SPANDIDOS PUBLICATIONS

Suppressor of cytokine signaling 1 interacts with oncogenic lymphocyte-specific protein tyrosine kinase

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Abstract. Lymphocyte-specific protein tyrosine kinase (Lck) plays a key role in T cell signal transduction and is tightly regulated by phosphorylation and dephosphorylation. Lck can function as an oncoprotein when overexpressed or constantly activated by mutations. Our previous studies showed that Lck-induced cellular transformation could be suppressed by enforced expression of suppressor of cytokine signaling 1 (SOCS1), a SOCS family member involved in the negative feedback control of cytokine signaling. We observed attenuated Lck kinase activity in SOCS1-expressing cells, suggesting an important role of SOCS in regulating Lck functions. It remains largely unknown whether and how SOCS proteins interact with the oncogenic Lck kinase. Here, we report that among four SOCS family proteins, SOCS1, SOCS2, SOCS3 and CIS (cytokine-inducible SH2 domain containing protein), SOCS1 has the highest affinity in binding to the oncogenic Lck kinase. We identified the positive regulatory phosphotyrosine 394 residue in the kinase domain as the key interacting determinant in Lck. Additionally, the Lck kinase domain alone is sufficient to bind SOCS1. While the SH2 domain in SOCS1 is important in its association with the oncogenic Lck kinase, other functional domains may also contribute to overall binding affinity. These findings provide important mechanistic insights into the role of SOCS proteins as tumor suppressors in cells transformed by oncogenic protein tyrosine kinases.

Introduction

Lymphocyte-specific protein tyrosine kinase (Lck) is an Src family protein tyrosine kinase essential for T cell development and activation (1). Like all Src family kinases, Lck has a unique amino-terminal region, an Src homology 3 (SH3) domain, an Src homology 2 (SH2) domain, a catalytic domain, and a short carboxy-terminal tail (2). Lck kinase activity is regulated by phosphorylation of tyrosine residues at 394 and 505. Phosphorylation at the negative regulatory Tyr505 induces an intra-molecular interaction with the SH2 domain and results in an inactive kinase with a closed conformation (2). Additional interactions between the SH3 domain and a polyproline sequence in the SH2-kinase linker region stabilize the inactive conformation of Lck (3). Dephosphorylation of Tyr505 opens the structure and makes the catalytic domain accessible to substrates. Subsequent auto-phosphorylation of the positive regulatory Tyr394 further augments the kinase activity (2). Lck with a tyrosine to phenylalanine mutation at 505 is unable to interact with the SH2 domain of the protein and renders the protein constitutively active (4).

Overexpression and constitutive activation of Lck kinase have been implicated in both lymphoid and non-lymphoid malignancies (5-7). The constitutively active Y505FLck is oncogenic as demonstrated by its ability to transform mouse fibroblast cells (8) and induce interleukin-3-independent growth of mouse BaF3 pro-B cells (4). We showed previously that, in Lck-transformed T and B cells, there was persistent activation of the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway (4,9). We further provided evidence that constitutive STAT5 activation is critical in Lck-mediated cellular transformation (4). This is consistent with many reports of constant JAK-STAT activation in tumor cells and identifies JAK-STAT signaling as a novel molecular target in cancer therapy (10).

Under normal physiological conditions, the JAK-STAT pathway is tightly regulated by a number of mechanisms including negative feedback inhibition by the suppressor of cytokine signaling (SOCS) proteins (11,12). Among the eight SOCS family members, CIS (cytokine-inducible SH2 domain containing protein), SOCS1, SOCS2 and SOCS3 are best characterized for their structures and functions. Apart from the carboxy-terminal SOCS box, they all share a central SH2 domain which binds phosphorylated tyrosine residues in the cytoplasmic tails of cytokine receptors or in the activation loop of JAK kinase domains (11). SOCS1 and SOCS3 also have a kinase inhibitory region (KIR) at their amino-terminal ends to inhibit JAK kinase activity.

