Association of the Ku autoantigen/DNA-dependent protein kinase holoenzyme and poly(ADP-ribose) polymerase with the DNA binding domain of progesterone receptors

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ABSTRACT

Ligand-activated progesterone receptors (PR) bind to DNA at specific progesterone response elements by means of a DNA binding domain (DBD_{PR}) containing two highly conserved zinc fingers. DNA-bound PRs regulate transcription via interaction with other nuclear proteins and transcription factors. We have now identified four HeLa cell nuclear proteins that copurify with a glutathione-S-transferase-human DBD_{PR} fusion protein. Microsequence and immunoblot analyses identified one of these proteins as the 113 kDa poly(ADP-ribose) polymerase. The three other proteins were identified as subunits of the DNA-dependent protein kinase (DNA-PK) holoenzyme: its DNA binding regulatory heterodimers consisting of Ku70 and Ku86, and the 460 kDa catalytic subunit, DNA-PK_{CS}. DNA-PK that was 'pulled-down' by DBD_{PR} on the affinity resin was able to

(1) autophosphorylate Ku70, Ku86, and DNA-PK_{CS}, (2) transphosphorylate DBD_{PR}, and (3) phosphorylate a DNA-PK-specific p53 peptide substrate. DNA-PK was also able to associate with the DBD of the yeast activator GAL4. However, neither a PR DBD mutant lacking a structured first zinc finger (DBD_{CYS}) nor the core DBD of the estrogen receptor (DBD_{ER}) copurified DNA-PK, suggesting the interaction is not non-specific for DBDs. Lastly, we found that DNA-PK copurified with full-length human PR transiently expressed in HeLa cells, suggesting that the human PR/DNA-PK complex can assemble *in vivo*. These data show that DNA-PK and DBD_{PR} interact, that DBD_{PR} is a phosphorylation substrate of DNA-PK, and suggest a potential role for DNA-PK in PR-mediated transcription.

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INTRODUCTION

Progesterone is a key hormone involved in the development and terminal differentiation of the mammary gland and other female target tissues (Clarke & Sutherland 1990, Horwitz 1992 and references therein). Progesterone receptors (PR) are ligand-activated nuclear transcription factors that bind specifically to DNA at progesterone response elements (PRE) (Tsai & O'Malley 1994 and references therein). Binding of the receptors to PREs occurs through a modular DNA binding domain (DBD_{PR}) present in the protein; it consists of two fingers, each formed by the co-ordination of

one zinc atom with four cysteine residues. Based upon mutational analyses and the crystal structure of the highly homologous glucocorticoid receptor (GR) DBD, residues located in the second zinc finger are postulated to make contact with proteins bound at the activated transcription complex (Skena *et al.* 1989, Hard *et al.* 1990, Luisi *et al.* 1991). In fact, it has been shown that at least one transcription factor, a TATA-binding protein (TBP)-associated factor-TAFII₁₁₀, interacts directly with the DBD of PR (Schwerk *et al.* 1995).

The DNA-dependent protein kinase (DNA-PK) holoenzyme is a trimeric protein complex composed of the DNA-binding heterodimeric regulatory

subunits Ku70 and Ku86, and the 460 kDa catalytic subunit (DNA-PK_{CS}) (Dvir *et al.* 1993, Gottlieb & Jackson 1993). Nuclear DNA-PK has been implicated in a variety of cellular processes including double-stranded DNA break repair and V(D)J recombination (Blunt *et al.* 1995, Zhu *et al.* 1996), DNA replication and growth (Feldmann & Winnacker 1993, Nussenzweig *et al.* 1996), and transcription (Giffin *et al.* 1994, 1996, Kuhn *et al.* 1995, Labhart 1995, Peterson *et al.* 1995a). Several DNA-binding transcription factors are known substrates for DNA-PK phosphorylation (Carter *et al.* 1990, Lees-Miller *et al.* 1992, Dvir *et al.* 1993), including glucocorticoid, estrogen, and progesterone receptors (Arnold *et al.* 1995, Giffin *et al.* 1997). In fact, a single DNA-PK phosphorylated residue of rat GR has been localized to Ser⁵²⁷, adjacent to the DBD (Giffin *et al.* 1997). PRs are phosphorylated in several stages, at least one of which is dependent on DNA binding of the receptors (Takimoto *et al.* 1992, Weigel *et al.* 1992).

As its name implies, DNA-PK is believed to have a unique catalytic requirement for direct DNA contact (Carter *et al.* 1990, Lees-Miller *et al.* 1990). Neither DNA-PK_{CS} nor the Ku heterodimer is active independently, but kinase activity is acquired following assembly of DNA-PK_{CS} onto DNA-bound Ku (Dvir *et al.* 1993, Gottlieb & Jackson 1993). Ku binding was originally thought to be DNA sequence-independent, until several recent studies demonstrated a high affinity of Ku heterodimers for specific DNA sequences (Okumura *et al.* 1994, Roberts *et al.* 1994, Genersch *et al.* 1995, Giffin *et al.* 1996). One such Ku-binding DNA element, NRE1, is present in the long terminal repeat of the mouse mammary tumor virus promoter (Giffin *et al.* 1997, 1996). NRE1 serves as a sequence-specific DNA binding site for Ku/DNA-PK_{CS}, and kinase activity correlates specifically with Kus binding to this element. Additionally, several heterodimeric transcription factors which bind to specific DNA sequences have been described, including proximal sequence element binding protein, transferrin receptor element binding protein, heat shock element binding factor, and nuclear factor IV, that are closely related to, or identical to Ku (Knuth *et al.* 1990, Stuver *et al.* 1990, Roberts *et al.* 1994, Kim *et al.* 1995). Thus, specific DNA binding sites for Ku on gene promoters may play a more important role in localizing Ku-associated kinase activity than had previously been thought.

Several recent studies suggest the possibility that the DNA binding specificity of Ku is dependent not solely on unique DNA binding sites, but also on interactions between the Ku heterodimers and

proteins. The redox factor protein REF1 binds Ku heterodimers specifically, while the proto-oncogene p95^{VA} has been shown to associate with Ku70 (Chung *et al.* 1996, Romero *et al.* 1996). Several other factors have been shown to interact with the Ku/DNA-PK_{CS} complex, including heat shock transcription factor 1, the epidermal growth factor receptor, the XRCC4 gene product, and antigen receptor response element binding proteins NF45 and NF90 (Huang *et al.* 1997, Bandyopadhyay *et al.* 1998, Leber *et al.* 1998, Ting *et al.* 1998). Thus, the mechanism by which DNA-PK phosphorylates an extensive array of upstream and basal transcription factors remains unknown, but may involve protein-DNA as well as direct protein-protein interactions.

