Tec and Jak2 Kinases Cooperate to Mediate Cytokine-Driven Activation of c-fos Transcription

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Although transcriptional activation of the c-fos proto-oncogene plays an intrinsic role in the mechanism of blood cell growth, it is still obscure how protein-tyrosine kinases (PTKs) regulate the cytokine-driven c-fos activation pathway. We present here that Tec PTK is tyrosine-phosphorylated and activated by granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation in a human GM-CSF-dependent cell line. Moreover, we could show that introduction of Tec into mouse BA/F3-hGMRβ cells can profoundly activate the c-fos promoter in response to GM-CSF or to interleukin-3 (IL-3). In contrast, introduction of a kinase-deleted Tec could suppress cytokine-driven c-fos activation, indicating that Tec is directly involved in the regulation of c-fos transcription. Interestingly, strong activation by Tec of the c-fos promoter was blocked by the co-expression of dominant negative Jak2. The molecular interaction between Tec and Jak2 was then investigated both in mammalian and insect cell systems, revealing that they can not only bind to each other, but also phosphorylate each other. Thus, Tec and Jak2 can “cross-talk” in a complexed way to mediate cytokine-driven c-fos activation.

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the c-fos gene and the luciferase cDNA, into mouse BA/F3-hGMRαβ cells 28 which express the high-affinity receptors for human GM-CSF. pSRα plasmids carrying the cDNAs of various nonreceptor PTKs were co-introduced to compare the ability of each PTK to modulate c-fos promoter activity. Interestingly, we could observe that Tec was one of the most potent PTKs in the ability of the reporter gene activation. On the contrary, introduction of a kinase-deleted Tec could suppress the cytokine-driven c-fos activation in a dose-dependent manner. Because Jak2 expression also activated the c-fos promoter in our assay, we next investigated the functional and physical interaction between Tec and Jak2 in the context of c-fos activation mechanism. Co-expression of dnJak2 could block the Tec-driven c-fos activation, suggesting that Jak2 may work at a point downstream of Tec. Surprisingly, in both 293 cells and insect cells we could show that Tec and Jak2 can not only associate with, but also phosphorylate, each other. Our data indicate that the Src-, Tec-, and Jak-family members functionally interact to transduce the cytokine-driven c-fos activation mechanism.

MATERIALS AND METHODS

Cell lines. BA/F3 cells 29 were maintained in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) and 25 U/mL of mouse IL-3. The BA/F3-hGMRαβ cells and a human GM-CSF–dependent cell line, UT-7, 30 were maintained in the same medium with 10% FCS and 1 ng/mL of human GM-CSF. 293 cells (American Type Culture Collection [ATCC], Rockville, MD) were maintained in Dulbecco’s modified Eagle medium/F12 (DMEM/F12; GIBCO-BRL) containing 10% FCS and 2 mmol/L L-glutamine. SF21 cells (Invitrogen, San Diego, CA) were grown in suspension at 28°C in the SF-900 II serum-free medium (GIBCO-BRL) without CO2 supply. For the stimulation experiments, UT-7 cells were cultured in the starvation medium (RPMI 1640 medium with 0.5% FCS, 100 µg/mL transferrin [Boehringer Mannheim, Mannheim, Germany] and 100 µg/mL bovine serum albumin [Boehringer Mannheim]) at the concentration of 5 × 106 cells/mL for 12 hours, then at the concentration of 1 × 106 cells/mL in the same medium for 0.5 hour. The cells were stimulated with 10 ng/mL of human GM-CSF for the period of 5 minutes unless otherwise indicated.

Immunoprecipitation and in vitro kinase assay. The cDNA of mouse Tec type IV, 12 mouse Jak2, 31 dominant negative Jak2, 24 mouse Lyn, 32 Syk with an N-terminal gp120 epitope tag, 33 or dominant negative Ras was ligated with the pSRα expression vector to generate pSRα-Tec, pSRα-Jak2, pSRα-dnJak2, pSRα-Lyn, pSRα-Syk, or pSRα-dnRas, respectively. To construct the cDNA encoding a kinase-deleted Tec (TecΔKD), Tec cDNA was digested by Bpu1102I, blunt-ended by Td DNA polymerase, and the 3′ fragment encoding the kinase domain was removed. Introduction of the expression plasmids into 293 cells was performed by the calcium phosphate method. UT-7 or 293 cells were rinsed once with ice-cold phosphate-buffered saline (PBS) supplemented with 0.1 mmol/L Na3VO4, and resuspended into the 1%-lysis buffer (1% Nonidet P-40, 50 mmol/L Tris-HCl, 7.4, 150 mmol/L NaCl, 1 mmol/L NaF, 1 mmol/L Na2VO4, 200 U/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). After incubation on ice for 30 minutes, cell extracts were centrifuged to remove insoluble materials. Tec or Jak2 was immunoprecipitated from 1 to 2 mg of the cell lysates by anti-Tec serum 32 or anti-Jak2 sera (Santa Cruz Biotechnology, Santa Cruz, CA and Upstate Biotechnology, Lake Placid, NY), respectively, and was eluted into the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Where indicated, cells were solubilized by the 0.1%-lys-2 buffer containing 0.1% of NP-40 instead of 1%.

