The molecular basis of the interaction between the proline-rich SH3-binding motif of PNRC and estrogen receptor alpha

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ABSTRACT

PNRC and PNRC2 are members of a new family of nuclear receptor coactivators. We systematically determined the molecular basis and the structure/function relationship for the PNRC–ERα interaction. PNRC was found to interact with ERα mainly through its C-terminus region, amino acids 270–327, and an SH3-binding motif within this region was shown to be essential for PNRC to interact with and function as coactivator of ERα. The importance of the flanking sequences of SH3-binding motif in the interaction between PNRC and ERα was also investigated. The PNRC-interacting domain(s) on ERα was also mapped. PNRC was found to interact with both AF1 and LBD of ERα, and to function as a coactivator for both AF1 and AF2 transactivation functions. The interaction of ERα mutants, I358R, K362A, V376R, L539R and E542K, with PNRC/PNRC2 was further investigated. ERα/HBD/V376R could bind to PNRC or PNRC2, with similar affinity as wild-type ERα/HBD, and the transactivation activity of ERα/V376R was enhanced 5-fold by PNRC. Since GRIP1, a well-characterized coactivator, was found not to be able to enhance the transactivation function of this mutant, our results indicate that the PNRC–ERα interaction interface is not exactly identical to that of GRIP1–ERα interaction.

INTRODUCTION

Nuclear receptor-mediated signaling is an important regulatory pathway in breast tissue. Estrogen and progesterone are critical hormones that regulate normal mammary gland development during puberty and pregnancy, and are associated with the initiation, development and progression of breast cancer (1,2). These hormones function via binding to their corresponding nuclear receptors, ER and PR. Ligand-bound nuclear receptors are transcriptional factors that regulate the expression of various genes by binding to the specific hormone-responsive elements located in the target gene promoters, thus playing essential roles in development, differentiation, cell proliferation and metabolism. For the past few years, a great deal of progress has been made in understanding the mechanisms by which the nuclear receptors regulate gene transcription. The function of nuclear receptors can be regulated by a number of factors including ligand binding, DNA binding, interaction with other members in the family, interaction with basal transcription factors, and interaction with coactivators and corepressors. Most of these coactivators of nuclear receptors have molecular weights of ∼160 kDa and interact with the liganded nuclear receptors using a short hydrophobic motif called NR-box or LXXLL-motif (3,4).

During the studies of mechanisms that regulate the expression of the human aromatase gene in breast cancer, we have identified and characterized a new family of coactivator proteins, PNRC (proline-rich nuclear receptor coregulatory protein) (5) and PNRC2 (6). PNRC and PNRC2 were identified as SF-1 (steroidogenic factor 1)-interacting proteins in a yeast two-hybrid screening of a human mammary gland cDNA expression library. These coactivators are unique in that they are significantly smaller than most of the coregulatory proteins previously identified and are proline-rich. We have found that PNRC and PNRC2 interact with the ligand-binding domains of all the nuclear receptors tested, including ER, PR, GR, TR, RAR and RXR, in a ligand-dependent manner. They were also found to interact in a ligand-independent manner with the orphan receptors SF1 and estrogen receptor-related receptor alpha (ERRα). Recently, PNRC2 was also isolated from a human brain cDNA phage display library by its interaction with the AF1 domain of ERRγ (7). Furthermore, through an automated yeast two-hybrid screening method to identify nuclear receptor-interacting proteins (8), PNRC was found to interact with RARβ, RARγ, RORα, RORβ, HNF4α, HNF4γ, ERβ, ERRγ and LRH1, and PNRC2 was found to interact with RARγ, RORα, RORβ, HNF4α, HNF4γ, ERα, ERβ, ERRγ and LRH1. These authors

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state that PNRC2 (and PNRC) appears to be a more general cofactor for nuclear receptor than previously appreciated. Unlike most of the coactivators that interact with nuclear receptors through their LXXLL motif, PNRC and PNRC2 interact with nuclear receptors mainly through a proline-rich Src homology domain-3 (SH3)-binding motif, S-D (E)-P-P-S-P-S (5,6). In addition to interacting with nuclear receptor and functioning as coactivator, PNRC also interacts with SH3-domains of Grb2 through its two SH3-binding motifs, located at its N-terminus and C-terminus, and down-regulates the activation of Ras and MAP kinase (9). Therefore, PNRC modulates both nuclear receptor-mediated pathways and growth factor-mediated pathways.

The molecular basis of the interaction between ERα and a number of coactivators has been extensively investigated (10–17). As summarized above, our previous studies indicate that PNRC is a novel coactivator protein. In this study, we carried out a systemic analysis to determine the key regions in PNRC and ERα for their interaction and function. We believe that such information will help us understand better the regulatory function of PNRC on the activity of ERα that plays a very important role in normal mammary gland and breast cancer development.

## MATERIALS AND METHODS

Cell lines and reagents

HeLa cells, human epithelial cells derived from a cervical carcinoma, were purchased from ATCC and were grown in Earle's MEM supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin per ml, 100 μg of streptomycin per ml and nonessential amino acid and sodium pyruvate.

The MATCHMAKER Two-Hybrid System kit was purchased from Clontech (Palo Alto, CA). Yeast transformation kit was purchased from Bio 101 (La Jolla, CA), and the yeast culture media were purchased from Clontech. Various restriction endonuclease were purchased from New England Biolabs (Beverly, MA). Hot start *Taq* polymerase was purchased from Qiagen (Valencia, CA). Lipofectamine 2000 was purchased from Invitrogen Corporation (Carlsbad, CA). Mouse ER antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PNRC antiserum was generated from our laboratory (9). Luciferase assay system was purchased from Promega Corporation (Madison, WI). TNT Quick Coupled Transcription/Translation System was purchased from Promega Corporation (Madison, WI). Bug Buster GST-bind purification kit was purchased from Novagen Inc. (Madison, WI). Oligonucleotide primers were synthesized in the DNA/RNA chemistry laboratory at the City of Hope. DNA sequencing was done in City of Hope Sequencing Core facility.

Construction of plasmids

All recombinant DNA and plasmid constructions were prepared according to standard procedures, and the sequences and orientations of inserted DNA fragments in plasmid constructs were verified by standard DNA sequencing. Yeast expression plasmids in pACT2 vector for the fusion proteins of AD_{Gal4} and PNRC or PNRC2 fragments including

