TRIM8/GERP  RING  Finger Protein Interacts with SOCS-1

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Running title: TRIM8/GERP, a RING Finger Protein that Interacts with SOCS-1
Summary

Members of the SOCS family of signaling molecules regulate the activation of cytokine signaling. Experimental evidence indicates that SOCS expression is induced by cytokines and pro-inflammatory stimuli and is controlled at both the transcriptional and post-transcriptional levels. SOCS proteins are unstable and appear to be rapidly degraded by proteasomal pathways. However, the mechanisms by which SOCS protein levels are regulated remain unclear. Here we show that TRIM8/GERP, a RING finger protein interacts with SOCS-1 in vitro and in vivo. TRIM8/GERP, previously identified as a new member of the family of proteins containing a Tripartite motif (TRIM), is a 551 amino acid RING finger protein conserved across species. TRIM8/GERP expression can be induced by IFN-γ in epithelial and lymphoid cells. Co-expression of TRIM8/GERP with SOCS-1 decreases SOCS-1 protein stability and levels. Functionally, expression of TRIM8/GERP decreases the repression of IFN-γ signaling mediated by SOCS-1. These data suggest that TRIM8/GERP may be a regulator of SOCS-1 function.
INTRODUCTION

Cytokines control many different cellular functions including proliferation, differentiation, and gene expression (1, 2). Moreover, they participate in the pathophysiology of viral infections, play a central role in the development of the hematopoietic system, and have been implicated in the pathogenesis of autoimmune diseases (3, 4). The biologic response of a cell to cytokines involves a complex network of signal transduction machinery. Signaling is initiated by the oligomerization of cognate cytokine receptors expressed on the surface of target cells (5), which triggers the activation of members of the JAK family of protein tyrosine kinases (PTK) that constitutively associate with the cytokine receptor. Subsequently, JAKs can phosphorylate tyrosine residues present in the cytoplasmic regions of the receptors. These phosphorylated tyrosines then act as docking sites for signaling molecules, such as members of the STAT family of transcription factors (6).

It appears that the intensity and duration of cytokine signaling is regulated by several mechanisms. It is now known that at least three different classes of negative regulators contribute to cytokine inhibition: (i) the SH2-containing protein tyrosine phosphatases 1 and 2, (SHP-1 and SHP-2) (7, 8); (ii) the protein inhibitors of activated STATs (PIAS) and (iii) the Suppressor of Cytokine Signaling (SOCS protein) (9-13). Experimental data suggest that: (i) SOCS genes are induced by cytokines; (ii) SOCS molecules can inhibit cytokine signaling by binding to downstream signaling molecules such as JAK kinases (14-16); (iii) deregulated expression of SOCS proteins perturbs cytokine-related cellular proliferation and hematopoietic differentiation in murine tissues (17). Mice lacking SOCS-1 die shortly after birth from hepatic necrosis (18-20). Data suggest that this lethality is due, at least in part, to hypersensitivity to IFN-γ signaling. SOCS-2 deficient mice appear healthy after birth but develop a much larger body than control littermates, suggesting an important role for SOCS-2 in regulating growth hormone signaling (21). SOCS-3 knock-out mice, on the other hand, die as embryo at 12-16 day and demonstrate massive erythrocytosis in the fetal liver (22). Thus, different SOCS proteins appear to regulate, by auto-feedback inhibition, signal transduction of various cytokines in vivo.

The levels of SOCS appear to be tightly regulated at the transcriptional level as well as at the posttranscriptional level (23-26). SOCS proteins are extremely labile and proteasome
inhibitors decrease their degradation (27). Experiments also suggest that a domain conserved among SOCS family members, termed the SOCS box, may regulate the degradation process. This domain can interact with the Elongin B / Elongin C complex, which may regulate targeting of the SOCS proteins to the proteasome (27, 28). However, studies designed to examine the importance of the SOCS box in regulating SOCS protein stability have yielded conflicting results. Therefore, the mechanism by which SOCS protein stability is regulated remains unclear.

In order to identify proteins that regulate SOCS-1 stability, we used the yeast-two hybrid screen to identify and clone SOCS-1 interacting proteins. One of these proteins, termed TRIM8/GERP, is a RING finger protein. TRIM8/GERP is a newly described member of the so called Tripartite motif family (also known as the RBCC subclass of the Ring family containing proteins) (29, 30). The RBCC proteins, which include members implicated in a variety of processes, such as development and cell growth regulation, possess two zinc-binding domains between an N terminal Ring finger and a coiled-coil region which is immediately N-terminal to a variable C-terminal region.

