Nuclear Receptor Coactivator Thyroid Hormone Receptor-binding Protein (TRBP) Interacts with and Stimulates Its Associated DNA-dependent Protein Kinase*

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Nuclear receptors mediate gene activation through ligand-dependent interaction with coactivators. We previously cloned and characterized thyroid hormone receptor-binding protein, TRBP (NcoA6; AIB3/ASC-2/RAP250/PRIP/TRBP/NRC), as an LXXLL-containing coactivator that associates with coactivator complexes through its C terminus. To search for protein factors involved in TRBP action, we identified a distinct set of proteins from HeLa nuclear extract that interacts with the C terminus of TRBP. Analysis by mass spectrometric protein sequencing revealed a DNA-dependent protein kinase (DNA-PK) complex including its catalytic subunit and regulatory subunits, Ku70 and Ku86. DNA-PK is a heterotrimeric nuclear phosphatidylinositol 3-kinase 3-kinase that functions in DNA repair, recombination, and transcriptional regulation. DNA-PK phosphorylates TRBP at its C-terminal region, which directly interacts with Ku70 but not Ku86 in vitro. In addition, in the absence of DNA, TRBP itself activates DNA-PK, and the TRBP-stimulated DNA-PK activity has an altered phosphorylation pattern from DNA-stimulated activity. An anti-TRBP antibody inhibits TRBP-induced kinase activity, suggesting that protein content of TRBP is responsible for the stimulation of DNA-independent kinase activity. Furthermore, in DNA-PK-deficient scid cells, TRBP-mediated transactivation is significantly impaired, and nuclear localization of TRBP is altered. The activation of DNA-PK in the absence of DNA ends by the coactivator TRBP suggests a novel mechanism of coactivator-stimulated DNA-PK phosphorylation in transcriptional regulation.

The functional properties of coactivators include the following: direct protein-protein interactions with transcriptional complexes (1); enzymatic activities of certain coactivators, such as histone acetyltransferase in CBP1 and steroid receptor coactivator-1 (SRC-1) family or arginine transmethylase in coactivator-associated arginine methyltransferase 1 (CARM1) (3, 4); RNA interactions, with RNA recognition motifs such as in peroxisome proliferator-activated receptor-γ-coactivator-1 (PGC-1), CoAA, and SMRT/HDAC1-associated repressor protein (SHARP); and RNA alone such as steroid receptor RNA activator (SRA) (5–8). It is becoming increasingly important to understand how coactivator-targeted molecules, such as interacting proteins or enzyme substrates, are regulated by coactivators.

We previously cloned and characterized thyroid hormone receptor-binding protein (TRBP) as a nuclear receptor coactivator (9). TRBP, designated as NcoA6 by the National Center for Biotechnology Information (NCBI) nomenclature committee, was concurrently identified by several groups as AIB3/ASC-2/RAP250/PRIP/TRBP/NRC (1, 2). TRBP is a high molecular weight, ubiquitously expressed coactivator. A single LXXLL motif is required for the ligand-dependent interaction with a number of nuclear receptors and subsequent transcriptional activation. The Ser-884 residue adjacent to the TRBP LXXL motif was shown to regulate the selectivity of TRBP for different nuclear receptors (10). TRBP also coactivates multiple transcriptional factors including AP-1 and NF-κB (1). In addition, gene amplification was observed for TRBP in human breast cancers (11). Furthermore, the C terminus of TRBP was shown to interact with coactivator CoAA (6), CBP/p300, and DRIP complexes (9).

In a search for additional nuclear factors that might be targeted by TRBP, we identified a distinct set of TRBP-bound proteins using a mass spectrometric protein sequencing approach. Proteins identified include the DNA-dependent protein kinase (DNA-PK) components. DNA-PK is a nuclear serine/threonine protein kinase that belongs to the PI3K family (12–16). Previous biochemical and genetic studies revealed DNA-PK to be a heterotrimeric enzyme composed of a catalytic subunit, DNA-PKcs, and two regulatory subunits, Ku86 and Ku70. Although DNA-PK is known to be activated by DNA