For the in vitro kinase assay, the immune complexes were washed three times with the 1%-lys-2 buffer, three times with the kinase buffer (20 mmol/L Tris-HCl, 7.4, 50 mmol/L NaCl, 10 mmol/L MgCl2, 2 mmol/L MnCl2), and finally incubated with 0.37 MBq of [γ-32P]ATP (Amersham, Arlington Heights, IL) for 15 minutes at 30°C. For the assay of Jak2 activity, a synthetic substrate of Jak2 (Upstate Biotechnology) was added to the reaction (20 µg/experiment). Samples of the Jak2 kinase assay were subjected to Tricine-SDS-PAGE.

Immunoblotting. Total cell lysates (10 µg/tube) and the immune complexes were separated through 7.5% SDS-PAGE and electrophoblotted onto polyvinylidine difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA). The membranes were incubated for 1 hour at room temperature in TBST (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20) with 4% bovine serum albumin (Fraction V; Sigma, St Louis, MO). The membranes were then incubated with anti-Tec serum (1:10,000 dilution), anti-Jak2 serum, anti-Lyn serum, 26 anti-gp120 epitope tag antibody (H902), or anti-phosphotyrosine antibody (4G10; Upstate Biotechnology) for 1 hour at room temperature in TBST. Specific bindings of the antibodies were visualized by the ECL detection system (Amersham) according to the manufacturer’s instructions.

Metabolic labeling and phosphoamino acid analysis. UT-7 cells were cultured at the concentration of 1 × 107 cells/mL in phosphate-free RPMI 1640 medium (GIBCO-BRL) supplemented with 5% dialyzed FCS (GIBCO-BRL) and 37 MBq/mL of [32P]orthophosphate for 1 hour, and then stimulated with human GM-CSF for 5 minutes. Tec was immunoprecipitated from the cells, blotted onto a PVDF membrane, and incubated in 1 N KOH at 55°C according to the method of Kamps and Seflon. 24 Phosphoamino acid contents of p70S6K were determined as described earlier.13

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-hGMRαβ cells by electroporation according to the method of Watanabe et al 35 with minor modifications. Briefly, 1 × 107 of BA/F3-hGMRαβ cells were resuspended into 200 µL of OPTI-MEM I medium (GIBCO-BRL) and mixed with the expression vector DNAs (5 µg per construct unless otherwise indicated) plus the pGAL4-reporter plasmid (2 µg). Total amounts of plasmid DNAs in each set of electroporation were adjusted to be equal by adding the appropriate amounts of the blank vector DNA. After electroporation with the GenePulsar apparatus (BioRad, Hercules, CA) at the concentration of 200 V and 960 µF, cells were resuspended into 30 mL of RPMI 1640 medium with 10% FCS and cultured for 5 hours. The samples were further cultured for 5 hours either unstimulated or stimulated with 25 µg/mL of mouse IL-3 or 5 ng/mL of human GM-CSF. The luciferase activities were measured by using the Luciferase Assay System (Promega, Madison, WI), and are shown as relative light units/min/µg of protein. The Elk activity was assayed in BA/F3 cells by using the PathDetect in vivo reporting system (Stratagene, La Jolla, CA). The MEK1 inhibitor (PD98059; New England Biolabs, Beverly, MA) was dissolved in dimethyl sulfoxide (DMSO) and added to the culture at the concentration of 50 mmol/L. The pfos/luc mutants were constructed by inserting the mutant promoter fragments 46 into pGL3-Basic plasmid (Promega).

Recombinant baculoviruses. The cDNAs of Tec and Jak2 were inserted into the pFastBacHT and pFastBac1 plasmids (both from GIBCO-BRL), respectively. The recombinant baculoviruses based on these plasmids were generated by the Bac-to-Bac baculovirus expression systems (GIBCO-BRL), and were used to infect SF21 cells at the multiplicity of infection (MOI) of 1.0. After 48 to 72 hours of culture, cells were harvested and lysed as described above.
RESULTS

Tec is involved in the signaling pathway of GM-CSF receptor.

