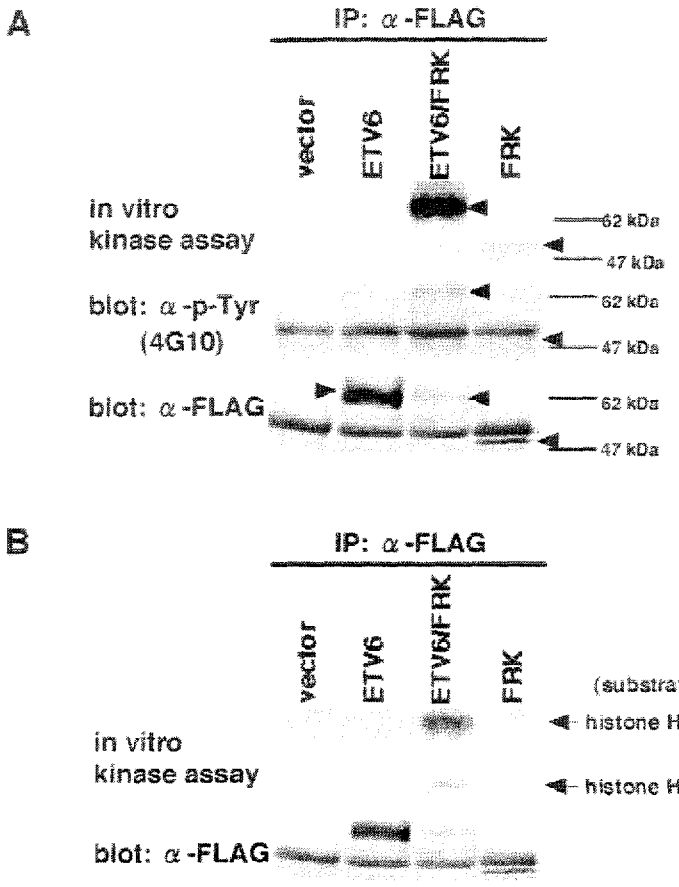

Figure 3. The ETV6/FRK tyrosine kinase is constitutively activated in HeLa cells. (A) Lysates of HeLa cells transfected with the indicated expression vectors were immunoprecipitated with an anti-FLAG-M2 monoclonal antibody and then analyzed by immune complex kinase assay (top) or immunoblotting with an antiphosphotyrosine antibody 4G10 (middle). The total amount of each protein was also assessed by immunoblotting with anti-FLAG-M2 antibody (bottom). Arrowheads show the proteins expressed or phosphorylated at an expected size. (B) Results of kinase assay performed with histones H2B (top) and H4 (middle).

Constitutive Activation of the ETV6/FRK Tyrosine Kinase

Because the ETV6/FRK fusion protein retained the kinase domain but lacked the SH3 domain and most of the SH2 domain, we examined its kinase activity. First, we compared the autophosphorylation status of ETV6/FRK and wild-type FRK. Either the ETV6/FRK fusion protein, wild-type FRK, or wild-type ETV6 FLAG-tagged at the N-terminus was introduced into HeLa cells, immunoprecipitated with an anti-FLAG-M2 monoclonal antibody, and then analyzed by the kinase assay or immunoblotting with an antiphosphotyrosine antibody 4G10 (Fig. 3A, top and middle). To compare expression levels, the same amounts of immunoprecipitate were also subjected to anti-FLAG blot (Fig. 3A, bottom). A high level of tyrosine phosphorylation occurred only in the ETV6/FRK protein (Fig. 3A, top and middle). A basal level of autophosphorylation also was detectable in the wild-type FRK (Fig. 3A, top), a finding in agreement with the previous data (Cance et al., 1994). However, the level of autophosphorylation was significantly lower than that of ETV6/FRK (Fig. 3A, top and middle). Next, we compared the ability of ETV6/FRK and wild-type FRK to phosphorylate exogenous substrates. When histone H2B or H4 was added to the kinase reaction, they were found to be phosphorylated to a greater extent in ETV6/FRK-expressing cells than in FRK-expressing cells (Fig. 3B), suggesting that the ETV6/FRK protein had elevated tyrosine kinase activity.

Cell Transformation by ETV6/FRK in a Kinase-Dependent Manner

To assay the transforming activities of ETV6/FRK, we stably expressed the cDNA-encoding ETV6/FRK or other proteins into the fibroblast cell line NIH3T3. We established 3 NIH3T3 clones expressing ETV6/FRK, 2 clones expressing FRK/ETV6, 2 clones expressing FRK, 2 clones expressing ETV6, and 2 clones expressing ETV6/FRK(K262R) (Fig. 4A), the kinase-inactive

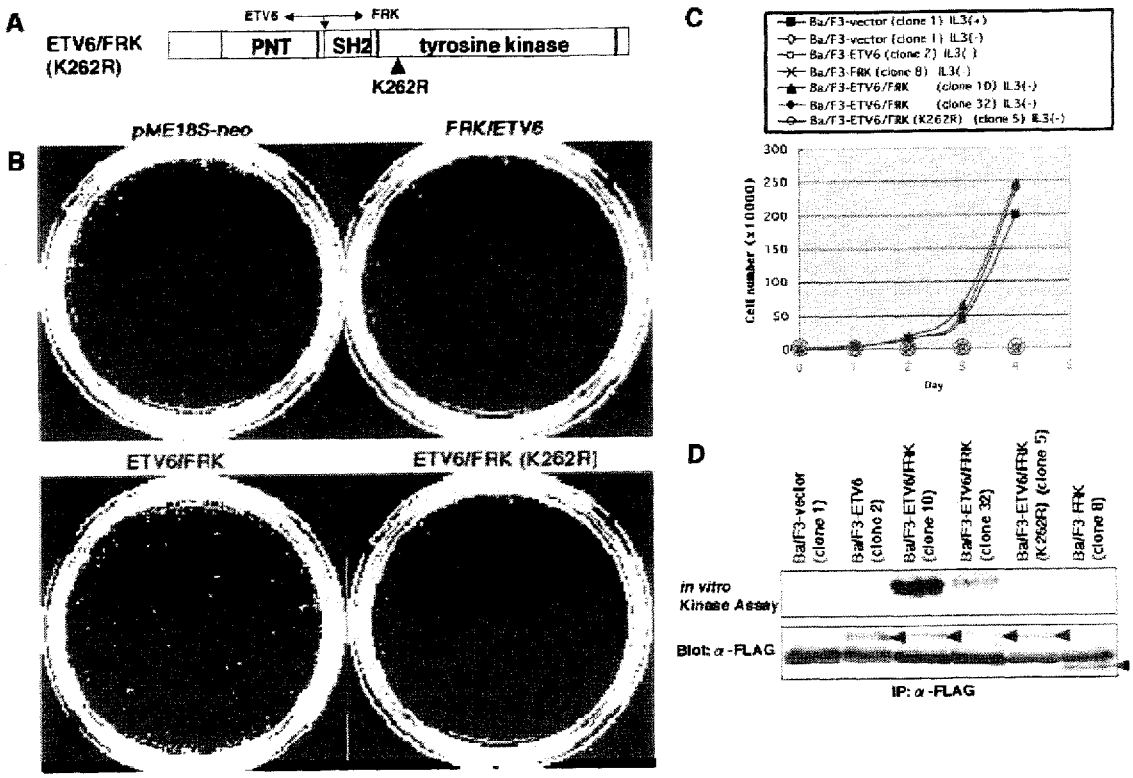

Figure 4. ETV6/FRK transforms NIH3T3 cells and Ba/F3 cells in a kinase-dependent manner. (A) Schematic representation of the kinase-inactive ETV6/FRK(K562R) mutant with a lysine-to-arginine mutation at the ATP binding site. (B) Soft-agar assay demonstrating macroscopic colony formation in ETV6/FRK-expressing NIH3T3 cells. (C) 2×10^4 Ba/F3 cells stably transfected with the indicated expression vectors were washed free of IL-3 and plated on day 0 in growth medium without IL-3. Viable cells were counted each day. Data of the representative clone(s) for each protein are presented. (D) Cell lysates of the indicated Ba/F3 clones were immunoprecipitated with an anti-FLAG-M2 antibody and then subjected to kinase assay (top) and immunoblotting with anti-FLAG-M2 antibody (bottom). Arrowheads show the proteins expressed at an expected size.

mutant of ETV6/FRK, confirmed by immunoblotting analysis (data not shown). The soft-agar assay was performed on each clone. Comparable results were obtained for the clones expressing the same proteins, and the representative data are presented. Only the NIH3T3 cells expressing intact ETV6/FRK were able to produce macroscopic colonies, whereas the NIH3T3 cells transfected with the empty vector or cells expressing the kinase-inactive mutant ETV6/FRK(K262R), the reciprocal FRK/ETV6 fusion protein, wild-type FRK, or wild-type ETV6 failed to grow colonies (Fig. 4B, Table 2). These results suggest that ETV6/FRK but not FRK/ETV6 contributes to neoplastic transformation in a kinase-dependent manner.

Next, we also examined the ability of ETV6/FRK to transform the murine hematopoietic cell line Ba/F3, which is strictly dependent on IL-3 for survival and proliferation. Following stable transduction by electroporation, we obtained 6 Ba/F3 clones expressing ETV6/FRK, 2 clones expressing FRK, 2 clones expressing ETV6, and 3 clones expressing ETV6/FRK(K262R), confirmed by immunoblotting analysis (data not shown). To assay the ability to confer independent proliferation of IL-3, each Ba/F3 clone was switched to growth medium without IL-3. Comparable results were obtained for the clones expressing the same proteins, and the representative data are presented. The Ba/F3 clones expressing ETV6/FRK showed sustained proliferation in the absence of IL-3 (Fig. 4C). In contrast, Ba/F3 cells transfected with the empty vector or cells expressing kinase-inactive mutant ETV6/FRK(K262R), wild-type FRK, and wild-type ETV6 were all unable to proliferate in the absence of IL-3 (Fig. 4C). Although the ETV6/FRK proteins expressed in the stable clones were constitutively autophosphorylated, the ETV6/FRK(K262R) mutants were not (Fig. 4D). These observations indicate that ETV6/FRK is a dominant oncoprotein and that constitutive activa-

TABLE 2. Transformation of NIH3T3 Cells By ETV6/FRK

Transfected DNA	No. of colonies[a]
pME18S-neo (vector)	0
pME18S-neo-ETV6	0
pME18S-neo-FRK	0
pME18S-neo-ETV6/FRK	15
pME18S-neo-ETV6/FRK(K262R)	0
pME18S-neo-FRK/ETV6	0

NIH3T3 cells were transfected with the indicated constructs, and stable transfectants were selected in G418. Cells were plated in soft agar. Macroscopic colonies were counted at day 21.
[a]Average of four experiments.

tion of the ETV6/FRK tyrosine kinase is necessary for ETV6/FRK-induced transformation.

Inhibition of ETV6-Mediated Transcription Repression by ETV6/FRK

Because ETV6 is an ETS transcription factor that acts as a transcriptional repressor (Lopez et al., 1999), we also investigated the transcriptional regulatory property of ETV6/FRK and its ability to modulate the function of wild-type ETV6. We transfected a previously described (EBS)3tkLuc reporter, in which the luciferase gene is placed under the control of an ETS responsive promoter (Waga et al., 2003), along with either wild-type ETV6, ETV6/FRK, or FRK/ETV6 into HeLa cells and evaluated luciferase activity. The results showed, in agreement with the previous finding (Waga et al., 2003), that there was decreased luciferase activity after cotransfection of (EBS)3tkLuc with the wild-type ETV6 expression plasmid (Fig. 5A). In contrast, no repression was observed when ETV6/FRK or FRK/ETV6 was expressed with the (EBS)3tkLuc reporter (Fig. 5A).

Because the oncoprotein ETV6/FRK lacks the ETS DNA binding site but still retains the PNT oligomerization domain, it is possible that it might affect ETV6-mediated transcriptional repression by heterodimerizing with ETV6. Notably, coexpression of ETV6/FRK abolished the transcriptional repression by ETV6 in a dose-dependent manner (Fig. 5B), suggesting that ETV6/FRK has a dominant-negative effect on ETV6-mediated transcriptional repression. In contrast, coexpression of the reciprocal FRK/ETV6 protein did not affect ETV6-mediated transcriptional repression (Fig. 5B). In control experiments, dose-dependent expression of the ETV6, ETV6/FRK, or FRK/ETV6 protein was confirmed by immunoblotting analysis (data not shown).

