

Interaction of estrogen receptor α with proliferating cell nuclear antigen

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Received May 7, 2007; Revised June 21, 2007; Accepted June 26, 2007

ABSTRACT

The ability of estrogen receptor α (ER α) to modulate gene expression is influenced by the recruitment of a host of co-regulatory proteins to target genes. To further understand how estrogen-responsive genes are regulated, we have isolated and identified proteins associated with ER α when it is bound to DNA containing the consensus estrogen response element (ERE). One of the proteins identified in this complex, proliferating cell nuclear antigen (PCNA), is required for DNA replication and repair. We show that PCNA interacts with ER α in the absence and in the presence of DNA, enhances the interaction of ER α with ERE-containing DNA, and associates with endogenous estrogen-responsive genes. Interestingly, rather than altering hormone responsiveness of endogenous, estrogen-responsive genes, PCNA increases the basal expression of these genes. Our studies suggest that in addition to serving as a platform for the recruitment of DNA replication and repair proteins, PCNA may serve as a platform for transcription factors involved in regulating gene expression.

INTRODUCTION

Estrogen receptor alpha (ER α) is a ligand-activated transcription factor that alters the expression of a wide variety of estrogen-responsive genes in target cells (1,2). It is essential for development of the reproductive tract and maintenance of reproductive function (3,4).

ER α is comprised of six functional domains (A–F) that have been evolutionarily conserved (5,6). The most highly conserved region is domain C, the DNA-binding domain (DBD), which is comprised of two zinc finger domains. The DBD is necessary and sufficient for specific interaction of the receptor with its DNA recognition sequence, the estrogen response element (ERE). Domain E, the

ligand-binding domain (LBD), is also highly conserved and directs the specific interaction of the receptor with hormone. In addition to these two highly conserved domains are regions with considerable variation in amino acid sequence, including the amino terminal A/B domain, the carboxy terminal F domain, and the centrally located hinge region, domain D. Sequence analysis of ER α from different species in combination with functional studies of mutant receptors have identified two regions of the receptor that are important in enhancing estrogen-responsive gene expression (7,8). The ligand-independent activation function 1, AF-1, is localized in the amino terminal A/B domain of the receptor and the hormone-inducible activation function 2, AF-2, is present in the LBD (9,10).

Upon binding hormone, ER α undergoes a conformational change, binds to EREs residing in estrogen-responsive genes, and recruits co-regulatory proteins to initiate changes in gene expression (11,12). These co-regulatory proteins include chromatin remodelers, modifiers of post-translational acetylation and phosphorylation, and an increasing number of cell-cycle and DNA repair-related factors (13–22). This extensive array of co-regulatory proteins, which possess a wide variety of functional activities, helps to ensure fine-tuned control of estrogen-responsive gene expression.

In order to identify novel co-regulatory proteins involved in ER α -mediated gene expression, we utilized a modified gel mobility shift assay to isolate proteins associated with the DNA-bound receptor and then identified the isolated proteins by mass spectrometry analysis (22,23). One protein of particular interest was proliferating cell nuclear antigen (PCNA), which is required for DNA replication and repair. Interestingly, PCNA interacts directly with the DNA repair protein flap endonuclease-1 [FEN-1 (24–26)], which we recently identified as a modulator of ER α -mediated transcription (22). In addition, PCNA has been used as an independent marker of breast, renal and skin cancer (27–30).

We have characterized the association of PCNA with ER α and find that PCNA interacts with ER α , enhances

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the receptor–DNA interaction *in vitro*, and associates with endogenous, estrogen-responsive genes. Rather than influencing estrogen responsiveness in MCF-7 breast cancer cells, PCNA helps to maintain the basal expression of estrogen-responsive genes.

MATERIALS AND METHODS

Isolation and identification of PCNA

Nuclear extracts (20 µg) from HeLa cervical cancer cells were incubated with annealed, ³²P-labeled oligos containing the *Xenopus laevis* vitellogenin A2 ERE (5'-GAT TAA CTG TCC AAA GTC AGG TCA CAG TGA CCT GAT CAA AGT TAA TGT AA-3' and 5'-TTA CAT TAA CTT TGA TCA GGT CAC TGT GAC CTG ACT TTG GAC AGT TAA TC-3') in the absence or presence of 400 fmol of purified, baculovirus-expressed ER α . Incubations were performed in agarose-binding buffer (15 mM Tris pH 7.9, 56 mM KCl, 0.2 mM EDTA, 4 mM DTT, 5 mM MgOAc, 0.05 mM ZnCl₂) with 10% v/v glycerol, 100 ng of poly dI/dC, 1 µg salmon sperm DNA and 10 nM 17 β -estradiol (E₂) in a final volume of 12.5 µl for 10 min on ice. Proteins associated with the ERE-bound ER α were separated on a 1.75% low melt agarose gel with modified TBE buffer (4.5 mM Tris pH 7.9, 44.3 mM boric acid, 5.2 mM MgOAc and 1 mM EDTA).

