The ets Family Member Tel Binds to the Fli-1 Oncoprotein and Inhibits Its Transcriptional Activity*

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The tel gene, recently shown to be translocated in a spectrum of acute and chronic human leukemias, belongs to the ets family of sequence-specific transcription factors. To determine the role of Tel in normal hematopoietic development, we used the tel gene as the bait in the yeast two-hybrid system to screen a hematopoietic stem cell library. Two partners were identified: Tel binds to itself, and Tel binds to the ets family member Fli-1. In vitro and in vivo assays confirmed these interactions. In transient transfection assays, Fli-1 activates megakaryocytic specific promoters, and Tel inhibits this effect of Fli-1. Transactivation studies using deletion mutants of Tel, and the Tel-AML-1 fusion protein, indicate that the helix-loop-helix domain of Tel only partially inhibits transactivation and that complete inhibition requires the full-length Tel molecule, including the DNA binding domain. The Tel and Fli-1 proteins are expressed early in hematopoiesis, and the inability of Tel fusion proteins such as Tel-AML-1 to counteract Fli-1 mediated transactivation may contribute to the malignant phenotype in human leukemias where this fusion protein is present.

EXPERIMENTAL PROCEDURES

Twoc-hybrid Library Screening—Two-hybrid screen and parent vectors pBTM116 and pVP16 were as described by (8). Plasmid pLexA-Tel-(1–371) was constructed by insertion of the Tel polymerase chain reaction (PCR) product encoding amino acids from 1 to 371 into EcoRI-BamHI sites of pBTM116, resulting in an open reading frame encoding a LexA-Tel fusion protein. Tel was constructed by PCR with the following primers: 5′-CGGAATTCCTGTGAGACTCCTGCTCA-GT-3′ (coding strand) and 5′-CGGGATCCGAGTGGACTGTGTTGGAATATTT-3′ (non-coding strand). The PCR product was cut with EcoRI and BamHI, and the 1125-bp fragment was cloned. The EML-1 cell line cDNA library was amplified once in DH5α and transformed into yeast containing pLexA-Tel-(1–371) (7). Plasmids pVP-Tel-(1–163), pVP-Fli-(32–334), and pVP-Fli-(14–230) were identified as clones that activated lacZ transcription and conferred histidine prototrophy in the presence of pLexA-Tel-(1–571). Binding specificity was confirmed in the mating test with specific bait pLexA-Tel-(1–371) versus nonspecific bait pLexA-Lamin (pBTM116 expressing Lamin fusion protein). Plasmids were sequenced by automatic fluorescent sequencing (Applied Biosystems).

In Vitro Transcription Translation and Protein Binding Assay—In vitro transcription-translation was performed in TNT rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of [35S]methionine (specific activity 37.0 TBq/mmol) (Amersham Pharmacia Biotech). All clones were transcribed under the control of T7 promoter. In each case, 2 μl of a 25 μl translation reaction was analyzed by SDS-PAGE (see Fig. 2A.). Radiolabeled Fli-1 protein (5 μl of translation reaction) was mixed with one of each radiolabeled proteins, PU.1, CD18, CD11a, RARα, Luciferase, or Tel-(1–371) (5 μl of translation reaction in each case) and immunoprecipitated with mouse monoclonal anti-human Fli-1 antibody (PharMingen, San Diego, CA) and assayed by SDS-PAGE followed by autoradiography.

GST Fusion Protein Binding Assay—Plasmids pGST-Fli and pGST-Tel-(1–371) were cloned by linking the full-length coding sequence of Fli-1 protein (amino acids 1–452) and the coding sequence for amino acids from 1 to 371 into pGEX-2T and pGEX-2T-M1, respectively. The GST fusion protein binding assay was performed essentially as described previously (9) using glutathione-agarose beads (Pharmacia Biosystems) and elution buffer (10).

Acute and chronic human leukemias are frequently associated with chromosomal translocations which result in the formation of chimeric proteins. Considerable evidence indicates that these chimeric proteins play a role in transformation (1). At least one member of the chimeric protein is frequently a transcription factor, implicating disordered regulation of target genes as a mechanism of transformation. In most cases the role of these transcription factors in normal hematopoiesis has not been determined.

Recently a member of the ets family of transcription factors, Tel, was identified at the site of chromosomal breakage in chronic myelomonocytic leukemia (CMML) where it forms a chimeric protein with the transmembrane and tyrosine kinase domains of the platelet-derived growth factor receptor β chain (PDGFRβ) (2). Tel has now been shown to be involved in a number of chromosomal translocations in human leukemias. The Tel-AML-1 fusion is present in approximately 40% of cases of childhood pre-B cell acute lymphoblastic leukemia (ALL),1 making it the most common molecular abnormality in childhood cancer (3–5). In a number of cases involving tel translocations, the remaining tel allele is deleted, suggesting that loss of functional Tel may contribute to leukemic transformation.