The 113 kDa poly(ADP-ribose), or PARP, is a eukaryotic nuclear protein that recognizes and binds to DNA strand breaks and is automodified by synthesis of poly(ADP-ribose) (Lindahl *et al.* 1995 and references therein). The enzyme is induced following DNA damage produced by alkylating agents or ionizing radiation (Durkacz *et al.* 1980). Multiple functions have been proposed for this protein, including involvement in DNA repair (Lindahl *et al.* 1995), a role in apoptosis (Kaufmann *et al.* 1993), binding to histones with concomitant changes in chromatin structure (Althaus *et al.* 1994), signaling in response to environmental stress (Masutani *et al.* 1995), and regulation of transcription (Oei *et al.* 1998). Despite its proposed roles in multiple processes, PARP-deficient mice develop normally and are fertile (Wang *et al.* 1995). Two recent studies have shown evidence for the coincidence of progesterone-mediated events with PARP activity during oocyte maturation and ovulation (Aoufouchi & Shall 1997, Murdoch 1998). In this study, we demonstrate an interaction between both DNA-PK and PARP with the DNA binding domain of human (h) PRs.

MATERIALS AND METHODS

Plasmid construction

Human PR fragments (DBD_{PR}, DBD_{CYS}, AF3, AF3-DBD_{PR}) were amplified by PCR using pSG5-hPR1 (a gift from P Chambon, CNRS, Strasbourg, France), pSG5-hPR1_{CYS} (Takimoto *et al.* 1992), and pSG5-AF3-DBD (Sartorius *et al.* 1994b) as templates. Human ER DBD (DBD_{ER}), and the 147 amino acid GAL4 DNA binding domain (GAL4¹⁻¹⁴⁷) were amplified from pSG5-HEGO (a gift from P Chambon) and pGBT9 (Clontech, Palo Alto, CA, USA) respectively. Oligonucleotide primers contained either a BamHI or BglII restriction site at the 5' end and an EcoRI

site at the 3' end. Amplified fragments were inserted into the corresponding BamHI/EcoRI cloning sites of the pGEX-4T-1 bacterial glutathione-S-transferase (GST) gene fusion expression vector (Pharmacia, Piscataway, NJ, USA). Correct fragment insertions and sequences were verified by automated sequencing. Plasmids containing the Ku70 and Ku86 cDNA inserts for *in vitro* translation were kindly provided by W Reeves (University of North Carolina, Chapel Hill, NC, USA) (Reeves & Stthoeger 1989, Yaneva *et al.* 1989).

Gel mobility shift assay

Gel mobility shift assays were performed as described (Sartorius *et al.* 1994b) using bacterial extracts containing overexpressed GST-fusion proteins. [³²P]Labeled oligonucleotide probes were a 27 bp perfect palindromic consensus PRE, and a 35 bp estrogen response element (ERE) from the *Xenopus* vitellogenin A2 gene promoter (Takimoto *et al.* 1992).

[³⁵S]Methionine labeled HeLa cell extracts

Subconfluent T175 cm² flasks of HeLa cells were washed for 30 min at 37 °C in methionine-free (modified Eagle's medium) MEM (Sigma Cell Culture, St Louis, MO, USA), and then incubated for 4 h at 37 °C in methionine-free MEM supplemented with 100 µCi/ml methionine TRANS³⁵S-LABEL (ICN Radiochemicals, Costa Mesa, CA, USA). Cells were collected and homogenized at 4 °C in 0.25 ml TEDG (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol) per flask. The cell homogenate was centrifuged for 30 min at 100 000 *g*, and the supernatant was stored at -70 °C as the cytosol fraction at ~3.0 mg/ml. The protein pellets were resuspended in 0.2 ml TEDG, 0.4 M NaCl plus protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN, USA) per flask and incubated for 1 h at 4 °C. The resuspended pellet was then centrifuged for 30 min at 100 000 *g* and the supernatant dialyzed overnight in TEDG containing 0.1 M NaCl. The resulting dialysate was stored at -70 °C as the nuclear extract, at ~2.5 mg/ml protein. To prepare unlabeled HeLa cell extracts, the same procedure was followed without the addition of [³⁵S]methionine.

GST-fusion protein expression and GST 'pull-down' assays

E. coli strain BL21(DE3)pLysS (Novagen, Madison, WI, USA) was employed for GST-fusion

protein expression according to the method described by the manufacturer (Pharmacia). Briefly, overnight cultures were grown from a single colony and diluted 1:10 in 2XYT-G media containing 100 µg/ml ampicillin. Cultures were grown at 37 °C until an OD₆₀₀ of 0.6 was reached, at which time isopropyl-β-D-thiogalactoside was added to a final concentration of 1.0 mM. Cultures were then grown for an additional 2 h at 30 °C and pelleted for 10 min at 10 000 *g*. Bacterial pellets were resuspended in 1 ml NETGT (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 5% glycerol, 0.1% Tween-20) plus protease inhibitors (Complete; Boehringer Mannheim) per 10 ml culture and lysed by mild sonication. The sonicate was cleared by centrifugation for 10 min at 10 000 *g* and the supernatant stored at -70 °C.

Bacterial extracts (100 µl) containing overexpressed GST-fusion proteins (~10 mg/ml) were rotated with 25 µl pelleted glutathione-Sepharose 4B affinity matrix (Pharmacia) for 1 h at 4 °C. The beads were repelleted by centrifugation at 100 *g* for 3 min, and washed four times with 1 ml NETGT. The immobilized fusion proteins were then rotated with 100 µl HeLa nuclear extract for 4 h at 4 °C. The beads were again washed four times with 1 ml NETGT. The bound proteins were eluted by boiling for 1 min in 100 µl protein loading buffer (0.01% bromophenol blue, 2.5% SDS, 25 mM DTT, 25% glycerol) and resolved by SDS-PAGE on 5–15% gradient or 11% gels. For [³⁵S]methionine-containing samples, the gel was dried onto Whatman paper and visualized by autoradiography.

DNase I treatment of HeLa cell and bacterial extracts

HeLa cell nuclear or bacterial extracts were desalted over G-25 Sephadex spin columns. Desalted extracts were then diluted with an equal volume of 2 × DNase I buffer (300 mM NaCl, 100 mM Tris pH 7.5, 4 mM MgCl₂). DNase I (Sigma) was added to a final concentration of 50 µg/ml and samples were incubated at 4 °C or 25 °C for 30 min, as described previously (Wang *et al.* 1994). Samples were then diluted with an equal volume of 2 × NETGT and incubated with the fusion protein bound to the Sepharose 4B affinity matrix, as described above.

Protein microsequencing

GST 'pull-down' assays with GST-DBD_{PR} were performed as described. Proteins were resolved on a 5–15% gradient SDS-polyacrylamide gel and

transferred to Immobilon P membrane (Millipore, Bedford, MA, USA) in CAPS-methanol buffer (10 mM CAPS, 10% MeOH, pH 11.0). The membrane was stained with Coomassie Blue and the appropriate bands were excised for microsequence analysis. The p110 protein band was N-terminally blocked, which necessitated digestion of the purified protein with lysine endopeptidase. This was accomplished by excising the p110 band directly from the gel, eluting it from the gel slice, and performing the digest in solution. Microsequencing and protease enzyme digestions were performed by the NYS Center for Advanced Technology, Cornell University (Ithaca, NY, USA), and the Molecular Resource Center, National Jewish Hospital (Denver, CO, USA).

