CCDC62/ERAP75 functions as a coactivator to enhance estrogen receptor beta-mediated transactivation and target gene expression in prostate cancer cells

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Human prostate cancer (PCa) and prostate epithelial cells predominantly express estrogen receptor (ER) β, but not ERα. ERβ might utilize various ER coregulators to mediate the E2-signaling pathway in PCa. Here, we identified coiled-coil domain containing 62 (CCDC62)/ERAP75 as a novel ER coactivator. CCDC62/ERAP75 is widely expressed in PCa cell lines and has low expression in MCF7 cells. Both in vitro and in vivo interaction assays using mammalian two-hybrid, glutathione S-transferase pull-down and coimmunoprecipitation methods proved that ERβ can interact with the C-terminus of CCDC62/ERAP75 via the ligand-binding domain. The first LXXLL motif within CCDC62/ERAP75 is required for the interaction between ERβ and CCDC62/ERAP75. Electrophoretic mobility shift assay showed that CCDC62/ERAP75 can be recruited by the estrogen response element–ER complex in the presence of ligand. Furthermore, a chromatin immunoprecipitation assay demonstrated the hormone-dependent recruitment of CCDC62/ERAP75 within the promoter of the estrogen-responsive gene cyclin D1. In addition, using silencing RNA (siRNA) against endogeneous CCDC62/ERAP75, we demonstrated that inhibition of endogeneous CCDC62/ERAP75 results in the suppression of ERβ-mediated transactivation as well as target gene expression in LNCaP cells. More importantly, using the tet-on overexpression system, we showed that induced expression of CCDC62/ERAP75 can enhance the E2-regulated cyclin D1 expression and cell growth in LNCaP cells. Together, our results revealed the role of CCDC62/ERAP75 as a novel coactivator in PCa cells that can modulate ERβ transactivation and receptor function.

Introduction

The estrogen actions are mediated by the estrogen receptors (ERs), members of the nuclear receptor (NR) superfamily, which are encoded by two distinct genes, ERα and ERβ (1). ERs share a common structural architecture with other NR superfamily members and consist of three main functional domain: an N-terminal A/B domain containing a ligand-independent transactivation function (AF1), a DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) containing a ligand-dependent transactivation function (AF2) (1). Recent studies have shown that a wide range of ER coregulators can modulate ERs at target promoters (2). The most characterized ER coactivators are members of the p160/SRC family including SRC-1 (3), GRIP/TIF2/NCaA-2/SRC-2 (4, 5) and SRC-3/AIB1/pCIP/RAC3 (6–8). The interaction between ERs and the p160 family is mediated by short amphipathic α-helices, which consist of leucine-rich motifs: LXXLL [where L is leucine and X is any amino acid (aa)] (9). The ligand-activated ERs lead to conformational changes that reposition the Helix 12 and form a hydrophobic groove on the receptor surface and interact with the coactivator LXXLL motif (9,10). The other important group of ER coactivators are the p300 and the highly related CBP (11,12), which are histone acetyltransferases that mediate the acetylation of nucleosomal histones. p300/CBP, as a bridge factor, forms a complex with other coactivators and ERs and countergulates the signals at distinct steps in ER-mediated transcription activation (13). Other coactivators have been suggested to function at different steps by directly binding to the basal transcriptional machinery and promoting the assembly of transcription initiation complexes (14, 15).

Prostate cancer (PCa) is the second leading cause of cancer deaths in American men. An estimated 28 660 men in the USA will lose their lives because of this malignancy in 2008 (16). Estrogens, in combination with androgens, play critical roles in prostate carcinogenesis (17, 18). Recent studies have shown that the ERα gene is transcriptionally inactivated by promoter DNA methylation in most PCa cell lines and specimens (19,20); therefore, ERβ is the predominant ER subtype expressed in normal prostate epithelial cells, PCa cell lines and PCa tissue (19, 21). Although ERβ is found lost during the prostate cancer initiation, Ho et al. (22) suggested that re-expression of ERβ in metastatic PCa might confer survival advantages to the advanced stage of PCa cells. To elucidate estrogen action in the PCa cells, it is important to understand the ERβ-mediated-signaling pathway and the proteins that interact with ERβ and might influence the ERβ transactivation and target gene expression.

