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Cloning and Functional Analysis of New Members of STAT Induced STAT Inhibitor (SSI) Family: SSI-2 and SSI-3

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Upon the corresponding ligand's stimulation, the cytokine receptors activate several signal pathways: JAK-STAT pathway, Ras-MAP kinase pathway and so on. Recently, we demonstrated that one of the STAT3 (signal transducer and activator of transcription-3) target genes could suppress the function of STAT3 and designated as SSI-1(STAT induced STAT inhibitor-1). SSI-1 is thought to play a critical role in negative feedback control of JAK-STAT signaling pathway. In the present study, we identified two novel human genes which products have homologous region in their SH2 domain and its COOH-terminal region to mouse SSI-1. Northern blotting analysis and functional studies demonstrated that SSI-2 and SSI-3 mRNA were also induced by cytokine stimulation and their forced expression in mouse myeloid leukemia cell, M1, suppressed the apoptotic effect of LIF, like SSI-1. We also demonstrated the structure of human SSI-1.

The interaction of cytokine with their receptor causes the activation of JAK (Janus kinase) family kinases which associate cytokine receptors constitutively. This step is critical in triggering the downstream signal transduction. One of these signal is JAK-STAT pathway (1-3). The activated JAK kinases phosphorylate the corresponding receptor and STAT (signal transducers and activators of transcription), resulting in specific gene expression, cell proliferation, cell differentiation and apoptosis. Genes transcriptionally activated by STAT determine stimulation-induced response. For example, STAT3 plays a critical role in the expression of acute phase proteins in hepatocytes (4, 5) and the differentiation of mouse myeloid leukemic cell M1 into macrophage by IL-6 or LIF stimulation (6, 7).

Recently, we demonstrated that one of the STAT3 target genes worked as an inhibitor of STAT3 and named as mouse SSI-1(STAT induced STAT inhibitor-1) (8). SSI-1 inhibits the IL-6-induced tyrosine phosphorylation of gp130 and STAT3 by direct binding to JAK kinases. From the structure of SSI-1, we found that SSI-1 showed significant homology with CIS (Cytokine Inducible SH2 protein) (9). CIS was reported to be induced by IL-2, IL-3, GM-CSF or Epo stimulation and bind to the tyrosine phosphorylated sites of βc or Epo receptor and regulate the cytokine signal negatively.

In the present study, we speculated that structural related genes with SSI-1 might work as negative regulators of cytokine signal. As a result of computer-aided homology search, we identified two novel human genes, SSI-2 and SSI-3, homologous to mouse SSI-1. Both of them had similar function to SSI-1, although their induction pattern by cytokines and expression pattern in various tissue are different. We also cloned human SSI-1 gene and showed its structure. These results indicate that these genes form a new gene family.

MATERIALS AND METHODS

Isolation of SSI-2 and SSI-3 cDNAs. Using ^{32}P-labelled PCR fragments derived from the partial sequences of human SSI-2 and human SSI-3 (GenBank accession number, D31101 and W60547 for SSI-2 and AA018910 for SSI-3), the SSI-2 and SSI-3 cDNAs were isolated from a human activated Jurkat cDNA library (lambda ZAP express, Stratagene, La Jolla, CA). Concerning the 3′ region of SSI-3 cDNA, we employed 3′ RACE technique to obtain it. Human SSI-1 cDNA was also obtained by screening the human activated Jurkat cDNA library using ^{32}P-labelled mouse SSI-1 cDNA fragment.

Cell culture. Myeloid leukemia M1 cells were cultured in Eagle's minimal essential medium supplemented with twice the normal con-

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Abbreviations used: IL, interleukin; JAK, Janus kinase; STAT, signal transducer and activator of transcription; SSI, STAT induced STAT inhibitor; CIS, cytokine inducible SH2 protein; Epo, erythropoietin; GM-CSF, granulocyte macrophage colony stimulating factor; PBL, peripheral blood lymphocyte.

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centrations of amino acids and vitamins and 10% [vol/vol] fetal calf serum [FCS]. The IL-3-dependent myeloid NFS60 cells (10) were maintained in RPMI 1640 medium supplemented with 10 % FCS and 10 % conditioned medium from the WEHI-3B cell line as a source of IL-3. The IL-2/IL-4-dependent CT4S cells (11) were cultured in RPMI 1640 medium supplemented with IL-4 (10 U/ml) and 10 % FCS.

