Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation

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Provirus insertion in the last intron of the Tpl-2 gene in retrovirus-induced rat T-cell lymphomas results in the enhanced expression of a carboxy-terminally truncated Tpl-2 kinase. Here we show that the truncated protein exhibits an approximately sevenfold higher catalytic activity and is two- to threefold more efficient in activating the MAPK and SAPK pathways relative to the wild-type protein. The truncated Tpl-2 protein and a GST fusion of the Tpl-2 carboxy-terminal tail interact when coexpressed in Sf9 cells. Their interaction down-regulates the kinase activity of the truncated protein suggesting that tail-directed intramolecular interactions regulate the Tpl-2 kinase. Tpl-2 transgenic mice expressing the wild-type protein from the proximal Lck promoter fail to show a biological phenotype, whereas mice expressing the truncated protein develop large-cell lymphoblastic lymphomas of T-cell origin. These results show that Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation.

[Key Words: Tpl-2/Cot proto-oncogene; protein kinase; MAPK; SAPK; oncogenesis; transgenic mice]

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Tumors are composed of cells with substantial genetic and phenotypic heterogeneity that are subject to continuous selection. The basis of selection may be the invasive and metastatic properties of the cells or perhaps their proliferative advantage in all or unique microenvironments. The outcome of this selection is tumor progression [Yeatman and Nicolson 1993]. Using an insertional mutagenesis-based genetic strategy we have searched for genes [tumor progression loci (Tpl)] that, when activated, enhance the proliferative capacity of T-cell lymphomas induced in rats by Moloney murine leukemia virus (MoMuLV) [Bear et al. 1989; Patriotis et al. 1993; Tsichlis et al. 1994]. One of the genes identified through this strategy, Tpl-2 [Patriotis et al. 1993], also known as Cot [Miyoshi et al. 1991], encodes a 55-kD serine–threonine protein kinase that is expressed primarily in spleen, thymus, and lung [Patriotis et al. 1993; Makris et al. 1993] and activates the mitogen-activated protein kinase (MAPK) [Patriotis et al. 1994] and stress-activated protein kinase (SAPK) [Salmerón et al. 1996] cascades. Tpl-2, one of five proto-oncogenes encoding serine–threonine protein kinases [Blair et al. 1986; Bonner et al. 1986; van Lohuizen et al. 1989; Bellacosa et al. 1991; Patriotis et al. 1993], is the target of provirus integration in 22.5% of the thymomas [Patriotis et al. 1993]. Cells with rearrangements in Tpl-2 grow better than parental cells both in situ, in the primary tumor-bearing animal, as well as in culture [Patriotis et al. 1993; C. Tsatsanis, C. Patriotis, and P.N. Tischlis, unpubl.]. Tpl-2 provirus insertions always target the last intron of the gene and elicit a complex set of events leading to the enhanced expression of a carboxy-terminally truncated protein [Makris et al. 1993; Patriotis et al. 1993]. First, the steady-state level of Tpl-2 mRNA is increased dramatically, perhaps because of a combination of transcriptional activation attributable to enhancer insertion, and mRNA stabilization attributable to the removal of destabilizing sequences from its 3'-untranslated region. Second, provirus integration is associated with a shift in promoter usage from promoter P1, which is used primarily in normal quiescent lymphocytes, to promoter P2. Use of promoter P2 gives rise to RNA transcripts containing an alternate 5'-untranslated region that appears to be translated with higher efficiency [C. Patriotis, C. Tsatsanis, A. Makris, and P.N. Tischlis, unpubl.]. Finally, the mRNA of the activated Tpl-2 locus terminates in the proviral long terminal repeat (LTR), and therefore it lacks sequences derived from its 3' exon. As a result, the kinase encoded by the activated Tpl-2 locus is carboxy-terminally truncated.
Data presented in this report indicate that carboxy-terminal truncation enhances the activity of the Tpl-2 kinase and potentiates its ability to activate the MAPK and SAPK cascades. The truncated Tpl-2 protein was shown to interact with a glutathione S-transferase (GST) fusion of its carboxy-terminal tail when the two were coexpressed in Sf9 cells. The Tpl-2 kinase activity was down-regulated via this interaction. The enhanced activity of the truncated protein correlates with its oncogenic potential. Transgenic mice expressing the truncated protein in thymocytes develop T-cell lymphomas, whereas transgenic mice expressing the wild-type protein do not. Therefore, Tpl-2 encodes an oncogenic kinase that is activated by carboxy-terminal truncation.

Results

Tpl-2 activation by provirus insertion in MoMuLV-induced rat T-cell lymphomas

The molecular mechanism leading to the expression of a carboxy-terminally truncated Tpl-2 protein is shown diagrammatically in Figure 1. Provirus insertion occurs reproducibly within intron vii, giving rise to mRNA transcripts that terminate in the proviral LTR and therefore lack exon 8. The Tpl-2 protein encoded by these transcripts lacks the 43 carboxy-terminal amino acids specified by exon 8 that are replaced by seven amino acids encoded by in-frame sequences of intron vii.

