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INCREASED TYROSINE-PHOSPHORYLATION OF 55KDA PROTEINS IN β-ACTIN/*TEC* TRANSGENIC MICE

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Protein-tyrosine kinases are considered to play important roles in cell proliferation and differentiation. *Tec* is a cytoplasmic protein-tyrosine kinase expressed in liver and hematopoietic tissues. To better understand Tec function *in vivo*, we generated transgenic mice expressing *tec* driven by the cytomegarovirus enhancer and β-actin promoter. Among six transgenic lines generated, a particular line, named 2-11, expressed *tec* transgene product more widely and abundantly than the other lines. In the tissues of 2-11, the kinase activity of Tec was enhanced in accordance with the high expression of *tec* transgene product. Interestingly, tyrosine-phosphorylation of approximately 55KDa proteins in the tissues was induced. These results suggest that cellular proteins of 55KDa might be potential substrates of Tec *in vivo*.

Protein-tyrosine kinases (PTKs) have been considered to be essential in cell proliferation and differentiation (1). A large number of protein-tyrosine kinases (PTKs) have been cloned, well-classified, and their biological functions have been intensively studied. On the basis of their primary structures, PTKs can be divided into two groups, namely, transmembrane-type (receptor-type) PTKs and cytoplasmic-type (non-receptor-type) PTKs. Some of growth factor receptors are transmembrane-type PTKs which, when bound to their ligands in the extracellular domain, phosphorylate tyrosine residue(s) in the cytoplasmic domain. This event results in sequential activation of other cellular molecules and eventually causes cell proliferation or differentiation (2). Comparing to transmembrane-type PTKs, the functions of cytoplasmic PTKs are rather obscure. However, evidences have been accumulated that cytoplasmic PTKs also play important roles in signal transduction. For example, in T-lymphocytes, lck and fyn have been shown to be associated with cell surface antigens CD4/CD8 and T cell receptor, respectively. (3,4,5). In B-cells, lyn has been demonstrated to interact with surface immunoglobulin (6).

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Recently, jak2 was proved to be associated with erythropoietin (Epo) receptor and play an important role in Epo-induced signal transduction (7).

Tec was originally cloned from a mouse liver cDNA library as a new member of cytoplasmic PTKs (8). A subsequent study showed that *tec* is abundantly expressed in liver and hematopoietic cell lines (8, 9). These results suggest that Tec is implicated in signaling pathways both in liver cells and in hematopoietic system. Indeed, we have recently revealed that Tec is involved in IL-3-mediated signal transduction in an IL-3-dependent mouse cell line, 32D (10). However, in contrast to the progressing studies of Tec function *in vitro*, little has been known about the role that Tec plays *in vivo*. To better understand the Tec role *in vivo*, we generated transgenic mice expressing Tec driven by the cytomegarovirus enhancer and β-actin promoter. Among six transgenic lines generated, we isolated a line, named 2-11, in which *tec* transgene product was more widely and abundantly expressed than the other lines in all tissues examined. In that line, the kinase activity of Tec was examined by the *in vitro* immunecomplex kinase assay and an alteration of tyrosine-phosphorylation of cellular proteins was investigated by Western blot analysis using anti-phosphotyrosine antibody.

Materials and Methods

Generation of β- actin/tec transgenic mice. Mouse *tec* cDNA (8) was subcloned into the EcoRI site of a mammalian expression vector, pUCCAGGS, which contains the cytomegarovirus (CMV) enhancer and β-actin promoter as a regulatory element (11). The SalI-BamHI fragment encompassing the CMV enhancer, β-actin promoter, *tec* cDNA, and a part of rabbit globin gene (named β-actin/*tec*) was purified and microinjected into the pronuclei of eggs derived from F_2 of C57BL/6XDBA2 mice (Clea, Japan). The schematic model of the injection fragment is shown in Fig. 1. The method for microinjection and embryonal transfer was essentially as described previously (12).

