Vol. 195, No. 1, 1993
August 31, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 428-434

p53 AND SV40 T ANTIGEN BIND TO THE SAME REGION OVERLAPPING THE CONSERVED DOMAIN OF THE TATA-BINDING PROTEIN

David W. Martin, Mark A. Subler, Rubén M. Muñoz, Doris R. Brown, Swati P. Deb, and Sumitra Deb*

Department of Microbiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284

Received July 16, 1993

Summary In this report we demonstrate that the cloned human TATA-binding protein (TBP) interacts with T antigen. TBP co-immunoprecipitates with T antigen when incubated with the T antigen-specific monoclonal antibody PAb419, and Protein-A agarose. Gel retention analysis with a radiolabeled TATA box-containing probe showed that the complex of TBP and T antigen can bind to the TATA box. Recently, p53 has also been shown to interact with TBP. Using TBP deletion mutants and co-immunoprecipitation experiments with p53 or T antigen, we show that both p53 and T antigen bind to the same region, amino acids 203-275, within the conserved C-terminal domain of TBP. Binding of p53 and T antigen to the same domain on TBP may lead to competition between the two proteins for transcriptional function.

The nuclear phosphoprotein p53 was first identified in association with simian virus 40 (SV40) large T antigen (1,2). p53 functions as a tumor suppressor and is the most common genetic defect associated with cancer (3,4). A growing body of experimental evidence indicates that p53 is also involved in transcription. Wild-type p53 activated a promoter with p53-binding sites both *in vivo* and *in vitro* (5-9). Wild-type p53 has also been shown to inhibit a number of cellular and viral promoters (10-15). Interestingly, mutants of p53 activated the human proliferating cell nuclear antigen (PCNA) and the multiple drug resistance gene (MDR-1) promoters significantly (11,15). Recent work has demonstrated that p53 binds to the TATA-binding protein (TBP) (16, 19) as well as the TBP/TATA complex (19). Taken together, these observations suggest that p53 may influence gene expression through a direct interaction with the transcriptional machinery.

The SV40 T antigen is a multifunctional protein that is the initiator of SV40 DNA replication and also plays a role in transcriptional regulation (20). p53 and T antigen are antagonistic to each others function. p53 inhibits T antigen-mediated initiation of SV40 DNA replication (21,22). T antigen antagonizes the transcriptional function of p53 both *in vitro* and *in vivo* (23,24). The molecular mechanisms which mediate the antagonism between p53 and T antigen are not yet fully defined. As a step towards this definition, we have tested if T antigen could also interact with

*To whom correspondence should be addressed.

0006-291X/93 $4.00

TBP. In the present study we demonstrate that the cloned human TBP interacts with T antigen and the complex of TBP and T antigen can bind to the TATA box. Using TBP deletion mutants and co-immunoprecipitation experiments with p53 or T antigen, we show that both p53 and T antigen bind to the conserved C-terminal region of TBP, between amino acids 203-275. Binding of p53 and T antigen to the same domain on TBP may lead to competition between the two proteins for transcriptional function.

MATERIALS AND METHODS

Expression and purification of wild-type p53 and SV40 T antigen. Recombinant baculoviruses containing wild-type p53 and SV40 T antigen, and the purification of the corresponding protein by immunoaffinity chromatography have been described earlier (19).

Construction of TBP mutants. For the construction of TBP deletion mutants, we have used a wild-type cDNA copy of human TBP (a generous gift of Drs. Horikoshi and Roeder) cloned in pGEM7zf(+). Two C-terminal deletion mutants of TBP (del 275-335) and (del 100-335) were generated using the restriction sites StuI and PstI, respectively, inside the TBP gene. These mutants were constructed by inserting an NheI linker with stop codons in all the three reading frames. One C-terminal deletion mutant (del 294-335) and one N-terminal deletion mutant (del 1-203) were generated by polymerase chain reaction (PCR) using primers with a stop codon or initiation codon, respectively, in frame. The schematic representation of the mutants are provided in Figure 3A.

