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Functional regulation of TEL by p38-induced phosphorylation

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Abstract

TEL is a nuclear phosphoprotein that belongs to a member of the ETS family transcription factors. TEL acts as a tumor suppressor and is essential for establishing hematopoiesis in neonatal bone marrow. Because TEL possesses multiple putative mitogen-activated protein (MAP) kinase phosphorylation sites, we here investigated functional regulation of TEL via stress signaling pathways. We showed that TEL becomes phosphorylated in vivo by activated p38 but not by JNK1. The constitutive and inducible phosphorylation sites were found to be Ser^{22} and Ser^{257}, respectively. TEL bound to p38 and was directly phosphorylated in vitro by p38. In vivo p38-dependent phosphorylation reduced trans-repressional abilities of TEL through ETS-binding consensus site. These data indicate that TEL's functions are potentially regulated by p38 which is activated by various kinds of stresses. TEL could be a constituent downstream of the specific MAP kinase in the signal transduction system.

Keywords: JNK; Leukemia; MAP kinase; p38; Stress signal; TEL; Transcription factor; Translocation; Tumor suppressor

TEL is a member of the ETS family transcription factors [1] that acts as a transcriptional repressor [2,3]. TEL contains two functional domains: the helix–loop–helix (HLH) domain (also called the pointed domain) at the N-terminus, and the ETS domain at the C-terminus. The HLH domain is necessary for homodimerization [4] and heterodimerization with other ETS family members such as FLI-1 [2,5] or TEL2 [6,7]. The ETS domain is responsible for DNA binding to the ETS-binding consensus site (EBS) that contains a purine-rich GGAA/T core motif. Being a transcriptional repressor, TEL is known to interact with relevant cofactors mSin3A and N-CoR and also directly bind to histone deacetylase (HDAC)-3 [8]. By interacting with HDAC directly or indirectly, TEL is believed to mediate transcriptional repression of target genes such as *FLI-1* [3], *Id1* [9], and *stromelysin-1* [10].

The *TEL* gene, mapped at 12p13, was originally identified as a fusion partner for the platelet-derived growth factor receptor β (*PDGFRβ*) gene in the t(5;12)(q33;p13) translocation found in chronic myelomonocytic leukemia [1]. Since then, various 12p13 translocations, involving the *TEL* gene and thus generating the *TEL*-related chimeric genes, have been discovered in many types of hematological malignancies. In some translocations, receptor-type or non-receptor-type tyrosine kinases are fused to the N-terminal portion of TEL and thus activated by homodimerizing through the HLH domain in the TEL moiety. Examples include PDGFRβ in t(5;12)(q33;p13) [11], ABL in t(9;12)(q34;p13) [12], JAK2 in t(9:12)(p24;p13) [13], and Syk in t(9;12)(q22;p13) [14]. In other cases, transcription factors are functionally modified by fusing with the N- or C-terminal part of TEL. Examples are shown in AML1 in t(12;21)(p13;q22) [15–17] and MN1 in t(12;22)(p13;q11) [18]. Thus, perturbing original functions of the partner genes could be a mechanism of causing leukemia in patients with such translocations.

TEL is widely expressed in embryonic and adult tissues. TEL knockout mice die between E10.5 and E11.5 with defective yolk sac angiogenesis and intraembryonic apoptosis of mesenchymal and neural cells, though hematopoiesis in the yolk sac is unaffected [19]. Because of

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the embryonic lethality, roles of TEL in fetal liver and after-birth bone marrow hematopoiesis are not fully understood. However, the analysis of chimeric mice with TEL(−/−) ES cells showed that TEL is dispensable for the intrinsic proliferation and differentiation of adult-type hematopoiesis in the yolk sac and fetal liver, but is essential for the establishment of after-birth hematopoiesis for all lineages in the bone marrow [20].

p38 mitogen-activated protein (MAP) kinase and c-Jun N-terminal kinase (JNK) are members of stress-activated protein kinase subfamily lying within the larger MAP kinase superfamily [21]. They are signaling molecules at the heart in the pathways that govern inflammation, cell differentiation, and cell growth and death in diverse cell types [22–24], and are activated by a wide range of stimuli such as ultraviolet (UV), irradiation, heat shock, high osmotic stress, proinflammatory cytokines, and certain mitogens. Several nuclear transcription factors have been identified as in vivo substrates for the MAP kinases, molecular functions of which are altered through phosphorylation. TEL is also a nuclear phosphoprotein that possesses multiple putative MAP kinase phosphorylation sites [2]. However, functional significance of the phosphorylation has not yet been elucidated. In the present study, we investigated the regulation of TEL's functions through MAP kinase-induced phosphorylation in the stress signals. TEL became phosphorylated in vivo by p38 but not by JNK1 on one serine residue in the internal domain between the HLH and ETS domains. The p38-dependent phosphorylation attenuated molecular functions of TEL. This is the first report describing a regulatory mechanism of TEL via the signal transduction pathway.

