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The FLI-1 Transcription Factor Is a Short-Lived Phosphoprotein in T Cells

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The FLI-1 transcription factor is a member of the ETS gene family, most closely related to ERG. In this study, the FLI-1 protein products were characterized using a specific monoclonal antibody previously developed against bacterially expressed protein. In the human T-cell line Jurkat, both isoforms of FLI-1, p51 and p48, are phosphorylated, primarily on serine residues. FLI-1 phosphorylation is increased by a Ca^{2+}-mediated process, and inhibitor studies indicate that protein phosphatase 2A, at least in part, controls FLI-1 phosphorylation level. FLI-1 phosphorylation is not stimulated by phorbal 12-myristate 13-acetate (PMA), a protein kinase C activator, and in this it differs from ERG protein phosphorylation. The p51 isoform has a half-life of 105 min, and p48 has a half-life of 80 min; in contrast, the ERG protein is much more stable with a half-life of 21 h. Newly synthesized FLI-1 protein decreased during human T cell activation. Our data suggest that although the FLI-1 and ERG genes are highly homologous, their distinct properties may contribute to their different roles in gene regulation.

Key words: FLI-1-1 transcription factor, phosphatase 2A, phosphoprotein, protein kinase, T cell activation.

Abbreviations: mAb, monoclonal antibody; PMA, phorbal 12-myristate 13-acetate; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B.

FLI-1, a member of the ETS gene family of transcription factors (*1*), performs functions critcal for normal development and oncogenesis (*2*). FLI-1 is preferentially expressed in cells of hematopoietic lineages and vascular endothelial cells. Murine FLI-1 was discovered as an activated oncogene in erythroleukemias induced as a result of retroviral integration by the Friend Leukemia Virus, F-MuLV (*3*). Similarly, viral integration and insertional activation of FLI-1 is associated with other hematological cancers. Ectopic FLI-1 expression promotes megakaryocytic differentiation in K562 cells by transcriptionally activating megakaryocytic genes, including the thrombopoietin receptor (c-mpl), glycoprotein IX and glycoprotein IIb (*4–6*). Mice deficient in FLI-1 expression displayed a significant reduction in megakaryocyte numbers (*7*, *8*), consistent with a critical role for FLI-1 in megakaryopoiesis. We recently used morula-stage aggregation as a strategy to assess further the hematopoietic defects of the FLI-1 gene–targeted mice and identified previously unknown physiological roles of FLI-1-1 in granulocytic, erythroid and NK cell proliferation and differentiation (*9*). FLI-1 expression is also detected in endothelial cells (*10*), and the vascular phenotype and embryonic lethality of mice deficient in FLI-1 expression are consistent with a possible role for FLI-1 in proper regulation of angiogenesis.

Clinical relevance of FLI-1 is demonstrated by the Ewing's sarcoma-associated chromosomal translocation [t(11;22)], which results in an in-frame fusion of the EWS region to the DNA binding domain of the FLI-1 protein (*11*, *12*). In the resultant EWS/FLI-1 chimeric protein, the weak transcriptional activation domain of FLI-1 is replaced by a strong transcriptional activation domain of EWS, which has been shown to be necessary for cellular transformation (*13*, *14*). A possible role for FLI-1 in autoimmunity is supported by our observation of elevated expression of FLI-1 mRNA in lymphocytes from patients with systemic lupus erythematosus (*15*). Such a role is further supported by the lupus-like disease that occurs in transgenic mice with overexpression of FLI-1, which die from progressive immunological renal disease associated with an increased number of auto-reactive T- and B-lymphocytes (*16*) and decreased expression of FLI-1 significantly reduced renal disease and prolonged survival in MRL/*lpr* mice, an animal model of autoimmune disease (*17*).

FLI-1 encodes two isoforms, p51 and p48, (51 and 48 kDa, respectively). In this report, we demonstrate that both FLI-1 isoforms are phosphorylated proteins, with serine serving as the predominant phosphoacceptor. FLI-1 phosphorylation is enhanced by Ca^{2+} stimulation, and FLI-1 dephosphorylation is dependent, at least in part, upon protein phosphatase 2A (PP2A). Newly synthesized FLI-1 protein is transiently decreased during T cell activation and expressed in many hematopoietic cell types. Human FLI-1 was identified based upon presence of ETS

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sequences and was named ERGB based upon its homology with the ERG gene, with 68% identity and 80% similarity at the amino acid level (*18*). The ETS (DNA-binding) domain is most highly conserved between FLI-1 and ERG (98% homology). Comparison of our results with those previously reported for ERG indicate that, although the FLI-1 is more closely related to ERG than others of the ETS family, the products of these two genes have distinct properties that are likely to contribute to their differential roles in gene regulation.