Expression of wild-type and mutant human TBP *in vitro*. Wild-type and mutant human TBP proteins were expressed by *in vitro* transcription and translation using the TNT system (Promega). All constructs were under the control of the T7 promoter. Aliquots from programmed extracts were analyzed on 10% SDS-polyacrylamide gels. Gels were treated with Enhance (DuPont) and the ^{35}S-labeled proteins were visualized by autoradiography.

Immunoprecipitation. Immunoprecipitation using wild-type human p53 were performed by adding 100 ng of immunoaffinity purified protein to 2 ul of human TBP-programmed extracts under conditions as described by Martin *et al.* (19). Incubations were done in a 60 ul reaction volume for 30 minutes at 30 C. PAb419 (a monoclonal antibody against T antigen) hybridoma supernatant was then added to the reaction and incubated at room temperature for one hour. The immune complexes were precipitated by gently rocking for two hours at room temperature with Protein A-agarose (Calbiochem). Pellets were washed as described (25) and resuspended in 2X Laemmli sample buffer (26), boiled ten minutes, and run on 10% SDS-polyacrylamide gels. Gels were treated with Enhance (DuPont) and visualized by autoradiography. Labeled human TBP in 2X Laemmli buffer was boiled ten minutes and run in parallel with the reactions to mark the position of migration of the precipitated bands. Immunoprecipitation of human TBP (wild-type and mutants) by p53 was performed as described above. p53 was precipitated using the monoclonal antibody PAb421, a monoclonal against p53 and Protein A-agarose (27).

Gel retardation assays. Gel shift assays were performed as described by Martin *et al.* (19). A TATA-containing probe was derived from the TATA-only construct described by Wang and Gralla (28), containing the TATA box from the adenovirus major late promoter. The fragment for DNA binding was generated by HindIII digestion and labeled with ^{32}P-dCTP. Purified, wild-type human TBP was a bacterially expressed product, acquired from Promega. Gel shift assays to determine the effect of T antigen on binding of TBP to TATA (Figure 2) used 20 ng of human TBP and 100 and 200 ng of T antigen, respectively. The antibody shift experiment to test for the presence of T antigen in the TBP/TATA complex used 200 ng of PAb101 (Pharmingen), 200 ng of T antigen, and 10 ng of human TBP.

RESULTS

TBP co-immunoprecipitates with SV40 T antigen. SV40 T antigen interacts with p53 and inhibits *in vitro* sequence-specific transactivation of promoters containing upstream p53-binding sites (23). SV40 T antigen is dominant to normal p53 in cells transformed with SV40, so that although the p53 protein remains wild-type in sequence, it cannot protect cells from transformation (29). T antigen also plays a role in transcription activating SV40 late transcription while inhibiting SV40 early transcription (20). In order to define the factors that may be involved in T antigen-mediated transcription and the functional antagonism between p53 and T antigen, we wished to check whether T antigen can bind to TBP. Increasing amounts of affinity purified T antigen (Molecular Biology Resources) were incubated with *in vitro* translated TBP and the mixture immunoprecipitated with PAb419 (a monoclonal antibody against SV40 T antigen), and Protein-A agarose. As demonstrated in Figure 1, PAb419 could specifically coimmunoprecipitate increasing amounts of TBP in the presence of increasing amounts of T antigen. No TBP was precipitated in the absence of T antigen demonstrating that PAb419 was not precipitatin TBP directly. In addition, no TBP is precipitated in the presence of T antigen and PAb421, a monoclonal antibody specific for p53 (data not shown). Taken together, these results suggest that T antigen forms a specific complex with TBP. The observation that SV40 T antigen forms a complex with TBP may be of significance in mediating T antigen's role in transcription (20). While our work was in progress, Gruda, *et al.* (30) also reported that T antigen binds to TBP, and that multiple protein-protein interactions result in SV40 late promoter activation by T antigen.