Immunoblotting

GST 'pull-down' assays were performed as described above. Proteins were then transferred to nitrocellulose using a wet transfer apparatus (Bio-Rad, Richmond, CA, USA) in CAPS-methanol buffer for 5 h at 0.7 amps in a 4 °C cold room. The membrane was blocked with 5% non-fat milk in phosphate-buffered saline and 0.1% Tween for 1 h. The mouse monoclonal antibodies N3H10 and N9C1 against Ku (kindly provided by R Burgess, McArdle Labs, Madison, WI, USA), and 18-2, 25-4, and 42-27 against DNA-PK_{CS} (kindly provided by T Shenk, Princeton University, Princeton, NJ, USA) were used at a concentration of 1 µg/ml as previously described (Knuth *et al.* 1990, Kaufmann *et al.* 1993). A rabbit polyclonal antibody against PARP was purchased from Boehringer Mannheim. Bands were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) as previously described (Sartorius *et al.* 1994a).

Kinase assays

GST 'pull-down' assays were performed as described above to purify the Ku/DNA-PK complex. Aliquots (30 µl) of the glutathione-Sepharose 4B matrix containing bound fusion proteins and associated proteins from HeLa cell nuclear extracts were transferred to siliconized 0.65 ml tubes and resuspended in 50 µl phosphorylation buffer (20 mM Tris pH 7.5, 100 mM KCl, 12 mM MgCl₂, 1 mM DTT). Samples were incubated in the absence of DNA, in the presence of a final concentration of 0.5 µg/ml of a ~250 bp DNA fragment containing two tandem PREs linked upstream of a truncated thymidine kinase (*tk*) promoter sequence, or in the presence of a ~200 bp

DNA fragment containing only the truncated *tk* promoter sequence (Sartorius *et al.* 1994a). Three microliters of [γ -³²P]ATP (12 Ci/mmol) were added to give a final concentration of 5 µM. The reaction mixture was incubated at 30 °C for 30 min. The resin was washed once with 0.5 ml NETGT, the proteins were eluted with protein loading dye, and separated by SDS-PAGE. The gel was dried onto Whatman paper and visualized by autoradiography.

The filter binding assay for detecting a phosphorylated synthetic peptide used conditions identical to the kinase assay described above except that 17 µg of a DNA-PK-specific synthetic peptide substrate was added to the reactions. The fusion protein/DNA-PK complex was incubated with the peptide-containing kinase mixture for 10 min at 30 °C, after which matrix-bound proteins were pelleted and ³²P-labeled peptide separated from free [γ -³²P]ATP by a filter binding assay described previously (Lees-Miller *et al.* 1992). The synthetic peptide substrate (Promega, Madison, WI, USA) is identical to E¹¹ through L²⁵ of the tumor suppressor protein p53 (E¹¹PPLSQEAFADLWKK) except that three substitutions were made (T¹⁸A, S²⁰A, L²⁵K) which inhibit phosphorylation by non-DNA-PK serine/threonine kinases and promote binding to P-81 paper (Lees-Miller *et al.* 1992). The phosphorylated residue is S¹⁵, located in the DNA-PK-specific SQ¹⁶ phosphorylation motif (Lees-Miller *et al.* 1992).

In vitro translation of Ku proteins

The TNT T7 Quick Coupled Transcription/Translation System kit (Promega) with [³⁵S]L-methionine (ICN Radiochemicals) was used to *in vitro* translate and label Ku70 and Ku86 from their cDNAs.

Immunoprecipitation assay

To identify proteins interacting with hPR *in vivo*, a vector expressing a FLAG epitope (GGDYKD-DDK) tagged hPR at the C-terminal end of the receptor (f:hPR_B) was used (Richer *et al.* 1998). The f:hPR_B plasmid was transiently transfected into HeLa cells, treated with 10 nM of the synthetic progestin R5020 for 1 h, and whole cell lysates were prepared as described previously (Takimoto *et al.* 1992). Lysates were dialyzed into 0.1 M NaCl and 100 µl of a 50% slurry of α -FLAG M2 affinity gel (Kodak, New Haven, CT, USA) was added to 2 mg protein and rotated at 4 °C for 4 h. The affinity gel was then washed twice with 1 ml 0.3 M KCl in TEDG, twice with 1 ml TEDG containing 0.1% NP40, and twice with TEDG. Immunoprecipitated

f:hPR_B and associated proteins were boiled in loading buffer, resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with α -FLAG M2 monoclonal antibody (Kodak), antibodies directed against Ku70, Ku86, and DNA-PK_{CS}, or an antibody directed against PARP.

RESULTS

Vectors expressing GST linked to several domains of the hPR, hER, and yeast GAL4 proteins were constructed as described in Materials and Methods. GST-PR chimeras encode the DBD_{PR} alone (amino acids 551–647), the 165 amino acid N-terminal activation function of PR (AF3) alone (Sartorius *et al.* 1994b), AF3 linked upstream of the DBD_{PR} (AF3-DBD_{PR}), and a DNA binding deficient DBD_{PR} mutant in which the structure of the first zinc finger is destroyed by a single missense mutation (Cys⁵⁸⁷→Ala) that specifically replaces one of the cysteine residues required for zinc co-ordination (DBD_{CYS}) (Takimoto *et al.* 1992). The DBD of hER (DBD_{ER}, amino acids 185–250) and the DBD of the yeast transcriptional activator GAL4 which include the N-terminal 147 amino acids (GAL4^{1–147}) were also linked to the C-terminal end of GST. All vectors expressed high levels of the fusion proteins in *E. coli* strain BL21(DE3)pLysS.

The ability of several of the receptor constructs to bind DNA at a PRE or ERE was tested using the gel mobility shift assay (Fig. 1). Bacterial extracts containing overexpressed GST-fusion proteins (0.5 or 2.0 μ l) were incubated with [³²P]labeled double-stranded oligonucleotides containing either a PRE (lanes 1–7) or an ERE (lanes 8–14). The DNA/protein complexes were resolved by non-denaturing gel electrophoresis, dried onto Whatman paper, and visualized by autoradiography. GST fusions containing AF3-DBD_{PR} (Fig. 1, lane 1) or the wild-type DBD_{PR} alone (Fig. 1, lanes 6 and 7) bound well to the PRE. However, GST-fusion proteins containing the zinc finger mutant, DBD_{CYS} (Fig. 1, lanes 2 and 3), or the DBD_{ER} (Fig. 1, lanes 4 and 5) did not bind to the PRE, as expected. GST-DBD_{ER} did, however, bind to an ERE (Fig. 1, lanes 11 and 12), to which none of the GST-PR fusion proteins could bind (Fig. 1, lanes 8–10, 13 and 14). Note that in lanes 8–14 the doublet bands represent non-specific ERE binding proteins present in bacterial extracts which did not bind to the PRE. This study demonstrates that the bacterially expressed GST-fusion proteins used in these experiments retained appropriate DNA binding abilities and specificities.