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The abbreviations used are: CBP, CREB-binding protein (where CREB is cAMP-response element-binding protein); TRBP, thyroid hormone receptor-binding protein; CoAA, coactivator activator; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; PARP, poly(ADP-ribose) polymerase; GST, glutathione S-transferase; CTD, C-terminal domain; DRIP, vitamin D receptor-interacting protein; scid, severe combined immune deficiency; aa, amino acid; Ab, antibody; MMTV, mouse mammary tumor virus; GR, glucocorticoid receptor; DTT, dithiothreitol; PI3K, phosphatidylinositol 3-kinase; ds, double strand.
ends, recent compelling evidence suggests that its kinase activity can also be stimulated by protein interactions (17–19). Supported by a large body of evidence, DNA-PK has been shown to be involved in transcriptional regulation as well as in recombination and DNA repair (12, 13, 19–21). DNA-PK is unique among nuclear protein kinases because it associates with DNA templates and phosphorylates a variety of protein factors that are important for transcriptional regulation. The identified in vitro substrates of DNA-PK include DNA-binding transcriptional factors such as c-Myc, c-Jun, Sp1, Oct-1, and nuclear receptors GR, progesterone receptor, tumor suppressor p53, HMG proteins, Ku subunits, as well as DNA-PKcs itself through autophosphorylation (12). The close relationship of DNA-PK with transcriptional regulation is also illustrated by its ability to phosphorylate the C-terminal domain (CTD) of RNA polymerase II (22–24), which might be important for coupled transcription and pre-mRNA processing (25). In addition, mouse cells with severe combined immune deficiency (scid) that lack functional DNA-PK showed the defects not only in DNA recombination and DNA repair (26) but also in Ku phosphorylation and transcriptional activation (21, 27). Thus, DNA-PK kinase activity might be essential for transcriptional control.

We report here the identification of DNA-PK components as TRBP-interacting proteins. Through its C-terminal region, TRBP interacts with DNA-PK via a direct interaction with the regulatory subunit, Ku70. TRBP can be phosphorylated by DNA-PK in vitro. Our results also suggest that TRBP stimulates DNA-PK kinase activity in the absence of DNA ends. This DNA-independent activity may result in potentially altered substrate phosphorylation specificity. In addition, DNA-PK-deficient cells have altered TRBP nuclear localization and exhibit a defect in TRBP-mediated transcription activation. Our studies suggest a novel connection between coactivator-stimulated DNA-PK phosphorylation and transcriptional regulation.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Human TRBP in pcDNA3 vector and its derived GST fusion fragments have been described previously (9). Briefly, GST-TRBP-1s were subcloned into Smal/XhoI or EcoRl/XhoI sites of pGEX-4T-2 (Amersham Biosciences), as indicated in Fig. 1. Full-length human Ku70 and Ku80 were cloned by RT-PCR and inserted into the HindlII/XhoI sites of pcDNA3. Mouse mammary tumor virus (MMTV) promoter luciferase reporter was generated by inserting MMTV promoter into PXP2 vector, and pCMV-GR was generated by inserting human GR into the BamHI/XhoI sites of pcDNA3 as described previously (6). Monoclonal anti-Ku70 (Ab-4, clone N3H10), anti-Ku86 (Ab-2, clone N111), and anti-DNA-PKcs (Ab-4) antibodies were obtained from NeoMarkers. Polyclonal anti-TRBP antibodies were produced from two rabbits (TRBP-1652 and TRBP-1653) using the GST-TRBP-6 (aa 1641–2063) as antigen (Covance). The TRBP antibody was affinity-purified by Affi-Gel 10 according to the manufacturer’s protocol (BioRad).

Isolation of DNA-PK Complexes—The GST and GST fusion TRBPs were produced in Escherichia coli BL21(DE3) and purified by glutathione-Sepharose resin (Amersham Biosciences). Nuclear extracts were isolated from a large quantity of HeLa or GH3 cells, approximately six 15-cm plates. Briefly, cells were lysed in buffer A (20 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 1 mM DTT) with the above protease inhibitors for 30 min on ice. The nuclei were then collected by centrifugation at 4 °C and extracted with buffer B (20 mM HEPES, pH 7.4, 420 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM MgCl2, 1 mM DTT) with the above protease inhibitors for 30 min on ice. After centrifugation, the supernatants containing nuclear extracts were further filtered with a 0.65 μm spin column (Millipore) to remove completely any insoluble cellular debris. This step is important to prevent nonspecific protein contamination in the subsequent binding assay. The binding assays were carried out with 5–10 μl of GST fusion proteins on beads plus 600–800 μg of freshly prepared nuclear extracts in 12 ml of binding buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05% Triton X-100, 10% glycerol, 1 mM DTT, and protease inhibitors) with shaking at 4 °C overnight. Bound resins were washed three times with binding buffer and resolved by SDS-PAGE, followed by Coomassie Blue R-250 staining (Bio-Rad).

Protein Microsequencing—Protein bands of interest identified by preparative SDS-PAGE were carefully excised and washed with 50% acetonitrile according to the Harvard Microchemistry Facility protocol (28, 29). Sequence analysis performed at the Harvard Microchemistry Facility included the proteolytic digestion of peptides, analysis by microcapillary high pressure liquid chromatography nanoelectrospray tandem ion trap mass spectrometry, and mass spectrometry/mass spectrometry peptide sequence interpretation, which was facilitated with the Algorithm Sequest and programs developed at the Harvard Microchemistry Facility. Multiple peptides determined by sequence analysis were matched to the known GenBank™ entries.