As endogenous inhibitors of JAK-STAT signaling, SOCS proteins are potential tumor suppressors. This theory is supported by previous reports on the tumor-suppressing activity of SOCS in cells transformed by oncogenic protein

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tyrosine kinases, such as focal adhesion kinase (FAK) (13), breast tumor kinase (14) and TEL-JAK2 (15). Both SOCS1 and SOCS3 bind to the phosphorylated tyrosine residue in the activation loop of FAK and inhibit FAK-induced cellular transformation (13). Consistent with the role of SOCS as tumor suppressors, loss of SOCS gene expression has been reported in several forms of solid tumors and lymphoid malignancies (16-18). We also showed that multiple SOCS family members, including CIS, SOCS1 and SOCS3, were not expressed in Lck-transformed cells (19,20).

Our earlier studies further demonstrated that exogenous expression of SOCS1 and SOCS3 led to reduced cell proliferation and increased apoptosis in Lck-transformed cells (20). While we observed attenuated Lck kinase activity in SOCS-expressing cells, it is not known if SOCS proteins interact with the oncogenic Lck kinase. In the study reported here, we examined the binding of oncogenic Y505FLck kinase with four closely related SOCS family members. Through mutational analysis, we further characterized the molecular details of SOCS1-Lck interaction.

Materials and methods

Reagents. Glutathione sepharose 4B beads were purchased from Amersham Biosciences (Piscataway, NJ, USA). Recombinant protein G beads, Alexafluor-conjugated secondary antibodies and DAPI were purchased from Invitrogen Corp. (Calsbad, CA, USA). Anti-Lck 3A5 and anti-GST B-14 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Myc 4A6 monoclonal and anti-Lck polyclonal antibodies were from Millipore (Billerica, MA, USA). Fish skin gelatin and anti-FLAG M2 antibody were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Anti-GAPDH antibody was from Cell Signaling (Danvers, MA, USA). Paraformaldehyde was purchased from Electron Microcopy Sciences (Hatfield, PA, USA).

Construction of plasmids. A *Bam*HI-*Spe*I restriction fragment containing the full-length SOCS1 cDNA with a FLAG tag was generated by PCR using pEF-FLAG-I/mSOCS1 as the template and then inserted into the multiple cloning site in pEBG to make the pEBG/mSOCS1 expression construct. Similar approach was used to construct pEBG expressing mSOCS2, mSOCS3 and mCIS with PCR-generated *Bam*HI-*Not*I restriction fragments. SOCS1 point mutations were introduced in pEBG/mSOCS1 using the GeneTailor site-directed mutagenesis system (Invitrogen) to make pEBG/mF59DSOCS1 and pEBG/mR105KSOCS1. The same site-directed mutagenesis system was also used to make pcDNA3.1/hY394FY505FLck and pcDNA3.1/hK273RY505 FLck from pcDNA3.1/hY505FLck. A *Xho*I-*Eco*RI restriction fragment containing either the N-terminal, SH3 and SH2 domains of Lck (N32 Lck) or the kinase and C-terminal region of Lck (kinase Lck) was generated by PCR using pcDNA3.1/wild-type hLck as the template and then inserted into the multiple cloning site in pcDNA3.1/Myc-His. All constructs were verified by sequencing for accuracy.

Cell culture and transfections. COS7 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37˚C in a 5% CO_2 incubator. Cells were transiently transfected with 10 μg of total DNA in 60-mm cell culture dishes using calcium phosphate precipitation and harvested after 48 h.

Glutathione pulldown, immunoprecipitation and Western blotting. Preparation of whole cell lysates was done using 1% Brij 35 lysis buffer as described before (20). For glutathione pulldown experiments, equal amounts of total protein were incubated overnight with glutathione beads at 4˚C. Beads were washed in wash buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100). For immunoprecipitation experiments, equal amounts of total protein were precleared using protein G beads at 4˚C for 30 min. Immunoprecipitation was then performed with anti-Lck 3A5 antibody as described previously (4). Samples from glutathione pulldown and immunoprecipitation were subjected to SDS-PAGE and Western blotting. Ponceau S staining and immunoblotting with anti-GAPDH antibody confirmed equal protein loading in whole cell lysate blots. Standard molecular weight markers were included and are shown in kilodaltons (kDa) in figures when applicable. Antibody dilutions were prepared as recommended by the manufacturers. Signals were detected and quantified using the LI-COR Odyssey infrared imaging system (Lincoln, NE, USA).