AD_{Gal4}–$PNRC_{1–327}$, AD_{Gal4}–$PNRC_{96–327}$, AD_{Gal4}–$PNRC_{102–327}$, AD_{Gal4}–$PNRC_{120–327}$, AD_{Gal4}–$PNRC_{157–327}$, AD_{Gal4}–$PNRC_{167–327}$, AD_{Gal4}–$PNRC_{184–327}$, AD_{Gal4}–$PNRC_{243–327}$, AD_{Gal4}–$PNRC_{263–327}$, AD_{Gal4}–$PNRC_{277–327}$ and AD_{Gal4}–$PNRC_{270–327}$ (used in Figure 1) or AD_{Gal4}–$PNRC2_{5–139}$, AD_{Gal4}–$PNRC2_{12–139}$, AD_{Gal4}–$PNRC2_{45–139}$, AD_{Gal4}–$PNRC2_{85–139}$ and AD_{Gal4}–$PNRC2_{110–139}$ (used in Figure 3) were isolated from yeast two-hybrid screening of human mammary gland cDNA library using bovine SF1 as bait (4). To test the importance of NR box sequence, LKTLL, located in amino acid residues 319–323, the plasmid pACT2–$PNRC_{270–327}/del_{319–323}$ (deletion of NR box) was prepared by inserting the PCR-amplified $PNRC_{270–327}/del_{319–323}$-coding region into pACT2 vector through the EcoR1 and Xho1 sites. The sequences of primers for amplifying the $PNRC_{270–327}/del_{319–323}$-coding region are 5′-GCC GAATTC GA CTA ACT GAA GTG AGC CAA AAG-3′ and 5′-CGG CTCGAG CTA AGT TTG AAC TTT GAA GAG-3′. To examine the importance of sequences of SH3-binding motif, SDPPSPS, located in amino acids region 285–291 of PNRC, a modified QuikChange site-directed mutagenesis (Stratagene) method was used to introduce internal deletion (18). Two primers, 5′-AA AAG GAA AAT TAT GCT GGG GCA AAG TTT GTT CTT CCA AAG CCT CCT A-3′ and 5′-CCA GTG ACT AGG AGG CTT TGG AAG AAC AAA CTT TGC CCC AGC ATA ATT-3′, were used in PCR to amplify the coding region for $PNRC_{270–327}/del_{285–319}$ (deletion of SH3-binding motif) and the PCR product was inserted into pACT2 vector through EcoR1 and Xho1 sites. To examine the importance of the flanking sequences of SH3-binding motif, amino acid residues 270–284, in the interaction between PNRC and ERα, the amino acid region 270–284 was either deleted by generating the plasmid pACT2–$PNRC_{285–327}$ or replaced with $PNRC2_{85–98}$ in the plasmid pACT2 $PNRC2_{85–98}/PNRC_{285–327}$. To construct pACT2–$PNRC_{285–327}$, the coding region for $PNRC_{285–327}$ was generated by PCR and inserted into pACT2 vector through EcoR1 and Xho1 sites. To prepare the pACT2–$PNRC2_{85–98}/PNRC_{285–327}$, the coding region for $PNRC2_{85–98}$ was first amplified by PCR with primers 5′-GAG GCC CCG GGG ATC CGA ATT CGA TTT AAA TCT CAA GCT AAT CAG-3′ and 5′-AAC ACT AGG AGA AGG TGG ATC ACT AAA TTT GGC ACC AGC ATA GTT-3′ and inserted into the N-terminal of $PNRC_{285–327}$ in pACT2–$PNRC_{285–327}$ vector using an adaptation of the QuikChange mutagenesis approach (19). To examine the importance of the region between SH3-binding motif and NR box, amino acid residues 292–318, in the PNRC–ERα interaction, the region 292–318 was either deleted as in pACT2–$PNRC_{270–327}/del_{282–318}$ or replaced with $PNRC2_{106–131}$ as in the pACT2–$PNRC_{270–291}/PNRC2_{106–131}/PNRC_{319–327}$. The pACT2–$PNRC_{270–327}/del_{292–318}$ was prepared by QuikChange mutagenesis method with two complementary mutagenic oligonucleotide primers: 5′-CA AAG TTT AGT GAT CCA CCT TCT CCT AGT TTA AAA ACC CTC CTC AAA G-3′ and 5′-AGT TTG AAC TTT GAG GAG GGT TTT TAA ACT AGG AGA AGG TGG ATC ACT-3′. These two mutagenic oligonucleotides were also used in another PCR mutagenesis reaction using pCI–PNRC as template to generate pCI–$PNRC/del_{292–318}$ construct. This construct was used in transient transfection to examine whether the $PNRC_{292–318}$ region is required for its
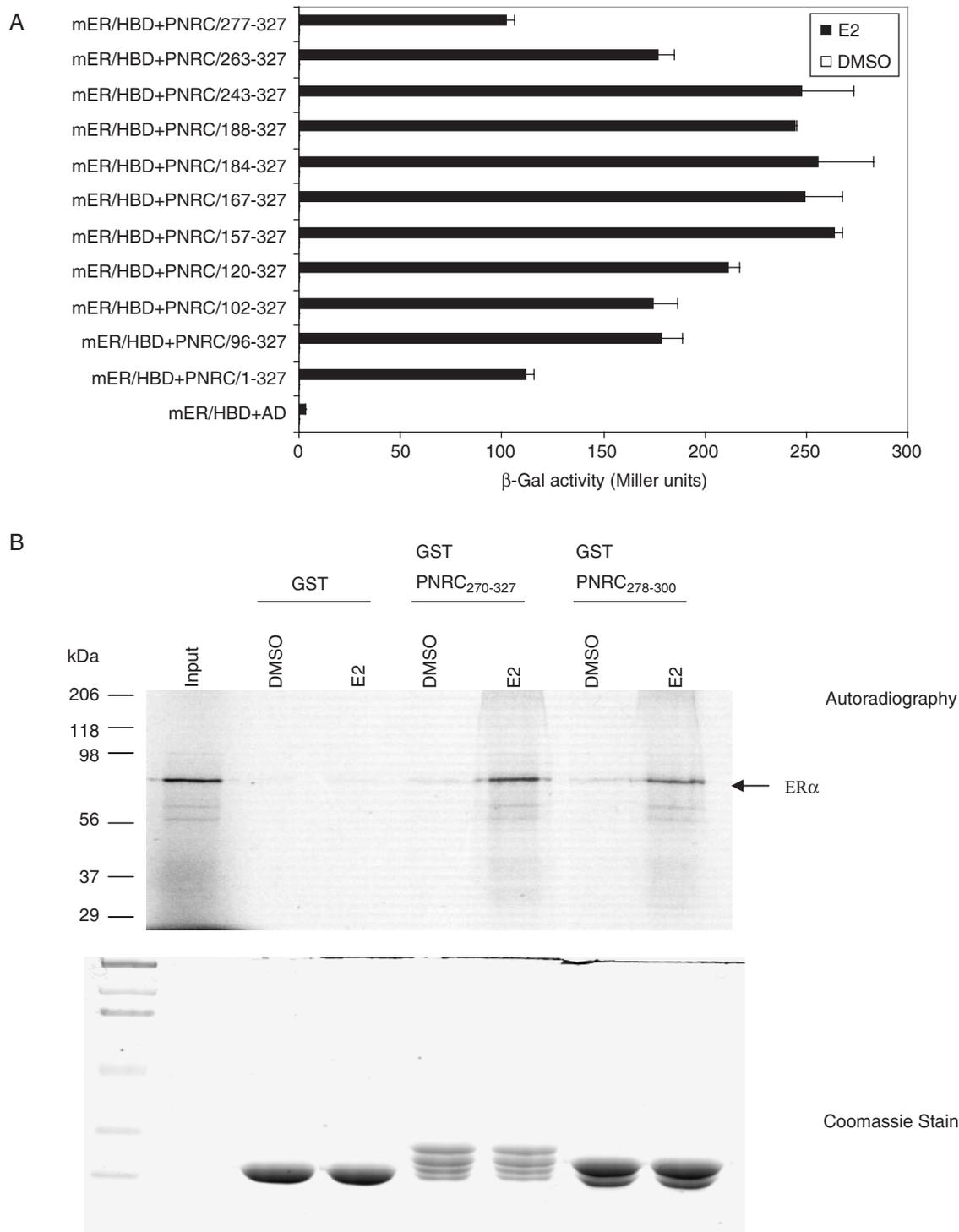
Figure 1. PNRC interacts with ERα through its C-terminus. (**A**) Interaction between mERα/HBD and PNRC or PNRC fragments in yeast two-hybrid assays. The expression plasmids (in pACT2 vector) for AD_{Gal4} and PNRC fragment fusion proteins were isolated from a human mammary gland expression library screening using DBD_{Gal4}–SF1 (in pGBT9 vector) as bait (5). Yeast strain Y187 was cotransformed with pGBT9–mERα/HBD (for the expression of DBD_{Gal4}–mERα/HBD fusion protein) and each of the expression plasmids for AD_{Gal4}–PNRC fragments as indicated, and transformants containing these plasmids were selected by growth on SD/−Leu/−Trp agar plates. The expression of interacting hybrid proteins in Y187 transformants was analyzed for *Lac*Z expression as described in Materials and Methods. AD_{Gal4} (AD) was included as background control. β-Galactosidase activities in liquid cultures in the presence of 17-β-estradiol (10 nM) are expressed in Miller units as mean ± SD of three independent assays. (**B**) Direct binding between ERα and PNRC in GST pull-down assay. Binding of ^{35}S-labeled ERα with GST (lanes 2 and 3), GST–PNRC$_{270–327}$ (lanes 4 and 5) and GST–PNRC$_{278–300}$ (lanes 6 and 7) was tested using the pull-down assay in the presence of 10 nM E$_2$ or DMSO (the vehicle control). The input (lane 1) represents 10% of the labeled ERα used in each reaction. The bound ^{35}S-ERα was detected by autoradiography (upper panel). One-third of GST proteins eluted from pull-down reaction are shown by Coomassie blue stain at the lower panel.

coactivation function. To examine the importance of SH3-binding motif and NR box sequence in the coactivation function of PNRC, pCI–PNRC/del SH3 motif and pCI–PNRC/del NR box were generated by QuickChange Mutagenesis approach on pCI–PNRC template using the mutagenic primers mentioned above. To examine the cellular localization of PNRC/del SH3-binding motif, the coding region for PNRC/del SH3-binding motif was amplified by PCR on

SH3-binding motif containing region) and AD_{Gal4}–$PNRC_{NR \ box}$, were previously described (5).

The mammalian expression plasmids for mutated $ER\alpha$ in pSG5 vectors, including pSG5–$ER\alpha$/I358R, pSG5–$ER\alpha$/V376R, pSG5–$ER\alpha$/L539R, pSG5–$ER\alpha$/K362A and pSG5–$ER\alpha$/E542K, were generated by QuikChange Site-directed mutagenesis method on pSG5–$ER\alpha$ template plasmid using following primers:

Mutation	Forward primer	Reverse primer
I358R	5′-GAGCTGGTTCACATG**CGC**AACTGGGCGAAG-3′	5′-CTTCGCCCAGTT**GCG**CATGTGAACCAGCTC-3′
V376R	5′-ACCCTCCATGATCAGC**CGC**CACCTTCTAGAAT-3′	5′-ATTCTAGAAGGTG**GCG**CTGATCATGGAGGGT-3′
L539R	5′-GTGCCCCTCTATGAC**CGG**CTGCTGGAGATG-3′	5′-CATCTCCAGCAGC**CGG**TCATAGAGGGGCAC-3′
K362A	5′-ATGATCAACTGGG**CGG**CGAGGGTCCAGGC-3′	5′-GCCTGGCACCCTCGCC**GC**CCAGTTGATCAT-3′
E542K	5′-TATGACCTGCTGCTGA**AAG**ATGCTGGACGCC-3′	5′-GGCGTCCAGCATC**TT**CAGCAGCAGGTCATA-3′

pCI–PNRC/del SH3-binding motif plasmid template, and the PCR product was subcloned in frame into pEGFP-C1 vector (Clontech) through HindIII and Xba1 sites.

To generate the plasmid pACT2–$PNRC_{270–291}$/$PNRC2_{106–131}$/$PNRC_{319–327}$, the coding region for $PNRC2_{106–130}$ was first amplified by PCR using pBluescript-PNRC2 as template and primer set: 5′-AGT GAT CCA CCT TCT CCT AGT GTT CTT CCC AAA CCA CCA AGC-3′ and 5′-G AAC TTT GAG GAG GGT TTT TAA TTG AAA TGT CAT TAT TTC CTT ATC-3′. The 5′ end of one of the two oligonucleotides used for the PCR was designed by having at least 20 nt corresponding exactly to the DNA sequence upstream from the point of insertion in the destination vector, followed by the first 20 nt of the DNA fragment to be amplified. Accordingly, the 5′ end of the other oligonucleotide consisted of at least 20 nt exactly downstream from the point of insertion, followed by the last 20 nt of the target DNA fragment. The latter oligonucleotide was derived from the complementary DNA strand. After PCR, the PCR product was separated on agarose gel and purified. The insertion of this PCR product, $PNRC2_{106–130}$, into pACT2–$PNRC_{270–327}$ to replace $PNRC_{292–318}$ was achieved by QuikChange Mutagenesis PCR using pACT2–$PNRC_{270–327}$ as template and the PCR product, in this case, $PNRC2_{106–130}$.