Materials and methods

Plasmid construction. Flag tag (DYKDDDDK) was created upstream from the translation initiation site of wild-type-TEL cDNA by the method of PCR amplification. The resultant cDNA was inserted into the *Eco*RI site of pME18S plasmid [25] in the sense orientation to give pME18S-FLAG-TEL. To construct the deletion mutants, new restriction sites, *Pma*CI (190) and *Sma*I (403) for ΔHLH-TEL, *Sma*I (403) for Δ5′ID-TEL, and *Pst*I (1040) and *Hin*cII (1282) for ΔETS-TEL (numbers adopted by Golub et al. [1]) were created using Chameleon Double-Stranded Site-Directed Mutagenesis Kit (Stratagene) in FLAG-tagged wild-type-TEL cDNA cloned into the pBluescript SK plasmid (Stratagene). FLAG-tagged ΔHLH-TEL and Δ5′ID-TEL cDNAs were generated by deleting internal fragments from mutagenic *Pma*CI (190) to mutagenic *Sma*I (403) and from mutagenic *Sma*I (403) to *Pvu*II (826), respectively. To construct FLAG-tagged ΔETS-TEL cDNA, an internal fragment from mutagenic *Pst*I (1040) to mutagenic *Hin*cII (1282) was deleted by digesting with the corresponding enzymes, blunting with T4 DNA polymerase, and religating. In FLAG-tagged ΔHLH + 5′ID-TEL and ΔHLH + ID-TEL cDNAs, ΔHLH + 5′ID (190–642) and ΔHLH + ID (190–873) regions were deleted, respectively. These mutated cDNAs were inserted into the *Eco*RI site of pME18S. The TEL mutants S22A, S213A, S238A, S257A, and S22A/S257A were obtained by replacing the serine residues with alanines with the site-directed mutagenesis as described above. The FLAG-tagged wild-type-TEL cDNA was also cloned into the *Eco*RI site of pGEX-1 (Pharmacia). pcDL-pSRα456-HA-p38 [26] and pSRα-HA-JNK1 [27] were described previously. The reporter plasmid $(EBS)_3$tkLuc was generated by introducing double-stranded oligonucleotides containing three tandemly repeated EBS (5′-ATAAACA GGAAGTGG-3′) [28] upstream of the *tk* promoter in tkLuc plasmid.

Cell culture. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). HeLa cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% FCS. To activate p38 or JNK1, COS-7 cells were treated with 0.7 M NaCl for 30 min or 60 J/m^2 UV.

Western blot analysis and immunoprecipitation for metabolic labeling. COS-7 cells were transfected with FLAG-tagged TEL expression plasmid alone or in combination with p38 or JNK1 expression plasmid by the DEAE–dextran method as described previously [29]. Western blot analyses were performed as described previously [30], using anti-FLAG M2 (Sigma–Aldrich), anti-p38 (Santa Cruz Biotechnology), or anti-HA (BabCO) antibody. The blots were visualized by using Pro-blot AP system (Promega). For metabolic labeling, COS-7 cells were cultured for 48 h after transfection in DMEM containing 10% FCS, transferred to methionine- or phosphate-free DMEM supplemented with 10% FCS (dialyzed against 150 mM NaCl) plus 100 μCi/ml [^{35}S]methionine (Tran-^{35}S label; ICN) or 400 μCi/ml of [^{35}P]phosphate (Phosphorus-32; Amersham), and then cultured for more 3–4 h. After that, they were either left untreated or treated with 0.7 M NaCl for 30 min and harvested in 750 μl lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.05% sodium dodecyl sulfate, 1% deoxycholic acid, 1% Triton X-100, 500 U/ml Ulinastatin, and 2 mM phenylmethylsulfonyl fluoride) per 100 mm-diameter culture dish. Immunoprecipitation was carried out with anti-FLAG M2 antibody conjugated with protein G–Sepharose (Pharmacia) and immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequent autoradiography, by the standard methods [31].

In vitro kinase and pull-down assays. GST, GST–c-Jun, and GST–wild-type-TEL proteins were expressed in the bacterial strain BL21 and purified using glutathione–Sepharose beads (Pharmacia), as described previously [32]. For an in vitro kinase assay, COS-7 cells that were transfected with p38 or JNK1 expression plasmid were incubated in DMEM containing 10% FCS for 48 h, then left untreated or treated with 0.7 M NaCl for 30 min or 60 J/m^2 UV, and harvested. Cell lysates were incubated with anti-p38 or anti-HA antibody conjugated with protein G–Sepharose, subsequently immunoprecipitated, and subjected to an in vitro kinase reaction with ATF2 (Sigma–Aldrich), GST–c-Jun, GST, or GST–wild-type-TEL fusion protein as a substrate, as described previously [33]. SB203580 (CN Biosciences Inc.) was used as a p38-specific inhibitor. For a pull-down assay, GST or GST–wild-type-TEL fusion proteins were collected on glutathione–Sepharose beads, incubated for 3 h with cell lysates containing endogenous or overexpressed p38, and analyzed by SDS–PAGE.

Luciferase assay. HeLa cells were transfected with 1 μg $(EBS)_3$tkLuc reporter plasmid, containing the *Photinus luciferase* gene (Promega), alone or along with 0.5–1.0 μg TEL expression plasmid with or without p38 expression plasmid by lipofectin method using Tfx-20 (Promega). To activate endogenous p38, TEL-transfected HeLa cells were incubated in either 0.7 M NaCl for 30 min, MEM without FCS for 8 h, MEM (10% FCS) plus 10 μg/ml anisomycin (Sigma) for 3 h, or MEM (10% FCS) plus 50 μg/ml sodium *m*-arsenite (Sigma) for 12 h before harvest. To keep the transfection efficiency as constant as possible among the samples to be compared, the total amount of DNA in terms of weight was adjusted to be equal by adding the expression plasmid pME18S. Luciferase assays were performed using Dual-Luciferase Reporter Assay System (Promega). Twenty ng pRL-CMV plasmid (Promega) was cotransfected as an internal control of transfection efficiency and the data were normalized to the *Renilla* luciferase activity. All transfection experiments were performed at least twice and similar results were obtained.

