Oncoprotein TLS Interacts with Serine-Arginine Proteins Involved in RNA Splicing*

Liu Yang‡§, Lisa J. Embree‡§, Schickwann Tsai§, and Dennis D. Hickstein‡§* From the ‡Medical Research Service, Veterans Affairs Puget Sound Health Care System, Seattle, Washington 98108, §Fred Hutchinson Cancer Research Center, Seattle, Washington 98104, and ¶Division of Oncology and Molecular Medicine, University of Washington School of Medicine, Seattle, Washington 98195

The gene encoding the human TLS protein, also termed FUS, is located at the site of chromosomal translocations in human leukemias and sarcomas where it forms a chimeric fusion gene with one of several different genes. To identify interacting partners of TLS, we screened a yeast two-hybrid cDNA library constructed from mouse hematopoietic cells using the C-terminal region of TLS in the bait plasmid. Two cDNAs encoding members of the serine-arginine (SR) family of proteins were isolated. The first SR protein is the mouse homolog of human splicing factor SC35, and the second SR member is a novel 183-amino acid protein that we term TASR (TLS-associated serine-arginine protein). cDNA cloning of human TASR indicated that mouse and human TASR have identical amino acid sequences. The interactions between TLS and these two SR proteins were confirmed by co-transfection and immunoprecipitation studies. In vivo splicing assays indicated that SC35 and TASR influence splice site selection of adenovirus E1A pre-mRNA. TLS may recruit SR splicing factors to specific target genes through interaction with its C-terminal region, and chromosomal translocations that truncate the C-terminal region of TLS may prevent this interaction. Thus TLS translocations may alter RNA processing and play a role in malignant transformation.

Chromosomal translocations are found frequently in leukemias as well as in malignancies of non-hematopoietic tissues. These translocations usually give rise to novel fusion genes and novel fusion proteins. To understand the role that these fusion proteins play in cellular transformation, knowledge of the function of the wild-type protein involved in the translocations is required.

We have focused these studies on understanding the function of the TLS gene. TLS (translocated in liposarcoma), also called FUS, was originally identified through its fusion to the CHOP gene (a member of the C/EBP family of transcription factors) in human myxoid liposarcoma with the t(12;16) chromosomal translocation (1, 2). In human acute myeloid leukemia with the t(16;21) translocation, the TLS gene is fused with the ERG gene (a member of the ETS family of transcription factors) (3). In translocations involving TLS, the N-terminal part of TLS is retained in the fusion protein and the C-terminal region is deleted.

TLS is a member of a closely related family of genes, including the EWS gene, which was originally identified in Ewing’s sarcoma (4). Both TLS and EWS are involved in various types of cancers through chromosomal translocation either with other genes of the ETS family or with other transcription factors (see Ref. 5 for a review). The evidence that transcriptional activation plays a role in transformation mediated by TLS (or EWS) fusion proteins stems from the observations that the N terminus of the TLS protein is rich in glutamine, serine, and tyrosine and is a potent transactivator when fused with various transcription factors (6).

Two observations suggest that the transactivational activity of the TLS fusion protein may not be sufficient to explain the role of the fusion protein in transformation. First, a correlation between transactivation and transformation by these fusion proteins has not been demonstrated (7). Second, the C terminus of TLS contains several motifs that are suggestive of other potential TLS functions. The TLS C-terminal region contains an evolutionarily conserved RNP1 consensus sequence and Arg-Gly-Gly (RGG) repeats, both of which have been implicated in RNA binding (8). In previous studies, TLS has been shown to bind to RNA in both the nucleus and the cytoplasm and was hypothesized to be a heterogeneous ribonuclear protein-like chaperone of RNA (9). In a recent study, TLS was found to interact with PU.1, an ETS protein capable of regulating transcription and RNA splicing. Overexpression of TLS in IW1–32 erythroid cells was shown to promote the use of the distal 5′-splice site during E1A pre-mRNA splicing. This site preference is counterpoised by PU.1, suggesting that TLS may be part of a protein network involved in the regulation of RNA processing (10).