Recombinant Protein Binding Assays—In vitro binding assays were performed by incubating GST resin (20 μl, 2 μg) and (32)S-methionine-labeled, in vitro translated proteins (5 μl) produced by rabbit reticulocyte lysate (Promega). Proteins were incubated at room temperature for 1 h in the binding buffer (20 μM HEPES, pH 7.4, 50 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05% Triton X-100, 10% glycerol, 1 mM DTT). Bound proteins were washed 3 times with binding buffer and subjected to SDS-PAGE and autoradiography.

Immunoblotting—GST-TRBP resins (20 μl, 2–5 μg) were incubated with 200 μl of HeLa or GH3 nuclear extracts at 4 °C overnight in the binding buffer as above with additional protease inhibitors. Bound proteins were transferred onto SDS-PAGE and detected by Western blotting (ECL). Anti-DNA-PKcs and anti-Ku70 monoclonal antibodies were obtained from NeoMarkers.

DNA-PK Kinase Assay—Phosphorylation of TRBP recombinant fragments in vitro by DNA-PK was assayed using the SigmaTACT DNA-dependent Protein Kinase Assay System from Promega with modifications. Briefly, DNA-PK enzyme purified from HeLa nuclear extract (Promega) was incubated with [γ-32P]ATP, with or without linear double stranded (ds) DNA as activator, and with GST-TRBP fusion proteins as substrates. Labeled proteins were washed and resolved by SDS-PAGE and autoradiography. Full-length GST-p53 from Santa Cruz Biotechnology was used as a positive control substrate for the DNA-dependent phosphorylation, and GST alone as an unphosphorylated protein was used as a negative control. When TRBP was measured for its stimulating activity, p53 peptide was used as a substrate. The amount of purified DNA-PK used (5–10 units) in each assay was titrated and determined with a biotinylated p53 peptide substrate prior to use. Alternatively, when endogenous DNA-PK from HeLa cell nuclear extracts was assayed, GST-TRBP-5 and -6 were preincubated with HeLa nuclear extracts, and washed beads containing TRBP bound DNA-PK were used in the assays. DNA-PK kinase activities were measured using the same SigmaTACT system in the presence or the absence of DNA, except a biotinylated p53 peptide was used as substrate. Phosphorylated p53 peptides were captured by the streptavidin-cellulose papers, and the papers were washed and counted in a scintillation counter. GST alone and GST-TRBP-3 were used as negative controls.

Immunofluorescence—TRBP antibodies were affinity-purified by Affi-Gel 10 chromatography according to the manufacturer’s protocol (BioRad). Mouse cell lines SCSV3 (DNA-PK-deficient, scid) and SCHR-1 (scid plus human DNA-PK) were methanol-fixed and double-stained with affinity-purified polyclonal anti-TRBP (1:50) and monoclonal anti-Ku70 (1:100, clone N3H10, NeoMarkers). Anti-rabbit Cy3- and mouse fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch) were applied at a dilution of 1:200. Images were obtained using a Nikon E800 fluorescent microscope.

Cell Culture and Transient Transfection—HeLa cells, mouse SCSV3 (DNA-PK−/−), and SCHR-1 (DNA-PK−/+; +/+) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 5 μg/ml penicillin/streptomycin in 5% CO2 at 37 °C. 24-well plates were transfected with MMTV-luc (0.1 μg), GR (0.01 μg), and TRBP (0.2 μg) plus 2 μg of plasmids per well using lipofectamine (Invi). Cells were incubated with fresh medium containing the indicated concentrations of ligands and wortmannin 16–24 h after transfection. After another 24 h, cells were harvested, and luciferase activities were assayed. Total amounts of DNA for each well were equalized by adding vector pcDNA3 (Invitrogen). Data are shown as means of triplicate transfections ± S.E.
RESULTS

DNA-PK Complexes Interact with TRBP C Terminus—Several lines of evidence support the identification of the C terminus of coactivator TRBP (aa 1641–2063) as a binding domain for the interactions with multiple nuclear proteins, including coactivator CBP/p300, DRIP complex component DRIP130 (9), and coactivator activator CoAA (6). In addition, the majority of the transcriptional activity of TRBP is mediated through its C terminus (9). To identify in vivo protein complexes in close contact with the TRBP C-terminal region, we carried out mass spectrometric analysis of nuclear proteins precipitated with immobilized recombinant TRBP fragments (Fig. 1). HeLa nuclear extracts were incubated with selected TRBP regions including its C terminus, and binding proteins were identified by SDS-PAGE (Fig. 2). Two overlapping recombinant TRBP-C fragments with different sizes (TRBP-5, aa 1237–2063, and TRBP-6, aa 1641–2063) were used, in order to detect the binding proteins in same size range of TRBP-C that might otherwise be obscured by size overlap.