Results

TEL is phosphorylated in vivo with dependence on the activation of p38

MAP kinase-induced phosphorylation of several transcription factors is frequently detected as size-shifted bands of the proteins in SDS–PAGE [34–38]. The starting point of this study was the observation that TEL shows a faint size shift in Western blot analysis with anti-FLAG antibody when COS-7 cells transfected with FLAG-tagged TEL expression plasmid are stimulated with hypertonic saline (0.7 M NaCl) but not with UV stress (Fig. 1A, lanes 3 and 7). To clarify a role of TEL in stress signaling pathways, we examined whether TEL becomes phosphorylated by the activation of p38 or JNK1. First, kinase activities of endogenous or overexpressed p38 and JNK1 were evaluated in COS-7 cells by an in vitro kinase assay with immunoprecipitates with the corresponding antibodies (anti-p38 and anti-HA antibodies) and their known substrates (ATF2 for p38 and c-Jun for JNK1) (Fig. 1B). Endogenous p38 was slightly activated upon the hypertonic stimulation, as judged by the phosphorylation status of ATF2. The most prominent p38 kinase activity was detected when p38-transfected COS-7 cells were treated with hypertonic saline. A p38 inhibitor SB203580 [39] markedly repressed its kinase activity. On the other hand, UV stress stimulated overexpressed but not endogenous JNK activity. We thus concluded that osmotic and UV stresses potentiate the kinase activities of overexpressed p38 and JNK1 in COS-7 cells, respectively. Therefore, TEL seems to be phosphorylated through the activation of p38 but not through that of JNK. To confirm in vivo phosphorylation of TEL proteins by activated p38, we next employed [^{35}S]methionine and [^{35}P]orthophosphate labelings. FLAG-tagged TEL was transiently expressed with or without overexpressed p38 in COS-7 cells and immunoprecipitated with anti-FLAG antibody. When COS-7 cells were labeled with [^{35}S]methionine, we observed two TEL-derived bands (a broad slow-migrating and a narrow fast-migrating bands) without the hypertonic treatment, and a size-shifted band with the treatment (Fig. 1C). When [^{32}P]orthophosphate labeling was carried out with COS-7 cells, the slow- and fast-migrating bands before the hypertonic treatment turned out to be derived from phosphorylated and unphosphorylated forms, respectively. The shifted-band after the treatment was derived from hyper-phosphorylated forms. These data indicate that approximately two-thirds of overexpressed TEL molecules are constitutively phosphorylated without p38 activation and almost all are inducibly hyper-phosphorylated upon p38 activation.