MATERIALS AND METHODS

Cell Culture—All cell lines, including the T cell leukemia cell lines, Molt4, Jurkat and H9, the primitive leukemia cell line KG-1, the promyeloid cell line HL60, the B lymphoma cell lines P3HR1 and Raji, and the erythroleukemia blast cell line K562, were obtained from American Tissue culture collection (ATCC, Manassas, VA). Cells were maintained in RPMI 1640 medium with 15% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a humidified 5% CO_2, 95% air atmosphere.

Antibodies—The FLI-1 specific monoclonal antibody (mAb) B3 and rabbit specific polyclonal antibody were developed as described previously (*7*, *19*).

Radiolabeling of Cells and Immunoprecipitation—Cells were labeled with either [^{35}S]methionine (300 μCi/ml, Amersham, USA) for 3 h, or [^{32}P]orthophosphate (1 mCi/ml, Amersham, USA) for 4 h, as described previously (*20*, *21*), except where indicated. For pulse chase experiments, cells were washed with methionine-free medium and incubated in the same medium with 1 mCi/ml [^{35}S]methionine for 15 min. An excess amount of unlabeled methionine was added to stop the labeling. The cells were collected by centrifugation, suspended in pre-warmed culture medium and collected after various lengths of time. Immunoprecipitation was carried out as described previously (*20*, *21*). Briefly, the cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1 mM EGTA, 1.0 mM EDTA, 0.1% SDS in the presence of proteinase inhibitors). The clarified cell lysate was immunoprecipitated with FLI-1 mAbs-coupled protein A agarose. The immunoprecipitated proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography. In the antigen competition experiments, 2 μg of B3 mAb-agarose was preincubated at 4°C for 30 min with the indicated amounts of recombinant FLI-1 protein, and then used for immunoprecipitation of FLI-1 from Jurkat cells.

Immunoblot Analysis—Jurkat cells were lysed with RIPA buffer and immunoprecipitated with B3 mAb-agarose. After multiple washes with RIPA buffer, immunocomplexes were boiled in RIPA buffer containing 1% SDS for 5 min and analyzed by SDS-PAGE as described previously (*19*). The immunocomplexes were transferred onto a nitrocellulose membrane and incubated with normal rabbit Ig or rabbit specific polyclonal antibody against FLI-1 (*7*). Antibody localization was detected using an Amersham ECL Western blotting detection system.

Chemicals—All chemicals were purchased from Sigma (USA), except where indicated. PMA was used at 10 nM for stimulation. The general protein phosphatase inhibitor okadaic acid was added to the culture medium for 2 h at 100 nM concentration (*22*). Cantharidin was used at 1 μM concentration for inhibition of protein phosphatase 2A (PP2A), and cypermethrin was added at 1 nM concentration for inhibition of protein phosphatase 2B (PP2B) (*23*, *24*).

Phospho-Amino Acid Analysis—FLI-1 protein was labeled *in vitro* with [^{32}P]orthophosphate and fractionated by immunoprecipitation and electrophoresis as described previously (*25*). Briefly, after being transferred to an Immobilon filter, phosphorylated FLI-1 protein was cut out from the filter and hydrolyzed with 6 N HCl for 1 h. After hydrolysis, the supernatants were speed vacuum dried, resuspended in 5 ml of electrophoresis buffer [pyridine:acetic acid:water, 1:10:189 (v/v)] containing unlabeled phosphoserine, phosphothreonine and phosphotyrosine standards (3 μg each), and spotted onto a Sigma thin layer chromatography sheet. One-dimensional electrophoresis was performed at pH 3.5 and 140 V for 20 min and radiolabeled phospho-amino acids were detected by autoradiography (*25*).