For large-scale isolation of protein complexes, reactions were increased 10-fold and proteins were identified using mass spectrometry analysis essentially as previously described (23). Nine discrete peptide fragments with amino acid sequence identical to that found in PCNA (LVQGSILKK, NLAMGVNLTSMK, FSASGELGNG NIK, LMDLDVEQLGIPEQEYSCVVK, YLNFFTK, ATPLSSTVTLMSADVPLVVEYK, DLSHIGDAVVIS CAK, FSASGELGNGNIKLSQTSNVDKEEEAVTIEM NEPVQLTFALR, AEDNADTLALVFEAPNQEK) were identified in two independent experiments. These peptides comprised 57% of the total PCNA amino acid sequence. Control lanes lacking ER α were run on the agarose gels in parallel to ensure that PCNA was associated with the DNA-bound ER α and did not simply co-migrate with the receptor–DNA complex.

Expression and purification of his-tagged PCNA

A bacterial expression vector encoding his-tagged PCNA (pHKEp-PCNA) was graciously provided by Zvi Kelman [University of Maryland Biotechnology Institute, Rockville, MD, USA (31)]. Expression and purification of his-tagged PCNA was performed as previously described (23). Protein purity was assessed on Coomassie stained gels and protein concentration was determined using the BioRad protein assay (BioRad, Hercules, CA, USA) with BSA as a standard.

Pull-down assays using truncated ER α proteins

Pull-down assays using *in vitro* transcribed and translated ³⁵S-labeled full-length ER α or truncated ER α proteins ABC, AB, CD and DEF (32–34) were performed essentially as described (23). Expression vectors for

Δ CD1 (amino acids 180–292), Δ CD2 (amino acids 180–272) and C (amino acids 180–262) were provided by Kendall Nettles (The Scripps Institute, Jupiter, FL, USA) and synthesized as described in Stols *et al.* (35). Full length and truncated ER α proteins were synthesized using the TNT T7 Quick Coupled Transcription/Translation system (Promega, Madison, WI, USA) and incubated with immobilized, his-tagged PCNA. For E domain interaction studies, pET15b-ER α (304–554) [kindly provided by Benita Katzenellenbogen, University of Illinois, Urbana, IL (36)] which encoded the his-tagged E domain of ER α , was transformed into *Escherichia coli*, expressed and immobilized on Ni-NTA beads as previously described (23), followed by incubation with full length, untagged PCNA (a gift from John Bruning and Kendall Nettles, The Scripps Institute, Jupiter, FL, USA). Incubations were done at 4°C for 45 min in binding buffer (15 mM Tris pH 7.9, 20 mM KCl, 0.2 mM EDTA, 4 mM DTT) with or without 10 µM E₂.

Bound proteins were washed once with binding buffer, once with wash buffer (15 mM Tris pH 7.9, 100 mM KCl, 0.2 mM EDTA, 4 mM DTT), and then eluted with 2 \times loading buffer (125 mM Tris pH 6.8, 4% v/v SDS, 20% v/v glycerol, 1.44 M β -mercaptoethanol). Eluted proteins were separated by SDS–PAGE and subjected to autoradiography (for ³⁵S-labeled proteins). For pulldowns using purified PCNA, eluted proteins were separated by SDS–PAGE and subjected to western blot analysis with antibodies specific for PCNA, his-tag and ER α (sc-7907, sc-803 and sc-8002, respectively, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were probed with a horseradish peroxidase-conjugated secondary antibody and developed using a chemiluminescent detection system as previously described (37).

Pull-down assays using full-length proteins

Flag-tagged full-length ER α was expressed in Sf9 cells as previously described (38,39), immobilized on M2-agarose (Sigma, St Louis, MO, USA), and washed with purification buffer (20 mM Tris pH 7.5, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 10% v/v glycerol). Twenty micrograms of MCF-7 nuclear extracts or purified, untagged PCNA (a gracious gift from John Bruning and Kendall Nettles, The Scripps Institute, Jupiter, FL, USA) were incubated with immobilized ER α in the absence or presence of DNA oligos containing an ERE (described above for PCNA isolation) or non-specific DNA sequence (5'-CTA GAT TAC TTC TCA TGT TAG ACA TAC TCA GAT CTA GAC ATA CTC AGA TC-3' and 5'-GAT CTG AGT ATG TCT AGA TCT GAG TAT GTC TAA CAT GAG AAG TAA TCT AG-3') in binding buffer, followed by washing and elution as described. Eluted proteins were separated by SDS–PAGE and subjected to western blot analysis with PCNA (sc-56 or sc-7907) or ER α (sc-8002) specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were probed with a horseradish peroxidase-conjugated secondary antibody and developed using a chemiluminescent detection system as previously described (37).

Polyacrylamide gel mobility shift assays

ER α was expressed and purified to near homogeneity as we have previously described (38). Purified ER α measuring 10–50 fmol were incubated without or with 0.5–2.5 μ g of purified his-PCNA in binding buffer with 10% v/v glycerol, 100 ng of poly dI/dC and 10 nM E₂ in a final volume of 20 μ l for 10 min on ice. BSA and His elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole) were included as needed to maintain constant protein and salt concentrations. For antibody supershift experiments, an ER α - or PCNA-specific antibody (sc-8002 or sc-56, respectively, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the binding reaction and incubated for 10 min on ice prior to addition of DNA. Radiolabeled ERE-containing oligos were added to the binding reactions and incubated for 10 min at room temperature prior to fractionation on low ionic strength polyacrylamide gels (40) at 4°C with buffer re-circulation. Radioactive bands were visualized by autoradiography or were quantitated by phosphorimager analysis with Image Quant software (GE Healthcare, Piscataway, NJ, USA).