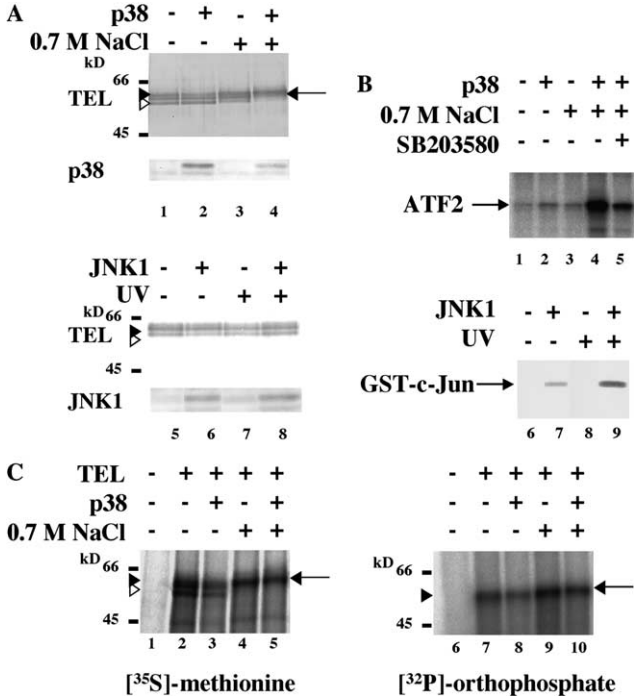

Fig. 1. (A) TEL shows a size shift upon activation of p38 but not JNK1. COS-7 cells (1.0×10^6) were transfected with 5 μg pME18S-FLAG-TEL alone (lanes 1, 3, 5, and 7) or in combination with 5 μg pcDL-SRα456-HA-p38 (lanes 2 and 4) or pSRα-HA-JNK1 (lanes 6 and 8), and left untreated (lanes 1, 2, 5, and 6) or treated with 0.7 M NaCl for 30 min (lanes 3 and 4) or 60 J/m^2 UV (lanes 7 and 8). Western blot analyses were performed with anti-FLAG M2, anti-p38 or anti-HA antibody. (B) p38 and JNK1 activities in COS-7 cells. COS-7 cells (1.0×10^6) were not transfected (lanes 1, 3, 6, and 8) or transfected with 5 μg pcDL-SRα456-HA-p38 (lanes 2, 4, and 5) or pSRα-HA-JNK1 (lanes 7 and 9), and left untreated (lanes 1, 2, 6, and 7) or treated with 0.7 M NaCl for 30 min (lanes 3, 4, and 5) or 60 J/m^2 UV (lanes 8 and 9). In vitro kinase assays were performed with ATF2 (lanes 1–5) or GST–c-Jun (lanes 6–9) as a substrate. A p38-specific inhibitor SB203580 was also added to an in vitro kinase reaction (lane 5). (C) [^{35}S]Methionine (lanes 1–5) and [^{32}P]orthophosphate (lanes 6–10) labelings of TEL proteins. COS-7 cells (1.0×10^6) were not transfected (lanes 1 and 6) or transfected with 5 μg pME18S-FLAG-TEL alone (lanes 2, 4, 7, and 9) or together with 5 μg pcDL-SRα456-HA-p38 (lanes 3, 5, 8, and 10), subjected to metabolic labeling, and left untreated (lanes 1–3 and 6–8) or treated with 0.7 M NaCl for 30 min (lanes 4, 5, 9, and 10), and immunoprecipitated with anti-FLAG M2 antibody. The open arrowheads, solid arrowheads, and solid arrows in (A) and (C) indicate unphosphorylated, phosphorylated, and hyper-phosphorylated forms of TEL, respectively. Positions of size markers (in kilodaltons) are shown.

Ser^{257} is a major p38-dependent phosphorylation site

Regardless of the presence or absence of exogenous p38, overexpressed TEL proteins were detected as three differently migrating bands in COS-7 cells without the hypertonic stimulation, by using Western blot analysis with anti-FLAG antibody (Fig. 1A, lanes 1 and 2). Compared with the results from the [^{35}S]methionine and [^{32}P]orthophosphate labeling experiments, two slow-

migrating bands were considered to be derived from phosphorylated forms and a fast-migrating band from unphosphorylated forms. When TEL was coexpressed with exogenous activated p38, a retarded band was observed (lane 4), mirroring hyper-phosphorylated forms. In order to determine phosphorylation sites in TEL, a set of deletion mutants shown in Fig. 2A were expressed with p38 in COS-7 cells and subsequently analyzed for size shifts after the hypertonic stimulation in SDS–PAGE. When three types of TEL mutants (ΔHLH-, ΔHLH + 5′ID-, and ΔETS-TEL) were expressed in the presence of activated p38, retarded bands were induced with almost the same pattern as in wild-type-TEL (Fig. 2B). However, we did not detect such a size shift induction by p38 when ΔHLH + ID- and Δ5′ID-TEL were expressed. These data suggest that major p38-dependent phosphorylation sites exist within the region of amino acids 206–267 in TEL. We cannot completely rule out the existence of other phosphorylation sites outside this region, because phosphorylation on some residues could not be detected as size shifts in SDS–PAGE.

Ser/Thr–Pro is a minimal consensus sequence for phosphorylation by all MAP kinases [21]. Thus, there are three potential phosphorylation sites (Ser^{213}, Ser^{238}, and Ser^{257}) within the region identified above, if TEL is directly phosphorylated by p38 (Fig. 3A). To determine whether these candidate sites become phosphorylated depending on p38 activation, we replaced each serine residue with alanine by in vitro mutagenesis and made the TEL mutants S213A, S238A, and S257A (Fig. 3B). In addition, we also constructed S22A mutant because Ser^{22} is equivalent to Thr^{38} in ETS1 and Thr^{72} in ETS2 [40] that are phosphorylated by another MAP kinase ERK [41,42]. COS-7 cells were again transfected with each TEL mutant expression plasmid alone or in combination with p38 expression plasmid, and left untreated or stimulated with the osmotic stress. All of these mutants gave equivalent levels of expression (Fig. 3C). Although S213A, S238A, and S257A mutants showed the slow- and fast-migrating bands that could correspond to the phosphorylated and unphosphorylated forms as wild-type-TEL before the stimulation, S22A mutant revealed only the fast-migrating band. It is conceivable that almost two-thirds of overexpressed wild-type-TEL molecules are constitutively phosphorylated on Ser^{22} without p38 stimulation. S213A and S238A mutants showed the same pattern of shifted bands as wild-type-TEL after the hypertonic treatment. In contrast, S257A mutant hardly induced the band shift by the p38 activation. Thus, Ser^{257} is a major phosphorylation site depending on p38 activation, but Ser^{213} and Ser^{238} are not detectable phosphorylation sites. S22A mutant that has Ser^{257} gave a slightly shifted band after the p38 activation. A double mutant S22A/S257A showed only the fast-migrating band either before or after the p38 activation and was thought to remain unphosphorylated. Taken together, we conclude that Ser^{22} and Ser^{257} in TEL are constitutive and p38-inducible phosphorylation sites, respectively.