A number of proteins ranging from 70 to 400 kDa were observed. However, only the predominant bands were isolated for further microsequence analysis. The identified TRBP-associated proteins are p70, p80, p90, p100, p200, and p400. These proteins were carefully isolated from the preparative gel (Fig. 2B), and their identities were determined by proteolytic peptide sequencing (Table I). Remarkably, among the six analyzed proteins, four of them belong to the DNA-PK complex, including DNA-PK catalytic subunit (p400), DNA-PK regulatory subunits, Ku 70 (p70) and Ku 86 (p80), and PARP (p100). The other two proteins were DNA topoisomerase I (p90) and NuMA (p200). The profiles of associated proteins for TRBP-5 and TRBP-6 were overlapping, particularly regarding DNA-PKcs, Ku70 and Ku86. PARP was more closely associated with TRBP-6, although the reason is unclear. DNA-PK subunits were not found to be associated with other TRBP regions, suggesting that the binding of these proteins was specific to the C-terminal region of TRBP (Fig. 2A). The stoichiometry of Ku and TRBP-5 interaction is very high (Fig. 2B), indicating Ku subunits might have higher affinities toward TRBP. In general, the number of the peptides sequenced to match to a GenBank access number correlates with the reliability of the identity of a given protein (Table I). Data also revealed the presence of contaminating GST-TRBP, which was shown with a small number of peptides. It should be noted that proteins present in lower abundance may not be identified by this sequencing approach, yet may still bind to TRBP either directly or indirectly. Other approaches, such as the yeast-two hybrid screen, may be more suitable for the isolation of these proteins (6). Nevertheless, we conclude from our data that DNA-PK complexes interact with TRBP at its C terminus.

FIG. 2. Identification of DNA-PK complex components that associate with TRBP C terminus. A, analytical SDS-PAGE for TRBP-associated proteins. Selected GST-TRBP fragments were incubated with or without HeLa nuclear extracts as indicated. Bound proteins were washed and resolved by SDS-PAGE followed by Coomassie Blue staining. Open arrows indicate GST-TRBP-5 and GST-TRBP-6. Small open circles denote the TRBP-interacting proteins. B, the preparative gels from which the TRBP-interacting proteins were excised for sequence analysis. Open arrows indicate GST-TRBP fragments; closed arrows indicate the multiple protein bands before excision for protein sequencing. Proteins were designated according to their molecular sizes, and their revealed identities are shown in parentheses.

TRBP Directly Interacts with DNA-PK Ku70 in Vitro—We decided to focus our current studies on DNA-PK and investigate its relationship with TRBP because the role of DNA-PK in transcriptional regulation is becoming increasingly appreciated (19, 30). To confirm the interaction of the DNA-PK complex with TRBP observed during peptide sequencing, we performed Western blot analysis using anti-DNA-PKcs and anti-Ku70 antibodies. As shown in Fig. 3A, recombinant GST fusion TRBPs were incubated with HeLa nuclear extract, and bound proteins were probed with indicated antibodies. These results suggest that DNA-PKcs and Ku70 interact with TRBP, but only at its C terminus. Similar results were obtained with anti-Ku86 antibodies and with GH4 nuclear extracts (not shown).

Because regulatory subunits Ku70 and Ku86 often interact with proteins that regulate DNA-PK kinase activity (17, 19), an in vitro GST pull-down assay was carried out to compare the potential direct interactions of Ku subunits with TRBP. Inter-
Interestingly, as shown in Fig. 3B, whereas interaction of TRBP with Ku 70 was very strong, interaction with Ku86 was absent. Negative controls including luciferase and GST alone did not yield signals. Notably, the strongest binding of TRBP was not to the full-length Ku70 but rather to fragments of Ku70. This may due to a regulated binding of full-length Ku70 with TRBP. These data confirm that TRBP interacts with DNA-PK, possibly through its Ku70 subunit.

