v-SRC Specifically Regulates the Nucleo-cytoplasmic Delocalization of the Major Isoform of TEL (ETV6)*

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TEL is a frequent target of chromosomal translocations in human cancer and an alleged tumor suppressor gene. TEL encodes two isoforms: a major TEL-M1 isoform as well as TEL-M43, which lacks the first 42 amino acid residues of TEL-M1. Both isoforms are potent transcriptional repressors that can inhibit RAS-induced transformation. Here we show that the v-SRC protein-tyrosine kinase relieves the repressive activity of TEL-M1, an activity that is associated with the v-src-induced delocalization of TEL-M1 from the nucleus to the cytoplasm. TEL-M1 delocalization requires the kinase activity of v-SRC and is not induced by oncogenic RAS or AKT. Cytoplasmic delocalization of TEL-M1 in response to v-SRC critically depends upon its unique amino-terminal domain (SRCD domain) because (i) v-SRC did not inhibit the repressive properties of TEL-M43, nor affected TEL-M43 nuclear localization; (ii) fusion of the first 42 amino acid residues of TEL-M1 to FLI-1, an ETS protein insensitive to v-SRC-induced delocalization, is sufficient to confer v-SRC-induced delocalization to this TEL/FLI-1 chimeric protein. The v-SRC-induced nucleo-cytoplasmic delocalization of TEL-M1 does not involve phosphorylation of the SRCD and does not require TEL self-association and repressive domains. Finally, enforced expression of the v-SRC-insensitive TEL-M43, but not of TEL-M1, inhibits v-SRC-induced transformation of NIH3T3 fibroblasts. These results identify a regulatory domain in TEL that specifically impinges on the subcellular localization of its major TEL-M1 isoform. They, furthermore, indicate that inhibition of TEL-M1 nuclear function is required for v-SRC to induce cellular transformation.

TEL, a member of the ETS family of transcriptional regulators, was discovered by virtue of its implication in a number of chromosomal translocations in leukemias and solid tumors (for review, see Ref. 1). These chromosomal translocations result in the fusion of TEL to unrelated genes and the expression of fusion oncoproteins. Moreover, in some of these cases the second allele of TEL was also deleted, suggesting a tumor suppressor function for TEL (2–7).

Despite its frequent involvement in human cancers, little is known about TEL function and regulation. The inactivation of TEL by homologous recombination caused defects in mouse yolk sac angiogenesis and consecutive death of the embryos at E9.5–E11.5 of development (8). Although not essential for fetal liver hematopoiesis, TEL is required for the development of the hematopoietic lineage in adult bone marrow (9). We recently showed that, in contrast to the majority of ETS transcription factors, TEL is a sequence-specific transcriptional repressor (10). This repressive activity is mediated by several functional domains of TEL: (i) its DNA binding domain (ETS domain), which targets TEL to specific response elements (ETS binding sites); (ii) its amino-terminal B/pointed/SAM self-association domain; and (iii) two autonomous repression domains in the central region of the protein. At the molecular level, oligomeric TEL and in particular its central repression domain interacts with several co-repressor complexes including mSin3A, SMRT, N-CoR, L(3)MBT, a member of the Polycomb group of chromatin-associated proteins, and Tip60, indicating that TEL represses transcription through both histone deacetylase-dependent and -independent mechanisms (11–16).

The regulation of TEL activity is, however, still poorly understood. Available evidence indicates that TEL is regulated at the translational and post-translational levels. First, TEL mRNA encodes two naturally occurring isoforms, TEL-M1 and TEL-M43, resulting from the use of two successive translation initiation codons, encoding methionine 1 and 43, respectively (17). These two isoforms are both transcriptional repressors (10) with TEL-M1 being by far the major isoform in a variety of cell types (17). In addition, TEL is modified in vivo by phosphorylation (17) and sumoylation events (18). The role of TEL phosphorylation is still elusive, whereas its sumoylation at a lysine residue in its self-association domain is involved in TEL steady state nucleo-cytoplasmic shuttling and its targeting to specific nuclear structures (TEL bodies (18, 19)).

We report here that the repressive activity of TEL-M1, but not that of TEL-M43, is down-regulated by the protein-tyrosine kinase v-SRC. This effect is associated with the specific, v-SRC-induced nucleo-cytoplasmic delocalization of TEL-M1. v-SRC tyrosine kinase activity is required to induce TEL-M1 delocalization to the cytoplasm but neither the RAS/MEK nor the PI3-kinase/akt pathways, two major pathways activated downstream of v-SRC seem to be involved. Deletion and substitution

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1 The abbreviations used are: PI, phosphatidylinositol; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; SRCD, v-SRC regulated cytoplasmic delocalization; STAT, signal transducer and activator of transcription; MEK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; JNK, c-Jun NH2-terminal kinase.
mutant analyses show that the amino terminus domain of TEL is critical to its v-SRC-induced regulation. Finally, we show that overexpression of the v-SRC-insensitive, nuclear TEL-M43, but not the regulatable TEL-M1, inhibits v-SRC-induced transformation of NIH3T3 fibroblasts.