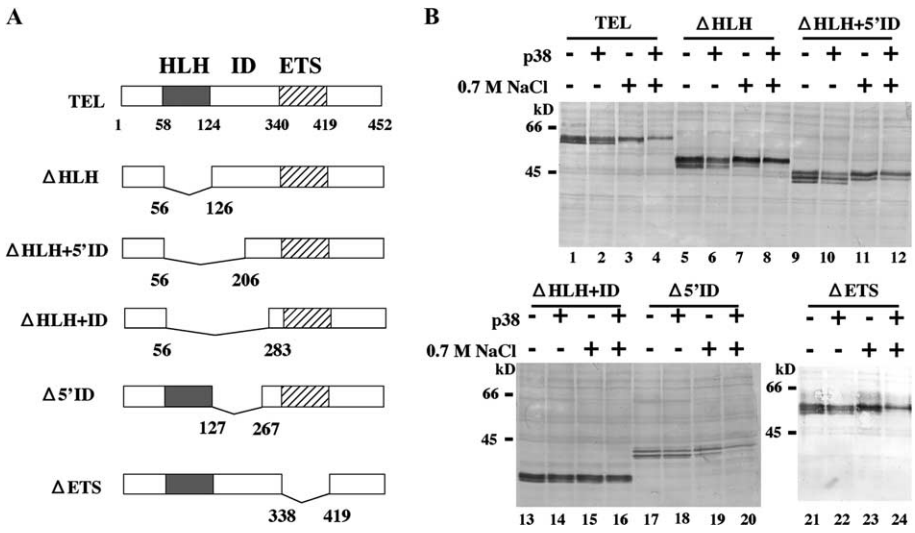

Fig. 2. (A) Structures of TEL deletion mutants are schematically presented. The HLH and ETS domains (see text) are shown by shaded and hatched boxes, respectively. Numerals show amino acid numbers in TEL protein. (B) p38-dependent size shifts of wild-type-TEL and its deletion mutants. COS-7 cells (1.0×10^6) were transfected with 5 μg pME18S-FLAG-TEL (lanes 1–4), pME18S-FLAG-ΔHLH-TEL (lanes 5–8), pME18S-FLAG-ΔHLH + 5′ID-TEL (lanes 9–12), pME18S-FLAG-ΔHLH + ID-TEL (lanes 13–16), pME18S-FLAG-Δ5′ID-TEL (lanes 17–20), or pME18S-FLAG-ΔETS-TEL (lanes 21–24) alone (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23) or in combination with 5 μg pcDL-SRα456-HA-p38 (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24), left untreated (lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, and 22) or treated with 0.7 M NaCl for 30 min (lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, and 24). Western blot analyses were performed with anti-FLAG M2 antibody. Positions of size markers (in kilodaltons) are shown.