Five HeLa cell nuclear proteins associate with DBD_{PR}

In Fig. 2A, the GST-DBD_{PR} fusion protein was purified on a glutathione-Sepharose 4B affinity matrix, then incubated with either cytosol (C, Fig. 2A, lane 1) or nuclear (N, Fig. 2A, lane 2) HeLa cell extract. After several washes, matrix-bound HeLa cell proteins were eluted, resolved on an SDS-polyacrylamide gel, transferred to nitrocellulose, and visualized with Coomassie Blue stain. Five major protein bands of approximately 62, 80, 110, 200, and >400 kDa were pulled down from HeLa nuclear extract (Fig. 2A, lane 2), but not from cytosol extract (Fig. 2A, lane 1). Since a DNA binding protein was used in these experiments, we tested whether DNase I treatment of HeLa cell nuclear and bacterial cell extracts would affect these interactions (Fig. 2B). DNase I treatments were performed under conditions shown to release chromatin-bound proteins from isolated nuclei (Wang *et al.* 1994). GST-DBD_{PR} was able to associate with HeLa proteins with equal efficiency in extracts that were untreated (Fig. 2B, lane 1) or treated with DNase I (Fig. 2B, lane 2). In Fig. 2C, *in vivo* [³⁵S]methionine labeled HeLa cell nuclear or cytosol extracts were used in a pull down assay with several GST-PR fusion proteins. The experiment was performed as described in Fig. 2A, except that bands were visualized by autoradiography of the dried gel. Using GST-DBD_{PR}, three major labeled nuclear proteins of 62, 80, and >400 kDa were purified (Fig. 2C, lane 2) that were absent in the cytosol fraction (Fig. 2C, lane 1). Neither GST alone (Fig. 2C, lane 3) nor GST-AF3 (Fig. 2C, lane 5) bound these nuclear proteins, suggesting that they are bound specifically to the DBD of hPR. AF3, when cloned upstream of the DBD_{PR} (Fig. 2C, lane 4), did not affect binding of the HeLa cell nuclear proteins. The p110 band was only weakly labeled with [³⁵S]methionine, due to its slow metabolic turnover rate (see below).

Identification of GST-DBD_{PR}-associated proteins by microsequencing and immunoblotting

The p62, p80, and p110 bands were excised from the Coomassie-stained membrane and processed for N-terminal microsequence analysis. Protein sequence obtained for the p80 band, RSGNKA AV VLCMDVGFT, was perfectly matched in the protein database using the BLAST algorithm, by the 86 kDa subunit of the Ku autoantigen (Yaneva *et al.* 1989). Proteins from the p62 and p110 bands were N-terminally blocked. However, after

FIGURE 1. GST-fusion proteins containing DBD_{PR} or DBD_{ER} bind their respective hormone response elements in a gel mobility shift assay. GST-fusion proteins containing AF3-DBD_{PR}, DBD_{CYS}, DBD_{ER}, and DBD_{PR} were expressed in *E. coli* strain BL21(DE3)pLysS. Bacterial extracts (0.5 µl or 2.0 µl) were added to reaction mixtures containing either a [³²P]labeled double-stranded PRE (lanes 1–7) or ERE (lanes 8–14) oligonucleotide. NS: non-specific bands obtained with the ERE probe. Free DNA: migration of the unbound oligonucleotide shown at the bottom of the gel.

FIGURE 2. Five major HeLa cell nuclear proteins associate with DBD_{PR}. (A) Immobilized GST-DBD_{PR} was incubated with cytosolic (C, lane 1) or nuclear (N, lane 2) HeLa cell extracts. GST-DBD_{PR} and associated proteins were eluted, resolved by SDS-PAGE on a 5–15% gradient gel, transferred to nitrocellulose, and stained with Coomassie Blue. GST-DBD_{PR} is indicated at approximately 35 kDa. The five associated proteins are labeled according to their approximate size (p62, p80, p110, p200, p400). (B) HeLa cell nuclear extract and the GST-fusion protein-containing bacterial extract were either untreated (–, lane 1) or treated with DNase I (50 µg/ml) (+, lane 2) prior to incubation with the glutathione-Sepharose 4B matrix. After incubation, fusion protein-associated proteins were eluted, resolved by SDS-PAGE, and stained with Coomassie Blue. (C) [³⁵S]Methionine labeled HeLa cell cytosolic (C, lane 1) and nuclear (N, lanes 2–5) extracts were prepared and incubated with immobilized GST alone (lane 3) or GST fusion proteins (lanes 1–2, 4–5). Associated proteins were eluted, resolved by SDS-PAGE, transferred to nitrocellulose, and detected by autoradiography. Approximate sizes of major [³⁵S]labeled proteins associated with GST-DBD_{PR} (lane 2) and GST-AF3-DBD_{PR} (lane 4) are shown (p62, p80, p400).

lysine endopeptidase digestion of the p110 band, sequence was obtained from four internal peptides: HSIKHPDVE, QVRLSK, ANIRV VSEDFLQDV, and THATTHNAYDEVI. These were perfectly matched in the protein database to the 113 kDa poly(ADP-ribose) polymerase, or PARP (Cherney *et al.* 1987). Its weak [³⁵S]methionine labeling is consistent with a slow turnover over rate *in vivo* (Ding *et al.* 1992). The identity of the p200 band is under investigation.

Since Ku86 is known to assemble into a trimeric protein complex that includes Ku70 and the 460 kDa DNA-PK_{CS}, we postulated that the p62 and p400 proteins were the other two components of this complex. This was confirmed by the immunoblots shown in Fig. 3. After extensive washing, HeLa cell nuclear proteins that copurified with the GST-fusion proteins on the glutathione-Sepharose 4B affinity matrix were eluted, and aliquots of the eluate were resolved on four parallel

FIGURE 3. Immunoblot analyses identifying the DBD_{PR}-associated proteins as the trimeric DNA-PK subunits and PARP. HeLa cell cytosol (C, lanes 2, A–C) and nuclear (N, all others) extracts were incubated with GST alone (lane 1, A–C) or GST fusion proteins (lanes 2–7) as indicated, immobilized on glutathione-Sepharose 4B matrix. Bound proteins were eluted, resolved by SDS-PAGE, and transferred to nitrocellulose. (A) Coomassie Blue stain of GST-fusion and associated proteins. Molecular mass markers are indicated on the left. Immunoblots (B–D) were probed with: (B) antibodies directed against Ku70 and Ku86 (α Ku70+86); (C) antibodies against DNA-PK_{CS} (α DNA-PK_{CS}); and (D) an antibody specific for PARP (α PARP).