Influence of SV40 T antigen on the binding of TBP to the TATA box. To determine whether the interaction of T antigen with TBP influences the ability of TBP to bind to the TATA box, we

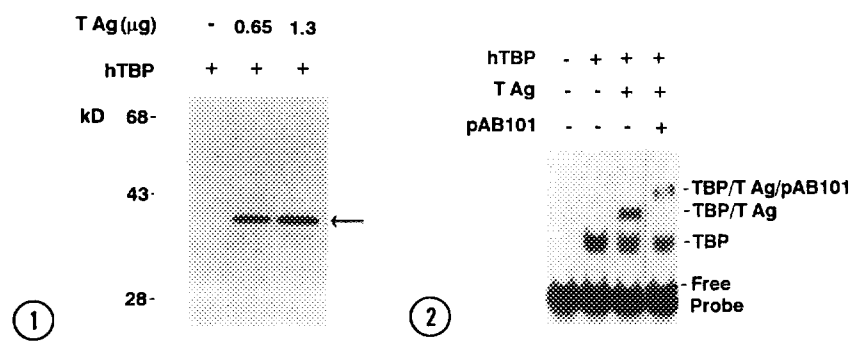

Figure 1. Co-imminuoprecipitation of T antigen with human TBP. *In vitro*-expressed TBP was incubated in the absence or presence of immunoaffinity-purified T antigen as described in Materials and Methods and precipitated using PAb419 and Protein-A agarose. The arrow indicates the presence of ^{35}S-labeled TBP. Note that no TBP is precipitated in the absence of T antigen.

Figure 2. Effect of T antigen on human TBP DNA binding. T antigen was used to test for any effect on human TBP binding to a TATA-containing probe in a gel retention assay as described in the text. The positions of the TBP and TBP/T antigen complexes are shown. PAb101 was added to confirm the presence of T antigen in the complex. The position of a TBP/T Antigen/PAb101 complex is shown.

examined TBP/TATA box interactions by gel retention assays (Figure 2). We have used a ^{32}P-labeled Hind III fragment containing a TATA box that corresponds to the TATA element of the adenovirus major late promoter (28). Under these conditions, described in Materials and Methods, TBP forms a prominent complex with the TATA probe (lane 2). In the presence of T antigen, the TBP-DNA complex formed a slower migrating band in the gel retardation assay (compare lanes 2 and 3). These results demonstrate that T antigen binding to TBP does not prevent the protein complex from binding to the TATA box. The presence of T antigen in the upper complex was demonstrated by the super shift observed in the presence of purified PAb101 (lane 4), a monoclonal antibody against T antigen. The migration of the TBP/TATA complex alone was unaffected by PAb101(data not shown).

Generation and expression of TBP mutants. It is known that a number of transactivators interact with TBP, particularly within the conserved domain of TBP (25,31). To determine whether T antigen and p53 also interact on this conserved domain, we have generated a number of deletion mutants of TBP for use in immunoprecipitation assays. Four mutant TBP DNA clones were generated by using restriction sites inside the TBP gene or by PCR-mediated mutagenesis as described in Materials and Methods. Figure 3A schematically shows the mutants. The deletion mutants were then utilized to generate synthetic proteins with the help of TNT lysate (Promega). Figure 3B represents the SDS-polyacrylamide gel electrophoretic analysis of the *in vitro* synthesized TBP mutants. The sizes of the major bands correspond to the expected size of mutant proteins.