Chromatin immunoprecipitation assays

MCF-7 cells were maintained in phenol red-containing MEM supplemented with 5% v/v calf serum, placed on phenol red-free MEM with 5% v/v CDCS for at least 72 h and exposed to ethanol vehicle or 10 nM E₂ for 2 h. Chromatin immunoprecipitation assays were carried out essentially as recommended by Millipore (Charlottesville, VA, USA) except that pelleted cells were washed three times in lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂) with 0.5% v/v NP-40, re-suspended in lysis buffer with 10 mM CaCl₂ and 4% v/v NP-40, and treated with 75 U micrococcal nuclease (USB, Cleveland, OH, USA) for 10 min prior to sonication. The ER α - and PCNA-specific antibodies sc-8002 and sc-56, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were used for immunoprecipitation of protein–DNA complexes. PCR primers (Table 1) flanking the pS2 ERE or a region 2.8-kb upstream of this site, which contains no known ER α -binding sequence, were used for quantitative PCR analysis with iQ SYBR Green Supermix and the iCycler PCR thermocycler according to manufacturer's directions for 40 amplification cycles (BioRad, Hercules, CA, USA). One thousand, 5000, 10 000 and 25 000 genomic copies were run in parallel with each primer set during each experiment to derive a standard curve. The relative copy number for each sample was determined from the standard curve. Each sample was run in triplicate and data from four independent experiments is reported as the relative number of copies of specific DNA sequence. Significant changes in induction were calculated using the student's *t*-test.

For agarose gel analysis, MCF-7 cells were maintained as above and treated with ethanol vehicle or 10 nM E₂ for 15, 45 or 120 min before chromatin was isolated as described above using antibodies specific to ER α , PCNA or non-specific mouse IgG (sc-8002, sc-56 and sc-2025, respectively, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Purified ChIP DNA or 10% of input was subjected

Table 1. PCR primer sequences

ChIP analysis	
pS2 ERE-containing region	5'-CCCGTGAGCCACTGTTGTC-3'
pS2 control region	5'-CCTCCCGCCAGGGTAAATAC-3' 5'-GTATGGTGTGGTCTGGGTTC-3' 5'-GGGTTGGAGCGGCTGGAG-3'
Oxytocin ERE-containing region	5'-AAGGCACCTCACCTTCTGTG-3'
36B4 control region	5'-TCGGTGGAGCTCTGTTAAAGA-3' 5'-GTGTTTCGACAATGGCAGCAT-3' 5'-GACACCCTCCAGGAAGCGA-3'
RT-PCR analysis	
PCNA mRNA	5'-CCTGTAGCGGCGTTGTTG-3' 5'-CGTTGATGAGGTCCTTGAGTG-3'
PR mRNA	5'-GTGCCTATCCTGCCTCTCAATC-3' 5'-CCCGCCGTCGTAACCTTTCG-3'
pS2 mRNA	5'-GCTGTTTCGACGACACCGTT-3' 5'-TTCTGGAGGGACGTCGATG-3'
ER α mRNA	5'-TGCCCTACTACCTGGAGAAC-3' 5'-CCATAGCCATACTTCCCTTGTC-3'
36B4 control mRNA	5'-GTGTTTCGACAATGGCAGCAT-3' 5'-GACACCCTCCAGGAAGCGA-3'

to 30 cycles of PCR with 0.5–1.0 mM MgCl₂ and *iTaq* DNA polymerase (BioRad, Hercules, CA, USA) in a 20 μ l reaction according to manufacturer's directions using primers flanking the oxytocin ERE-containing gene region, or a control region of the non-estrogen-responsive 36B4 gene which lacks an ER α -binding sequence (Table 1). The entire PCR reaction was run on a 1.5% agarose gel and visualized using SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR, USA) under UV light on a Gel Doc with Quantity One software (BioRad, Hercules, CA, USA).

RNA interference assays

For siRNA experiments, MCF-7 cells were maintained as stated above and seeded in 12 well plates 24 h prior to transfection. Cells were transfected with 50 pmol of control (renilla luciferase, 4630, Ambion, Austin, TX, USA) or PCNA-specific siRNA oligos (Silencer validated siRNA ID 42853, Ambion, Austin, TX, USA) in the absence of antibiotics using siLentFect (BioRad, Hercules, CA, USA) for 48 h. Medium was replaced with phenol red-free MEM containing 5% v/v CDCS for an additional 24 h, followed by treatment with 10 nM E₂ or ethanol vehicle for 24 h. Preliminary time course experiments were performed with 0–72 h of siRNA exposure to determine the amount of time required to maintain reduced PCNA protein levels. Protein knockdown was monitored by western blot analysis of whole cell lysates as described above using antibodies to PCNA and Sp1 (sc-56 and sc-59, respectively, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). RNA was harvested using Trizol (Invitrogen, Carlsbad, CA, USA) and processed according to manufacturer's directions. cDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA). Real-time PCR was performed using iQ SYBR

Green Supermix and the iCycler PCR thermocycler (BioRad, Hercules, CA, USA) according to manufacturer's directions with primer sequences specific for the PCNA, progesterone receptor (PR), pS2 and ER α mRNA (Table 1). The 36B4 gene, which is not regulated by E $_2$, was used as a control. Samples were run in duplicate for each primer set during each experiment and standard curves were derived using serial dilutions of cDNA equivalent to 0.02, 0.2, 2 and 20 ng of input RNA. The relative nanogram of RNA was determined from the standard curve. Significant changes in RNA levels due to specific siRNA or hormone exposure were calculated by analysis of variance (ANOVA) with ezANOVA (C. Rorden, www.mmicro.com, Columbia, SC, USA).