We report here that TRIM8/GERP interacts with SOCS-1, and that this interaction requires the SH2 domain and the SOCS box of SOCS-1. Interestingly, co-expression of TRIM8/GERP with SOCS-1 decreases the stability and thus the levels of SOCS-1. In addition the reduced expression of SOCS-1 correlates with decreased inhibition of IFN γ-induced JAK-STAT activation. Together, these results suggest that TRIM8/GERP may be a regulator of IFN-γ signaling.
EXPERIMENTAL PROCEDURES

Yeast two-hybrid screen
Full-length murine SOCS-1 was subcloned into the pAS2 vector (Clonetech, USA), and transformed into the yeast strain Y190. A murine macrophage cDNA library made in the pGADNOT vector was a gift from Steve Goff (Columbia University). Yeast transformation was carried out using the EZ Yeast Transformation kit from Zymo Research (Orange, CA), and yeast two-hybrid screen was conducted essentially as described by Durfee et al. (31).

Molecular hybridization
Screening of a murine pre-B full-length cDNA 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 (S & S), fixed by UV crosslinking and hybridized to 50% formamide, 5X SSC (1X SSC = 0.15 Mol/L sodium chloride, 0.015 Mol/L sodium citrate), 0.02 Mol/L sodium phosphate and 10% dextran sulfate at 42 °C for 16 hrs with a 32P-labeled cDNA fragment, p612 corresponding to 612 bp of the 5' coding portion of TRIM8/GERP. Filters were subsequently washed and exposed at -80 °C for two days using an intensifying screen. Autoradiograms were obtained and used for phage isolation and further purification.

Generation of anti-TRIM8/GERP antibodies.
In order to generate anti-TRIM8/GERP polyclonal antibodies, a recombinant expression vector containing a portion of the TRIM8/GERP cDNA was constructed as follows. First a 343 bp PstI-PstI segment from plasmid pGAD-Not50 (containing the NH2-portion of TRIM8/GERP) was cut and inserted into the Pst I site of the pBlueScriptSK. Subsequently, the TRIM8/GERP portion was cut from pBS as a RI-Sma fragment and cloned in-frame into the pGST-5X IPTG-inducible expression vector for generating the chimeric GST-TRIM8/GERP fusion protein. Large scale growth of competent bacteria was achieved after transformation of bacteria with the recombinant pGST-TRIM8/GERP vector, and expression of recombinant GST-TRIM8/GERP protein was induced by incubating bacteria with 1 mM IPTG (Isopropyl-beta-D-Thiogalactoside) for 4 hrs at 37 °C. GST-TRIM8/GERP polypeptides were further purified using the Glutathione sepharose procedure and
A recombinant product was separated on a protein gel for analysis and quantitation. Approximately 1 mg of GST-TRIM8/GERP proteins was used for immunization of rabbits.

### Generation of recombinant TRIM8/GERP expression vectors

Plasmid p612 was obtained by inserting a Bam-Bgl II 612 bp fragment from the original pGAD-NOT containing the RING domain of TRIM8/GERP into the Bam HI site of the pcDNA3.1 (HisC)-Xpress tagged vector (Invitrogen, San Diego, CA). The Ring-TRIM8/GERP construct was obtained by amplifying a 240 bp segment from the TRIM8/GERP full length cDNA template using PCR and primers: TRIM8/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. Ring-TRIM8/GERP was made by cutting an EST clone (Sal-Not 1.3 Kb cDNA) with Sall-Xho I giving a fragment of 420 bp and inserting it into the pBSSK+. Then, a RI-Xho from pBS was cut and inserted in-frame into pcDNA3.1-Xpress tagged for functional expression.

For constructs TRIM8/GERP-R5, TRIM8/GERP-R6 and TRIM8/GERP-R7, a PCR-based strategy was used employing upstream primer TRIM8/GERP-Bam-F1 and downstream primers R5, R6, and R7 located at +762, +1,000 and +1292 from ATG, respectively.

For mSOCS-1 constructs, an HA-tagged vector was used except full-length and the ΔSH2-SOCS-1 which contained both HA and Xpress epitopes. ΔN-SOCS-1 (deleting residues 1-79), ΔSH2-SOCS-1 (deleting residues 80-166) and ΔC-SOCS-1 (deleting residues 167-212) were generated by PCR.