The carboxy-terminally truncated Tpl-2 exhibits enhanced catalytic activity in vitro

To determine the significance of the tumor-specific Tpl-2 truncation we examined whether the wild-type and truncated proteins differed with regard to their kinase activities. To this end, pCMV-5 expression constructs of the wild type, the kinase deficient (K167M) (Patriotis et al. 1994), and the carboxy-terminally truncated Tpl-2, tagged at their carboxyl terminus with a hemagglutinin (HA) epitope tag, were transfected into COS-1 cells. Following 24 hr of serum starvation, the cells were lysed and the expression of the transfected constructs was determined by probing Western blots of total cell lysates with the anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim) (Fig. 2a). Tpl-2 immuno-precipitates of the same lysates were used for in vitro kinase assays. Quantitation of the autophosphorylation of Tpl-2 (Fig. 2b) and the Tpl-2-mediated transphosphorylation of exogenously added substrates (Fig. 2c), coupled to the expression of Tpl-2, revealed that the specific activity of the truncated protein was considerably higher than that of the wild-type protein (Fig. 2d). Specifically, the truncated protein was approximately five times more active in autophosphorylation and five and seven times more active in the phosphorylation of the histone substrates H3 and H2A, respectively (Fig. 2d).

The carboxy-terminally truncated Tpl-2 activates the MAPK and SAPK cascades with higher efficiency than the wild-type Tpl-2

Tpl-2 is a potent activator of the MAPK and SAPK cascades (Patriotis et al. 1994; Salmerón et al. 1996). We therefore addressed the question as to whether the truncated protein, which exhibits enhanced kinase activity, activates these cascades more efficiently. To this end, COS-1 cells were cotransfected with expression constructs of the wild-type or truncated Tpl-2 and

Figure 1. Activation of Tpl-2 by provirus insertion. The Tpl-2 gene is composed of eight exons. Provirus integration occurs reproducibly in intron vii and results in mRNA transcripts that lack exon 8. These transcripts encode a carboxy-terminally truncated protein that lacks 43 amino acids encoded by exon 8 but contains seven alternative amino acids derived from sequences of intron vii fused in frame to Tpl-2. [N] Amino-terminal domain; [C] catalytic domain; [T or T'] carboxy-terminal tail; [MoMLV] Moloney murine leukemia virus.
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Figure 2. The carboxy-terminally truncated Tpl-2 exhibits enhanced catalytic activity relative to the wild-type protein. [a] Western blot of Tpl-2 wt-HA, Tpl-2tr-HA, or Tpl-2trK167M-HA (kinase deficient) transfected COS-1 cells probed with the anti-HA antibody 12CA5. [b] Immunocomplex in vitro kinase reactions of Tpl-2. Autophosphorylation of wild-type and truncated Tpl-2 immunoprecipitated from equal amounts of the COS-1 lysates shown in a. [c] Phosphorylation of histone H3 + H2B + H2A or MBP exogenous substrates in the in vitro kinase reactions presented in b. [d] Specific activity of the wild-type and truncated Tpl-2 kinase. The specific activity was determined by dividing the Phosphorimager (Fuji)-derived values of \(^{32}P\) incorporation in Tpl-2 or the Tpl-2 substrates (histone H3 or H2A) by the relative expression of Tpl-2. The relative expression of Tpl-2 was measured by densitometry (Ambis) of the Tpl-2 bands in Western blots of the same lysates. The data shown are representative of the results obtained from four independent experiments of which two were carried out with the untagged Tpl-2 cDNAs. The results obtained with both the tagged and untagged Tpl-2 constructs were indistinguishable. The untagged proteins were immunoprecipitated using an anti-Tpl-2 peptide specific (LFDRKRLSRIKELEL) antibody (PT492).

with an expression construct of the MAPK ERK1 tagged at its amino terminus with a c-myc epitope tag [MT-p44ERK1] or with an expression construct of the SAPK JNK1 tagged at its amino terminus with an HA epitope tag [HA-JNK1]. Thirty hours later the cells were serum-starved and after an additional 18-hr period they were lysed. MT-p44ERK1 and HA-JNK1 were immunoprecipitated from these lysates using the anti-c-myc tag monoclonal antibody 9E10.2 [MT-p44ERK1] (Evan et al. 1985), or the anti-HA tag monoclonal antibody 12CA5 [HA-JNK1]. To determine the MAPK activity in the anti-c-myc immunoprecipitates, we employed an in-gel kinase assay using myelin basic protein (MBP) as the exogenous substrate (Patriotis et al. 1994) (Fig. 3A,a). Quantitation of the phosphorylation of MBP, coupled to the expression of MT-p44ERK1 (Fig. 3A,b) and Tpl-2 (Fig. 3A,c), revealed that the truncated protein activates the MAPK ~2.5 times more efficiently than the wild-type protein (Fig. 3A,d). To determine the activity of the SAPK JNK1 in the anti-HA immunoprecipitates we carried out immunocomplex in vitro kinase assays using GST/N5-89Jun as the exogenous substrate (Fig. 3B,a). Quantitation of the phosphorylation of GST/N5-89Jun, coupled to the expression of HA-JNK1 (Fig. 3B,b) and Tpl-2 (Fig. 3B,c), revealed that the truncated Tpl-2 activates the SAPK approximately two times more efficiently than the wild-type protein (Fig. 3B,d).