RESULTS

Isolation and identification of PCNA as a potential ER α -interacting protein

To isolate novel proteins associated with the DNA-bound ER α , we performed agarose-based gel mobility shift assays with 32 P-labeled ERE-containing oligos, purified ER α and HeLa nuclear extracts. Although ER α slightly altered the migration of the ERE-containing oligos (Figure 1A, compare lanes 1 and 2), HeLa nuclear extracts alone did not alter migration of the radiolabeled probe (compare lanes 1 and 3). However, when both ER α and HeLa nuclear extracts were utilized, a higher order protein-DNA complex was formed (lane 4). We have shown previously that these higher-order complexes are supershifted by an ER α -specific antibody (23). This large protein-DNA complex was excised from the gel, proteins were subjected to trypsin digestion and the peptides were subjected to mass spectrometry analysis. Nine discrete peptides, which had amino acid sequence identical to that found in PCNA, were identified. As seen in Figure 1B, these peptides, which were identified in two independent experiments, represented a major portion (57%) of the PCNA protein. Since we had previously identified two DNA repair proteins, *N*-methylpurine DNA glycosylase (MPG) and FEN-1, which alter the ability of ER α to activate gene expression (21,22), we were intrigued by the identification of another DNA repair protein associated with the DNA-bound receptor.

PCNA and ER α interact

Because PCNA was isolated as a component of a large, multi-protein complex associated with the DNA-bound ER α , it was possible that other proteins were required for the ER α -PCNA interaction. To determine whether purified PCNA and ER α could interact, flag-tagged ER α was immobilized and incubated with purified, untagged PCNA. The purified PCNA was able to interact with the immobilized ER α in the absence and in the presence of E $_2$ (Figure 2A, lanes 3 and 4), but did not interact with the resin alone (lane 2). Thus, PCNA interacts directly with ER α .

To define which regions of the receptor are required for interaction with PCNA, bacterially expressed, his-tagged PCNA was immobilized and incubated with *in vitro*

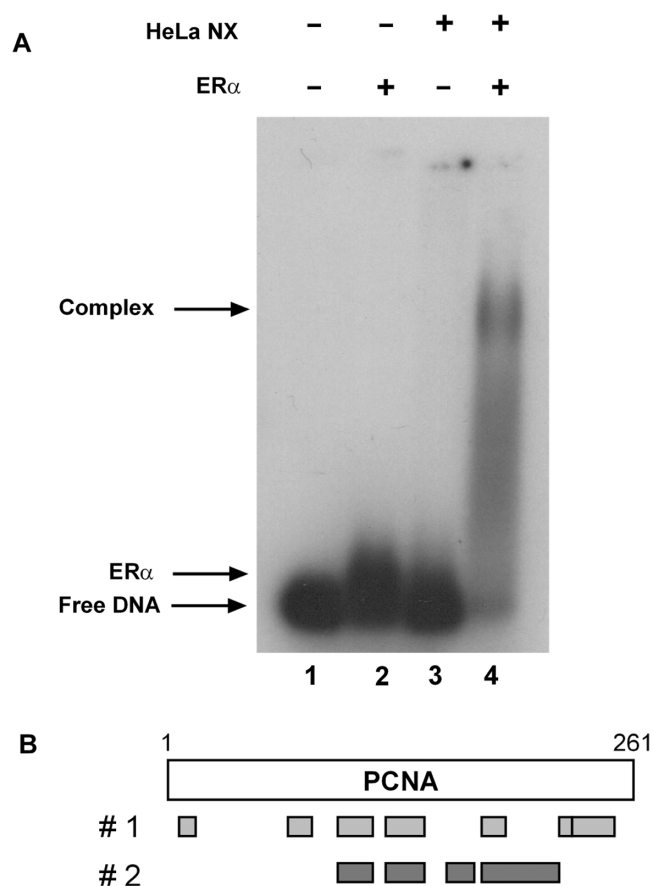


Figure 1. Isolation of PCNA. (A) Radiolabeled ERE-containing oligos were incubated with purified ER α in the absence or presence of HeLa nuclear extracts as indicated. Protein-DNA complexes were resolved on an agarose gel, excised and analyzed by mass spectrometry. (B) The locations of peptides identified by mass spectrometry analysis in two independent experiments are indicated by gray bars.

transcribed and translated full-length or truncated ER α . As shown in Figure 2B, full-length ER α and the amino-terminal ABC interacted with PCNA in the absence and in the presence of E $_2$. Deletion of the C domain abolished the interaction with PCNA (AB). The failure of AB alone to interact with PCNA may reflect the relatively unstructured nature of the AB domains in the absence of the C domain (41-43).