Northern blotting. Cells were factor-depleted for 4 hrs in RPMI medium containing 1 % BSA, and then stimulated with cytokines at the following concentrations for various periods: 10 ng/ml of IL-2, 5 ng/ml of IL-3, 10 U/ml of IL-4 or 20 ng/ml of G-CSF. Cytoplasmic RNA was extracted using Iso-Gen (Nippon Gene, Tokyo, Japan). Total RNA was subjected to agarose gel electrophoresis and transferred to a nylon membrane Hybond N+, (Amersham, Buckinghamshire, England). The membrane was hybridized with radiolabeled human SSI-2 cDNA probes. After dehybridization, the same membrane were hybridized with radiolabeled human SSI-3 cDNA probes, again. In the same way, human MTN blot (Clonthec, Palo Alto, CA) was hybridized with radiolabeled human SSI-1, SSI-2, SSI-3 and mouse β-actin probes.

Plasmid construction and DNA transfection. The expression vectors for SSI-2 and SSI-3 were constructed using pEF-BOS (12), a mammalian expressing vector. Briefly, pEF-BOS was digested with XbaI, and ligated with cloned cDNA fragment for SSI-2 or SSI-3. M1 cells were transfected by electroporation method with each expression vector and pSTneoB selection marker vector (13), and neomycin-resistant clones were selected in growth medium containing Geneticin (Gibco BRL, Rockville, MD) at 600 μg/ml.

Western blotting. Cells (5×10^6 cells) were treated with 0u, 40u, 200u, or 1000u/ml of LIF for 5 mins or 1000u/ml of LIF for 60 mins, and solubilized with lysis buffer (0.5% Nonidet P-40, 10mM Tris, pH 7.4,150 mM NaCl, 1 mM EDTA, 1 mM Na_2VO_4) containing protease inhibitors. The immunoprecipitates obtained with anti-STAT3 antibody (4) were resolved by SDS/PAGE under reducing condition and immunoblotted with anti-phosphotyrosine monoclonal antibody: 4G10 (Upstate Biotechnology, Lake Placid, NY). Bound antibody was visualized with an enhanced chemiluminescence system (Amersham, Buckinghamshire, England).

RESULTS

Computer-aided homology search for the SH2 domain of SSI-1 in DDBJ/EMBL/GenBank database showed us partial sequences of several candidates for SSI gene family. In order to clone the full-length cDNAs encoding two of these candidates (GenBank accession number, D31101 and W60547 for SSI-2 and AA018910 for SSI-3), we made specific primers and used to amplify about 540 bp fragment for SSI-2 and 360 bp for SSI-3 from cDNA library of human activated PBL.

Using human DNA fragments, we screened a ZAP express activated Jarkat cDNA library (Stratagene, La Jolla, CA) and obtained several clones for human SSI-2 and one clone for human SSI-3. We also obtained human SSI-1 cDNA in the same way using mouse SSI-1 cDNA fragment. In addition, we obtained 3′ region of SSI-3 cDNA using 3′ RACE technique because we failed to obtain a full-length cDNA for SSI-3 from the ZAP express activated Jarkat cDNA library. Sequencing analysis of these clones revealed that SSI-2 and SSI-3 have 594 and 675 nucleotides of open reading frame encoding 198 amino acids and 225 amino acids,