### Northern analysis.

Northern blot was performed as described (32). Briefly 10 µg of total RNA was 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 and 10% dextran sulfate at 42 °C for 16 hrs with a ³²P-labeled probes.
**Transfections.**

Briefly, 293 kidney embryonic cells were plated at ~ 10-15% confluency 1 day prior to transfection and cultured with DMEM supplemented with 10% Fetal Calf Serum (FCS) and antibiotics. On day 2 appropriate amount of plasmids with the gene of interest and 1 µg of Xpress-LacZ vector as internal control were transfected into the cells using calcium phosphate precipitation method. DNA was mixed into 2 M CaCl2, added to 2X HBS buffer (280 mM NaCl, 1.5 mM Na2HPO4, 50 mM Hepes, pH 7.05) and let it stand for 30’ at RT. Media was removed from the cells and the DNA precipitates were added to the cells and incubated for 20’ at 37 °C. Then, the precipitation mixture was diluted by adding 3.5 ml of pre-warmed 10% FCS DMEM medium for the cells incubated overnight. Cells were then washed three times with serum-free DMEM and supplemented with fresh medium for additional 8 hrs before cell harvesting. For functional studies, transfections were performed in the human HeLa cell line, using the manufacture’s lipofectamine-plus procedure (GIBCO-BRL, Gaithersburg, MD).

**Immunoprecipitation and immunoblots.**

For western blotting, cells were washed three times with cold PBS and lysed using 0.5 ml of Lysis buffer (50 mM Tris.Cl pH 7.6, 1 % NP40, 150 mM NaCl, 5 mM EDTA, 2 mM Na3VO4, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 1% glycerol). Protein extracts were quantified using the micro-assay Bio-Rad and 40 µg of total protein was run on a denaturing protein gel and electrotransfered onto membrane. Filters were blocked with 5 X BLOTTO (2% non-dry fat milk, 1X TBST) and immuno-blotted with 1:1000 diluted primary antibody.

For immunoprecipitation experiments, 293T cells were transfected as described above. For immunoprecipitation of endogenous SOCS-1/TRIM8/GERP complexes, the murine L929 fibroblasts were used (24). Cells were washed three times with 1X PBS, scraped, spun down and resuspended in 200 µl of lysis buffer (50 mM Tris.Cl pH 7.6, 1 % NP40, 150 mM NaCl, 5 mM EDTA, 2 mM Na3VO4, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 1% glycerol). Samples were left in ice for 20 min and spun down at 15,000 rpm for 15'. Supernatants were recovered and precleared using 5 µl of normal serum plus 100 µl 50% protein-G sepharose for at least 1 hr. Then after spinning, protein concentration was evaluated by Biorad kit and 1 mg of total extracts was incubated with 5 µl of specific
antibody and rocked at 4 °C for 3 hours. Beads were washed three times with lysis buffer, resuspended with sample buffer, boiled for 5 min, and loaded into SDS-PAGE.

**Luciferase assay.**

Luciferase assays were performed as previously described (33) with some modifications. Twenty-four hours after transfection, HeLa cells were separated into two plates. One plate was cultured with human IFN-γ and the other plate served as untreated control. Cells were harvested 18 hr after treatment and luciferase activity was measured.
RESULTS

Cloning of TRIM8/GERP cDNA

To identify proteins that could interact with SOCS-1, we performed a yeast-two hybrid screen using full length SOCS-1 cDNA as a bait. One of the clones identified contained a 612 bp cDNA insert encoding a RING finger protein. Re-screening in yeast confirmed that this interaction was specific for SOCS-1 (data not shown). To clone the full length cDNA of the gene, this initial fragment was used to screen a murine pre-B lymphoid cDNA library. Hybridizing recombinant phages were further purified and isolated. Several recombinants were obtained which contained the 5' end of the gene, which included 821 bp of untranslated region (UTR) and a potential ATG translation initiation site flanked by a consensus Kozak sequence. Subsequently, several murine and human ESTs (Expressed Sequence Tags) which contain sequences more 3' were identified. No ESTs were identified that extend beyond a Not I site in the 3' end of the gene. Therefore, we performed a 3' RACE strategy using specific primers located 3' at the end of the consensus and an engineered downstream primer designed to anneal to poly-dT tails associated to an adapted oligo sequence. After PCR, we obtained several clones encompassing the Not I site and extending more 3'. The cloning and further sequencing of the RACE products completed the cDNA sequence of the entire gene, which included a 700 bp 3' untranslated region with two putative non-canonical (AGAAA) polyadenylation sites. Sequencing of the genomic locus further confirmed the sequence and structure of the gene (data not shown). The sequence of this gene turned out to be identical to the TRIM8/GERP gene, independently identified by two groups using a functional genomic approach, based on systematic data gathered from screening the dbEST database with oligos derived from conserved RING domain sequences (29, 30).