To investigate whether Tec is involved in the signaling mechanism mediated by GM-CSF receptor (GMR), Tec was immunoprecipitated from a human GM-CSF–dependent cell line, UT-7, with or without the GM-CSF stimulation, and was immunoblotted with anti-phosphotyrosine antibody (α-P-Tyr Ab). As shown in the upper panel of Fig 1A, GM-CSF stimulation of UT-7 cells for 5 minutes could clearly induce tyrosine-phosphorylation of Tec (indicated by an arrow) and a Tec-associated p56. The identity of this p56 is yet to be determined although we confirmed that p52shc and p56lyn, both of which are known to be associated with Tec, have the same electrophoretic mobility with that of the “p56.” The same membrane was reblotted with anti-Tec serum to prove that equivalent amounts of Tec were precipitated (lower panel). We could not detect a significant level of Btk expression in UT-7 cells by using an anti-Btk antibody (M-138; Santa Cruz Biotechnology). We next examined the time course of Tec phosphorylation. Tec was immunoprecipitated from UT7 cells with various periods of GM-CSF stimulation, and probed with α-P-Tyr Ab. As shown in Fig 1B, tyrosine-phosphorylation of Tec was induced as rapidly as 1 minute after the stimulation, reached to the maximum level in 5 to 10 minutes, and decreased thereafter. Thus, the phosphorylation of Tec in response to GM-CSF is rapid and transient. To examine whether the kinase activity of Tec is also affected in response to GM-CSF, Tec was immunoprecipitated from UT-7 cells with or without GM-CSF stimulation and subjected to an in vitro kinase assay without exogenous substrates. As shown in Fig 1C and D, stimulation with GM-CSF for 5 minutes could enhance the auto-phosphorylation activity of Tec.

To directly estimate the phosphotyrosine contents, Tec was immunoprecipitated from UT-7 cells metabolically labeled with [32P]orthophosphate, separated through 7.5% SDS-PAGE, blotted onto a PVDF membrane, and incubated in 1 N KOH to enrich the signals of phosphotyrosine. Autoradiography of the membrane could show that GM-CSF can induce phosphorylation of pp70Tec were then examined by thin-layer chromatography, confirming that p52shc and p56lyn, both of which are known to be Tec (indicated by an arrow) and a Tec-associated p56. The identity of this p56 is yet to be determined although we confirmed that p52shc and p56lyn, both of which are known to be associated with Tec, have the same electrophoretic mobility with that of the “p56.” The same membrane was reblotted with anti-Tec serum to prove that equivalent amounts of Tec were precipitated (lower panel). We could not detect a significant level of Btk expression in UT-7 cells by using an anti-Btk antibody (M-138; Santa Cruz Biotechnology). We next examined the time course of Tec phosphorylation. Tec was immunoprecipitated from UT7 cells with various periods of GM-CSF stimulation, and probed with α-P-Tyr Ab. As shown in Fig 1B, tyrosine-phosphorylation of Tec was induced as rapidly as 1 minute after the stimulation, reached to the maximum level in 5 to 10 minutes, and decreased thereafter. Thus, the phosphorylation of Tec in response to GM-CSF is rapid and transient. To examine whether the kinase activity of Tec is also affected in response to GM-CSF, Tec was immunoprecipitated from UT-7 cells with or without GM-CSF stimulation and subjected to an in vitro kinase assay without exogenous substrates. As shown in Fig 1C and D, stimulation with GM-CSF for 5 minutes could enhance the auto-phosphorylation activity of Tec.

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Tec is involved in cytokine-driven activation of c-fos transcription.

To examine whether Tec mediates cytokine-driven activation of the c-fos gene, the pfos/luc plasmid in which the luciferase expression is controlled by the c-fos promoter was transfected into BA/F3-hGMROβ cells by electroporation together with the pSRα-Tec expression plasmid of Syk, Lyn, Jak2, or Tec. As shown in Fig 2A, stimulation of the vector-transfected BA/F3-hGMROβ cells with either GM-CSF or IL-3 could enhance the luciferase reporter activity. Co-introduction of the Syk kinase with an N-terminal tag33 did not affect the luciferase activity, suggesting Syk is not involved in the c-fos activation mechanism in BA/F3 cells. In contrast, introduction of Lyn kinase significantly elevated the luciferase activity of the unstimulated basal level. However, cytokine stimulation of the cells could not further enhance the reporter activity. This lack of cytokine-responsiveness in Lyn-transfected cells was confirmed in repeated experiments. As previously reported, introduction of Jak2 could elevate the reporter activity of the unstimulated state as well as of cytokine-stimulated states. Interestingly, Tec introduction elevated the reporter activity of the unstimulated state similar to the level obtained by the Lyn-transfection. In contrast to the case of Lyn, Tec expression could also strongly enhance the reporter activity in response to GM-CSF or IL-3. Appropriate expression of each kinase was confirmed by the immunoblot analysis of the total cell lysates (Fig 2B).