**DNA-independent Phosphorylation of TRBP C Terminus by DNA-PK**—The data presented above indicated that TRBP might have a close relationship with DNA-PK. To understand this interaction further, we first examined the possibility that TRBP is a substrate of the kinase. Recombinant TRBP frag-

| Size (kDa) | Identified proteins | GenBank™ accession no. | No. of peptides |
|-----------|--------------------|-----------------------|----------------|
| p400      | DNA-PK catalytic subunits | U47077.5  | 24            |
|           | TRBP               | D8003                | 3             |
| p200      | NuMA               | CAA77689             | 24            |
|           | TCOF1              | 22304673A            | 11            |
|           | TRBP               | D800063             | 7             |
|           | GST                | 1GTB                 | 3             |
| p100      | PARP               | A29725               | 50            |
|           | TRBP               | D80003               | 9             |
| p90       | DNA topoisomerase I | J03250               | 39            |
|           | TRBP               | D00083               | 22            |
|           | GST                | 1GTB                 | 12            |
| p80       | Ku86               | A32626               | 60            |
|           | TRBP               | D00083               | 17            |
|           | GST                | 1GTB                 | 8             |
| p70       | Ku70               | P12956               | 72            |
|           | TRBP               | D00083               | 14            |

Proteins isolated from HeLa nuclear extracts were initially designated according to their apparent molecular sizes.

Number of peptides sequenced that matched the respective proteins available in GenBank™.

Mixture of NuMA and TCOF1.

**Fig. 3.** TRBP-interacts with DNA-PK complex through Ku70. A, TRBP C terminus interacts with endogenous DNA-PK complex. GST-TRBP fusion protein fragments were incubated with HeLa nuclear extracts, and TRBP-bound proteins were subjected to Western blot analysis. Anti-DNA-PKcs and anti-Ku70 antibodies are indicated. B, GST, GST-TRBP-5, or TRBP-6 was incubated with in vitro translated, [35S]methionine-labeled full-length Ku70 or Ku86. Luciferase was a negative control. Bound proteins were resolved by SDS-PAGE and detected by autoradiography.

**Fig. 4.** DNA-independent phosphorylation of TRBP C terminus by DNA-PK. A, GST-TRBP fusion proteins were used as substrates in DNA-PK kinase assays in the absence or presence of linear double strand (ds) DNA as DNA-PK activator. GST alone and GST-p53 were negative and positive controls. Phosphorylated GST fusion proteins ([γ-32P]ATP-labeled) were washed and resolved on the gel followed by autoradiography. B, comparison of TRBP-3 and TRBP-6 in DNA-PK phosphorylation assays in the absence of DNA (−) or the presence of double strand (ds) or supercoiled plasmid (sc) DNA as activators. Wortmannin (1 μM) was used as a DNA-PK inhibitor. C, similar phosphorylation assays were performed using different amounts of DNA-PK (10, 50, and 250 units). Double strand DNA and wortmannin (1 μM) used are as indicated. Closed arrows indicate the bands produced by DNA-dependent phosphorylation, and open arrow indicates the lower migrating bands produced by DNA-independent phosphorylation.
ments were used as substrates in a DNA-PK kinase assay, as shown in Fig. 4A. Whereas the control GST alone was not phosphorylated, GST-TRBP C terminus was phosphorylated by DNA-PK. The GST-p53 was used as a positive control for DNA-dependent phosphorylation. Remarkably, the phosphorylation of TRBP-5 or TRBP-6, which overlaps at C terminus, was DNA-independent. The TRBP middle region (TRBP-3) can be phosphorylated but in a DNA-dependent manner. Other TRBP regions appeared not to be good substrates for DNA-PK (data not shown). Together, these results suggest that TRBP-C can be phosphorylated by DNA-PK in a DNA-independent manner. This differs from the phosphorylation of p53 or TRBP-3, which is largely DNA-dependent.

A closer examination of the TRBP phosphorylation patterns revealed that the DNA-independent phosphorylation produced a slower migrating band than DNA-dependent phosphorylation (Fig. 4, A and B). Due to the smaller size of TRBP-6, which was more obvious for the upper band than TRBP-5, we compared phosphorylation patterns of TRBP-3 and TRBP-6 in assays shown in Fig. 4, B and C. The DNA-independent phosphorylation of TRBP-6 had two phosphorylation bands. Both phosphorylation bands can be inhibited by wortmannin, a PI3K inhibitor that blocks the kinase activity of DNA-PK, indicating the phosphorylation was DNA-PK-specific. The data suggest that the TRBP C terminus may, by itself, function as an activator to stimulate DNA-independent kinase activity. In addition, the TRBP-activated kinase may phosphorylate TRBP at different site(s) than the DNA-activated kinase to yield a band with slower mobility on the gel. This indicates that there is a potential differential substrate specificity of DNA-PK and, depending on the activator, is either TRBP or linear DNA ends.