The catalytic activity of the carboxy-terminally truncated Tpl-2 is down-regulated by a GST/carboxy-terminal tail fusion protein coexpressed in trans in Sf9 cells

To explain the preceding findings we hypothesized that the carboxy-terminal tail folds onto the kinase domain of Tpl-2 and inhibits its catalytic function. To address this question, wild-type or carboxy-terminally truncated Tpl-2 were coexpressed with a GST fusion of the carboxy-terminal tail (GST/C-tail) or GST in Sf9 cells using baculovirus constructs. Immunoprecipitation of the protein encoded by Tpl-2 [wild-type or truncated] led to the cophosphorylation of the carboxy-terminal tail (Fig. 4A). These results provided support to the hypothesis that the kinase activity of Tpl-2 may be modulated by intramolecular interactions between the carboxy-terminal tail and the remainder of the molecule. To address this question, we carried out in vitro kinase assays on Tpl-2 immunoprecipitates from lysates of Sf9 cells infected with the combinations of baculoviruses shown in Figure 4B. The results showed that coexpression of GST/C-tail with truncated Tpl-2 down-regulates the Tpl-2 kinase activity to the wild-type level (Fig. 4B,c). The interaction of the Tpl-2 carboxy-terminal tail with the remainder of the molecule may therefore play an important regulatory role. This interaction may be regulated by phosphorylation of the tail by Tpl-2 or other kinases. Examination of the amino acid sequence of the tail for kinase target motifs indeed revealed several potential phosphorylation sites [Table 1].

Transgenic mice expressing the carboxy-terminally truncated, but not the wild-type Tpl-2 from the proximal Lck promoter develop T-cell lymphomas

To determine whether the Tpl-2 kinase is oncogenic in T cells and whether its oncogenic potential is modulated by carboxy-terminal truncation, we established Tpl-2 transgenic mice using wild-type and carboxy-terminally truncated constructs generated as described in Materials
and Methods. In these constructs, Tpl-2 was placed under the control of the proximal Lck promoter and was expressed specifically in thymocytes [Abraham et al. 1991a,b; Wildin et al. 1991]. Forty-eight transgenic founders, 24 with the wild-type and 24 with the truncated construct, were established. Of 16 founders carrying the wild-type transgene that were analyzed, five exhibited high and two exhibited low to intermediate levels of expression. Expression of the carboxy-terminally truncated transgene that were analyzed, three exhibited high and two exhibited low to intermediate levels of expression. In the remaining founders expression of the transgene was undetectable. Of 12 founders carrying the truncated construct, developed lymphoblastic lymphomas with an 88% incidence by 3 months of age (Table 3). Neoplasms developing in these mice were comprised of homogeneous populations of large lymphoid cells with a scant, weakly-staining cytoplasm [Fig. 5C,D]. The neoplastic proliferation was confined to the thymus, with obliteration of thymic architecture and infiltration of the adjacent extracapsular area. In animals with clinical manifestations of disease, the neoplastic proliferation was disseminated widely in many tissues [Fig. 5E–H].

**Phenotypic characterization of T-cell lymphomas arising in Tpl-2 transgenic mice**

To determine whether the tumors were of T-cell origin, Southern blots of tumor genomic DNA were hybridized to DNA probes of the T-cell receptor (TCR) and the immunoglobulin heavy or light chain rearrangements by this analysis (data not shown) confirmed that the tumors were of T-cell origin [Gilbert et al. 1993]. FACS analyses were also carried out on lymphoblastic lymphomas from them (3456), expressing the truncated construct, developed metastatic lymphoblastic lymphomas with an 88% incidence by 3 months of age (Table 3).

**Figure 3.** The carboxy-terminally truncated Tpl-2 activates the MAPK and SAPK pathways more efficiently than the wild-type protein. (A) Activation of the MAPK ERK1 by wild-type and carboxy-terminally truncated Tpl-2. [A,a] In-gel kinase assay of MT • p44ERK1 immunoprecipitated from Tpl-2wt • HA-, Tpl-2tr • HA-, and Tpl-2trK167M • HA-cotransfected and noncotransfected COS-1 cell lysates with the anti-c-myc tag monoclonal antibody 9E10.2. The kinase substrate in this reaction was MBP. Stimulation with EGF was used as a control. [A,b] Western blot of MT • p44ERK1 immunoprecipitated from the lysates shown in a with antibody 9E10.2 and probed with the anti-ERK1 polyclonal antibody SC93 (Santa Cruz). [A,c] Western blot of the same lysates shown in a, probed with the anti-HA tag monoclonal antibody 12CA5. [A,d] Specific activity of MT • p44ERK1 in the same lysates normalized relative to the expression of Tpl-2. The specific activity of MT • p44ERK1 was determined as described in the legend to Fig. 2. This was normalized based on the expression of Tpl-2 in the same lysates. [B] Activation of the SAPK JNK1 by wild-type and carboxy-terminally truncated Tpl-2. [B,a] In vitro kinase assay of HA • JNK1 immunoprecipitated from Tpl-2wt • MT and Tpl-2tr • MT cotransfected and noncotransfected COS-1 cell lysates with the anti-HA tag monoclonal antibody 12CA5. The kinase substrate was GST/N5-89Jun. Stimulation with TNF-α was used as a control. [B,b] Western blot of HA • JNK1 immunoprecipitated from the lysates shown in a with the anti-HA tag antibody 12CA5 and probed with the anti-JNK1 rabbit polyclonal antibody SC474 (Santa Cruz). [B,c] Western blots of the same lysates shown in a, probed with the anti-c-myc tag monoclonal antibody 9E10.2. [B,d] Specific activity of MT • p44ERK1 in the same lysates normalized relative to the expression of Tpl-2. The specific activity of MT • p44ERK1 was determined as described in the legend to Fig. 2. This was normalized based on the expression of Tpl-2 in the same lysates.
11 animals derived from the transgenic line 3456. Single-cell suspensions from the spleen and thymus of tumorbearing transgenic and tumor-free control mice were stained with antibodies specific for T cells (Thy 1.2, CD4, CD8) [Ledbetter et al. 1980; Bierer et al. 1989; Janeway 1992; Schlossman et al. 1995], B cells [B220] [Coffman 1982; Hardy et al. 1991], and myeloid cells [Mac1, Gr1] [Springer et al. 1979; Fleming et al. 1993]. The results showed that four of the tumors consisted primarily of CD4/CD8 double-positive cells (Fig. 6B). The spleen of the animal, shown in Figure 6B (right), contained 100-fold more double-positive cells than the spleen of control animals (Fig. 6A, right), suggesting that it was almost completely replaced by infiltrating tumor cells. Five of the tumors consisted primarily of CD8 single-positive cells (Fig. 6C), and one tumor consisted primarily of CD4 single-positive cells (Fig. 6D). These results confirmed that the tumors were of T-cell origin and demonstrated that they were derived from cells at different stages of T-cell development.