Northern blot analysis. Total RNA was extracted using the acid guanidine/phenol-chloroform method (13). Twenty μg of total RNA of each sample were electrophoresed in a 1.2% formaldehyde gel and blotted to a Hybond-N nylon membrane (Amersham). Hybridization was carried out in a solution containing 50% formamide, 5X SSC (1XSSC: 150mM NaCl, 15mM Na-Citrate), 5X Denhart's solution (1mg/ml polyvinylpyrrolidone, 1mg/ml bovine serum albumin, and 1mg/ml Ficoll), 0.5% SDS, 50 mM sodium phosphate (PH 7.0), 100 ng/ml salmon sperm DNA, and ^{32}P-labeled injection fragment at 42°C for overnight. The filters were washed twice in 2X SSC/0.1% SDS at 50°C for 20 minutes and then twice in 0.2X SSC/0.1% SDS at 55°C for 20 minutes. The signals were detected with Kodak XAR Films (Kodak) or using Fujix Bas 2000 image analyser (Fujifilm).

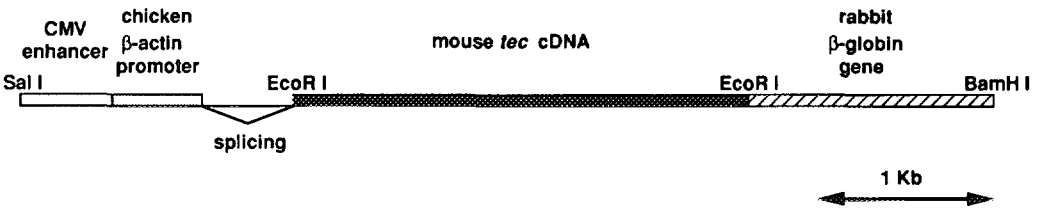

Figure 1. Schematic model of the injected fragment for generating β-actin/*tec* transgenic mice. Mouse *tec* cDNA was inserted in the EcoRI site of the expression vector pUCCAGGS. The SalI-BamHI fragment encompassing the cytomegarovirus enhancer (white box), chicken β-actin promoter (dotted box), mouse *tec* cDNA (black box), and a part of rabbit globin gene (shaded box) was used for microinjection. A splicing signal is also indicated. DNA at a concentration of approximately 5ng/ml was injected to the pronuclei of eggs derived from F_2 of C57BL/6XDBA2 mice.

Immunoprecipitation and Western blot analysis. Proteins were extracted by homogenizing the tissues in 1% NP40 lysis buffer (150mM NaCl, 50mM Tris-Cl (pH 7.4), 1% Nondet P-40 (NP40; Bio-rad), 1% sodium deoxycholate, and 50U/ml of aprotinin). For detecting the *tec* transgene product, 1mg of total cell lysates (TCL) were incubated with 1:200 diluted anti-Tec polyclonal antibody, anti-NTec2 (14). The immunoprecipitated proteins were separated by 10% SDS-PAGE, electroblotted to a polyvinylidene membrane (Immobilon; Millipore), and probed with 1:500 diluted anti-NTec2. The membrane was subsequently incubated with 1:7500 diluted anti-rabbit immunoglobulin antibody conjugated with alkaline phosphatase (Promega), and the specific binding was visualized by the combination of bromochloroindolyl phosphate (BCIP; Promega) and nitro blue tetrazolium (NBT; Promega). For detecting tyrosine-phosphorylated proteins, 30 μg of TCL were separated by 7.5% SDS-PAGE, electroblotted to a polyvinylidene membrane (Immobilon; Millipore), and probed with anti-phosphotyrosine monoclonal antibody, 4G10, according to the manufacturers' instructions (15). The following procedures were as described above except that anti-mouse immunoglobulin was used instead of anti-rabbit immunoglobulin as the second antibody.

In vitro kinase assay. Total cellular proteins (1mg/experiment) were incubated with 1:200 diluted anti-NTec2 followed by protein A (Sigma). The immunoprecipitated proteins were washed five times with the lysis buffer, followed by five times with the kinase buffer (50mM Tris-Cl (pH 7.4), 10mM $MgCl_2$, and 10mM $MnCl_2$) and incubated with 10μCi of γ^{32}P-ATP (Amersham) at room temperature for 15 minutes. The phosphorylated proteins were separated in a 10% SDS-PAGE, dried, and autoradiographed.