Interestingly, a small portion of ER α containing only the DBD and hinge region interacted strongly with PCNA (CD). To further identify the central region of ER α required for PCNA interaction, sequential truncations of the hinge region were made. When 15 (Δ CD1) or 35 (Δ CD2) amino acids of the D domain were deleted, the interaction of ER α with PCNA was maintained. However, deletion of the entire D domain to amino acid 262 (C) eliminated the interaction of the two proteins. Thus, the inclusion of amino acids 262-272, which were previously defined as the C-terminal extension (CTE) of the DBD (44,45), is required for interaction with PCNA. Interestingly, the CTE not only stabilizes the receptor-DNA interaction, but also serves as a site for ER α acetylation, which in turn influences ER α -mediated transcription (46-48).

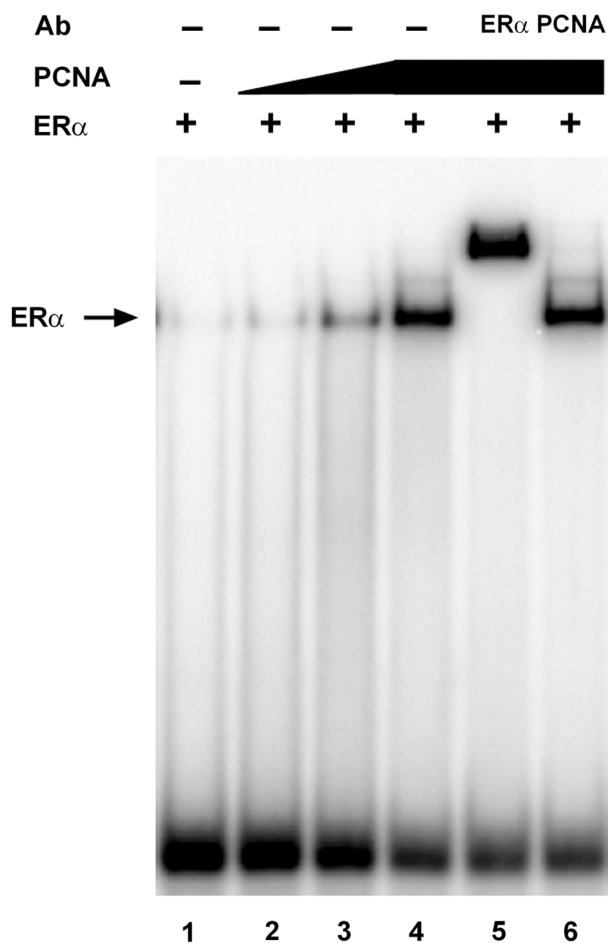


Figure 4. PCNA enhances ER α -ERE complex formation. 32 P-labeled oligos containing the consensus ERE were incubated with 10 fmol of purified ER α in the presence of E₂. Purified his-labeled PCNA (lanes 2–6) and ER α - (lane 5) or PCNA- (lane 6) specific antibody (Ab) were added to the binding reactions as indicated. Bound and unbound 32 P-labeled oligos were fractionated on a non-denaturing polyacrylamide gel and visualized by autoradiography. Complexes containing ER α are indicated (ER α -). Results are representative of three independent experiments.

PCNA enhances the ER α -ERE interaction

Although the addition of DNA did not influence the ER α -PCNA interaction, it was possible that the interaction of PCNA with ER α might alter the ability of the receptor to bind efficiently to DNA. To determine whether PCNA influenced ER α -ERE complex formation, gel mobility shift assays were performed with purified ER α in the absence and in the presence of increasing amounts of purified, his-tagged PCNA. When 10 fmol of purified ER α was combined with radiolabeled ERE-containing oligos, the receptor-DNA complex was barely detectable (Figure 4, lane 1). However, a dose-dependent increase in complex formation was seen when increasing amounts of PCNA were included in the binding reactions (lanes 2–4). Inclusion of an ER α -specific antibody (lane 5), but not a PCNA-specific antibody (lane 6), supershifted the receptor-DNA complex. Thus, although PCNA dramatically increased the ER α -ERE interaction, it was not present in

the receptor-DNA complex. The failure of PCNA to form a stable ternary complex with the DNA-bound ER α could result from the absence of other nuclear proteins required for stabilization of the PCNA-receptor-DNA interaction and/or the extended period of electrophoresis required for gel mobility shift assays, which could prohibit the formation of a stable ternary complex containing these two purified proteins. The ability of purified co-regulatory proteins to enhance the receptor-DNA complex, but not form a stable ternary complex in gel mobility shift assays, has been reported with a number of ER α -associated proteins by our laboratory and others (23,53–57), including the DNA repair proteins MPG and FEN-1 (21,22). Furthermore, these findings are consistent with the ability of PCNA to enhance the interaction of FEN-1 with DNA but not form a ternary complex with the DNA-bound FEN-1 (58).