DISCUSSION

The t(6;12)(q21;p13) is a rare but recurrent reciprocal chromosome translocation in human leukemia (Hayashi et al., 1990; Katz et al., 1991; Raimondi et al., 1997). In this article, we report our finding that it generated novel fusion genes *ETV6/FRK* and *FRK/ETV6* in a case of AML. FRK belongs to a family of SRC kinases, as at the amino acid level, it has the highest homology, 50%, with FYN (Cance et al., 1994; Lee et al., 1994). Although several tyrosine kinase (TK) genes have been identified as fusion partners of *ETV6* (Golub et al., 1994; Papadopoulous et al., 1995; Lacronique et al., 1997; Peeters et al., 1997; Cazzaniga et al., 1999; Eguchi et al., 1999; Iijima et al., 2000; Kuno et al., 2001), this is the first report of a SRC-family tyrosine kinase gene being fused with *ETV6* and structurally altered in human cancers. In the resultant ETV6/FRK fusion protein, the entire PNT oligomerization domain of ETV6 and the kinase domain of FRK are fused in frame. We demonstrated that this ETV6/FRK fusion protein constitutively underwent autophosphorylation on its tyrosine residues. ETV6/FRK had elevated kinase activity compared to that in wild-type FRK. ETV6/FRK showed transforming activities in two cell lines, Ba/F3 and NIH3T3, indicating that ETV6/FRK is a dominant transforming oncoprotein. The kinase-inactive mutant ETV6/FRK(K262R) transformed neither of these two cell lines, indicating that the kinase activity of ETV6/FRK was essential for transformation. The reciprocal fusion protein FRK/ETV6, whose mRNA also was transcribed in the patient sample, did not have transforming activity. These data strongly suggest that the elevated kinase activity of the ETV6/FRK fusion protein directly contributes to the pathogenesis of leukemia with a t(6;12)(q21;p13).

Although activated variants of the SRC family kinases show transforming activities (Parker et al., 1984; Cartwright et al., 1987), the *SRC* and its family of genes rarely have been reported as being mutated or structurally altered in primary human tumors. Irby et al. (1999) reported that 12% of advanced human colon cancers in the United States had a truncating mutation at codon 531 of the *SRC* gene and that the mutation elevated kinase activity and promoted the potential for malignancy. However, three subsequent large-scale studies on advanced colorectal cancers in Japanese, northern European, Chinese, and Italian patients failed to detect the mutation (Daigo et al., 1999; Wang et al., 2000; Laghi et al., 2001), making the

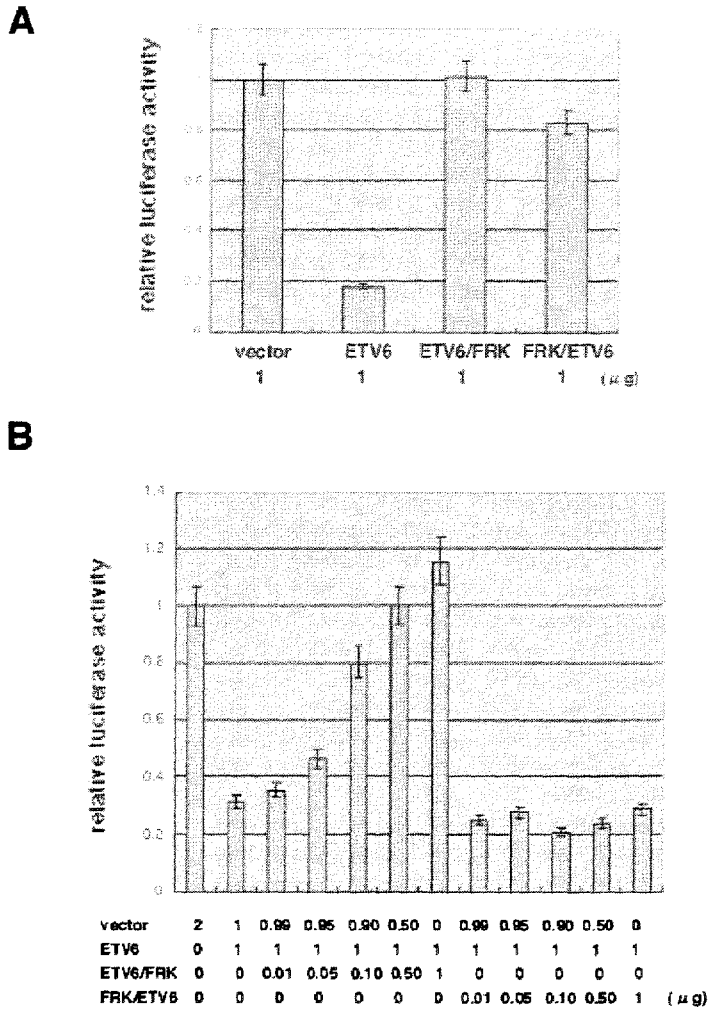

Figure 5. ETV6/FRK is a dominant-negative regulator of ETV6-mediated transcriptional repression in HeLa cells. (A) HeLa cells were transfected with 1 μg of (EBS)3tkLuc reporter plasmid along with 1 μg of the indicated expression vector. Bars show relative luciferase activities to the level when a control plasmid pME18S-neo was cotransfected with the corresponding reporter plasmid, and they present average results of duplicate experiments. (B) HeLa cells were transfected with 1 μg of (EBS)3tkLuc reporter plasmid along with 1 μg of pME-18S-neo-FLAG-ETV6 expression vector together with indicated amounts of pME18S-neo-FLAG-ETV6/FRK or pME18S-neo-FLAG-FRK/ETV6 expression vector. The results are presented as relative luciferase activities.

importance of this mutation controversial. In hematopoietic malignancies, two human T-cell acute lymphoblastic leukemia cell lines have been shown to have rearrangement of *LCK*, a SRC-family kinase gene (Tycko et al., 1991; Wright et al., 1994). In these two cell lines, HSB-2 and SUP-T12, the upstream promoter of the *LCK* gene was juxtaposed to the *TCRB* locus without any accompanying large structural abnormality of the LCK protein. *LCK* mRNA was elevated in the two cell lines (Tycko et al., 1991), and the HSB-2 cell line was later shown to carry several activating point mutations in the *LCK* gene (Wright et al., 1994), indicating that overexpression and/or activation of the LCK kinase would lead to cell transformation. On the other hand, the involvement of SRC family members in primary leukemia has not been reported previously. In this study, we showed that the structural abnormality of an SRC-like kinase gene, *FRK*, through translocation with *ETV6* can directly contribute to leukemogenesis through activation of the altered tyrosine kinase. In addition to the analysis of the current case with a t(6;12), we also performed a mutation analysis of the *FRK* gene in 20 hematopoietic cell lines but failed to detect activating mutations or structural abnormalities (data not shown). Thus, it is currently unclear whether FRK could be activated through other mechanisms such as activating mutations or translocations with other partner gene(s), although more intensive analyses may be required.

Two mechanisms could contribute to the constitutive activation of the ETV6/FRK kinase. First, in the ETV6/FRK fusion protein, the SH3 and SH2 domains of FRK are lost or disrupted, respec-

tively. Both the SH2 and SH3 domains are required to maintain the SRC family kinases in an inactive state: the SH2 domain binds to the C-terminal tyrosine residue in a phosphorylation-dependent manner, and the SH3 domain interacts with a short polyproline type II helix located between the SH2 domain and the kinase domain (Schindler et al., 1999; Xu et al., 1999; Young et al., 2001). These intramolecular interactions are believed to lock the molecule in a closed, inactive state, resulting in repression of kinase activity. In this regard, disruption of this closed conformation would activate the SRC family kinases and lead to cell transformation. In fact, some deletions or mutations in either the SH2 or the SH3 domain of SRC have been shown to activate its catalytic and/or transforming activities (Hirai and Varmus, 1990). Thus, the disruption of the SH3 and SH2 domains in ETV6/FRK may contribute to deregulation of kinase activity. Secondly, in the ETV6/FRK fusion protein, the entire PNT domain of ETV6 is fused to the kinase domain of FRK. As is the case with other ETV6/TK fusion proteins (Carroll et al., 1996; Golub et al., 1996; Jousset et al., 1997), the PNT domain would force dimerization of the ETV6/FRK protein and lead to constitutive tyrosine autophosphorylation and activation of the ETV6/FRK kinase.

The downstream signaling pathway mediated by ETV6/FRK still remains to be elucidated. The wild type FRK is expressed primarily in epithelial tissues (Cance et al., 1994), but also weakly in various hematopoietic cell line (data not shown). However, its functions or downstream signaling pathways remain largely unknown, especially in hematopoietic systems. The only known candidate endogenous downstream component of FRK is the SH2-domain adaptor protein SHB. According to recent reports, GTK, a rodent homologue of FRK, induces neurite outgrowth in PC12 cells and insulin stimulated signaling pathways in pancreatic insulin-producing cells via SHB (Anneren et al., 2000; Anneren and Welsh, 2002). In the present study, however, immunoblotting analysis failed to detect expression of the SHB protein in ETV6/FRK-expressing cells (data not shown). Thus, involvement of SHB in transformation by ETV6/FRK remains unclear. We also tested the phosphorylation status of several signaling molecules, including signal transducer and activator of transcription (STAT1, STAT3, STAT5, STAT6, extracellular signal-regulated kinase 1/2 (ERK1/2), P38 mitogen-activated protein kinase (P38 MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C (PLC)-gamma, in ETV6/FRK-expressing cells. However, we failed to detect any aberrant phosphorylation of these molecules in ETV6/FRK-expressing cells in comparison to FRK-expressing cells (data not shown). Future identification of the target substrate of ETV6/FRK might provide a novel insight into the mechanism of ETV6/FRK-induced transformation as well as of wild-type FRK-mediated signal transduction.

Finally, we demonstrated that ETV6/FRK had a dominant-negative effect over ETV6-mediated transcriptional repression. Because ETV6/FRK retains the PNT oligomerization domain of ETV6, ETV6/FRK may interfere with the transcriptional repression activity of ETV6 by heterodimerizing with wild-type ETV6. Our results indicate that ETV6/FRK is a novel oncoprotein with dual functions: deregulated tyrosine kinase activity and a dominant-negative modulation of transcriptional repression by ETV6. Because wild-type ETV6 appears to have tumor-suppressive activity (Romparey et al., 2000), its suppression by ETV6/FRK also could contribute to oncogenesis. It may be possible that ETV6/FRK can contribute to oncogenesis through two independent mechanisms: activation of the ETV6/FRK tyrosine kinase, which would lead to aberrant stimulation of the downstream signaling pathway, and inhibition of the tumor-suppressive functions of ETV6. This model suggests potential strategies for reversion of transformation by ETV6/FRK. Because the kinase-inactive mutant of ETV6/FRK is nontransforming, a specific inhibitor of the SRC family kinases may inhibit transformation by ETV6/FRK. Alternatively, overexpression of wild-type ETV6 also would interfere with the ability of ETV6/FRK to transform cells. Further experiments will explore these possibilities.

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TEL/ETV6 accelerates erythroid differentiation and inhibits megakaryocytic maturation in a human leukemia cell line UT-7/GM

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TEL/ETV6 accelerates erythroid differentiation in the murine erythroleukemia cell line. To clarify the effects of TEL on megakaryocytic maturation as well as erythroid differentiation, we chose the human leukemia cell line UT-7/GM that differentiates into the erythroid and megakaryocytic lineages by treatment with erythropoietin and thrombopoietin, respectively. Upon erythropoietin exposure, overexpressed TEL stimulated hemoglobin synthesis and accumulation of the erythroid differentiation-specific transcripts such as γ-globin, δ-aminolevulinic acid synthase-erythroid, and erythropoietin receptor. Moreover, the glycophorin A(+)/glycoprotein IIb(–) fraction appeared more rapidly in the TEL-overexpressing cells. Interestingly, overexpression of TEL was associated with lower levels of the megakaryocytic maturation-specific glycoprotein IIb and platelet factor 4 transcripts under the treatment with thrombopoietin. Consistently, the glycophorin A(–)/glycoprotein IIb(+) fraction increased more slowly in the TEL-overexpressing cells. Finally, expression of endogenous TEL proteins in UT-7/GM cells was down-regulated following erythropoietin and thrombopoietin exposure. All these data suggest that TEL may decide the fate of human erythrocyte/megakaryocyte common progenitors to differentiate towards the erythroid lineage and against the megakaryocytic lineage. (*Cancer Sci* 2005; 96: 340–348)

TEL (also known as ETV6) is a member of the E26 transformation-specific (ETS) family of transcription factors.[1] The highly conserved ETS domain is located at the C-terminal region, while a distinct domain with weak homology to the well-described helix-loop-helix (HLH) domain (also referred to as the pointed domain) is located at the N-terminal region. The former serves for DNA binding to the ETS-binding consensus site (EBS) (GGAA/T) and the latter for homodimerization and heterodimerization with other ETS family members.[2,3] Through interacting with relevant corepressors mSin3A, N-CoR and SMRT, and histone deacetylase-3,[4] TEL mediates transcriptional repression on its target genes such as *FLI-1*,[2] inhibitor of differentiation/DNA binding-1 (*Id-1*),[5] stromelysin-1[6] and *Bcl-X$_L$*.[7] Transcriptional activities of TEL are regulated through phosphorylation with mitogen-activated protein kinases[8,9] and small ubiquitin-like modifier conjugation.[10,11]