All the PCR mutagenesis reactions were performed in a reaction volume of 50 μl that consisted of 100–500 ng of template plasmid DNA, 200 nM each primer or 200–300 ng of PCR fragment (for insertion and replacement), 200 μM dNTPs, 2.5 U *Pfu* enzyme in 1× *Pfu* DNA polymerase reaction buffer. A preliminary step of denaturation at 95°C for 3 min was followed by 25 cycles of PCR. These PCR cycles consisted of 30 s of denaturation at 95°C, 1 min of annealing at a temperature 2°C higher than melting temperature (T_m) of the primer and 2 min/kb template plasmid DNA of extension at 68°C. At the end of PCR, the PCR product was treated with 10 U Dpn1 for 3 h at 37°C and 2 μl of Dpn1-digested DNA was transformed into DH5α-competent cells using electroporation. The construction of the plasmids for the expression of AD_{Gal4}–$PNRC_{270–317}$ (with deletion of last 10 amino acid that contains NR box sequence), AD_{Gal4}–$PNRC_{278–300}$ (contains SH3-binding motif), AD_{Gal4}–$PNRC_{278–300}$/2P>2A (mutated SH3-binding motif), AD_{Gal4}–$PNRC_{301–327}$ (deletion of

The yeast expression plasmids for the fusion proteins of DBD_{Gal4} and $ER\alpha$/$HBD_{297–554}$ or mutated $ER\alpha$/HBD including $ER\alpha$/HBD/I358R, $ER\alpha$/HBD/V376R, $ER\alpha$/HBD/L539R, $ER\alpha$/HBD/K362A and $ER\alpha$/HBD/E542K were prepared as follows. The coding regions for wild-type $ER\alpha$/HBD and mutated $ER\alpha$/HBD were PCR-amplified using forward primer, 5′-GCG GAATTC ATG ATC AAA CGC TCT AAG AAG-3′ and reverse primer, 5′-GAA GGATCC TCA GCT AGT GGG CGC ATG TAG-3′ and pSG5–$ER\alpha$/WT and corresponding $ER\alpha$ mutation containing plasmids as templates, respectively. The PCR products were then digested with EcoR1 and BamH1, and subcloned into pGBT9 vector through EcoR1 and BamH1 sites.

The deletion fragments of $ER\alpha$, including $ER\alpha$/1–185 (AF1), $ER\alpha$/185–251 (DBD), $ER\alpha$/252–355 (Hinge), $ER\alpha$/356–549 (HBD), $ER\alpha$/550–595 (F domain) and $ER\alpha$/252–288 (CTE domain) were PCR-amplified from pSG5–$ER\alpha$ with the primers incorporating EcoR1 at the 5′ ends and BamH1 at the 3′ ends, and inserted into pGBT9 vector. The $ER\alpha$ fragments, $ER\alpha$/1–549, $ER\alpha$/185–595, and $ER\alpha$/1–251, were PCR-amplified with the primers incorporating EcoR1 and BamH1 sites, and inserted into the mammalian expression vector pSG5.

Yeast two-hybrid assay

The yeast MATCHMAKER two-hybrid system (Clontech) was used to examine protein–protein interaction *in vivo*. Briefly, the yeast strain Y187 was co-transformed with the AD vector alone or AD–PNRC wild-type or its fragments along with pGBT9 (DBD_{Gal4} vector only), pGBT9–$ER\alpha$/HBD or mutated $ER\alpha$/HBD plasmids or DBD–hLC negative control plasmid. The transformants that carry both plasmids were selected by plating them onto SD/−Leu/−Trp plates and were further analyzed for β-galactosidase activity by liquid β-galactosidase activity measurement as essentially described in the protocol. Briefly, the transformants that grew on SD/−Leu/−Trp agar plates were cultured overnight in liquid SD/−Leu/−Trp medium at 30°C. This overnight culture was diluted in liquid YPD and then continued to incubate for 4–6 h in the presence of 17-β-estradiol (E2) (10 nM) or Dimethyl sulfoxide (DMSO) (vehicle control). At the end of this incubation, a liquid β-Galactosidase activity assay,

using 2-Nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate, was performed according to the manufacturer's protocol (Clontech).

Cell culture, transfection and luciferase assays

HeLa cells, a human cervical carcinoma cell line, were cultured in MEM Earle's Salts medium and supplemented with 5% charcoal dextran-treated fetal bovine serum (CD-FBS). Twenty-four hours before transfection, cells were seeded in 6-well plates at 5×10^4/per well. Each well of cells were transfected with 2.0 µg of lipofectamine 2000 (Invitrogen) and total 1.25 µg of plasmid DNA in 1 ml of Opti-MEM containing various amounts of the test plasmids as indicated in each experiment and appropriate amounts of empty vector, pSG5 or pCI, to maintain the same overall amount of total DNA in all transfections. After 4 h of incubation with medium containing lipofectamine 2000/DNA, 2 ml of MEM/5% CD-FBS medium containing 10 nM E_2 or DMSO (vehicle control) was added to each well.

Luciferase activity was assayed 24 h after transfection. Cell extracts were prepared by lysing the cells with 400 µl of 1× reporter lysis buffer (Promega). The lysate was centrifuged at 12 000 g for 20 s to pellet the cell debris. The supernatants were transferred to a fresh tube and their protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's protocol. The luciferase activities in the cell lysates with same amount of total protein from each well were measured using Luciferase assay substrate (Promega). Each transfection was performed in triplicate. When necessary, 20 µg of crude cell lysates from each transfected reaction were subjected to western blot analysis to examine the expression levels of various ER mutants and PNRC deletion forms (described below).

Western blot analysis

The procedures have been previously described (5). Briefly, the lysis buffer (ice cold, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and the complete set of protease inhibitor cocktail) was added to the phosphate-buffered saline (PBS)-washed cells. After 20 min of incubation on ice, the cells were then scraped and transferred into Eppendorf tubes, sonicated and centrifuged at 12 000 g at 4°C for 10 min. Twenty-five micrograms of cell lysate in the supernatant were analyzed by 10% SDS–PAGE. The proteins were transferred onto PVDF membranes under 18 V for 60 min using a Semi Dry Transfer Cell (Bio-Rad). Membranes were blocked with 5% non-fat milk in 1× Tris-buffered saline Tween-20 (TBST) buffer at room temperature for 1 h and then incubated overnight with mouse monoclonal antibody against ERα (Santa Cruz, sc-8002, 1:500 dilution) or 1:500 diluted PNRC antiserum. The membrane was then incubated with 1:5000 diluted goat anti-rabbit (for PNRC) or anti-mouse (for ERα) horseradish peroxidase conjugate (Pierce Chemical Co.) at room temperature for 1 h and followed by SuperSignal West Pico Chemiluminescent (Pierce) substrate visualization.

GST pull-down assay

The glutathione *S*-transferase (GST) pull-down assays were performed as previously described (5). Briefly, the full-length ERα coding region in pSG5 vector was translated *in vitro* in the presence of [^{35}S]methionine, using the TNT Coupled Transcription/Translation System (Promega Corp., Madison, WI). The construction of expression plasmids for GST–PNRC$_{270–327}$ and GST–PNRC$_{278–300}$ was previously described (5). GST and GST-deleted PNRC fusion proteins were prepared using BugBuster GST Bind Purification kit (Novagen, Madison, WI) following the supplier's instructions. Briefly, 10 µg of purified GST, GST–PNRC$_{270–327}$ or GST–PNRC$_{278–300}$ was incubated with 20 µl of settled GST bind resin for 30 min at room temperature. The washed beads were incubated for 1 h at 4°C with 4 µl of *in vitro* translated, [^{35}S]methionine-labeled ERα in a total volume of 150 µl binding buffer (50 mM KPi, pH 7.4, 100 mM NaCl, 1 mM MgCl$_2$, 10% glycerol, 0.1% Tween-20, 1.5% BSA) in the presence of 10 nM E_2 or DMSO (the vehicle control). Beads were collected and washed four times with binding buffer without BSA. Washed beads were resuspended in 50 µl of 1× SDS sample buffer, boiled in water for 5 min and pelleted briefly in a microfuge. Supernatant (25 µl), along with 1/10 of input [^{35}S]-labeled ERα, was then subjected to 8% SDS–PAGE and autoradiography. To control the equal loading of GST and GST fusion proteins, 15 µl of eluate was separated on another 8% SDS–PAGE gel, and the gel was subjected to Coomassie blue staining.

Fluorescence imaging

HeLa cells were cultured in MEM Earle's Salts medium supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin for 24 h. The day before transfection, cells were divided and cultured in 6-well plate. Cells were then transfected with 2 µg of Lipofectin (Invitrogen) and 2 µg of pEGFP–PNRC$_{wt}$, pEGFP–PNRC/del SH3-binding motif, or pEGFP-C1 vector (as control). After 4 h transfection, the medium containing Lipofectin and DNA was discarded, and the cells were cultured in regular culture medium for HeLa cells. After 20 h incubation, the fluorescent signal was detected by an inverted fluorescence microscope, Nikon Eclipse, TE 2000-S. Standard filter sets were used for all imaging. All images were processed using Image-Pro Plus 5.1 software and standard image processing techniques.