Immunofluorescence microscopy. COS7 cells were cultured on the surface of 12-mm coverslips in 35-mm cell culture dishes to 60% confluency. Cells were cotransfected with 2 μg of pEF-FLAG-I/mSOCS1 and 2 μg of pcDNA3.1/hY505FLck using calcium phosphate precipitation. After 48 h, cells were fixed with 4% paraformaldehyde at 4˚C for 10 min and incubated in blocking buffer (2% fish skin gelatin and 0.2% Triton X-100 in 1X phosphate-buffered saline) for 15 min at room temperature. Immunostaining was performed with anti-Lck polyclonal antibody and anti-FLAG monoclonal antibody (diluted 1:100 in blocking buffer) at room temperature for 1 h. Alexafluor 488-conjugated donkey anti-rabbit and Alexafluor 594-conjugated donkey anti-mouse antibodies were diluted 1:2000 in blocking buffer and used for secondary staining at room temperature for 30 min. Nuclei were marked by DAPI staining. Stained cells were visualized with appropriate filters using a fluorescence microscope with a Nikon Metamorph digital imaging system.

Statistical analysis. Signals from Western blots were quantified using the LI-COR Odyssey system. Relative affinity for Y505FLck or SOCS1 was expressed as a ratio between proteins in the pulldown to proteins in the input, and then normalized to glutathione S-transferase (GST) fusion proteins in the pulldown. Statistical significance was calculated from multiple experiments using one-way ANOVA and the Holm-Sidak post-test (Sigmaplot, Systat Software Inc., CA, USA).

Results

SOCS1 has the highest affinity for oncogenic Y505FLck. To determine if different SOCS proteins interact with oncogenic

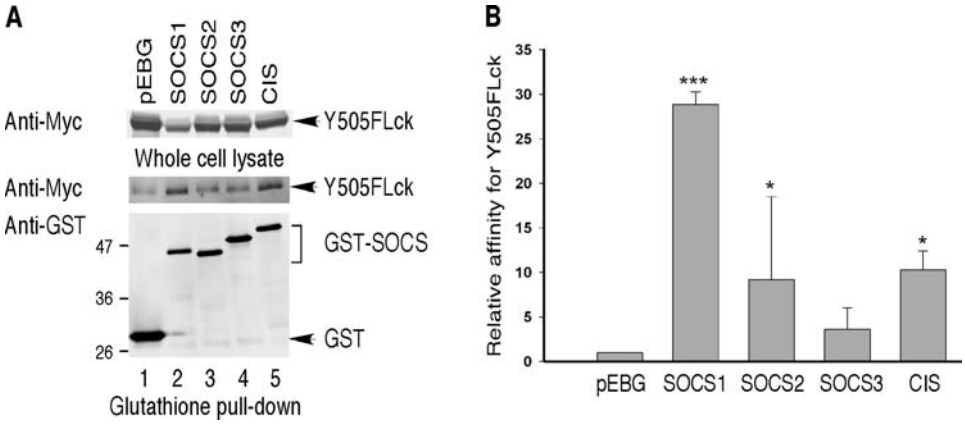

Figure 1. SOCS1 has the highest affinity for oncogenic Y505FLck kinase. (A) COS7 cells were cotransfected with 5 μg of pcDNA/Y505FLck and 5 μg of pEBG (empty vector), pEBG/SOCS1, pEBG/SOCS2, pEBG/SOCS3 or pEBG/CIS. A small aliquot of normalized whole cell lysate was analyzed by immunoblotting with anti-Myc antibody to detect Y505FLck (top panel). Following glutathione pulldown, anti-Myc and anti-GST immunoblotting was performed to detect Y505FLck (middle panel) and GST or GST-SOCS fusion proteins (bottom panel), respectively. (B) The relative affinity of SOCS1, SOCS2, SOCS3 and CIS for Y505FLck was calculated as described in Materials and methods. The affinity of GST for Y505FLck was set as 1 and values represent the means ± SD (n=3; $^{*}p<0.05$; $^{***}p<0.001$).