Because the C terminus of TLS is replaced in fusion proteins as the result of chromosomal translocation, loss of TLS interactions with cellular regulators may play an important role in transformation. To identify protein molecules that interact with TLS, we used the C-terminal part of TLS protein as the bait to screen a yeast two-hybrid library derived from mouse multipotential hematopoietic cells (11). We isolated two cDNAs that encode TLS-interacting proteins with serine-arginine (SR)-rich domains. The first cDNA corresponds to the mouse homolog of the human splicing factor SC35, and the second cDNA encodes a novel protein that we term TASR (TLS-associated serine-arginine protein). The SR family of proteins has been shown previously to be involved in constitutive and regulated RNA splicing.

* This work was supported by grants from the Department of Veterans Affairs and the National Institutes of Health. 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF077858, AF042383, and AF047448.

** To whom correspondence should be addressed: VA Puget Sound Health Care System, 1660 S. Columbian Way, GMR 151, Seattle, WA 98108. Tel: 206-764-2705; Fax: 206-764-2527; E-mail: dennishi@u.washington.edu.

The abbreviations used are: RNP, ribonucleoprotein; SR, serine-arginine; PCR, polymerase chain reaction; EML, erythroid, myeloid, and lymphoid; RT, reverse transcription; PAGE, polyacrylamide gel electrophoresis; DOTAP, N-dioleoyloxy)propyl-trimethylammonium methylsulfate.

This paper is available on line at http://www.jbc.org

27761
**EXPERIMENTAL PROCEDURES**

**Plasmid and Library Constructions**—To generate a TLS bait plasmid for yeast two-hybrid screening, human TLS cDNA was PCR-amplified, digested with EcoRI, and cloned into the EcoRI-StuI sites of the pBTM116 vector (12) to generate the bait plasmid pBTM-TLS in which the C-terminal region of TLS protein is fused to LexA DNA-binding protein (see Fig. 1). For expression of Flag-tagged TLS protein in COS-7 cells, the coding region of the TLS DNA was subcloned in-frame into the EcoRI-StuI sites of pSG5-Flag vector (CLONTECH) to generate pSG5-Flag-TLS. Flag-tagged luciferase expression plasmid pSG5-Flag-Luc was constructed by ligation of the pSG5-Flag vector with the luciferase insert from pGL3 plasmid (Promega). Myc-tagged expression plasmids pCS2-Myc-Flag and pCS2-Myc-TASR were generated by in-frame ligation of mouse SC35 and TASR cDNA sequences into the EcoRI-StuI sites of pCS2-MT vector. For overexpression of the SC35 and TASR proteins in HeLa cells, full-length mouse SC35 and TASR cDNAs were cloned into the EcoRI-EcoRV sites of pCR3 vector (Invitrogen) to generate plasmids pCR3-SC35 and pCR3-TASR. Plasmid pCS3-MT-E1A was made by Dr. M. Gacheli and described previously (10). The yeast two-hybrid library was constructed with mRNAs from the murine hematopoietic cells with erythroid, myeloid, and lymphoid (EML) potential (11, 12).

**Two-hybrid Screen**—The yeast two-hybrid screen was performed as described previously (11).

**cDNA Cloning**—To obtain full-length mouse SC35 and TASR cDNA sequences, the SC35 and TASR inserts obtained using the yeast two-hybrid screen were used as the probe in the hybridization screening of a Uni-ZAP phage cDNA library derived from EML cells. pBluescript phagemids containing full-length SC35 and TASR cDNAs were prepared after in vivo excision from the Uni-ZAP XR vector and were used in sequencing reactions with Dye terminators (Applied Biosystems). To obtain human TASR cDNA, total RNA was isolated from K562 leukemia cells and used as a template for amplification by RT-PCR.