**EXPERIMENTAL PROCEDURES**

**TEL Mutants and Expression Vector Constructs**—The SV40-based expression plasmids (ΔEβ) encoding TEL-M1, TEL-M3, TEL-ΔB, TEL-ΔC, and FLI-1 have been described previously (10, 20). The pEFBoS-v- SRC, pCDNA3-(HA)-RAS-V12, and pCDNA3-(Myc)-v-AKT expression plasmids were generous gifts of Dr. M. Owen (ICRF, London), Dr. A. Yehuene (CNRB, Paris, France), and Dr. A. Bellacosa (Fox Chase Cancer Center, Philadelphia, PA), respectively. The E74.7k_Luc reporter gene was previously described (10).

Single amino acids point mutants were generated using the QuickChange site-directed mutagenesis kit according to the manufacturer instructions (Stratagene). For the mutagenesis/amplification step, we used the following conditions: 1 cycle of 30 s at 95 °C and 16 cycles of 30 s at 95 °C, 1 min at 55 °C, and 12 min at 68 °C. The amplimers used were: for TEL-M1S22A, 5′-CATATACACTCCAGAGGCCCAAGTGCGAGGATACG-3′ (sense) and 5′-CTGAAATCCTGGCACTGGGGCCTCTGCGAGGTTATAG-3′ (antisense); for TEL-M1(Y17F), 5′-GCGAGAACGAATTTTCTTACCTCAGAGGCCCAGTGCC-3′ (sense) and 5′-GCCCTCTGGCTGAAAAAGGACTCAGCTCTGCG-3′ (antisense); for TEL-M1(Y27F) and TEL-M1(Y17F/Y27F), 5′-GCGAGACTGAGGGTGTTATATG-3′ (sense) and 5′-GGCTCTGGAGGAAGCAAATCTGGCACTGGGC-3′ (antisense); and for v-SrcK7, 5′-CGCAAGTGCTCAAAAGACTGACCGCCAGGCTG-3′ (sense) and 5′-CGCTGCTTGGGCTCAGCTCTG-3′ (antisense). The matrix was ΔE-TEL-M1 for TEL-M1 mutants, ΔE-TEL-M1(Y17F) for the TEL-M1(Y17F) mutant, and pEFBoS-v-Src for the v-SRCΔ mutant. The retrovirual expression vectors pRevTRE-TEL-M1 and pRevTR-E-TEL-M43 were generated by standard molecular biology techniques from pRevTRE (Clontech).

To generate the TEL/FLI-1 substitution mutant, a BamHI and HindIII-fragment encoding FLI-1 amino acids residues 127 to 452 was obtained by PCR amplification of the human FLI-1 cDNA and subcloned into BamHI + HindIII-restricted pRevTRE-TEmod (10). This resulted in the in-frame substitution of the first 126 amino acid residues of FLI-1 for the first 52 amino acids residues of TEL. The amplimers used were: 5′-CGAGTTACG-3′ (antisense) and 5′-CATATACACTCCAGAGGCCCAAGTGCGAGGATACG-3′ (sense); for TEL-M1(Y27F) and TEL-M1(Y17F/Y27F), 5′-GCGAGACTGAGGGTGTTATATG-3′ (sense) and 5′-GGCTCTGGAGGAAGCAAATCTGGCACTGGGC-3′ (antisense); and for v-SrcK7, 5′-CGCAAGTGCTCAAAAGACTGACCGCCAGGCTG-3′ (sense) and 5′-CGCTGCTTGGGCTCAGCTCTG-3′ (antisense). The matrix was ΔE-TEL-M1 for TEL-M1 mutants, ΔE-TEL-M1(Y17F) for the TEL-M1(Y17F) mutant, and pEFBoS-v-Src for the v-SRCΔ mutant. The retroviral expression vectors pRevTRE-TEL-M1 and pRevTR-E-TEL-M43 were generated by standard molecular biology techniques from pRevTRE (Clontech).