RESULTS

PNRC interacts with ERα through its C-terminus

PNRC has been demonstrated previously to interact with ERα in a ligand-dependent and AF-2-dependent manner by yeast two-hybrid assay, GST pull-down, and Co-immunoprecipitation analyses (5). To systematically map the ERα-interacting domains of PNRC, the interaction between ERα/HBD and the full-length PNRC or its fragments was analyzed and compared by yeast two-hybrid assays. A series of PNRC fragments in pACT2 (AD$_{Gal4}$) vector was isolated from our previous yeast two-hybrid screening of a human mammary gland cDNA expression library using

bovine SF1 as bait. Thirty-five out of a total 90 His[+] and β-Gal[+] clones isolated from the screening code for the wild-type PNRC protein or PNRC fragments with different length of deletion at its N-terminus, with the shortest clone coding for $PNRC_{277-327}$. The yeast strain, Y187, was cotransformed with pGBT9–ERα/HBD plasmid along with either pACT2 vector alone (as background control) or each pACT2–PNRC/fragment plasmid (test plasmid). The transformants that grew on SD/−Leu/−Trp plates were picked and cultured in liquid YPD containing 10 nM E_2 or DMSO (vehicle control) to the log phase, and the β-galactosidase activity of the cultured cells were analyzed by liquid β-galactosidase activity measurement as described in the Materials and Methods. As shown in Figure 1A, the shortest fragment, $PNRC_{277-327}$, interacted with ERα/HBD with the same affinity as that of full-length PNRC, suggesting that PNRC interacts with ERα through its C-terminus. Furthermore, the interaction between ERα and PNRC or its fragment was detected only in the presence of estradiol (Figure 1A), demonstrating its ligand dependence.

The C-terminus of PNRC binds to ERα directly as demonstrated in GST pull-down analysis

To further confirm the interaction between the C-terminus of PNRC and ERα detected in yeast two-hybrid assays, a GST pull-down binding assay was performed to study the direct interaction between ^{35}S-labeled full-length ERα and the PNRC C-terminal fragments *in vitro*. PNRC fragments, including $PNRC_{270-327}$ and $PNRC_{278-300}$, were expressed as fusion protein with GST in *Escherichia coli* BL21, purified with glutathione Sepharose 4B beads, and tested for their ability to bind *in vitro* translated [^{35}S]methionine-labeled ERα in pull-down assays. As shown in Figure 1B, both GST–$PNRC_{270-327}$ and GST–$PNRC_{278-300}$ were found to bind ERα in the presence of 10 nM estradiol (E_2) (Figure 1B, lanes 5 and 7). These results demonstrate a direct binding of PNRC to ERα and the interaction is ligand dependent, because GST alone or in the absence of E_2 (vehicle DMSO) retains only very small amounts of ^{35}S-labeled ERα (Figure 1B, lanes 2–4 and 6).

PNRC/NR box plays role, but not essential, in the interaction between PNRC and ERα

A short conserved peptide motif LXXLL (referred to as the NR box) has been identified and reported to be necessary and sufficient to mediate the binding of several coactivators to liganded nuclear receptors (3). There is one NR box-like sequence, LKTLL (amino acids 319–323), at the very end of the C-terminus in PNRC. Our previous deletion and mutation experiments suggested that this NR box was not essential for PNRC to interact with SF1 (5). To confirm our previous finding, a series of PNRC fragments were prepared to examine their interaction with ERα/HBD in yeast two-hybrid assays. Compared with $PNRC_{270-327}$, $PNRC_{270-317}$ fragment has a deletion of the last 10 amino acid segment that contains the NR box. As shown in Figure 2, the interaction intensities, as expressed by β-Gal activity, between $PNRC_{270-327}$ or $PNRC_{270-317}$ and ERα/HBD are about the same, indicating that the NR box sequence in PNRC is not essential for the
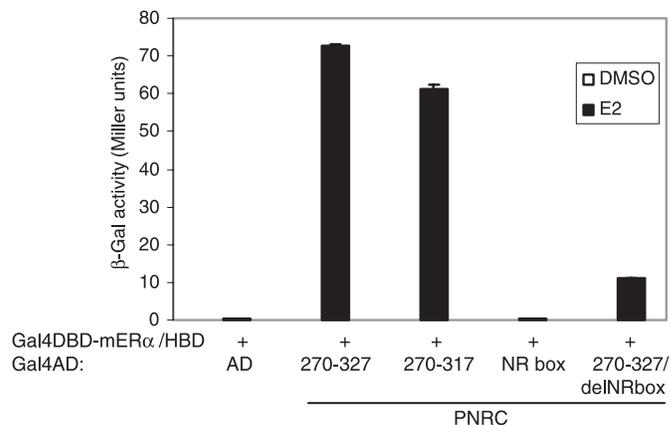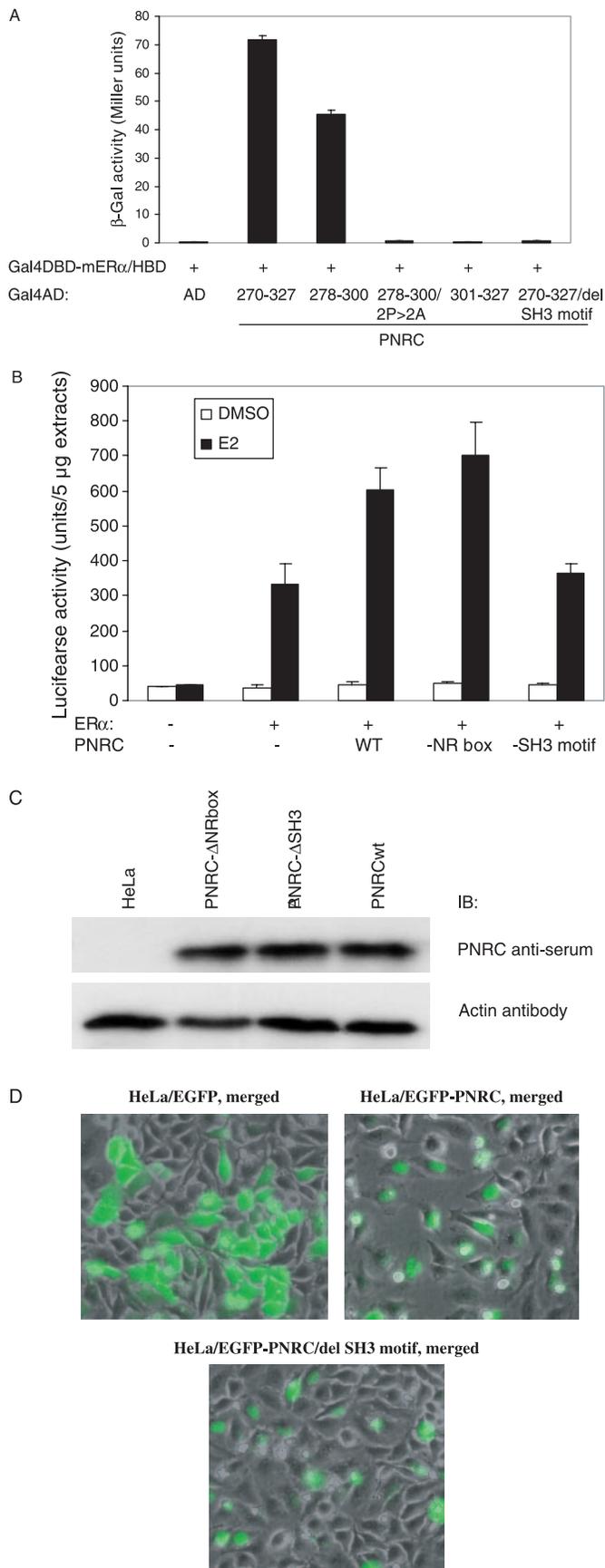

Figure 2. NR box sequence plays a role, but not essential, in the interaction between PNRC and ERα. Yeast strain, Y187, was cotransformed with pGBT9–mERα/HBD and the expression plasmid for AD_{Gal4} (AD, in pACT2 vector) or an yeast expression plasmid for the fusion proteins of AD_{Gal4} and various deletion mutants of PNRC as indicated. The yeast transformants bearing both plasmids were cultured in the absence (DMSO) or presence of 10 nM 17-β-estradiol (E2). β-Galactosidase activities were determined and expressed as the mean (units) ± SD of three independent colonies.

interaction. We also expressed this NR box as a fusion protein with AD_{Gal4} domain and tested its interaction with ERα/HBD in yeast. No interaction was detected between NR box and ERα/HBD, suggesting again that this NR box sequence is not responsible for the interaction. However, when the NR box sequence, amino acids 319–323, was deleted from $PNRC_{270-327}$ template ($PNRC_{270-327}/del_{319-323}$), it resulted in the reduction of the interaction strength between $PNRC_{270-327}$ and ERα/HBD (Figure 2). Taken together, these results indicated that NR box sequence may participate, but is not essential and not sufficient to mediate the binding of PNRC to ERα/HBD. This conclusion was further supported by the fact that deletion of NR box sequence from PNRC did not affect the coactivation activity of PNRC on the transcription mediated by ERα (Figure 3B).

SH3-binding motif in the amino acid region 278–300 is essential for PNRC to interact with and to function as a coactivator of ERα

Sequence comparison revealed that there is a 23-amino acid region with a great homology between PNRC and PNRC2, and this 23-amino acid sequence is within the C-terminal fragment, $PNRC_{270-327}$. Our previous results from deletion and mutation analysis demonstrated that the SH3-binding motif in the amino acid region 278–300 is essential for PNRC to interact with SF1 (5). To test whether this is also true for the interaction between PNRC and ERα, the interaction between ERα/HBD and $PNRC_{278-300}$ was examined in yeast two-hybrid assays. As shown in Figure 3A, this 23-amino acid peptide was found to retain most of the interaction of the $PNRC_{270-327}$ to ERα/HBD. Deletion of amino acid region 270–300 that contains this 23-amino acid segment from $PNRC_{270-327}$ (construct pACT2–$PNRC_{301-327}$) totally eliminated the ability of PNRC to interact with ERα/HBD (Figure 3A). Together, these data demonstrated that the amino acid region from 278 to 300 in PNRC is critical and sufficient for interaction with ERα.