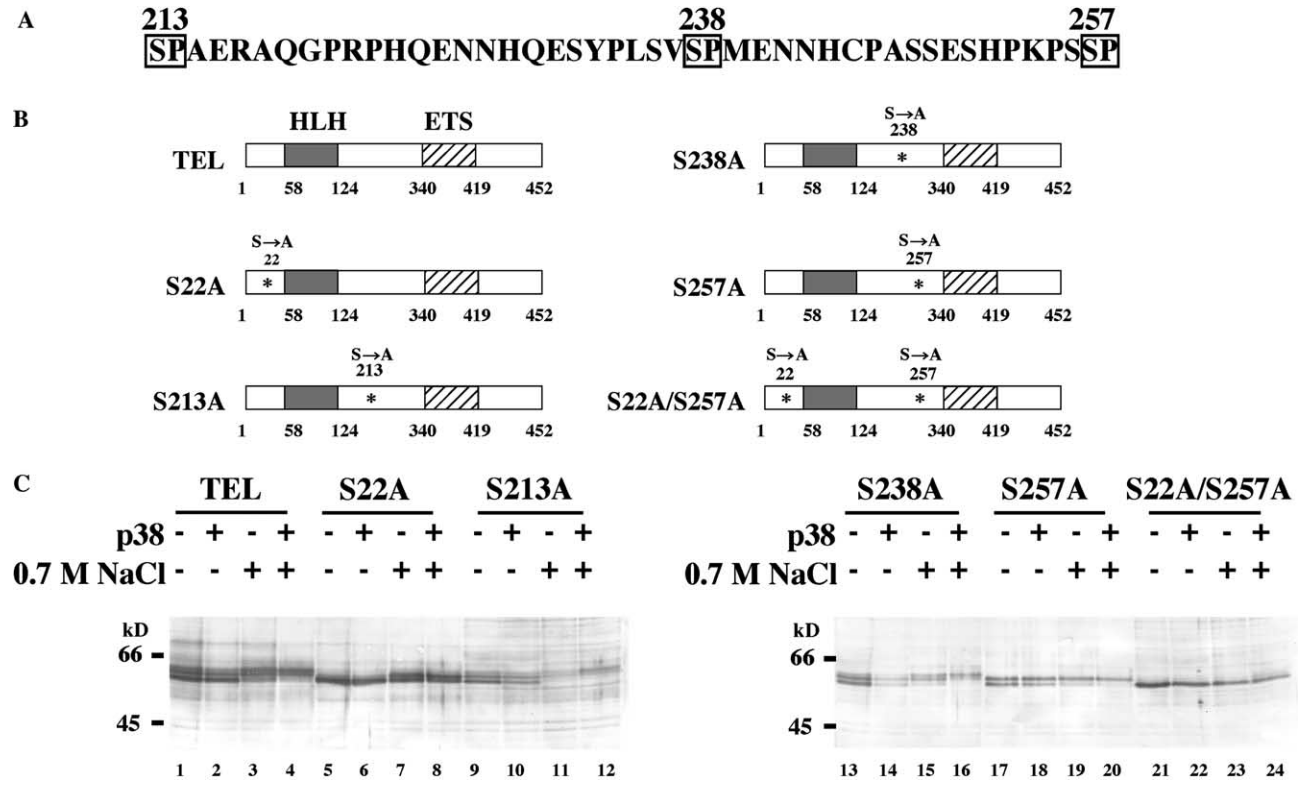

Fig. 3. (A) Potential phosphorylation sites by p38 in TEL. Potential phosphorylation sites that meet a minimal consensus Ser/Thr–Pro and reside within amino acid 206–267 are boxed. Numerals show amino acid numbers in TEL protein. (B) Structures of alanine mutants. The potential serine residues for phosphorylation were replaced with alanines. Asterisks show positions of the replaced serine residues. S and A indicate serine and alanine, respectively. (C) p38-dependent size shifts of wild-type-TEL and its alanine mutants. COS-7 cells (1.0×10^6) were transfected with 5 μg pME18S-FLAG-TEL (lanes 1–4), pME18S-S22A (lanes 5–8), pME18S-S213A (lanes 9–12), pME18S-S238A (lanes 13–16), pME18S-S257A (lanes 17–20), or pME18S-S22A/S257A (lanes 21–24) alone (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23) or in combination with 5 μg pcDL-SRα456-HA-p38 (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24), and left untreated (lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, and 22) or treated with 0.7 M NaCl for 30 min (lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, and 24). Western blot analyses were performed with anti-FLAG M2 antibody. Positions of size markers (in kilodaltons) are shown.

TEL is directly phosphorylated by p38 in vitro

It is quite likely that TEL is directly phosphorylated by p38, since the identified phosphorylation residue Ser^{257} meets the minimal consensus sequence (Ser/Thr–Pro) for phosphorylation by p38. In order to confirm direct phosphorylation of TEL by p38, a fusion protein between GST and wild-type-TEL was produced in *Escherichia coli*, affinity purified, and used as a substrate for an in vitro p38 kinase assay. p38 expressed in COS-7 cells was immunoprecipitated with anti-p38 antibody and subjected to the assay in the presence of an equivalent amount of GST–wild-type-TEL. Prior to the assay, we observed that GST was not phosphorylated by p38 in vitro (data not shown). In contrast, GST–wild-type-TEL became phosphorylated in vitro by endogenous or overexpressed p38 activated with the hypertonic stimulation (Fig. 4). It is conceivable that the Ser^{257} residue in TEL is a direct target for p38-induced phosphorylation.

p38 physically interacts with TEL

MAP kinases, including p38, have been reported to physically interact with some of their substrates [27,38,43–46]. Thus, we investigated whether p38 asso-

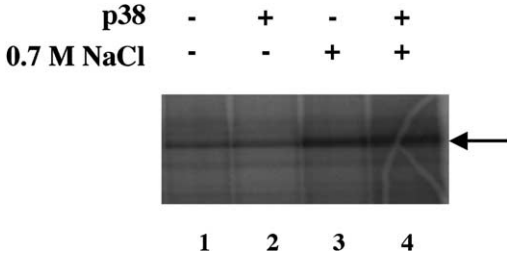

Fig. 4. In vitro p38 kinase assay with wild-type-TEL as a substrate. COS-7 cells (1.0×10^6) were not transfected (lanes 1–3) or transfected with 5 μg pcDL-SRα456-HA-p38 (lanes 2–4), left untreated (lanes 1 and 2) or treated with 0.7 M NaCl for 30 min (lanes 3 and 4), immunoprecipitated with anti-p38 antibody, and subjected to in vitro kinase assays with purified GST–wild-type-TEL protein as a substrate. The arrow indicates phosphorylated GST–wild-type-TEL fusion protein.

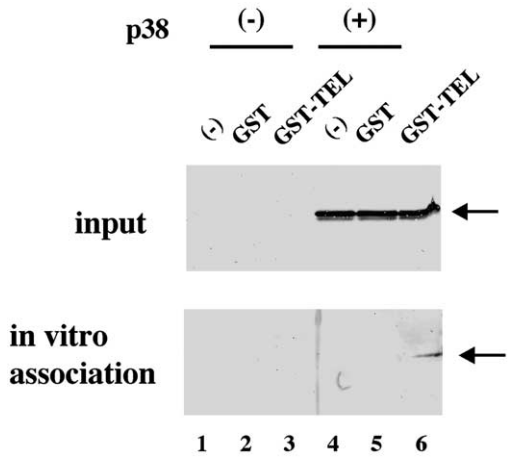

Fig. 5. Physical interaction between p38 and wild-type-TEL. COS-7 cells (1.0×10^6) were not transfected (lanes 1–3) or transfected with 5 μg pcDL-SRα456-HA-p38 (lanes 4–6), harvested, and mixed with GST (lanes 2–5) or GST–wild-type-TEL (lanes 3–6) conjugated with glutathione–Sepharose beads, or glutathione–Sepharose beads alone (lanes 1–4). Western blot analyses were performed with anti-p38 antibody to detect p38 proteins expressed in COS-7 cells (upper panel) and bound to GST–wild-type-TEL (lower panel). The arrows indicate p38 protein.

ciates with TEL. For this purpose, p38 was overexpressed in COS-7 cells and its association with immobilized GST–wild-type-TEL or GST as a control was examined. p38 significantly associated with GST–wild-type-TEL but not with GST (Fig. 5). We concluded that p38 phosphorylates TEL by binding to it.