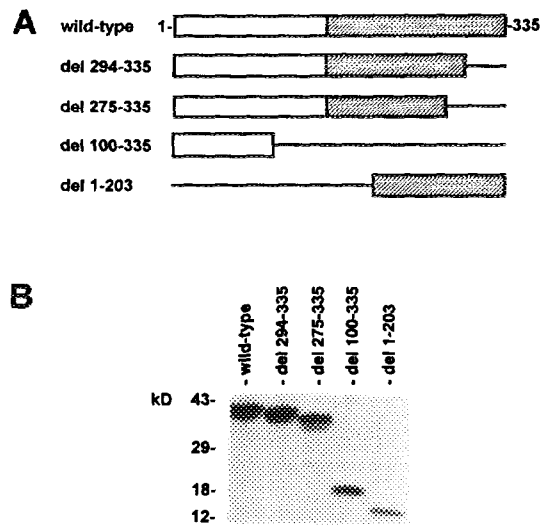

Figure 3. Expression of wild-type and mutant TBP. A. Schematic showing the nature of mutations in TBP. Boxed regions indicate the portion of TBP that remains. Thin, solid lines indicate the region deleted. Shaded areas indicate the extent of the conserved domain. B. Expression of proteins. Proteins were expressed *in vitro* as described in Materials and Methods. An aliquot of the ^{35}S-labeled protein was run on 10% SDS-polyacrylamide gels. The resulting autoradiograms indicates the migration of each of the proteins at their expected molecular weight. The position of molecular weight markers run in parallel is shown.

Binding of p53 and SV40 T antigen with wild-type and mutant TBPs. The interaction between wild-type p53 and SV40 T antigen with wild-type and mutant human TBP was examined by coimmunoprecipitation experiments. Wild-type p53 or T antigen was incubated with *in vitro* TBP (wild-type or mutant) expressed *in vitro*, and then immuoprecipitated by anti-p53 or anti-T antigen monoclonal antibodies and Protein A-agarose as described in the Materials and Methods. Figure 4 represents a SDS-polyacrylamide gel electrophoretic analysis of the immunoprecipitates. Two C-terminal deletion mutants, del 294-335 and del 275-335, both bound to p53 and T antigen. Thus, the amino acid residues from 275-335 are dispensable for interaction with these proteins. A further C-terminal deletion from amino acids 100-335 resulted in a loss of binding to both p53 and T antigen. However, the N-terminal deletion, del 1-203, was able to be immunoprecipitated with both p53 and T antigen. These results indicate that p53 and T antigen bind to the same region of TBP from amino acids 203-275. This region overlaps with the conserved domain on TBP (33).

DISCUSSION

In this communication we report that SV40 T antigen can associate with human TBP. The association of T antigen with TBP may be related to the fact that T antigen can act as a powerful transactivator of transcription (20). While our work was in progress, an interaction between TBP and T antigen has been reported using a glutathione S-transferase fusion protein (30). Previously, we and others have reported that human p53 binds to TBP (16-19). The domain on TBP that binds to p53 and T antigen contains the conserved C-terminal region of TBP (33). This region includes the segment between two homologous repeats in TBP (residues 165-225 and 255-316). This domain contains a repeat of basic residues and has been postulated to be a site of interaction between TBP and acidic activation domains (32). The same region binds to adenovirus E1A (25). Our results suggest that same basic domain on TBP is also responsible for binding to two other transactivators, p53 and T antigen.

The ability of viral oncoproteins such as SV40 T antigen to function in cell transformation correlates with their ability to interact physically with cellular proteins such as p53 or Rb (3). It is

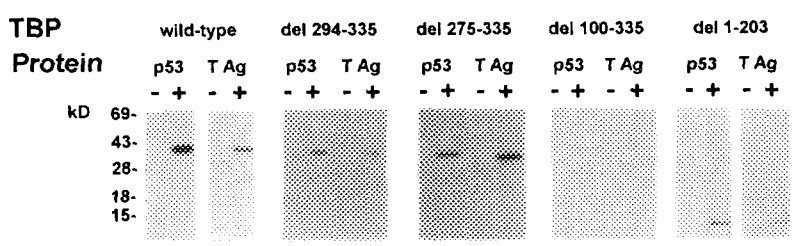

Figure 4. Immunoprecipitation of wild-type and mutant TBP with p53 and T antigen. Immunoprecipitations were carried out as described in Materials and Methods. The identity of the respective TBP used in each panel is indicated. Negative controls, designated (-), were immunoprecipitated with the desired TBP derivative and the monoclonal PAb421 or PAb419, in the absence of p53 or T antigen, respectively. The position of molecular weight markers run in parallel is shown.