A

Gal4DBD-mERα/HBD	+	+	+	+	+	+
Gal4AD:	AD	270-327	278-300	278-300/ 2P>2A	301-327	270-327/del SH3 motif

PNRC

B

ERα:	-	+	+	+	+
PNRC	-	-	WT	-NR box	-SH3 motif

C

IB:

PNRC anti-serum

Actin antibody

D

HeLa/EGFP, merged

HeLa/EGFP-PNRC, merged

HeLa/EGFP-PNRC/del SH3 motif, merged

There is a putative core binding motif for SH3, SDPPSPS (amino acid residues 286–291), in the amino acid region 278–300 of PNRC. Double mutations of P287A and P290A in the putative SH3-binding motif, i.e. SDPPSPS to SDAP-SAS, in amino acid region 278–300 of PNRC was found to completely eliminate the interaction between PNRC$_{278-300}$ and ERα/HBD (Figure 3A). The importance of SH3-binding motif in the interaction between PNRC and ERα was also confirmed by deletion of SH3-binding motif in the content of PNRC$_{270-327}$. As shown in Figure 3A, deletion of SH3-binding motif, amino acid residues 285–291, from PNRC$_{270-327}$ completely abolished its interaction with ERα/HBD. These results clearly demonstrated that the SH3-binding motif is essential for PNRC to interact with ERα. Consequently, deletion of SH3-binding motif, amino acid residues 286–291, completely eliminated the coactivation activity of PNRC on the transcription mediated by ERα, while the deletion of NR box motif from PNRC did not affect the coactivation function (Figure 3B), demonstrating a crucial role of SH3-binding motif in ERα interaction and coactivation function of PNRC. To rule out the possibility that the difference in coactivation activities among various PNRCdeleted forms might be due to the different levels of protein expression, western blot analysis was performed to examine the protein levels of PNRC, PNRC/del SH3 motif and PNRC/del NR box in the transfected HeLa cells. As shown in Figure 3C, PNRC and its deleted forms were found to be expressed at similar levels in the transfected HeLa cells, demonstrating that the changes in the coactivation function among PNRC and its deletion forms are indeed the consequences of corresponding mutation. To further validate that the loss of coactivator activity of PNRC/del SH3 motif is due to the loss of interaction with ERα, instead of an alteration of cellular localization, the expression plasmids for EGFP–PNRC

Figure 3. SH3-binding motif within amino acid region 278–300 is essential for PNRC to interact with ERα and function as coactivator of ERα. (**A**) Yeast strain, Y187, was cotransformed with pGBT9–mERα/HBD and the expression plasmid for AD$_{Gal4}$ (AD, in pACT2 vector) or an yeast expression plasmid for the fusion proteins of AD$_{Gal4}$ and various deletion mutants of PNRC as indicated. The yeast transformants bearing both plasmids were cultured in the absence (data not shown) or presence of 17-β-estradiol (10 nM). β-Galactosidase activities were determined and expressed as the mean (units) ± SD of three independent colonies. (**B**) HeLa cells cultured in 6-well plates were transfected with pGL3(ERE)_SV40_Luciferase reporter (0.25 μg) alone or along with pSG5–ERα (50 ng) or pSG5–ERα (50 ng) plus pCI–PNRC (WT), pCI–PNRC/del NR box (−NR box), or pCI–PNRC/del SH3 motif (−SH3 motif) (1 μg each). Four hours after transfection, cells were cultured in the presence of 10 nM 17-β-estradiol (E$_2$) or DMSO (the vehicle control) for additional 20 h. Twenty-four hours after transfection, cells were harvested, lysed and the luciferase activities in the cell lysate from triplicate wells were measured as described in the Materials and Methods, and expressed as mean ± SD of three independent assays. (**C**) Western blot analysis. Twenty-five microgram extract prepared from non-transfected HeLa cells (HeLa) or from pCI–PNRC/del NR box (PNRC − NR box), pCI–PNRC/del SH3 (PNRC − SH3), or pCI–PNRC WT (PNRCwt) transfected HeLa cells was subjected to 10% SDS–PAGE and western blot analysis using PNRC antiserum and β-actin antibody, respectively, as described in the Materials and Methods. (**D**) Cellular localization of wild-type PNRC and PNRC/del SH3 motif. HeLa cells in 6-well dish were transfected with pEGFP, pEGFP-PNRCwt or pEGFP–PNRC/del SH3 motif. Twenty-four hours after transfection, the images of the expressed fluorescent proteins in transfected HeLa cells were taken using a fluorescence microscope. The images shown are the merged images of bright field images (whole cells) and green fluorescence image (expressed fluorescent proteins).

and EGFP–PNRC/del SH3 motif were constructed and transfected into HeLa cells. The cellular localizations of EGFP (control), EGFP–PNRC, and EGFP–PNRC/del SH3 motif in the transfected HeLa cells were examined using an inverted fluorescence microscope. As shown in Figure 3D, both EGFP–PNRC/WT and EGFP–PNRC/del SH3 motif were localized in the nuclei of HeLa cells while EGFP protein was present in the cytoplasm.

Importance of the flanking sequences of SH3-binding motif in the interaction between PNRC and ERα

The second member of PNRC coactivator family, PNRC2, was also identified in the same yeast two-hybrid screening of a human mammary gland cDNA library through its interaction with SF1. The amino acid region 85–130 of PNRC2 contains a proline-rich 23-amino acid region (amino acids 99–105) and an NR box-like sequence (amino acids 132–136), with 96% amino acid sequence identity between PNRC and PNRC2. The locations of the SH3-binding motif and NR-box in PNRC and PNRC2 molecules are shown in Figure 4A. The results from our previous deletion and mutagenesis study demonstrated that SH3-binding motif in the amino acids 85–139 was very important for PNRC2 to interact with nuclear receptors including SF1, ERα and ERRα1. Intriguingly, however, the NR box-containing sequence in PNRC2 is differentially required for its interaction with different nuclear receptors (6). Our previous results suggest that the sequences between the SH3-binding motif and NR box in PNRC and PNRC2 may have an important impact as to how these proteins interact with nuclear receptors. Therefore, the importance of the sequences flanking SH3-binding motif and NR box for the interaction of PNRC and ERα was examined by deletion mutagenesis or by replacement of the interested PNRC region with corresponding region of PNRC2.

To examine the importance of the amino acid region 270–284 in the interaction, it was either deleted from $PNRC_{270–327}$ fragment (in construct pACT2–$PNRC_{285–327}$) or replaced with the corresponding amino acid region 85–98 of PNRC2 (in construct pACT2–$PNRC2_{85–98}$–$PNRC_{285–327}$). As shown in Figure 4B, three fragments, $PNRC_{270–327}$, $PNRC_{285–327}$ and $PNRC2_{85–98}$–$PNRC_{285–327}$ interacted with ERα/HBD to similar degrees. These results demonstrated that deletion or replacement of amino acids 270–284 in the $PNRC_{270–327}$ content did not affect its interaction with ERα/HBD, suggesting that the region 270–284 is not essential for the interaction.

The importance of sequences between SH3-binding motif and NR box of PNRC, amino acids 292–318, was also examined by a combination of deletion and replacement mutagenesis and yeast two-hybrid assays. As shown in Figure 4C, deletion of amino acids 292–318 from $PNRC_{270–327}$ fragment totally abolished the interaction of PNRC and ERα. Replacement of this region with corresponding PNRC2 sequence, amino acids 106–131, restored ERα-interacting ability of PNRC. Sequence comparison of amino acid residues between $PNRC_{292–318}$ and $PNRC2_{106–131}$ showed a high homology. These results indicate that the amino acid sequences from 292 to 318, are critical for the interaction between PNRC and ERα/HBD. To further investigate whether this region
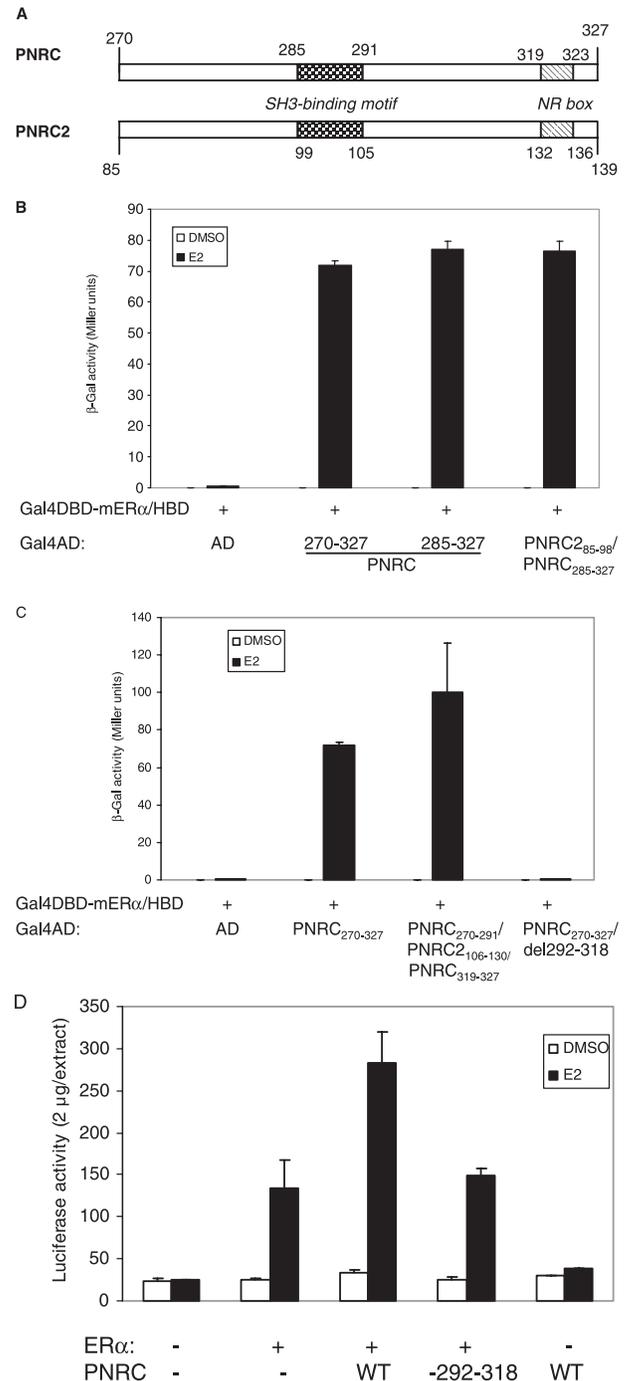
Figure 4. Importance of the flanking sequences of SH3-binding motif in the interaction between PNRC and ERα. (**A**) Diagram of the residue location of SH3-binding motif and NR box sequence on PNRC and PNRC2 molecules. (**B**) $PNRC_{270–284}$ region is not required for the PNRC–ERα interaction. The yeast expression plasmids for AD_{Gal4} and $PNRC_{270–327}$, $PNRC_{285–327}$ or $PNRC2_{85–98}$–$PNRC_{285–327}$ fusion protein were constructed as described in the Materials and Methods. The procedures for yeast transformation and β-Galactosidase activity measurement are the same as described in Figure 1A. (**C**) The sequences between NR box and SH3-binding motif is critical for the PNRC–ERα interaction. The construction of the plasmids used in this figure was described in the Materials and Methods. The procedures for yeast two-hybrid assays are the same as described in (B). (**D**) the sequences between NR box and SH3-binding motif is required for the PNRC coactivator function. The procedures for transient transfection and luciferase assay were the same as described in Figure 3B. ERα, pSG5-ERα, 50 ng; PNRCwt, pCI–PNRC, 1.0 μg; $PNRC_{292–318}$, pCI–PNRC/del$_{292–318}$, 1.0 μg.