We then directly tested whether Tec is an intermediate in the cytokine-driven c-fos activation pathway by using a kinase-deleted Tec (TecΔKD). As shown in Fig 2C, introduction of pSRα-TecΔKD into BA/F3-hGMROβ cells suppressed the c-fos promoter activity stimulated by GM-CSF or IL-3 in a dose-dependent manner. These data strongly support the idea that Tec directly mediates the cytokine-driven c-fos activation. It is widely known that c-fos transcription is regulated via the Ras-MAPK pathway. Therefore, we checked whether the Tec-driven c-fos activation is transduced through Ras by coexpressing a dominant negative form of Ras (dnRas). As shown in Fig 2D, coexpression of dnRas could totally block the Tec-driven activation of the c-fos gene. Thus, Tec is likely to drive the c-fos activation through a Ras-regulated mechanism. By using the PathDetect in vivo reporting system (Stratagene), we then asked whether Elk, a transcriptional factor acting downstream of Ras, is involved in the Tec-mediated c-fos activation. The pFA-Elk plasmid, encoding the fusion protein consisting of the DNA binding domain of yeast GAL4 and the activation domain of Elk, was transfected into BA/F3 cells together with Tec-expression plasmids and the reporter pFR-luc plasmid in which expression of luciferase is controlled by a promoter containing the GAL4-binding sites (Fig 2E). In the cis-regulatory elements, namely, the c-fos element (Ca/CRE), the serum response element (SRE), the c-fos element (SIE), and the c-fos activation domain of Elk, was transfected into BA/F3 cells together with Tec-expression plasmids and the reporter pFR-luc plasmid in which expression of luciferase is controlled by a promoter containing the GAL4-binding sites (Fig 2E). In the cis-regulatory elements, namely, the c-fos element (Ca/CRE), the serum response element (SRE), the c-fos element (SIE), and the c-fos activation domain of Elk, was transfected into BA/F3 cells together with Tec-expression plasmids and the reporter pFR-luc plasmid in which expression of luciferase is controlled by a promoter containing the GAL4-binding sites (Fig 2E). In the cis-regulatory elements, namely, the c-fos element (Ca/CRE), the serum response element (SRE), the c-fos element (SIE), and the c-fos activation domain of Elk, was transfected into BA/F3 cells together with Tec-expression plasmids and the reporter pFR-luc plasmid in which expression of luciferase is controlled by a promoter containing the GAL4-binding sites (Fig 2E). In the cis-regulatory elements, namely, the c-fos element (Ca/CRE), the serum response element (SRE), the c-fos element (SIE).
Fig 1. Tec is involved in the signaling pathway of GM-CSF receptor. (A) UT-7 cells (1 x 10^7) were cultured in the starvation medium for 12 hours and then stimulated with 10 ng/mL of human GM-CSF (+) for 5 minutes or left unstimulated (−). Tec was immunoprecipitated from each fraction (αTec), subjected to 7.5% SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody (α-P-Tyr). Total cell lysates (TCL; 10 μg/lane) and the immunoprecipitates by normal rabbit serum (NRS) prepared from the same set of cells were also analyzed. The position of Tec is indicated by an arrow. The molecular weight standards (× 10^2) are shown at the left. The same membrane was reblotted with anti-Tec serum to show the amounts of Tec precipitated (lower panel). (B) UT-7 cells were stimulated with GM-CSF (10 ng/mL) for 0, 1, 5, 10, or 20 minutes as indicated at the top. Tec was immunoprecipitated from each fraction (1 x 10^7 cells), and was immunoblotted with anti-phosphotyrosine antibody (α-P-Tyr) or anti-Tec serum (αTec). (C) Tec was immunoprecipitated from 1 x 10^7 of UT-7 cells with (+) or without (−) 5 minutes of GM-CSF stimulation, and was subjected to an in vitro kinase assay. Autophosphorylation of pp70^Tec is shown. (D) Specific kinase activity of the Tec protein (32P-incorporation/protein amount) with (+) or without (−) the GM-CSF stimulation was calculated by densitometric analysis and shown as arbitrary units. (E) Tec was immunoprecipitated from UT-7 cells (1 x 10^7), with (GM) or without (−) the GM-CSF stimulation (10 ng/mL), metabolically labeled with [32P]orthophosphate (37 MBq/mL), and was analyzed by 7.5% SDS-PAGE. The proteins were blotted onto a PVDF membrane, and heated in 1 N KOH to decrease the backgrounds of serine- and threonine-phosphorylation. The position of Tec is indicated. (F) pp70^Tec in (E) was subjected to the phosphoamino acid analysis. The positions of free phosphate (Pi), phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) are indicated at the right.
Fig 2. Tec is involved in the cytokine-driven activation of c-fos proto-oncogene. (A) BA/F3-hGMR<sub>ab</sub> cells (1 x 10<sup>7</sup>) were transfected with the pfos/luc reporter plasmid (2 μg) together with 5 μg each of the pSRα (Vector), pSRα-Syk (Syk), pSRα-Lyn (Lyn), pSRα-J ak2 (J ak), or pSRα-Tec (Tec). After 5 hours of incubation in cytokine-free medium, the cells were further cultured for 5 hours without (no factor) or with 5 ng/mL of human GM-CSF (+ GM-CSF) or 25 U/mL of mouse IL-3 (+ IL-3). Luciferase activity was assayed in each fraction and calculated as relative light units (RLU)/min/μg of protein. The mean value plus SD of the luciferase activities in triplicate samples from each fraction is shown as arbitrary units. (B) BA/F3-hGMR<sub>ab</sub> cells were transfected with pSRα-Syk, pSRα-Lyn, pSRα-J ak2, or pSRα-Tec, and cultured for 24 hours in the presence of IL-3. Total cell lysates (10 μg/lane) were prepared from each set (+) and untransfected BA/F3-hGMR<sub>ab</sub> cells (−), and were immunoblotted with the antibodies against the corresponding kinases.
introduced into individual regulatory elements (kind gifts of T. Curran), we here analyzed how each element contributes to the Tec-driven c-fos activation (Fig 2H). In accordance with the results in Fig 2E, mutations at SRE, the binding site of the Elk/TCF complex, significantly decreased the Tec-driven activation of c-fos transcription. These lines of evidence support the idea that Tec activates c-fos promoter through, at least in part, the Ras-MEK1-Elk pathway.

