The Transcription Factor Spi-1/PU.1 Interacts with the Potential Splicing Factor TLS*

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Spi-1/PU.1 is an Ets protein deregulated by insertional mutagenesis during the murine Friend erythroleukemia. The overexpression of the normal protein in a pro-erythroblastic cell prevents its terminal differentiation. In normal hematopoiesis Spi-1/PU.1 is a transcriptional factor that plays a key role in normal myeloid and B lymphoid differentiation. Moreover, Spi-1/PU.1 binds RNA and interferes in vitro with the splicing process. Here we report that Spi-1 interacts in vivo with TLS (translocated in liposarcoma), a RNA-binding protein involved in human tumor-specific chromosomal translocations. This interaction appears functionally relevant, since TLS is capable of reducing the abilities of Spi-1/PU.1 to bind DNA and to transactivate the expression of a reporter gene. In addition, we observe that TLS is potentially a splicing factor. It promotes the use of the distal 5′ splice site during the E1A pre-mRNA splicing. This effect is counterpoised in vivo by Spi-1. These data suggest that alteration of pre-mRNA alternative splicing by Spi-1 could be involved in the transformation of an erythroblastic cell.

The transcription factor Spi-1/PU.1 (1) is an Ets protein that plays a central role in the differentiation of macrophages and B cells during normal hematopoiesis. Indeed, the disruption of the Spi-1/PU.1 gene in mouse (2, 3) induces an early lethality of animals that lack mature macrophages, neutrophils, B and T cells, and also osteoclasts (4). These animals present no alteration in erythroidic and megacaryocytic lineages. Consistent with this phenotype, Spi-1-responsive elements have been identified in transcriptional promoters and enhancers of many myeloid and B lymphoid genes (for review, see Ref. 5). In murine Friend acute erythroleukemia, the mutation of the spi-1 gene by retroviral insertional mutagenesis induces its transcriptional activation. The erythroleukemic process developed in spi-1 transgenic mice reveals that Spi-1 is involved in blocking the differentiation of the proerythroblast (6). The molecular mechanisms that lead Spi-1 to arrest the erythroid differentiation are not yet understood. The up-regulation of Spi-1 related to the transformation of the proerythroblast suggests that Spi-1 may induce a transcriptional dysregulation of some erythroid genes or/and may be abnormally associated with erythroid partners.

Some nuclear proteins interacting with Spi-1 have already been identified such as the transactivator NF-IL6/C/EBPβ (7), the transcription factor Pip/EM-4 (8), the retinoblastoma protein (9), and the basal transcription factor TFIIID (9). We reported previously that Spi-1 interacts with the RNA-binding protein p54nrb (nuclear RNA-binding protein, 54 kDa), binds to RNA by its DNA-binding domain and interferes in vitro with the splicing of a β-globin minigene (10). Here, we describe that Spi-1/PU.1 interacts with another RNA-binding protein TLS and that this association impedes the transcriptional functions of Spi-1. In addition, TLS influences, in vivo, the selection of the 5′ splice site during the alternative splicing of the adenovirus E1A pre-mRNA, and Spi-1 interferes with this selection process. These data suggest that the transcription factor Spi-1 may change the alternative selection of pre-mRNA splicing sites and may consequently modulate the splicing of some pre-mRNA.

