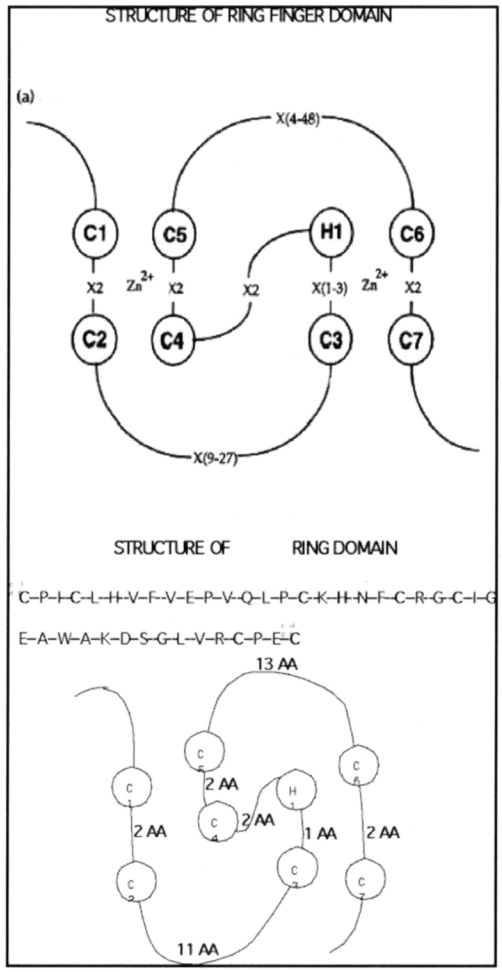
Luciferase assay

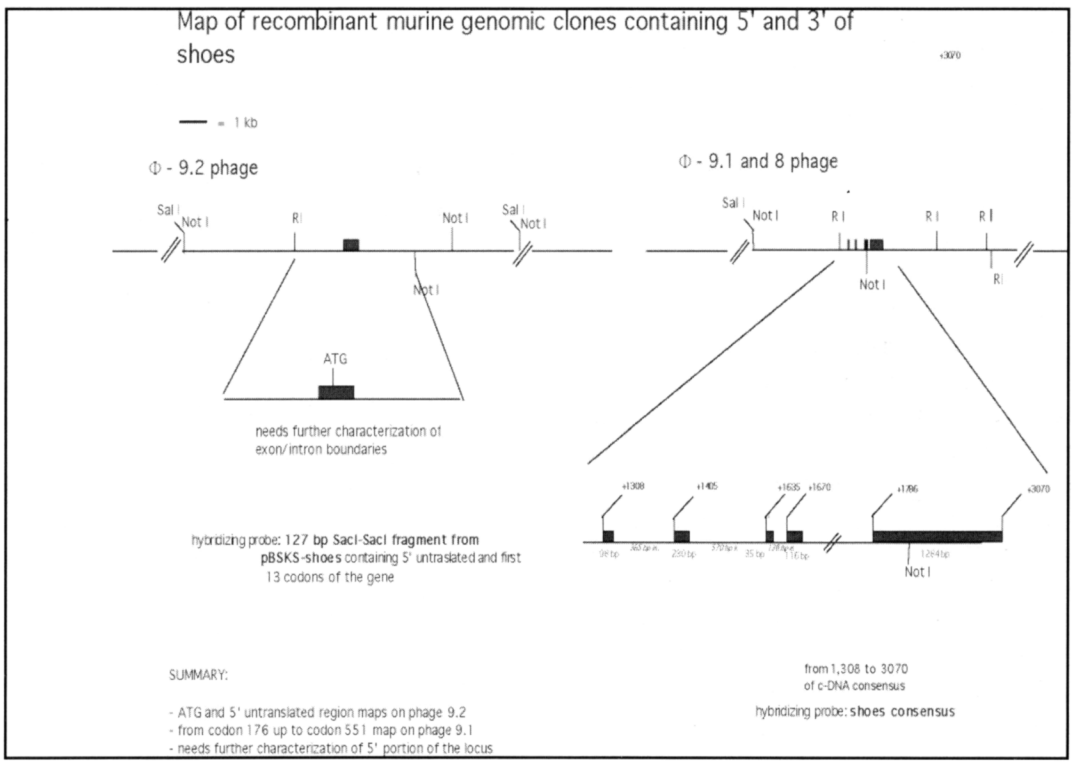
Luciferase assay was performed as described (15) with some modifications: 24 hrs after transfection cells of each transfection were split into half. One half was treated with human IFN-γ and the other half serve as control. Cells were harvested 18 hr after treatment and luciferase activity was measured.