5–15% gradient gels. The gels were either stained with Coomassie Blue (Fig. 3A) or transferred to nitrocellulose and probed with antibodies directed against Ku70 and Ku86 (Fig. 3B) (Knuth *et al.* 1990), DNA-PK_{CS} (Fig. 3C) (Clarke & Sutherland 1990), or PARP (Fig. 3D).

In Fig. 3C, when nuclear proteins associating with GST-DBD_{PR} were probed with a mixture of antibodies to Ku70 and Ku86, two strongly labeled immunoreactive bands appeared (Fig. 3B, lanes 3), suggesting that these proteins are, indeed, Ku70 and Ku86. These bands comigrated with the Coomassie Blue-stained p62 and p80 bands shown in Fig. 3A (lane 3). Only a small amount of immunoreactive Ku70 was seen to associate with DBD_{PR} from cytosol extract (Fig. 3B, lane 2). In contrast, no immunoreactive Ku70 or Ku86 were seen to associate with purified GST alone (Fig. 3B, lane 1). An immunoblot probed with antibodies directed against DNA-PK_{CS} resulted in the appearance of a single immunoreactive band of >400 kDa with GST-DBD_{PR} (Fig. 3C, lane 3). This

>400 kDa protein corresponds to the expected molecular weight of DNA-PK_{CS} (Hartley *et al.* 1995). Again, little or no DNA-PK_{CS} was seen with GST-DBD_{PR} when it was incubated with a HeLa cell cytosol extract (Fig. 3C, lane 2), consistent with the predominantly nuclear localization of this protein (Jackson *et al.* 1990). No immunoreactive DNA-PK_{CS} was seen to associate with purified GST alone (Fig. 3C, lane 1). These results confirm that all three components of the DNA-PK holoenzyme complex are associated with DBD_{PR}.

The association between the trimeric DNA-PK complex and GST-DBD_{PR} was effectively abolished by a single C⁵⁸⁷A point mutation in the DBD to produce GST-DBD_{CYS} (Fig. 3B and C, lane 4) (Takimoto *et al.* 1992). This mutation destroys the structural integrity of the first zinc finger, resulting in a DNA binding deficient mutant. Interestingly, each component of DNA-PK also associated strongly with GST-GAL4¹⁻¹⁴⁷ (Fig. 3B and C, lane 6), which is known to stimulate DNA-PK-mediated phosphorylation of several different substrates

(Peterson *et al.* 1995b). Surprisingly, DNA-PK did not associate with GST-DBD_{ER} (Fig. 3B and C, lane 5), even though DBD_{ER} shares approximately 50% sequence similarity with DBD_{PR} (Koelle *et al.* 1991), has the same two zinc finger structure, and full-length human ER are *in vitro* substrates of DNA-PK (Arnold *et al.* 1995). A GST fusion containing a larger region of the ER (90 amino acids, 171–260) was found to give the same results as the 68 amino acid DBD_{ER} (data not shown). We also confirmed by immunoblot that GST-AF3 lacking DBD_{PR} was unable to pull down the DNA-PK complex (Fig. 3B and C, lane 7). Association with DNA-PK was not limited exclusively to the DBD_{PR} fragment, since other constructs, including GST-AF3-DBD_{PR} (see Fig. 2), and GST-PR fusions containing various N-terminal PR flanking sequences linked to DBD_{PR} retained the ability to associate with DNA-PK (data not shown).

Figure 3D shows an immunoblot probed with an anti-PARP antibody. Immunoreactivity was seen in HeLa nuclear extracts pulled down with GST-DBD_{PR}, confirming the identity of p110 as PARP (Fig. 3D, lane 2). PARP did not associate with GST-DBD_{CYS} (Fig. 3D, lane 1) or GST-AF3 (Fig. 3D, lane 6). PARP did, however, associate strongly with GST-AF3-DBD_{PR} (Fig. 3D, lane 5), and with GST-GAL4^{1–147} (Fig. 3D, lane 4). GST-DBD_{ER} also appeared to have some ability to bind PARP (Fig. 3D, lane 3).

Kinase properties of the DBD_{PR}-associated DNA-PK complex

To determine if the DNA-PK that copurified with GST-DBD_{PR} was catalytically active, we measured both the autophosphorylation of Ku, which has previously been shown to be dependent on the activated trimeric kinase complex (Lees-Miller *et al.* 1990), and the phosphorylation of the GST-fusion proteins. The DNA-PK complex was copurified from HeLa cell nuclear extracts on immobilized GST-fusion proteins as described. DNA-PK bound to GST-DBD_{PR} on the glutathione-Sepharose 4B matrix was then incubated with kinase buffer containing [γ -³²P]ATP at 30 °C for 10 min, followed by several washes to remove free [γ -³²P]ATP. The bound proteins were eluted from the Sepharose beads and resolved on a 5–15% gradient SDS-polyacrylamide gel (Fig. 4). As visualized by Coomassie Blue staining, GST-GAL4^{1–147} (Fig. 4A, lane 2) and GST-DBD_{PR} (Fig. 4A, lane 4) effectively pulled down Ku70, Ku86, and to a lesser extent DNA-PK_{CS}, as described. The parallel [³²P]autoradiogram (Fig. 4B) demonstrates that the

presence of DNA-PK was associated with autophosphorylation of both Ku subunits as well as of DNA-PK_{CS} (Fig. 4B, lanes 2 and 4). In contrast, GST-DBD_{CYS} (Fig. 4B, lane 3) and GST-DBD_{ER} (Fig. 4B, lane 5), with which DNA-PK does not copurify, exhibited no kinase activity. Note that a low level of kinase activity copurified with GST-AF3 (Fig. 4B, lane 1, open arrowhead), the identity of which is unknown. Figure 4B shows not only the autophosphorylation of the three DNA-PK subunits, but also that the DNA-PK_{CS} transphosphorylated the GST-fusion proteins to which the kinase is bound (solid arrowheads).

To determine which portions of the GST-fusion proteins were phosphorylated, the proteins were eluted following the kinase assay, and GST was dissociated from the bait peptides by thrombin cleavage. Cleaved proteins were resolved on an 11% gel (Fig. 4C), and the position of the [³²P]label was monitored by autoradiography of the dried gel (Fig. 4D). Free GST migrated as a 29 kDa band (Fig. 4C, lanes 1–5) and the released proteins, which ranged in size from approximately 10 to 40 kDa, are marked by solid arrowheads. AF3 migrated as a triplet (open arrowheads). The [³²P]label comigrated with the released proteins AF3-DBD (Fig. 4D, lane 2), GAL4^{1–147} (Fig. 4D, lane 3), and DBD_{PR} (Fig. 4D, lane 4), which are indicated by solid arrowheads. GST itself was not phosphorylated by DNA-PK. Note that incomplete thrombin cleavage and/or proteolysis occurred with GST-AF3-DBD_{PR} (Fig. 4D, lane 2) and GST-DBD_{PR} (Fig. 4D, lane 4), accounting for the additional [³²P]labeled bands. The unknown kinase that copurifies with AF3 (Fig. 4B, lane 1) generated a triplet banding pattern at the 21 kDa marker (Fig. 4D, lane 1) that is characteristic of the phosphorylated state of this receptor domain (Sartorius *et al.* 1994b).