The *TEL* gene that is mapped to 12p13 is most frequently rearranged and fused to various partner genes by chromosomal translocations in human leukemias and myelodysplastic syndromes. The partners include receptor type or non-receptor type tyrosine kinases and transcription factors. Providing tyrosine kinases, such as platelet-derived growth factor receptor β (PDGFRβ) in t(5;12) (q33;p13),[12] ABL1 in t(9;12) (q34;p13),[13] ARG (ABL2) in t(1;12) (q25;p13),[14] JAK2 in t(9;12) (p24;p13)[15] and Syk in t(9;12) (q22;p13),[16] with the HLH domain, TEL homodimerizes them and thereby stimulates their kinase activities. In contrast, TEL gives corepressor-binding domains to a transcription factor AML1 in t(12;21) (p13;q22) and interferes with its transcriptional abilities.[17] Therefore, dysregulation of the partner proteins by TEL functional domains seems to cause leukemia in patients with 12p13 translocations. Moreover, inactivation of the *TEL* gene is speculated to be the second hit in t(12;21) (p13;q22) type leukemia, because the wild-type-*TEL* allele is deleted in the vast majority of the patients.[18,19] Thus, TEL appears to be a tumor suppressor. Consistent with its roles as a putative tumor suppressor, expression of TEL in Ras-transformed NIH3T3 cells inhibits cell growth in liquid and soft agar cultures,[6] and in serum-starved NIH3T3 cells induces apoptosis.[6]

TEL is required for mouse development as its inactivation by homologous recombination results in embryonic lethality at E10.5–11.5.[18] The knockout embryos show defects in yolk sac angiogenesis and intraembryonic apoptosis of mesenchymal and neural cells, while they present normal yolk sac hematopoiesis. Analyzing chimeric mice with *TEL*(–/–) ES cells, an essential role of TEL in establishing hematopoiesis of all lineages in neonatal bone marrow has been uncovered, although *TEL*(–/–) ES cells contributed to both primary and definitive fetal hematopoiesis.[19] As for lineage-specific roles in hematopoietic systems, we have reported that TEL accelerates erythroid differentiation of mouse erythroleukemia (MEL) cells induced by hexamethylene bisacetamide (HMBA) or dimethylsulfoxide (DMSO).[20] Because both erythroblasts and megakaryocytes arise from common progenitors, this observation prompted us to search for TEL's roles in lineage commitment of the bi-potential progenitors.

A human tri-factor dependent hematopoietic cell line UT-7/GM[21] is a subline of UT-7 that was originally established from a patient of acute megakaryoblastic leukemia.[22] UT-7/GM cells show absolute dependence for growth and survival on granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO) or thrombopoietin (TPO). They differentiate into the erythroid or megakaryocytic lineage in the presence of EPO or TPO, while they keep immature phenotypes and proliferate in the presence of GM-CSF.[21] Thus, UT-7/GM cells are considered to mimic erythrocyte/megakaryocyte common progenitors and differentiate along two distinct lineages in relatively physiological conditions. We employed this cell line and examined influences of TEL overexpression on erythroid differentiation and megakaryocytic maturation. As judged from higher percentages of benzidine positivity in TEL-overexpressing cells under treatment with EPO, TEL accelerated erythroid differentiation in UT-7/GM cells similar as in MEL cells. The TEL-overexpressing cells showed increased expression of the transcripts for γ-globin, δ-aminolevulinic acid synthase-erythroid (*ALAS-E*) and EPO receptor (*EPO-R*) during the erythroid differentiation. Moreover, accumulation of the glycophorin A(+)/glycoprotein (GP) IIb(–) fraction was more prompt in these cells. Interestingly, expression

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levels of the transcripts for *GPIIb* and platelet factor 4 (*PF 4*) under the treatment with TPO were lower in the TEL-overexpressing cells. Consistent with this, accumulation of the glycophorin A(–)/GPIIb(+) fraction was delayed and appearance of platelet peroxidase (PPO)-positive cells was reduced in these cells. Endogenous TEL proteins disappeared after 14 and 21 days upon EPO and TPO exposure, respectively. We conclude that TEL stimulates erythroid differentiation while opposing megakaryocytic maturation in human hematopoietic system.

Materials and Methods

Cell culture. Parental UT-7/GM cells, the mock (M-1 and M-4) and the TEL-overexpressing (T-5 and T-6) clones were maintained in Isocove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 1 ng/mL of recombinant human (rh) GM-CSF. To physiologically induce erythroid or megakaryocytic differentiation, these cells were cultured in IMDM supplemented with 10% FCS, and 10 U/mL of rhEPO or 100 ng/mL of rhTPO. Light microscopic examination was performed on Wright-Giemsa-stained cytospin preparations. Erythroid differentiation was evaluated by counting percentages of benzidine-positive cells.

Isolation of stable transfectants. The expression of plasmid pCXN2-FLAG-TEL was described in a previous study.[20] To establish stable transfectants, 1×10^7 of UT-7/GM cells were electroporated with 20 μg of pCXN2-FLAG-TEL at 380 V and 975 μF using Gene Pulser (Bio-Rad, Hercules, CA). Transfected cells were selected with 0.8 mg/mL of G418 (Sigma-Aldrich, St. Louis, MO) and cloned by limiting dilution. Expression of FLAG tagged-TEL proteins was confirmed by the western analysis method using anti-FLAG antibody (Sigma-Aldrich).

Immunoprecipitation and western analysis. UT-7/GM cells were lyzed on ice in lysis buffer composed of 20 mM Tris pH 8.0, 50 mM sodium fluoride (NaF), 2 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EDTA), 1% NP-40, 500 U/mL aprotinin, 1 mM sodium orthovanadate (Na_3VO_4), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Immunoprecipitation and western analysis were performed as described in a previous study,[20] using anti-TEL (N-19 for immunoprecipitation and H-214 for western analysis; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-FLAG monoclonal (Sigma-Aldrich) antibodies. The blots were visualized by ProtoBlot AP system (Promega, Madison, WI).

Northern analysis. Total RNA was extracted from the mock and the TEL-overexpressing cells using ISOGEN (Nippon Gene, Tokyo, Japan) under the manufacturer's instruction. Twenty μg of each RNA sample was resolved by electrophoresis on agarose formaldehyde gels, transferred to Hybond-N+ nylon membranes (Amersham, Piscataway, NJ) in 20 × standard sodium citrate (SSC) and hybridized to human cDNA fragments for *ALAS-E*, *EPO-R*, γ-globin, *GPIIb* and *PF 4* that were labeled with [α-^{32}P] dCTP using the Megaprime DNA labeling system (Amersham). Human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA probe was used as a control. After overnight incubation at 42°C in the presence of 50% formamide, membranes were washed with 0.1 × SSC containing 0.1% sodium dodecyl sulfate (SDS) at 42°C and autoradiographed using Fujix BAS2500 Bio-image Analyzer (Fuji Photo Film, Tokyo, Japan). Relative expression levels to the level at day 0 in each clone were quantified.

Fluorescence activated cell sorter (FACS) analysis. The mock and the TEL-overexpressing cells were incubated for 30 min at 4°C with appropriately diluted fluorescein-labeled antiglycophorin A and anti-GPIIb (CD41b) antibodies (Beckman Coulter, Fullerton, CA). After washing, cells were analyzed using Becton Dickinson FACS Calibur.

Electron microscopic analysis. Ultrastructural PPO activity was detected by a conventional method.[23]

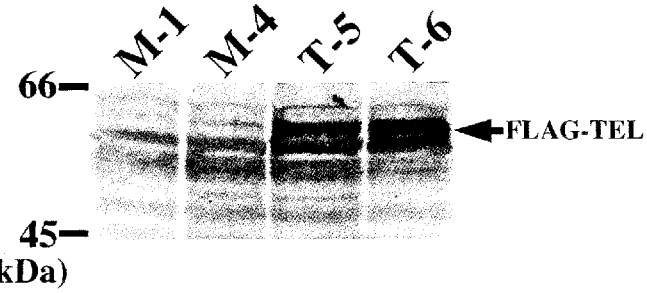

Fig. 1. Establishment of UT-7/GM sublines overexpressing FLAG-tagged TEL proteins. Clones T-5 and T-6 were obtained from UT-7/GM cells that were transfected with pCXN2-FLAG-TEL and selected by G418 resistance. Clones M-1 and M-4 were established from UT-7/GM cells that were transfected with the empty pCXN2 vector and selected by G418 resistance. Expression of FLAG-tagged TEL proteins was confirmed by western analysis with anti-FLAG antibody. An arrow indicates overexpressed FLAG-TEL proteins.

Results

TEL accelerates erythroid differentiation upon EPO treatment in UT-7/GM cells. Human leukemia UT-7/GM cells differentiate into either erythroblasts or megakaryocytes upon cytokine exposure. Thus, this cell line provides a useful tool to analyze the effects of TEL on erythroid differentiation and megakaryocytic maturation in human hematopoietic cells. We established UT-7/GM clones stably overexpressing FLAG-tagged TEL by electroporating the expression plasmid containing *TEL* cDNA and selecting cells with G418 resistance. Western analysis with anti-FLAG antibody demonstrated that representative clones T-5 and T-6 expressed TEL proteins at high levels (Fig. 1). Mock clones M-1 and M-4 were also isolated by introducing the empty expression plasmid. Overexpression of TEL slightly retarded growth of the cells under treatment with EPO or TPO, but did not influence proliferation of the cells maintained in GM-CSF (data not shown).

We previously reported that TEL acts as an accelerator of erythroid differentiation induced by chemical compounds such as HMBA and DMSO in MEL cells.[20] To confirm this effect of TEL under a more physiological condition in human hematopoietic cells, we treated the mock and the TEL-overexpressing clones with EPO. We observed no morphological differences between them, except a faint color difference in the cytoplasm. Figure 2

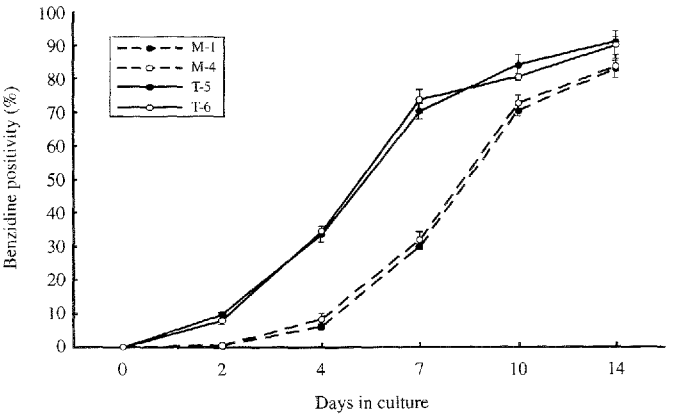

Fig. 2. TEL accelerates hemoglobin synthesis induced by treatment with erythropoietin (EPO) in the UT-7/GM clones. The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones were cultured in the presence of EPO (10 U/mL). Hemoglobin synthesis was evaluated by the proportions of benzidine-positive cells and their averages in three independent experiments were indicated with standard deviations.

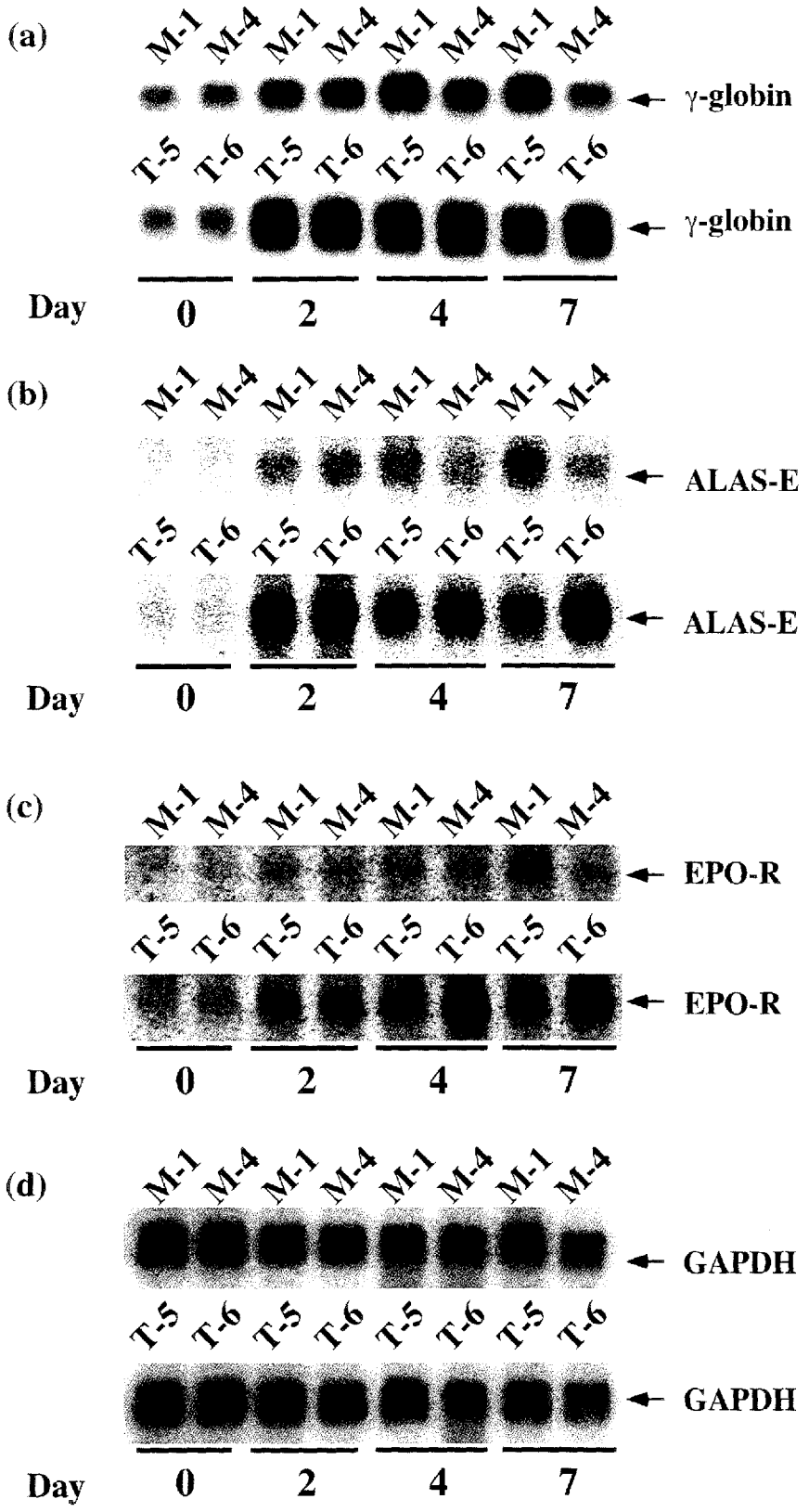

Fig. 3. Erythroid lineage-specific gene transcription in the UT-7/GM clones under treatment with erythropoietin (EPO). The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones cultured in the presence of EPO (10 U/mL) were harvested at each time point indicated (days 0, 2, 4, 7). Total mRNA was extracted and subjected to northern analysis with *γ-globin* (A), *ALAS-E* (B), *EPO-R* (C) and *GAPDH* (D) probes.