The TRIM8/GERP gene encodes a 551 amino acid protein (Figure 1). Comparison of murine, rat and human sequences (obtained by scanning the NCBI database) revealed a 90% identity at the protein level. The carboxyl end of the protein contains a consensus nuclear localization motif (30), whereas the amino terminus of the predicted polypeptide sequence contains a canonical RING motif. The TRIM8/GERP RING domain spans 41 amino acids with canonical consensus residues along with cysteine/histidine contrapositions similar to other C2H2 category RING fingers (29, 30) (Figure 1). C2H2 RING finger proteins have
been implicated in a wide range biologic processes including the regulation of protein
stability (for review, see 34).

**TRIM8/GERP transcription is induced by IFN-γ.**

Northern blotting demonstrated that TRIM8/GERP is expressed in many murine
tissues including heart, brain, lung, liver, muscle, thymus and spleen (Figure 2). Highest
expression was noted in heart, liver, and thymus. To determine if the expression of
TRIM8/GERP is regulated, different cell lines were stimulated with IFN-γ and expression of
TRIM8/GERP was examined by Northern blotting. TRIM8/GERP mRNA can be induced by
IFN-γ in murine B lymphoid M12 cells, murine fibroblasts, and HeLa cells (Figure 2 and
data not shown). These data demonstrate that TRIM8/GERP is expressed in many tissues
and its expression can be further induced by IFN-γ.

**SOCS-1 and TRIM8/GERP associate in mammalian cells**

To further define the interaction between SOCS-1 and TRIM8/GERP, the ability of
recombinant TRIM8/GERP and SOCS proteins to interact in mammalian cells was analyzed.
An expression vector containing the cDNA encoding the amino 204 aa of TRIM8/GERP,
along with the Xpress epitope, was overexpressed in 293T cells along with vectors
expressing different SOCS proteins (tagged with HA epitope). The interaction of these
proteins was then analyzed by co-immunoprecipitation experiments.

When SOCS-1 (HA) was immunoprecipitated, TRIM8/GERP co-immunoprecipitated
with it (Figure 3A, lane 5). Note that SOCS-1 is tagged with Xpress and HA and is seen as
the slower migrating band above TRIM8/GERP in lane 5. In contrast, TRIM8/GERP did not
coop-immunoprecipitate with either SOCS-2 or SOCS-3 (lanes 6 and 7). The specific
interaction between TRIM8/GERP and SOCS-1 was confirmed when the reciprocal
experiment was performed (data not shown). These data demonstrate that SOCS-1 and
TRIM8/GERP specifically interact when co-expressed in mammalian cells.

In order to detect endogenous TRIM8/GERP protein in the cells, the murine L929
fibroblast cells were treated for 30 min with 800 U/ml of IFN-γ (Figure 3B, lane 2). LLnL, a
specific proteasome inhibitor was also added alone (Figure 3B, lane 3), immediately prior to
(Figure 3B, lane 5) or after (Figure 3B, lane 4) IFN-γ treatment. The cells were then lysed
and subject to immunoprecipitation using an anti-SOCS-1 antibody (33). While SOCS-1 was
present constitutively in L929 cells, the association between SOCS-1 and TRIM8/GERP was
only evident upon IFN-γ treatment (Figure 3B, lane 2), and was further enhanced when LLnL was added following a 30 min treatment with IFN-γ. These data demonstrate the interaction between endogenous SOCS-1 and TRIM8/GERP proteins.

To further define the region of TRIM8/GERP required for the association with SOCS-1, SOCS-1 was co-expressed with various mutants of TRIM8/GERP: the N-terminal 204 amino acids alone (TRIM8/GERP -204AA), the N-terminal 204 amino acids without the RING motif (TRIM8/GERP-∆RING), or the RING motif alone (TRIM8/GERP-RING) (Figure 3C). SOCS-1 was immunoprecipitated with HA and co-immunoprecipitation of TRIM8/GERP was determined by Western blotting (Xpress) (Figure 3D). Both the TRIM8/GERP -204AA (lane 4) and the TRIM8/GERP-∆RING (lane 6) interacted with SOCS-1, while the RING motif alone did not (lane 5). These data indicate that the RING motif is not essential for the interaction of TRIM8/GERP with SOCS-1, and that the region immediately C-terminal to the RING motif is sufficient to mediate this interaction.