Tel contains the highly conserved ETS DNA-binding domain at the carboxyl-terminal region as well as a distinct 5′ region with weak homology to the well described helix-loop-helix (HLH) domain (also referred to as the pointed domain) (2). The predicted HLH secondary structure in the amino-terminal region suggests that this region may be involved in protein-protein interactions (6).

To identify potential protein partners of Tel, we screened a hematopoietic stem cell library using Tel in the bait plasmid (7). We identified two partners of Tel, Tel itself, and the ets protein Fli-1.

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‡ The abbreviations used are: ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CMML, chronic myelomonocytic leukemia; ETS, ETS family of transcription factors; Fli-1, Friend leukemia oncogene-1; HLH, helix-loop-helix; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; SCP, stem cell factor; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction.
Fusion proteins were bound to the glutathione-Sepharose by incubating
tions from the GST gene fusion system (Amersham Pharmacia Biotech).
respectively. This
at 4 °C in cell lysis buffer (150 mM NaCl, 20 m M Tris (pH 8.0), 5 m M
hybrid screening.
LexA DNA-binding domain (vector pBTM116). fragments of Tel and
coding sequences from the EML cell line cDNA library in pVP16
vector interacting with the Tel bait clone in yeast. Positive clones
include: 2 clones coding for Tel protein fragment ranging from amino
acids 19 to 163; 2 clones coding for Fli-1 protein fragment amino acids
32–334, and one clone coding for Fli-1 protein region covering amino
acids 14–230. Relative position of the isolated fragments to full-length
proteins is retained. D, schematic representation of full-length Fli-1
protein with the striped bar representing helix-loop-helix domain from
amino acids 106 to 201 and the solid dark bar representing DNA
binding domain (from amino acids 271 to 367).

**Fig. 1.** Tel bait plasmid and Tel partners isolated by two-
hybrid screening. A, schematic of Tel protein; the striped bar indicates
the amino-terminal helix-loop-helix domain (pointed domain) at
amino acids 58–124, and the solid dark bar represents the carboxy-
terminal DNA binding domain at amino acids 340–419. B, diagram of
the bait plasmid consisting of amino acids 1–371 of Tel fused to the
LexA DNA-binding domain (vector pBTM116). C, fragments of Tel and
Fli-1 coding sequences from the EML cell line cDNA library in pVP16

To identify potential partners of Tel in normal hematopoietic
cell development, we used a LexA-Tel fusion protein consisting of
the first 371 amino acids of Tel to screen a yeast two-hybrid
library constructed from a murine pluripotent hematopoietic
cell line (EM2-1) (7). The fragment of Tel used in the bait
plasmid contains the HLH domain. Five positive clones
were identified from 2 × 10^6 transformants screened. Two ets family
members were identified as partners of Tel, Tel itself and Fli-1
(Fig. 1). The two 435-bp Tel cDNA clones that were isolated
include the entire HLH domain, and the three positive clones
coding for Fli-1 also include the HLH domain (Fig. 1) (12). These results
suggest that the amino-terminal HLH domain in each protein is involved in
the interaction.

To confirm the specificity of the Tel/Tel and Tel/Fli-1 interaction,
we conducted both in vitro and in vivo experiments. Initially, we
expressed several proteins known to be involved in
protein-protein interactions using rabbit reticulocyte lysates
(Fig. 2A). These proteins were then used in a co-immunopre-
cipitation assay (Fig. 2B). Monoclonal antibodies directed
against Fli-1 immunoprecipitated the human Tel protein (ami-
no acids 1–371) but none of the control proteins (Fig. 2B, lane
Fli-I + Tel). Additional in vitro experiments addressed the specificity of
protein-protein interactions. In *in vitro* studies,
GST-Fli-I bound to 35S-Met-Tel protein (amino acids 1–371)
but not to control 35S-Met-Luciferase (Fig. 2C). Similarly, GST-
Tel associated with 35S-Met-Tel (1–371 amino acid fragment),
but not with 35S-Met-Luciferase (Fig. 2C).