Results and Discussion

To investigate Tec function *in vivo*, we attempted to generate transgenic mice expressing Tec in a wide variety of tissues. To this aim, mouse *tec* cDNA was subcloned into a mammalian expression vector pUCCAGGS containing the CMV enhancer and β-actin promoter, and a fragment encompassing the enhancer/promoter unit, *tec* cDNA, and the rabbit globin gene was excised and used for microinjection. As a result, we generated six transgenic founder mice which carried ten to thirty copies of transgene in a tandem manner and transmitted the transgene stably to their progeny. To examine Tec expression among the transgenic lines, Northern blot analysis of liver was initially carried out, since Tec was reported to be abundantly expressed in liver. As shown in Fig. 2, a particular line, named 2-11, expressed *tec* mRNA in liver more abundantly than other transgenic lines. Subsequent analyses for *tec* expression in other tissues also showed that the line 2-11 expressed equally or more abundant message than other lines (data not shown). For investigating tissue distribution of *tec* transgene product in line 2-11, Northern and Western blot analyses were performed in various tissues of a transgenic mouse and a non-transgenic littermate. RNAs and proteins extracted from tissues including brain, thymus, lung, heart, liver, spleen, and kidney were probed with *tec* cDNA and anti-NTec2 (14), respectively. As shown in Fig. 3, all tissues of a transgenic mouse expressed *tec* transgene much more abundantly at both mRNA and protein levels comparing to those of a non-transgenic littermate. These results indicate that *tec* transgene is widely and abundantly expressed in the tissues of 2-11, therefore, the line 2-11 is considered to be a good candidate to investigate Tec function *in vivo*.

The reason why the particular line, 2-11, expressed more abundant message than the other lines is not clear. One possibility is that 2-11 carried high transgene copy number (approximately thirty copies). However, other lines, such as line 2-8 and line 2-9, carried approximate the same copy number as line 2-11 (data not shown) but expressed much less message (see Fig 2). Therefore, the high copy number in 2-11 might not be a reason. The genomic sequence around the integration site might be responsible for the high and wide expression of the transgene in 2-11.

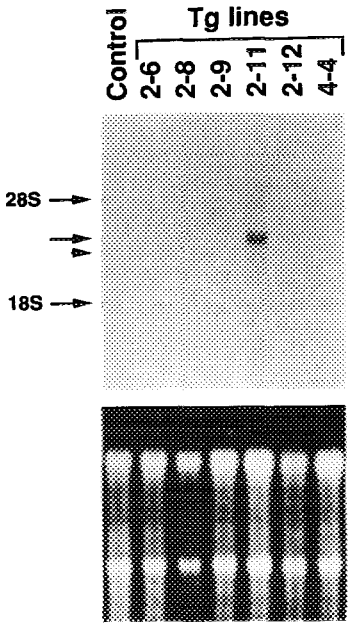

Figure 2. Northern blot analysis for *tec* transgene expression in liver among six transgenic mouse lines. Twenty mg of total RNA extracted from liver of a transgenic mouse of each line was separated in a 1.2% agarose gel, transferred to a nylon membrane (Hybond-N; Amersham), and hybridized with radiolabeled *tec* cDNA as a probe. The *tec* transcripts are indicated by an arrow (for larger transcript) or an arrowhead (for smaller transcript). 28S and 18S are shown as size markers. Ethidium bromide stained RNAs are also shown for quality and quantity of the RNAs.

In Northern blotting for *tec* transgene expression, two different sizes of the messages are observed. The size of the smaller message, which is observed in heart of a transgenic mouse and in spleen of a non-transgenic littermate, corresponds to the size of *tec* cDNA (indicated by an arrowhead in Fig. 3). On the other hand, the size of the larger message, which is observed ubiquitously in the tissues of a transgenic mouse, is a little larger than that of *tec* cDNA (indicated by an arrow in Fig. 3) . Since the *tec* transgene is expected to be transcribed as a fusion message of *tec* cDNA and a part of rabbit globin gene (see Fig. 1), it is reasonable that *tec* transgene transcript is larger than the native *tec* transcript. The smaller massage is considered as the message transcribed using its own poly (A) signal.

We then examined whether overexpression of Tec increases its kinase activity by the *in vitro* kinase assay. Hearts were used for this experiment, since *tec* transgene was demonstrated to be most abundantly expressed in heart (see Fig. 3). As shown in Fig. 4, the proteins immunoprecipitated with anti-NTec2 in a transgenic mouse showed increased phosphorylation comparing to those in a non-transgenic littermate. This result indicates that the kinase activity of Tec in a transgenic mouse was elevated in accordance with the high expression of *tec* transgene product. The increased phosphorylated protein of approximately 70KDa might be an autophosphorylated *tec* transgene product (indicated as an arrow in Fig. 4).