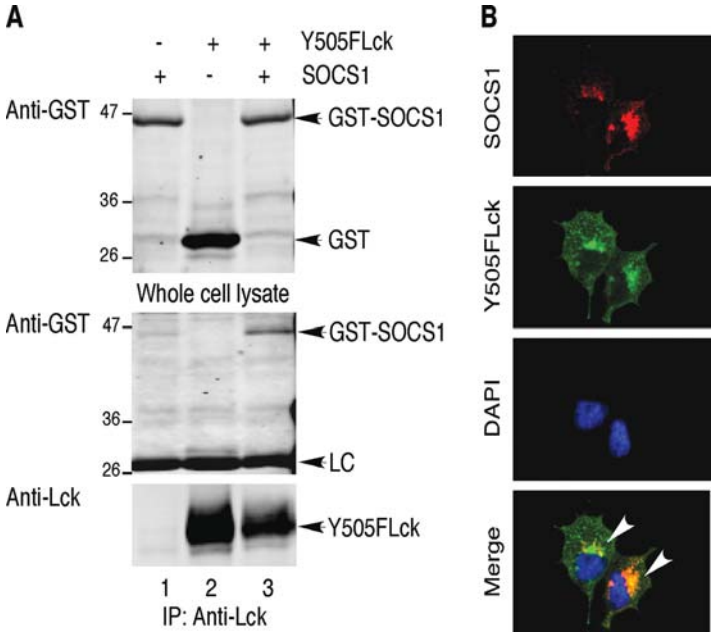

Figure 2. SOCS1 interacts with the oncogenic Y505FLck kinase. (A) COS7 cells were cotransfected with 5 μg of pcDNA/Y505FLck (+) or pcDNA (-) and 5 μg of pEBG/SOCS1 (+) or pEBG (-). A small aliquot of normalized whole cell lysate was analyzed by anti-GST immunoblotting to detect GST and GST-SOCS1 (top panel). Following Lck immunoprecipitation, Western blotting was performed with anti-GST antibody to detect GST and GST-SOCS1 (middle panel) and anti-Lck antibody to detect Y505FLck (bottom panel). The position of immunoprecipitating antibody light chain (LC) is also indicated. (B) COS7 cells were cotransfected with SOCS1 and Y505FLck expression constructs. Immunofluorescence microscopy was performed as described in Materials and methods to visualize SOCS1 (red), Y505FLck (green), and DAPI-stained nuclei (blue). In the three-color merge image (bottom panel), arrows point to regions of interaction between SOCS1 and Y505FLck (yellow). Original magnification, x60.

Lck kinase, SOCS1, SOCS2, SOCS3 and CIS were expressed as GST fusion proteins for glutathione pulldown analysis in COS7 cells co-expressing Y505FLck. As shown in Fig. 1A, all 4 SOCS proteins brought down various amounts of Y505FLck (middle panel). To account for different expression levels of Y505FLck (Fig. 1A, top panel) and GST-SOCS fusion proteins (Fig. 1A, bottom panel), we quantitated our results by comparing different GST-SOCS fusion proteins to the GST alone control (Fig. 1A, lane 1). Statistical analysis shows that SOCS1 has the highest affinity in binding to Y505FLck (Fig. 1B). Affinities of SOCS2 and CIS for Y505FLck are also significantly higher than that of the GST alone control (Fig. 1B).

To further confirm the interaction between SOCS1 and Y505FLck, we performed a reciprocal immunoprecipitation experiment and immunofluorescence microscopy. As shown in Fig. 2A (middle panel), significant amounts of GST-SOCS1 fusion proteins were co-precipitated in anti-Lck immunoprecipitates (lane 3) compared to the GST alone control (lane 2) and no Lck control (lane 1). Expression of GST