Association of PCNA with estrogen-responsive genes

Although we were unable to isolate a ternary complex containing purified ER α and PCNA with DNA in our gel mobility shift experiments, it seemed possible that PCNA might be able to interact with an ERE-containing region of an endogenous estrogen-responsive gene in its native chromatin environment. Thus, chromatin immunoprecipitation assays were performed to examine the interaction of endogenously expressed ER α and PCNA with the native pS2 gene in MCF-7 cells using real-time PCR analysis. Consistent with previous studies (22,23,59,60), more ER α was associated with the ERE-containing region of the pS2 gene in the presence than in the absence of E₂ (Figure 5A). In contrast, no change was observed in the association of ER α with a region 2.8-kb upstream of the pS2 ERE (Control), which lacked an ER α -binding site. Likewise, when a PCNA-specific antibody was utilized, there was no change in the association of PCNA with the upstream pS2 region in the absence or presence of E₂. However, a modest, statistically significant increase was observed in the association of PCNA with the ERE-containing region of the pS2 gene in the presence of E₂.

We also examined another ERE-containing gene region using agarose gels to visualize the PCR products. While both ER α and PCNA were present at the ERE-containing region of the oxytocin gene in the absence of hormone, more ER α and PCNA were associated with this gene region when cells had been treated with E₂ for 45 min or 2 h. The protection of the pS2 ERE and the association of ER α with the ERE-containing region of the pS2 gene in the absence of hormone have been reported previously (38,61). In contrast, neither ER α nor PCNA was associated with the 36B4 gene, which contains no ER α -binding site and is unaffected by hormone treatment. When a non-specific IgG control antibody was utilized, the amount of amplicon produced was far less than observed when an ER α - or PCNA-specific antibody was used. Thus, PCNA was present at the ERE-containing region of the estrogen-responsive oxytocin gene in the absence and in the presence of hormone. Combined with our gel shift assays, which demonstrated that PCNA

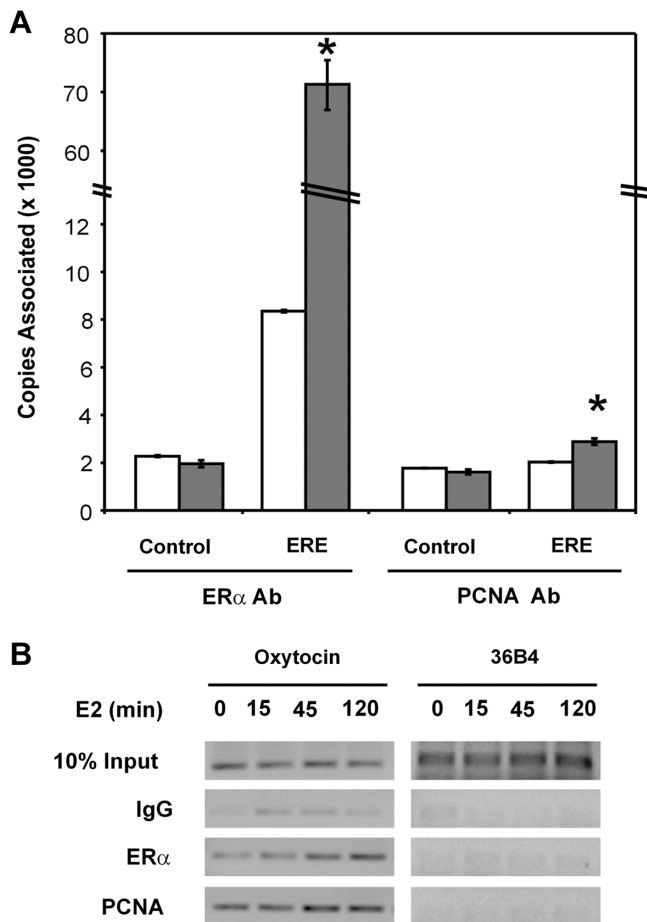


Figure 5. PCNA associates with endogenous estrogen-responsive genes. (A) Sheared chromatin from MCF-7 cells, which had been treated with ethanol vehicle (white bars) or 10 nM E₂ for 2 h (gray bars), was immunoprecipitated with an ER α - or PCNA-specific antibody. DNA was isolated and real-time PCR was performed in triplicate to monitor the association of ER α and PCNA with the region of the pS2 gene containing an imperfect ERE or a region 2.8-kb upstream of the pS2 ERE (Control). Standard curves were derived for each primer set and the relative copy number for each sample was obtained based on the standard curve. Data from four independent experiments are expressed as the mean \pm SEM. A significant change in the copies associated induction in the presence of E₂ was determined by Student's *t*-test and is indicated by an asterisk (*, $P < 0.05$). (B) Sheared chromatin from MCF-7 cells, which had been treated with ethanol vehicle or 10 nM E₂ for 15, 45 or 120 min, was immunoprecipitated with an ER α - or PCNA-specific antibody or non-specific IgG. DNA was isolated and subjected to PCR amplification to monitor the association of ER α and PCNA with the ERE-containing region of the oxytocin gene or the non-estrogen-responsive 36B4 gene. Ten percent of input DNA was included as a control. PCR products were run on 1.5% agarose gels, stained and visualized using UV light. Results are representative of three independent experiments.

enhances the ER α -ERE interaction, these findings suggest that PCNA may help to stabilize the interaction of ER α with endogenous, ERE-containing DNA regions in the absence and in the presence of E₂.