Finally we investigated whether overexpression of Tec tyrosine kinase affects tyrosine-phosphorylation of other cellular proteins. As shown in Fig. 5, the tyrosine-phosphorylation

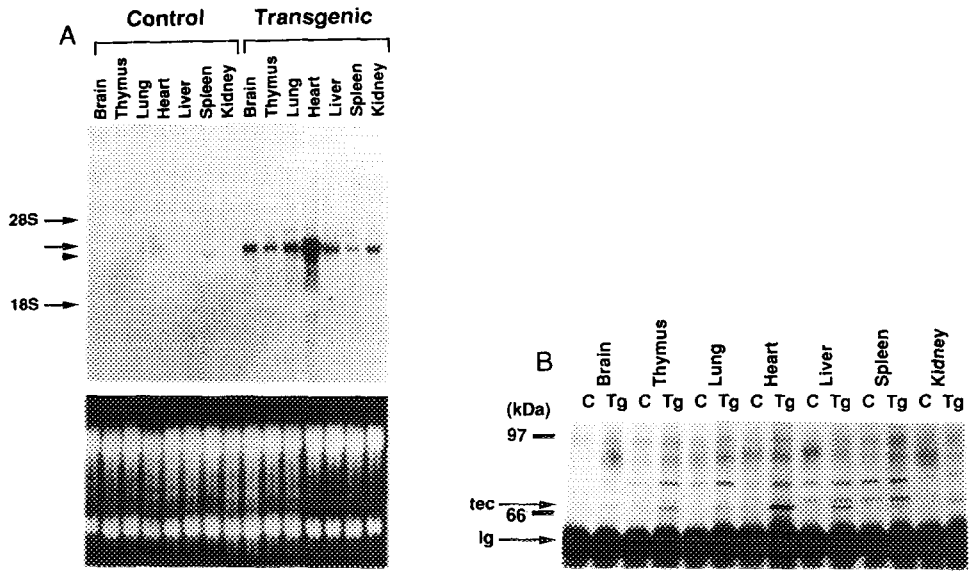

Figure 3. *Tec* transgene expression in the tissues in line 2-11 by Northern blot (A) or Western blot analysis (B). (A) Total RNA was extracted from brain, thymus, lung, heart, liver, spleen, or kidney of a transgenic mouse (Tg) or a non-transgenic littermate (C) in line 2-11. Twenty μg of RNAs were separated in a 1.2% formaldehyde gel, transferred to a nylon membrane (Hybond-N; Amersham), and hybridized with radiolabeled *tec* cDNA as a probe. The *tec* transcripts are indicated by an arrow or an arrowhead as shown in Fig. 2. 28S and 18S are shown as size markers. Ethidium bromide stained RNAs are also shown for quality and quantity of the RNAs. (B) Proteins were extracted from brain, thymus, lung, heart, liver, spleen, and kidney of a transgenic mouse (Tg) or a non-transgenic littermate (C) in line 2-11 and incubated with anti-NTec2. The immunoprecipitated proteins were separated by 7.5% SDS-PAGE, electroblotted to a polyvinylidene membrane (Immobilon; Millipore), and probed with 1:500 diluted anti-NTec2. *Tec* transgene products (Tec) and immunoglobulins (Ig) are indicated by arrows. Molecular standard markers are shown on the left.

level around 55KDa was apparently increased in the tissues of a transgenic mouse comparing to those of a non-transgenic littermate. This result suggests that overexpression of Tec induces tyrosine-phosphorylation of other cellular proteins around 55KDa via its increased tyrosine kinase activity. Since the tyrosine-phosphorylation levels of the 55KDa proteins are elevated when and where Tec is overexpressed, the proteins are considered to be functionally associated with Tec-mediated signal transduction and might be potential substrates of Tec *in vivo*. Molecular weights of several protein-tyrosine kinases are known to be around 55KDa, including lyn, lck, and yes. We recently demonstrated that Tec is constitutively associated with Lyn through its N-terminal domain (14). This observation suggests that Lyn might be included in the hyper-phosphorylated 55KDa proteins observed in the tissues of a transgenic mice in line 2-11.