** RESULTS AND DISCUSSION**

The structural features of TLS, fusion proteins TLS/CHOP and TLS/ERG, and the C-terminal region of TLS used in the yeast bait plasmid are shown (Fig. 1). TLS sequence features implicated in RNA binding include the Arg-Gly-Gly repeats (RGG) and the evolutionarily conserved ribonucleoprotein consensus sequence (RNP-CS). In the initial yeast two-hybrid screen of $1 \times 10^7$ transformants, we identified two independent clones that were positive for interaction with the TLS bait. Sequencing of the first clone indicated that it was the mouse homolog of human SC35, one of the SR-rich mammalian splicing factors (14). Sequencing of the second positive clone revealed the presence of C-terminal serine-arginine repeats, whereas the remainder of the sequence did not align with sequences in the GenBank database. In-frame translation of the cDNA insertion that it represented a novel TLS-associated protein containing multiple serine-arginine repeats; therefore the protein was named TASSR (TLS-associated protein with serine-arginine repeats). To obtain full-length mouse SC35 and TASSR cDNAs, the corresponding cDNA inserts were used to screen an EML cDNA phage library. Several clones containing full-length SC35 and TASSR cDNAs were isolated. Nucleotide sequence analysis revealed that mouse SC35 cDNA is approximately 1.9 kilobases in length (GenBank accession no. AF077856) and encodes a protein nearly identical to human SC35. Mouse TASSR cDNA is approximately 3.4 kilobases in length (GenBank accession no. AF042383) and human TASSR (GenBank accession no. AF047448) are identical at the amino acid level. RNPs2 and RNPl consensus sequences are underlined. Arg-Ser or Ser-Arg dipeptide repeats are shown in boldface. B, schematic comparison of TASSR to SC35. The RNP consensus sequences are shown in gray boxes. The glycine-rich hinge is in a hatched box, and the RS domains are in boxes with wavy lines.
the GenBank protein data base with the BLASTp program (16) revealed that the TASR protein shares similar structural motifs with the SR family of splicing factors such as SC35, SF2/ASF (17, 18), and SRp20 (19), the conserved glycine-rich hinge in SC35 and SF2/ASF is absent in TASR protein (Fig. 2B).

Using RT-PCR, we have also cloned the human TASR cDNA (GenBank accession no. AF047448) from K562 leukemia cells. Sequencing analysis revealed that both mouse and human TASSR coding regions are 93% identical at the nucleotide level and 100% identical at the amino acid level, indicating that TASSR protein is evolutionarily conserved.

The finding that SC35 interacts with TLS in the yeast two-hybrid system suggested that these two proteins may also associate with each other in mammalian cells. To confirm their intracellular association, plasmids expressing Myc-SC35 and Flag-TLS or Flag-luciferase were co-transfected into COS-7 cells, and lysates from the co-transfected cells were used for immunoprecipitation. A polyclonal rabbit anti-Flag antibody was used in the experiment, neither Flag-TLS nor Myc-SC35 associated with Myc-SC35 and could not be detected in the immunoprecipitates (Fig. 3A, lane 5). When a normal mouse IgG was used in the experiment, neither Flag-TLS nor Myc-SC35 was present in the immunoprecipitates (Fig. 3A, lane 6).

To confirm the intracellular association between TASSR and TLS, plasmids expressing Myc-TASR and Flag-TLS or Flag-luciferase were also transfected into COS-7 cells for co-immunoprecipitation studies. Myc-TASR and Flag-TLS formed a protein complex that is recognized by specific antibodies directed against the epitope tags (Fig. 3B, lanes 1 and 4). Myc-TASR did not bind to Flag-luciferase (Fig. 3B, lanes 2 and 5), indicating the specificity of the interaction between TLS and TASSR. Normal IgG did not immunoprecipitate Myc-TASR or Flag-TLS (Fig. 3B, lanes 3 and 6).

To investigate the functions of TLS, SC35, and TASSR as splicing factors, we analyzed the effects of their overexpression on the alternative splicing of the adenovirus E1A pre-mRNA in HeLa cells. Differential splicing of E1A pre-mRNA generates five major isoforms (13, 12, 11, 10, and 9 S) (Fig. 4A) (20). When overexpressed in HeLa cells, TLS promoted splice site selection generating the 13 and 12 S isoforms (Fig. 4B, compare lanes 1 and 2). In agreement with previous reports, expression of SC35 did not increase 13 S isoform but decreased the 12 S isoform (Fig. 4B, compare lanes 1 and 3) (21). When both TLS and SC35 were co-expressed, TLS was unable to promote the use of splice sites leading to 13 and 12 S isoforms (Fig. 4B, compare lanes 2 and 4). The TASSR protein also functioned as a splicing factor. In contrast to SC35, however, TASSR expression decreased the 13 S isoform and promoted the use of distal 5′-splice sites leading to the 11, 10, and 9 S isoforms (Fig. 4B, compare lanes 1 and 5). When TLS and TASSR were co-expressed, again the distal 5′-splice sites resulting from TASSR selection were favored (Fig. 4B, compare lanes 2 and 6). Together, these results
indicate that SC35 and TASS display opposing effects on E1A pre-mRNA splicing and that the influence of TLS on E1A pre-mRNA splicing is abrogated in the presence of overexpressed SC35 or TASS.