Correlation of tumor induction with the expression of the transgene

To determine whether tumor induction correlates with the expression of the transgene, the relative levels of Tpl-2 in founder mice as well as in the offspring of established transgenic mouse lines were measured by RNase protection. The results showed that, overall, the mice carrying the truncated Tpl-2 transgene exhibit higher levels of transgene expression. Of 24 founder mice carrying the wild-type and 24 carrying the truncated Tpl-2 transgenes only 5 expressing wild type and 3 expressing truncated Tpl-2 were shown to express approximately equivalent levels of Tpl-2 [Fig. 7A; data not shown]. Both the founder mice and transgenic mouse

| Amino acid position | Protein kinase | Phosphorylation sequence |
|---------------------|----------------|--------------------------|
| 431                 | CKII           | STEE                     |
| 440                 | PKA            | RQRS                     |
| 463                 | Cas phosphor.  | TLE                      |

*CKII Casein kinase II; {PKA} protein kinase A.
lines, established from founders expressing easily detectable levels of the transgenes, were observed for tumor incidence. The results showed that even when the level of transgene expression was approximately equivalent, only mice carrying the truncated Tpl-2 transgene developed tumors. However, in mice carrying the truncated transgene, tumor induction correlated with transgene expression [Fig. 7B].

The higher level of expression of the carboxy-terminally truncated Tpl-2 transgene suggested that Tpl-2 may activate the Lck promoter. Because the specific activity of the carboxy-terminally truncated Tpl-2 is higher than that of the wild-type protein, we hypothesized that if Lck is regulated by Tpl-2 its expression will be higher in the presence of the carboxy-terminally truncated Tpl-2 protein. To address this hypothesis, Western blots of total cell lysates from the T-cell lymphoma line 2769 [Lazo et al. 1990], engineered to express wild-type or truncated Tpl-2, were probed with the anti-Lck antibody SC13 [Santa Cruz]. The results [Fig. 8A] showed that Lck expression is higher in cells expressing the truncated Tpl-2 protein. To determine whether the effects of Tpl-2 on Lck gene expression was a result of the effects of Tpl-2 on the activity of the proximal Lck promoter, EL4 cells were transiently transfected with an Lck promoter/CAT reporter construct [Muise and Rosen 1995] and with expression constructs of the wild-type or truncated Tpl-2. Cotransfection of the vector only, followed by treatment with PMA plus ionomycin, was used as a control. The results [Fig. 8B] confirmed that Tpl-2 induces expression from the proximal Lck promoter and that the truncated Tpl-2 is more efficient in activating the promoter.

Discussion

Tpl-2 is one of five serine-threonine protein kinases encoded by cellular proto-oncogenes [Blair et al. 1986; Bonner et al. 1986; van Loohuizen et al. 1989; Bellacosa et al. 1991; Patriotic et al. 1993]. The kinase encoded by the Tpl-2 proto-oncogene regulates the activity of several signaling pathways. Thus, it has been shown to activate the MAPK and SAPK pathways in a variety of cell types [Patriotic et al. 1994; Salmerón et al. 1996]. Moreover, it has been shown to activate NF-κB and the calcium-dependent nuclear factor of activated T cells [NF-AT] pathway and to induce interleukin-2 [IL-2] expression in T cells [C. Tsatsanis, S. Patriotic, S.E. Bear, J. Caamano, and P.N. Tischlis, in prep.]. Provirus integration in the last intron of Tpl-2 occurs during the progression stage of MolMuLV-induced rodent T-cell lymphomas and induces high levels of expression of a carboxy-terminally truncated protein [Makris et al. 1993; Patriotic et al. 1993]. The work presented in this report confirmed the oncogenic properties of Tpl-2 and demonstrated the importance of its carboxy-terminal tail in regulating its catalytic activity and biological function. Moreover it showed that the enzymatic activity of Tpl-2 correlates well with its ability to activate the MAPK and SAPK pathways and with its oncogenic potential.

Analysis of the crystal structure of several kinases has shown that they are all composed of two lobes linked together by a loop. ATP and substrate binding take place in the cleft between the two lobes [De Bondt et al. 1993; Han et al. 1995; Datta et al. 1996]. The role of the tails inhibit rather than enhance kinase activity. The data presented in this report show that, similar to the Src kinases, tail-directed intramolecular interactions regulate the kinase activity of Tpl-2 and its ability to activate signaling pathways involved in the regulation of cellular signaling pathways and with its oncogenic potential.

Table 3. Tumor incidence in established transgenic lines

| Line  | Construct | No. aged | No. of lymphoblastic lymphoma | Age of animals of time of diagnosis in days (mean ± S.D.) | No. of metastatic tumors |
|-------|-----------|----------|-------------------------------|----------------------------------------------------------|-------------------------|
| 1     | wt        | 14       | 0                             | N.A.                                                     | 0                       |
| 2     | wt        | 10       | 0                             | N.A.                                                     | 0                       |
| 3     | tr        | 26       | 23                            | 100 ± 26                                                 | 23                      |
| 4     | tr        | 13       | 0                             | N.A.                                                     | 0                       |
| control | nontransgenic | 15      | 0                             | N.A.                                                     | 0                       |

*a(wt) Wild-type construct; (tr) truncated construct; line 3 is line 3456.