indicates time courses of hemoglobin synthesis estimated by proportions of benzidine-positive cells in these UT-7/GM clones. In the mock clones, proportions of benzidine-positive cells reached to 80% within two weeks. Interestingly, the TEL-overexpressing clones showed rapid onset and higher saturation of benzidine positivity in comparison with the mock clones. Eighty percent of the cells became positive for benzidine staining after 10 days of culture and 90% after 14 days. We thus conclude that TEL is also an accelerator for erythroid differentiation upon cytokine stimulation in human hematopoietic cells.

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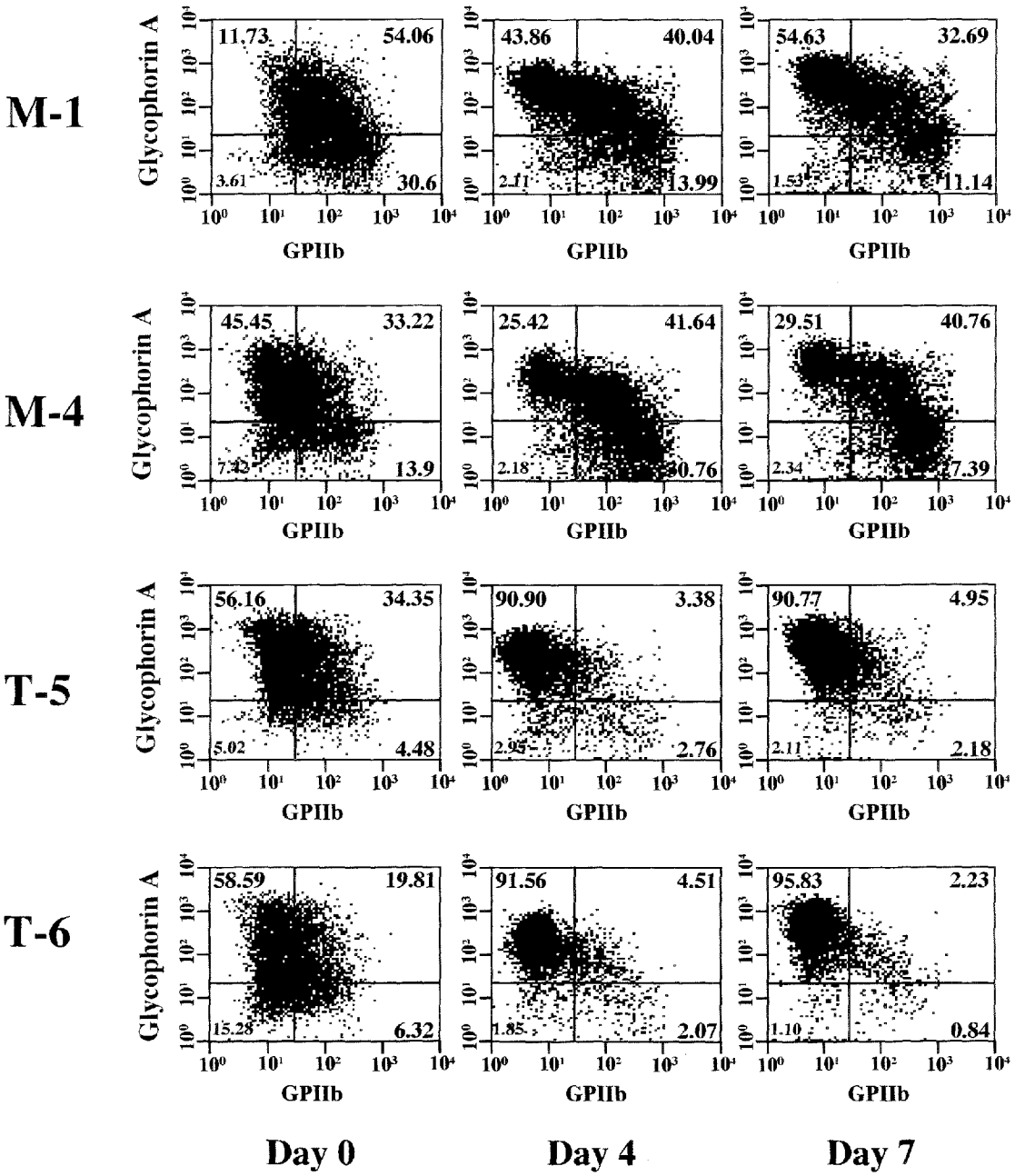

Fig. 4. Erythroid and megakaryocytic lineages-specific surface antigen expression in the UT-7/GM clones under the treatment with erythropoietin (EPO). The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones cultured in the presence of EPO (10 U/mL) were harvested at each time point indicated (days 0, 4, 7) and subjected to flow-cytometric analysis. GPIIb on *X* axis and glycophorin A on *Y* axis were megakaryocyte- and erythrocyte-specific markers, respectively.

To further obtain evidence for erythroid differentiation exaggerated by TEL in UT-7/GM cells, erythroid differentiation-specific transcripts were analyzed using northern analysis. As shown in Fig. 3, transcripts for γ-globin, *ALAS-E* and *EPO-R* increased upon EPO exposure in both cell types. However, even before the treatment (at day 0), expression of these genes appeared to be stimulated by overexpressed TEL proteins. This tendency was maintained at all the time points examined. Next, we performed flow cytometric analysis to assess expression levels of erythrocyte-specific glycophorin A and megakaryocyte-specific GPIIb in the cell surface during the course of erythroid differentiation. Proportions of the glycophorin A(+)/GPIIb(−) fractions were significantly higher at days 4 and 7 in the TEL-overexpressing cells than in the mock cells (Fig. 4). The glycophorin A(−)/GPIIb(+) fractions disappeared more rapidly in the TEL-overexpressing cells. These results collectively confirm the TEL functions as an erythroid differentiation stimulator and indicate the possibility that TEL might concomitantly accelerate erythroid differentiation and repress megakaryocytic maturation.

TEL inhibits megakaryocytic maturation upon TPO treatment in UT-7/GM cells. To clarify the roles of TEL in megakaryocytic maturation of human hematopoietic cells, we induced megakaryocytic maturation by treatment with TPO in the mock and the TEL-overexpressing clones and first analyzed their morphological changes. Differing from the mock clones, the TEL-overexpressing clones hardly maturated into megakaryocyte-containing multilobulated nuclei even after 28 days of culture with TPO

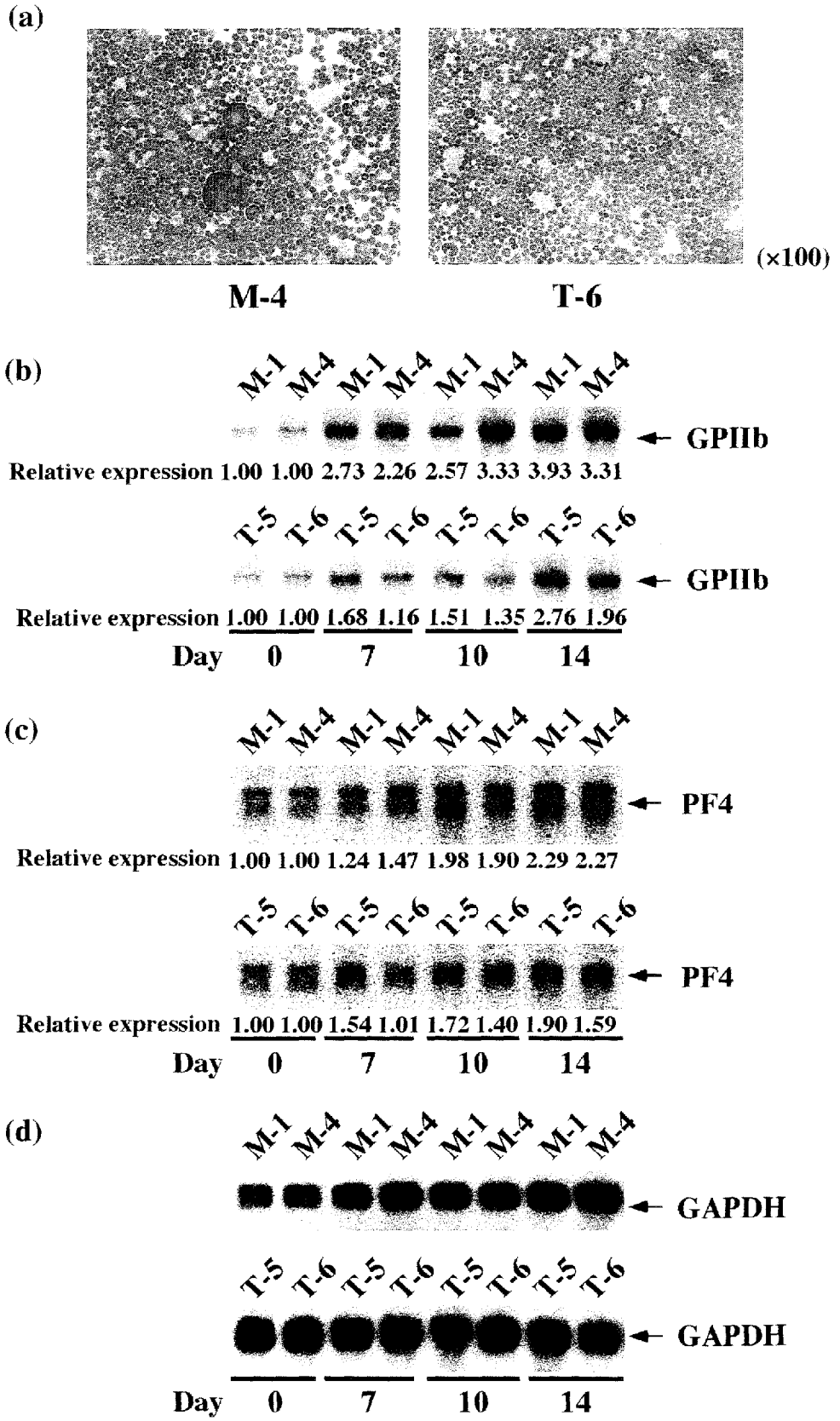

Fig. 5. Morphology and megakaryocytic lineage-specific gene transcription in the UT-7/GM clones under treatment with thrombopoietin (TPO). The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones cultured in the presence of TPO (100 ng/mL) were harvested at each time point indicated (days 0, 7, 10, 14, 28). (a) Cytospin preparations of M-4 and T-5 at day 28. Wright-Giemsa staining, ×100. (b–d) Total mRNA was extracted and subjected to northern analysis with GPIIb (b), *PF 4* (c) and *GAPDH* (d) probes. Signal ratios between day 0 and the indicated time points were quantified and presented below each lane.