SOCS-1 contains several structural motifs important for its function. The SH2 domain is required for SOCS-1 interaction with the JAK kinases. The SOCS box motif has been shown to interact with the Elongin B/C complex which may regulate stability of SOCS-1 protein. To determine the domains of SOCS-1 required for the interaction with TRIM8/GERP, TRIM8/GERP (Xpress tagged) was co-expressed with different deletion mutants of SOCS-1 (HA). The different SOCS-1 proteins were immunoprecipitated with anti-HA and co-immunoprecipitation of TRIM8/GERP was examined (Figure 3E). TRIM8/GERP was co-immunoprecipitated with the SOCS-1 lacking the amino terminus (lane 7). In contrast, SOCS-1 lacking the SOCS box or the SH2 domain did not co-immunoprecipitate with TRIM8/GERP (lanes 5 and 6). Note that ΔSH2-SOCS-1 is both HA and Xpress tagged and is detected in lane 5. These data suggest that both the SOCS box and the SH2 domain are required for the interaction between SOCS-1 and TRIM8/GERP.

**TRIM8/GERP Protein is Unstable**

Full length TRIM8/GERP protein expression was barely detectable by Western blotting (Figure 4, lane 6). Deletion mutants of TRIM8/GERP, however, could be efficiently expressed in 293T cells (Figure. 4). Transfection of recombinants bearing longer portions of the TRIM8/GERP gene resulted in a gradual decrease of expression levels (Figure. 4). These data suggest that the carboxyl region of TRIM8/GERP contain motifs that alter its expression. To determine the half life of TRIM8/GERP protein, cells transfected with the short form of TRIM8/GERP, TRIM8/GERP_{(204AA)}, were grown in the absence or presence of...
cycloheximide. The levels of TRIM8/GERP over time were examined by Western blotting (Figure 5). TRIM8/GERP was extremely unstable and was rapidly eliminated from the cells (Figure 5, lanes 9-12). These data indicate that TRIM8/GERP is an extremely labile protein.

**SOCS-1 stability is regulated by TRIM8/GERP interaction**

RING finger proteins such as Cbl are thought to function as ubiquitin ligases, which regulate protein stability. The presence of a RING motif in TRIM8/GERP and its inherent instability raise the possibility that TRIM8/GERP may function to regulate the stability of other proteins. To examine this possibility, TRIM8/GERP and SOCS-1 were co-transfected into 293T cells. The cells were then cultured in the absence or presence of cycloheximide to inhibit new protein synthesis and the stability of SOCS-1 examined by Western blotting. When TRIM8/GERP was expressed with SOCS-1, the levels of SOCS-1 were decreased (Figure 5). In addition, the stability of SOCS-1 was decreased when it was co-expressed with TRIM8/GERP (Figure 5). This was also evident by transfecting increasing amount of TRIM8/GERP(204AA) along with SOCS-1 in a dose-dependent manner (data not shown). These data suggest that TRIM8/GERP can regulate SOCS-1 protein levels.

**Functional role of TRIM8/GERP in the IFN-γ-mediated biological response**

Expression of TRIM8/GERP is induced by IFN-γ. Furthermore, SOCS-1 has been shown to be an inhibitor of IFN-γ signaling. These data raise the question of whether TRIM8/GERP can affect the function of SOCS-1. To address this question we examined the ability of TRIM8/GERP to alter the IFN-γ-mediated induction of transcription from the IFN-γ responsive GAS element. This element is found in the promoter of many IFN-γ inducible genes and binds homodimers of Stat1 (35). SOCS-1 has been shown to inhibit transcription driven by this element through inhibition of JAK-STAT activation. HeLa cells were transfected with a luciferase reporter construct driven by upstream GAS elements. When cells were stimulated with IFN-γ, luciferase activity was induced over 500 fold (Figure 6). As previously reported, when an expression vector containing SOCS-1 was co-transfected, the induction of luciferase activity was greatly inhibited. Interestingly, when increasing amounts of an expression vector containing TRIM8/GERP was transfected along with SOCS-1 and the GAS-reporter, the inhibition of luciferase activity by SOCS-1 could be reversed in a dose dependent manner. These data suggest that TRIM8/GERP can inhibit SOCS-1 function.
DISCUSSION