EXPERIMENTAL PROCEDURES

Plasmids Construction—GST-Spi-1, GST-158Spi-1, GST-DBD-Spi-1, GST-Fli-1, GST-Nter-Fli-1, and GST-DBD-Fli-1 fusion proteins have been described elsewhere (10). pBS-TLS SK+ plasmid was a gift from D. Ron (11). pGEX-TLS, pGEX-AD-TLS, pGEX-AD-RGG1-TLS, pGEX-RMM-TLS, and pGEX-RGG2–3–TLS plasmids encode fusion proteins between GST and TLS-amino acids 1–526, 1–165, 1–271, 271–392, or 392–526, respectively. The GST fusion proteins were produced and immobilized on GST-Sepharose beads as described previously (10). The pCS3-MT (Myc-Tag) expression vector contains the simian cytomegalovirus IE94 promoter/enhancer, the SP6 promoter and six copies of the 9E10 Myc epitope.2 pCS3-MT-TLS, pCS3-MT-AD-RGG1-TLS, pCS3-MT-RMM-TLS, and pCS3-MT-RGG2–3–TLS encode fusion proteins between Myc epitope and TLS-amino acids 1–526, 1–165, 1–271, 271–392, or 392–526, respectively. pCS3-MT-A1 was generated by cloning the blunted BamHI-EcoRI fragment of hRNP A1 from pGEX-2T-A1 vector (a gift of S. Riva (12)) into the blunted XhoI pCS3-MT-Vector. The blunted Spi1-EcoRI Fli-1 cDNA was cloned into the blunted StuI pCS3-MT vector to generate pCS3-MT-Fli-1. pCS3-MT-E1A was generated by inserting the EcoRI-XhoI fragment of E1A from Spi plasmid (a gift of J. Stevenin (13)) into the EcoRI-XhoI sites of pCS3-MT-vector.

Cellular Protein Purification—74A Friend cells (1010 cells) were collected and resuspended in HN buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 2 mM MgCl2, 0.5% Nonidet P-40, 2 mM PMSF, and antiproteases). After 10 min on ice cells were centrifuged for 5 min at 4 °C. The pellet was resuspended in HNN buffer (HN buffer with 420 mM

* This work was supported in part by grants from the Ligue Nationale Française contre le Cancer and the Association pour la Recherche sur le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a fellowship from the Fondation pour la Recherche Médicale.

‡ Supported by the Institut National de la Santé et de la Recherche Médicale.

§ Supported by a grant from the Ministère de l’Education Supérieure et de la Recherche.

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1 The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; DBD, DNA-binding domain; RRM, RNA recognition motif; AD, transcriptional activation domain; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; hRNP, heterogeneous nuclear ribonucleoprotein.

2 A. Vojtek, personal communication.
Interaction of Spi-1 with TLS

NaCl, stirred gently for 30 min at 4 °C, and centrifuged for 20 min at 45,000 rpm (TLA-45 rotor, TL100 ultracentrifuge). The supernatant (nuclear extracts) was diluted in HNTBSA buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1.5 g/l BSA, 0.3% Nonidet P-40, 0.05% Triton X-100, 2 mM PMSF, and antiproteases), precleared three times with GST-Sepharose beads, and then incubated with GST-Spi-1 beads for 1 h at 4 °C. After washes with HNTBSA buffer, bound proteins were eluted with HNTBSA buffer containing 1.0 M KCl. The resulting eluate was diluted in HNTBSA buffer, precleared with GST-Sepharose beads and rechromatographed on GST-Spi-1 beads. The beads were washed with HNT buffer (HNTBSA buffer without BSA). Proteins were eluted with HNT buffer containing 1.0 M KCl, separated on SDS-PAGE, visualized by Amido Black staining, purified, and subjected to internal amino acid sequence determination.

**In Vitro Protein Interaction**—In vitro translated TLS and Spi-1 proteins were synthesized using TNT-coupled reticulocyte lysates (Promega) and [35S]methionine. Labeled proteins were incubated 1 h in NDT buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.1% Triton X-100, 0.2 mM DTT) with GST fusion proteins bound to glutathione-Sepharose beads. After extensive washing, the bound proteins were analyzed by electrophoresis on a 10% SDS-PAGE and electrotransferred to a nitrocellulose filter. The filter was incubated with affinity-purified anti-TLS or anti-Spi-1 antibodies, and Western blot analysis was performed according to the ECL protocol (Amersham Corp.).

**Electromobility Shift Assay**—Electromobility shift assay results were performed as described elsewhere (15) with in vitro translated Spi-1, TLS, AD-I-D1-TLS, RRM-TLS, and RGG2–3-TLS proteins and [35S]-labeled c-fes (15) DNA probe.