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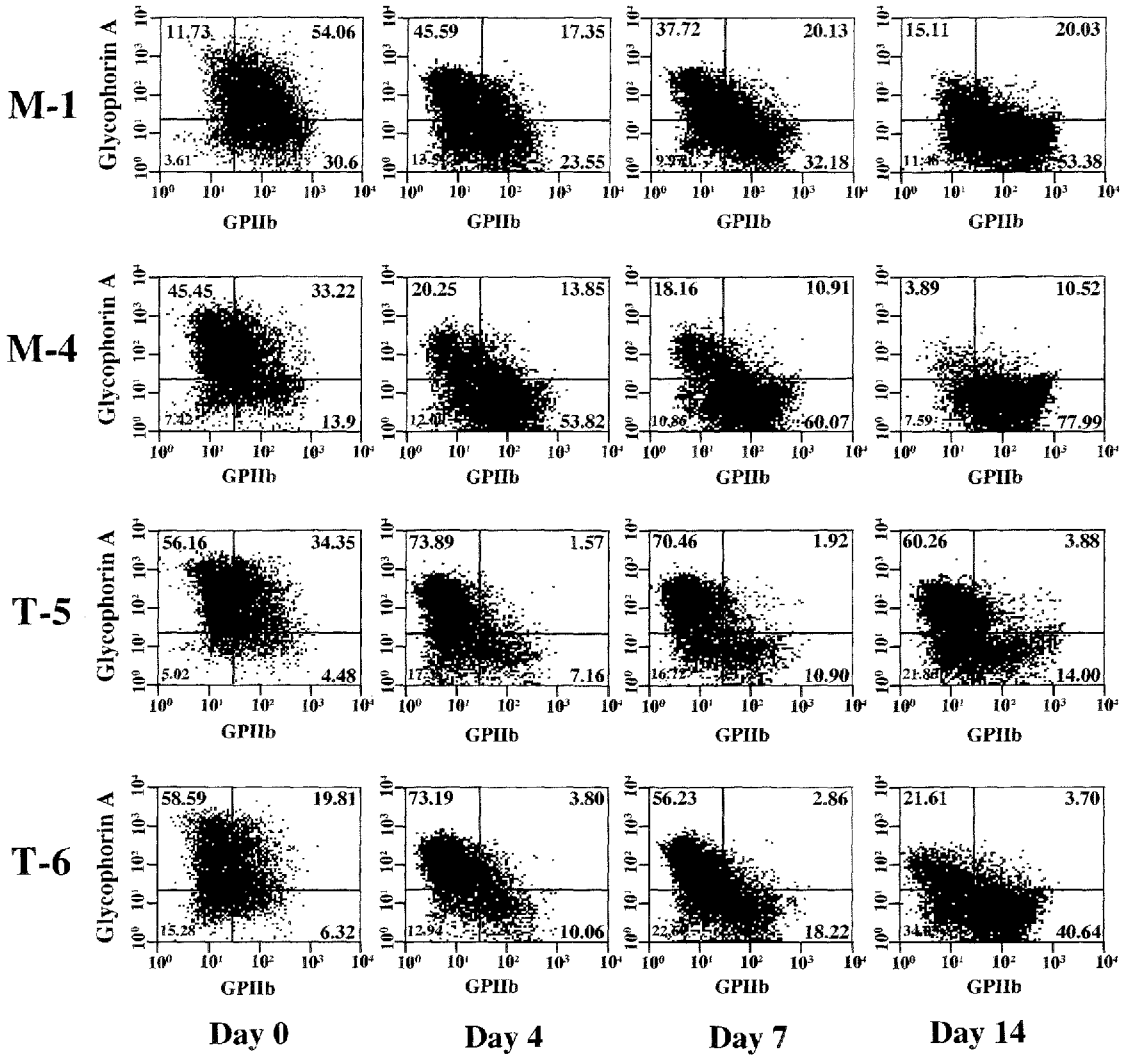

Fig. 6. *Erythroid and megakaryocytic lineage-specific surface antigen expression in the UT-7/GM clones under the treatment with thrombopoietin (TPO). The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones cultured in the presence of TPO (100 ng/mL) were harvested at each time point indicated (days 0, 4, 7, 14) and subjected to flow-cytometric analysis. GPIIb on X axis and glycophorin A on Y axis were megakaryocyte- and erythrocyte-specific markers, respectively.*

(Fig. 5a). Expression of megakaryocytic maturation-specific genes such as *GPIIb* and *PF 4* was also examined using northern analysis. The TEL-overexpressing cells expressed these transcripts at almost comparable levels to mock cells before the treatment (Fig. 5b–d). As expected, they increased upon TPO exposure in both cell types. It is interesting to note that levels of these transcripts were lower in the TEL-overexpressing cells than in the mock cells at least until day 14. We again examined cell surface expression of glycophorin A and GPIIb during the course of megakaryocytic maturation. Proportions of the glycophorin A(–)/GPIIb(+) fractions were markedly lower until day 14 in the TEL-overexpressing cells than in the mock cells, whereas proportions of the glycophorin A(+)/GPIIb(–) fractions higher (Fig. 6). Furthermore, fewer percentages of the cells became positive for electron microscopic PPO in the TEL-overexpressing clones after 14 days treatment with TPO (Fig. 7). We hypothesize that TEL could prevent megakaryocytic maturation and maintain expression of erythroid markers in erythrocyte/megakaryocyte common progenitors even when induced towards the megakaryocytic lineage.

Expression of endogenous TEL proteins decreases upon both EPO and TPO treatments in UT-7/GM cells. Finally, we examined changes of endogenous TEL expression during both the courses of erythroid and megakaryocytic differentiation in parental UT-7/GM cells to further obtain findings for the physiological roles of TEL. Under the presence of GM-CSF, endogenous TEL proteins were detected at almost the same size as overexpressed TEL proteins in the T-5 clone (lane 4, Fig. 8a) using western analysis (lane 3) and self-immunoprecipitation assay (lane 1). When the cells were induced to erythroid differentiation by treatment with EPO, endogenous TEL proteins maintained steady expression until 3 days of culture and then began to decline (Fig. 8b). At day 14, endogenous TEL proteins almost completely disappeared. When induced to megakaryocytic maturation by treatment with TPO, UT-7/GM cells kept constant expression of endogenous TEL proteins until 14 days of culture and lost their expression at day 21 (Fig. 8c). These data suggest that endogenous TEL may work in the early phase of differentiation to either lineage and accelerate erythroid differentiation and actively repress megakaryocytic maturation.

Discussion

We demonstrated in the present study that TEL accelerates erythroid differentiation induced by a physiological cytokine

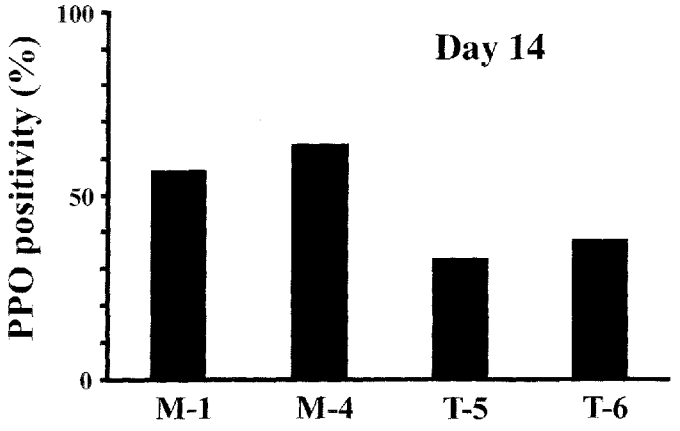

Fig. 7. TEL represses ultrastructural platelet peroxidase (PPO) reactions after 14 days of treatment with thrombopoietin (TPO) in the UT-7/GM clones. The mock (M-1 and M-4) and TEL-overexpressing (T-5 and T-6) UT-7/GM clones were cultured in the presence of TPO (100 ng/mL) for 14 days. PPO reactions were evaluated by electron microscopic analysis.

EPO in human leukemia cell line UT-7/GM. Associated with increased expression of erythroid differentiation-specific transcripts γ-globin, *ALAS-E* and *EPO-R*, and a surface antigen glycophorin A, the TEL-overexpressing cells accumulate hemoglobin more rapidly than the mock cells. A megakaryocyte maturation-specific surface marker GPIIb disappears more quickly during the course of erythroid differentiation in the TEL-overexpressing cells. Importantly, morphological maturation towards megakaryocyte with multilobulated nuclei and induction of megakaryocyte maturation-specific transcripts *GPIIb* and *PF 4* after TPO treatment are weaker when TEL is overexpressed. Moreover, GPIIb accumulates and glycophorin A disappears more slowly in the cell surface of these cells. Electron microscopic PPO reaction is detected at fewer ratios. All these data collectively suggest that TEL might drive erythroid differentiation and suppress megakaryocytic maturation in erythrocyte/megakaryocyte common progenitors. Consistently, endogenous TEL proteins are expressed only in the early phase of either differentiation in which TEL is expected to function, and thereafter disappear. This paper is the first describing the unique role of TEL in the megakaryocytic lineage.

Because TEL is a transcriptional regulator for EBS-containing promoters, it is interesting to know whether the *cis*-regulatory elements actually exist in the erythrocyte or megakaryocyte-specific genes, the expression of which was found in this study to be altered by overexpressed TEL. Numerous megakaryocyte-specific genes contain EBS and GATA-1 binding sites in their promoters.[24] Of note, both ETS-1 and GATA-1 are reported essential for positive regulation of *GPIIb* and *PF 4* gene transcription.[25] Moreover, ETS-1 is demonstrated to directly bind to their promoters by chromatin precipitation assays.[26] Although it remains undetermined whether TEL binds to EBS in the promoters of *GPIIb* and *PF 4* genes, overexpressed TEL could repress it directly or indirectly. In the latter case, TEL may

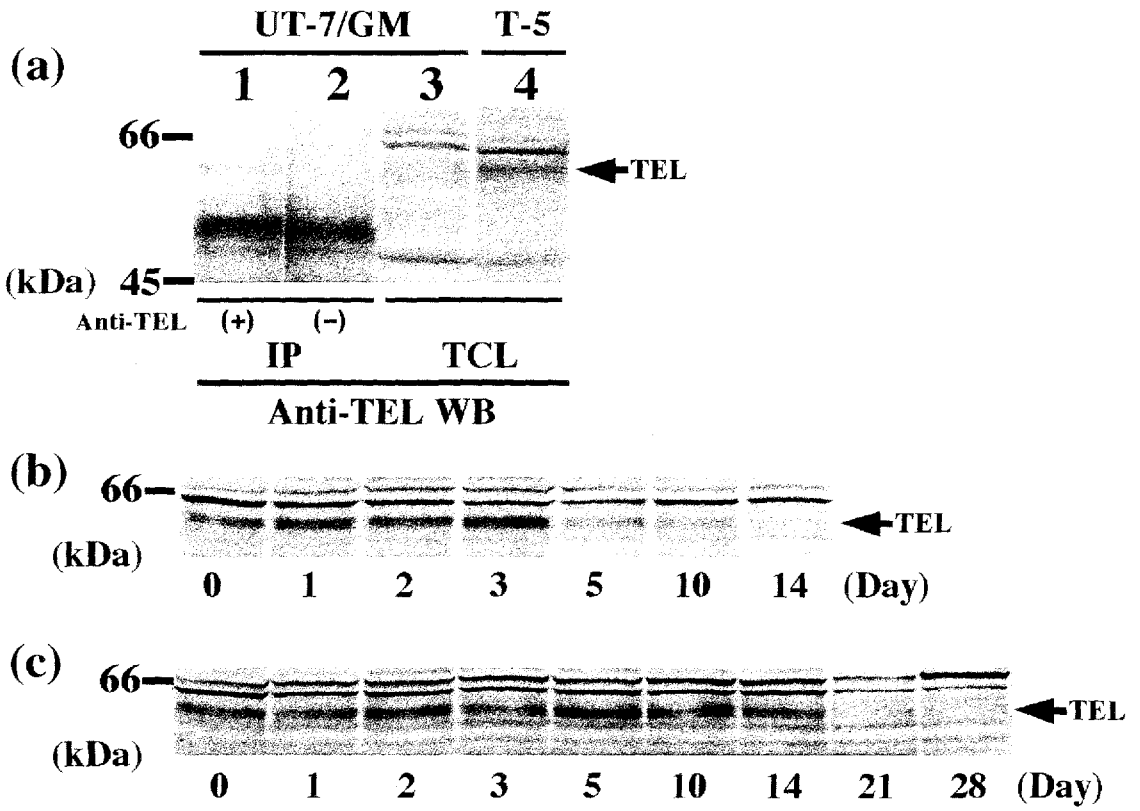

Fig. 8. Expression of endogenous TEL proteins in parental UT-7/GM cells. (a) Expression of endogenous TEL proteins in parental UT-7/GM cells was confirmed under the presence of GM-CSF (1 ng/mL) by western analysis (lane 3) or immunoprecipitation assay (lane 1) with anti-TEL antibody. Overexpressed FLAG-tagged TEL proteins in clone T-5 were shown in lane 4. An arrow indicates endogenous TEL or overexpressed FLAG-tagged TEL proteins; (b,c) Parental UT-7/GM cells cultured in the presence of erythropoietin (10 U/mL); (b) or thrombopoietin (100 ng/mL); (c) were harvested at each time point indicated (days 0, 1, 2, 3, 5, 10, 14, 21, 28). Cell lysates were extracted and subjected to western analysis with anti-TEL antibody. Arrows indicate endogenous TEL proteins.

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dominantly suppress functions of other ETS family members such as ETS-1 that show transactivation abilities on the promoters through heterodimerizing with them by the HLH domain. However, because EBS is not identified in the promoters of the erythrocyte-specific genes examined in this study, we have no ground to speculate that TEL could be involved in their transcriptional regulation.