**Metastatic tumors in transgenic line 3456 were observed in spleen, heart, liver, lung, bone, kidney, prostate, pancreas, bone marrow, bladder, adrenal gland, thyroid gland, ovaries, pituitary gland, and salivary gland.
Figure 5. Histopathology of neoplasms arising in Tpl-2tr transgenic mice. [A] Hematoxylin-eosin (H and E) stain (original magnification 132×) of wild-type thymus with normal cortical (c) and medullary (m) areas. [B] Higher magnification (264×) of wild-type thymus. Arrows indicate occasional macrophages. [C] H and E stain of a representative thymic neoplasm from a Tpl-2tr transgenic mouse (132×). (ec) Extracapsular area infiltrated by neoplastic lymphocytes. [D] Higher magnification (264×) of the tumor in C. Arrows indicate debris-laden macrophages. [E] Lymphoblastic infiltrates in liver portal triads [n] and hepatic sinusoids [arrows] (H and E, 264×). [F] Lymphoblastic infiltrate in the splenic red pulp (rp). [If] A splenic lymphoid follicle [H and E, 132×]. [G] Lymphoblastic infiltrate [n] in the hilus adjacent to the bronchus (arrow) [H and E, 132×]. [H] Lymphoblastic infiltrate [n] in the myocardium adjacent to the atrium [a]. [V] Ventricle [H and E, 132×].

proliferation and oncogenesis. Alternatively, the tail-directed regulation of the Tpl-2 catalytic activity may be operating through yet unidentified effector molecules. The data presented are remarkable in that they show that the carboxy-terminal tail inhibits the activity of Tpl-2 even when expressed in trans. This suggests that truncated Tpl-2 and the carboxy-terminal tail either colocalize in the same subcellular compartment, thus achieving high intermolecular concentrations, and/or interact with very high affinity.

The dramatic difference in oncogenic potential between the wild-type and the carboxy-terminally truncated Tpl-2 shows clearly that the regulation of the activity of the kinase by the carboxy-terminal tail is likely to play an important physiological role. Therefore, it will be important to define the exact sequences involved and to determine how the intramolecular interactions we described here are regulated. Based on the presence of several potential phosphorylation sites in the Tpl-2 carboxy-terminal tail (Table 1) our working hypothesis is that the interaction may be regulated by phosphorylation.

Whereas the wild-type Tpl-2 protein is not oncogenic independently of its level of expression, the carboxy-terminally truncated protein is oncogenic only when expressed at high levels. Therefore, the oncogenic potential of Tpl-2 depends on the deregulation of its kinase activity combined with high levels of expression. This suggests that the effects of Tpl-2 on the signaling pathways it activates can be repressed by other regulatory mechanisms unless the active Tpl-2 protein is expressed at sufficiently high levels to overcome these regulatory networks. Our data on the effects of Tpl-2 on the activity of the Lck promoter suggest that one of the factors contributing to tumor induction by the carboxy-terminally truncated Tpl-2 kinase could be the induction of Lck.

The tumors induced when the carboxy-terminally truncated Tpl-2 is overexpressed in murine thymocytes are of T-cell origin. With regard to their differentiation status, they are composed of CD4/CD8 double-positive or CD8 or CD4 single-positive cells. The induction of tumors with different differentiation profiles suggests that the expression and activation of Tpl-2 does not inhibit T-cell differentiation. In this respect the tumors induced in Tpl-2 transgenic mice express a phenotype that is similar to the phenotype of MoMuLV-induced rat tumors with rearrangements in the Tpl-2 locus (Lazo et al. 1990; Patriotis et al. 1993).

In summary, the results presented in this report confirmed that Tpl-2 is a highly oncogenic kinase and that its carboxy-terminal tail regulates its kinase activity and, secondarily, its biological function. The dramatic differences of the wild-type and truncated proteins in kinase activity and oncogenic potential indicate that the regulation of the Tpl-2 kinase by its carboxy-terminal tail plays an important physiological role. Our working hypothesis, currently under investigation, is that the tail-directed intramolecular interactions described here may be regulated by phosphorylation.

Materials and methods

Growth factors and monoclonal antibodies

Recombinant human epidermal growth factor (EGF) was purchased from GIBCO-BRL, and human or murine tumor necrosis factor-α (TNFα) was purchased from Genzyme. The monoclonal anti-HA tag antibody 12CA5 was purchased from Boehringer Mannheim. Hybridoma cells producing the anti-c-myc tag monoclonal antibody 9E10.2 (Evan et al. 1985) were purchased.
Activation of the Tpl-2 kinase

Figure 6. FACS analyses of thymus and spleen from control and transgenic animals with lymphoblastic lymphomas. Eleven tumors from offspring of the transgenic line 3456 expressing the truncated Tpl-2 were analyzed by FACS. Single-cell suspensions prepared from spleen and thymus were examined by flow cytometry using CD4 and CD8 antibodies as described in Materials and Methods. (A) Thymus and spleen from a nontransgenic littermate; (B–D) samples from transgenic animals; (B) thymus and spleen from animal 10070; (C) thymus and spleen from animal 10035; (D) thymus from animal 8595 (no spleen sample was available from this animal).

