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Deletion of *Src* Homology 3 Domain Results in Constitutive Activation of Tec Protein-Tyrosine Kinase

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Tec protein-tyrosine kinase (PTK) is the prototype of a new subfamily of non-receptor type PTKs, and is abundantly expressed in hematopoietic tissues. We have revealed that Tec is inducibly tyrosine-phosphorylated and activated by stimulation with a wide range of cytokines. To get more insight into the signaling mechanism through Tec, we have generated a constitutively active form of Tec PTK. Deletion of the Src homology (SH) 3 domain gave rise to a hyperphosphorylated and activated Tec kinase (TecΔSH3). The activity of TecΔSH3 was confirmed in 293 cells, as well as in cytokine-dependent hematopoietic cells (BA/F3). TecΔSH3 should be a useful tool to study the *in vivo* substrates of Tec PTK.

Key words: Protein-tyrosine kinase — Tec — Cytokine — SH3

Protein-tyrosine kinases (PTKs) play essential roles in cell growth and oncogenic transformation. PTKs can be divided into two groups, namely, receptor-type PTKs and non-receptor type PTKs.[1] The *Tec* PTK was originally identified in mouse liver,[2] and subsequently shown to be abundantly expressed in hematopoietic tissues.[3] Recently, other researchers have reported four novel PTKs, all of which are highly homologous to *Tec*. This group represents a novel subfamily among non-receptor type PTKs, the *Tec* family, consisting of *Tec*, *Btk*,[4,5] *Itk/Tsk/Emt*,[6–8] *Txk*[9] and *Bmx*.[10]

We and other groups have examined whether *Tec* is involved in the intracellular signaling mechanisms of cytokines. *Tec* was indeed shown to be inducibly tyrosine-phosphorylated and activated in response to stimulation with a wide range of cytokines, including interleukin (IL)-3,[11] IL-6,[12] stem cell factor (SCF),[13] G-CSF,[14] erythropoietin[15] and thrombopoietin.[16] In the cases of IL-6 and SCF, *Tec* was further demonstrated to bind to the corresponding receptors. Therefore, *Tec* is presumed to be implicated in the signaling pathway mediated by cytokine receptors.

To investigate further the cytokine signaling through *Tec* PTK, we decided to construct a constitutively active form of *Tec* kinase. As shown in Fig. 1, Tec protein is composed of, from its N-terminus, a pleckstrin homology (PH) domain,[17] a *Tec* homology (TH) domain,[18] a *Src* homology (SH) 3 domain,[19] an SH2 domain and a kinase domain. Since *Tec* does not have C-terminal tyrosine residues as the negative regulatory site,[20] another approach to generate activated *Tec* was needed. Since internal deletions of SH3 domains were previously shown to activate c-*Src*[21] and c-*Abl*[22] PTKs, we constructed a mouse *tec* cDNA, by using PCR-based mutagenesis, encoding the Tec protein (TecΔSH3) lacking amino acid positions 186–233 of mouse Tec type IV.[11] TecΔSH3 and normal *Tec* cDNAs were then subcloned into the pSRα expression vector having a blasticidin S-resistance gene[23] as a selectable marker, giving rise to pSR*bsr*/TecΔSH3 and pSR*bsr*/Tec, respectively. Both cDNAs were also inserted into the pTagCMV-neo vector[24] to produce Tec proteins with an N-terminal tag of the human immunodeficiency virus gp120 epitope. The resultant plasmids are referred to as pTag/TecΔSH3 and pTag/Tec in this manuscript.

By using the transient expression system in 293 cells (American Type Culture Collection, Rockville, MD), we first examined whether TecΔSH3 protein is hyperphosphorylated and activated. pTag/TecΔSH3 and pTag/Tec plasmids as well as the wild pTagCMV-neo vector were introduced into 293 cells by the calcium phosphate method.[25] After 48 h culture, cells were lysed in 1.0% Lysis buffer (50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1 m*M* NaF, 1 m*M* Na_3VO_4, 200 U/ml aprotinin, 1 m*M* phenylmethylsulfonyl fluoride and 1% Nonidet P-40) and insoluble materials were removed by centrifugation at 10000*g* for 10 min. The Tec proteins were then precipitated by the combination of anti-tag antibody (H902 obtained from NIH AIDS Research and Reference Reagents Program) and protein-G Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). As shown in the upper panel of Fig. 2A, total cell lysates and anti-tag immunoprecipitates were electrophoresed and immunoblotted with anti-phosphotyrosine antibody (4G10; Up-