T Cell Isolation and Stimulation—Human T cells were isolated from blood donated by volunteers. Mononuclear cells were first isolated by Ficoll-Pague plus (Sigma, USA). T cells were further isolated using the Dynabeads negative T isolation kits from Dynal Inc. (Lake Success, NY) following the manufacturer's instructions. Purity of the resultant T cell population was determined to be over 90% by staining with CD3 marker and flow cytometry. The T cells were cultured at 10^6 cells/ml/well in RPMI medium with 10% fetal bovine serum. PMA (10 nM, Sigma) and ionomycin (2 μM, Sigma) were added to the cultures for T cell activation. Each well of T cells was radiolabeled with [^{35}S]methionine (300 μCi/ml for 1 h) and harvested at 1, 6, 24 and 48 h after activation. The cells were lysed and immunoprecipitated with the FLI-1 mAbs-coupled protein A agarose. The immunoprecipitated proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

RESULTS

FLI-1 Proteins Were Specifically Immunoprecipitated by B3 mAb from T Cells—When T cell line Jurkat cells were labeled with [^{35}S]methionine and the cell lysate was immunoprecipitated using B3 mAb, only two components with molecular masses of 51 kDa (p51) and 48 kDa (p48) were detected, as previously reported (*16*, *19*) (Fig. 1A, lane 2). The specificity of B3 mAb for FLI-1 protein was further demonstrated by the dose-dependent competition and complete abolishment of each of the two bands by incubation with purified recombinant FLI-1 protein (Fig. 1A, lanes 3 and 4) and the lack of any band with normal mouse IgG1 (Fig. 1A, lane 1). Furthermore, IP/Western analysis indicates that the B3 mAb and a rabbit polyclonal antibody against FLI-1 recognize the same polypeptide. Specifically, rabbit polyclonal antibody against FLI-1 (Fig. 1B, lane 2), but not normal rabbit Ig control (Fig. 1B, lane 1) specifically detects FLI-1 protein in B3 mAb

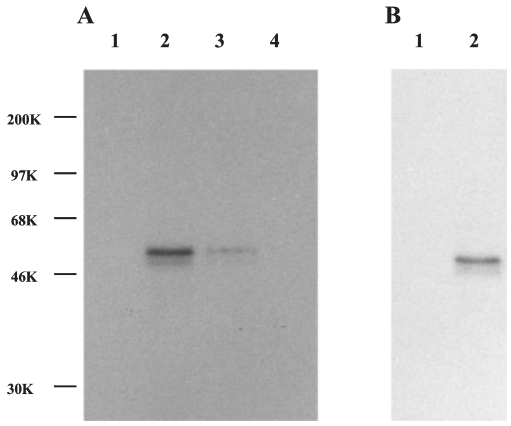

Fig. 1. **FLI-1 proteins are immunoprecipitated from Jurkat cell with B3 mAb.** (A) Jurkat cells were labeled with [^{35}S]methionine, and lysate was immunoprecipitated with normal mouse IgG1-agarose control (lane 1), B3 mAb-agarose (lane 2), B3 mAb-agarose preincubated with 5 μg of recombinant FLI-1 protein (lane 3) or B3 mAb-agarose preincubated with 20 μg of recombinant FLI-1 protein (lane 4). The immunoprecipitates were analyzed by SDS-PAGE. All lanes show immunoprecipitates from equal number of cells (5×10^6). Relative mobilities of the protein size markers are shown at left in kilodaltons (k). (B) The Jurkat cells were lysed with RIPA buffer and immunoprecipitated with B3 mAb-agarose. The immunocomplexes were boiled in RIPA buffer containing 1% SDS for 5 min and analyzed by SDS-PAGE. The immunocomplexes were transferred onto a nitrocellulose membrane and incubated with normal rabbit Ig (lane 1) or rabbit specific polyclonal antibody against FLI-1 (lane 2). Each lane shows immunocomplexes from an equal number of cells (5×10^6).

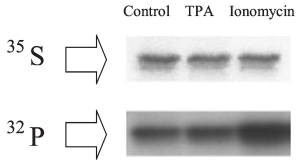

Fig. 2. **FLI-1 proteins are phosphorylated.** Jurkat cells were labeled with [^{35}S]methionine (Top Panel) or [^{32}P]orthophosphate (bottom panel). The cells were lysed with RIPA buffer and immunoprecipitated with B3 mAb against FLI-1 protein. The immunoprecipitates were analyzed by SDS-PAGE. Cells were treated with PMA (10 nM, lane 2) or ionomycin (2 μM, lane 3) for 30 min. The untreated cells serve as a control (lane 1). The same number of cells was used for each immunoprecipitation.