Since DNA-PK has been shown to have a unique catalytic requirement for direct DNA contact (Carter *et al.* 1990, Lees-Miller *et al.* 1990), the extensive kinase activity we observed in the apparent absence of DNA was surprising. As shown in Fig. 2B, DNase I treatment of both HeLa and bacterial extracts had no effect on subsequent copurification of the Ku/DNA-PK_{CS} complex, suggesting the absence of DNA. However, as we have demonstrated, DNA-PK retains its kinase activity when copurified from HeLa cell extracts with specific GST-fusion proteins. We therefore asked whether addition of exogenous DNA, specifically an ~250 bp fragment containing *tk* promoter sequence with or without two tandem palindromic PREs, augments the catalytic activity of DNA-PK (Sartorius *et al.* 1994b). To determine whether

exogenously added DNA affected DNA-PK activity, the trimeric complex was first copurified from a HeLa nuclear extract with either affinity matrix bound GST-DBD_{PR} (Fig. 5, lanes 1–3) or GST-DBD_{CYS} (Fig. 5, lanes 4–6). The copurified proteins were then washed extensively and both autophosphorylation of the matrix-bound DNA-PK and phosphorylation of the DBD_{PR} substrate were measured. Kinase activity was measured in the absence of exogenously added DNA (Fig. 5, lanes 1 and 4), in the presence of a DNA fragment containing *tk* promoter sequence (Fig. 5, lanes 2 and 5), or in the presence of the same *tk* promoter fragment with two tandem PREs inserted (Fig. 5, lanes 3 and 6). As shown, no kinase activity copurified with the mutant DBD_{CYS} (Fig. 5, lanes 4–6). In contrast, with wild-type DBD_{PR} in the absence of added DNA (Fig. 5, lane 1), DNA-PK was extremely catalytically active, transphosphorylating the GST-DBD_{PR} fusion protein and autophosphorylating its own Ku70, Ku86, and DNA-PK_{CS} subunits. Addition of DNA fragments without (Fig. 5, lane 2) or with two PREs (Fig. 5, lane 3) had no significant effect on this catalytic activity. A minimal amount of DNA may therefore be present in the reaction bound to DNA-PK which is sufficient to support kinase activity.

To determine whether the DNA-PK heterotrimer that copurifies with the hPR DBD can phosphorylate an exogenous substrate, we measured phosphorylation of Ser¹⁵ in the N-terminus of the tumor suppressor protein p53. These experiments used the modified peptide EPPLSQEAFADLWKK, which is a specific DNA-PK substrate (Lees-Miller *et al.* 1992). To this end, the DNA-PK heterotrimer was copurified with GST alone, or GST-fusion proteins (GST-DBD_{CYS}, GST-DBD_{PR}, GST-GAL4^{1–147}, GST-DBD_{ER}) on a glutathione-Sepharose 4B affinity matrix as

described (Fig. 6). The beads were washed, then incubated with the peptide substrate and [γ -³²P]ATP at 30 °C for 10 min. Reactions were stopped, and transfer of [³²P]phosphate to the peptide was quantified by a phosphocellulose filter binding assay (Lees-Miller *et al.* 1992). Compared with the low background phosphorylation of the peptide seen with GST alone, phosphorylation of the peptide by DNA-PK associated with GST-DBD_{PR} or GST-GAL4^{1–147} was induced 16- and 24-fold respectively. Only minimal kinase activity was detected with the GST-DBD_{CYS} mutant. A small (fourfold) induction was seen with GST-DBD_{ER}. These data indicate that the DNA-PK heterotrimer, purified with the DBDs of transcription factors, retains its specificity for an exogenously added substrate.

Association of *in vitro* translated Ku70 and Ku86 with DBD_{PR}

To determine if the Ku components alone (without the DNA-PK catalytic subunit) could interact with DBD_{PR}, we used an *in vitro* translation system in which each Ku subunit could be synthesized individually. For these studies, [³⁵S]methionine labeled Ku70 and Ku86 were prepared by *in vitro* translation of each subunit alone (Fig. 7, lanes 1–4 and 9–12), or by cotranslation of both subunits (Fig. 7, lanes 5–8). Lysates containing translated proteins were copurified with bacterially expressed, glutathione-Sepharose 4B-immobilized GST-DBD_{PR} (wt) or GST-DBD_{CYS} (cys). Eluted proteins were separated by SDS-PAGE and visualized by autoradiography of the dried gel. Experiments were performed in the absence (–) or presence (+) of 5 µg/ml of the PRE₂*tk* DNA fragment described above. *In vitro* translated Ku did not associate with GST alone (data not shown).

FIGURE 4. The association of DNA-PK with DBDs correlates with autophosphorylation of Ku and DNA-PK_{CS} and transphosphorylation of the DBDs. GST-fusion and associated HeLa proteins bound to the glutathione-Sepharose matrix, as described, were incubated with a kinase mixture containing [γ -³²P]ATP. After extensive washing, [³²P]labeled proteins were eluted from the matrix, resolved on a 5–15% SDS-polyacrylamide gradient gel, and visualized by autoradiography of the dried gel. (A) Coomassie Blue stain of eluted proteins resolved by SDS-PAGE. (B) Autoradiogram of GST-fusion and associated proteins phosphorylated after incubation with [γ -³²P]ATP. Arrows denote positions of Ku70, Ku86, and DNA-PK_{CS}. Solid arrowheads in panels A and B denote positions of the Coomassie-stained and [³²P]labeled fusion proteins. The open arrowhead in panel B denotes activity of an unknown kinase bound to AF3. (C) Incubation with the kinase mixture was followed by thrombin (25 U/ml) cleavage prior to elution from the matrix. Eluted proteins were resolved on an 11% gel and stained with Coomassie Blue. Arrows denote the GST protein liberated from the cleaved fusion protein (GST), and a 70 kDa bacterial protein that copurifies with the fusion protein. (D) Autoradiogram showing [³²P] incorporation of the gel described in (C). Solid arrowheads denote the derivative protein fragments liberated by thrombin cleavage (AF3-DBD_{PR}, lane 2; GAL4^{1–147}, lane 3; DBD_{PR}, lane 4; DBD_{CYS}, lane 5) that are Coomassie stained in panel C and phosphorylated in panel D. Open arrowheads denote the AF3 triplet that is Coomassie stained in panel C and phosphorylated in panel D by the unknown kinase (see panel B).

