The Transcription Factor Spi-1/PU.1 Binds RNA and Interferes with the RNA-binding Protein p54nrβ*

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The protooncogene for Spi-1/PU.1 is an Ets-related transcription factor overexpressed during Friend erythroleukemia. The molecular basis by which Spi-1/PU.1 is involved in the erythroleukemic process remains to be elucidated. By using an immobilized protein binding assay, we have identified a 55-kDa protein as a putative partner of Spi-1/PU.1 protein. Microsequence analysis revealed that this 55-kDa protein was p54nrβ (nuclear RNA-binding protein, 54 kDa) a RNA-binding protein highly similar to the splicing factor PSF (polymeradenylic tract-binding protein-associated splicing factor). In this paper, we show that Spi-1/PU.1 impedes the binding of p54nrβ to RNA and alters the splicing process in vitro. Moreover, we present evidence that the transcriptional factor Spi-1/PU.1, unlike other Ets proteins, is able to bind RNA. Altogether, these results raise the intriguing possibility that the functional interference observed between Spi-1/PU.1 and RNA-binding proteins might represent a novel mechanism in malignant erythropoiesis.

In the Friend spleen focus forming virus-induced erythroleukemia, the insertional mutagenesis of the spi-1 gene appears to be related to the emergence of a clonal population of tumorogenic erythroid cells arrested in their differentiation at the proerythroblast stage. Such activation of spi-1 results in an overexpression of the normal Spi-1/PU.1 protein in the Friend tumor cells (1). Spi-1 encodes the PU.1 protein, a member of the Ets family of transcription factors (2). Its DNA binding domain targets specific sequences around a central core 5’−GGAAG−3’ in transcriptional promoters and enhancers of some myelomonocytic, mastocytic, and B lymphoid genes (3). Spi-1/PU.1 contains also an amino-terminal transactivation domain and a central PEST region that could be involved in interactions with proteins like the retinoblastoma protein (4), the transcription factor TFIID (4), and the factor NF-EMS (5) or Pip (6). The down-regulation of spi-1 during the chemically induced differentiation of the Friend tumor cells (7) and the severe anemia developed by the Spi-1/PU.1 transgenic mice (8) suggest that spi-1 plays a role in the transformation of the proerythroblast by blocking its differentiation. The oncogenic potential of Spi-1/PU.1 may result from targeting of inappropriate regulatory elements of some erythroid genes and/or an abnormal association with erythroid partners. In order to determine whether Spi-1/PU.1 interacts specifically with nuclear proteins from Friend cells, a glutathione S-transferase-Spi-1/PU.1 fusion protein was used as affinity chromatographic reagent. We report here that the RNA-binding protein p54nrβ (9) was identified by this procedure as a putative partner of Spi-1/PU.1. In addition, this study reveals the ability of Spi-1/PU.1 to bind RNA and to interfere in vitro with splicing process. This novel property of Spi-1/PU.1, characterized as a DNA-binding transcriptional regulator, provides new insights into the molecular mechanism involved in malignant hematopoiesis.

EXPERIMENTAL PROCEDURES

Protein Expression Vectors—The pGEX-Spi-1 vector encoding the GST-Spi-1 fusion protein has been described elsewhere (7). The pGEX-185Spi-1 (1−158) and the pGEX-DBD-Spi-1 (154−266) vectors contain polymerase chain reaction fragment encoding Spi-1/PU.1-amino acids 1−158 and 154−266, respectively, doned in pGEX-3X. The pEXV-Spi-1 eukaryotic expression vector has been described elsewhere (7). The murine gene for p54nrβ was amplified by reverse transcriptase-polymerase chain reaction and cloned into pGEX-AT1, pGEX-AT1/RBDp54nrβ and pGEX-AT1/Cterp54nrβ encode fusion proteins between GST and p54nrβ amino acids 1−238 or 226−473, respectively. p54nrβ was doned in frame with a tag-Myc epitope in the mammalian expression vector pJ 3 tagMyc (10). The pGEX-3X/Fli-1 plasmid was constructed by insertion of the entire Fli-1 cDNA into pGEX-3X. The pGEX-3X/nterFli-1 and pGEX-3X/DBD-Fli-1 encode fusion proteins between GST and Fli-1 amino acids 1−276 and 273−452, respectively.