is required for the coactivator activity of PNRC on ERα's transactivation function, a mammalian expression plasmid for PNRC/del$_{292-318}$, pCI–PNRC/del$_{292-318}$, was prepared and transfected into HeLa cells. The ability of this mutant to potentiate the transcription mediated by ERα was examined and compared with that of wild-type PNRC. As shown in Figure 4D, deletion of amino acid region 292–318 from PNRC abolished its coactivator activity, demonstrating the requirement of this region in PNRC coactivation function.

Both AF1 and LBD domain of human ERα interacts with PNRC

As most of coactivators, PNRC and PNRC2 interact with ERα/HBD domain in a ligand- and AF2-domain dependent manner (5). To determine whether the interaction between PNRC and ERα is also involved in the other domains of ERα, i.e. the AF1 domain (amino acids 1–185), DNA-binding domain (DBD, amino acids 186–251), hinge region (amino acids 252–355), CTE (C-terminal Extension) domain (amino acids 252–288), hormone-binding domains (HBDs, amino acids 356–549 or 297–554 or 274–595) and F domain (amino acids 550–595), as shown in the diagram of Figure 5A, of human ERα were expressed as DBD$_{Gal4}$ fusion proteins and examined for their interaction with PNRC$_{270-327}$ fragment in yeast two-hybrid assays. As shown in Figure 5B, in addition to ERα/HBD that interacts with PNRC$_{270-327}$ in a ligand-dependent manner, AF1 region also interacts with PNRC$_{270-327}$. However, this interaction is much weaker and is in a ligand-independent manner. We noticed that in the presence of estradiol, PNRC also interacted weakly with ERα$_{252-355}$. No interaction was detected between PNRC and other domains of ERα, including hinge region, CTE and F domains. Additional experiments will be performed to explore the interaction between ERα/AF1 and PNRC in detail.

PNRC is a coactivator of both AF1 and AF2 domains of ERα

Nuclear receptors contain two transcription activation functions, AF1 in the NTD and AF2 in the HBD. PNRC was previously demonstrated to interact with ERα and function as ERα coactivator in a ligand and AF2-dependent manner. In this study, PNRC was found to interact with both AF1 domain and HBD of ERα. Therefore, the capacity of PNRC in stimulating the two activation functions of ERα was analyzed in transient transfection experiments. Full-length PNRC expression plasmid was cotransfected in HeLa cells with expression vectors for the full-length ERα or for the deletion constructs for ERα along with a (ERE)$_3$_luciferase reporter, pGL3-(ERE)$_3$_Luciferase. The deletion constructs AF1/DBD (amino acids 1–251) and DBD/HBD (amino acids 185–595) each retains the ERα DNA-binding domain but is missing the HBD and the N-terminal domain that contains AF1 function, respectively. The transfected cells were exposed to either 100 nM estradiol or vehicle control, DMSO. As shown in Figure 5C, the luciferase activity in the cells transfected with wild-type ERα was enhanced 2.5-fold by the coexpression of PNRC in the presence of estradiol, not in the presence of DMSO. In addition, both AF1/DBD and DBD/HBD fragment of ERα slightly activated the luciferase gene transcription, and coexpression of PNRC enhanced the reporter activity mediated by ERα/AF1–DBD in a ligand-independent manner and the reporter activity mediated by ERα/DBD–HBD in a ligand-dependent manner. Together, this study demonstrated that PNRC interacts with both AF1 and HBD of ERα and functions as coactivator for both AF1 and AF2 transactivation functions.