RESULTS

Cloning and characterization of the Genomic TRIM-8/GERP locus

In order to give completion of the partial TRIM-8-DNA segment pulled out by the recombinant yeast vector, we first used the subcloned pc50 fragment as a probe for screening a murine





pre-B lymphoid full-length c-DNA library. Approximately 2 X 10^6 recombinant phages were hybridized with ^{32}P-labelled p50 and few positive clones re-hybridized, further purified and isolated. A couple of *bona fide* recombinants were obtained containing the most 5' part of the gene with 821bp of untranslated region (UTR) and first ATG translation initiation site. Subsequently, several murine and human EST (Expressed Sequence Tags) were identified at high score of alignment using the BlastP program for sequence search in the Genebank (NCBI, NIH). Some of those characterized and sequenced allowed elongation at the 3' portion of the TRIM-8 transcript with several PCR cycles and we obtained several clones encompassing the Not I site and extending more 3'. The cloning and further sequencing of the RACE products denoted the complete c-DNA consensus of TRIM-8 gene resembling the endogenous transcript, giving a 551 AA of predicted polypeptide sequence and a 700 bp 3' untranslated region with two putative non-canonical (AGAAA) polyadenylation sites.

Furthermore, we use EST and bona fide sequenced c-DNA clones in order to arrange the genomic organization of the murine TRIM-8/GERP locus.

Accordingly to identified genomic map related to Gerp/TRIM-8 locus, the gene spans approximately 12 kb and is organized as shown on Fig.1.

Functional role of Shoe in the IFN-γ mediated biological response

Next, in order to test a functional role of Shoe in the IFN-γ dependent signal transduction, we co-transfected the human HeLa cells with a GAS-Luc reporter plasmid together with SOCS-1 and/or TRIM-8 expression vectors. As detailed on Fig. 4 and 5 tiny amount of SOCS-1 as little as 0.25 ug is able to inhibit the IFN-γ induced transcription of GAS-Luc on HeLa recipient cells. However, the co-incubation of p50 expressing a partial TRIM-8 polypeptide able to bind SOCS-1 counteract such inhibition, stating that at least a antagonist effect of Shoe attenuates the potent SOCS-1-mediated inhibition of the cytokine response. In addition, different amounts of TRIM-8 p50 protein act on SOCS-1 in a typical dose-dependent manner being the relative mass of the protein a critical component of the signaling regulation.

DISCUSSION

On this report we have shown the genomic cloning and characterization of a new Ring interactor, namely TRIM-8/GERP, capable to bind specifically the Suppressor of Cytokine Signaling–1 (SOCS-1) in-vitro and in-vivo. TRIM-8/GERP is expressed almost ubiquitously in murine and human tissues and detects two transcripts, which are both regulated by IFN-γ nd IL-4 in a different cohort of epithelial ad lymphoid cell lines. The new gene has an ORF of 551 amino acid with a predictive molecular weight of 60 KD, whose sequence appears to be highly conserved during evolution suggesting an important role of TRIM-8 as modulator and regulator of evolutionarily conserved signal transduction pathways. In spite of the intrinsic capacity of TRIM-8/Gerp to act as a partner of SOCS-1, a similar scenario evicts a possible complex mechanism undergoing cytokine response regulation. The SOCS proteins, indeed, work as cytoplasmic inhibitors of signaling triggered by cytokine/receptor interactions. In particular regulation of SOCS-1 has always been difficult to define because of the intrinsic instability of the protein and the difficulty to detect stable SOCS-1 complexes by standard assays. Moreover, the genomic organization of TRIM-8/Gerp reflects a distribution of exons that stand in two different portion along the 10q24.3 chromosome site. The first two exons are more than 10 kb apart from the remaining portion of the gene with the second exon coding for the NH-2 terminal portion of the Ring domain. Such long site maps within the fragile locus on 10q24.3 that has been associated to rearrangement and deletions found on different tumoral cells in particular lymphoma and glioblstomas. The feature of such location for Gerp/TRIM-8 may suggest that the SOCS-1 interactor could play a role in tumorigenesis since the identity of the coding part of the gene may be disrupted during tumoral development. In addition SOCS-1 has been associated to specific methylation on the regulatory region resulting in silencing and loss of negatively regulation of proliferativo events.

The effort could be at this stage to structurally investigate the TRIM-8/Gerp locus in tumor or related diseases.

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