SOCS-1/JAB/SSI-1 Can Bind to and Suppress Tec Protein-tyrosine Kinase*

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Tec is the prototype of a recently emerging subfamily of protein-tyrosine kinases and is known to become tyrosine-phosphorylated and activated by a wide range of cytokine stimulations in hematopoietic cells. Although Tec was recently shown to be involved in the cytokine-driven activation mechanism of c-fos transcription, it is yet obscure how Tec relays the signals from cell surface receptors to the nucleus. To identify signaling molecules acting downstream of Tec, we have looked for Tec-interacting proteins (TIPs) by using the yeast two-hybrid system. Here we report the identification and characterization of a novel protein, TIP3, which has been simultaneously identified by other groups as SOCS-1, JAB, or SSI-1. TIP3 carries one Src homology 2 domain with a sequence similarity to that of CIS. In 293 cells, TIP3 associates with Tec and suppresses its kinase activity. Interestingly, TIP3 can also down-regulate the activity of Jak2 but not that of Lyn. We propose that SOCS-1/JAB/SSI-1/TIP3 is a novel type of negative regulator to a subset of protein-tyrosine kinases.

Tec is the prototype of a recently emerging subfamily of cytoplasmic protein-tyrosine kinases (PTKs), the Tec family comprised of Tec (1), Btk (2, 3), Itk/Tsk/Emt (4–6), Txk (7), and Bmx (8). Many members of this family are abundantly expressed in hematopoietic tissues, and Tec has been shown to be involved in the intracellular signaling systems of a wide range of cytokines including interleukin (IL)-3, IL-6, stem cell factor, granulocyte colony-stimulating factor, erythropoietin, and thrombopoietin (9–14). In addition to cytokine receptors, recent studies have indicated that other cell surface receptors on lymphocytes, such as CD28 (15) and CD38 (16), can also stimulate the Tec family kinases. It remains, however, unclear what the in vivo roles of these Tec family members are. Do they participate specifically in cell growth mechanism of blood cells, differentiation mechanism, or both? What kind of signaling molecules do they interact with in vivo?

We have recently revealed that transient introduction of Tec into an IL-3-dependent cell line, BA/F3 (17), resulted in marked elevation of the promoter activity of the c-fos proto-oncogene and that introduction of kinase-dead Tec suppressed the IL-3-driven activation of the c-fos promoter, suggesting that Tec is directly involved in the cytokine-driven activation mechanism of c-fos proto-oncogene. To investigate how Tec regulates c-fos transcription, we have searched for Tec-interacting proteins (TIPs) by using yeast two-hybrid screening and have identified seven TIPs. Here we report the molecular cloning and characterization of a novel protein, TIP3. The predicted TIP3 protein contains one Src homology (SH) 2 domain with the highest similarity to that of mouse CIS (18). In the reconstitution system in 293 cells, TIP3 was shown to bind to Tec and suppress its activity. We could further reveal that TIP3 is a negative regulator of Jak2.

EXPERIMENTAL PROCEDURES

Isolation of TIP3 cDNA—By using the MATCHMAKER two-hybrid system (CLONTECH, Palo Alto, CA), we conducted a yeast two-hybrid screen in which human Tec kinase domain (amino acids 357–630) fused to the DNA-binding domain of GAL4 was used as a “bait” to identify cDNA clones from a variety of cDNA libraries. From more than three million transformants, we could obtain seven independent cDNA groups, the products (TIP 1–7) of which could interact with Tec in yeast cells. TIP3 cDNAs were initially identified in the human B-cell cDNA library (CLONTECH), and the full-length cDNAs were subsequently isolated from a cDNA library of UT-7 cells (19) constructed in the pAZAP1 phage vector (Stratagene, La Jolla, CA). The nucleotide sequence of the longest TIP3 cDNA was determined on both strands.