Because both of the Tec and Jak2 kinases could enhance cytokine-driven c-fos activation, we then tried to clarify whether Tec and Jak2 work in the same pathway or in a parallel manner to drive the c-fos promoter. First, Tec was introduced into...
Tec can phosphorylate Jak2 in cells. To understand how Tec and Jak2 can functionally interact with each other, we first examined the possibility that the former directly phosphorylates the latter. A kinase-dead Jak2 (Jak2\textsuperscript{KE}; Lys-882 in the ATP-binding site is replaced with Met) could slightly suppress the Jak2-driven activation of the c-fos gene. Although we could reproducibly observe this weak suppression (about 20% reduction), we do not yet have a strong proof that Tec is involved in a part of the Jak2-driven mechanism in the c-fos regulation.

We then tried to map the phosphorylation site of Tec by Jak2. Yamashita et al.\textsuperscript{37} previously demonstrated that the deletion of the internal SH3 domain results in hyperphosphorylation and activation of Tec in vivo. In addition, Tec kinase has a tentative autophosphorylation site (Tyr-518) in the activation loop of its catalytic domain, corresponding to Tyr-416 in c-Src. Therefore, we investigated the possibility that either of the SH3 domain or Tyr-518 position in Tec-family kinases are well conserved. In these experiments, Jak2-phosphorylation by Tec was clearly and reproducibly observed when we used, for immunoprecipitation, anti-Jak2 serum against the C-terminal tail of Jak2 (C-20; Santa Cruz Biotechnology), not the one against amino acid positions 758-776 (Upstate Biotechnology), which may imply that the target site(s) of Tec is localized within or very close to the 758-776 region.