In *in vivo* experiments confirmed the interaction of Tel and
Fli-1. When the Tel protein containing the Myc domain at its
amino terminus and wild type Tel protein were expressed in
293 cells and immunoprecipitated with anti-Myc antibody, the
anti-Myc antibody immunoprecipitated wild type Tel protein as
well as the Myc-Tel protein (Fig. 2D). Neither mouse nonspe-
specific IgG (Fig. 2 D) nor anti-Myc antibody (data not shown) were able to immunoprecipitate the wild type Tel protein alone. The Tel-Fli-1 interaction in vivo was tested in K562 cells electroporated with expression vectors harboring wild type Fli-1 and Tel protein expression during differentiation of human CD34+ cells. A, cell lysates were made from $5 \times 10^5$ column-purified human CD34+ cells from the indicated day of differentiation. The CD34+ cells were incubated with IL-3, IL-6, SCF, G-CSF, and GM-CSF, and lysates were made from aliquots of cells. The cell lysates were resolved by SDS-PAGE (lyaste made from $2.5 \times 10^5$ cells was loaded in each lane) and blotted onto PVDF (Bio-Rad) membrane. Polyclonal rabbit anti-human Fli-1 antibody was used to detect the presence of the Fli-1 protein. B, cell lysates were processed as in panel A, and blots were detected with polyclonal rabbit anti-human Tel antibody.

amounts of the labeled proteins indicated at the top of each lane were loaded. B, co-immunoprecipitation with anti-Fli-1 antibody. [35S]Met-Fli-1 protein was mixed with [35S]Met-labeled protein indicated and then immunoprecipitated with anti Fli-1 antibody. Precipitated complexes were purified on protein A-Sepharose CL 4B (Amersham Pharmacia Biotech), resolved on SDS-polyacrylamide gel electrophoresis, and identified by autoradiography. The arrow indicates the truncated Tel protein (fragment corresponding to that used as a bait in two-hybrid screening). The control line on the right represents Fli-1 from the TNT system. C, GST fusion protein immunoprecipitation assay using GST-Fli-1 and GST-Tel. In this in vitro system, GST-Fli-1 and GST-Tel (in the GST protein expressing vector pGEX4T1, Amersham Pharmacia Biotech) were incubated with Tel and luciferase labeled using [35S]Met. The interacting complexes were purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech). The bound proteins were resolved on SDS-PAGE followed by autoradiography. D,E, in vivo Myc-Tel-Tel interaction with wild type Tel. The Tel cDNA was cloned into pCS2+MT vector and expressed as an amino-terminal Myc-tagged Tel protein. Tel was also cloned into the pSG5 expression vector with mouse anti-Myc antibody (amyc 9E10, Sigma) and anti-mouse IgG-linked agarose (Sigma). Precipitated complexes were resolved by SDS-PAGE, blotted onto PVDF (Bio-Rad) membrane, and detected with rabbit anti-Myc antibody, which recognizes both wild type and Myc-tagged Tel protein. In the control experiment, proteins were immunoprecipitated with a corresponding amount of nonspecific mouse IgG. Control lane at right of the gel represents corresponding cell lysate (293T cells cotransfected with pCS2+MT-Tel and pSG5-Tel) loaded directly onto the gel. E, in vivo interaction of Fli-1 and Tel. K562 cells were electroporated with pL(Fli-1)SN vector (expressing wild type Fli-1 protein) together with pSG5-Tel vector (expressing wild type Tel protein). Cell lysates were immunoprecipitated with mouse anti-Fli-1 antibody (PharMingen) and washed on protein A-Sepharose. Precipitates were resolved on SDS-PAGE, blotted onto PVDF membrane, and detected with rabbit anti-Tel antibody.
wild type Tel. Cell lysates immunoprecipitated with an anti-Fli-1 antibody revealed the presence of the accompanying Tel protein on Western blots using an anti-Tel antibody (Fig. 2E).

To identify the stage of hematopoietic cell development at which Tel and Fli-1 are expressed, human CD34+ cells were differentiated with IL-3, IL-6, SCF, G-CSF, and GM-CSF, and the appearance of Tel and Fli-1 proteins was detected by Western blotting. Fli-1 protein was expressed at day 0, peaked at day 2 of differentiation, and decreased to undetectable levels by day 4 (Fig. 3A). Interestingly, the Tel protein displayed a similar pattern of appearance with a peak at 2 days of differentiation and a decrease to undetectable levels by day 4 (Fig. 3B).

To determine whether Tel and Fli-1 displayed a functional interaction, the transactivation abilities of Tel and Fli-1 proteins were tested individually and in concert on promoters containing ETS binding sites (Fig. 4A). Recombinant Fli-1 has been shown to bind to DNA in a sequence-specific manner and to transcriptionally activate the platelet glycoprotein IIb promoter (12, 13). In this study, we used the promoters from the related megakaryocytic genes GPIba (14) and GPIX (15) which contain ETS sequences. The GPIba promoter region extended to −567 bp upstream from the transcriptional start site, and the GPIX promoter region covered 203 bp proximal to the transcriptional start site. Transfection of Fli-1 resulted in a 3.5-fold increase of activity of the GPIba promoter and a 7-fold increase in the activity of the GPIX promoter (Fig. 4, B and C). Expression of Tel alone did not activate either promoter; however, co-transfection of Tel with Fli-1 together resulted in complete inhibition of Fli-1-mediated transactivation (Fig. 4, B and C). Transfection of Fli-1, Tel, or the two ets factors together did not transactivate a GPIX promoter construct in which the ETS site was deleted (pGPIX-EETS-Luc) (Fig. 4D). Thus, Tel inhibits Fli-1-mediated transactivation of megakaryocytic promoters