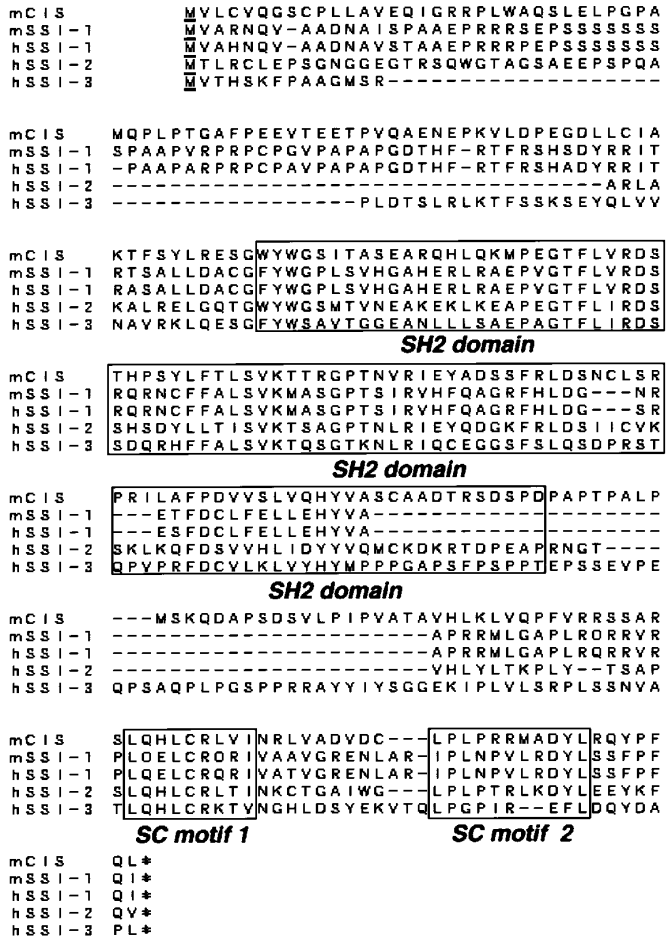

FIG. 1. The amino acid sequences deduced from human SSI-2 and human SSI-3 cDNAs and their comparison with human and mouse SSI-1 and mouse CIS. The first methionine were underlined and the asterisk indicates the stop codon. The dashed line indicates a gap. SH2 domain and conserved alignments are boxed and indicated as SH domain, SC motif-1 and SC motif-2.

respectively (Fig.1). The deduced amino acids sequences of human SSI-2 and human SSI-3 showed significant homology with human and mouse SSI-1 and mouse CIS at the COOH-terminal region as well as the SH2 domain (Fig.1). Comparison of the amino acids alignments among these molecules revealed two conserved motifs at COOH-terminal region. We named these conserved motifs as SSI COOH-terminal motif 1 and 2 (SC motif 1 and 2). At present, it is not clear whether these motifs are important in their function. Mutational analysis will clarify the functional roles of these motifs.

In order to examine whether these genes are inducible by cytokine stimulation, we stimulated CT4S or NSF60 cells with IL-2, IL-4 or IL-3, G-CSF, respectively. As shown in Fig. 2, both of these genes were induced by IL-2 or IL-4 stimulation in CT4S cells, and by IL-3 and G-CSF in NSF60 cells, although the bands

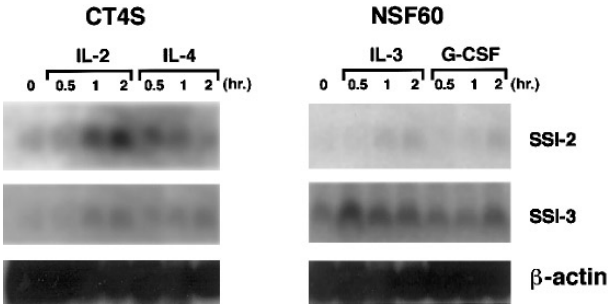

FIG. 2. Induction of mRNA for SSI-2 and SSI-3 by cytokines. CT4S cells were stimulated with IL-2 (10 U/ml) or IL-4 (10 ng/ml) and NFS60 cells were stimulated with IL-3 (10 U/ml) or G-CSF (20 ng/ml) for the indicated periods. Total RNA was extracted from the cells and Northern blotting analyses were performed with human SSI-2, human SSI-3 and β-actin probes.

showing SSI-2 in NSF60 cells or SSI-3 in CT4S cells were very faint. The comparison of the detected bands clearly demonstrated that the induction ratio of SSI-2 gene to SSI-3 gene was different between the stimulated cell type. We also examined the expression pattern of these genes in human tissue (Fig.3). SSI-1 was strongly expressed in spleen, small intestine and PBL. However, strong expression of SSI-2 was observed in heart, placenta, lung, kidney and prostate; and that of SSI-3 in heart, placenta, lung, skeletal muscle and PBL. These results indicate they have a different distribution of their expression in the body, even if they showed the similar function as shown below.

Myeloid leukemia M1 cells were previously demonstrated to differentiate into macrophages and cause apoptotic cell death in response to IL-6 or LIF (6, 7). In order to examine their function in the cytokine signal transduction, we constructed the expression vector, pEF-BOS/SSI-2 and pEF-BOS/SSI-3, and introduced them into M1 cells (M1/SSI-2 clones, M1/SSI-3 clones), because they were structurally homologous to SSI-1 and the forced expression of SSI-1 abrogated LIF-induced M1 cell death (8). As shown in Fig.4, M1/SSI-2 or M1/SSI-3 clones did not show any growth arrest in response to LIF stimulation as the control Neo-resistant M1 clones did. These results strongly suggest that the function of SSI-2 or SSI-3 is similar to that of SSI-1.