**Cell Transfection and CAT Assay**—HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and transfected, using the calcium phosphate procedure, at 60% confluence in 60-mm plates. 2 μg of reporter plasmid, various amounts of expression vectors, and DNA carrier up to 10 μg were used for each plate. The total amount of expression vector was kept constant by addition of the control expression plasmid pCS3. Conditions for CAT assay and determination of CAT activity have been described previously (15). Transfection efficiencies were standardized by cotransfection of a β-galactosidase reporter construct, and all experiments were repeated three times.

**In Vivo Splicing Assay**—IWI–32 cells (2 × 10⁶ cells) were resuspended in 0.8 ml of Opti-MEM. Each transfection experiment was performed with 0.3 μg of the adenovirus E1A reporter vector pCS3-MT-E1A and 1 μg of pCS3-MT, pCS3-MT-A1, pCS3-MT-Spi-1, pCS3-MT-TLS, pCS3-MT-Fli-1, or pCS3-MT. The total amount of expression vector was kept constant by addition of the control expression plasmid pCS3. 10 μg of DNA in water were diluted in 93 μl of Opti-MEM and mixed with 100 μl of OPTI-MEM containing 12.5 μl of LipofectAMINE (Life Technologies, Inc.). The DNA/LipofectAMINE mixtures were added to the IWI–32 cells, and after 4 h at 37 °C, 3.5 ml of minimum Eagle's medium, 10% fetal calf serum were added to the transfected cells. Cells were harvested 20 h after transfection and total RNAs were purified using guanidine hydrochloride extraction procedure and DNase treatment. RT-PCR detection of E1A isoforms was performed as described by Caceres et al. (16). Detection was carried out by autoradiography and quantification by a PhosphorImager (Molecular Dynamic).

**RESULTS AND DISCUSSION**

A glutathione S-transferase-Spi-1 fusion protein was used as affinity chromatography reactant to search for proteins interacting with Spi-1 from nuclear extracts of murine Friend erythroleukemic cells 745A (17). Analysis of the Spi-1-bound proteins by SDS-PAGE revealed that several proteins with apparent molecular masses between 120 and 34 kDa bind specifically to the GST-Spi-1 fusion protein (Fig. 1A). The 70-kDa protein was purified and an internal peptide was subjected to amino acid sequence determination. This sequence (GEATVSFDPPSAK) allowed to identify in GenBank™ the 70-kDa protein as TLS (translocated in lymphoma) (11, 18), a protein involved in various human tumor-specific chromosomal translocations; such as the translocation observed in an acute myeloid leukemia where the 5’ region of TLS is fused to the DNA-binding domain of the Ets protein Erg (19). To determine whether the interaction of Spi-1 with TLS occurs in vivo, we looked for the presence of a TLS/Spi-1 complex in nuclear
extracts from 745A cells. Spi-1 was immunoprecipitated at reduced stringency with anti-Spi-1 antibodies, and the presence of TLS in Spi-1 immunoprecipitates was assessed with anti-TLS antibodies. Fig. 1B revealed the presence of TLS in the Spi-1 immune complexes. To assess the specificity of the immunoprecipitation with anti-Spi-1 antibodies, the same procedure was carried out with nuclear extracts from the erythroid cell line IW1–32 (20) that do not express Spi-1 (Fig. 1B, Input). TLS was not detected, even though it is expressed in these cells (Fig. 1B, Input). Reciprocally, TLS was first immunoprecipitated from 745A nuclear extracts with anti-TLS antibodies, and the presence of Spi-1 and TLS was searched by immunoblotting of the TLS immunoprecipitates with anti-Spi-1 or anti-TLS antibodies. Spi-1 was present in anti-TLS immunoprecipitates (Fig. 1B). Thus, protein complexes that contain both Spi-1 and TLS occur in vivo.