proposed that binding of the viral oncoproteins to the tumor suppressors disrupts the normal suppressor function exerted by these proteins. Consistent with this antagonistic behavior, the viral oncogene product T antigen and the tumor suppressor p53 counteract each other's biochemical functions. For example, T antigen affects p53's sequence-specific DNA-binding ability, transcriptional inhibitory and activation functions (23,24,34). As mentioned earlier, p53 inhibits SV40 DNA replication mediated by T antigen (21,22). Wild-type p53 inhibits the helicase activity of T antigen correlating with its inhibition of DNA replication (22). T antigen activates SV40 late transcription (35). However, overexpression of wild-type p53 inhibits this activation (Sumitra Deb, personal communication). Interestingly, both T antigen and p53 bind to the same domain on TBP, suggesting that they may compete with each other for binding to TBP. This may constitute another form of antagonism between these two proteins. It is also worth noting that the association of TBP with T antigen may also have important implications for SV40 DNA replication. Such speculation is especially tempting in light of recent reports that demonstrate that p53 as well as other proteins with acidic activating domains have the capacity to bind replication factor A (RPA) (36,37). This further indication of the potential link between transcription and DNA replication may have interesting implications for the possible function of the T antigen/TBP complex. In addition, it has been shown that TBP is required for RNA polymerase I (38) and RNA polymerase III transcription (39,40). Thus, the interaction of TBP with T antigen may be highly significant in that T antigen could potentially effect transcription by all three RNA polymerases.

ACKNOWLEDGMENTS

This work was supported by grants from the Elsa U. Pardee Foundation, United States Department of Agriculture (No. 91-37204-6820), and a research grant from the March of Dimes to Sumitra Deb. This work was done by Sumitra Deb during the tenure of an Established Investigatorship of the American Heart Association. Ruben M. Munoz is supported in part by a NIH training grant in microbial pathogenesis (AI07271-08). We thank Dr. A.J. Levine for providing us with wild-type and mutant p53 constructs and Drs. M. Horikoshi and R.G. Roeder for TFIID cDNA clones. We thank Dr. Kathy Partin for stimulating discussion and encouragement.

REFERENCES

1. Lane, D.P. and Crawford, L.W. (1979) Nature 278, 216-263.
2. Linzer, D.H.H. and Levine, A.J. (1979) Cell 17, 43-52.
3. Levine, A.J., Momand, J., and Finlay, C.A. (1991) Nature 351, 453-456.
4. Vogelstein, B. and Kinzler, K.W. (1992) Cell 70, 523-526.
5. Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E., and Shay, J.W. (1992) Mol. Cell. Biol. 12, 2866-2871.
6. Farmer, G., Bargonetti, J., Zhu, H., Friedman, P., Prywes, R., and Prives, C. (1992) Nature 358, 83-86.
7. Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, A., Kinzler, K.W., and Vogelstein, B. (1992) Science 256, 827-830.
8. Weintraub, H., Hauschku, S., and Tapscott, S.J. (1991) Proc. Natl. Acad. Sci. USA 88, 4570-4571.