Plasmid Construction—The EcoNI-KpnI fragment of human TIP3 cDNA containing the full coding region was blunt-ended by T4 DNA polymerase and inserted into the pTagCMV-neo vector (20) to encode the TIP3 protein with an NH2-terminal tag of His, plus human immunodeficiency virus (HIV) gp120 epitope (His-HIV-TIP3). The EcoNI-NotI or EcoNI-ApaI fragment of the TIP3 cDNA was also blunt-ended and ligated with the same vector to encode His-HIV-TIP3ΔI or His-HIV-TIP3Δ2, respectively. The HIV-TIP3 cDNA and its derivatives were further isolated from these plasmids by BamHI digestion and were subcloned into the pEBG vector (21), giving rise to pGST-HIV-TIP3, pGST-HIV-TIP3ΔI, and pGST-HIV-TIP3Δ2. Expression plasmids of nontagged TIP3 and PTKs were all constructed on the pSR vector.

Immunoprecipitation and Western Blot Analyses—Expression plasmids (10 µg of each) of TIP3, Tec, Lyn, or Jak2 were introduced into 2 × 10⁶ of 293 cells (American Type Culture Collection, Rockville, MD) by...
the calcium phosphate method. Cells were harvested at 48 h post-transfection and solubilized by the 1% lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl2, and 2 mM MnCl2) and incubated with 0.37 MBq of [γ-32P]ATP (American Corp.) for 15 min at 30 °C. When analyzing the Jak2 activity, a synthetic substrate of Jak2 (Upstate Biotechnology) was added to the reaction mixture (20 μg/μl). The precipitates irrespective of the Tec activity. Because our anti-Tec serum was raised against the GST-Tec SH3 fusion protein, the serum can recognize GST as well as Tec. Therefore, GST-HIV-TIP3 protein was precipitated in each fraction. Expression of Tec in each fraction was also verified by probing the total cell lysates with anti-Tec serum (Fig. 2B, lower panel). To determine which domain of TIP3 is responsible for the binding to Tec, GST, GST-HIV-TIP3, GST-HIV-TIP3Δ1 (lacking the COOH-terminal 39 residues), or GST-HIV-TIP3Δ2 (lacking the COOH-terminal 129 residues) was expressed in 293 cells with Tec. GST or GST-HIV-TIP3 derivatives were precipitated and probed with anti-Tec serum (upper panel of Fig. 2C). Deletion of the SH2 and COOH-terminal domains could not block the binding between TIP3 and Tec, suggesting that the NH2-terminal region of TIP3 is responsible for the binding to Tec. Again, the precipitated GST-HIV-TIP3 and its derivatives were identified by the serum in the same blot (Fig. 2C, middle panel). Appropriate expression of Tec in each set was confirmed in Fig. 2B (lower panel).

We next tested whether TIP3 can regulate the tyrosine phosphorylation or kinase activity of Tec. Tec was expressed in 293 cells with or without GST-HIV-TIP3 and its deletion mutants, immunoprecipitated, and probed with anti-phosphotyrosine antibody (α-P-Tyr Ab). As shown in the upper panel of Fig. 2D, co-expression of GST-HIV-TIP3 suppressed the tyrosine phosphorylation of Tec. Truncation of the COOH-terminal domain in TIP3 further enhanced this suppressive effect (T+G-TIP3Δ1

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**RESULTS AND DISCUSSION**

To identify signaling molecules acting downstream of Tec, we carried out a yeast two-hybrid screen in which human Tec kinase domain (amino acids 357–630) was used as a bait to identify TIPs. From the cDNA library of human B-cells transformed by Epstein-Barr virus, we could isolate one cDNA group, the product (TIP3) of which can interact with the Tec kinase domain in yeast cells. Northern blot analysis with the TIP3 cDNA probe revealed that a human granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent cell line, UT-7, expresses the TIP3 message of about 1.3 kilobases under normal (not shown). Therefore, we tried to isolate full-length TIP3 cDNAs from a conventional cDNA library of UT-7 cells containing the 5′ and 3′ ends of the cDNA, and were used to infect Sf21 cells at the multiplicity of infection of 1.0.

**Recombinant Baculoviruses**—The cDNA of His-HIV-TIP3 or Jak2 was inserted into the pFastBac1 plasmid (Life Technologies, Inc.). The recombinant baculoviruses based on these plasmids were generated by the Bac-to-Bac baculovirus expression system (Life Technologies, Inc.) according to the manufacturer’s protocol. 293 cells at a multiplicity of infection of 1.0.