In Vitro Protein Interactions—GST fusion proteins were prepared as described previously (7). 35S-Labeled proteins were produced by TNT-coupled Reticulocyte Lysate Systems according to the Promega Protocols. In vitro translated Spi-1/PU.1, Fli-1, and Ets-2 proteins were incubated for 1 h in HN 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) with GST fusion proteins bound to glutathione-Sepharose beads, extensively washed, eluted, and fractionated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Comunprecipitation—The pEXV-Spi-1 and pJ 3-tag-Myc-p54nrβ expression vectors were transfected into COS1 cells by electroporation (7). COS1 nuclear extracts (11) were adjusted to a final concentration of 20 μg/ml HEPES (pH 7.4), 120 mM NaCl, 2 mM MgCl2, 0.1 mM EDTA, 0.5% Nonidet P-40, preclarified 30 min with protein G-Sepharose at 4 °C, and immunoprecipitated overnight with 9E10 anti-Myc antibody (12) bound to protein G-Sepharose. After three washes in HN buffer, immunoprecipitates were separated on 10% SDS-PAGE and transferred to a nitrocellulose filter (Schleicher & Schuell). Western blot analysis was performed according to the ECL protocol (Amersham Corp.), using the anti-Myc antibody (9E10) or an affinity-purified anti-Spi-1/PU.1 antibody (7).

Electrophoretic Mobility Shift Assay—In vitro translated Spi-1/PU.1 protein was incubated with 102P-5’-end-labelled eE3 DNA probe (5) in 20 mM HEPES (pH 7.5), 50 mM KCl, 2 mM MgCl2, 10% glycerol with 1.5 μg of poly(dI-dC)/20-μl reaction. Reactions were incubated at 25 °C for 15 min. For competitive experiments, the eE3 probe was incubated with Spi-1/PU.1 before addition of 250 ng of homoribopolymers (Pharmacia Biotech Inc.). RNA were transcribed in vitro using [α−32P]UTP and SP6

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1The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; DBD, DNA binding domain; RBD, RNA binding domain; PSF, polymeradenylic tract-binding protein-associated splicing factor; RRM, RNA recognition motif; EMSA, electrophoretic mobility shift assay.
polymerase following the recommendations of the supplier (Promega). The gel mobility shift assay was carried out with \(^{32}\)P-labeled RNA polyuridylinide tract of the \(\beta\)-tropomyosin intron probe (13) and GST fusion proteins. The reaction mixture for binding was 20 mM HEPES (pH 7.5), 50 mM KCl, 10% glycerol, and 1 \(\mu\)g of yeast transfer RNA.

**Northwestern Blot—**Northwestern blotting was carried out according to Crozet et al. (14).

**Homoribopolymer Binding Assays—**RNA binding studies were carried out as described by Ohno et al. (15) in the binding buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM MgCl\(_2\), 0.5 mM ATP, 20 mM creatine phosphate, 1% (w/v) polyvinyl alcohol, and 4 ng of \(^{32}\)P-labeled \(\beta\)-globin pre-mRNA. After 3 h of incubation at 30 \(^\circ\)C the RNA was purified and analyzed on 6% polyacrylamide gel in 8M urea, as described elsewhere (16).

**RESULTS AND DISCUSSION**

A glutathione S-transferase fusion protein containing the murine Spi-1/PU.1 protein (GST-Spi-1) bound to glutathione-Sepharose was used as affinity chromatographic reagent to search for putative partners of Spi-1/PU.1. GST-Spi-1 was incubated with nuclear extracts from the murine Friend erythroleukemia cell line 745A. The proteins recovered on GST-Spi-1-glutathione-Sepharose beads were analyzed by SDS-PAGE. One protein, with apparent molecular mass around 55 kDa, was retained specifically by the GST-Spi-1 fusion protein (data not shown). This 55-kDa protein was purified on SDS-polyacrylamide gel. A 15-amino acid internal peptide was subjected to amino acid sequence determination. A data base search revealed that this sequence (LEMEMEAARHEHQVW) perfectly matched to the nuclear RNA-binding protein p54nrb (9). p54nrb is highly related to the human splicing factor PSF (PTB-associated splicing factor) (17) in the binding buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM MgCl\(_2\), 0.5 mM ATP, 20 mM creatine phosphate, 1% (w/v) polyvinyl alcohol, and 4 ng of \(^{32}\)P-labeled \(\beta\)-globin pre-mRNA. After 3 h of incubation at 30 \(^\circ\)C the RNA was purified and analyzed on 6% polyacrylamide gel in 8M urea, as described elsewhere (16). GST and GST-fusion proteins were incubated for 10 min with HeLa nuclear extract to equilibrate GST-Spi-1 fusion proteins with their responsive proteins (Fig. 1). Only Spi-1/PU.1 and Spi-B bound GST-p54nrb, revealing that in vitro association of p54nrb with Spi proteins is not a general property of the Ets proteins.

**Spi-1/PU.1 and its related protein Spi-B (18), are the most phylogenetically divergent members of the Ets family. Their Ets domains are 70% homologous and present only 35-40% sequence identity with that of Fli-1 (19) and Ets-2 (20). The specificity of binding of p54nrb to Spi-1/PU.1 was approached by investigating whether other Ets proteins: Fli-1, Ets-2, and Spi-B, were able to interact with p54nrb. GST-p54nrb fusion protein was incubated in the presence of 35S-labeled Spi-1/PU.1, Spi-B, Ets-2, and Fli-1 proteins translated in reticulocyte lysates (Fig. 1B). Only Spi-1/PU.1 and Spi-B bound GST-p54nrb, revealing that in vitro association of p54nrb with Spi proteins is not a general property of the Ets proteins.