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state Biotechnology Inc., Lake Placid, NY) as described previously.[11] The tagged TecΔSH3 is hyperphosphorylated compared with the tagged normal Tec. It should be noted that the cellular proteins of 293 cells expressing TecΔSH3 are more intensively tyrosine-phosphorylated than those of normal Tec-expressing cells ("TCL" part of Fig. 2A, upper panel). The same membrane was re-

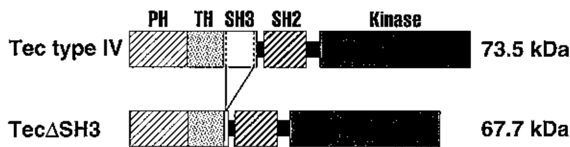

Fig. 1. Structure of Tec and TecΔSH3 proteins. Pleckstrin homology (PH)-, *Tec* homology (TH)-, *Src* homology (SH) 3-, SH2- and kinase (kinase) domains of mouse Tec type IV[11] and TecΔSH3 are schematically shown. A part of the SH3 domain (amino acids 186–233, indicated by dotted lines) of Tec type IV is deleted in TecΔSH3. The calculated molecular weight of each kinase is shown on the right.

blotted with H902 to prove that equivalent amounts of Tec were immunoprecipitated (Fig. 2A, lower panel). To study directly the kinase activity of the tagged proteins, the H902 immunoprecipitate prepared from each transfection was rinsed with Kinase buffer (20 m*M* Tris-HCl, pH 7.4, 50 m*M* NaCl, 10 m*M* $MgCl_2$ and 2 m*M* $MnCl_2$) and finally incubated with 0.37 MBq of [γ-^{32}P]ATP (Amersham, Arlington, IL) for 15 min at 30°C. As shown in Fig. 2B, autophosphorylation of tagged TecΔSH3 is enhanced in comparison to that of tagged normal Tec. Thus, we conclude that deletion of the internal SH3 domain activates the *Tec* PTK in 293 cells.

To examine whether TecΔSH3 can be similarly hyperphosphorylated in the hematopoietic system, we transfected pSR*bsr*/TecΔSH3 into an IL-3-dependent cell line, BA/F3,[26] by electroporation. Several blasticidin S-resistant clones were obtained, and two of them, "ΔSH3(1)" and "ΔSH3(2)," were used for further investigation. Each cell clone and vector-transfected BA/F3 cells were stimulated with IL-3 for 5 min, and then lysed with the 1.0% Lysis buffer. From each fraction, Tec proteins were immunoprecipitated with a polyclonal anti-