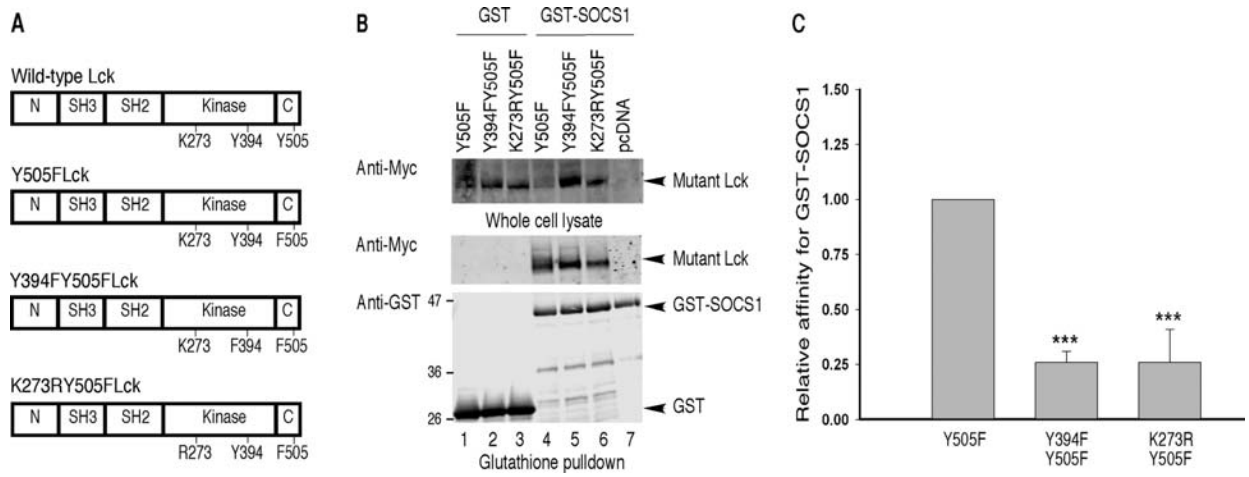

Figure 3. Phosphorylated Tyr394 in the Lck kinase domain is important in SOCS1 association. (A) Schematic diagrams of Lck functional domains and point mutations. (B) COS7 cells were cotransfected with 5 μg of pEBG or pEBG/SOCS1 and 5 μg of pcDNA without or with various Lck mutant constructs. A small aliquot of normalized whole cell lysate was analyzed by anti-Myc immunoblotting to detect Myc-tagged mutant Lck (top panel). Following glutathione pulldown, Western blotting was performed with anti-Myc antibody to detect mutant Lck (middle panel) and anti-GST antibody to detect GST and GST-SOCS1 (bottom panel). (C) The relative affinity of mutant Lck for GST-SOCS1 was calculated as described in Materials and methods. The affinity of Y505FLck for GST-SOCS1 was set as 1 and values represent the means ± SD (n=3; $^{***}p<0.001$).