**Spi-1/PU.1 contains three domains: the transactivation domain (amino acids 1-111), the PEST domain (amino acids 111-158), and the DNA-binding domain (DBD) including the Ets motif (amino acids 158-266). The Spi-1/PU.1 domain (Fig. 1A) involved in the association of Spi-1/PU.1 with p54nrb was mapped by testing interactions of 35S-labeled in vitro translated p54nrb with various deleted forms of GST-Spi-1 (Fig. 1A). Data in Fig. 1C showed that only the entire Spi-1/PU.1 (GST-Spi-1) and its DNA-binding domain (GST-DBD-Spi-1) interacted with p54nrb protein. The same results were obtained for Spi-B (data not shown). In contrast, the DBD of Fli-1 fused to GST did not interact with p54nrb. In these experiments, we ascertained that the DNA binding domains of Spi-1/PU.1 and Fli-1 fused to GST were able to bind their respective responsive element in band shift assay (data not shown). p54nrb, like PSF (17), contains a central RNA binding domain with two RNA recognition motifs (RRM). Different truncated GST-p54nrb fusion proteins (Fig. 1A) were tested for their abilities to bind in vitro 35S-translated Spi-1/PU.1 protein. Interactions occurred only between the entire GST-p54nrb protein or the GST-DBD-p54nrb suggesting that p54nrb bound Spi-1/PU.1 by its RNA binding domain. The RNA binding activities of GST-p54nrb and GST-DBD-p54nrb were controlled on a Northwestern blot probed with \(^{32}\)P-labeled poly(A)\(^+\) mRNAs from 745A cells (Fig. 4A). Altogether, these data suggested that the interaction of
the transcription factor Spi-1/PU.1 with the RNA-binding protein p54nrb involved their respective DNA- and RNA-binding domains.

Then, we searched whether p54nrb and Spi-1/PU.1 could be co-immunoprecipitated when coexpressed in COS cells (Fig. 2). Nuclear extracts from transfected COS cells were immunoprecipitated under low stringency conditions with the antibody against 9E10 Myc epitope (12) used to tag p54nrb (tag-Myc-p54nrb). The presence of Spi-1/PU.1 in Myc immunoprecipitates was assessed by immunoblotting with an antibody against Spi-1/PU.1 (7) and was detected only in COS cells transfected with both Spi-1/PU.1 and p54nrb expression vectors. This provided evidence that Spi-1/PU.1 was associated with p54nrb in vivo.

The interaction between the DBD of Spi-1/PU.1 and the RBD of p54nrb suggested that it could alter the function of each partner. p54nrb altered neither the binding of Spi-1/PU.1 on various DNA-responsive elements nor the transcriptional activity of Spi-1/PU.1 in CAT assay (data not shown). We sought to discover whether Spi-1/PU.1 might change the behavior of the RNA-binding protein p54nrb. The pyrimidine-rich sequence of the branchpoint/polypyrimidine tract RNA, which is part of most 3' splice sites in mammalian introns, is targeted by PSF (17). Since p54nrb presents 70% identity with PSF in its RRM, we tested whether p54nrb could bind such RNA sequence. We observed (Fig. 3) that, like PSF, p54nrb binds the pyrimidine-rich sequence of the 3' splice site in the intron of the β-tropomyosin pre-mRNA (13). Thus, this RNA sequence was used as probe in EMSA (Fig. 3). Increasing amounts of GST-Spi-1 mixed with an equal input of GST-p54nrb reduced the formation of the GST-p54nrb-RNA complex in a dose-dependent manner, revealing that Spi-1/PU.1 impedes the binding of p54nrb to RNA.

The binding of Spi-1/PU.1 to the RNA-binding protein p54nrb prompted us to check whether Spi-1/PU.1 binds RNA. Various deletion mutants of Spi-1/PU.1 and p54nrb fused to GST were analyzed by Northwestern blot using the labeled poly(A) plus RNA as a probe. Fig. 4A revealed that Spi-1/PU.1, by its DNA binding domain, was able to interact with RNAs as the RNA-binding domain of p54nrb. To further investigate the interaction of Spi-1/PU.1 with RNA, we tested its ability to bind homoribonucleotide polymers fixed to agarose beads. Spi-1/PU.1 (and Spi-B not shown) bound preferentially the homoribopoly(G) (Fig. 4B). This affinity of Spi-1/PU.1 for poly(G) appeared functionally relevant since in EMSA performed with the Spi-1/PU.1 DNA-responsive element xE3' (5), an excess of poly(G) competed the binding of Spi-1/PU.1 to DNA sequence (Fig. 4C). Elsewhere, in competitive experiments (Fig. 3), p54nrb exhibited affinity for poly(G) and poly(U) revealing that Spi-1/PU.1 and p54nrb display a similar ability to bind poly(G). In agreement with the absence of in vitro interaction between p54nrb and Fli-1 or Ets-2, we observed that the DNA binding activity of Fli-1 fused to GST was not affected by an excess of homoribopolymers (data not shown) and that Fli-1 and Ets-2 did not bind any homoribopolymers (Fig. 4B). These data brought a first evidence that the transcription factor Spi-1/PU.1 was able to bind RNA.