**Western Blot Analysis and Used Antibodies**—For immunofluorescence analysis, HeLa cells were grown on glass coverslips and 300 ng of the indicated TEL mutants expression plasmids together with 1 μg of v-SRC, v-SRCK7, (HA)-RAS-V12, or (Myc)-v-AKT expression plasmids co-transfected by the calcium phosphate precipitation method. After 20 h, cells were washed twice and grown for 24 h before analysis. Where indicated, transfected cells were treated with 10 μM LY294002 (50 μM), or with both compounds for 1, 2, 4, 6, 8, or 24 h prior to fixation. Where indicated, transfected cells were treated with leptins (10 μg/mL) or with 1 μg/mL of a rabbit polyclonal antibody to ERK1 (C-16, Santa Cruz; used at 1:100 dilution), a rabbit antibody raised against the amino-terminal half of FLI-1 antigen, a mouse monoclonal antibody to v-SRC (Ab-1, Oncogene Research Products; used at 1:100 dilution); a monoclonal anti-FLI-1 antibodies to FLI-1 (C-19, Santa Cruz; used at 1:100 dilution); or with 0.1% crystal violet (Sigma) in 10% methanol in PBS. Fluorescence was analyzed using a Zeiss epifluorescence microscope.

**RESULTS**

**v-SRC Specifically Inhibits TEL-M1 Transrepressive Activity**—Both the TEL-M1 and TEL-M43 isoforms of TEL are...
TEL Subcellular Localization Is Regulated by v-SRC

**v-SRC Specifically Induces TEL-M1 Nucleo-cytoplasmic De-localization**—To investigate the possible mechanisms by which v-SRC could specifically affect the repressive activity of TEL-M1, we first compared the subcellular localization of TEL-M1 and TEL-M43 both in the absence or presence of v-SRC. HeLa cells were transiently transfected with expression plasmids for either TEL-M1 or TEL-M43 both in the absence or presence of a v-SRC expression vector. The subcellular localization of the respective proteins was analyzed by immunofluorescence using a SRC-specific monoclonal antibody and a rabbit antiserum to the carboxyl-terminal domain of TEL (serum number 68; Ref. 17). In the absence of v-SRC, both TEL-M1 and TEL-M43 localized to the nucleus of transfected cells (Fig. 2A, −v-SRC), as previously reported for the endogenous TEL protein (17). Of note, TEL-M43 formed mostly nuclear, rod-like speckles, whereas TEL-M1 localized as thinner speckles throughout the entire nucleoplasm. Such TEL nuclear bodies have been previously described (18), but their composition and function remain to be characterized. When TEL-M1 was co-expressed with v-SRC, it delocalized from the nucleus to the cytoplasm (Fig. 2A, TEL-M1 + v-SRC). Importantly, only cells co-expressing v-SRC and TEL-M1 showed cytoplasmic TEL-M1, whereas cells from the same co-transfection but only expressing TEL-M1 presented a nuclear TEL-M1 localization (Fig. 2A, TEL-M1 + v-SRC). This shows that v-SRC-induced delocalization of TEL-M1 is an intrinsic consequence of the activity of v-SRC and does not result from a paracrine effect resulting from the production of a diffusible factor by v-SRC-transformed cells. The same results were observed using two other antibodies, namely antibodies 70 and 71 (10, 21) (data not shown), which are directed to the amino-terminal region of TEL. This shows that these observations are not specific to the detection of a particular TEL antigenic epitope. In contrast, co-expression of v-SRC with TEL-M43 failed to promote the subcellular delocalization of TEL-M43 as TEL-M43 remained clearly nuclear in v-SRC expressing cells (Fig. 2A, TEL-M43 + v-SRC).

To investigate whether this specific regulation was not restricted to transiently transfected HeLa cells, stable clones of v-SRC-transformed NIH3T3 cells were generated that expressed either TEL-M1 or TEL-M43 in an inducible fashion, using the tetracyclin (doxycyclin)-regulatable Tet-off system (see below and “Experimental Procedures”). As shown in Fig. 3A, TEL-M1 presented a cytoplasmic localization in the majority of v-SRC NIH3T3 cells, being either almost exclusively in the cytoplasm (Fig. 3, B, C > N; and pink arrow in A), or distributed between nucleus and cytoplasm (Fig. 3, B, N + C; yellow arrow in A). In contrast, TEL-M43 in v-SRC NIH3T3 cells was almost exclusively nuclear (Fig. 3B, N; green arrow in A), accumulating in typical TEL bodies. We conclude from these experiments that v-SRC activates the nucleo-cytoplasmic delocalization of TEL-M1, without affecting the nuclear localization of the TEL-M43 isoform.

To investigate if the nucleo-cytoplasmic delocalization of TEL-M1 in response to v-SRC was mediated by the Crm-1/Exportin-1 receptor, transfected cells were treated for 8 h with leptomycin B, a specific inhibitor of this nuclear export receptor (for review, see Ref. 22). The subcellular localization of TEL-M1 was then determined by immunofluorescence analysis. As shown in Fig. 2B, leptomycin B treatment failed to inhibit the v-SRC-induced nucleo-cytoplasmic delocalization of TEL-M1. In contrast, under these conditions and in line with published data (23), leptomycin B efficiently blocked the Crm-1-dependent nuclear export of endogenous IκBα as evidenced by its nuclear accumulation in leptomycin B-treated cells as com-