**Fig. 4.** Transactivation of GPIba and GPIX promoters by Fli-1 and Tel proteins in transient transfection experiments. A, schematic representation of the GPIba and GPIX promoter constructs. GPIba promoter spans the proximal 567 bp, with identified ETS and GATA binding sites at positions −150 and −93, respectively. GPIX wild type promoter consisted of −203 bp proximal to transcriptional start site and includes GATA and ETS binding sites at positions −67 and −45, respectively. GPIX(EETS) promoter contains a mutated ETS binding site generated by PCR mutagenesis. B, 293T cells were cotransfected by the calcium phosphate technique. The graph shows luciferase activity (relative light units + S.D.) generated by the constructs divided by the amount of growth hormone constitutively expressed by the pCMV-GH plasmid to normalize for variations in transfection efficiency. The transactivation magnitudes were confirmed in at least three separate experiments with each sample in triplicate. C, the proximal −203-bp GPIX promoter was used in transactivation assay performed as in panel C. D, the effect of disruption of the ETS binding site in −203-bp GPIX promoter on Fli-1 and Tel transactivation abilities.
Tel inhibits Fli-1 transactivation.

As GPIIbα and GPIIb (data not shown), and the ETS binding site in the promoter sequence is required for transactivation.

To analyze the mechanism whereby Tel inhibits Fli-1-mediated transactivation in more detail, we generated deletion mutants of Tel. The Tel H protein consists of the HLH domain (striped bar) and DNA binding domain (solid dark bar); Tel truncation constructs: Tel H and Tel D and Tel-AML-1 protein with its RUNT homology and transactivation domains (as indicated). Each construct plasmid DNA (Fli-1 and Tel constructs expressing) have been used. C, Western blot detection of Fli-1 and Tel constructs expression in transactivation lysates. Bands representing protein expression in each sample have been aligned appropriately to the bar graph representation in panel B.

Tel constructs were tested in transactivation assays with Fli-1 on the GPIX promoter. Transfection of the Tel and Fli-1 constructs were adjusted to achieve expression of equivalent amounts of respective proteins (Fig. 5C). In these experiments, wild type Tel was able to completely abrogate transactivation (Fig. 5B). The Tel H, Tel D, and Tel-AML-1 constructs partially inhibited Fli-1-mediated transactivation by approximately 50%, indicating that protein-protein interaction between Tel and Fli-1 most likely accounts for this effect. The mechanism whereby the Tel D construct inhibits transactivation has not been established. We and others have not been able to demonstrate DNA-binding activity by Tel (data not shown). It is possible that the Tel DNA-binding domain may recruit a generalized transcriptional repressor to the promoter.

To determine whether overexpression of the Tel truncation constructs abrogated Fli-1-mediated transactivation of the GPIX promoter, titration experiments were performed. Transactivation experiments were conducted using increasing molar ratios of Tel expression constructs (Tel, Tel H, Tel D, and Tel-AML-1) to Fli-1 expressing plasmid DNA (Fig. 6). Three sets of transactivations were performed with each ratio of Tel to Fli-1 expressing plasmid DNAs: 1:1, 2.5:1, and 5:1. Each marked curve point represents the average value from three samples.
these studies may not represent the physiological level of the protein in cells from ALL patients because, in at least one ALL cell line expressing Tel-AML-1 protein (the REH cell line), the level of expression of Fli-1 was more than 10-fold higher then the expression of Tel-AML-1 (data not shown). The wild type Tel protein is not expressed by REH cells. In this cell line, it is possible that the low molar ratio of Tel-AML-1 protein to Fli-1 protein expression may not be sufficient to inhibit the activation of Fli-1 target genes in these ALL cells.

Ets family members have been identified at the site of chromosomal translocations in primitive stem cell tumors including leukemia (16). The identification of Fli-1 as a partner of Tel is of interest for several reasons. First, the interaction of Tel with Fli-1 was unexpected since ets family members have previously not been demonstrated to form heterodimers with others ets family members (17). Second, Fli-1 is involved in malignancies not demonstrating to form heterodimers with others ets family members (17). The identification of Fli-1 as a partner of Tel is consistent with the loss-of-function hypothesis of Tel as a result of the generation of the chimeric protein (3–5). Loss of Tel would also result in the loss of its ability to dimerize with Fli-1.

Future studies will address the effect of Tel and Fli-1, and their respective fusion proteins, on the process of malignant transformation.

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