In this report we present evidence that TRIM8/GERP, previously identified as a new member of the Ring Finger B-Box Coiled-coil subclass of the Ring family, is capable of specifically binding SOCS-1. SOCS proteins work as cytoplasmic inhibitors of signaling triggered by cytokine/receptor interactions. One of the predominant features of SOCS-1 has been the stringent regulation of SOCS-1 expression at multiple levels: (i) SOCS-1 mRNA is present at low levels in resting cells, and stimulation of cells by a variety of signals, including cytokines and crosslinking of antigen receptors, induces transcription of SOCS-1 mRNA; (ii) SOCS-1 mRNA stability is also regulated; (iii) Initiation of translation is another mechanism by which the protein levels of SOCS-1 are regulated. We and others have demonstrated that SOCS-1 protein appears to be constitutively degraded by the proteasomes. Prior work has suggested that degradation of SOCS-1 can be regulated by binding (via the SOCS box domain) to Elongin B and C(28).

Here we have identified another protein involved in the regulation of SOCS-1 stability, TRIM8/GERP. We have demonstrated that the TRIM8/GERP protein can bind to SOCS-1 in co-immunoprecipitation assays, and that TRIM8/GERP can modulate SOCS-1 mediated suppression of cytokine signaling. As shown by co-immunoprecipitation in Fig. 3, exogenously expressed TRIM8/GERP specifically binds SOCS-1, but not other members of the SOCS family (i.e., SOCS-2 and SOCS-3). In addition, the RING portion of TRIM8/GERP is not required for binding SOCS-1, suggesting that the interaction domain resides along the B box Coiled-Coil region of the protein. By contrast, the interaction between SOCS-1 and TRIM8/GERP appears to require both the SOCS box and the SH2 domain.

It has previously been shown that SOCS-1 binds JAK kinases in an SH2 dependent manner. The mechanism by which SOCS-1 inhibits JAK-STAT signaling is unclear. SOCS-1 may just bind JAK kinases and prevent access of interacting proteins and/or ATP to the catalytic pocket. Alternatively, SOCS-1 may target activated JAK kinases to protein degradation, similar to what has been shown for TEL-JAK (36). It is possible that the binding of TRIM8/GERP to the SH2 domain of SOCS-1 alters the ability of SOCS-1 to interact with JAK kinases. In addition, binding of TRIM8/GERP to the SOCS box may alter the
interaction between SOCS-1 and other components of the degradation machinery, which may result in altered function and/or protein stability of SOCS-1. We have shown that co-expression of TRIM8/GERP and SOCS-1 in 293 cells increases the turnover of both proteins, suggesting that stability may be affected by their interaction.

It is interesting that the conserved B-box Coiled-Coil domain of TRIM8/GERP is sufficient, for efficient interaction with SOCS-1, suggesting that the RING domain may bind to other proteins. Recent reports have identified a subclass of Ring Finger proteins containing the RING CH2CH signature motif which may be part of a ubiquitin-ligase (37-39). In our report several lines of evidence suggest that TRIM8/GERP may play such a role. First, the carboxyl-terminal portion of TRIM8/GERP has C domains that principally affect its protein stability. Secondly, the NH2 portion of TRIM8/GERP has features which resemble other ubiquitin-ligase components; Lastly, co-expression of TRIM8/GERP and SOCS-1 drastically decreases the stability of SOCS-1 in vitro.

Definition of TRIM8/GERP as a new member of the RBCC subclass of RING protein also suggests a putative role in cellular transformation and differentiation. Several RBCC members, including PML (40), TIF1 (41), KAP-1 (42) which like TRIM8/TRIM8/GERP/GERP possess two B boxes between the RING finger and the Coiled-Coil domain have been associated to pathogenesis of tumors, others, like Rpt-1 (43) RFP (44) and BERP (45), which retain only a single B box, are controller of developmentally regulated differentiation. The human TRIM8/GERP gene is ubiquitously expressed in adult tissues and in a variety of tumors, including anaplastic oligodendroglioma and maps at chromosome 10q24.3 within a region mostly involved in deletions and rearrangements in human glioblastoma, raising the possibility that the gene may be involved in the pathogenesis of such tumor.