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**Fig. 1.** v-SRC specifically inhibits the repressive activity of TEL-M1. A, HeLa cells were cotransfected with 1 μg of the E74,tk6Luc reporter construct together with 300 ng of ΔEB, ΔEB-TEL-M1, or ΔEB-TEL-M43 and 1 μg of pEFBos (−) or pEFBos-v-SRC (+) expressing plasmids. Luciferase activity was assayed 48 h after transfection and the results are expressed in -fold repression relative to the control gene expression vector in the absence or presence of v-SRC. B, schematic description of TEL functional domains and mutants. The ETS domain of TEL is depicted in *hatched black* and the B/Pointed/SAM domain in *black*. The corresponding domain of FLI-1 are in *hatched gray* and *gray*, respectively. The other TEL domains are depicted in *white*, and the ones of FLI-1 in *dotted gray*. Tyrosine to phenylalanine residue mutations are represented by a *black star* and serine to alanine by a *black arrow*. The amino-terminal region of TEL is essential for v-Src to negatively impinge on TEL repressive activity.
TEL Subcellular Localization Is Regulated by v-SRC

**Fig. 2.** The TEL-M1 isoform is specifically delocalized from the nucleus to the cytoplasm in response to v-SRC. A, HeLa cells grown on coverslips were cotransfected with 300 ng of ΔEB-TEL-M1 (TEL-M1) or ΔEB-TEL-M43 (TEL-M43) and 1 μg of either control pEFBos (−v-SRC) or pEFBos-v-SRC (+v-SRC) expressing plasmids. After 48 h, cells were fixed, permeabilized, and analyzed by immunofluorescence using a polyclonal antibody to the carboxyl-terminal half of TEL (serum 68) and a monoclonal antibody to SRC (Ab-1) as primary antibodies. An antibody to rabbit Ig coupled to Texas Red and an antibody to mouse Ig coupled to fluorescein isothiocyanate were used to detect TEL and v-SRC immune complexes, respectively. Nuclei are visualized by Hoescht staining. Pictures were taken using a camera coupled to a fluorescence microscope. In transfected cells co-expressing TEL-M1 and v-SRC, TEL-M1 displayed cytoplasmic delocalization in virtually 100% of v-Src co-expressing cells. In cells expressing TEL-M1 alone, TEL-M1 was almost exclusively nuclear. TEL-M43 was exclusively nuclear in all transfected cells, irrespective of the fact that they co-express v-SRC or not. B, top: HeLa cells transfected as in A and either left untreated or treated for 8 h in the presence of 20 nM leptomycin B (LMB). Cells were processed for immunofluorescence analysis as in A. Bottom, mock transfected HeLa cells were left treated or untreated for 2 h in the presence of 20 nM leptomycin B. Cells were processed for immunofluorescence analysis using a rabbit polyclonal antibody to IκBα and an antibody to rabbit Ig coupled to Texas Red. Nuclei were stained with Hoescht.

pared with its predominantly cytoplasmic localization in untreated cells (Fig. 2B). We conclude from these experiments that the v-Src-induced cytoplasmic delocalization of TEL-M1 is independent of the Crm-1/Exportin-1 nuclear export receptor. TEL-M1 Nucleo-cytoplasmic Delocalization Is Dependent on the Kinase Activity of v-SRC, but Does Not Depend upon Activation of the RAS and PI 3-Kinase Pathways—The tyrosine kinase activity of SRC is essential to many of its functions. However, SRC also functions as an adaptor protein, independently of its protein kinase activity to regulate specific downstream signaling pathways (for reviews, see Refs. 24 and 25).

To study the importance of the tyrosine kinase activity of v-SRC in its ability to induce TEL-M1 delocalization, we generated a kinase-inactive mutant of v-SRC (v-SRCK*) by substituting lysine residue 295 in its ATP binding site by methionine, a mutation that was previously shown to inactivate the tyrosine kinase activity of v-SRC (26).

Expression plasmids for TEL-M1 and either v-SRC or v-SRCK* were co-transfected in HeLa cells, and the subcellular localization of both v-SRC and TEL-M1 analyzed by immunofluorescence analysis. The results of Fig. 4A show that, unlike wild type v-SRC, v-SRCK* was unable to promote the nucleo-cytoplasmic delocalization of TEL-M1.

Two well characterized signaling pathways downstream of the v-SRC tyrosine kinase activity are the RAS/MAP kinase and the PI 3-kinase/AKT pathways, respectively (for reviews, see Refs. 24 and 25). Activation of these pathways has been shown to regulate the subcellular localization of many transcription factors or their cofactors, including specific ETS family members (27–30). We therefore investigated if the subcellular delocalization of TEL-M1 was dependent upon these pathways. HeLa cells were co-transfected with expression plasmids for v-SRC and TEL-M1 and 24 h later, transfected cells were either left untreated or were treated with specific inhibitors of components of these signaling pathways and TEL-M1 subcellular localization was analyzed by immunofluorescence. As shown in Fig. 4B, treatment of cells with UO126, an inhibitor of the MEK/ERK signaling pathway, or LY294002, an inhibitor of PI 3-kinase, or a mixture of both compounds failed to affect the nucleo-cytoplasmic delocalization of TEL-M1 in response to v-SRC.