Considerable evidence now implicates TLS in binding to RNA. First, TLS protein contains structural motifs such as RGG and RNP-CS, which are conserved in many RNA-binding proteins (8). Second, bacterially expressed full-length TLS and its C terminus both bind to in vivo labeled RNA (1). The fact that this binding is enriched in the poly(A)+ fraction of RNA suggests that the targets for TLS are in the mRNA fraction. Third, in vivo UV cross-linking studies indicate that TLS binds to RNA species that turn over rapidly inside cells (9). Fourth, immunocytochemical analysis localizes TLS protein to nuclear regions outside of the nucleolus, further supporting the notion that TLS binds to non-ribosomal RNA transcripts (1, 22).

The evidence linking TLS to splicing of nascent pre-mRNA is also accumulating. TLS has been found to be associated with proteins, such as human nuclear RNP A1 and SF1, that are implicated in splicing (23, 24). Also, as mentioned in the introduction, TLS induces the preferential use of the most distal 5′-splice site during E1A pre-mRNA splicing in WI–32 erythroid cells (10). At the present time, the mechanism by which TLS influences pre-mRNA splicing remains unclear. This study, for the first time, links TLS to the SR family of proteins, which have been extensively investigated for their role in RNA splicing.

RNA processing is clearly a primary function of SR proteins. The N-terminal RNP consensus sequence of SR proteins is required for binding to RNA, whereas the C-terminal arginine-serine-rich domain is important in mediating association with other cellular proteins (25). In addition, phosphorylation of SR proteins has also been reported to affect both protein-protein and protein-RNA interactions, thus exerting further control over the enzymatic activity and intracellular localization of these splicing factors (26, 27). Although different SR proteins may have overlapping functions in constitutive splicing, they differ from each other in their ability to regulate alternative splicing as shown by previous studies (21, 28) and by the in vivo splicing assays of this paper.

Important regulatory roles for SR proteins have been demonstrated by their functions in Drosophila development and sex determination (29–31), their involvement in T-cell activation (32), their close association with cell cycle control (33), and their interaction with key cellular enzymes such as DNA topoisomerase I (34). SR proteins have been found to be associated with various transcription units (35), linking SR proteins to the tightly coupled processes of transcription and splicing in mammalian cells (36). The association of the SR family of splicing factors with RNA polymerase II has also been described by several groups (37–39), suggesting interactions between SR proteins and the C-terminal repeat domain of RNA polymerase II.

Because of the potential functions of TLS and SR proteins, interactions between the SR family of proteins and TLS-related proteins may be an integral part of the control of normal cell growth and differentiation. As our yeast two-hybrid screen suggests that the C-terminal region of TLS is involved in binding to SR proteins, interactions between TLS and SR proteins are likely to be altered or disrupted when this part of TLS protein is replaced through chromosomal translocations. The role that the TLS fusion proteins play in the TLS-SR protein interaction and their effect on RNA processing of TLS target genes are important questions for further study. Alterations of RNA splicing for several genes are associated with various tumor types (40). This study thus suggests that truncation of the TLS protein by translocation may result in the alteration or loss of critical RNA processing capability, leading cells on the path to malignant transformation.

Acknowledgments—We thank Dr. David Ron for TLS cDNA, Dr. Moreau-Gachelin for pCS3-MT-E1A construct, and Dr. Dan Wu for pSG5-Flag vector.