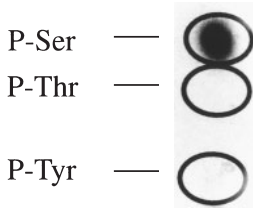

Fig. 3. **Phosphoamino acid analysis of the FLI-1 proteins.** The FLI-1 proteins labeled with [^{32}P]orthophosphate were isolated by immunoprecipitation and SDS-PAGE. The protein was transferred to an Immobilon filter, cut out and hydrolyzed with 6 N HCl for 1 h. One-dimensional electrophoresis was performed at pH 3.5 and 1,400 V for 20 min. The radiolabeled phosphorylated amino acids were detected by autoradiography.

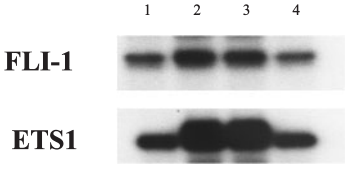

Fig. 4. **Protein phosphatase 2A modulates FLI-1 phosphorylation.** Jurkat cells were labeled with [^{32}P]orthophosphate, lysed with RIPA buffer and immunoprecipitated with B3 mAb again FLI-1 protein (top panel) or E44 mAb against ETS1 (bottom panel). Lane 1, untreated cells; lanes 2, cells cultured with okadaic acid at 100 nM concentration for 2 h; lane 3, cells were cultured with cantharidin at 1 μM concentration for 2 h for inhibition of PP2A; lane 4, cells were cultured with cypermethrin at 1 nM concentration for 2 h for inhibition of PP2B. The same number of cells was used for each immunoprecipitation.

immunoprecipitated proteins from unlabeled Jurkat cells lysates.

FLI-1 Phosphorylation Is Increased Following Ca^{2+} Mobilization—It has been reported that several members of the ETS family are phosphoproteins. To determine whether the FLI-1 is phosphorylated, Jurkat T-cells were labeled with [^{32}P]orthophosphate or [^{35}S]methionine. The FLI-1 proteins were purified by immunoprecipitation and analyzed by SDS-PAGE. As shown in Fig. 2, both p51 and p48 are phosphorylated. Addition of the Ca^{2+} ionophore ionomycin to the culture medium resulted in increased phosphorylation of FLI-1; in contrast, PMA, a protein kinase activator, did not cause an increase in phosphorylation of FLI-1 (Fig. 2). To determine which amino acids were phosphorylated, [^{32}P]phosphate-labeled FLI-1 was isolated, hydrolyzed and characterized by thin layer chromatography. As shown in Fig. 3, FLI-1 is phosphorylated primarily on serine residues.

Phosphatase 2A Contributes to the Phosphorylation of FLI-1 Protein—Processes that are reversibly controlled by protein phosphorylation require a balance between protein kinase and protein phosphatase (PP) activities (*26*). Thus, it was important to assess whether specific PP(s) are associated with dephosphorylation of FLI-1 protein. As shown in Fig. 4, the general phosphatase inhibitor okadaic Acid and the PP2A-specific inhibitor cantharidin greatly increased the steady state phosphorylation level of FLI-1. In contrast, FLI-1 phosphorylation level was not significantly altered upon incubation with the PP2B inhibitor cypermethrin. Phosphorylation of ETS1 was also increased when cells were grown in the presence of cantharidin, but not cypermethrin (Fig. 4). These results suggest that PP2A may be responsible for dephosphorylation of both FLI-1 and ETS1.

Turnover of the FLI-1 Proteins—To examine the half-life of the p51 and p48 isoforms of FLI-1, FLI-1 proteins were pulse-labeled with [^{35}S]methionine and chased in the presence of excess unlabeled methionine, and the level of FLI-1 protein was measured by immunoprecipitation (Fig. 5A). The relative levels of FLI-1 were quantified by NIH Image densitometry software, and half-life was calculated (Fig. 5B). The p51 has a half-life of 105 min, and the p48 has a half-life of 80 min.