We also analyzed if activated forms of RAS (RAS-V12) or AKT (v-AKT) were able to induce the nucleo-cytoplasmic delocalization of TEL-M1. HeLa cells were co-transfected with the expression plasmid for TEL-M1 together with expression plasmids for either an HA-tagged version of RAS-V12 or a MYC epitope-tagged form of v-AKT and the subcellular localization of the respective proteins was analyzed by immunofluorescence. As shown in Fig. 4C, neither RAS-V12 nor v-AKT promoted the nucleo-cytoplasmic delocalization of TEL-M1. We conclude from these experiments that the v-SRC-induced delocalization of TEL-M1 is dependent on v-SRC tyrosine kinase activity but does not involve the activation of the RAS/MAPK and PI 3-kinase/AKT pathways.

**The Amino-terminal Domain of TEL Is a Transportable Regulatory Domain**—The results presented so far indicate that an intact TEL amino-terminal domain is essential for its v-SRC-induced subcellular nucleo-cytoplasmic delocalization. We next analyzed whether other remarkable domains of TEL, namely its self-association B/pointed/ISAM domain or its central repressive domain, were also involved in v-SRC-induced delocalization of TEL-M1.

As shown by immunofluorescence analysis, TELΔAB, a monomeric mutant because of deletion of the TEL self-association
domain and TELΔC, in which the central repressive domain was deleted (10) (see Fig. 1B for a schematic description of these mutants) were both nuclear in the absence of v-SRC (Fig. 5A). In contrast, when co-expressed with v-SRC, both TELΔB and TELΔC delocalized from the nucleus to the cytoplasm (Fig. 5A), indicating that neither the TEL self-association, nor its
central repressive domains are required for the v-SRC-induced delocalization of TEL-M1. These data also indicate that the v-SRC-induced delocalization of TEL is not mediated by the co-repressors associated with TEL repression domains.

To analyze whether the v-SRC-regulated activity of the amino-terminal domain of TEL can be transported to another protein, we generated a fusion protein in which the first 127 amino acid residues of TEL (TEL/FLI-1; see Fig. 2B and data not shown). Therefore, the integrity of the tyrosine residues of the SRCD domain of TEL is not essential for its v-SRC-induced regulation.

In the SRCD domain, a serine residue (Ser-22) is found in a consensus sequence motif (PXXS/TP) for phosphorylation by proline-directed serine/threonine kinases (17) and was found to be phosphorylated in vivo by several members of the MAPK family (data not shown). This residue is highly conserved in the TEL proteins from man to zebrafish (31), suggesting that it plays a major role in TEL function. A TEL-M1 mutant was therefore generated in which this serine residue was changed to a non-phosphorylatable alanine (TEL-M1(S22A)) and its repressive activity was relieved in the presence of v-SRC. The results of Fig. 6A show that the repressive activity of TEL-M1(S22A) was not abrogated by the v-SRC-induced nucleo-cytoplasmic delocalization of TEL-M1.

These mutants were next studied for their ability to respond to v-SRC-mediated inhibition of TEL-M1 trans-repressive activity. HeLa cells were transiently transfected with E743tk80Luc reporter together with expression plasmids for proline-directed serine/threonine kinases (17) and was found to be phosphorylated in vivo by several members of the MAPK family (data not shown). This residue is highly conserved in the TEL proteins from man to zebrafish (31), suggesting that it plays a major role in TEL function. A TEL-M1 mutant was therefore generated in which this serine residue was changed to a non-phosphorylatable alanine (TEL-M1(S22A)) and its subcellular localization was analyzed in the absence or presence of v-SRC. As shown in Fig. 6A, this mutation did not abrogate the v-SRC-induced nucleo-cytoplasmic delocalization of TEL-M1.

These mutants were next studied for their ability to respond to v-SRC-mediated inhibition of TEL-M1 trans-repressive activity. HeLa cells were transiently transfected with E743tk80Luc reporter together with expression plasmids for either TEL-M1 or the different TEL-M1 mutants, both in the presence or absence of v-SRC. The results of Fig. 6B show that TEL-M1(Y17F), TEL-M1(Y27F), and TEL-M1(Y17F/Y27F), respectively. These mutants were next analyzed for their subcellular localization either in the absence or presence of v-SRC. All three mutants localized to the nucleus in the absence of v-SRC expression, but presented a nucleo-cytoplasmic delocalization in response to v-SRC (Fig. 6A and data not shown). Therefore, the integrity of the tyrosine residues of the SRCD domain of TEL is not essential for its v-SRC-induced regulation.