Effect of PCNA on transcription

The ability of PCNA to enhance the ER α -ERE interaction and associate with estrogen-responsive genes

suggested that it might be able to influence ER α -mediated transcription. To examine the potential effect of PCNA on transcription of endogenous, estrogen-responsive genes in their native chromatin environment, siRNA was employed to knock down PCNA expression in MCF-7 cells. In addition, siRNA directed against renilla luciferase was used as a control. When PCNA-specific siRNA was used, PCNA protein levels were decreased and remained low for 72 h after siRNA treatment (Figure 6A). In contrast, PCNA levels were not affected by control siRNA. The level of Sp1 protein was also monitored to help ensure that neither of the siRNAs utilized had an overall effect on protein expression. Sp1 is a transcription factor that plays an important role in regulating expression of a number of estrogen-responsive genes (62) including the progesterone receptor (PR) gene (63–68). The level of PCNA mRNA was also examined using quantitative real-time PCR. Exposure of MCF-7 cells to E₂ increased PCNA mRNA levels when control siRNA was used (Figure 6B). When PCNA-specific siRNA was used, significant decreases in PCNA mRNA were observed in the absence and in the presence of E₂.

To determine whether decreasing endogenous PCNA expression would alter the expression of endogenous estrogen-responsive genes, we examined the expression of the well-studied pS2 gene. Exposure of MCF-7 cells to E₂ increased pS2 mRNA in the presence of control siRNA as has been reported previously (22,23). Interestingly, the PCNA-specific siRNA decreased basal pS2 mRNA levels, but did not affect estrogen-induced pS2 mRNA levels. We also examined expression of the human PR gene, which lacks a palindromic ERE sequence, but instead derives at least part of its estrogen responsiveness from multiple AP-1 and Sp1 sites (63–68). In agreement with previous studies (22,23), PR mRNA levels increased when MCF-7 cells were treated with E₂ and control siRNA. Interestingly, as we had observed with the pS2 gene, basal PR mRNA levels were significantly decreased in the presence of PCNA-specific siRNA, but no change was observed in the level of PR mRNA when MCF-7 cells were treated with hormone.

Since ER α regulates estrogen responsiveness, we also examined the ER α mRNA levels. When control siRNA was used, an E₂-induced decrease in ER α mRNA level was observed as has been reported previously (65,69). However, when PCNA-specific siRNA was utilized, ER α mRNA levels were decreased in the absence, but not in the presence of E₂ as we had observed with the pS2 and PR genes. In contrast, no change was observed in the level of 36B4 mRNA, which is constitutively expressed, in the absence or in the presence of hormone when control siRNA was used. A slight increase in the level of 36B4 mRNA was detected in the presence of E₂ when the PCNA-specific siRNA was used, but the magnitude of this increase was less than the decreases in basal mRNA levels observed for the estrogen-responsive genes.

These experiments demonstrate that although decreased PCNA levels did not alter the estrogen responsiveness of the PR, pS2 and ER α genes, significant decreases in basal mRNA levels were observed. Combined with our ChIP experiments, our findings suggest that PCNA may help to