Due to the extensive homology between p54nrb and PSF, a factor involved in RNA splicing, we asked whether Spi-1/PU.1 might interfere with the splicing process. In vitro splicing reactions were performed with HeLa cell nuclear extracts and a pre-mRNA transcribed from a minigene containing human β-globin exons 1 and 2. The addition of GST-Spi-1/PU.1 inhibited the formation of the spliced transcript, whereas addition of another Ets protein, like GST-Fli-1 protein, did not (Fig. 5). This suggests that alteration of splicing process did not result from an excess of an Ets protein in splicing extracts. Moreover, the DNA-binding domain of Spi-1/PU.1 that contains the RNA-binding activity appeared sufficient to inhibit the formation of the spliced transcript (Fig. 5). Noteworthily, when the HeLa cell nuclear extracts were precleared on GST-Spi-1 column before splicing reactions, their splicing activity was lost (Fig. 5). This suggests that the alteration of β-globin splicing by Spi-1/PU.1 occurred through a direct trapping of proteins involved in the splicing process. Altogether, these data suggest that Spi-1/PU.1 could interfere with splicing events.

The molecular mechanism by which the transcription factor Spi-1/PU.1 blocks the differentiation of proerythroblast and promotes their malignant transformation in the Friend erythroleukemia is not understood. Until now Spi-1/PU.1 was considered as a transcriptional regulator, targeting purine-rich DNA sequences in promoters and enhancers of some hematopoietic genes. The finding that Spi-1/PU.1 interacts with RNA-binding proteins and binds RNAs raises the possibility that in vivo, an elevated level of Spi-1/PU.1 may lead Spi-1/PU.1 to change the activities of RNA-binding proteins, like p54nrb. Interestingly, some human sarcoma (21, 22) and myeloid leukemia (23) are associated with chromosomal translocations that lead to the fusion of the two highly similar RNA-binding proteins TLS (14, 24) and EWS (21), deleted in their RRM, with proteins that either mediate DNA binding like Fli-1 and Erg. Only the fusion proteins that exhibit altered RNA binding and
transcriptional activities as compared to the native proteins (15, 25) are oncogenic. It can be speculated that Spi-1/PU.1 normally binds DNA and regulates transcription of some hematopoietic genes through its cooperation with the transcriptional machinery. Unphysiological high concentration of Spi-1/PU.1, consequent to insertional mutagenesis, may promote its interaction with proteins involved in premRNA splicing. Although the function of p54 is unknown, its high homology with PSF and its ability to bind polypyrimidine sequences are indicative of a putative role in post-transcriptional modifications of RNA. Thus, Spi-1/PU.1 might disturb the post-transcriptional gene regulation by sequestering some RNA-binding proteins like p54nrb. This alteration in alternative splicing, by preventing normal or promoting abnormal splicing complex formation, could be a mechanism involved in leukemogenesis.

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Fig. 4. Spi-1/PU.1 binds RNA. A, GST fusion proteins were separated by gel electrophoresis and blotted onto nitrocellulose. The blot was stained with Ponceau Red (left panel) and then probed with 32P-end-labeled poly(A)+ RNAs (right panel). Molecular mass markers are indicated on the right. B, RNA binding activities of Spi-1/PU.1, Fli-1, and Ets-2. 35S-labeled in vitro translated proteins were bound to homoribopolymers linked to agarose beads. 50% input are shown. Molecular mass markers are indicated on the right. C, EMSA of in vitro translated Spi-1/PU.1 incubated with 32P-labeled c-Fes-RE probe in the presence of homoribopolymer competitor.

Fig. 5. Spi-1/PU.1 alters the splicing of a b-globin minigene in vitro. 32P-Labeled b-globin mRNA was incubated with HeLa cell nuclear extracts (except lanes 1, 8, and 9) under splicing conditions in the absence or presence of 1 mg of GST or GST fusion proteins. In lanes 8 and 9, HeLa cell nuclear extracts were first preclered on GST (lane 8) or GST-Spi-1 (lane 9) coated beads before the splicing reaction. Unspliced and spliced RNAs, separated on a 6% denaturing polyacrylamide gel, are indicated.
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