We also investigated whether overexpression of *tec* transgene product induces its own tyrosine-phosphorylation in 2-11 by immunoprecipitating cellular proteins with anti-NTec2 and probing the immunoprecipitants with anti-phosphotyrosine antibody, 4G10. However, we failed to demonstrate increased tyrosine-phosphorylation of *tec* transgene product (data not shown). In Fig. 5, tyrosine-phosphorylation around 70KDa, which corresponds to the molecular weight of *tec* gene product (8), does not seem to be increased in the tissues of a transgenic mouse comparing to those of a non-transgenic littermate. Of course, there is a possibility that the tyrosine-phosphorylation of the *tec* transgene product was increased in a transgenic mouse, but

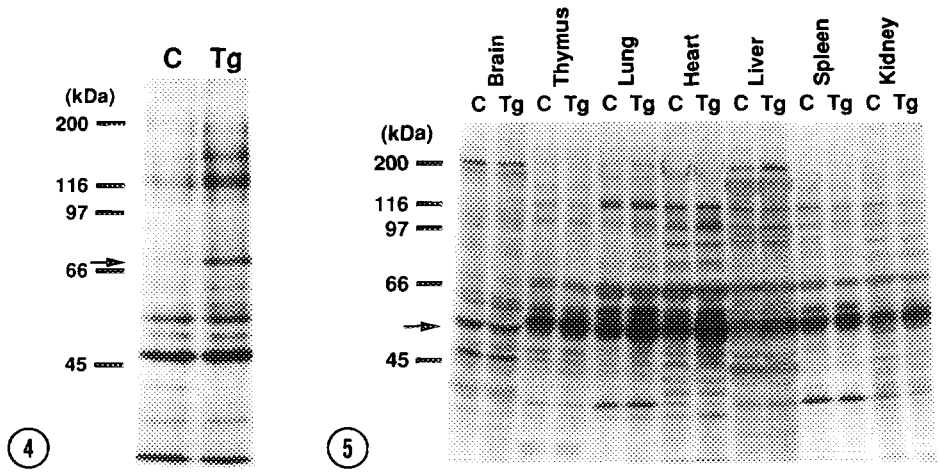

Figure 4. The *in vitro* kinase activity of *tec* transgene product in 2-11. Proteins extracted from hearts of a transgenic mouse (Tg) or a non-transgenic littermate (C) were incubated with anti-NTec2 and the immunoprecipitated proteins were incubated with γ^{32}P-ATP. The phosphorylated proteins were separated by 7.5% SDS-PAGE, dried, and autoradiographed. An increased phosphorylated band of approximately 70KDa observed in a transgenic mouse, which corresponds to the molecular weight of the *tec* transgene product, is indicated by an arrow. Molecular size markers are shown on the left.

Figure 5. Western blot analysis of tyrosine-phosphorylated cellular proteins in a transgenic mouse (Tg) or a non-transgenic littermate (C) in line 2-11. Thirty μg proteins extracted from brain, thymus, lung, heart, liver, spleen, and kidney were separated by 7.5% SDS-PAGE and probed with anti-phosphotyrosine antibody, 4G10. A band of approximately 55-KDa with increased phosphorylation in tissues of a transgenic mouse is indicated by an arrow. Molecular size markers are shown on the left.

the increased phosphorylation was below the detection level of Western blotting, since the *in vitro* kinase assay showed that the phosphorylation of approximately 70KDa protein was increased (see Fig. 4).

Several transgenic mice carrying cytoplasmic PTKs under various promoters have been reported and their phenotypical changes have also been described. For example, transgenic mice expressing v-fps tyrosine kinase under β−globin promoter was shown to develop not only lymphoid and mesenchymal tumors but also cardiac and neurological abnormalities (16,17). On the other hand, transgenic mice carrying wild-type lck ($p56^{lckY505}$) or mutated lck ($p56^{lckF505}$) under the lck proximal promoter were reported to develop thymic tumors after different periods of time (18). These results suggest that overexpression or aberrant expression of cellular or activated cytoplasmic PTKs might be responsible to the neoplastic transformation in transgenic mice. As for the phenotypical changes in β-actin/*tec* transgenic mice, no obvious tumor has so far been observed. It is not clear yet that overexpression and/or aberrant expression of Tec is not sufficient to cause malignant transformation in transgenic mice. An extensive and long-term study for β-actin/*tec* transgenic mice will be required to clarify a possible relationship between Tec expression and tumor pathogenesis, and that study will give us further insights for Tec function *in vivo*.

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