Newly Synthesized FLI-1 Protein Level Is Decreased Following T Cell Activation—It has been reported that the expression of FLI-1 mRNA is decreased dramatically

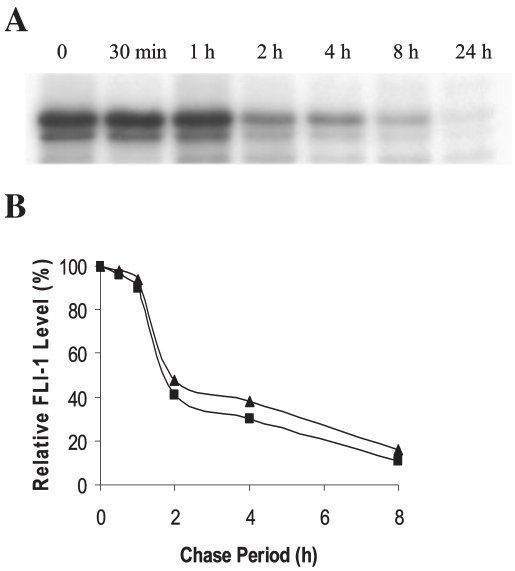

Fig. 5. **Turnover of the FLI-1 proteins.** (A) Jurkat cells were pulse labeled with [^{35}S]methionine (1 mCi/ml) for 15 min (lane 1) and chased with excess unlabeled methionine for 30 min, 1 h, 2 h, 4 h, 8 h and 24 h (lanes 2–7). All lanes show immunoprecipitates from an equal number of cells (5×10^6). (B) Relative levels of FLI-1 during chase period. Levels of the labeled FLI-1 at various chase periods were determined by a densitometer and normalized to the 100% value obtained at the zero time. Solid triangles, turnover of the FLI-1 p51 isoform; solid squaresturnover of the FLI-1 p48 isoform.

during the T cell activation (*27*). Next, we examined whether newly synthesized FLI-1 protein level is similarly reduced during the T cell activation. Isolated human T cells were simulated with PMA and ionomycin. As shown in Fig. 6, the FLI-1 protein level decreased to undetectable levels at 1 and 6 h after T cell activation. FLI-1 was expressed at reduced levels at 24 h after stimulation, returning to control levels at 48 h after stimulation. Since [^{35}S]methionine is only incorporated into newly synthesized protein, our results indicate the synthesis of FLI-1 protein was greatly reduced upon T cell activation.

Expression of the FLI-1 Protein in Various Cell Lines—Specific ETS family members have been found to have either restricted expression profiles or can be found to have nearly ubiquitous expression (*1*, *28*, *29*). Next, we examined the expression of FLI-1 protein in a variety of hematopoietic cell lines. After the cells were labeled with [^{35}S]methionine, FLI-1 protein was purified by immunoprecipitation with B3 mAbs and analyzed by SDS-PAGE. As shown in Fig. 7, FLI-1 is highly expressed in the primitive leukemia cell line KG-1, relative highly expressed in the promyeloid cell line HL60, the T cell leukemia cell lines Molt4, Jurkat and H9, the B lymphoma cell line P3HR1, and poorly expressed in the B lymphoma cell line Raji. No detectable protein was found from the erythroleukemia blast cell line K562.

DISCUSSION

It is well known that protein phosphorylation is a ubiquitous mechanism involved in the regulation of most biological processes (*26*). In this report, we demonstrate that

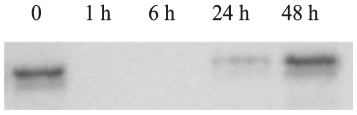

Fig. 6. **The level of newly synthesized FLI-1 proteins decreases in activated human T cells.** Human T cells were isolated and cultured in 24-well plates with 10^6 cells /well. PMA (10 nM) and Ionomycin (2 μM) were added to stimulate the cells. The cells were labeled at 1, 6, 24, and 48 h after stimulation with [^{35}S]methionine for 1 h (300 mCi/ml), lysed with RIPA buffer and immunoprecipitated with B3 mAb against FLI-1 protein. The immunoprecipitates were analyzed by SDS-PAGE. Lane 1, untreated T cell control; lanes 2–5, T cells after activation at 1, 6, 24, 48 h, respectively.

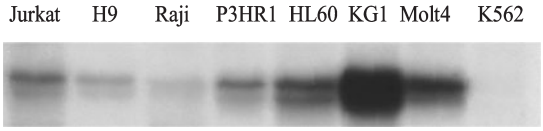

Fig. 7. **Expression of the FLI-1 proteins in hematopoietic cell lines.** The indicated cell lines were labeled with [^{35}S] methionine (300 μCi/ml) for 3 h, and the FLI-1 proteins were purified by immunoprecipitation with B3 mAb and analyzed by SDS-PAGE. Lane 1, Jurkat; lane 2, H9; lane 3, Raji; lane 4, P3HR1; lane 5, HL60; lane 6, KG1; lane 7, molt 4; lane 8, K562. The same cell number (5×10^6) was used for each immunoprecipitation.