Expression constructs

Wild-type Tpl-2 cDNA and the cDNA of the tumor-specific, carboxy-terminally truncated Tpl-2, containing the 21-bp-long open reading frame from intron vii, were subcloned between EcoRI and BamHI in the polylinker of the pCMV-5 expression vector (Anderson et al. 1989; Patriotis et al. 1994). Using site-directed mutagenesis the lysine 167 in the ATP-binding domain of Tpl-2 was mutated to methionine. Using overlap extension PCR an 11-amino-acid HA-epitope tag (YPYDVPDYASR) or a 10-amino-acid c-myc epitope tag (MT) (EQKLISEEDL) were fused in frame to the carboxyl terminus of the Tpl-2 open reading frame. Baculovirus expression constructs of Tpl-2 were generated by subcloning the same Tpl-2 cDNAs into the pVL1392 baculovirus transfer vector (Pharmingen). To recombine Tpl-2 into baculovirus we cotransfected Sf9 cells with these constructs and wild-type baculovirus DNA using the BaculoGold expression kit (Pharmingen), and following the manufacturer's instructions. Similar procedures were employed to recombine an HA-tagged GST fusion of the Tpl-2 carboxy-terminal tail into

from the American Type Culture Collection (ATCC). 9E10.2 monoclonal antibody was purified from ascites induced in SCID mice inoculated intraperitoneally (IP) with hybridoma cells.
baculovirus. The GST fusion was generated by cloning the Tpl-2 tail 3’ of, and in-frame with the GST gene in the baculovirus expression vector pAcG3X [Pharmingen] using standard PCR procedures.

Cell culture

COS-1 cells, purchased from ATCC, were maintained in Dulbecco's modified Eagle medium [DME, Gibco-BRL] supplemented with 10% fetal calf serum [Gibco-BRL], penicillin [50 U/ml], streptomycin [50 µg/ml], and kanamycin [100 µg/ml]. COS-1 cells were transiently transfected as described previously [Patriotis et al. 1994], using the DEAE-dextran/chloroquine method [McCutchian and Pagano 1968]. Briefly, ~7 x 10^6 cells were seeded in 60-mm petri dishes and, 24 hr later, they were transfected with 6 µg of DNA (2 µg each of the expression constructs supplemented with vector DNA to a final total of 6 µg). Twenty-four hours later they were cultured in serum-free DMEM for an additional 24 hr and then they were harvested. Selected cultures were stimulated for 20 min prior to harvesting with EGF [20 ng/ml] or TNFα [20 ng/ml].

S99 insect cells, purchased from ATCC, were maintained at 27°C in Grace's media [GIBCO-BRL] supplemented with 10% fetal calf serum [GIBCO-BRL], 3.3 mg/ml of yeastolate extract [GIBCO-BRL], 3.3 µg/ml of lactalbumin hydrolysate [GIBCO-BRL], 2 mM L-glutamine [GIBCO-BRL], 25 U/ml of penicillin, 25 µg/ml of streptomycin, and 50 µg/ml of kanamycin. Baculovirus infections were carried out by exposing 2 x 10^6 S99 cells seeded into 60-mm petri dishes to the virus. The multiplicity of infection [m.o.i.] was 2-4. Twenty-four hours later, the cells were serum-starved for an additional 24 hr prior to harvesting.

Metabolic labeling of S99 cells with [35S]methionine

Forty-eight hours after infection with baculoviruses, S99 cells were washed twice with phosphate buffered saline [PBS] and incubated for 3 hr in l-methionine (-) Grace's media [Gibco-BRL]. The cells were then labeled for 6 hr in the same media with 250 µCi/ml of 35S-ProMix [Amersham]. The labeled cells were collected by centrifugation at 600g for 10 min, washed twice with ice-cold PBS and lysed in 500 µl of NP-40 lysis buffer [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% NP-40, 1 mM sodium-orthovanadate, 1 mM sodium pyrophosphate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml of aprotinin [Sigma], and 5 µg/ml of leupeptin [Sigma]] at 4°C for 15 min. The cell lysates were then centrifuged at 12,000g at 4°C for 10 min. The clarified lysates were used for immunoprecipitation as described in the following section.

Immunoprecipitation and in vitro kinase assays

Transfected COS-1 cells or baculovirus-infected S99 cells were washed with ice-cold PBS and lysed for 20 min at 4°C in 0.5 ml of the NP-40 lysis buffer. The cell lysates were clarified by centrifugation at 12,000g at 4°C for 10 min. The clarified lysates
were then mixed for 30 min at 4°C with 20 μl of a 50% suspension of protein A/protein G (1:1)–agarose (GIBCO-BRL). Subsequently, the agarose beads were removed by centrifugation for 10 min at 12,000g at 4°C, and the lysates were incubated for 3 hr at 4°C with anti-Tpl-2 antibody [1/500 dilution], anti-c-myc tag antibody [9E10.2] [1/500 dilution], or anti-HA antibody [12CA5] [1/500 dilution], in the presence of 40 μl 50% protein A/protein G [1:1] mixture. The immunoprecipitate/agarose bead complexes were collected by centrifugation at 6000g for 2 min at 4°C and washed three times with 1 ml of lysis buffer, once with 1 ml of 50 mM Tris-HCl at pH 7.5 at 4°C, and once with 0.5 ml of 20 mM HEPES at pH 7.5, 5 mM MgCl2, and 1 mM dithiothreitol at room temperature.