We hypothesize that TEL could trigger erythroid differentiation and prevent megakaryocytic maturation through repressing transcription of its target genes that play key roles in hematopoietic differentiation. Among the known target genes of TEL, *FLI-1* and *Id-1* are shown to have functions in erythrocyte/megakaryocyte differentiation. The *FLI-1* gene was first isolated as a common site for retroviral integration in Friend virus-induced erythroleukemia cells,[27] and also encodes a member of the ETS family of transcription factors. FLI-1 suppresses erythroid differentiation partly through inhibiting transcription of the genes such as *GATA-1*,[28] *Rb*[29] and β-globin[30] that promote erythroid differentiation. Moreover, *FLI-1* knockout mice are embryonic lethal around mid-gestation and display a marked reduction of megakaryocytes in the fetal liver as well as a vascular developmental aberration,[31] suggesting a critical role of FLI-1 in megakaryocytic maturation. FLI-1 binds and transactivates the promoters from megakaryocyte-specific genes including *GPIX*,[32] *GPIIb*[32] and *TPO* receptor.[33] Therefore, FLI-1 appears to play opposite roles in erythroid differentiation and megakaryocytic maturation. We analyzed expression levels of FLI-1 proteins before and after induction of erythroid differentiation or megakaryocytic maturation in the mock and TEL-overexpressing UT-7/GM cells. However, overexpressed TEL proteins did not affect the expression of FLI-1 in UT-7/GM cells (data not shown). In spite of this, there still remains the possibility that TEL could repress molecular functions of FLI-1 in these cells, because TEL has been proved to exert a dominant-negative effect on FLI-1 in reporter assays.[2] The *Id-1* gene was initially cloned from MEL cells by virtue of homology to the helix 2 subdomain in c-myc, MyoD and myogenin, and codes for the first member of Id-family that has the HLH domain.[34] Id-1 has been reported to be functionally implicated in differentiation of specific hematopoietic lineages including erythroid,[35] myeloid,[36] and B cells[37] and negatively control erythroid differentiation. We observed that expression of Id-1 proteins slightly increased after induction of erythroid differentiation or megakaryocytic maturation in UT-7/GM cells, but that overexpressed TEL proteins did not influence its expression levels. Therefore, we failed to obtain evidence that *FLI-1* or *Id-1* could be targets of TEL-induced transcriptional repression in UT-7/GM cells. Identification of novel target genes for TEL that regulate erythroid and/or megakaryocytic differentiation should provide new insights into molecular mechanisms in hematopoietic cell differentiation. Studies to determine the target genes of TEL in differentiating MEL and UT-7/GM cells are now in progress in our laboratory.

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Cloning and Characterization of the Novel Chimeric Gene *TEL/PTPRR* in Acute Myelogenous Leukemia with inv(12)(p13q13)

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Abstract

We have cloned a novel *TEL/protein tyrosine phosphatase receptor-type R* (*PTPRR*) chimeric gene generated by inv(12)(p13q13). PTPRR is the first protein tyrosine phosphatase identified as a fusion partner of TEL. The chimeric gene fused exon 4 of the *TEL* gene with exon 7 of the *PTPRR* gene, and produced 10 isoforms through alternative splicing. Two isoforms that were expressed at the highest level in the leukemic cells could have been translated into COOH-terminally truncated TEL protein possessing the helix-loop-helix domain (tTEL) and TEL/PTPRR chimeric protein linking the helix-loop-helix domain of TEL to the catalytic domain of PTPRR. These two mutant proteins exerted a dominant-negative effect over transcriptional repression mediated by wild-type TEL, although they themselves did not show any transcriptional activity. Heterodimerization with wild-type TEL might be an underlying mechanism in this effect. TEL/PTPRR did not exhibit any tyrosine phosphatase activity. Importantly, overexpression of TEL/PTPRR in granulocyte macrophage colony-stimulating factor–dependent UT7/GM cells resulted in their factor-independent proliferation, whereas overexpression of tTEL did not. After cytokine depletion, phosphorylated signal transducers and activators of transcription 3 (STAT3) significantly declined in mock cells, but remained in both tTEL- and TEL/PTPRR-overexpressing cells. Loss of tumor suppressive function of wild-type TEL and maintenance of STAT3-mediated signal could at least partly contribute to the leukemogenesis caused by inv(12)(p13q13). (Cancer Res 2005; 65(15): 6612-21)

Introduction

The 12p13 translocations are one of the most commonly observed chromosomal abnormalities in human leukemia and myelodysplastic syndrome and fuse the *TEL* gene on 12p13 with various partner genes. The *TEL* gene was originally cloned as a gene that was rearranged by t(5;12)(q33;p13) in chronic myelomonocytic leukemia, and encodes a member of the ETS family transcription factors (1). TEL shares with other ETS proteins an evolutionarily conserved ETS domain at the COOH terminus that is responsible for DNA binding to the ETS-binding consensus site (EBS; ref. 2). TEL also contains an NH_2-terminal domain that is referred to as the helix-loop-helix or pointed domain. The helix-loop-helix domain in TEL has the unique property of inducing its stable homodimerization or heterodimerization with other ETS family members (3–6). Associating with the relevant corepressors mSin3A and N-CoR as well as histone deacetylase-3 (7), TEL works as a transcriptional repressor. Known target genes of TEL are *FLI-1* (8), *Id1* (9), *stromelysin-1* (10), and *Bcl-X_L* (11).

Gene-engineered mice have highlighted critical roles of this transcription factor in the embryonic development and hematopoietic regulation. The ablation of the *TEL* gene by homologous recombination causes death in uteri between E10.5 and E11.5 (12). These knock-out embryos show defect in yolk sac angiogenesis and intraembryonic apoptosis of mesenchymal and neural cells, although they present normal yolk sac hematopoiesis. Generating chimeric mice with TEL−/− embryonic stem cells, the pivotal function of TEL in establishing hematopoiesis of all lineages in neonatal bone marrow has been clarified, whereas TEL−/− embryonic stem cells contributed to both primary and definitive fetal hematopoiesis (13). Moreover, a recent study shows that inactivation of TEL in adult mice leads to decrease of hematopoietic stem cells in bone marrow (14). On the other hand, TEL is believed to function as a tumor suppressor because its overexpression in NIH3T3 fibroblasts results in reduced cell growth in liquid and soft agar cultures (10, 15).

Molecular dissecting of the *TEL*-related chimeric genes has provided interesting clues to the pathogenesis of 12p13 translocation-type leukemia. In some translocations, receptor-type and non-receptor-type tyrosine kinases are fused to the NH_2-terminal portion of TEL and are thus catalytically activated by homodimerization through the helix-loop-helix domain in the TEL moiety. Examples for the former include platelet-derived growth factor receptor β in t(5;12)(q33;p13) (refs. 1, 16, 17) and tyrosine kinase C in t(12;15)(p13;q25) (ref. 18), and those for the latter Abl in t(9;12)(q34;p13) (refs. 19, 20), Janus-activated kinase (JAK)-2 in t(9;12)(p24;p13) (refs. 21, 22), Syk in t(9;12)(q22;p13) (ref. 23), and Abl-related gene in t(1;12)(q25;p13) (ref. 24). In other translocations, transcription factors are structurally and functionally modified by fusing with the NH_2- or COOH-terminal part of TEL. Examples include acute myelogenous leukemia (AML)-1 in t(12;21)(p13;q22) (refs. 25–29), MN1 in t(12;22)(p13;q11) (refs. 30, 31), Evi-1 in t(3;12)(q26;p13) (ref. 32), PAX5 in t(9;12)(q11;p13) (ref. 33), and CDX2 in t(12;13)(p13;q12) (ref. 34). Thus, perturbation of original functions of the partner genes could be a mechanism in causing leukemia in patients with such translocations. Furthermore, disruption of tumor-suppressive function of wild-type TEL itself

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seems to be another cause of leukemogenesis because some chimeric molecules such as TEL/AML1 are shown to dominantly interfere with function of wild-type TEL (35).

To obtain a new insight into the molecular mechanism in leukemogenesis by the 12p13 translocations, we cloned several species of novel chimeric cDNAs generated by inv(12)(p13q13) found in a patient with acute myelogenous leukemia [M2 according to the French-American-British (FAB) classification]. These cDNAs contained the NH_2-terminal *TEL* sequence followed by the COOH-terminal sequence from *protein tyrosine phosphatase receptor-type R* (*PTPRR*) and were expected to produce either truncated TEL or chimeric TEL/PTPRR protein. Both molecules lost DNA binding to and trans-repression through EBS, but blocked the molecular function of wild-type TEL probably by heterodimerizing with it. TEL/PTPRR showed no tyrosine phosphatase activity. Notably, overexpression of TEL/PTPRR in factor-dependent human leukemia cell line UT7/GM led to factor-independent growth, suggesting the oncogenic potential of this chimeric molecule.

Materials and Methods

Case presentation. A 24-year-old woman presented with slight fever in April 2001. Her hemoglobin level was 9.1 g/dL, WBC count 4,200/μL with 29% blasts, and platelet count 191,000/μL. The bone marrow aspirate disclosed 79% blasts positive for myeloperoxidase, and a diagnosis of acute myelogenous leukemia (M2 according to the FAB criteria) was made. Flow cytometric assay revealed that the blasts were positive for CD13, CD15, CD33, and CD34. Cytogenetic analysis showed 46, XX, inv(12)(p13q13) (12) /46, XX (8). The patient achieved complete remission after one course of induction chemotherapy and underwent bone marrow transplantation in the first remission from her HLA-matched sibling in November 2001. She has thus far been free from relapse. All the following experiments were done under the written informed consent of the patient.

Fluorescence *in situ* hybridization analysis. The metaphase samples that were subjected to conventional cytogenetic studies were also applied to fluorescence *in situ* hybridization (FISH) analysis. LL12NCO1 cosmid probes (2G8, 163E7, and 184C4) which are located within the *TEL* gene were used in the assay (36). The probes were labeled with biotin-11-dUTP or digoxigenin-11-dUTP using PCR labeling after sequence-independent amplification, and were hybridized to metaphase samples as previously described (37, 38). The hybridization was detected with avium fluorescein or anti-digoxigenin rhodamine and the metaphase cells were subsequently counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride. Images of the hybridized signals were captured under fluorescence microscopy.

Cloning of TEL/PTPRR. Total RNA was extracted from cryopreserved leukemic cells with inv(12)(p13q13) using RNeasy RNA miniprep system (Qiagen, Valencia, CA). We purified mRNA by oligo-dT column. The first-strand cDNA was synthesized from 2 μg of RNA using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) with random hexamers and Moloney murine leukemia virus reverse transcriptase. For 3′-rapid amplification of cDNA ends (RACE) procedure, we adopted Marathon cDNA amplification system (Clontech, Palo Alto, CA). Synthesis of the second-strand cDNA and adaptor ligation were carried out according to the instructions of the manufacturer. RACE-PCR was done for 40 cycles with primers TELf2 and activator protein (AP)-1, followed by nested PCR for 30 cycles with primers TELf4 and AP-2 (refer to the instructions of the manufacturer for the sequences of primers AP-1 and AP-2). PCR products were subcloned into the pCR2.1-TOPO cloning vector (Invitrogen) and nucleotide sequences were determined by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

PCR amplification. To amplify *TEL/PTPRR*, *PTPRR/TEL*, wild-type *TEL*, and wild-type *PTPRR* cDNAs, we used primer sets TELf2 and PTPRRr7a, PTPRRf6 and TELr5, TELf2 and TELr5, and PTPRRf6 and PTPRRr7a, respectively. PCR was done for 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Extension time was elongated to 2 minutes when we amplified full-length *TEL/PTPRR* cDNA with a set of primers TELf1 and PTPRRr14. Sequences of all PCR primers are given in the next section.

Oligonucleotides. TELf1 [nucleotide (nt) 25-47 of *TEL*]: 5′-ATGTC-TGAGACTCCTGCTCAGTG-3′; TELf2 (nt 88-111 of *TEL*): 5′-AGCC-CAGTGCCGAGTTACGCTTCC-3′; TELf3 (nt 328-347 of *TEL*): 5′-TTTCGCTATCGATCTCCTCA-3′; TELf4 (nt 376-405 of *TEL*): 5′-CAGCA-TATTCTGAAGCAGAGGAAACCTCGG-3′; TELr5 (complement, nt 637-660 of *TEL*): 5′-GAGGCGGCGGATCATGTTGTCCAG-3′; PTPRRf6 (nt 971-992 of *PTPRR*): 5′-ACCAGGAGATCCACCTATCGCC-3′; PTPRRr7a (complement, nt 1,313-1,334 of *PTPRR*): 5′-ACGACGTCCCTCAGCTGAGACC-3′; PTPRRr7b (complement, nt 1,193-1,216 of *PTPRR*): 5′-TCATGTCCAATGTAAGAGA-TACGT-3′; PTPRRf10 (nt 1,565-1,580 of *PTPRR*): 5′-CCACGCAGGGCCCCAT-3′; PTPRRr10 (complement, nt 1,565-1,584 of *PTPRR*): 5′-GATCATGGGGCC-CTGCGTGG-3′; PTPRRr14 (complement, nt 2,126-2,148 of *PTPRR*): 5′-TCA-CTGGACAGTCTCTGCTGAAA-3′.