We then tested whether this trans-phosphorylation of Jak2 by Tec affects the kinase activity of the Jak2 protein. Jak2 was expressed in SF21 cells with or without Tec, immunoprecipitated by anti-Jak2 serum, and subjected to an in vitro kinase assay with a synthetic substrate peptide. As shown in Fig 3C, coexpression of Tec did not affect the phosphorylation of the Jak2-substrate. Because the immunoprecipitated Jak2\textsuperscript{KE} could not phosphorylate the peptide at all (lane "JE"), phosphorylation of the peptide in the other lanes was supposed to be carried out by Jak2, not by the coprecipitated kinases from SF21 cells. We observed that Jak2 was expressed in equal amounts in each SF21 fraction, as judged from the immunoblotting of the total cell lysates with anti-Jak2 serum (data not shown). Similar results of the in vitro kinase assay were obtained with Jak2 expressed in 293 cells (data not shown). Phosphorylation of Jak2 without the modulation of its activity may be used in vivo for collecting signaling molecules to Jak2 protein. We checked this possibility by using the BA/F3 cells expressing an SH3-deleted active Tec (Tec\textsuperscript{ASH3}).\textsuperscript{37} Jak2 was immunoprecipitated from parental BA/F3 cells and two BA/F3 transfectants stably expressing Tec\textsuperscript{ASH3} and blotted with pTyr Ab. As shown in Fig 3D, many tyrosine-phosphorylated proteins become associated with Jak2 only when Tec\textsuperscript{ASH3} is coexpressed, which may indirectly support the possibility above.

Jak2 can phosphorylate Tec at Tyr-518. To investigate the phosphorylation reaction in the reverse direction between the two kinases, Tec\textsuperscript{KM} was introduced into 293 cells with or without Jak2, and was analyzed for tyrosine-phosphorylation (upper panel, Fig 4A). Because it was already known that Tec can be directly phosphorylated and activated by Lyn PTK, a coexpression experiment of Lyn kinase was used as a positive control. To our surprise again, Tec could be in vivo phosphorylated by Jak2 as well as by Lyn. The same membrane was then probed with anti-Tec serum to estimate the amounts of Tec precipitated (lower panel). Therefore, Tec and Jak2 can trans-phosphorylate each other.

Cells were expressing Tec alone or in combination with Jak2 and Lyn. The same membrane was then reblotted with anti-Jak2 serum to estimate the amounts of Jak2 precipitated (lower panel). Therefore, it would not be surprising if other members of the Tec-family are also controlled by Src- and Jak-family kinases through a similar phosphorylation mechanism.

Because Jak2 and Lyn can phosphorylate the same residue (Tyr-518) of Tec, we speculated that Jak2 may activate Tec as in the case of Lyn. Tec or Tec\textsuperscript{KM} was expressed in 293 cells either alone or in combination with Jak2. Tec was immunoprecipitated from each set and subjected to an in vitro kinase assay to test its
Fig 3. Tec can phosphorylate Jak2 in both mammalian and insect cells. (A) Jak2 was immunoprecipitated from 2 × 10⁶ of 293 cells expressing Jak2 and anti-Jak2 immunoprecipitates (Jak IP) were electrophoresed and probed with anti-phosphotyrosine antibody (αP-Tyr) or anti-Jak2 serum (αJak2). The positions of Jak2 (Jak2), Tec (Tec), and the Ig heavy chain (IgH) are indicated at the right. (B) Jak2 was immunoprecipitated from SF21 cells infected with Jak2 expressing baculovirus (JE) alone or in combination with Tec-expressing (T) or Tec KM-expressing (TM) virus. The immunoprecipitates were separated through 7.5% SDS-PAGE and probed with anti-phosphotyrosine antibody (αP-Tyr) or anti-Jak2 serum (αJak2). The positions of Jak2 (Jak2) and Tec (Tec) are indicated at the right. (C) Jak2 was immunoprecipitated from SF21 cells expressing Jak2 (J) or Jak2 alone or in combination with Tec (T). The immunoprecipitates were incubated with [γ-32P]ATP and the synthetic Jak2-substrate, and subjected to Tricine-SDS-PAGE. Phosphorylation of the Jak2-substrate is shown. (D) Total cell lysates (TCL; 10 μg/lane) and the anti-Jak2 immunoprecipitates (Jak IP) were prepared from parental BA/F3 cells (P) and two BA/F3 clones (1 and 2) stably expressing Tec ΔSH3, and immunoblotted with αP-Tyr Ab (upper panel) or anti-Jak2 serum (lower panel). The position of Jak2 is indicated at the right. The positions of molecular weight standards (× 10⁻³) are also shown at the left.
auto-phosphorylation activity. Unexpectedly, as shown in Fig 4D, autophosphorylation level of p70 Tec was not altered irrespective of the presence of Jak2 PTK. In contrast, coexpression of Lyn kinase could activate Tec as reported previously (data not shown). Thus, although Jak2 and Lyn can phosphorylate the same site of Tec, we observed only Lyn can activate the Tec kinase under the sensitivity of our assay.