To delineate the Spi-1 regions that interact with TLS, we investigated whether TLS could associate with GST fusion proteins containing either the transactivation domain with the PEST domain (aa 1–158) or the DNA-binding domain (aa 158–266) of Spi-1 (Fig. 2A). The 35S-translated TLS protein was retained on the entire Spi-1 (GST-Spi-1) and on its DNA-binding domain (GST-DBD-Spi-1) (Fig. 2B). To gain further insight into the specificity of TLS interaction with Spi-1, we analyzed whether another Ets family protein; the Fli-1 protein fused to GST containing the Ets domain; the Fli-1 protein fused to GST, could bind TLS (Fig. 2B). Neither GST-Fli-1 nor its functional domains interacted with TLS. The same result was observed with Ets-2 (data not shown). In these experiments, we ensured that the DNA-binding domains of Spi-1 and Fli-1 were functional when fused to GST in a band shift assay (data not shown). These experiments reveal that the interaction of TLS with the Ets domain of Spi-1 is not a general feature of the Ets proteins. The TLS protein contains a transcriptional activation domain rich in glutamine residues (AD) (aa 1–165), three RGG domains (repeats of the tripeptide Arg-Gly-Gly) (aa 165–246, 372–425, and 454–526) and an 80-amino acid RNA recognition motif (RRM) (aa 287–372), both involved in the binding to RNA (11). To identify the TLS domain(s) involved in the interaction with Spi-1, a series of deleted TLS proteins fused to GST (Fig. 2A) were evaluated for their abilities to retain in vitro 35S-translated Spi-1 protein (Fig. 2B). Spi-1 binds to the GST-TLS protein as well as to the GST-RRM-TLS (271–392) and the GST-RGG2–3-TLS (aa 392–526) proteins, showing that TLS interacts in vitro with Spi-1 through its central RRM domain and its two RGG COOH-terminal domains. Taken together, these results reveal that the Spi-1-TLS interaction occurs through its respective DNA- and RNA-binding domains. Moreover, addition of RNase A or micrococcal nuclease in such reactions reveal that Spi-1-TLS interaction did not depend on RNA as a third partner.

To investigate whether the interaction between TLS and Spi-1 could interfere with the transcriptional activity of Spi-1, we examined whether TLS affects the binding of Spi-1 to DNA in a band shift assay with the c-fes Spi-1 binding site (fes-RE) (15). In contrast to AD-RGG1-TLS, when increasing amounts of either TLS, RRM-TLS, or RGG2–3-TLS were mixed to a constant amount of Spi-1 protein, a progressive decrease in the formation of the fes-RE-Spi-1 complex was observed (Fig. 3A). This result reveals that TLS, by its 271–526-amino acid region, is able to prevent Spi-1 to bind its responsive element. We investigated whether TLS could interfere with the transactivator function of Spi-1 in cotransfection experiments performed in HeLa cells with a reporter vector containing three direct repeats of the fes-RE upstream of the thymidine kinase promoter and the CAT gene (15). TLS did not, per se, affect the expression of the reporter gene. Cotransfection of the Spi-1 expression vector with increasing quantities of the TLS expression vector resulted in a dose-dependent reduction of the CAT expression (Fig. 3B). It was ascertained, by Western blotting, that the Spi-1 protein level, in all cotransfected HeLa cells, was identical (data not shown). Moreover, the TLS-induced inhibition of Spi-1 transcriptional activity did not result from titration of a limited component of the basal transcriptional machinery by the TLS transactivator domain. Indeed, the expression of the TLS amino-terminal domain (AD-RGG1-TLS) did not affect this Spi-1 function (Fig. 3B). In light of our results on the alteration of Spi-1 DNA binding activity by TLS, we...
hypothesized that TLS could repress the transcriptional activity of Spi-1 by inhibiting its DNA binding.