Plasmid construction. Constructions of pME18S-HA-TEL, pME18S-FLAG-TEL, pcDNA3-HA-TEL, $(EBS)_3$tkLuc, and pSRαMSVtk*neo*-FLAG-wild-type-TEL were previously described (39, 40). Hemagglutinin or FLAG tag was inserted at both ends of *TEL/PTPRR* chimeric cDNAs by the PCR amplification method. The resultant cDNAs were subcloned into the *Eco*RI sites of pME18S, pcDNA3, and pCXN2 expression plasmids in the sense orientation. To obtain hemagglutinin-tagged wild-type *PTPRR* cDNA, a 5′ fragment spanning the initiation codon (nt 175 according to GenBank accession number U42361) and the *Apa*I site (nt 1,567) was amplified from a human brain total cDNA library (Clontech). Together with a 3′ *Apa*I (nt 1,567 in PTPRR cDNA)/*Xho*I (nt 355 in pCR2.1-TOPO vector) fragment derived from pCR2.1-TOPO-TEL/PTPRR-HA, it was subcloned into the *Xho*I site of pME18S. FLAG-tagged *human SUMO-1* cDNA was obtained by the PCR amplification method, and was subsequently cloned into the *Xba*I site of pME18S.

Cell culture. COS-7 cells were cultured in DMEM (Sigma, St. Louis, MO) containing 10% FCS and transfected with various expression plasmids by the DEAE-Dextran method. NIH3T3 fibroblasts were grown in DMEM with 10% bovine serum. HeLa cells were cultured in Eagle's MEM (Sigma) supplemented with 10% FCS and 1% nonessential amino acids. Human leukemia cells UT7/GM were maintained in Iscove's modified Dulbecco's medium (Invitrogen) containing 10% FCS and 1 ng/mL of human recombinant granulocyte macrophage-colony stimulating factor (GM-CSF; Kirin, Gunma, Japan). KASUMI-1 cells were cultured in RPMI 1640 (Sigma) with 10% FCS.

Establishing bulk transfectants of NIH3T3 cells. NIH3T3 bulk population constitutively overexpressing FLAG-tagged wild-type TEL and its control were obtained by retroviral infection and G418 (Sigma) resistance as previously described (41).

Generating bulk and stable transfectants of UT7/GM cells. To obtain bulk transfectants for experiments, 1×10^7 of UT7/GM cells suspended in 500 μL PBS were electroporated at 380 V and 975 μF with pCXN2 plasmid carrying the aimed gene. Forty-eight hours after the electroporation, cells were selected with 0.8 mg/mL of G418. Stable UT7/GM clones overexpressing TEL/PTPRR were established by the limiting dilution method. For factor deprivation, UT7/GM cells were washed with PBS at least thrice and were finally suspended in factor-free media.

Metabolic labeling. Forty-eight hours after transfection, COS-7 cells were washed with phosphate-free DMEM and then cultured for 2 hours in DMEM supplemented with 400 μCi [^{32}P]orthophosphate/mL (Amersham Biosciences, Piscataway, NJ) and 10% dialyzed FCS.

Subcellular localization. NIH3T3 cells were transiently transfected with expression plasmids using TransFast (Promega). Forty-eight hours later, cells were suspended in hypotonic suspension buffer [10 mmol/L sodium phosphate (pH 7.0), 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride], and were separated into nucleic and cytoplasmic fractions using Dounce homogenizer (Wheaton, Millville, NJ). Equal volumes of aliquots were applied to Western blot analysis.

Western blot analysis and immunoprecipitation. Western blot analysis and immunoprecipitation were done as previously described (42). Anti-FLAG (M2) and anti-hemagglutinin (CH-7 or rabbit) antibodies were purchased from Sigma, and antibodies against TEL (N-19 and C-20), histone H1, actin, Bcl-X_L, signal transducers and activators of transcription (STAT)-3, and phospho-STAT3 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Electrophoretic mobility shift assay. The procedures for electrophoretic mobility shift assays (EMSA) were previously described (43). Lysates were *in vitro* prepared by TNT-Coupled Wheat Germ Extract System (Promega). The EBS oligonucleotide used as a probe or a specific competitor and its mutant used as a nonspecific competitor were previously described (2). End labeling of the double-stranded oligonucleotide was carried out with [α-^{32}P]dCTP (Amersham Biosciences) and Klenow enzyme (Takara, Shiga, Japan) at room temperature for 30 minutes. Unincorporated nucleotides were removed by G-50 Sephadex columns (Amersham Biosciences). Protein-DNA complexes were separated on a 4% polyacrylamide gel and visualized by autoradiography. In competition studies, a 300-fold molar excess of unlabeled oligonucleotide was added to the reaction. In supershift assays, we employed anti-TEL (N-19) supershift antibody of which epitope is encoded by NH_2-terminal *TEL* sequence in *tTEL* and *TEL/PTPRR* cDNAs. The antibody was preincubated with the lysates at 4°C for 1 hour.

Luciferase assay. HeLa cells in 24-well tissue culture plates were transfected with 1 μg of $(EBS)_3$tkLuc or $(mEBS)_3$tkLuc along with 1 μg of expression plasmids by using Tfx-20 (Promega). To equalize transfection efficiencies, total amounts of expression plasmids were kept constant in terms of weight by adding empty pME18S vector. Luciferase assays were done with Dual-Luciferase reporter assay system (Promega) as previously described (39, 42).

***In vitro* phosphatase assay.** Lysates of COS-7 cells overexpressing hemagglutinin-tagged wild-type PTPRR, tTEL, or TEL/PTPRR were immunoprecipitated with anti-hemagglutinin (CH-7) antibody conjugated with Sepharose A beads (Amersham Biosciences). The immunoprecipitates were washed and finally suspended in 150 μL of assay buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride]. One third of the mixture (50 μL) was added to 100 μL of assay buffer containing 15 mmol/L *p*-nitrophenyl phosphate (Sigma-Aldrich), followed by incubation at 37°C for 10 minutes. The reaction was stopped by adding 25 μL of 2.5 N NaOH. The absorption at 405 nm was read on a 96-well microtiter plate.

Results

Cloning of the *TEL/PTPRR* chimeric gene. Because the *TEL* gene on 12p13 is fused to various partners in a variety of hematologic malignancies, we suspected that inv(12)(p13q13) implicated it. To look whether the *TEL* gene is rearranged by the chromosomal abnormality, we first did FISH analysis using some cosmid probes spanning the *TEL* gene (Fig. 1*A*; ref. 36). Split signals were observed in bone marrow metaphases with inv(12)(p13q13) when we used cosmid 184C4 (Fig. 1*B*) or 163E7 (data not shown) as a probe. Because the procedure with cosmid 2G8 did not yield split signals (data not shown), it was likely that the inversion breakage occurred between introns 5 and 6 of the *TEL* gene. Subsequently, we adopted the 3′-RACE method to identify a fusion partner of the *TEL* gene. RACE-PCR products shown in Fig. 1*C* were subcloned into the pCR2.1-TOPO vector and resultant five clones were sequenced. Among them, four clones contained the wild-type *TEL* sequence only, but the last one included *TEL* exon 4–derived sequence followed by an unknown sequence. BLAST database searching revealed that the chimeric cDNA joined *TEL* exon 4 and *PTPRR* exon 7 with frameshift (human *TEL* was referred to GenBank accession number U11732 and human *PTPRR* to U42361; Fig. 2*A*).

Then, we did reverse transcription-PCR (RT-PCR) analysis to examine whether *TEL/PTPRR*, *PTPRR/TEL*, wild-type *TEL*, and wild-type *PTPRR* transcripts were expressed in the leukemic cells with inv(12)(p13q13). To detect each transcript, we used primer sets TELf2 (in *TEL* exon 2) and PTPRRr7a (in *PTPRR* exon 7) for *TEL/PTPRR*, PTPRRf6 (in *PTPRR* exon 6) and TELr5 (in *TEL* exon 5) for *PTPRR/TEL*, TELf2 and TELr5 for wild-type *TEL*, and PTPRRf6 and PTPRRr7a for wild-type *PTPRR*. As for *TEL/PTPRR*, two smaller bands (416 and 251 bp) besides a product of the expected size (551 bp) were observed (Fig. 1*D*). Sequencing revealed that these smaller cDNAs lacked exon 4 or exons 3 and 4 of the *TEL* gene, strongly suggesting the presence of alternative splicing mechanisms. On the other hand, reciprocal *PTPRR/TEL* mRNA was not

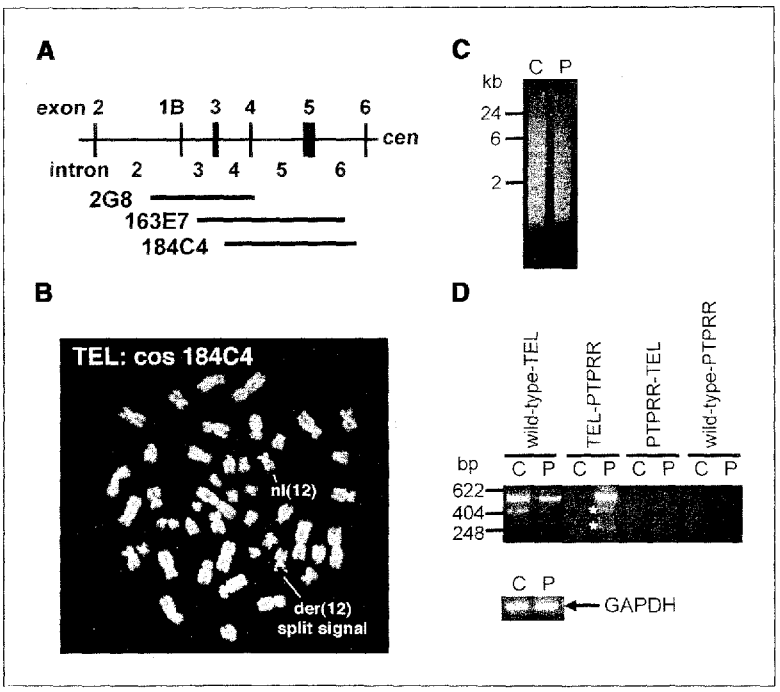

Figure 1. Detection of the fusion gene *TEL/PTPRR*. *A*, physical map of the *TEL* gene. The cosmid probes used in FISH analysis are shown. *B*, FISH analysis of metaphases with inv(12)(p13q13). Split signals of cosmid 184C4 were observed on the der(12) chromosome. *C*, 3′-RACE method adopted to identify the fusion partner for the *TEL* gene. A fraction of RACE-PCR products was electrophoresed on a 2% agarose gel. *C*, control cell line (HL60); *P*, patient's leukemic cells. *D*, RT-PCR analysis of inv(12)(p13q13)-carrying leukemic cells. Transcripts for wild-type *TEL*, *TEL/PTPRR*, *PTPRR/TEL*, wild-type *PTPRR*, and *GAPDH* were amplified. *Asterisks*, two minor *TEL/PTPRR* isoforms lacking exon 4 or exons 3 and 4 of the *TEL* gene. Primers used are described in Materials and Methods. *C*, control cell line (HL60); *P*, patient's leukemic cells.