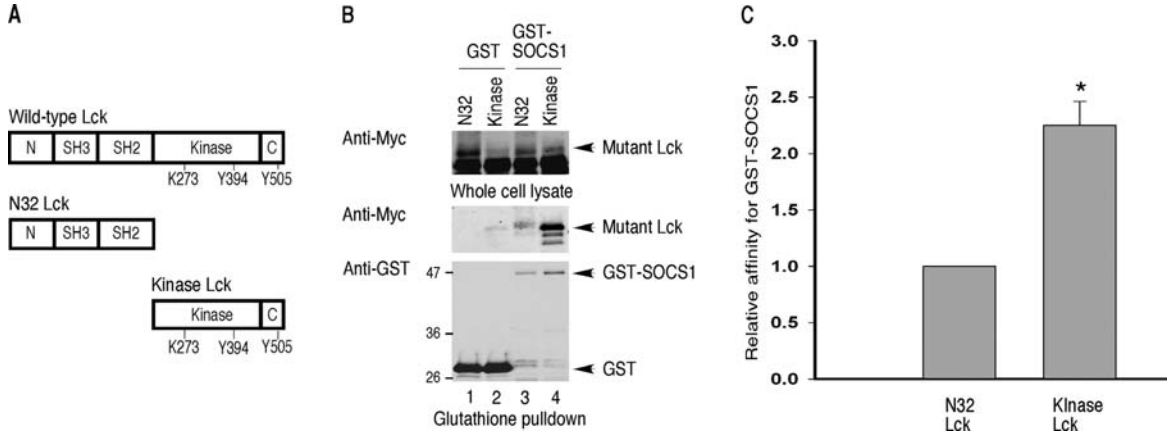

Figure 4. Kinase domain of Lck is sufficient to mediate interaction with SOCS1. (A) Schematic diagrams of Lck functional domains and truncation mutants. (B) COS7 cells were cotransfected with 5 μg of pEBG or pEBG/SOCS1 and 5 μg of pcDNA with various Lck mutant constructs. A small aliquot of normalized whole cell lysate was analyzed by anti-Myc immunoblotting to detect Myc-tagged mutant Lck (top panel). A strong non-specific band was detected below the truncated Lck proteins (top panel). Following glutathione pulldown, Western blotting was performed with anti-Myc antibody to detect mutant Lck (middle panel) and anti-GST antibody to detect GST and GST-SOCS1 (bottom panel). (C) The relative affinity of mutant Lck for GST-SOCS1 was calculated as described in Materials and methods. The affinity of N32Lck for GST-SOCS1 was set as 1 and values represent the means ± SD (n=2; $^{*}p<0.05$).

and GST-SOCS1 in whole cell lysate and Y505FLck in Lck immunoprecipitates was verified by anti-GST (top panel) and anti-Lck (bottom panel) immunoblotting, respectively (Fig. 2A). Immunofluorescence microscopy further revealed co-localization of SOCS1 and Y505FLck in the perinuclear region of cotransfected COS7 cells (Fig. 2B). These results suggest that SOCS1 may inhibit the oncogenic Y505FLck kinase through direct interaction.

Phosphorylated Tyr394 in the kinase domain of Y505FLck is important in SOCS1 interaction. Oncogenic Y505FLck mutant has constitutive kinase activity with constant phosphorylation of the positive regulatory tyrosine 394 (5). To test if interaction between SOCS1 and Y505FLck depends on Tyr394 phosphorylation, we mutated Tyr394 to Phe in the context of Y505FLck (Fig. 3A). Kinase-dead Lck is incapable of auto-phosphorylating Tyr394 and is expected to behave like the Y394F Lck mutant. Therefore, we also constructed the kinase-dead Lck mutant by mutating the conserved Lys273 in Y505FLck to Arg (Fig. 3A). All three Lck mutants were examined by co-precipitation assay with GST-SOCS1 in COS7 cells. As shown in Fig. 3B (lanes 4-6), glutathione pulled down similar amounts of GST-SOCS1 fusion proteins (bottom panel) and Lck mutants (middle panel) even though the expression level of Y505FLck was significantly less than the other two mutants (top panel). In order to account for the lower expression level of Y505FLck, we calculated relative affinity of all three Lck mutants for GST-SOCS1. Statistical analysis showed ~70% reduction in SOCS1-binding affinity of Y505FLck with the additional Y394F or K273R mutation as compared to Y505FLck (Fig. 3C). As negative controls (Fig. 3B), no Lck was pulled down by glutathione from cells