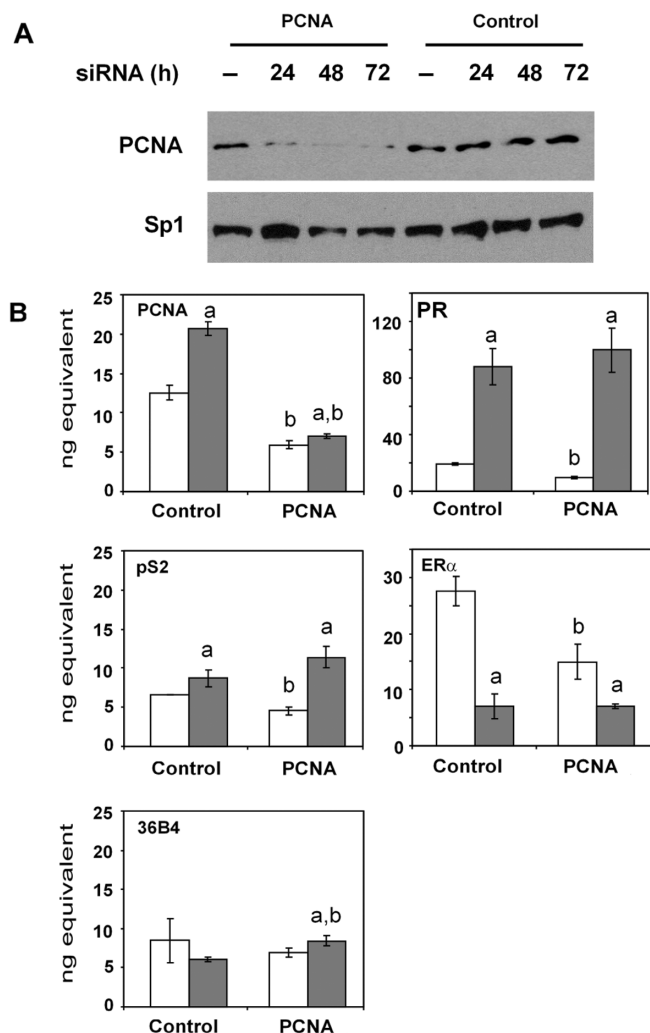


Figure 6. Knocking down endogenous PCNA expression alters gene expression. MCF-7 cells were transfected with 50 pmol of double-stranded control siRNA directed against renilla luciferase (Control) or PCNA-specific siRNA. (A) After 0–72 h, whole cell lysates were subjected to western analysis using a PCNA- or Sp1-specific antibody. Results are representative of two independent experiments. (B) After 72 h, cells were treated with ethanol vehicle (white bars) or 10 nM E₂ (gray bars) for 24 h, RNA was harvested and cDNA was synthesized. Real-time PCR was performed using primers specific to PCNA, PR, pS2, ER α or 36B4 mRNA sequences. Standard curves were derived for each primer set in each experiment. The relative amount of RNA obtained for each sample was calculated from the standard curve. Data are reported as the mean of three replicates \pm SEM. One representative of four independent experiments is shown. Significant differences ($P < 0.05$) in E₂-treated cells compared to the corresponding ethanol vehicle (a) or control siRNA (b) were determined by ANOVA and are indicated. Some error bars are too small to be visible.

maintain basal expression of estrogen-responsive genes by stabilizing the interaction of the receptor with DNA in the absence of hormone.

DISCUSSION

We have identified a novel interaction between ER α and PCNA, a protein required for DNA replication and repair. We have shown that PCNA interacts with ER α ,

enhances the ER α –ERE interaction and helps to maintain basal expression of estrogen-responsive genes.

PCNA is present in cells as a homotrimer comprised of three PCNA monomers that encircle the DNA helix in a head-to-tail orientation (70). While associated with DNA, PCNA serves as a loading dock for proteins involved in DNA replication and repair. It binds to DNA polymerase δ and FEN-1 and increases their catalytic activities thereby enhancing DNA replication and processing of Okazaki fragments (58,71). PCNA participates in numerous DNA repair pathways including nucleotide excision repair, base excision repair, mismatch repair and double-strand break repair (72–77). The extraordinary versatility of PCNA is evident in its ability to interact with more than 20 polymerases, ligases, endonucleases and helicases involved in DNA replication and repair (78).

PCNA functions as a sliding clamp that advances along template DNA while recruiting DNA replication and repair factors and tethering them to DNA (79). Although the association of many replication factors with DNA is transient, the interaction of PCNA with DNA is more sustained (80). It has been suggested that the continued association of PCNA with DNA and the transient association of its binding partners enables PCNA to simultaneously coordinate DNA replication, DNA repair and cell-cycle progression (74,78). This persistent association of PCNA with DNA might explain the presence of PCNA at the endogenous ERE-containing gene regions in the absence of E₂ and the modest increases we observed in the presence of E₂.

In addition to its interaction with ER α , previous studies have documented the interaction of PCNA with other transcription factors including the retinoic acid receptor (RAR) and the nuclear receptor coactivator p300 (81,82). Since the E₂-occupied ER α interacts with p300 (18,47,83,84) and p300 interacts with PCNA, PCNA may help to form an interconnected network of regulatory proteins associated with nuclear receptors to modulate gene expression in the presence of hormone. Our ChIP assays (Figure 5) suggest that PCNA may also be essential in stabilizing the receptor–DNA interaction and maintaining basal expression of estrogen-responsive genes in the absence of hormone, when fewer co-regulatory proteins are present to stabilize the ER α –ERE interaction. Thus, in addition to serving as a platform for the recruitment of proteins involved in DNA replication and repair, PCNA may serve as a platform for nuclear receptors and co-regulatory proteins involved in modulating transcription.

Recently, Ivanov *et al.* (85) used computer modeling to examine PCNA–DNA interaction and showed that PCNA contacts the minor groove of the DNA helix and causes the DNA to tilt. Previous work from our laboratory demonstrated that ER α CTE induces conformational changes in DNA structure resulting in compression of the major groove and expansion of the minor groove (39,86). Since the ER α CTE is required for interaction with PCNA and stabilizes the ER α –ERE interaction (46), it is possible that PCNA may foster the ER α –ERE interaction by altering DNA structure.

Although increased PCNA expression has been reported in proliferating normal cells (73,74,87–89) and at the site of uterine implantation (90,91), increased expression of PCNA has also been linked to decreased survival of breast cancer patients (29,92–94). Interestingly, a post-translational modification of PCNA that lowers its DNA replication fidelity has been found in MCF-7 cells and in malignant breast and ovarian cancers, but not in normal tissues (95,96). It has been suggested that this decreased fidelity of the modified PCNA in cancer cells may play a role in tumor progression and decreased survival, whereas the unmodified PCNA in normal cells may help to protect the integrity of the genome.

While PCNA is required for DNA replication and repair and has been used as a marker of proliferation, our studies expand the functional repertoire of PCNA from the realm of DNA replication and repair and highlight its involvement in altering gene expression.

ACKNOWLEDGEMENTS

We thank W.L. Kraus and J. Kadonaga for providing ER α viral stock, B. Katzenellenbogen, Z. Kelman and K. Nettles for expression plasmids, and J. Bruning and K. Nettles for purified PCNA. This work was supported by NIH grant R01 DK 53884 (to A.M.N.) and NIH P41 RR11823-10 (to J.R.Y.). J.R.S.-N. was supported by the NIH Reproductive Biology Training Grant (T32 HD07028). Funding to pay the Open Access publication charges for this article was provided by R01 DK 53884.

Conflict of interest statement. None declared.

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