9. Zambetti, G.P., Bargonetti, J., Walker, K., Prives, C., and Levine, A.J. (1992) Genes Dev. 6, 1143-1152.
10. Ginsberg, D., Mechta, F., Yaniv, M., and Oren,M. (1991) Proc. Natl. Acad. Sci. USA 88, 9979-9983.
11. Chin, K., Ueda, V. K., Pastan, I., and Gottesman, M.M. (1992) Science 255, 459-452.
12. Santhanam, U., Roy, A., and Sehgal, P.B. (1991) Proc. Natl. Acad. Sci. USA 88, 7605-7609.
13. Subler, M.A., Martin, D.W., and Deb,S. (1992) J. Virol. 66, 4757-4762.
14. Mercer, W.E., Shields, M.T., Lin, D., Apella, E., and Ullrich, S.J. (1991) Proc. Natl. Acad. Sci. USA 88, 1954-1962.
15. Deb, S., Jackson, C.T., Subler, M.A., and Martin, D.W. (1992) J. Virol. 66, 6164-6170.
16. Seto, E., Usheva, A., Zambetti, G.P. Momand, J., Horikoshi, N., Weinman, R., Levine, A.J., and Shenk, T. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 12028-12032.
17. Truant, R., Xiao, H., Ingles, C.J., and Greenblatt, J. (1993) J. Biol. Chem. 268, 2284-2287.
18. Liu, X., Miller, C.W., Koeffler, P.H., and Berk, A.J. (1993) Mol. Cell. Biol. 13, 3291-3300.
19. Martin, D.W., Munoz, R.M., Subler, M.A., and Deb, S. (1993). J. Biol. Chem. 268, 13062-13067.
20. Fanning, E. and Knippers, R. (1992) Ann. Rev. Biochem. 61, 55-85.
21. Braithwaite, A.W., Sturzbecher, H., Addison, C., Palmer, C., Rudge, K., Jenkins, J.R. (1987) Nature 329, 458-460.
22. Wang, E.H., Friedman, P.N., Prives, C. (1989) Cell 57, 3790-392.
23. Farmer, G., Bargonetti, J., Zhu, H., Friedman, P., Prywes, R., and Prives, C. (1992) Nature 358, 83-86.
24. Mietz, J.A., Unger, T., Huibregtse, J.M., and Howley, P.M. (1992) EMBO J. 11, 5013-5020.
25. Lee, W.S., Cheng-Kao, C., Bryant, G.O., Liu, X., and Berk, A.J. (1991) Cell. 67, 365-376.
26. Laemmli, U.K. (1970) Nature 227, 680-685.
27. Harlow, E., Crawford, L.V., Pim, D.C., and Williamson, N.M. (1981) J. Virol. 39, 861-869.
28. Wang, W.D. and Gralla, J.D. (1991). Mol. Cell. Biol. 11, 4561-4571.
29. Moore, M., Teresky, A.K., Levine, A.J., and Seiberg, M. (1992) J.Virol. 66, 641-649.
30. Gruda, M.C., Zabolotny, J.M., Xiao, J.H., Davidson, I., and Alwine, J.C. (1993) Mol. Cell. Biol. 13, 961-969.
31. Stringer, K.F., Ingles, C.J., and Greenblatt, J. (1990) Nature 345, 783-786.
32. Horikoshi, M., Wang, C.K., Fujii, H., Cromlish, J.A., Weil, P.A., and Roeder, R.G. (1989) Nature 345, 783-786.
33. Kao, C.C., Lieberman, P.M., Schmidt, M.C., Zhou, Q., Pei, R., and Berk, A.J. (1990) Science 248, 1646-1650.
34. Bargonetti, J., Reynisdottir, F., Friedman, P.N., and Prives, C. (1992) Gen. Dev. 6, 1886-1898.
35. Keller, J.M. and Alwine, J.C. (1984) Cell 36, 381-389.
36. Li, R. and Botchan, M.R. (1993) Cell 73, 1207-1221.
37. He, Z., Brinton, B.T., Greenblatt, J., Hassell, J.A., and Ingles, C.J. (1993) Cell 73, 1223-1237.
38. Comai, L., Tanese, N., and Tjian, R. (1992) Cell 68, 965-976.
39. Margottin, F., Dujardin, G., Gerard, M., Egly, S.M., Huet, J., and Sentenac, A. (1991) Science 251, 424-426.
40. White, R.J., Jackson, S.P., and Rigby, P.W. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 1949-1953.