Important residues in ERα/LBD for the interaction between PNRC and ERα

Site-directed mutagenesis in combination with transient transfection and yeast two-hybrid assays was employed to examine the importance of residues in the HBD of human ERα in the interaction for PNRC. Our mutagenesis design was based on a crystal structure of the ERα HBD complexed with diethylstilbestrol and an NR box-containing 13-amino acid peptide derived from GRIP1 coactivator (10). The interaction surface in the ER consists of 16-amino acid residues from helices 3, 4, 5 and 12: L354, V355, I358, A361, K362 (helix 3); L372 (helix 3, 4 turn); F367, V368 (helix 4); Q375, V376, L379 E380 (helix 5); and D538, L539, E542, M543 (helix 12). ER mutants including I358R, K362A, V376R, L539R and E542K have been reported to completely lose the ability to interact with coactivator GRIP1 (10,11). Since PNRC interacts with ERα mainly through SH3-binding motif, not NR box sequence, one would expect a different intersurface between ERα and PNRC from that between ERα and P160 coactivator such as GRIP1. Therefore, the same set of ER mutations, including I358R, K362A, V376R, L539R and E542K were prepared and examined for their transactivation function by transient transfection. As shown in Figure 6A, the transactivation activities of all the mutated ERα were much lower than that of the wild-type ERα, except that V376R still retained 50% activity of wild-type ERα. Cotransfection with PNRC expression plasmid enhanced the transactivation activities of the wild-type ERα and ERα/V376R by 2- and 5-fold, respectively, but not the activities of other ERα mutants, including I358R, K362A, L539R and E542K (Figure 6A). The changes in the transactivation activities of those mutated ERα are indeed the consequences of mutation, not due to the differences in the expression levels, as demonstrated by western blot using ERα antibody (Figure 6B). To further determine whether these specific ERα mutants also cause loss of interaction with PNRC, as all of these mutants were found to lose the interaction with GRIP1 (10), the wild-type ERα/HBDs and ERα/HBD mutants carrying each of above five specific mutations were expressed as fusion protein with DBD$_{Gal4}$, and their abilities to interact with PNRC were examined in yeast two-hybrid assays. The wild-type ERα/HBD and ERα/HBD/V376R were found to interact with PNRC (Figure 6B) and PNRC2 (data not shown). No interaction was detected between PNRC and ERα/HBD/I358R, ERα/HBD/K362A, ERα/HBD/L539R or ERα/HBD/E542K (Figure 6B). These results are in agreement with the results obtained from our transactivation function analysis. The residue V376 was found to be in the ERα–GRIP1/NR box interaction surface and V376R was designed to perturb the
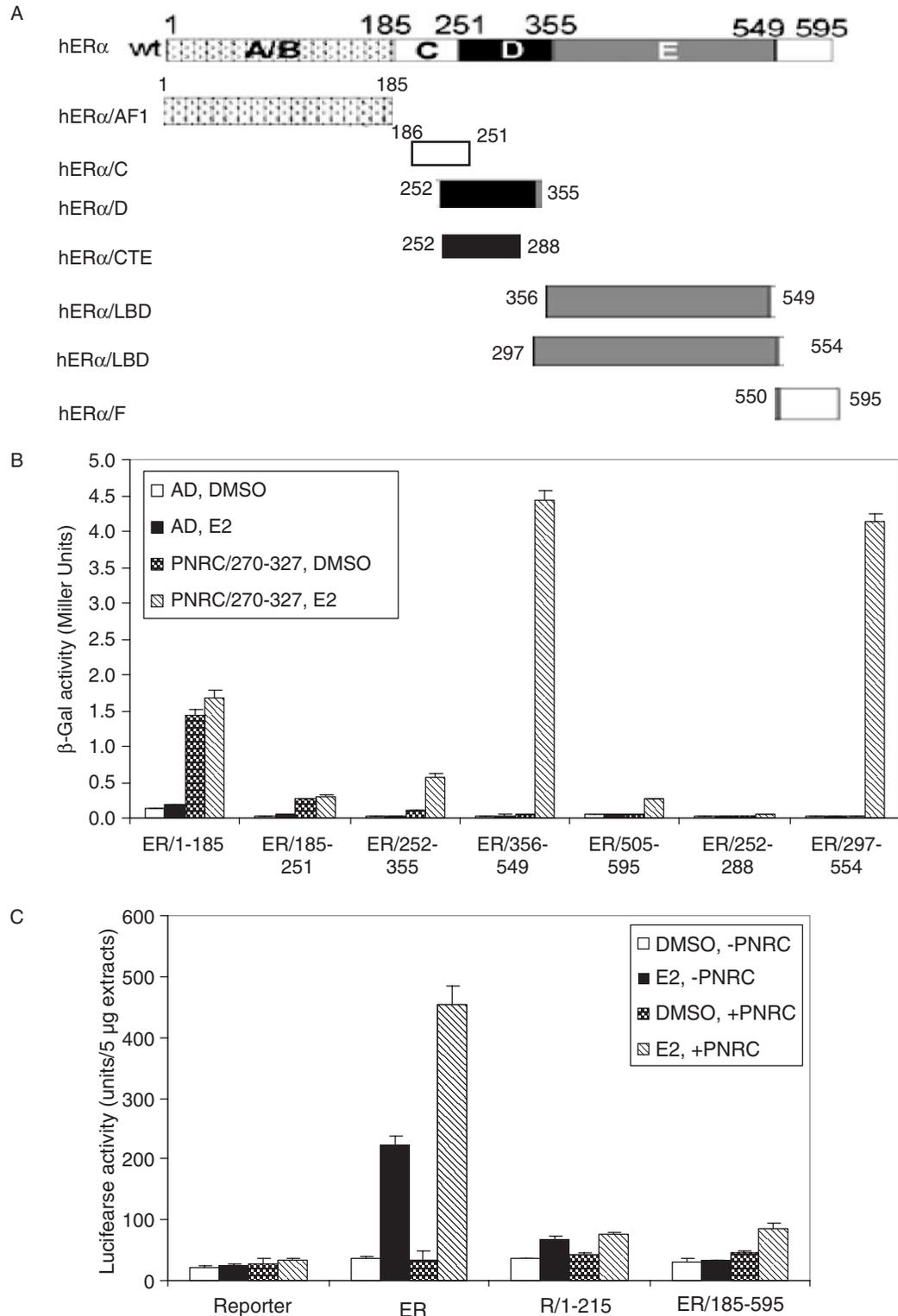
Figure 5. PNRC interacts with AF1 and LBD domains of human ERα and functions as coactivator for both transactivation domains. (**A**) A schematic diagram of human ERα domains and the representation of the various ERα deletion constructs prepared and used. (**B**) Yeast strain Y187 was cotransformed with pACT2 (for the expression of AD_{Gal4}) or pACT2–$PNRC_{270–327}$ (for the expression of AD_{Gal4}–$PNRC_{270–327}$ fusion protein) and an yeast expression plasmid for the fusion proteins of DBD_{Gal4} and various deletion mutants of ERα as indicated. The yeast transformants bearing both plasmids were cultured in the absence (DMSO) or presence of 10 nM E_2. β-Galactosidase activities were determined and expressed as the mean (units) ± SD of three independent colonies. (**C**) HeLa cells were transfected with pGL3 (ERE)$_3$_SV40_Luciferase reporter (0.25 µg) alone or along with pSG5–ERα wt, pSG5–$hERα_{1–251}$, or pSG5–$hERα_{185–595}$ (50 ng each) and with (+PNRC) or without pSG5–PNRC (1.0 µg) (−PNRC). Four hours after transfection, cells were cultured in the absence (DMSO, vehicle control) presence of 10 nM E_2 for additional 20 h. Twenty-four hours after transfection, cells were harvested, lysed and the luciferase activities in the cell lysate from triplicate wells were measured as described in the Materials and Methods, and expressed as mean ± SD of three independent assays.
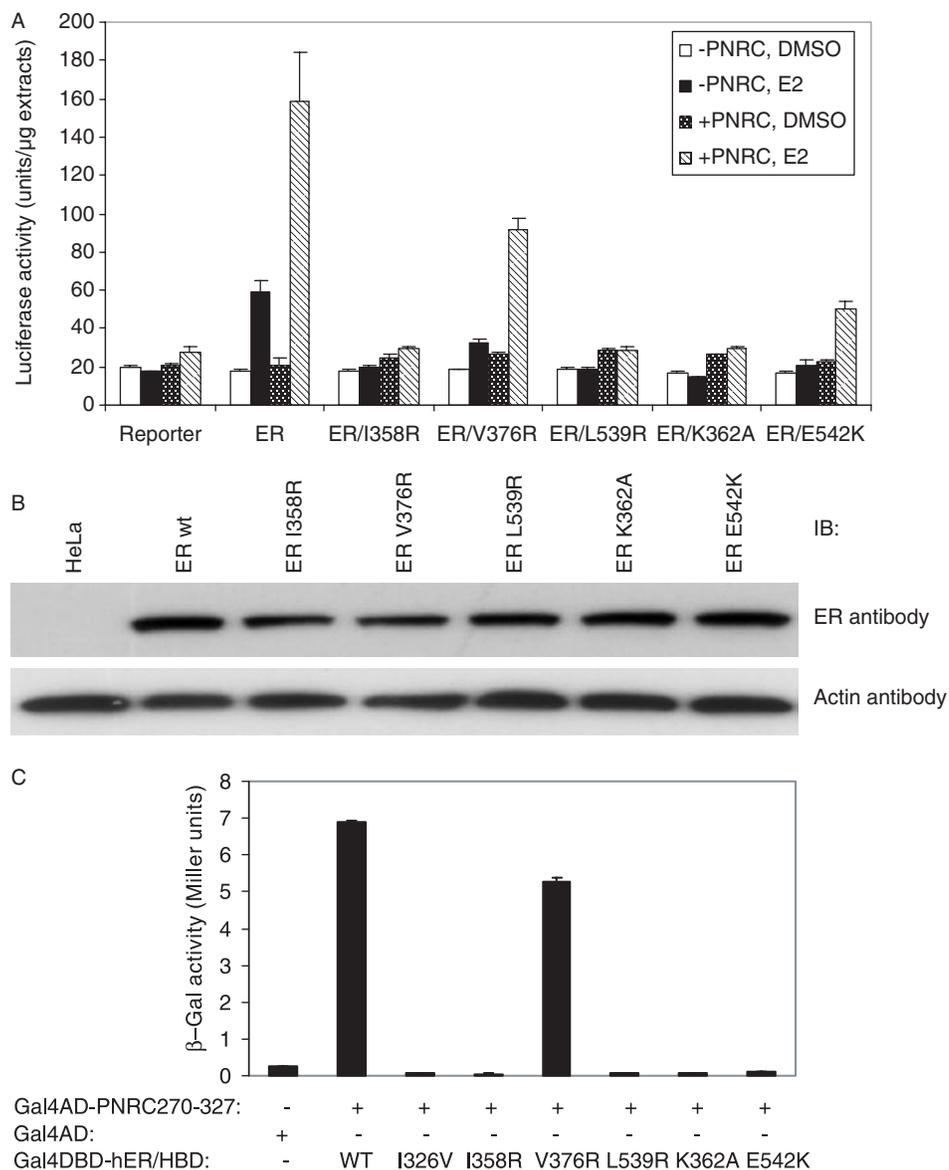
Figure 6. Important residues in ERα/HBD for its interaction with PNRC. (**A**) HeLa cells were transfected with pGL3(ERE)₃_SV40_Luciferase reporter (0.25 µg) alone or along with an expression plasmid for wild-type ERα or its mutants (in pSG5 vector, 50 ng each) and with (+PNRC) or without pSG5–PNRC (1.0 µg) (−PNRC). Four hours after transfection, cells were cultured in the absence (DMSO) or presence of 10 nM E₂ for additional 20 h. Twenty-four hours after transfection, cells were harvested, lysed and the luciferase activities in the cell lysate from triplicate wells were measured as described in the Materials and Methods, and expressed as mean ± SD of three independent assays. (**B**) Western blot analysis. Twenty-five microgram extract, prepared from non-transfected HeLa cells (HeLa) and from the wild-type ERα or ERα mutant expression plasmids transfected HeLa cells, was subjected to 10% SDS–PAGE and western blot analysis using mouse monoclonal antibody against ERα and β-actin antibody, respectively, as described in the Materials and Methods. (**C**) Y187 cells were cotransformed with pACT2–PNRC₂₇₀₋₃₂₇ (AD_Gal4–PNRC₂₇₀₋₃₂₇) or pACT2 vector (AD_Gal4) and a yeast expression plasmid for the DBD_Gal4 and ERα/HBD carrying various mutations as indicated. The yeast transformants bearing both plasmids were cultured in the absence (data not shown) or presence of E₂ (10 nM). β-Galactosidase activities were determined and expressed as the mean (units) ± SD of three independent assays

nonpolar character of the floor of the binding groove. V376R mutant was found to lose its interaction with GRIP (10) and therefore the transactivation function (11). However, in our study, ERα/HBD/V376R still interacts with PNRC or PNRC2, with similar affinity as the wild-type ERα/HBD and the transactivation activity of ERα/V376R mutant was enhanced 5-fold by PNRC while GRIP1 has no effect on the transactivation function of this mutant, implying that the PNRC–ERα interaction interface is not exactly identical to that of GRIP1–ERα interaction.

DISCUSSION

Results from this and other laboratories (5,7,8) have demonstrated that PNRC acts as a coactivator for a number of nuclear receptors, and experiments from this laboratory (5) have found that PNRC is a novel coactivator in that its proline-rich SH3-binding motif is important for the interaction with nuclear receptors. In this study, we have carefully characterized the structural determinants of the interaction between PNRC and ERα, as well as the functional consequences of their interaction. Specifically, we showed that

PNRC interacts with ERα through its C-terminus, amino acid region 270–327. There is one NR box-like sequence, LKTLL (amino acids 319–323), at the very end of the C-terminus in PNRC. It is well known that most of the coactivators interact with AF2 in LBD (ligand-binding domain) of many nuclear receptors via conserved, helical LXXLL motifs, named NR box (3). This sequence forms part of an amphipathic α-helix, which binds in a conserved hydrophobic cleft on the surface of liganded LBDs. However, the NR box was found to be nonessential for ERα-interaction although it may be required for higher affinity binding between PNRC and ERα. The amino acid region 270–327 of PNRC contains a 23-amino acid region with 100% identity between PNRC and PNRC2. The SH3-binding motif in the amino acid region 278–300 was found to be essential for PNRC to interact with ERα. The importance of NR box motif and SH3-binding motif on the coactivation function of PNRC on ERα's transactivation activity was further compared. In agreement with the observation for the ERα interaction, the NR box motif is not required for PNRC to function as a coactivator for ERα while the SH3-binding motif is critical for PNRC to enhance the transcription mediated by ERα. Thus, PNRC is a unique coactivator that interacts with nuclear receptors through its proline-rich SH3-binding motif which is both structurally and functionally important. The importance of the sequences flanking SH3-binding motif and NR box was also investigated by deletion and/or insertion mutagenesis. The 5′ flanking sequences of SH3-binding motif, amino acids 270–284, was found to be not required for the PNRC–ERα interaction, while the residues between NR box and SH3-binding motif, from 292 to 318, are critical for the interaction between PNRC and ERα/HBD as well as its coactivator activity.