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v-SRC differentially affected TEL-M1 and TEL-M43 subcellular localization and repressive properties, we next analyzed whether enforced expression of these TEL isoforms would also differentially inhibit v-SRC-induced transformation.

We made use of the tetracycline-regulatable Tet-Off system to conditionally express either TEL-M1 or TEL-M43 in control and v-SRC-transformed NIH3T3 cells (see “Experimental Procedures”; Fig. 7A). In line with previously reported results (33), induction of the expression of both TEL-M1 and TEL-M43 inhibited the proliferation of control NIH3T3 cells, with TEL-M43 being more potent as compared with TEL-M1 (Fig. 7B, left panel). TEL-M43 also inhibited the proliferation rate of v-SRC-transformed NIH3T3 cells (Fig. 7B, right panel). In contrast, TEL-M1 did not affect the proliferation of v-SRC-transformed NIH3T3 cells (Fig. 7B, right panel). TEL-M43-induced inhibition of proliferation was accompanied by reversion of the transformed phenotype of these cells. Indeed, in the presence of doxycyclin, both 3T3-v-SRC/TEL-M1 and 3T3-v-SRC/TEL-M43 cells failed to express detectable levels of exogenous TEL proteins as compared with parental v-SRC cells (Fig. 7A) and displayed the characteristic fusiform morphology of v-SRC-transformed cells (Fig. 7C). Upon removal of doxycyclin and ensuing induction of the expression of TEL-M1 or TEL-M43 in the respective cultures (Fig. 7A), 3T3-v-SRC/TEL-M1 cells remained morphologically transformed, whereas TEL-M43-expressing cells adopted a flat morphology (Fig. 7C) and restored cell contact inhibition.

Another characteristic of v-SRC-transformed cells is their loss of proper contact inhibition control as evidenced by their ability to proliferate as foci of densely packed cells when grown in the midst of a large excess of normal cells. The differential effect of TEL-M1 and TEL-M43 on v-SRC-induced transformation was therefore assessed on the focus forming ability of v-SRC-transformed cells. Specifically, control v-SRC-transformed cells, v-SRC/TEL-M1, and v-SRC/TEL-M43 NIH3T3 cells were plated together with an excess of normal NIH3T3 cells and cultures were maintained in either the presence or absence of doxycyclin. As expected, whereas NIH3T3 cells formed no foci, v-SRC-transformed cells formed numerous foci under these conditions, irrespective of the presence or absence of doxycyclin (Fig. 8, rows 1 and 2). Both v-SRC/TEL-M1 and v-SRC/TEL-M43 cells formed foci with an efficiency similar to the parental v-SRC cells when maintained in the presence of doxycyclin, i.e. when expression of exogenous TEL proteins is repressed (Fig. 8, rows 3–4, −dox). Induction of TEL-M43 expression severely inhibited the ability of v-SRC-transformed cells to form foci (Fig. 8, rows 5 and 6, −dox). In contrast, under these conditions, the focus forming ability of TEL-M1 expressing cells was essentially unaffected as compared with cells maintained in the presence of doxycyclin (Fig. 8, rows 3–4, −dox) or to v-SRC parental cells (Fig. 8, row 2, −dox).

We conclude from these experiments that in line with its ability to accumulate in the cytoplasm in v-SRC-transformed cells, enforced expression of TEL-M1 does not affect transformation of NIH3T3 by v-SRC. In contrast, expression of the v-SRC-insensitive, nuclear TEL-M43 isoform severely inhibits v-SRC-induced transformation of NIH3T3 fibroblasts.

**DISCUSSION**

TEL is a repressor of ETS binding site-directed transcription activity that (i) depends upon its binding to specific ETS binding sites through its ETS domain; (ii) requires its self-association through its BiPointed/SAM domain; (iii) relies upon autonomous repression domains that ultimately target several co-repressors and repressive mechanisms (10–14). We show here that the amino-terminal 52 residues of TEL (SRCD domain) is a regulatory domain that enables the specific nucleo-cytoplasmic delocalization of TEL-M1, the major isoform of TEL in response to the protein-tyrosine kinase activity of v-SRC. These conclusions rely on the fact that (i) TEL-M1 relocalizes from the nucleus to the cytoplasm in v-SRC expressing cells, an activity that requires a catalytically proficient v-SRC protein; (ii) deletion of known functional domains of TEL-M1, including its self-association and major central repression domains fails to affect its v-SRC-induced relocalization; (iii) in contrast, the absence of the SRCD in TEL-M43, a natural
variant of TEL, results in a protein insensitive to v-SRC-induced nucleo-cytoplasmic relocalization; (iv) fusion of the TEL SRCD to FLI-1, an ETS protein normally insensitive to v-SRC-induced relocalization, is sufficient to induce the relocalization of this chimeric protein from the nucleus to the cytoplasm in response to v-SRC.