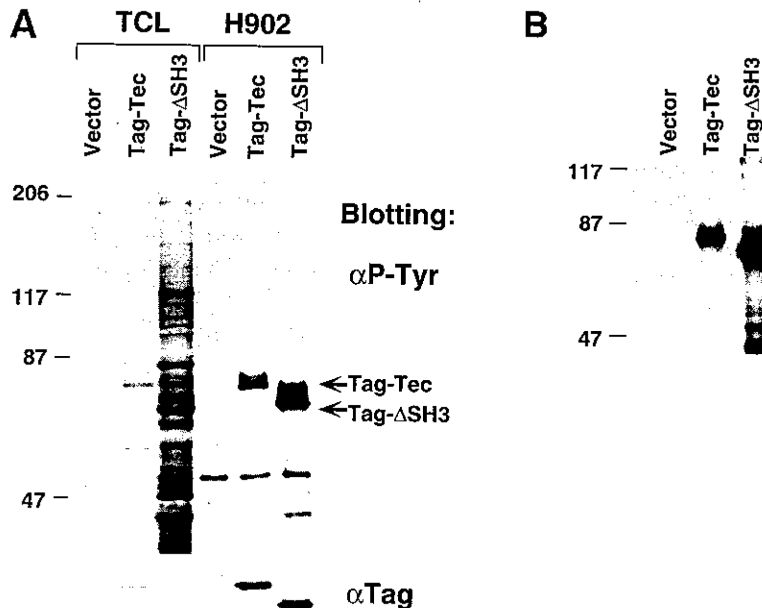

Fig. 2. TecΔSH3 is hyperphosphorylated and activated. (A) Ten micrograms of pTagCMV-neo (Vector), pTag/Tec (Tag-Tec) or pTag/TecΔSH3 (Tag-ΔSH3) plasmid was introduced into 2×10^6 293 cells by the calcium phosphate method. Total cell lysates (TCL, 10 μg/lane) and anti-tag immunecomplexes (H902) prepared from each transfection were electrophoresed through 7.5% SDS-PAGE, and blotted with either anti-phosphotyrosine antibody (αP-Tyr) or anti-tag antibody (αTag). The positions of tagged normal Tec and tagged TecΔSH3 are indicated at the right. The positions of molecular weight markers ($\times10^{-3}$) are shown on the left. (B) Anti-tag immunecomplexes prepared as described above were subjected to an *in vitro* kinase assay without exogenous substrates. Autophosphorylation of TecΔSH3 (Tag-ΔSH3) is increased compared with that of normal Tec (Tag-Tec).

Tec C serum (raised in rabbits against the synthetic peptide corresponding to the C-terminal 19 amino acids of mouse Tec protein), and probed with anti-phosphotyrosine antibody. As shown in the "Vector" part of Fig. 3, IL-3 stimulation of BA/F3 cells could induce tyrosine-phosphorylation of endogenous $pp70^{Tec}$. In both ΔSH3 (1) and ΔSH3(2) cells, the immunoprecipitated TecΔSH3 is intensively tyrosine-phosphorylated. Interestingly, endogenous $pp70^{Tec}$ in these cells was also profoundly phosphorylated compared with the $pp70^{Tec}$ in the "+" lane of the "Vector" part. Furthermore, the phosphorylation level of endogenous $pp70^{Tec}$ and TecΔSH3 in ΔSH3(1) and ΔSH3(2) cells was no longer controlled by IL-3 stimulation. Therefore, TecΔSH3 is a constitutively active form of the *Tec* kinase.

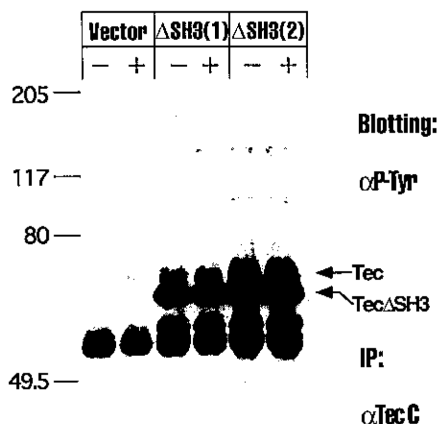

Fig. 3. TecΔSH3 is hyperphosphorylated in BA/F3 cells. The pSR*bsr*/TecΔSH3 plasmid was introduced into BA/F3 cells by electroporation with a Gene Pulser (Bio-Rad, Hercules, CA) at 280V, 960 μF. The cells were then cultured with 20 μg/ml of blasticidin-S (KAKEN Seiyaku, Co., Tokyo). Two of the blasticidin-S resistant clones (ΔSH3(1) and ΔSH3 (2)) were examined here. The vector-transfected cells (Vector), ΔSH3(1) cells and ΔSH3(2) cells were starved of cytokine, and treated with (+) or without (−) 250 U/ml of mouse IL-3 for 5 min. Tec and TecΔSH3 were immunoprecipitated by anti-Tec C serum (αTec C) and then probed with anti-phosphotyrosine antibody (αP-Tyr). The positions of normal Tec and TecΔSH3 are indicated on the right. The positions of molecular weight markers ($\times 10^{-3}$) are also shown on the left.