The Spi-1-TLS interaction interferes with the functions of Spi-1. Conversely, it was more difficult to determine the consequences of this interaction on TLS function. Indeed, little is known about the function of this ubiquitously expressed protein. TLS presents 95% amino acids identity with the small nuclear RNP-associated protein 69KD (21) and forms in vivo a complex with the hnRNP A1, a splicing factor capable to favor the selection of the most distal 5′ splice site during alternative splicing of some pre-mRNA (16, 22). These data prompted us to address the role of TLS in regulation of alternative splicing in vivo. As a first approach, we analyzed the effect of transient overexpression of TLS in the IW1–32 erythroid cells on the pattern of alternative splicing of a cotransfected adenovirus E1A gene (Fig. 4). The E1A pre-mRNA contains alternative 5′ splice sites that lead to the formation of three primary isoforms of RNA (13, 12, and 9 S) when expressed alone in IW1–32 cells (Fig. 4, A and B). Noteworthily, cotransfection of TLS resulted in a clear increase in the generation of the E1A 9 S isoform, which is correlated to a decrease of the 13 and 12 S isoforms formation. This result reveals that TLS induces the preferential use of the most distal 5′ splice site during E1A pre-mRNA splicing. The TLS effect is similar to that of hnRNP A1 (16) and Fig. 4C. We then wondered whether Spi-1 alters the effect of TLS in alternative splicing process. Since we showed previously that Spi-1 inhibits in vitro the splicing process of a β-globin minigene (10), we first investigated the consequences of overexpression of Spi-1 on E1A splicing in IW1–32 cells that do not express endogenous Spi-1. Spi-1 faintly increased the formation of 13...
and 12 S species and reduced the formation of the 9 S RNA as compared with control cells (Fig. 4B, lanes 1 and 3). In contrast, the Fli-1 expression in IW1–32 cells did not alter the pattern of expression of the E1A mRNA isoforms (Fig. 4B, lane 8). This was consistent with the absence of effect of Fli-1 on in vitro splicing (10). These results suggest that Spi-1 may modify the alternative splicing of the E1A gene in erythroid cells. In all the transfection experiments, the expressions of the transfected plasmids were ascertained by RT-PCR with primers that discriminate exogenous from the endogenous genes (data not shown). When Spi-1 and TLS were coexpressed in IW1–32 cells, the pattern of E1A mRNA isoforms became similar to the pattern observed in control cells (Fig. 4C). Thus Spi-1 counteracts in vivo the effect of TLS on promoting the use of the distal 5′ splice site. The biological significance of this Spi-1-TLS interaction in pre-mRNA splicing requires additional knowledge on the specific pathways of the interactions of Spi-1 and TLS with RNA. TLS contains RNA binding motifs and binds to homoribopolymers (23) and RNA (11), but no RNA target elements for TLS are known to assess its RNA binding specificity. In addition, TLS and Spi-1 exhibit the same binding ability to poly(G) (10, 11). This prevents the analysis of Spi-1 effect on the binding of TLS to homoribopolymers.

In Friend erythroleukemic cells, the up-regulation of Spi-1 is related to the arrest of proerythroblast differentiation. The prevailing view is that Spi-1 may deregulate the transcription of genes involved in the differentiation of the proerythroblast. Our previous data (10), showing the loss of splicing activity of HeLa nuclear extracts depleted on GST-Spi-1 affinity chromatography, suggested that Spi-1 may alter the splicing process by trapping splicing factors. We report here that interaction of Spi-1 and TLS alters, in vivo, the transactivator function of Spi-1 and counterpoises the TLS effect on 5′ splice site selection. Thus, we hypothesized that ectopic expression of Spi-1 in a proerythroblastic cell, such as in the Friend erythroleukemic cell or in the Spi-1-transgenic proerythroblast, leads Spi-1 to interfere with TLS or other RNA-binding proteins that together participate to the spliceosome assembly. Consequently, Spi-1 overexpression would disturb the balance of such splicing complexes involved in post-transcriptional regulation and could lead to an aberrant expression of some erythroid protein isoforms. This interaction of the protooncoproteins Spi-1/PU.1 and TLS could bring about a modulation of the alternative splicing process in vivo and establish an additional hypothesis implicating RNA processing alterations in the oncogenic processes.

Acknowledgments—We are grateful to D. Ron for the gift of TLS cDNA, S. Riva for the gift of hnKPN A1 cDNA, and J. Stevenin for providing us with the Spi 4 plasmid.

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*J. Biol. Chem. 1998, 273:4838-4842.*
doi: 10.1074/jbc.273.9.4838

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