Another point relevant for the function of TRIM8/GERP/SOCS-1 interaction is the capacity of TRIM8/GERP to modulate the inhibitory function of SOCS-1 in an IFN-γ biological response. As shown in HeLa cells transfected with a GAS-Luc reporter vector (Fig. 6), the exogenous co-expression of SOCS-1 and a RBCC-TRIM8/GERP mutant partially restore the IFN-γ-mediated induction of luciferase, which was inhibited by the deregulated expression of SOCS-1. Such data stress a putative role for TRIM8/GERP in inhibiting SOCS-1 function in vivo by binding and targeting it to proteasomal degradation. Furthermore, several studies
have already indicated that most RING protein function as ubiquitin-ligases involved in the protein degradation machinery. However, more detailed analysis of SOCS-1/TRIM8/GERP interactions will be necessary to determine if both proteins act as a single functional entity or may involve additional proteins.

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REFERENCES


**FIGURE LEGENDS**

**Fig. 1 Schematic representation of TRIM8/GERP protein.**
The 551 amino acid TRIM8/GERP protein sequence is shown. The RING domain is schematically displayed as a folding structure and the alignment with other canonical RING domains from different proteins was performed using the Prettybox program of the GCG software package.

**Fig. 2 Expression pattern of TRIM8/GERP mRNA**
Mouse tissue Northern blot was obtained from Clonetech. For M12 and HeLa blots, approximately 10 µg of mRNA was loaded in each lane. For all experiments, the 612 bp cDNA isolated from the initial two-hybrid screen was used as a TRIM8/GERP probe.
Murine M12 and Human HeLa cell lines were treated with IFN-γ in time-course experiments as indicated.

**Fig. 3 Co-Immunoprecipitation of TRIM8/GERP and SOCS-1.**

A. 293T cells were co-transfected with plasmid encoding the N-terminal 204 amino acids of TRIM8/GERP (tagged with Xpress) in combination with SOCS-1, 2 or 3. Total cell lysate obtained were subject to immunoprecipitation and immunoblot analysis. As an internal control for transfection and loading, a plasmid carrying the LacZ (tagged with Xpress) was included in every transfection. SOCS-1 was epitope-tagged with HA and Xpress, SOCS-2 and SOCS-3 were tagged with HA. Lanes 1-4 were loaded with total cell lysates, lanes 5-8 were loaded with immunoprecipitates.

B. L939 cells were treated with IFN-γ and/or LLnL as indicated. Cells were then lysed and total cell lysates were immunoprecipitated with an anti-SOCS-1 antibody (lanes 1-5) or with preimmune serum as a control (lanes 6-10). The immunoprecipitates were subject to immunoblot analysis using an anti-TRIM8/GERP antibody.

C. Diagrammatic view of the SOCS-1 and TRIM8/GERP mutants used for mapping the interaction domains.

D. Various deletion mutants of Xpress-tagged TRIM8/GERP (as depicted in 3C) were co-transfected into 293T cells with SOCS-1 (tagged with both HA and Xpress). Co-immunoprecipitation experiments were performed as described in 3A. Lanes 1-3 were loaded with total cell lysates, lanes 4-6 were loaded with immunoprecipitates.

E. Various deletion mutants of SOCS-1 (as depicted in 3C,) were co-transfected into 293T cells with Xpress-tagged TRIM8/GERP-204 AA. Both ∆C-SOCS-1 and ∆N-SOCS-1 were HA tagged, while the ∆SH2-SOCS-1 mutant was tagged with both HA and Xpress. Co-immunoprecipitation experiments were performed as described in 3A. Lanes 1-3 were loaded with total cell lysates, lanes 4-6 were loaded with immunoprecipitates.

**Fig. 4 Differential expression of TRIM8/GERP mutants.**

Various truncation mutants of TRIM8/GERP, as schematically represented in the bottom panel, were transfected into 293T cells along with a plasmid carrying the LacZ gene (lanes 2-6). Lane assignment are as follows: lane 1: mock, lane 2: TRIM8/GERP-204AA, lane 3: TRIM8/GERP-R5, lane 4: TRIM8/GERP-R6, lane 5: TRIM8/GERP-R7, lane 6, full length
TRIM8/GERP. The TRIM8/GERP mutants and LacZ were all tagged with Xpress epitope. Lysates from the transfectants were subject to immunoblot analysis. Comparable transfection efficiency and equal loading were indicated by the expression levels of β-galactosidase encoded by LacZ.