FIGURE 5. Exogenously added DNA has no effect on DBD_{PR}-associated DNA-PK activity. DNA-PK was purified from HeLa cell nuclear extract on immobilized GST-DBD_{PR} (lanes 1–3) or GST-DBD_{CYS} (lanes 4–6) as described. Matrix-bound proteins were incubated with a [γ -³²P]ATP-containing kinase mixture either in the absence of DNA (–, lanes 1 and 4), or in the presence of 0.5 μ g/ml of an $\sim$ 200 bp *tk* DNA fragment without (*tk*, lanes 2 and 5) or with a tandem PRE₂ linked upstream (PRE₂*tk*, lanes 3 and 6). Arrows denote major phosphorylated proteins including autophosphorylated Ku70, Ku86, and DNA-PK_{CS}, and transphosphorylated GST-DBD_{PR} fusion protein.

Each Ku subunit was, however, able to bind DBD_{PR} individually (Fig. 7: Ku70, lane 1; Ku86, lane 9) and addition of DNA appeared to have no effect on their individual binding (Fig. 7: Ku70, lane 3; Ku86, lane 11). A very low level (compared with wt) of each *in vitro* translated Ku subunit was able to bind to the DBD_{CYS} mutant, a phenomenon not observed with HeLa cell extracts (Fig. 7: Ku70, lanes 2 and 4; Ku86, lanes 10 and 12). Coexpression of the Ku subunits enhanced their binding to DBD_{PR} (Fig. 7, lane 5) compared with the binding

of each alone (Fig. 7, compare lanes 1, 5 and 9). Again, addition of DNA did not affect the levels of DBD_{PR}-bound Ku heterodimers (Fig. 7, lane 7). Coexpression of the Ku subunits did not increase their binding to DBD_{CYS} (Fig. 7, lanes 6 and 8).

***In vivo* association of DNA-PK components and PARP with DBD_{PR}**

To determine whether the hPR/DNA-PK multi-protein complex assembles *in vivo*, we transiently

FIGURE 6. A modified peptide substrate of the p53 tumor suppressor protein is phosphorylated by DBD_{PR}-associated DNA-PK. HeLa cell nuclear extracts were incubated with immobilized GST alone or GST-fusion proteins as indicated. GST-fusion and associated proteins bound to the matrix were washed, then incubated with a kinase mixture containing [γ -³²P]ATP and 17 μ g of a DNA-PK-specific peptide substrate derived from the N-terminus of the p53 tumor suppressor protein. The matrix-bound GST fusion protein/kinase complex was pelleted and the labeled peptide in the supernatant was separated from free [γ -³²P]ATP by filtration over P-81 cellulose phosphate circles. The circles were extensively washed and bound radioactivity was quantitated by liquid scintillation spectroscopy. The amount of peptide phosphorylation for each condition (bars) represents the average of two separate experiments with the range of the experimental values shown (vertical lines).

transfected HeLa cells with FLAG epitope-tagged, transcriptionally active, full-length hPR_B (f:hPR_B) (Richer *et al.* 1998). Extracts from HeLa cells transiently transfected with f:hPR_B were immunopurified using anti-FLAG M2 affinity gel. The immunoprecipitated f:hPR_B and associated proteins were transferred to nitrocellulose and probed with an α FLAG M2 monoclonal antibody (Fig. 8, lane 5). The immunoblot was then stripped and reprobed with a mixture of monoclonal antibodies directed against Ku70, Ku86 and DNA-PK_{CS} (Fig. 8, lanes 1 and 2). Again, the blot was stripped and reprobed with a polyclonal antibody directed against PARP (Fig. 8, lanes 3 and 4). Aliquots of crude HeLa cell extracts (1/10 volume used in assay) are shown in lanes 2 (α DNA-PK) and 4 (α PARP). All three proteins of the DNA-PK complex copurified with f:hPR_B (Fig. 8, lane 1), as did PARP (Fig. 8, lane 3). The percent of immunoreactivity of each subunit compared with

the crude HeLa cell extract that copurified with f:hPR_B was greatest for Ku70, followed by Ku86 and DNA-PK_{CS}, suggesting, perhaps, the order in which the three proteins assemble on PR. The possibility that Ku70 is the proximal PR binding protein is also suggested by the fact that it was independently isolated from a HeLa cell cDNA library when hPR were used as bait in a yeast two-hybrid interaction screen (data not shown).

DISCUSSION

We have identified four proteins that associate in a complex with GST-fusion proteins containing the DBDs of both hPR and yeast GAL4. These proteins were identified as the 113 kDa poly(ADP-ribose) polymerase enzyme, PARP, and the three components of the DNA-dependent protein kinase holoenzyme: its Ku70 and Ku86 regulatory subunits and the 460 kDa catalytic subunit. The association of these proteins with GST-DBD_{PR} was discovered *in vitro* using HeLa cell nuclear extracts, and was confirmed *in vivo* with wild-type hPR. Additionally, we show that DBD_{PR} is a substrate for DNA-PK. Possible mechanisms of DNA-PK association with DBD_{PR} and implications on PR-mediated transcription are discussed below.

Phosphorylation of DBD_{PR} by DNA-dependent protein kinase

In addition to its proposed involvement in DNA double-strand break repair and V(D)J recombination (Blunt *et al.* 1995, Zhu *et al.* 1996), DNA replication (Feldmann & Winnacker 1993), and growth control (Nussenzweig *et al.* 1996), DNA-PK plays a potential role in RNA polymerase-dependent transcription (Jackson *et al.* 1990, Dvir *et al.* 1993, Giffin *et al.* 1996). A variety of transcription factors including the RNA polymerase II enzyme itself are phosphorylated at Ser-Gln or Thr-Gln recognition sites by DNA-PK (Carter *et al.* 1990, Lees-Miller *et al.* 1992, Dvir *et al.* 1993). Glucocorticoid, estrogen, and progesterone receptors have all been described as DNA-PK substrates *in vitro* (Weigel *et al.* 1992, Arnold *et al.* 1995, Giffin *et al.* 1997). We have previously shown that PRs undergo two stages of ligand-dependent phosphorylation: a DNA-independent and a DNA-dependent stage (Takimoto *et al.* 1992), making DNA-PK a likely candidate for *in vivo* phosphorylation of PR.

Rat GR is phosphorylated by DNA-PK in the hinge region of the receptor at Ser⁵²⁷ (Giffin *et al.* 1997). Our DBD_{PR} construct contains four serine

FIGURE 7. *In vitro* translated Ku70 and Ku86 associate with DBD_{PR}. GST-DBD_{PR} was immobilized on a glutathione-Sepharose 4B matrix and incubated with [³⁵S]methionine labeled Ku70 and Ku86 that were *in vitro* translated separately (lanes 1–4 and 9–12) or cotranslated (lanes 5–8). Where indicated, 0.5 µg/ml of an ~250 bp DNA fragment from the *tk* gene promoter with a tandem PRE₂ insert (PRE₂*tk*), was added to *in vitro* translated Ku prior to incubation with the immobilized fusion protein. The matrix bound complex was eluted, resolved by SDS-PAGE, and proteins visualized by autoradiography. Arrows denote Ku70 and Ku86 bands. Note that the *in vitro* translated Ku migrated as doublets by SDS-PAGE. wt: GST-DBD_{PR}; cys: GST-DBD_{CYS}.

residues, all of which are located within the DBD (and does not contain the hinge region). A mutant PR DBD with two of these serines mutated (Ser⁵⁵⁸→Ala and Ser⁵⁶¹→Ala) retained its ability to pull down DNA-PK and be phosphorylated, albeit

at lower levels than wild-type DBD_{PR} (data not shown). It is possible that in full-length PR some or all of the *in vivo* target sites of DNA-PK may lie outside the DBD.