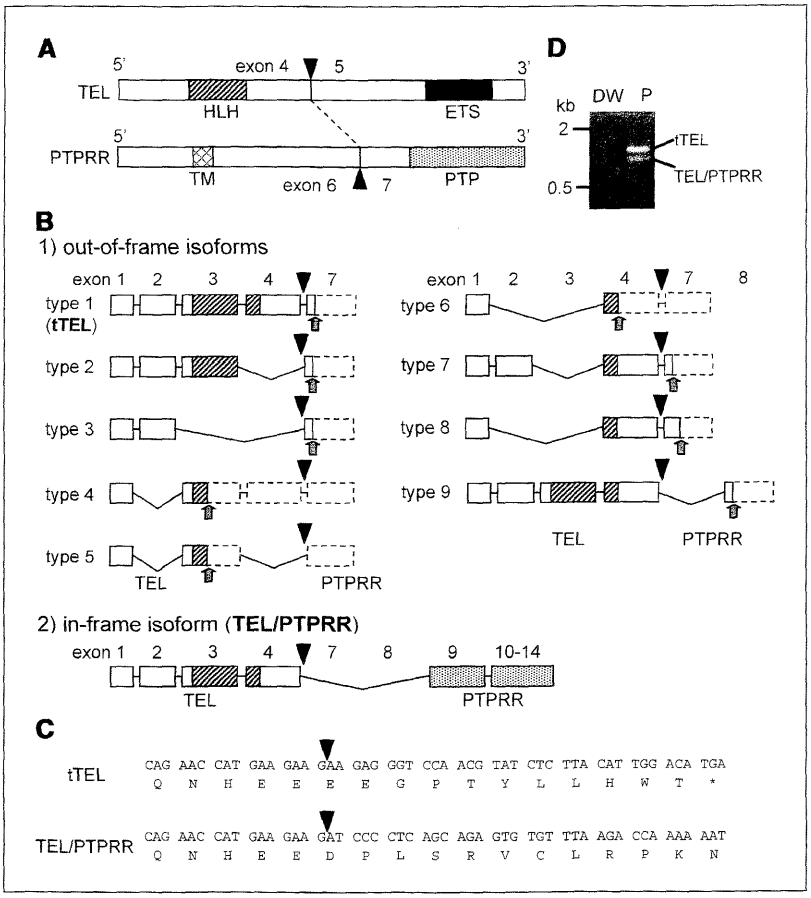

Figure 2. Ten isoforms of *TEL/PTPRR* cDNAs generated through alternative splicing. *A*, schematic structures of wild-type TEL and wild-type PTPRR. *Solid triangles*, breakpoints in each protein. *HLH*, helix-loop-helix oligomerization domain; *ETS*, ETS DNA-binding domain; *TM*, transmembrane domain; *PTP*, protein tyrosine phosphatase domain (catalytic domain). *B*, schematic presentation of 10 *TEL/PTPRR* isoforms. Exons surrounding the junctions are presented as boxes to emphasize exon skipping. *Solid triangles* and *arrows*, fusion points and locations of stop codon, respectively. *C*, nucleotide sequences and deduced amino acids around the breakpoints of the two dominant isoforms (tTEL and TEL/PTPRR). *D*, dominant amplification of *tTEL* and *TEL/PTPRR* in RT-PCR assay. A set of primers TELf1 (in *TEL* exon 1) and PTPRRr14 (in *PTPRR* exon 14) was used to amplify full-length *TEL/PTPRR* cDNA.

detected in spite of our efforts with several sets of primers including the one described above. Thus, we conclude that *TEL/PTPRR* is expressed in the leukemic cells of this patient and might therefore contribute to leukemogenesis. Wild-type *TEL* mRNA probably derived from the intact *TEL* allele was expressed in the leukemic cells, whereas wild-type *PTPRR* mRNA was not.

Alternative splicing leads to generation of 10 *TEL/PTPRR* chimeric cDNAs. Because the *TEL* gene was fused out-of-frame to the *PTPRR* gene, the resultant full-length *TEL/PTPRR* cDNA (type 1) represented an open reading frame encoding exons 1 to 4 of the *TEL* gene (154 amino acids) with additional 11 amino acids (Fig. 2*B* and *C*). This isoform expresses truncated TEL including the intact helix-loop-helix domain, but lacks the COOH-terminal ETS domain of TEL and any functional domains of PTPRR. We thus refer to it as "truncated TEL (tTEL)" in the following sections. To seek for other *TEL/PTPRR* isoforms in the inv(12)(p13q13)-carrying leukemic cells, we further did RT-PCR analysis with other combinations of primers. When we used a set of primers TELf1 (in *TEL* exon 1) and PTPRRr7b (in *PTPRR* exon 7), various *TEL/PTPRR* cDNAs of smaller sizes were amplified as well as a full-length cDNA (data not shown). Sequencing analysis showed that exon-skipping mechanisms in the *TEL* gene produced seven isoforms (types 2-8). All these isoforms were also out-of-frame and should express only the NH_2-terminal portion of TEL in which a part or the entire of the helix-loop-helix domain is spliced out. Moreover, using another set of primers TELf3 (in *TEL* exon 3) and PTPRRr10 (in *PTPRR* exon 10), we identified two other isoforms that lacked exon 7 (type 9) or exons 7 and 8 (TEL/PTPRR) of the *PTPRR* gene. Although type 9 isoform again contained an out-of-frame junction, TEL/PTPRR was the unique in-frame isoform with an open reading frame of 1,158 nucleotides coding for 385 amino acid residues that linked the helix-loop-helix domain of TEL and almost the entire protein tyrosine phosphatase domain of PTPRR (Fig. 2*B* and *C*). RT-PCR with a combination of primers PTPRRf10 (in *PTPRR* exon 10) and PTPRRr14 (in *PTPRR* exon 14) amplified only one kind of cDNA that contained exons 10 to 14 of the *PTPRR* gene without any deletions (data not shown), indicating that alternative splicing did not occur in this region. To examine which of these 10 isoforms were dominantly expressed in the leukemic cells, we then made PCR amplification with a set of primers TELf1 and PTPRRr14. Interestingly, two major bands which turned out to be derived from *tTEL* and *TEL/PTPRR* by sequencing analysis were observed (Fig. 2*D*). Thus, we decided to investigate molecular and biological functions of these two isoforms in the following experiments to establish the underlying mechanisms in inv(12)-type leukemia.

Truncated TEL and TEL/PTPRR affect nuclear localization of wild-type TEL. We first induced wild-type TEL, tTEL, or TEL/PTPRR expression in COS-7 cells by transfecting the corresponding cDNAs into them. As previously reported (42), slow-migrating bands were detected in addition to that of the expected size, when wild-type TEL was expressed (Fig. 3*A*, *left*). tTEL and TEL/PTPRR proteins also showed similar slow-migrating bands. When these proteins were metabolically labeled with [^{32}P]orthophosphate, all these size-shifted bands for wild-type TEL, tTEL, or TEL/PTPRR turned out to be hyperphosphorylated forms (Fig. 3*A*, *right*). The lowest band of

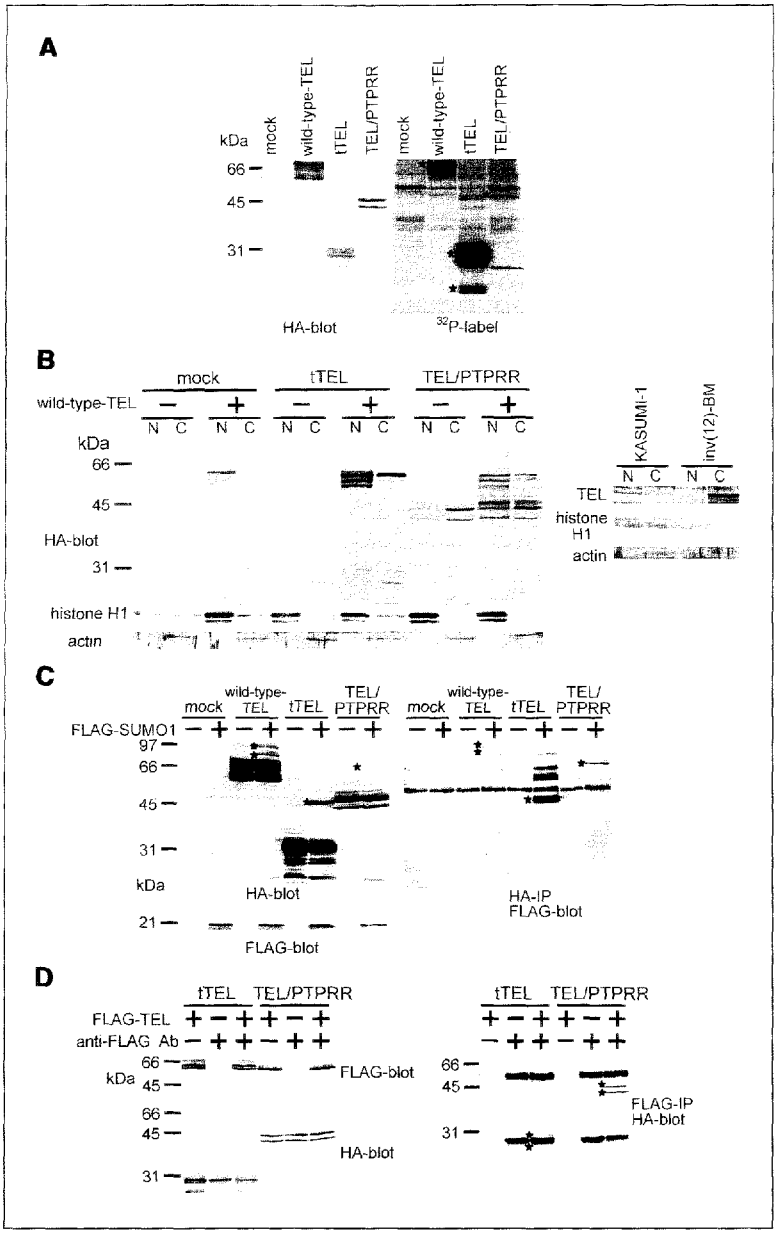

Figure 3. Subcellular localization, SUMO-1 modification, and heterodimerization with wild-type TEL of tTEL and TEL/PTPRR. *A*, truncated TEL and TEL/PTPRR are phosphorylated *in vivo*. *Left*, expression of hemagglutinin-tagged wild-type TEL, tTEL, and TEL/PTPRR in COS-7 cells was confirmed by Western blot analysis with anti-hemagglutinin antibody. *Right*, COS-7 cells expressing each protein were subjected to metabolic labeling with [^{32}P]orthophosphate. The lysates were immunoprecipitated with anti-hemagglutinin antibody. *Asterisks*, phosphorylated wild-type TEL, tTEL, and TEL/PTPRR. *B*, truncated TEL and TEL/PTPRR change subcellular localization of wild-type TEL. *Left*, hemagglutinin-tagged wild-type TEL, tTEL, and TEL/PTPRR were transiently expressed in NIH3T3 cells as indicated. Equal volumes of nuclear (*N*) or cytoplasmic (*C*) fraction were subjected to Western blot analysis with anti-hemagglutinin antibody. *Right*, non-inv(12)-carrying KASUMI-1 cells and inv(12)-carrying leukemic cells were also fractionated and subjected to Western blot analysis with anti-TEL antibody (N-19). Endogenous histone H1 and actin were immunoblotted as nuclear and cytoplasmic markers, respectively. *C*, both tTEL and TEL/PTPRR are sumoylated. *Left*, COS-7 cell lysates expressing hemagglutinin-tagged wild-type TEL, tTEL, or TEL/PTPRR, alone or along with FLAG-tagged SUMO-1, were immunoblotted with anti-hemagglutinin antibody. *Asterisks*, sumoylated wild-type TEL, tTEL, and TEL/PTPRR. Expression of FLAG-tagged SUMO-1 was confirmed by Western blot analysis with anti-FLAG antibody. *Right*, these lysates were subjected to immunoprecipitation with anti-hemagglutinin antibody, followed by Western blot analysis with anti-FLAG antibody. *D*, both tTEL and TEL/PTPRR associate with wild-type TEL *in vivo*. *Left*, COS-7 cell lysates expressing FLAG-tagged wild-type TEL, hemagglutinin-tagged tTEL or TEL/PTPRR, or both FLAG-tagged wild-type TEL and hemagglutinin-tagged tTEL or TEL/PTPRR were immunoblotted with anti-FLAG or anti-hemagglutinin antibody. *Right*, these lysates were subjected to immunoprecipitation with anti-FLAG antibody, followed by Western blot analysis with anti-hemagglutinin antibody. *Asterisks*, hemagglutinin-tagged TEL and TEL/PTPRR.

tTEL was likely to be derived from a degradation product. Given that Ser^{22} in wild-type TEL is a constitutive phosphorylation site (42), these aberrant TEL proteins could have been also phosphorylated at least on the same residue and showed larger-sized bands.

To get some insights into molecular functions of the aberrant TEL proteins, we next examined subcellular localization of tTEL and TEL/PTPRR by Western blot analysis with fractionated lysates overexpressing each protein. We confirmed that specific marker for nuclear or cytoplasmic fraction, histone H1 or actin, was exclusively located in the corresponding fraction (Fig. 3*B*, *left*). Because both tTEL and TEL/PTPRR lack ETS DNA-binding domain of TEL containing nuclear localization signal, and ETS-lacking mutant or isoforms of wild-type TEL have been reported to reside in the cytoplasm (41, 44), it is quite plausible that they show different distribution patterns from that of wild-type TEL. When wild-type *TEL* cDNA was transiently transfected to NIH3T3 cells, overexpressed wild-type TEL protein was predominantly localized in the nucleus. On the other hand, overexpressed tTEL protein was exclusively distributed in the cytoplasm and overexpressed TEL/PTPRR chimeric protein also chiefly resided in the cytoplasm. Surprisingly, when tTEL or TEL/PTPRR was coexpressed with wild-type TEL, all these proteins were almost equally expressed in both fractions. These data suggest that both tTEL and TEL/PTPRR prevent *de novo* translated wild-type TEL from entering the nucleus, whereas wild-type TEL draws tTEL and TEL/PTPRR into the nucleus. We further compared subcellular localization of endogenous wild-type TEL between the inv(12)-carrying M2 leukemic cells and t(8;21)-carrying M2 KASUMI-1 cells. Interestingly, whereas endogenous TEL proteins were predominantly distributed to the nucleus in KASUMI-1 cells, they were exclusively cytoplasmic in the inv(12)-carrying cells (Fig. 3*B*, *right*).

It has been reported that wild-type TEL is sumoylated on Lys^{99} and that sumoylated wild-type TEL is a target of CRM1-mediated nuclear export (44, 45). Because tTEL and TEL/PTPRR