p38-dependent phosphorylation reduces trans-repression by TEL

In order to get insights into functional modification of TEL through p38-induced phosphorylation, we examined whether the phosphorylation alters trans-repressional abilities of TEL through EBS. HeLa cells were used in this experiment because they have high endogenous activities for EBS-mediated transcription [3]. We have previously observed that TEL represses the transcription driven through $(ETS)_3$tkLuc reporter plasmid containing three tandem repeats of EBS [47] in dose- and sequence-dependent manner in these cells (in submission). Luciferase assays with $(ETS)_3$tkLuc reporter were performed

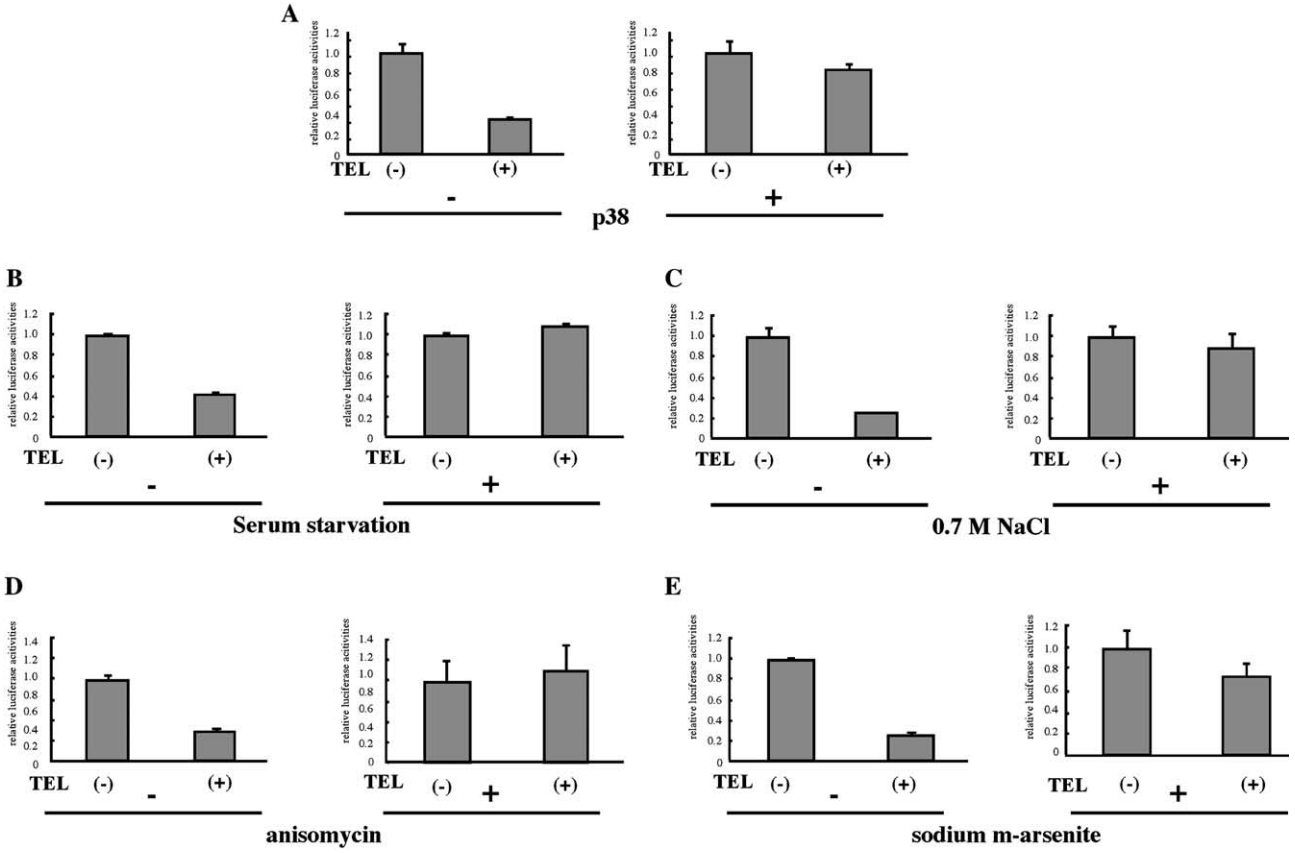

Fig. 6. (A) Overexpression of p38 inhibits TEL's trans-repressional ability. HeLa cells (3.0×10^4) were transfected with 1 μg $(ETS)_3$tkLuc reporter plasmid alone or along with 0.5 μg pME18S-FLAG-TEL with or without 0.5 μg of pcDL-SRα456-HA-p38, and cultured in MEM containing 10% FCS for 48 h. Bars show relative luciferase activities to the level when a control plasmid pME18S was cotransfected and present average results of duplicate experiments. (B–E) Activation of endogenous p38 also inhibits TEL's trans-repressional ability. HeLa cells (3.0×10^4) were transfected with 1 μg $(ETS)_3$tkLuc reporter plasmid alone or along with 1 μg pME18S-FLAG-TEL. After 36–48 h, they were incubated in either MEM without FCS for 8 h (B), 0.7 M NaCl for 30 min (C), MEM (10% FCS) plus 10 μg/ml anisomycin for 3 h (D), or MEM (10% FCS) plus 50 μg/ml sodium *m*-arsenite for 12 h before harvest. The results are presented as relative luciferase activities as in (A).

to evaluate the transcription through EBS by p38-modified TEL. A threefold decrease in luciferase activities was observed when wild-type-TEL was expressed without overexpressed p38. However, overexpression of p38 abolished the transcriptional suppression by wild-type-TEL (Fig. 6A). Treatment with serum starvation, hypertonic saline, anisomycin, or sodium *m*-arsenite that activates endogenous p38 also impaired the trans-repressional ability of wild-type-TEL in the same way (Figs. 6B–E). From these data, we concluded that p38-dependent phosphorylation inhibits the transcriptional repression by TEL.