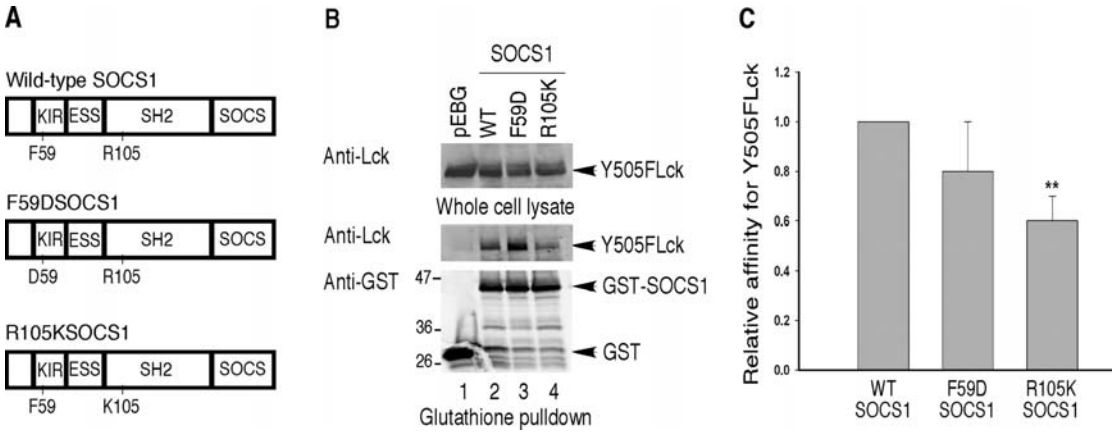

Figure 5. A functional SH2 domain in SOCS1 is involved in binding to the oncogenic Lck kinase. (A) Schematic diagrams of SOCS1 functional domains and point mutations. KIR and ESS represent the kinase inhibitory region and the extended SH2 subdomain, respectively. A highly conserved SOCS box in the carboxy-terminus is also illustrated. (B) COS7 cells were cotransfected with 5 μg of pcDNA/Y505FLck and 5 μg of pEBG without or with wild-type (WT) and mutant SOCS1 constructs. A small aliquot of normalized whole cell lysate was analyzed by anti-Lck immunoblotting to detect Y505FLck (top panel). Following glutathione pulldown, Western blotting was performed with anti-Lck antibody to detect Y505FLck (middle panel) and anti-GST antibody to detect GST and GST-SOCS1 (bottom panel). (C) The relative affinity of wild-type and mutant SOCS1 for Y505FLck was calculated as described in Materials and methods. The affinity of wild-type GST-SOCS1 for Y505FLck was set as 1 and values represent the means ± SD (n=3; $^{**}p<0.01$).

transfected with pEBG (lanes 1-3) or vector lacking Lck (lane 7). All together, these results demonstrate the critical role of phosphorylated Tyr394 in mediating SOCS1-Lck interaction.

Kinase domain of Lck is sufficient to mediate the interaction with SOCS1. The observation that phosphorylation of Tyr394 in Lck is important in Lck-SOCS1 association suggests that the Lck kinase domain may be sufficient in mediating its interaction with SOCS1. To test this hypothesis, we divided the full-length wild-type Lck into N32 Lck and kinase Lck (Fig. 4A). Due to the absence of intra-molecular interaction, the truncated kinase Lck mutant remains in its open conformation and is constitutively active (21). Consistent with our hypothesis, significant amounts of the kinase Lck truncation mutant co-precipitated with GST-SOCS1 as compared to the GST alone control (Fig. 4B, middle panel, lanes 2 and 4). It should be noted, however, that interaction of the N32 Lck truncation mutant with GST-SOCS1 was also above the GST alone control (Fig. 4B, middle panel, compare lanes 1 and 3). Statistical analysis shows that affinity of kinase Lck for GST-SOCS1 is significantly higher than that of N32 Lck (Fig. 4C). Therefore, while the kinase domain of Lck plays a key role in its association with SOCS1, other functional domains of Lck may also contribute to the overall high affinity binding to SOCS1.

SOCS1 SH2 domain is involved in the interaction with oncogenic Y505FLck kinase. To determine if the SOCS1 SH2 domain can recognize phosphorylated Tyr394 in Y505FLck, we mutated the highly conserved Arg105 to Lys, making the SH2 domain non-functional (Fig. 5A) (22). To evaluate the contribution of KIR in binding to Y505FLck, we also constructed a SOCS1 mutant with a defective KIR by mutating Phe59 to Asp (Fig. 5A) (23). Both wild-type and mutant SOCS1 were expressed as GST fusion proteins and tested for their ability to pull down Y505FLck in cotransfected COS7 cells. As shown in Fig. 5B, SOCS1 mutant with the defective SH2 domain, but not the KIR-deficient SOCS1 mutant, pulled down significantly less Y505FLck as compared to wild-type SOCS1 (middle panel, lanes 2-4). Statistical analysis confirmed that the SH2 domain mutation in SOCS1 caused a 40% reduction in its Lck-binding affinity (Fig. 5C). As a negative control, GST alone did not bring down detectable Y505FLck (Fig. 5B, middle panel, lane 1). We conclude that the interaction between the SOCS1 SH2 domain and the phosphorylated Tyr394 in Lck contribute greatly to their high affinity association.