In vitro kinase assays were carried out according to the following protocol. Pelleted immunoprecipitates were incubated with 10 μCi [γ-32P]ATP [Amersham; 3000 Ci/m mole] for 30 min at room temperature in a volume of 20 μl and buffer conditions optimum for the corresponding protein kinase: Tpl-2 kinase assays were carried out in 20 mM HEPES at pH 7.5, 10 mM MgCl2, 5 mM MnCl2, 1 mM dithiothreitol, 6 μM cold ATP, 1 μg aprotinin, and 1 μg of leupeptin; exogenous substrates added included MBP (0.75 μg/reaction), histone H2A, H2B, and H3 (2 μg/reaction). JNK1 kinase assays were carried out in 12.5 mM HEPES at pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM ethylene glycol-bisβ-aminomethyl ether-N,N,N',N'-tetraacetic acid (EGTA), 0.5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM dithiothreitol, 20 μg cold ATP, 1 μg of aprotinin, and 1 μg of leupeptin; the exogenous substrate was GST/N5-89Jun (2 μg/reaction). The kinase reaction was stopped by adding 7 μl of 4× sample loading buffer (1 M urea, 120 mM Tris-HCl at pH 6.8, 100 mM dithiothreitol, 3% SDS, 10% glycerol, and 0.02% bromphenol blue). ERK1 activity was measured by an in-gel assay as described previously [Patriotis et al. 1994].

**Immunoblotting**

Following size fractionation by SDS-PAGE, proteins were transferred onto Immobilon-P membranes (Millipore) with a Genie electroblotter (Idea Scientific, Corvallis, OR) using a Tris-glycine buffer at pH 8.3 (25 mM Tris, 190 mM glycine, and 20% methyl alcohol). Directly following electroblootting, or after overnight exposure to XAR film [Kodak] [in case the filter was used for the blotting of the products of an in vitro kinase reaction], the blotted membranes were briefly rewetted and rinsed once in methyl alcohol and Tris-buffered saline (TBS), containing 100 mM Tris-HCl at pH 7.5, 0.9% NaCl, and 0.05% MgCl2. The rewetted filters were blocked by overnight incubation at 4°C with 3% lyophilized, nonfat milk in TBS (milk/TBS). The blocked membranes were then incubated with one of the following antibodies [anti-Tpl-2, PT492 [1/1000 dilution], anti-c-myc tag [9E10.2] [1/1500 dilution], anti-HA tag [12CA5] [1/1000 dilution], and anti-Lck (SC13) [1/1000 dilution]] in milk/TBS at 4°C for 2–4 hr. The excess antibody was removed by two 10-min washes in TBS, a 10-min wash in TBS containing 3% Tween 20, and three final 10-min washes in TBS. Antigen-bound antibody was detected by a 60-min room temperature incubation in a 1/5000 to 1/10000 dilution of the corresponding secondary antibody conjugated with horseradish peroxidase [Amersham]. The unbound secondary antibody was removed with the same washing procedure we used to remove the excess primary antibody. The binding of the secondary antibody was detected by enhanced chemiluminescence (ECL, Amersham). Immunoblots were exposed to Reflection NEF film (NEN, DuPont) for 1–10 min.

**Transgenic constructs, microinjection, and Southern blotting**

Tpl-2 cDNAs containing exon 1B were placed under the control of the proximal Lck promoter that is specifically active in thymocytes [Abraham et al. 1991a, b; Wildin et al. 1991]. An SV40 large T antigen polyadenylation signal was inserted 3′ of the Tpl-2 cDNA to ensure proper termination of transcription. To generate these constructs we used the vector pLck-SV40, which was constructed as follows: A 3.1-kb BamHI–NotI fragment from pLck–hGH containing the proximal Lck promoter [Abraham et al. 1991a] was subcloned into the BamHI–NotI sites of

**Figure 8.** Tpl-2 activates the proximal Lck promoter. (A, top) Western blot of total cell lysates derived from independent 2769 cell mass cultures infected with the murine sarcoma virus (MSV)–SRa retrovirus (Vector, lanes 1, 2) or MSV–SRa-based constructs expressing wild-type [Tpl-2, lanes 3, 4] or carboxy-terminally truncated Tpl-2 [Tpl-2tr, lanes 5, 6]. (Bottom) Quantitation of the data in the top panel as determined by densitometry [Ambis]. (B) CAT activity in lysates of EL4 cells cotransfected with the Lck promoter/CAT reporter construct and pCMV-5 [Vector] or pCMV-5-based expression constructs of wild-type [Tpl-2] or truncated Tpl-2 [Tpl-2tr]. PMA plus ionomycin was used as a control. Activity was measured by Fuji Phosphorlmager as described in Materials and Methods. The difference between CAT expression in these cultures was statistically significant [P < 0.0002 by t-test analysis].
pBluescript KS+ [Stratagene]. A 1.5-kb ClaI-XhoI fragment derived from pSV1500 and containing the SV40 large T antigen polyadenylation signal was then cloned into this plasmid giving rise to the pLck-SV40 vector.

The tumor-specific, truncatedTpl-2 cDNA clone Tpl-2.19a, containing intron vii and viral LTR sequences at its 3' end [Makris et al. 1993; Patriotis et al. 1994], was cloned into the EcoRI site of the pLck-SV40 vector. The wild-type Tpl-2 cDNA expression construct was generated by combining the 5’ end of Tpl-2.19a, which encodes the truncated Tpl-2 expressed from promoter P2, and the 3’ end of Tpl-2.3 [Patriotis et al. 1993], which encodes the wild-type Tpl-2 expressed from promoter P1; Tpl-2.3 was digested with HindIII-BglII fragment corresponding to the 5’ end of the transcript. This region was then replaced with a 1.2-kb HindIII-BglII fragment from the 5’ end of Tpl-2.19a and called Tpl-2B. Following digestion of the Tpl-2B insert with NotI, blunt ends were created with T4 DNA polymerase, and EcoRI linkers were added. The 2.5-kb EcoRI insert of this plasmid was then cloned into the EcoRI site of pLck-SV40. The wild-type and truncated Tpl-2 cDNAs expressed in transgenic mice are illustrated in Figure 7C.