In M1 cells, the forced expression of dominant negative STAT3 also abrogated this response (6, 7), indicating that the activation of STAT3 is responsible for it. Therefore, we examined the tyrosine-phosphorylation of STAT3 using anti-phosphotyrosine antibody before and after LIF stimulation in M1 transformants. As shown in Fig. 5, partial suppression of LIF-induced STAT3 tyrosine phosphorylation was observed in both M1/SSI-2 clone and M1/SSI-3 clone, when compared with the control Neo-resistant M1 clones. After stripping of anti-phosphotyrosine antibody, the detected band showing STAT3 by anti-STAT3 antibody were also reduced in M1/SSI-3 clone, when compared with that of the control Neo resistant M1 clones. Another M1/SSI-3 clone also showed the same result (data not shown). These results indicate that SSI-2 and SSI-3 partially suppress LIF-induced STAT3 activation, and may suggest that the forced expression of SSI-3 reduces the protein level of STAT3 in M1 cells in an unknown manner. It is not clear whether the partial suppression of STAT3 is sufficient for the abrogation of LIF-induced growth arrest in M1 cells. Alternatively, SSI-2 and SSI-3 may also suppress another signaling pathways as well as JAK-STAT pathway, both of which are indispensable for M1 differentiation.

DISCUSSION

In the present study, we identified two novel genes, SSI-2 and SSI-3 homologous to SSI-1 and CIS, and raised the possibility that these genes may be included in a novel gene family, SSI family. Several characteris-

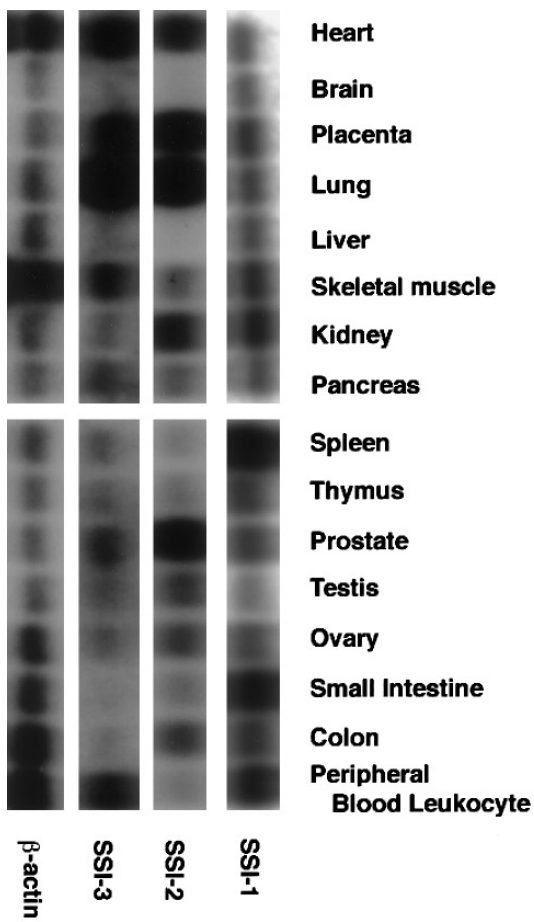

FIG. 3. Expression of SSI-2 and SSI-3 mRNA in various human tissues. Human MTN Blot membrane (Clontech, Palo Alto, CA) was hybridized with radiolabeled human SSI-1, human SSI-2, human SSI-3 or β-actin probes.

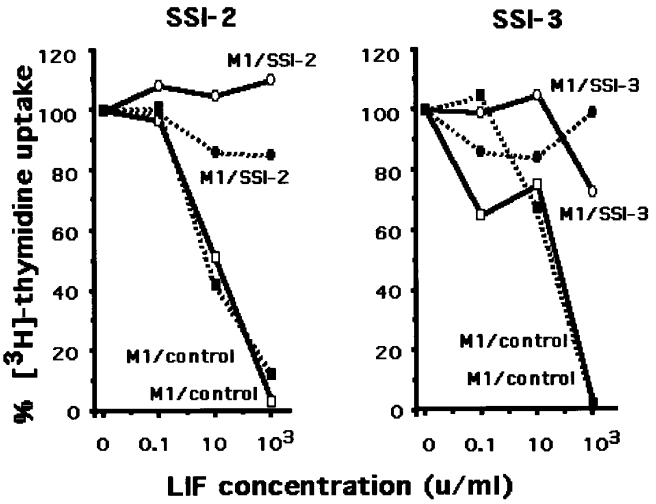

FIG. 4. Abrogation of LIF-induced growth arrest in Ml clones transfected with human SSI-2 or SSI-3 expression vector. SSI-2 or SSI-3 expressing M1 transfectants (1×10^4) were seeded with LIF (0, 0.1, 10,1000 U/ml) at day 0, and [^{3}H]-thymidine incorporation was measured at day 3. As a control, we used M1 clones transfected with Neo resistant gene alone. Each value was demonstrated as percentage of [^{3}H]-thymidine uptake against non-stimulated M1 transfectants and represents the mean of triplicated experiments.

tics of SSI family are as follows: (i) They have an SH2 domain in the middle of them and show homology in its COOH-terminal region. (ii) Their expression is induced by various cytokine stimulation (presumably, by activated STATs). (iii) They have inhibitory effects on, at least, STAT activation.