PNRC-interacting domain(s) on ERα was also mapped in this study. We found that PNRC interacts with both AF1 and LBD of ERα, and functions as a coactivator for both AF1 and AF2 transactivation functions (Figure 5C). Furthermore, the interaction between PNRC and ERα/HBD and the coactivation function of PNRC on the transcription mediated by ERα/HBD were found to be ligand dependent, while its interaction with ERα/AF1 and its coactivation function towards ERα/AF1-mediated transcription was found to be ligand independent (Figure 5B and C). This property of PNRC is similar to that of some p160 coactivators. In addition to the interaction, in a ligand-dependent manner, with the AF2 domain of NRs through the LXXLL motif, the C-terminal region of the p160 coactivators is also able to interact with the AF1 domain of some NRs, and then enhance the AF1 activities of those NRs in the absence of ligands. For example, SRC1 has been shown to interact with both the N-terminal A/B or AF1-containing domain and the C-terminal D/E or AF2-containing domain of PR, and hence to enhance the transcriptional activity of AF1 and AF2 of PR in a cooperative manner (20). SRC1 was also found to interact with AF1 of AR via a conserved, glutamine-rich region (21) and to interact with AB domain of ERα and potential ERα's AF1 transcription function (22). TIF2/GRIP1 was shown to be able to interact with both AF1 and AF2 domain of the two ERs, ERα and ERβ, and the concomitant interaction of TIF2/GRIP1 with both AFs results in synergistic activation of transcription mediated by ERs (23).

PNRC2, a closely related coactivator to PNRC, was also identified as activation function-1 (AF1) cofactor of the orphan nuclear receptor ERRγ from a human brain cDNA phage display system with the N-terminal domain of ERRγ2 as bait (7). In their transfection experiments, A/B region of ERRγ2, which encodes the AF-1 domain, was necessary for optimal PNRC2-dependent transactivation.

Since PNRC interacts with ERα mainly through SH3-binding motif, not NR box sequence, we expected to see an interface between ERα and PNRC which is different from that between ERα and p160 coactivator such as GRIP1. Therefore, the same set of ER mutations, including I358R, K362A, V376R, L539R and E542K were prepared and examined for their transactivation function by transient transfection. ERα/HBD/V376R, which has been shown to lose its ability to interact with GRIP1 (10), was shown to still interact with PNRC or PNRC2, with similar affinity as wild-type ERα/HBD (Figure 6B). Consequently, the full-length ERα carrying V376R mutation retains 50% of transactivation activity of wild type. Furthermore, the transactivation activity of ERa/V376R mutant is enhanced 5-fold by the cotransfection of PNRC but not be affected by GRIP1. These results imply that the PNRC–ERα interaction interface may be different from that of GRIP1–ERα interaction. The physiological significance of this finding is not totally known at the present time. However, one could speculate that this would allow the formation of more active coactivator complexes that include PNRC and other coactivators. Furthermore, it is possible that the structural requirements for the interaction of PNRC and nuclear receptors such as ERα would provide some levels of selectivity of nuclear receptors that interact with PNRC. As indicated by Albers *et al.* (8), PNRC (and PNRC2) only interacts with few nuclear receptors among those tested.

In conclusion, we believe that this is a thorough study to determine the molecular features of the interaction between PNRC and ERα. The results presented in this paper demonstrate that PNRC (and PNRC2) is an unusual coactivator that interacts with nuclear receptors such as estrogen receptor in a novel manner. Since results from this and other laboratories indicate that PNRC and PNRC2 are more general cofactors for nuclear receptor than previously appreciated, it is essential to understand how they interact and regulate the activity of nuclear receptors.

ACKNOWLEDGEMENTS

REFERENCES

1. Median,D. (2005) Mammary developmental fate and breast cancer risk. *Endocr. Relat. Cancer*, **12**, 483–495.
2. Russo,J., Hu,Y.F., Silva,I.D. and Russo,I.H. (2001) Cancer risk related to mammary gland structure and development. *Microsc. Res. Tech.*, **52**, 204–223.

3. Heery,D.M., Kalkhoven,E., Hoare,S. and Parker,M.G. (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature*, **387**, 733–736.

4. Xu,J. and Li,Q. (2003) Review of the in vivo functions of the p160 steroid receptor coactivator family. *Mol. Endocrinol.*, **17**, 1681–1692.

5. Zhou,D., Quach,K.M., Yang,C., Lee,SY, Pohajdak,B. and Chen,S. (2000) PNRC: a proline-rich nuclear receptor coregulatory protein that modulates transcriptional activation of multiple nuclear receptors including orphan receptors SF1 (steroidogenic factor 1) and ERRalpha1 (estrogen related receptor alpha-1). *Mol. Endocrinol.*, **14**, 986–998.

6. Zhou,D. and Chen,S. (2001) PNRC2 is a 16 kDa coactivator that interacts with nuclear receptors through an SH3-binding motif. *Nucleic Acids Res.*, **29**, 3939–3948.

7. Hentschke,M. and Borgmeyer,U. (2003) Identification of PNRC2 and TLE1 as activation function-1 cofactors of the orphan nuclear receptor ERRgamma. *Biochem. Biophys. Res. Commun.*, **312**, 975–982.

8. Albers,M., Kranz,H., Kober,I., Kaiser,C., Klink,M., Suckow,J., Kern,R. and Koegl,M. (2005) Automated yeast two-hybrid screening for nuclear receptor-interacting proteins. *Mol. Cell Proteomics*, **4**, 205–213.

9. Zhou,D., Chen,B., Ye,J.J. and Chen,S. (2004) A novel crosstalk mechanism between nuclear receptor-mediated and growth factor/Ras-mediated pathways through PNRC-Grb2 interaction. *Oncogene*, **23**, 5394–5404.

10. Shiau,A.K., Barstad,D., Loria,P.M., Cheng,L., Kusuner,P.J., Agard,D.A. and Greene,G.L. (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*, **95**, 927–937.

11. Feng,W., Ribeiro,R.C.J., Wagner,R.L., Nguyen,H., Apriletti,J.W., Fletterick,R.J., Baxter,J.D., Kushner,P.J. and West,B.L. (1998) Hormone-dependent coactivator Binding to a Hydrophobic Cleft on Nuclear Receptors. *Science*, **280**, 1747–1749.

12. Eng,F.C., Barsalou,A., Akutsu,N., Mercier,, Zechel,C., Mader,S. and White,J.H. (1998) Different classes of coactivators recognize distinct but overlapping binding sites on the estrogen receptor ligand binding domain. *J. Biol. Chem.*, **273**, 28371–28377.

13. Leduc,A.M., Trent,J.O., Wittliff,J.L., Bramlett,K.S., Briggs,S.L., Chirgadze,N.Y., Wang,Y., Burris,T.P. and Spatola,A.F. (2003) Helix-stabilized cyclic peptides as selective inhibitors of steroid receptor-coactivator interactions. *Proc. Natl Acad. Sci. USA*, **100**, 11273–11278.

14. Rodriguez,A.L., Tamrazi,A., Collins,M.L. and Katzenellenbogen,J.A. (2004) Design, synthesis, and in vitro biological evaluation of small molecule inhibitors of estrogen receptor alpha coactivator binding. *J. Med. Chem.*, **47**, 600–611.

15. Savkur,R.S. and Burris,T.P. (2004) The coactivator LXXLL nuclear receptor recognition motif. *J. Peptide. Res.*, **63**, 207–212.

16. Kong,E.H., Heldring,N., Gustafsson,J.A., Treuter,E., Hubbard,R.E. and Pike,A.C.W. (2005) Delineation of a unique protein-protein interaction site on the surface of the estrogen receptor. *Proc. Natl Acad. Sci. USA*, **102**, 3593–3598.

17. Galande,A.K., Bramlett,K.S., Trent,J.O., Burris,TP, Wittliff,J.L. and Spatola,A.F. (2005) Potent inhibitors of LXXLL-based protein-protein interactions. *Chembiochem.*, **6**, 1991–1998.

18. Makarova,O., Kamberov,E. and Margolis,B. (2000) Generation of deletion and point mutations with one primer in a single cloning step. *BioTechniques*, **29**, 970–972.

19. Geiser,M., Cebe,R., Drewello,D. and Schmitz,R. (2001) Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *BioTechniques*, **31**, 88–92.

20. Onate,S.A., Boonyaratanakornkit,V., Spencer,T.E., Tsai,S.Y., Tsai,M.J., Edwards,D.P. and O'Malley,B.W. (1998) The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J. Biol. Chem.*, **273**, 12101–12108.

21. Bevan,C.L., Hoare,S., Claessens,F., Heery,D.M. and Parker,M.G. (1999) The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. *Mol. Cell Biol.*, **19**, 8383–8392.

22. Webb,P., Nguyen,P., Shinsako,J., Anderson,C., Feng,W., Nguyen,M.P., Chen,D., Huang,S.M., Subramanian,S.S., McKinermey,E., Katzenellenbogen,B.S., Stallcup,M.R. and Kushner,P.J. (1998) Estrogen receptor activation function 1 works by binding to p160 coactivator proteins. *Mol. Endocrinol.*, **12**, 1605–1618.

23. Benecke,A., Chambon,P. and Gronemeyer,H. (2000) Synergy between estrogen receptor alpha activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2. *EMBO. Rep.*, **1**, 151–157.