**Luciferase Reporter Assay**—With the c-fos promoter-luciferase plasmid (pfos/luc) as a reporter, the expression plasmid of each kinase was introduced into BA/F3 cells by electroporation at the condition of 200 V and 960 microfarad, followed by the incubation in RPMI 1640 medium (Life Technologies, Inc.) with 10% fetal calf serum for 5 h. The samples were further cultured for 5 h either unstimulated or stimulated with 25 units/ml of mouse IL-3. Luciferase activities were measured by using the Luciferase Assay System (Promega, Madison, WI), and are shown as relative light units/min/μg of protein.

**FIG. 1. Sequence analysis of human TIP3 cDNA.** The nucleotide sequence of human TIP3 cDNA and its deduced amino acid sequence are shown. The stop codon of the open reading frame and the polyadenylation signal are indicated by an asterisk and a broken underline, respectively. Amino acid residues of TIP3 are shown in single-letter codes with its SH2 domain double underlined and a potential PEST sequence underlined. Nucleotides and amino acids are numbered at the right side of the figure.
Fig. 2. TIP3 binds to Tec and suppresses its kinase activity in cells. A, schematic representation of TIP3 and its derivatives used in this study. TIP3 constructs were tagged at the NH2 terminus with GST (hatched oval) plus the HIV gp120 epitope (closed diamond) or His (open oval) plus the HIV epitope. The first and the last amino acids of the fragments are numbered with respect to their positions in the full-length TIP3. B, GST-HIV-TIP3 associates with Tec in 293 cells. GST (G) or GST-HIV-TIP3 (G-TIP) was expressed in 2 x 10^6 of 293 cells without or with Tec (T) or kinase-inactive Tck (Tck, Lys-357 in the ATP binding site of Tec is replaced with Met) as indicated at the top. GST and GST-HIV-TIP3 were purified by glutathione-Sepharose beads from the cells lysed by the 0.1% lysis buffer, separated through 7.5% SDS-polyacrylamide gel electrophoresis, and immunoblotted with anti-Tec serum (Glutathione beads). The serum could simultaneously recognize Tec and GST-HIV-TIP3 (indicated at the right). Total cell lysates (10 μg/lane) of the same set were also probed with anti-Tec serum to estimate the expression level of Tec in each fraction (TCL). C, the NH2 domain of TIP3 is required for the binding to Tec. In 2 x 10^6 of 293 cells, Tec (T), GST, GST-HIV-TIP3 (G-TIP), GST-HIV-TIP3Δ1 (G-TIPΔ1), or GST-HIV-TIP3Δ2 (G-TIPΔ2) were expressed in the various combinations as indicated at the top. Cells were lysed by the 0.1% lysis buffer, and GST or the GST-TIP3 derivatives were purified from each fraction by glutathione-Sepharose beads (Glutathione beads). The precipitates were immunoblotted with anti-Tec serum, which reacted with Tec and GST-TIP3 derivatives (indicated at the right). Total cell lysates (10 μg/lane) of the same sample set were also blotted with anti-Tec serum to estimate the expression level of Tec in each fraction (TCL). D, TIP3 suppresses the tyrosine phosphorylation of Tec. Tec was immunoprecipitated from 293 cells expressing Tec (T) with GST (G), GST-HIV-TIP3 (G-TIP), GST-HIV-TIP3Δ1 (G-TIPΔ1), or GST-HIV-TIP3Δ2 (G-TIPΔ2) and immunoblotted with aP-Tyr Ab (4G10, Upstate Biotechnology, Inc.). In the bottom panel, the same membrane was reprobed with anti-Tec serum (αTec) to estimate the amounts of Tec protein precipitated. E, TIP3 suppresses the kinase activity of Tec. Tec was immunoprecipitated from the same set of 293 cells as in D and incubated in the kinase buffer with [γ-32P]ATP for 15 min at 30°C. Autophosphorylation of pp70SOCS-1/JAB/SSI-1 as a Suppressor of Tec (24). We thus tested whether co-expression of TIP3 suppresses this Jak2-mediated phosphorylation of the βc chain of human GM-CSF receptor. The βc chain was expressed in 293 cells either alone or in combination with Jak2 or Jak2 plus His-HIV-TIP3. As shown in the upper panel of Fig. 3D, Jak2 could phosphorylate the βc chain in cells, but this effect was abolished by the co-expression of TIP3. Therefore, TIP3 should modulate the Jak2-mediated signalings in vivo.