Tec can bind to Jak2 in insect cells. We then tested whether Tec and Jak2 can physically associate with each other in cells. Recombinant baculovirus expressing Tec or TecKM was used to infect Sf21 cells either alone or in combination with Jak2 (J) or Lyn (L) kinase. Tec was immunoprecipitated from each fraction, and probed with anti-phosphotyrosine antibody (α-P-Tyr) or anti-Tec serum (α-Tec). The positions of full-length Tec (T) and SH3-deleted (ΔSH3) forms are indicated at the right. (C) The amino acid sequences of the Tec-family kinases, surrounding the tyrosine residues corresponding to Tyr-518 in mouse Tec, are compared. The asterisk indicates the position of the phosphorylated tyrosine. At the left shown are the numbers of amino acid positions of mouse Tec, human Btk,10,11 mouse Emt/Itk/Tsk,9,41,42 human Bmx,43 and human Txk.44 (D) pSRa (V), pSRa-Tec (T), or pSRa-TecKM (TM) was transfected into 293 cells with or without pSRa-Jak2 (J). Tec was immunoprecipitated from each fraction, and incubated with [γ-32P]ATP without exogenous substrates. Autophosphorylation of pp70Tec in each sample is shown.

Fig 4. Jak2 can phosphorylate Tec at Tyr-518. (A) The kinase-dead TecKM (TM) was expressed in 293 cells either alone or in combination with Jak2 (J) or Lyn (L) kinase. Tec was immunoprecipitated from each fraction, and probed with anti-phosphotyrosine antibody (α-P-Tyr) or anti-Tec serum (α-Tec). (B) TecKM (TM), Tec-promoter SH3 (TMD3), or TecpromoterΔSH3 (TMD3SH3) was expressed in 293 cells either alone (−) or in combination with Jak2 (J) or Lyn (L). Tec was immunoprecipitated from each fraction, and blotted with anti-phosphotyrosine antibody (α-P-Tyr) or anti-Tec serum (α-Tec). The positions of full-length Tec (T) and SH3-deleted (ΔSH3) forms are indicated at the right. (C) The amino acid sequences of the Tec-family kinases, surrounding the tyrosine residues corresponding to Tyr-518 in mouse Tec, are compared. The asterisk indicates the position of the phosphorylated tyrosine. At the left shown are the numbers of amino acid positions of mouse Tec, human Btk,10,11 mouse Emt/Itk/Tsk,9,41,42 human Bmx,43 and human Txk.44 (D) pSRa (V), pSRa-Tec (T), or pSRa-TecKM (TM) was transfected into 293 cells with or without pSRa-Jak2 (J). Tec was immunoprecipitated from each fraction, and incubated with [γ-32P]ATP without exogenous substrates. Autophosphorylation of pp70Tec in each sample is shown.

Fig 5. Tec can constitutively associate with Jak2 in Sf21 cells. Sf21 cells were infected with baculovirus expressing Tec (T), Jak2 (J), and Jak2ΔSH3 (ΔJ) in the combinations indicated at the top. Jak2 was immunoprecipitated from each cells lysed by the 0.1%-lysis buffer (α Jak IP), and probed with either anti-Tec serum (α-Tec) or anti-Jak2 serum (α-Jak2). Total cell lysates (TCL; 10 μg/lane) of each fraction were also probed with anti-Tec serum to estimate the expression level of Tec.

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active Tec always reduced the expression level of Jak2 in S21 cells (middle panel, and also confirmed in other repeated experiments). Therefore, difference of the amounts of coprecipitated Tec may have arisen from the different expression level of Jak2 (compare the intensities of the bands between the top and middle panels).

We could not test the Tec/Jak2 interaction in the reverse direction, since Jak2 nonspecifically bound to protein-A-Sepharose beads and glutathione Sepharose 4B (and even to nickel agarose beads) when Jak2 was expressed abundantly in either 293 or S21 cells (data not shown).

**DISCUSSION**

In this report we have shown that Tec is involved in the signaling pathway of GMR, especially in the c-fos activation machinery. Because Jak2 was previously shown to be an intermediate in the cytokine-driven c-fos activation pathway, both of Jak2 and Tec should play a role in the regulation of c-fos transcription. Furthermore, our data with dnJak2 support an intriguing idea to place Jak2 downstream of Tec in the mechanism of c-fos activation.