We previously observed that Tec can associate *in vivo* with several tyrosine-phosphorylated cellular peptides, including Shc[11] and Vav.[15, 16] Therefore, we examined whether hyperphosphorylation and activation of Tec affected the binding between Tec and Shc proteins. TecΔSH3 and endogenous $pp70^{Tec}$ were immunoprecipitated by the anti-Tec C serum from vector-transfected BA/F3, ΔSH3(1) and ΔSH3(2) cells resuspended in Lysis buffer containing NP-40 at 0.1% instead of 1% (0.1% Lysis buffer). The immunecomplexes were then probed with anti-Shc antibody (Transduction Laboratories, Lexington, KY). As shown in Fig. 4A, at an exposure time where Shc protein could not be recognized in the vector-transfected cells, Shc was easily detectable in anti-Tec immunoprecipitates from ΔSH3(1) or ΔSH3 (2) cells. After a longer exposure, Shc was also identified in anti-Tec complexes from the vector-transfected BA/F 3 cells (data not shown). Therefore, we conclude that hyperphosphorylation or activation of Tec enhances the binding between Shc and Tec. We also investigated whether activation of Tec affected the phosphorylation of Shc protein. Endogenous Shc proteins were immunoprecipitated from vector-transfected BA/F3 or ΔSH3(1) cells, and probed with either anti-phosphotyrosine antibody or anti-Shc antibody. As shown in Fig. 4B, tyrosine-phosphorylation of Shc proteins is enhanced in ΔSH3(1) cells compared to that in the vector-transfected cells.

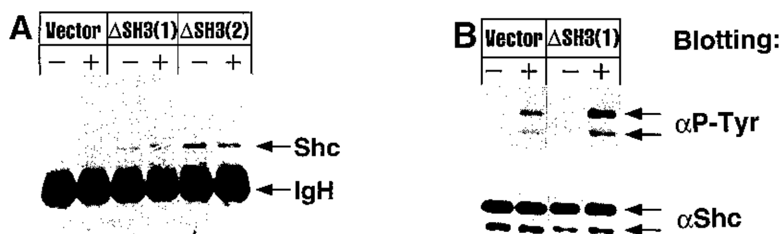

Fig. 4. Shc binds to TecΔSH3 *in vivo*. (A) The same set of cells as in Fig. 3 was lysed in 0.1% Lysis buffer. Tec and TecΔSH3 were immunoprecipitated from each fraction by anti-Tec C serum, and blotted with anti-Shc antibody. The positions of Shc (Shc) and immunoglobulin heavy chain (IgH) are indicated on the right. (B) Shc was immunoprecipitated from vector-transfected BA/F3 cells (Vector) or ΔSH3(1) cells (ΔSH3(1)), with (+) or without (−) IL-3 stimulation. The immunecomplexes were probed with either anti-phosphotyrosine antibody (αP-Tyr) or anti-Shc antibody (αShc). The positions of $p56^{Shc}$ and $p52^{Shc}$ are indicated by arrows.

Thus, activation of Tec should stimulate the intracellular signaling pathway mediated by Shc.

We have demonstrated that SH3-deletion results in constitutive activation of the *Tec* kinase. TecΔSH3 is hyperphosphorylated and has an elevated kinase activity. However, forced expression of TecΔSH3 in 3T3 fibroblasts did not induce transforming foci (data not shown). Similarly, expression of TecΔSH3 did not abrogate IL-3-dependency in BA/F3 cells (data not shown). Therefore mere deletion of the internal SH3 domain can not confer full oncogenic activity upon the Tec kinase. This is in contrast to the observation that SH3-deleted c-Src can transform chicken embryo fibroblasts.[21] The discrepancy may be due to the difference of assay systems, or due to the different *in vivo* roles of these PTKs. Interestingly, although BA/F3 cells expressing TecΔSH3 still require IL-3 for long-term growth, the expression of TecΔSH3 can protect these cells from apoptosis by IL-3-depletion to some extent (data not shown). Thus, Tec may be involved in the anti-apoptotic pathway driven by cytokines.

The exact mechanism by which deletion of the SH3 domain elevates the *Tec* kinase activity is still obscure. Since truncation of SH3 domains has also been shown to increase kinase activity in c-*Abl* and c-*Src*, the SH3 domain may act as a docking site for cellular peptides suppressing the activity of PTKs. We have recently revealed that a point mutation of a certain tyrosine residue in the Tec SH3 domain results in activation of Tec. Therefore, suppressive molecules may bind to the tyrosine-containing sequence of Tec SH3 domain, and be released from Tec when the internal SH3 is truncated. We have reproducibly observed that TecΔSH3 is more intensively tyrosine-phosphorylated in BA/F3 cells than in 293 cells. Therefore, the identity and/or quantity of the putative "PTK-suppresser" may vary among different tissues. As in the case of Tec-Shc association, TecΔSH3 may be a useful tool to study the intracellular substrates of *Tec* kinase.

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