DNA-PK binding to DBD: involvement of DNA?

DNA-PK appears to have the ability to bind a variety of proteins, many of which are transcription factors. One of the concerns with DNA-PK is that its affinity for DNA may copurify it with DNA binding proteins, which may be bound to DNA during purification processes. Nonetheless, most interactions with DNA-PK are postulated to involve protein–protein contacts, perhaps in addition to colocalization on DNA. The DNA binding mechanism of DNA-PK itself is controversial. The regulatory Ku heterodimer has traditionally been considered the DNA binding partner (reviewed in Dynan & Yoo 1998). The Ku70 subunit is capable of binding DNA alone; however, the Ku70/Ku86 dimer is much more efficient at interacting with DNA (Dynan & Yoo 1998).

Most interactions with DNA-PK have been described for the entire holoenzyme. In an attempt to map a particular subunit with the DBD_{PR} interaction, we used *in vitro* translated Ku70 and Ku86. In our hands, Ku70 and Ku86 each associated with DBD_{PR} individually *in vitro*, suggesting that both subunits are capable of binding

FIGURE 8. The hPR-DBD/DNA-PK complex assembles *in vivo*. Nuclear extract was prepared from HeLa cells transiently transfected with f:hPR_B (1 µg) (Richer *et al.* 1998). M2 affinity gel purified f:hPR_B and associated HeLa nuclear proteins (lanes 1, 3, and 5), and aliquots of the crude HeLa nuclear extract (lanes 2 and 4), were resolved by SDS-PAGE and transferred to nitrocellulose. Immunoblots were probed with antibodies specific for Ku70, Ku86, and DNA-PK_{CS} (lanes 1 and 2), PARP (lanes 3 and 4), and f:hPR_B (lane 5). Proteins on immunoblots are indicated by arrows.

DBD_{PR}. In fact, affinity for DBD_{PR} was increased with the Ku heterodimer, additionally suggesting that both subunits play a role in binding to DBD_{PR}, perhaps in a co-operative manner.

Treatment of bacterial and HeLa cell extracts with DNase I had no effect on binding of the DNA-PK complex to DBD_{PR} (Fig. 2B). However, multiple kinase assays showed no exogenous DNA requirement for DNA-PK activity. This suggests either that there is some residual DNA bound to the proteins that is not degraded by DNase I or that its interaction with DBD_{PR} stimulates DNA-PK activity. Recent proposals suggest that DNA and protein-protein interactions of DNA-PK are co-operative and that this augments its kinase activity (Leber *et al.* 1998, Yavuzar *et al.* 1998). In fact, even the binding of DNA-PK_{CS} to Ku has been shown to occur only on DNA and not in solution (Dvir *et al.* 1993, Suwa *et al.* 1994).

Interestingly, although DNA-PK interaction was strong for the unrelated DBDs of GAL4 and hPR, it was unable to associate with the DBD of hER. This was surprising since the GST-DBD_{ER} fusion bound efficiently to DNA *in vitro*, ER is a substrate of DNA-PK and, like the hPR DBD, it has a two zinc finger structure (Schwabe *et al.* 1993). A potential DNA-PK interaction with ER may require other regions of the receptor outside the DBD. The fact that DBD_{ER} had some ability to associate with PARP suggests that it is capable of protein interactions. These data support that not all DNA binding-efficient proteins copurify DNA-PK, and that at least part of the observed interactions must be due to direct contact between the proteins.

DNA-PK – relationship to transcription and progesterone

Immune precipitation of RNA polymerase II copurifies several DNA replication and repair proteins, including DNA-PK (Maldonado *et al.* 1996). Peterson *et al.* (1995a) have shown that, even in the presence of a specific DNA binding fragment, the basal kinase activity of purified DNA-PK_{CS} is low, but can be increased 20-fold by the presence of the DNA binding domain of the yeast transcription factor GAL4 (GAL4¹⁻¹⁴⁷). They did not assess possible interactions between GAL4¹⁻¹⁴⁷ and the DNA-PK holoenzyme. However, their observations of a role for transcription factors in stimulating DNA-PK activity could be extended by our data showing that such interactions can occur, and can stimulate phosphorylation of an exogenous substrate. This model also suggests the possibility that Ku/DNA-PK_{CS} binding both to DNA and to

transcription factors co-operatively enhances its binding affinity at a promoter, and enhances its catalytic activity for colocalized substrates.

Female Ku86-deficient mice, while fertile, cannot lactate (Nussenzweig *et al.* 1996). The lactational deficiency of females is interesting in the context of the association between DNA-PK and PR, since progesterone is a key hormone intermediate in mammary gland development and maturation, and its withdrawal at the end of pregnancy signals the onset of lactation (Clarke & Sutherland 1990, Horwitz 1992).

PARP – relationship to transcription and progesterone

PARP has been shown to affect steroid receptor-mediated transcription. More specifically, a PARP-GR fusion repressed GR-mediated transcription (Rosenthal *et al.* 1994). There have also been two articles implicating a role for PARP during ovulation and oocyte maturation (Aoufouchi & Shall 1997, Murdoch 1998). PARP activation during *Xenopus laevis* oocyte maturation was linked to progesterone induction and, furthermore, progesterone treatment led to a phosphorylation of PARP (Aoufouchi & Shall 1997). PARP activity and progesterone levels also coincide during sheep ovulation, and progesterone was proposed to play a role in PARP-stimulated DNA repair (Murdoch 1998). In a mouse model, young PARP-deficient mice appear to be healthy and fertile (Wang *et al.* 1995). However, as they age, the PARP-deficient females become obese (Wang *et al.* 1995). The restriction of obesity to females is interesting and may implicate a role for progesterone, a hormone that has no known function in males.

DNA repair enzymes and PR

DNA-PK and PARP are both proposed to be involved in DNA repair mechanisms and in silencing transcription (Giffin *et al.* 1994, Kuhn *et al.* 1995, Oei *et al.* 1998). Under conditions of DNA damage, this could be a mechanism to divert the cell from transcriptional and replicational tasks to DNA repair functions. In this regard, both DNA-PK and PARP may target transcription factors directly, in an effort to inhibit their activity. This makes members of the nuclear receptor family such as GR and PR likely targets for DNA-PK and PARP. The fact that females are specifically affected in both Ku86 and PARP knockout mice indicates an involvement of female-predominant hormones such as progesterone with these two enzymes.

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