Discussion

Although SOCS proteins are closely related to each other both structurally and functionally, there is a wide variation in their interaction with oncogenic protein tyrosine kinases (12). Previous studies have shown that SH2 domains of SOCS1 and SOCS3 are involved in their association with protein tyrosine kinases (22,24,25). We identify the SOCS1 SH2 domain as a mediator of the Lck-SOCS1 interaction. However, the SH2 mutation in SOCS1 can not completely abolish Lck-SOCS1 association (Fig. 5). It suggests that other SOCS1 functional domains may contribute to its interaction with the oncogenic Lck kinase. Yasukawa *et al* reported that the SH2 domain, the KIR domain, and the extended SH2 subdomain (ESS) in SOCS1 all contributed to the high-affinity binding of SOCS1 to JAK2 (22). Even though we did not observe significant loss of Lck binding to the KIR mutant (Fig. 5), it remains possible that the ESS domain may contribute to SOCS1-Lck association. It should also be noted that sequences of some regions in the SH2 domain of SOCS proteins are highly variable. Therefore, it is possible that sequence variation between the SH2 domains of different SOCS proteins may influence their affinity for oncogenic protein tyrosine kinases, such as Lck.

Previous studies showed that SOCS1 interaction with other protein tyrosine kinases, such as JAK and FAK, was solely dependent on phosphorylation of the conserved tyrosine

residues in their kinase domains (13,22). We also identify phosphorylated Tyr394 in the Lck kinase domain as one of the key determinants in mediating Lck-SOCS1 interaction (Fig. 3). However, Y394F mutation did not completely abolish Lck association with SOCS1. It suggests that other Lck functional domains contribute to its interaction with SOCS1. Consistent with this hypothesis, we detected low levels of interaction between the N32 Lck truncation mutant with SOCS1 (Fig. 4). It raises the possibility that the N-terminal, SH3 or SH2 domains of Lck may also contribute to the association between SOCS1 and full-length Lck. Furthermore, we consistently observed reduced expression of Y505FLck compared with Y394FY505FLck or K273RY505FLck (Fig. 3B). Giannini *et al* showed that Y505FLck was degraded more rapidly than wild-type Lck in COS7 cells (26). Based on this report, it is possible that the active Y505FLck kinase may exhibit faster turnover rate than the other two mutants that are less active (Y394FY505FLck) or inactive (K273RY505FLck).

We show here that SOCS1 has the highest affinity for the oncogenic Y505FLck kinase (Fig. 1). While the phosphorylated Tyr394 in the Lck kinase domain and the SOCS1 SH2 domain are two key determinants in mediating Lck-SOCS1 interaction, our data suggest that other Lck and SOCS1 functional domains may also contribute to the strong Lck-SOCS1 interaction. We can not exclude the possibility that other cellular proteins may be involved in the association between Lck and SOCS1. For example, previous studies in a yeast two-hybrid system showed that SOCS1 bound to the kinase domain of JAK2, but not Lck kinase (27). On the other hand, SOCS3 was capable of binding to Lck kinase in the same yeast two-hybrid system. It suggests that the cellular context may greatly affect how different SOCS family members interact with distinct protein tyrosine kinases.

It is likely that the interaction between SOCS1 and oncogenic Y505FLck is responsible for SOCS1-mediated inhibition of Lck kinase activity and subsequent suppression of cellular transformation that we reported previously (20). Earlier studies in v-Src-transformed fibroblasts showed that SOCS1 was unable to inhibit v-Src-induced STAT3 activation or v-Src-mediated cellular transformation (15,28). Lck and Src belong to the same protein tyrosine kinase family and have high degree of structural similarity. While the molecular details of interaction between SOCS1 and v-Src have yet to be determined, biological data from the above reports strongly suggest the delicate specificity of SOCS1 tumor-suppressing activity toward distinct oncogenic protein tyrosine kinases. Our studies reported here will help us better understand the molecular mechanisms of SOCS-mediated tumor suppression. They also reveal important insights into designing protein tyrosine kinase inhibitors with higher specificity.

Acknowledgments

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