Compared with DNA ends, TRBP might be a unique stimulator of DNA-PK. We reached this conclusion by excluding mechanisms that might stimulate DNA-PK other than TRBP. TRBP itself did not exert any kinase activity, as a smaller amount of DNA-PK enzyme was not able to produce the signal (Fig. 4C). Similar to ds DNA, supercoiled plasmid DNA did not produce a lower mobility band (Fig. 4B), suggesting that the phosphorylation site(s) might be different for the protein activator. We also washed GST-TRBP resin with high salt to prevent any protein contamination in the assay. In addition, we compared assays with pretreatment of TRBP resin with DNase or RNase to prevent potential bacterial nucleic acid contamination during the isolation of GST fusion protein. In any case, however, nonspecific DNA contamination would not produce a lower mobility band. Thus, these results are all consistent with our conclusion that TRBP protein is responsible, at least in part, for the DNA-independent activation of DNA-PK.

TRBP C Terminus Stimulates DNA-PK—To this end, we sought further evidence for TRBP-stimulated activity of DNA-PK. In TRBP-stimulated kinase assays, a biotinylated p53 peptide was used as a substrate, which can be separated from TRBP on streptavidin-cellulose paper before quantitation. As shown in Fig. 5A, the TRBP C

![Fig. 5. TRBP C terminus stimulates DNA-PK kinase activity in the absence of DNA ends. DNA-PK kinase assays were carried out using a biotinylated p53 peptide as substrate and GST-TRBP proteins as activators as described under “Experimental Procedures.” Briefly, after the reaction, the [γ-32P]ATP-labeled biotinylated p53 peptides were captured by the streptavidin-cellulose papers and separated from GST-TRBP proteins. The papers were then washed and counted in a scintillation counter. A, each TRBP fragment was compared for the activity that stimulates DNA-PK in the absence or the presence of DNA. B, polyclonal anti-TRBP antibodies 1652 and 1653, prepared using TRBP fragment as antigen (aa 1641–2063), were compared with anti-Ku antibodies for their capability to block TRBP-5-stimulated DNA-PK activities. Anti-TRBP antibodies but not anti-Ku antibodies blocked the DNA-independent activity. C, anti-TRBP antibody (1652) and its preimmune serum from the same rabbit were tested with TRBP-5 and TRBP-6 in a similar kinase assay. GST alone and GST-TRBP-3 were negative controls. Assays were performed in the absence or the presence of ds DNA.](image-url)
These data suggest that DNA-PK might be regulated by a kinase with activity comparable with DNA-induced activity. Formational change of DNA-PK and subsequently stimulate the TRBP and anti-DNA-PKcs, for example, might result in a conformational change that is necessary for the activation of DNA-PK. Anti-TRBP, its preimmune serum, anti-Ku, and anti-DN-PKcs antibodies were added as indicated. Anti-DNA-PKcs antibody alone can stimulate kinase activity, and the stimulation was synergistic with TRBP protein.

Unexpectedly, during the course of studies with the antibodies that might affect DNA-PK, we discovered that an anti-DNA-PKcs antibody alone was able to activate DNA-PK in the absence of any activators. Fig. 6 showed that the combination of this antibody and TRBP protein can produce a robust DNA-independent activity that is comparable with the level of DNA-stimulated activity. The anti-TRBP again blocked the kinase activity, and preimmune serum and anti-Ku70 or anti-Ku80 antibodies had no effect, which were used as controls. Although anti-DNA-PKcs-stimulated DNA-PK does not occur in vivo, the data indeed indicate that a combination of protein interactions, TRBP and anti-DNA-PKcs, for example, might result in a conformational change of DNA-PK and subsequently stimulate the kinase with activity comparable with DNA-induced activity. These data suggest that DNA-PK might be regulated in vivo by interacting proteins as well as linear DNA ends. Because TRBP plays an important role in transcription, the direct stimulation by TRBP, or combined with other potential stimulatory proteins, may activate DNA-PK in the absence of DNA ends in transcriptional regulation.

**Interrelationship of TRBP and DNA-PK in Cells**—Because TRBP can stimulate the activity of purified DNA-PK in vitro, we examined whether TRBP might also stimulate endogenous DNA-PK in cells. As shown in Fig. 7A, endogenous DNA-PK from HeLa nuclear extracts was isolated using TRBP resin and showed DNA-dependent activity. TRBP-bound DNA-PK showed DNA-independent activity. B, DNA-PK is required for TRBP activity. Mouse SCSV3 (DNA-PK−/−, scid) and SCSV1 (DNA-PK+/+) cells were cotransfected with MMTV luciferase reporter (100 ng), GR (10 ng), and full-length TRBP (200 ng). After transfection, cells were grown overnight in the presence or the absence of 100 nM dexamethasone (DEX). Total amounts of DNA for each well were equalized with additional vector pcDNA3. Data are shown as means of triplicate transfections ± S.E.
DNA-PK catalytic subunit and has restored DNA-PK phosphorylation (26, 27). When TRBP activity was compared in these two lines, TRBP activity was dramatically decreased in the DNA-PK-deficient SCSV3 cells compared with that observed in SCH8-1 cells, which has restored DNA-PK activity (Fig. 7B). Therefore, these results confirm the idea that TRBP may, in part, exert its transcriptional activity in vivo through the DNA-PK function, inasmuch as DNA-PK represents a critical enzymatic activity in the transcriptional complex.