To ensure that all fragments were cloned in the proper orientation, all junctions were sequenced using a Sequenase Kit (Amersham). Inserts from both constructs were isolated by digestion with XhoI-NotI, purified and microinjected into C57BL/6J × C3H/HeJ F2 mouse oocytes as described previously [Osborn et al. 1987; Ceci et al. 1990, 1991]. Genomic DNA was isolated from the tails of live-born mice at the time of weaning [Sircusca et al. 1987]. To identify transgenic founder mice, DNAs were digested to completion with PstI and analyzed by Southern blotting [Jenkins et al. 1982; Ceci et al. 1989, 1991] using an SV40 polyadenylation signal probe derived from pSV1500. Four transgenic lines were maintained by continuously backcrossing the offspring of the founders to C57BL/6J mice.

Histopathology

A complete necropsy was performed. All tissues were dissected, fixed in 10% formalin, and embedded in paraffin. Five micron sections were stained with hematoxylin and eosin and examined by light microscopy.

T-cell receptor and immunoglobulin heavy-chain and κ light-chain rearrangements

Genomic DNA was isolated from tumors as described previously [Sircusca et al. 1987]. Southern blot analysis was performed using λλ1 and λλ2 probes that detect TCR rearrangements, and immunoglobulin heavy chain and κ light chain probes as described previously [Mucenski et al. 1986].

FACS analysis

Single-cell suspensions of splenocytes and thymocytes from tumor-free control and tumor-bearing transgenic mice, in red cell lysis buffer [BRL/GIBCO], were pelleted, resuspended, and washed in RPMI media containing 10% fetal bovine serum and penicillin/streptomycin [BRL/GIBCO]. Then, cells were frozen in 50% RPMI, 40% fetal bovine serum, and 10% DMSO. Prior to FACS analysis, cells were quickly thawed at 37°C and spun in 10 ml of thawing buffer [RPMI containing 20% fetal bovine serum, 5 mM HEPES, and penicillin/streptomycin]. Twenty minutes later, 1 x 10^6 cells were incubated with FITC or PE conjugated antibodies to mouse B220 [FITC], Thy1.2 [FITC], CD4 [FITC], CD8 [PE], Mac1 [FITC], and Gr1 [PE]. All antibodies were purchased from Pharmingen. Following staining, cells were analyzed using a Coulter EPICS 753 FACS machine.

Ribonuclease protection assays

A 285-bp Accl-PstI fragment from Tpl-2 corresponding to nucleotides 886-1171 of the Tpl-2.19a cDNA was subcloned into pBluescript KS+ and used as a probe for RNase protection. A probe containing a fragment from the mouse β-actin gene (either pSP6-β-actin or pTPI-β-actin from Ambion) was used as an internal control for the amount of RNA loaded in each lane. The Tpl-2 and pSP6-β-actin clones were linearized by digestion with BspHI and Ddel, respectively, and purified by one phenol extraction, one phenol/chloroform extraction, and one chloroform extraction, followed by ethanol precipitation. pTPI-β-actin linearized plasmid was provided by the manufacturer. 32P-Labeled Tpl-2 (383 bases), pSP6-β-actin (215 bases), and pTPI-β-actin (304 bases) probes were synthesized using T7 or SP6 RNA polymerase [Boehringer Mannheim] and [32P]CTP. RNase protection assays were performed using gel-purified probes and an Ambion RNase protection kit according to the instructions provided by the manufacturer. Following 12-16 hr of hybridization, samples were digested with a 1:100 dilution of RNaseA + T1 for 30 min at 37°C. Samples were analyzed by electrophoresis in a 5% polyacrylamide sequencing gel. The Tpl-2 probe protected a 285-base-long RNA fragment. The pSP6-β-actin and pTPI-β-actin probes protected 130- and 250-base-long RNA fragments, respectively.

Transient transfections and chloramphenicol acetyltransferase (CAT) assays

EL4 cells [10^7] were electroporated with 4 μg of a Lck-promoter/CAT reporter construct DNA containing 600 bp upstream of the Lck initiation site [Muise and Rosen 1995] and 2.5 μg of a pCMV-5 Tpl-2 [wild-type or truncated] construct, or pCMV-5. pBluescript was used to supplement the amount of electroporated DNA to a total of 20 μg per transfection. Electroporations were carried out at 260 mV/960 μF in quadruplicate. Electroporated cells were harvested at 44 hr and CAT assays were carried out as described previously [Durand et al. 1986]. Prior to harvesting, selected cell cultures were treated with 10 nm phorbol 12-myristate 13-acetate (PMA) and 2 nm ionomycin for 8 hr. The acetyltransferase reaction was carried out for 2.5 hr. The acetylated and nonacylated forms of [14C]chloramphenicol (Amersham) were separated on TLC plates (Sigma). Chloramphenicol acetylation was quantitated using a Fuji PhosphorImager and Fuji MacBas V2.2 software. Data are presented as the mean ± standard deviation for each set of transfections. The statistical significance of the difference between CAT expression in the cultures was determined by t-test analysis using Microstat Excel.

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