One of the initial aims of this study was to identify signaling proteins that specifically bound to protein A-Sepharose beads and glutathione-Sepharose beads, we could not test the physical interaction of these two molecules in the reconstitution system in 293 cells. Instead, Jak2 and TIP3 were expressed in the insect cell system. The recombinant baculovirus expressing His-HIV-TIP3 (see Fig. 2A) was used to infect SF21 cells derived from Spodoptera frugiperda, either alone or in combination with the virus expressing Jak2 or kinase-dead Jak2KDF in which Lys-882 at the ATP-binding site is replaced with Glu. Jak2 was immunoprecipitated from each fraction and probed with anti-HIV tag antibody (H902), showing that TIP3 can interact with Jak2 in insect cells (upper panel of Fig. 3C). However, in contrast to the Tec-TIP3 interaction, Jak2KDF could not bind to TIP3, suggesting that Jak2 associates with TIP3 in a phosphorylation-dependent manner. Appropriately, expression of Jak2 and TIP3 in each set was confirmed by probing the same membrane with anti-Jak2 serum (middle panel) and by probing the total cell lysates with H902 (lower panel), respectively.

It is widely known that Jak kinases can phosphorylate the tyrosine residues of cytokine receptors (24). We thus tested whether co-expression of TIP3 suppresses this Jak2-mediated phosphorylation of the βc chain of human GM-CSF receptor. The βc chain was expressed in 293 cells either alone or in combination with Jak2 or Jak2 plus His-HIV-TIP3. As shown in the upper panel of Fig. 3D, Jak2 could phosphorylate the βc chain in cells, but this effect was abolished by the co-expression of TIP3. Therefore, TIP3 should modulate the Jak2-mediated signalings in vivo.
molecules involved in the c-fos regulation pathway. We therefore tested whether TIP3 can modulate the cytokine-driven c-fos activation mechanism. The pfos/luc reporter plasmid in which expression of luciferase is controlled by the c-fos promoter was introduced into IL-3-dependent BA/F3 cells by electroporation together with pSRα or the pSRα-based expression plasmid of Syk, Tec, or TIP3. After incubation with or without IL-3, cells were lysed and subjected to luciferase assay (Fig. 4A). Stimulation of the vector-transfected BA/F3 cells with IL-3 could elevate the reporter activity. Expression of Syk did not significantly affect the c-fos promoter activity, making it unlikely that Syk mediates c-fos activation in blood cells. Expression of Tec, however, could strongly enhance the luciferase activity both in the unstimulated and the IL-3-stimulated states. On the contrary, TIP3 almost abolished the IL-3-driven activity of the c-fos promoter. We then tested whether TIP3 can suppress the c-fos-driven c-fos activation. As shown in Fig. 4B, co-introduction of TIP3 could significantly suppress the Tec-mediated activation of the c-fos promoter. Similarly, expression of TIP3 strongly suppressed the Jak2-driven c-fos activation in BA/F3 cells (not shown).

Without the GST-tag, TIP3 expression in 293 cells was low and varied from experiment to experiment. Phosphorylation of Tec could not always be suppressed in cells by those amounts of TIP3. However, even with the nontagged TIP3, we could reproducibly observe the suppression of kinase activity and tyrosine phosphorylation of Jak2. Thus, TIP3 should have a higher binding affinity to Jak2 than to Tec, and Jak2 may be the major target of TIP3 in vivo. We also observed that cytokine stimulation induces the TIP3 transcription in cytokine-dependent hematopoietic cells (not shown). TIP3 might play as a physiological “brake” against overgrowth of blood cells, probably, via down-regulating the PTK activities. After submission of this manuscript, identification of TIP3 molecule has been reported by other groups under the name of SOCS-1 (25), JAB (26), or SSI-1 (27). We therefore use SOCS-1/JAB/SSI-1 as the name of TIP3 hereafter.

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