From their structure, we found a significant homology in SH2 domain and its COOH-terminal side among the molecules, and they don't have any characteristic motifs except SH2 domain. We can imagine that SH2 of SSI family will bind to the tyrosine phosphorylation sites of JAK kinases, cytokine receptors, STATs and so on, after cytokine stimulation. The association of SSI gene family products with these signal transducers may inhibit the cytokine signals by masking the signal transducer, destabilizing it or dephosphorylation of it. We cannot deny that other molecules, such as tyrosine phosphatase or protease, are involved in the inhibitory effects of SSI gene family products, because SSI family molecules seem to have any enzymatic activity. If such molecules exist, they may associate with SSI family molecules at COOH-terminal domain, which contains the conserved motifs (SC motif 1 and 2).

In this study, we did not show any direct evidences that SSI-2 and SSI-3 genes are induced by activated STATs. Cytokine stimulation activates several signaling pathways: JAK-STAT pathway, Ras-MAP kinase pathway and so on. However, in IL-4 system, STAT6 knockout mice abrogated all of IL-4 function (14-16), suggesting that IL-4 signal is mainly transduced by STAT6 activation. This finding suggests that the activated STAT6 may be responsible for, at least, SSI-2 expression and, presumably, SSI-3 expression.

It is surprising that the forced expression of all members of SSI family genes can prevent the LIF signal in M1 cells. However, the questions are remained whether these inhibitory effect of the SSI family is specific to gp130-mediated signaling. In addition, the inhibition of STAT3 activation was partial in the SSI-2 and SSI-3 transduced M1 cells, suggesting that the partial suppression of STAT3 activation is sufficient for the inhibition of gp130-mediated M1 cell differentiation, or SSI-2 and SSI-3 may also suppress another signaling pathways as well as JAK-STAT pathway, both of which are indispensable for M1 cell differentiation.

Physiological roles of the SSI gene family are not clear, although we can imagine that they play an important role on the cytokine signal negative regulation. The induction efficiency of these gene family seems to be different among cell types as observed in CT4S and NSF60 cells, and the expression pattern is also different among the various tissues, which suggests that their physiological roles are different. Knockout mice of the SSI family may be helpful to understand their physiological function and may show the overresponse (proliferation, differentiation, gene activation) to cytokine in a certain type of cells, even if SSI family products have similar function. In addition, SSI-1 binds to JAK kinase and suppress the STAT activation (8). However, CIS inhibits the STAT activation by competing with STAT at the step of its binding to the tyrosine-phosphorylated cytokine receptor (9). It is interesting to elucidate which step of cytokine signal is suppressed by SSI-2 or SSI-3. Further experiments will answer these questions.

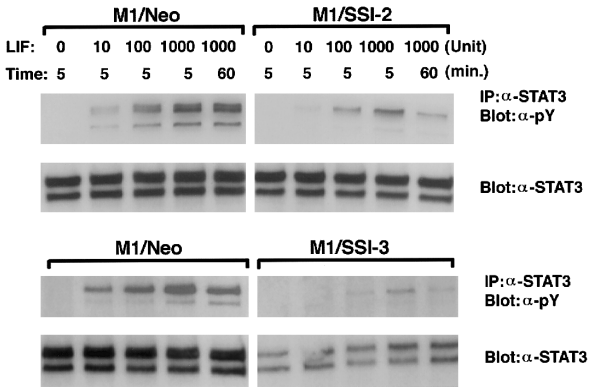

FIG. 5. Suppression of LIF-induced tyrosine-phosphorylation of STAT3 in M1 cells transfected with human SSI-2 or SSI-3 expression vector. Cells (5×10^6 cells) were treated with 0u, 40u, 200u, or 1000u/ml of LIF for 5 mins or 1000u/ml of LIF for 60 mins, and solubilized with NP-40 lysis buffer. Immunoprecipitates obtained with an anti-STAT3 antibody were immunoblotted with an anti-phosphotyrosine monoclonal antibody. After stripping of anti-phosphotyrosine antibody, the protein levels of STAT3 were also examined by using STAT3 antibody.

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