FLI-1 can be post-translationally modified by phosphorylation. FLI-1 phosphorylation level is increased following culture with a calcium ionophore, suggesting that intracellular Ca^{2+} concentration may modulate FLI-1 phosphorylation. Previously reports have demonstrated that many ETS factors are phosphorylated. Like FLI-1, both ETS1 and ETS2 undergo enhanced phosphorylation following Ca^{2+} mobilization (*20*, *21*); in contrast, phosphorylation of ERG proteins is increased by PMA, a protein kinase C activator, but not by Ca^{2+} ionophores (*30*). As end-effector molecules of multiple signal transduction pathways, the functions of ETS proteins can be controlled by phosphorylation-mediated effects on DNA binding, protein–protein interaction, transcriptional activation and subcellular localization (reviewed in Ref. *1*). Ca^{2+}-dependent phosphorylation of ETS1 reduces its DNA-binding ability (*31*, *32*), due to stabilization of an intramolecular autoinhibitory conformation (*33*). Phosphorylation of PU.1 is necessary for protein–protein interaction with NF-EM5 to regulation transcriptional activity (*34*). Phosphorylation has been shown to reduce the trans-repressional activities of TEL, an ETS family repressor (*35*). FLI-1 interactive proteins are able to enhance or inhibit transcriptional activation of specific target genes (*36–39*). Phosphorylation may regulate the ability of FLI-1 to bind DNA and interact with specific protein partners. In this study, we also determined that FLI-1 phosphorylation occurs primarily on serine residues. We have identified 16 serines as potential phosphorylation sites in FLI-1 protein using the NetPhos software (Technical University of Denmark, http://www.cbs.dtu.dk/services/NetPho). Since we found that FLI-1 phosphorylation was increased by Ca^{2+} mobilization, we defined 12 serines that are potential phosphorylation sites for Ca^{2+}/calmodulin-dependent protein

kinases using the NetPhos software. The specific kinase(s) responsible for FLI-1 phosphorylation and the correlation between phosphorylation status of FLI-1 and its stability in T-cells remain to be determined. Future studies will be directed towards determining the effect of specific phosphorylation events upon FLI-1 stability, interaction with other proteins, subcellular localization, DNA-binding affinity and target gene selectivity.

Protein phosphorylation plays a critical role in regulating many cellular processes in eukaryotes (*26*). The balance between protein kinases and protein phosphatases controls phosphorylation levels. Inhibitor studies demonstrate that PP2A may be one component that controls dephosphorylation of FLI-1 and ETS1. It remains to be determined whether PP2A directly dephosphorylates FLI-1. Increased FLI-1 phosphorylation observed following PP2A inhibition may result from the activation of a protein kinase, since PP2A is know to regulate the function of many protein kinases, including CaM kinases (*40*).

The FLI-1 protein expression pattern is consistent with the previously reported FLI-1 mRNA profiles (*17*). We demonstrate that FLI-1 protein in quiescent T-cells is high, and newly synthesized FLI-1 protein was reduced during T-cell activation, a pattern similar to that previously found for FLI-1 mRNA (*27*). However, in our study, we were able to evaluate both FLI-1 protein isoforms and determine that p51 and p48 are both preferentially expressed in quiescent cells.

The Novartis gene atlas display of "normal" mRNA gene expression for both mice and human is informative regarding the expression of FLI-1 [Genomics Institute of the Novartis Research Foundation (http://symatlas.gnf.org/SymAtlas/)]. Expression of FLI-1 was found in almost every organ, with highest expression in spleen, thymus, bone marrow, and kidney. In lymphocyte subsets, FLI-1 was found to be expressed at highest levels in both mice and human in B cells, with lower, but still appreciable expression in CD4 and CD8 T cells. Expression of FLI-1 was higher in activated B cells than naive B cells. Prominent mRNA expression is also found in tissue macrophages and NK cells.

Although alignment with all ETS family members demonstrates that FLI-1 is most related to ERG at the amino acid level, their protein products have several different properties. The FLI-1 proteins have a relatively short half-life (80–105 min), compared with 21 h for ERG proteins (*30*). Both ETS1 and ETS2 have a short half-life (85 min and 20 min, respectively) (*20*, *41*). Although both FLI-1 and ERG are phosphoproteins, phosphorylation appears to be mediated by different pathways. Our data suggests that although the FLI-1 and ERG genes are highly homologous, their protein products have unique properties that may facilitate differential regulation of specific target gene expression.

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