Discussion

In this study, we demonstrated that TEL is phosphorylated in vivo with dependence on p38 activation. Ser^{22} and Ser^{257} of TEL are constitutive and inducible phosphorylation sites, respectively. TEL is efficiently subjected to phosphorylation by p38 in vitro, suggesting that TEL is a direct target of p38 for phosphorylation. TEL associates with p38 in vitro, although it is still not clarified whether the association is direct or indirect. Importantly, phosphorylation of TEL by overexpressed or endogenous activated p38 results in the abolishment of its trans-repressional effects on the EBS promoter. Therefore, we propose that p38 is a physiologically important stress MAP kinase that induces phosphorylation of TEL and thereby potentially regulates its functions. No phosphorylation of TEL is induced by JNK1 activation with UV. Although JNK and p38 are coordinately activated by proinflammatory cytokines, environmental stresses, and hematopoietic cytokines [22–24], only p38 appears to be responsible for the phosphorylation and functional regulation of TEL.

Various ETS family transcription factors become phosphorylated by MAP kinases and thereby molecularly activated. The phosphorylating MAP kinases and phosphorylation sites differ among the molecules. The subclass members containing the HLH domain in the N-terminus and the ETS domain in the C-terminus such as ETS1 and ETS2 are phosphorylated by ERK in the N-terminal region [42] and those having the ETS domain in the N-terminus such as Elk-1, SAP-1, and SAP-2 are phosphorylated by both ERK and stress MAP kinases in the C-terminal transactivation region [47–53]. It is interesting to know whether TEL becomes phosphorylated by ERK. We are currently examining the possibility. In any case, phosphorylation by p38 is a unique property of TEL among the ETS family proteins structurally resembling TEL, such as ETS1 and ETS2. Notably, the identified inducible phosphorylation site Ser^{257} of TEL is not conserved among them. Therefore, phosphorylation by p38 might be characteristic of TEL in the subclass of the ETS transcription factors.

Certain transcription factors are negatively regulated through phosphorylation, although its mechanisms are diverse. For examples, members of the Forkhead family of transcriptional activators, such as FKHR, FKHR1, and AFK, are phosphorylated by protein kinase B (PKB)/Akt on three or more serine or threonine residues [54–60]. Interestingly, regulatory mechanisms in inhibiting their transcriptional activation are different depending on the phosphorylation sites. The phosphorylation of the residue equivalent to Ser^{256} in FKHR represses nuclear import through suppression of a nuclear localization signal [61–63], while that to Thr^{24} induces interaction with 14-3-3 proteins, leading to sequestration into the cytosol [54,63]. Another example is the nuclear factor of activated T cells (NFAT) complex. Inactivation of a phosphatase calcineurin causes phosphorylation of an NFAT component NFATp and in turn prevents its abilities of DNA binding and NFAT complex formation with Fos and Jun [64]. Among the ETS family transcription factors, ETS2 repressor factor (ERF) that exhibits strong transcriptional repressor activity on the EBS promoters becomes phosphorylated by ERK2 and cdc2/cyclin B kinases and loses its suppressive effects through export to the cytoplasm [65,66]. TEL is the same as ERF in that phosphorylation causes a decrease in trans-repressional effects. The possible mechanisms in preventing TEL's transcriptional effects through phosphorylation are as follows. First, the phosphorylation of TEL might change its binding affinity with co-repressors or DNA. However, the identified phosphorylation site resides outside the mSin3A- or N-CoR-binding region in the HLH or central repression domain (amino acids 268–303) [8], or the DNA-binding ETS domain, although the fact does not completely exclude the possibility. Second, the phosphorylation might affect the functional domains themselves that exert the trans-repression. Two trans-repressional regions have been identified in TEL molecule [3]. The first includes amino acids 171–215 in the internal domain and the second encompasses the ETS domain and the last 55 residues in the internal domain. However, the phosphorylation site is again located outside these regions. Lastly, it might be possible that the phosphorylation decreases protein stability or alters intracellular localization from the nucleus to the cytoplasm.

What biological functions of TEL would be regulated through p38-dependent phosphorylation? By and large, ERK and p38 mediate opposite signals for cell differentiation and proliferation. Activation of ERK links to cell survival, whereas that of p38 is related to apoptosis induction. TEL is suggested to be a tumor suppressor, because expression of TEL in Ras-transformed NIH3T3 cells inhibits cell growth in liquid and soft agar cultures, and suppresses tumor formation in nude mice [10,67]. Considering that activated p38 represses TEL's inhibitory effects on the EBS promoter and that it could lead to loss of tumor suppressive

functions, there is present an inconsistency in the functional regulation of TEL by p38. We should carefully interpret the present data, because experiments with an overexpression system might not correctly reflect normal physiology. Physiological relevance of p38-mediated TEL's phosphorylation remains open to be established in further studies.

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