How does Jak2 participate in the Tec-driven pathway to the c-fos gene? A simple hypothesis is that Jak2 becomes activated via the phosphorylation by Tec, and drives the c-fos transcription as an effector of the Tec kinase. However, this is unlikely because coexpression of Tec could not affect the activity of Jak2 in either mammalian or insect cells. The second explanation is that Jak2 may be required to fully activate Tec through the phosphorylation of Tec protein by Jak2. This assumption is again unlikely because (1) coexpression of Jak2 could not activate Tec in either 293 cells or S21 cells, and (2) coexpression of dnJak2 with Tec in 293 cells did not suppress the kinase activity or tyrosine-phosphorylation of Tec (data not shown). Therefore, Jak2 may not be a direct second messenger of Tec, but should be required for the appropriate function of Tec-substrates (“Substrate X” in Fig 6). There are several possible scenarios for such interaction. Jak2 may be, for instance, prerequisite to recruit the substrates of Tec into the cytokine receptor complex. It is well known that cytokine receptors are the good substrates of Jak-family kinases both in vitro and in vivo, and that a variety of signaling molecules become associated with the receptors through the phosphotyrosine-SH2 domain (or phosphotyrosine-binding [PTB] domain) interaction. Thus, it is possible that the second messengers for c-fos activation can become accessible to Tec through the phosphorylation of receptors by Jak2. Another explanation may be that Tec collects its substrates by phosphorylating Jak2 and thereby making it bound to the Tec-substrates. In this scenario, Jak2 plays as a “bridge” to connect Tec and its effector molecules. There would be, again, the other possibility that Jak2 is required to phosphorylate the Tec-substrates and to let them associated with Tec. To determine which interaction really takes place in vivo, we have to identify the “Substrate X” responsible for the c-fos activation, and we should also clarify the phosphorylation site(s) of Jak2 by the Tec kinase.

Analysis of various deletion mutants of human GMR common β chain (βc) showed that a central area in the cytoplasmic region of βc is necessary for cytokine-dependent Shc phosphorylation, activation of Ras, and induction of the c-fos gene. In fibroblasts, Shc is already known to bind to Grb2 in a phosphorylation-dependent manner, and thereby to trigger the recruitment of SOS guanine nucleotide exchanging factor and the Ras activation. Therefore, Shc/Ras may be a key component to drive the c-fos transcription also in blood cells. If this is the case, Shc would be an intriguing candidate for the “Substrate X” in Fig 6. Currently we have only a few data to support this hypothesis. First, because expression of dnRas could suppress the cytokine-driven as well as Tec-driven c-fos activation, Ras itself or the Ras-regulated machinery should be a relay point of the pathway to the c-fos gene. Second, we already proved that Shc can be associated (either directly or indirectly) with Tec in cells. However, it is yet to be shown whether Shc is a direct substrate of Tec in vivo, and whether Shc plays a central role in the activation of c-fos gene in the hematopoietic system.

Our mapping experiments evidenced that Tyr-518 is a major phosphorylation site of Tec by both Jak2 and Lyn. The fact that Jak2 is capable of phosphorylating Tec was also confirmed in another laboratory (T. Matsuda and J.N. Ihle, personal communication). However, only Lyn could enhance...
the kinase activity of Tec in our experiments. Although we do not have any evidence to explain this discrepancy, several possibilities can be raised. First, as shown in Fig 4A and B, stoichiometry of Tec-phosphorylation was always higher when coexpressed with Lyn than with Jak2. Thus, low level of Tyr-518 phosphorylation by Jak2 may not be sufficient to demonstrate the enhancement of autophosphorylation activity in the anti-Tec immunoprecipitates. On the other hand, a weak Tyr-518 phosphorylation by Jak2 may not be sufficient to coexpressed with Lyn than with Jak2. Thus, low level of possibilities can be raised. First, as shown in Fig 4A and B, these minor sites may have a pivotal role in the regulation of Tec activity. It is also possible that Lyn and Jak2 bind to Tec at different sites, and that these bindings may render distinct allosteric effects on Tec molecules.

Our report has shown the presence of a “cross-talk” between Tec and Jak2 PTKs. Although Tec is the first PTK among non-Jak kinases shown capable of phosphorylating Jak2, it would not be surprising if the members of other PTK-families are also able to phosphorylate Jak kinases. Growth of blood cells would be controlled through these complexed networks among various PTKs, and our observation would be an important information to decipher the control mechanisms.

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REFERENCES


29. Palacios R, Steinmetz M: IL-3 dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell 41:727, 1985


Tec and Jak2 Kinases Cooperate to Mediate Cytokine-Driven Activation of c- fos Transcription

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