We also performed immunofluorescent studies to analyze the nuclear localization of TRBP in DNA-PK-deficient cells. Mouse cell lines SCSV3 (DNA-PK −/−, scid) and SCH8-1 (DNA-PK +/+ ) were methanol-fixed and double-stained with affinity-purified polyclonal anti-TRBP (red) and monoclonal anti-Ku70 (green). Secondary antibodies were anti-rabbit Cy3- and anti-mouse fluorescein isothiocyanate-conjugated antibodies. The merged images of the two panels for TRBP and Ku70 are shown on the right. DNA-PK-deficient cells exhibit a more restricted distribution of TRBP and Ku70. Thus, TRBP exhibits less colocalization with Ku70 in DNA-PK-deficient cells.

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Inhibition of DNA-PK Represses TRBP-mediated Transcription—To examine further whether DNA-PK may influence TRBP transcriptional function, transient transfection assays, using HeLa cells, were carried out with wortmannin, a PI3K inhibitor that inhibits DNA-PK. Fig. 9 shows that increasing amounts of wortmannin inhibit TRBP-stimulated transcription when both full-length TRBP and TRBP fragments as indicated (200 ng). TRBP-MC contains the TRBP C-terminal region (amino acids 714–2063). TRBP-M does not contain the C-terminal region (amino acid 714–1242), which is used as a control. After transfection, cells were grown in the presence or the absence of 100 ng dexamethasone (DEX), and with different amounts of wortmannin (0, 100, 500 ng) as indicated. Total amounts of DNA for each well were equalized with additional vector pcDNA3. Data are shown as means of triplicate transfections ± S.E.

DISCUSSION

The discovery of the interaction of TRBP with the DNA-PK complex was initially serendipitous. During the analysis of coactivator CBP and DRIP130 interactions with TRBP fragments, a 400-kDa abundant protein from nuclear extracts, later revealed as DNA-PKcs by protein sequencing, was repeatedly seen to interact with the C terminus of TRBP. This compelled further analysis of additional interacting proteins, which ultimately identified associated components including the
DNA-PK complex. It does not, however, diminish the possibility that other nuclear factors, possibly detected as minor bands on the gel, could also directly or indirectly associate with TRBP, although these proteins may be insufficiently abundant to permit appropriate sequence analysis.

DNA-PK has been extensively characterized as a nuclear PKcs involved in regulating transcriptional activation, DNA repair, and V(D)J recombination (12, 15, 26). The two tightly associated regulatory subunits of DNA-PK, Ku70 and Ku80, were originally identified as the autoantigens in autoimmune diseases. Ku-deficient mice, phenotypically similar to DNA-PKcs-deficient mice, are immunodeficient and sensitive to DNA damage (32–34). Ku is a protein that binds DNA in a non-sequence-specific manner and regulates DNA-PKcs catalytic activity. Although DNA was originally shown as a potent activator of DNA-PKcs, recent studies (17, 19) indicate that the multiple protein factors recruited by the Ku subunit may also serve as activators to stimulate DNA-PK activity. For instance, several homeodomain proteins, including Oct-1, have recently been shown (17) to interact with Ku70 and enhance DNA-PK phosphorylation. Consistent with this, recent studies indicate that DNA-PK can be fully activated in the absence of DNA. It appears that although DNA-PK can be stimulated by DNA ends (36), or by other kinases (37, 38), protein interactions via Ku70 may be another important mechanism for DNA-PK activation, especially in transcriptional regulation.

In addition to DNA-PK subunits, several other proteins were also found in the TRBP-interacting complex, including DNA topoisomerase I, NuMA, PARP, and TCOF1, that have not been investigated in this study. These factors may also associate with TRBP directly or indirectly. PARP is a chromatin-associated protein that catalyzes the transfer of ADP-ribose from NAD$^+$ to nuclear proteins. PARP is the only nuclear ribosylating enzyme that can be phosphorylated by DNA-PK (39) and, in turn, ribosylates DNA-PK. PARP is up-regulated in human tumors (40, 41) and serves as a death substrate in apoptosis (42). PARP has also been shown to associate with DNA-PK and regulate transcription including gene activation mediated by nuclear receptors (43–45). It is possible that PARP associates with TRBP through the DNA-PK complex. In addition to the DNA-PK complex, the C terminus of TRBP has been shown previously (6, 9), by different approaches, to interact with co-activators such as CBP/p300, DRIP components, and coactivator CoAA. These interactions taken together may provide a mechanism for the function of TRBP as a coactivator acting, in part, via its C terminus.