TEL belongs to a small group of ETS family proteins with repressor activity that includes *Drosophila melanogaster* YAN/Pokkuri and vertebrate NET and ERF (for review, see Ref. 34). The molecular mechanisms involved in transcriptional repression by these factors are diverse, involving the recruitment of distinct co-repressor complexes (12, 13, 35, 36). However, YAN, NET, and ERF share the property of being effectors of RAS signaling and direct substrates of the MAP kinases ERK and/or JNK (27, 36–40). Direct phosphorylation of these factors by MAP kinases results in their export from the nucleus (27, 39–42). Our results show that unlike YAN or ERF, a constitutively active, oncogenic RAS mutant does not activate the nucleo-cytoplasmic transport of TEL. This indicates that specificity among otherwise ubiquitously expressed ETS family repressors like ERF or TEL involves their differential response to specific signaling events.

Our results show that the nucleo-cytoplasmic relocalization of TEL-M1 by v-SRC does not critically depend upon the activity of MEK or PI 3-kinase pathways, which are known to mediate the nucleo-cytoplasmic relocalization of many transcription factors (27, 28, 39). This indicates that the v-SRC-induced relocalization of TEL-M1 relies upon the activation of other pathway(s), acting downstream of v-SRC. Direct phosphorylation of the TEL-M1 SRCD on tyrosine residues does not appear to be involved because mutation of the tyrosine residues of the SRCD did not affect the v-SRC-induced relocalization of TEL. Indeed, a mutant TEL-M1 in which both serine 22 and these threonine residues were changed for alanine delocalized TEL-M43, but not TEL-M1, is able to inhibit v-SRC-induced transformation of NIH3T3 cells. A, normal NIH3T3 and v-SRC-transformed NIH3T3 cells expressing the iTA were obtained as described under “Experimental Procedures” and transduced by retroviral-mediated gene transfer using Rev-TRE (control), Rev-TRE-TEL-M1, or Rev-TRE-TEL-M43. Cells carrying the iTA and TRE-based transgene were selected in the presence of G418 and hygromycin. Cells were grown in these conditions and in the presence of 1 μg/ml doxycyclin to shut off TRE-driven expression of TEL proteins. To analyze for the expression of the expected TEL isoforms, cells were seeded for 4 days in the absence of G418 and hygromycin and maintained for 4 days in either the absence (even-numbered lanes) or presence (odd-numbered lanes) of doxycyclin. Extracts were prepared and processed for Western blot analyses using either a rabbit antibody to the amino-terminal half of TEL (serum 70) or an anti-ERK antibody to ensure for equal loading of the different samples. Top panels, representative NIH3T3-v-SRC control (number 2), TEL-M1- (number 3) and TEL-M43 (numbers 8 and 18)-expressing clones. Lower panels, representative NIH3T3 control (number 2), TEL-M1- (number 4), and TEL-M43 (number 13)-expressing clones. B, control NIH3T3 and v-SRC-transformed transfectants used in panel A were seeded in growth medium (105 cells/35-mm Petri dish) in the absence of doxycyclin to induce the expression of the respective TEL isoforms. Cells were counted daily for 3 days. The results show the mean ± S.D. in cumulative cell numbers of duplicate samples over time. C, v-SRC-transformed transfectants used above and maintained in either the absence (−dox) or presence (+dox) of doxycyclin were visualized by bright field microscopy and photographed. Top panels, note the fusiform morphology of v-SRC- and v-SRC/TEL-M1-expressing cells and the flat morphology of v-SRC/TEL-M43-expressing cells. Bottom panels, all clones show the fusiform morphology in the absence of TEL expression.
in response to v-SRC.\textsuperscript{2} TEL is subject to other post-translational modifications, in particular sumoylation, which appears to be associated with its accumulation into specific but ill-characterized nuclear bodies (18). Recent studies have shown that the steady state nucleo-cytoplasmic shuttling of TEL involves sumoylation of a lysine residue (Lys-99) in its self-association domain (19). Specifically, mutation of this TEL sumoylation site or mutations elsewhere in the B/Pointed/SAM domain that prevent TEL sumoylation were found to increase the export of TEL in a manner at least partially dependent upon the activity of the Crm-1 export system. The v-SRC-induced nucleo-cytoplasmic relocation of TEL-M1 does not appear to activate this steady state shuttling mechanism because (i) deletion of the self-association domain of TEL did not affect the v-SRC-induced relocalization of TEL (mutant TELΔB; Fig. 5A), whereas it impaired the steady state nuclear export of TEL (19); (ii) unlike steady state nuclear export, the v-SRC-induced relocation of TEL is insensitive to leptomycin B and is therefore probably not critically dependent upon Crm-1. Of note, even in steady state conditions, TEL-M43 is less efficiently exported from the nucleus than TEL-M1, indicating that determinants other than sumoylation of lysine 99 are involved in the control of TEL subcellular localization in these conditions (19).\textsuperscript{2} The extent to which these, possibly Crm-1-independent, determinants overlap with those involved in the v-SRC-induced relocalization of TEL clearly require further analyses. Such a dual mechanism for nuclear export is not uncommon as exemplified by β-catenin nuclear export, which can be exportin-dependent through its interaction with APC or exportin-independent through other unknown mechanism(s) (for review, see Ref. 43 and references therein).