**Fig. 5 TRIM8/GERP\textsubscript{(204AA)} accelerates the degradation of SOCS-1.**

293T cells were transfected with SOCS-1 and TRIM8/GERP\textsubscript{(204AA)} either alone or together. LacZ was included as an internal control. Cells were treated with cycloheximide as described (33). Lysates of cells harvested at different time points after cycloheximide treatment was subject to Western blotting with anti-Xpress antibody.

**Fig. 6 TRIM8/GERP\textsubscript{(204AA)} reverses SOCS-1 mediated inhibition of JAK-STAT activation.**

HeLa cells were transfected with a reporter GAS-Luc in combination with SOCS-1 and TRIM8/GERP\textsubscript{(204AA)} expression vectors. While the total amount of SOCS-1 DNA remained constant (0.25 µg) from samples #4 through #8, the amount of TRIM8/GERP\textsubscript{(204AA)} DNA was scaled down from 2 µg (#4) to 0.0625 µg (#8). Luciferase activity was assayed as described (33).
Translated product from TRIM8/GERP consensus

1 MAENWKNCFE EELICPICLH VFEVPEVLPC KHNCRCGCIC EAWEAKDSGLV
51 RCPECNQAYN QKPGEKNKL LTNIVEKFNA LHVEKPTAL HCFCRCRGPP
101 LPAQKVLRC EAPCCQSHVQ THLQPSTAR GHLLVADDV RAWSCPQHNA
151 YRLYHEAEQ VAVCVQCCYY SGAGHGHVSC DVEIRRNILR KMLMKQGERL
201 EEERQDIEDQ LKLESKRL VEEKVSQLKE EVRLQYEKLM QLLDEDLQT
251 VEVLQKAQAK FCSENAQAL HLGERMQEAK KLGLSLQRLF DKTEDVGFMK
301 NTKSVKILMD RTQCTGSSSL SFPKIHLNS KLFLEVAKK EKQLRKMLEG
351 PFPSTVPFLQ SVPLPGCVSN SSQERKHKIS TAPFEEASFL TSSGPVGGQY
401 GAAGTSASSEG QSQPLGPCS STQLVLALPG GTQPVHSSPV FPFPQYPNGS
451 TTQQPMLQQ GGRKILVCSV DNCYCSSVAN HGHHQPYPRS GHFPWTVPSEQ
501 EYSHPFLPTP SVPOSLPGLA VRDNLDASQQ PGHQDFYRVY GQPSTKHVT
551 S*

Total AA = 551

Fig. 1
Murine tissues

M12

HeLa

<table>
<thead>
<tr>
<th>Heart</th>
<th>Brain</th>
<th>Lung</th>
<th>Liver</th>
<th>Skeletal</th>
<th>Kidney</th>
<th>Gypsy</th>
<th>Riplen</th>
</tr>
</thead>
</table>

TRIM8/GERP

GAPDH

IFN-γ time

0      15'     30'     1hr     3hr    6hr

TRIM8/GERP

GAPDH

TRIM8/GERP

GAPDH

TRIM8/GERP

GAPDH
Fig. 3A

<table>
<thead>
<tr>
<th></th>
<th>Lysates</th>
<th>IP: SOCS (anti-HA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacZ</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TRIM8 (204AA)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SOCS-1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SOCS-2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SOCS-3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

IB: anti-Xpress

IB: SOCS (anti-HA)
Fig. 3B

IP: anti-SOCS-1  IP: preimmune serum

IB: TRIM8

1,6 = Untreated
2,7 = 30' muIFN-γ
3,8 = 30' LLnL
4,9 = 30' muIFN-γ treatment then 30' LLnL
5,10 = 30' LLnl, then 30' muIFN-γ treatment

IB: anti-SOCS-1
Fig. 4

IB: TRIM8(Xpress)

TRIM8

NH2 __________________ COOH

__________________________

TRIM8-R5 (1-254 AA)

__________________________

TRIM8-R6 (1-333 AA)

__________________________

TRIM8-R7 (1-431 AA)
Fig. 5

SOCS-1       + + + + + + + - - - -
TRIM8(204AA) - - - - + + + + + + + +

LacZ

IB: Xpress
TRIM8(204AA)

GAS-Luc.       -         +        +         +        +        +       +        +
SOCS-1           -         -         +        +        +         +      +        +

Relative light units

Control
IFN-gamma

TRIM8(204AA)