The substrate specificity of DNA-PK displays fewer sequence characteristics. A number of phosphorylation sites, including sites in the p53 substrate, are at serines or threonines followed by glutamines (SQ or TQ) (46). However, many other substrates of DNA-PK have phosphorylation sites other than (S/T)Q. RNA polymerase II CTD can be heavily phosphorylated in vitro at serines that are not followed by glutamine (12, 23). It is currently unclear whether the substrate specificity of DNA-PK has any correlation with its stimulation properties, i.e. whether DNA-activated phosphorylation results in distinct patterns than those protein-activated. Interestingly, we observed that the TRBP-stimulated DNA-independent phosphorylation produced an additional lower mobility band that is distinguishable from products of DNA-stimulated phosphorylation (Fig 4). A similar observation was reported using RNA polymerase II CTD as substrate and Gal4 as an activator (23). The substrate specificity and phosphorylation sites in these cases may be altered, and the phosphorylation sites might be worth mapping in future studies. These results, nevertheless, indicate a possible regulation of DNA-PK substrate specificity by coactivators, which might be important for the in vivo function of DNA-PK.

In addition to DNA repair and recombination, the involvement of DNA-PK in transcription is evident (15, 19, 21). DNA-PK phosphorylates many transcriptional factors including glucocorticoid receptor (GR) and regulating the MMTV promoter (47). The DNA-PKcs- or Ku-deficient cells are defective in transcription on multiple promoters tested in vitro, and the deficiency of Ku leads to severe defects in transcription (20). It is also noteworthy that there are examples where protein factors are involved in the regulation of DNA-PK in transcription. A transcriptionally active Gal4 domain was previously shown to stimulate DNA-PK phosphorylation of RNA polymerase II CTD (22, 23). Homeodomain-containing proteins stimulate DNA-PK via Ku70 (17). A recent report (30) also suggested that a limited Ku-dependent protein factor, which is not template-associated, may be responsible for the reinitiation of transcription. Together with our current studies, it appears that DNA-PK is targeted by multiple stimulating factors, such as coactivators, and functions as a signaling kinase for a variety of nuclear functions including transcription.

Mice with scid have a nonfunctional immune system due to a defect of V(D)J recombination and double strand break repair (16). Genetic analysis of scid mice revealed the presence of mutations in the catalytic domain of DNA-PK. Mouse scid cells are also defective in phosphorylation of DNA-PK substrates such as Ku (27). In scid cells, both TRBP phosphorylation by DNA-PK and TRBP-stimulated DNA-PK activation may be abolished. This likely explains the severe defect of TRBP-stimulated transactivation (Fig 7B), because DNA-PK may synergistically be responsible for the regulation of transcription. Consistent with this, previous evidence has suggested that transcription level of multiple genes is profoundly decreased in scid cells (21). It is also interesting that the localization of nuclear components, including TRBP, may be altered in scid cells (Fig 8). DNA-PK activity might be required for the phosphorylation of a number of nuclear proteins including TRBP and Ku70. Hence, the phosphorylation states may determine nuclear localization, which, in turn, may affect function. Restored DNA-PK activity in SCID-1 cells, however, rescues these defects and promotes the coactivator function of TRBP.

In summary, as the machinery for transcription, recombination, and DNA repair may functionally overlap and share similar factors (31, 48), DNA-PK may be one of the critical and versatile components. Consistent with this view, TRBP may stimulate DNA-PK and, in turn, phosphorylate proteins in the transcriptional complex. The substrate specificity of DNA-PK displays fewer sequence characteristics. A number of phosphorylation sites, including sites in the p53 substrate, are at serines or threonines followed by glutamines (SQ or TQ) (46). However, many other substrates of DNA-PK have phosphorylation sites other than (S/T)Q. RNA polymerase II CTD can be heavily phosphorylated in vitro at serines that are not followed by glutamine (12, 23). It is currently unclear whether the substrate specificity of DNA-PK has any correlation with its stimulation properties, i.e. whether DNA-activated phosphorylation results in distinct patterns than those protein-activated. Interestingly, we observed that the TRBP-stimulated DNA-independent phosphorylation produced an additional lower mobility band that is distinguishable from products of DNA-stimulated phosphorylation (Fig 4). A similar observation was reported using RNA polymerase II CTD as substrate and Gal4 as an activator (23). The substrate specificity and phosphorylation sites in these cases may be altered, and the phosphorylation sites might be worth mapping in future studies. These results, nevertheless, indicate a possible regulation of DNA-PK substrate specificity by coactivators, which might be important for the in vivo function of DNA-PK.

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