The SRCD domain of TEL contains a lysine residue at position 11 in a context (IKQE) that conforms to the consensus modification motif for sumoylation (44). Further analyses are required to investigate whether TEL is actually sumoylated at this site \textit{in vivo}, whether sumoylation of lysine 11 is regulated in response to v-SRC, and whether such a regulation is involved in v-SRC-induced delocalization of TEL-M1. Such a modification may unmask a nuclear export sequence in the SRCD, or induce the recruitment to the SRCD of either an adaptor protein carrying a nuclear export sequence or of a protein capable of impairing the nuclear import of TEL. Although the SRCD does not contain a classical leucine-containing nuclear export sequence, it carries a hydrophobic stretch that resembles the sequence involved in the nuclear export of histone deacetylase 4 (45). Alternatively, v-SRC signals may impinge not on TEL itself but upon cellular factors interacting with the SRCD to regulate their nucleo-cytoplasmic transport.

TEL, TEL-M1, or TEL-M43 were previously shown to inhibit RAS-induced NIH3T3 transformation and tumorigenesis through mechanism(s) involving TEL direct or indirect transcriptional targets that remain to be identified (32, 33). Our results show that only the constitutively nuclear TEL-M43 inhibits v-SRC-induced transformation of NIH3T3 cells and tumor formation \textit{in vivo} (data not shown), whereas TEL-M1 is unable to do so presumably because of its v-SRC-induced cytoplasmic relocalization. This indicates that inhibition of TEL function in the nucleus is a critical determinant for NIH3T3 cells to be transformed by v-SRC. v-SRC-induced cellular transformation involves both RAS-dependent and -independent events (for review, see Ref. 25). It is therefore possible that inhibition of v-SRC transformation by TEL-M43 results from the inhibition of the same pathways as those involved in RAS-induced transformation. The requirement for transcriptionally active ETS proteins in RAS-induced transformation is well established (46, 47) and TEL could interfere with the activity of these proteins either by direct competition for DNA binding to the promoter region of responsive genes or through direct protein-protein interaction (48). However, in addition to their inhibitory effects on cell transformation both TEL-M1 and TEL-M43 induce cell aggregation of v-RAS-transformed cells (32, 33, 49). In contrast, TEL-M43 induces the spreading of v-SRC-transformed cells and restores contact inhibition (Figs. 7 and 8), suggesting that TEL targets additional pathways in these cells than in RAS-transformed cells. A pathway critical to cell transformation by v-SRC and other oncogenic tyrosine kinases is the direct tyrosine phosphorylation and activation of STAT3, a member of the signal transducer and activator of transcription family of transcriptional regulators (for review, see Refs. 50–52). Interestingly, the sequence of a subset of STAT3 DNA binding sites (53) overlaps with the consensus DNA binding sites of TEL (17), suggesting that TEL could interfere with the regulation of a subset of the STAT3 target genes essential to v-SRC transformation. This notion is in accordance with the fact that TEL-M43 but not TEL-M1 inhibits the v-SRC-induced activation of a STAT3-specific reporter gene in transient transfection assays.\textsuperscript{3}

The ability of v-SRC to specifically relocalize the major TEL isoform out of the nucleus and the fact that a v-SRC-insensitive, constitutively nuclear TEL mutant inhibits v-SRC transformation suggests that inhibition of TEL function is essential for v-SRC to transform cells. In addition to be the target of >15

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\textsuperscript{2} R. G. Lopez, C. Carron, and J. Ghysdael, unpublished observations.
chromosomal translocations in acute leukemias and sarcomas to generate dominant fusion oncoproteins (for review, see Ref. 1), loss or mutation of the nontranslocated TEL allele has been reported in several of these cases (2, 4, 54, 55). This, together with the fact that the 12p13 region including the TEL locus frequently shows LOH in leukemia (Ref. 56 and references therein) suggest that TEL might have a tumor suppressor function. The observation that overexpressed TEL can revert oncogenic RAS-mediated transformation and constitutively nuclear TEL interferes with v-SRC-mediated transformation lend further support to this notion and suggest that inhibition of TEL function in cancer can take forms other than deletion or mutation of its gene.

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