REFERENCES

1. Crozat, A., Aman, P., Mandal, N., and Ron, D. (1993) Nature 363, 640–644
2. Rabbits, T., Forster, A., Larson, R., and Nathan, P. (1993) Nat. Genet. 4, 175–180
3. Ichikawa, H., Shimizu, K., Hayashi, Y., and Ohki, M. (1994) Cancer Res. 54, 2865–2868
4. Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Koval, H., Houbert, I., Desjung, P., Rouleau, G., Aurias, A., and Thomas, G. (1992) Nature 359, 162–165
5. Bertolotti, A., Melot, T., Acker, J., Vigneron, M., Delattre, O., and Tora, L. (1998) Mol. Cell. Biol. 18, 1489–1497
6. Prasad, D. D., Ouchida, M., Lee, L., Yao, V. N., and Reddy, E. S. (1994) Oncogene 9, 3717–3729
7. Lessnick, S. L., Braun, B. S., Denny, C. T., and May, W. A. (1995) Oncogene 10, 423–431
8. Borrow, C. G., and Dreyfuss, G. (1994) Science 265, 615–621
9. Zinszner, H., Sok, J., Immanuel, D., Yin, Y., and Ron, D. (1997) J. Cell Sci. 110, 1741–1750
10. Hallier, M., Larga, A., Barnache, S., Tavitchian, A., and Moreau-Gachelin, F. (1998) J. Biol. Chem. 273, 4838–4842
11. Tsai, S., Bartelmes, S., Sitnicka, E., and Collins, S. (1994) Genes Dev. 8, 2831–2841
12. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
13. Caceres, J. F., Stamm, S., Helfman, D. M., and Krainer, A. R. (1994) Science 265, 1706–1709
14. Fu, X. D., and Maniatis, T. (1992) Science 256, 555–558
15. Kozak, M. (1991) J. Biol. Chem. 266, 19867–19870
16. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
17. Ge, H., Zou, P., and Manley, J. L. (1991) Cell 66, 373–382
18. Krainer, A. R., Mayeda, A., Kozak, D., and Birnstiel, G. (1991) Cell 66, 383–394
19. Ayane, M., Preuss, U., Kohler, G., and Nielsen, P. J. (1991) Nucleic Acids Res. 19, 1273–1278
20. Stephens, C., and Harlow, E. (1987) EMBO J. 6, 2027–2035
21. Wang, J., and Manley, J. L. (1995) RNA 1, 335–346
22. Hackl, W., and Luhrmann, R. (1996) J. Mol. Biol. 264, 843–851
23. Zhang, D., Paley, A. J., and Childs, G. (1998) J. Biol. Chem. 273, 18096–18091
24. Valsecchi, J., and Green, M. R. (1996) Trends Biochem. Sci. 21, 296–301
25. Cahard, D., and Roth, M. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2642–2646
26. Chang, J. H., and Baker, B. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 115–120
27. Scraton, R. G., Caceres, J. F., Mayeda, A., Bell, M. V., Plebanski, M., Jackson, D. G., Bell, J. I., and Krainer, A. R. (1995) EMBO J. 14, 4336–4349
28. Jumaa, H., Groenen, L. J., and Nielsen, P. J. (1997) Mol. Cell. Biol. 17, 3116–3124
29. Rossi, F., Labourier, E., Ferne, T., Divita, G., Derancourt, J., Rius, J. F., Antonie, E., Cathala, G., Brunel, C., and Tazi, J. (1996) Nature 381, 80–82
30. Neugebauer, K. M., and Roth, M. B. (1997) Genes Dev. 11, 3279–3285
31. McCracken, S., Feng, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., and Bentley, D. (1997) Nature 385, 357–361
32. Yuryev, A., Patturajan, M., Litingtung, Y., Joshi, R. R., Gentle, C., Gebara, M., and Corden, J. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6975–6980
33. Jumaa, H., Blencowe, B. J., Wei, X., Nakayasu, H., Du, L., Warren, S. L., Sharp, P. A., and Berezymy, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8255–8257
34. Kim, E., Du, L., and Warren, S. L. (1997) J. Cell Biol. 136, 19–28
35. Cote, G. J., Huang, E. S.-C., Jin, W., and Morrison, R. S. (1997) J. Biol. Chem. 272, 1054–1060