Similar to the positive expression in prostate, ERβ is expressed in the human testis tissues (23). ERβ exists as five isoforms due to alternative splicing. The current report used the ERβ1 to collect all data. Using the yeast two-hybrid system and ERβ1-LBD as bait, we screened a human testis complementary DNA (cDNA) library and identified an unreported ER-associated protein, designated ERAP75 (ER-associated protein with a predicted molecular mass of 75 kDa) (24). The sequence blast research revealed that ERAP75 matched the EREα binds to the ERα-containing complex via the LXXLL motif (9). The full-length open reading frame of CCDC62 before we identified and characterized it as an ER-interacting protein and a coactivator to modulate ERα function in the prostate stromal cells (24). In the current study, we unify the nomenclature as CCDC62/ERAP75. The coiled-coil domain in the CCDC62/ERAP75, consisting of two to five amphipathic α-helices that wind around one another into a superhelix, is expected to fulfill a function in protein–protein interactions, DNA–protein interactions and dimerization (25). We showed that CCDC62/ERAP75 is widely expressed in the normal prostate epithelial cells and PCa cell lines. Since most PCa cell lines express ERβ, we decided to use the human PCa epithelial cells to further elucidate the possible roles of CCDC62/ERAP75 involved in the ERβ-mediated-signaling pathways. Here, we demonstrate that CCDC62/ERAP75 functions as an ERβ coactivator to enhance ERβ-mediated transactivation, target genes expression and cell growth in the PCa epithelial cells.

Materials and methods

Plasmids

pM2-ERα/ERβ, pDNA3.1(+)–ERα/ERβ, pSG5–ERα/ERβ, estrogen response element (ERE)–Luc, pSG5–AR, pSG5–GR, pSG5–PR, pSG5–VDR, MMVT–Luc, CYP21–Luc, pGADT7–SRC-1 and pSG5–SRC-1 have been described previously (24, 26). The full-length open reading frame of CCDC62/ERAP75 was determined by direct DNA sequencing of the PCR products.

Introduc
was cloned by polymerase chain reaction (PCR) using the human testis cDNA library (Clontech, Mountain View, CA) as the template and subsequently constructed into pcDNA3-flag vector using EcoRI and BamHI enzyme site. pVP16-CCDC62/ERAP75, psg55-CCDC62/ERAP75, PGADT7-CCDC62/ERAP75, pRev-TRE-CCDC62/ERAP75 and different glutathione S-transferase (GST)-C-terminal-fusion protein. Replacement of lysine residues with alanine fragments of CCDC62/ERAP75 from pcDNA3-flag-CCDC62/ERAP75 via PCR and inserted into the target vectors with the proper enzymes. Site-directed mutagenesis on lysine residues of ‘LXXLL’ motifs in the plasmid pGEX-CCDC62/ERAP75 or pVP16-CCDC62/ERAP75 was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s protocol. Replacement of lysine residues with alanine was made by using mutagenic primers. All the plasmids, after construction, were verified by sequencing. The expression of plasmids was either confirmed by TNT<sup>TM</sup> in vitro expression or western blotting.

**Antibodies**

Cyclin D1 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERβ antibody (60-629) was purchased from Upstate Biotechnology (Lake Placid, NY), and anti-Flag antibody M2 was purchased from Sigma, (St. Louis, MO). CCDC62/ERAP75 monoclonal antibody was produced by injecting the purified human CCDC62/ERAP75-carboxyl terminus (338–684 aa) antigen into a mouse, and the antigen purified as described previously (27).

**Yeast two-hybrid system**

The LBD of ERβ (β1 isofrom) CDNA (233–530 aa) was cloned into the EcoRI and BamHI enzyme sites in the pGBK7 vector (Clontech). Yeast strain AH109 was transformed with the plasmid pGBK7-ERβ-LBD encoding the Gal4DBD-ERβ-LBD fusion protein. Transformants AH109 was then mated with yeast strain Y187 that had been pretransformed with the human tests MATCHMAKER cDNA library (Clontech). The library was screened following the manufacturer’s protocol. The detailed method has been described previously (28).

**Commmunoprecipitation assays and western blotting**

LNCaP cells were seeded on 10 cm diameter cell culture dishes and treated with vehicle (0.01% ethanol) or 10 nM E2 for 24 h in 10% charcoal-dextran (CD)–fetal bovine serum (FBS) phenol red-free media. The cells were then lysed using lysis buffer (1% NP-40, 10% glycerol, 135 mM NaCl, 40 mM Tris pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol and 1X protease inhibitor cocktail) (Roche, Indianapolis, IN) in the presence or absence of E2. Lysates were centrifuged and supernatants were precipitated by 2 μg anti-ERβ antibody or normal mouse IgG for 4 h at 4°C with agitation followed by adding 40 μl protein A/G plus agarose for another 2 h. Lysates were centrifuged, the immunoprecipitates were washed three times with phosphate-buffered saline and re- 

**Chromatin immunoprecipitation assay**

LNCaP cells were grown in phenol red-free RPMI supplemented with 10% FBS for 24 h and then incubated in fresh medium without or with 10 nM E2 for 3 h. Cells were cross-linked with 1% formaldehyde at room temperature for 10 min and lyzed with lysis buffer. Sonication was performed to shear DNA to 200–1000 bp, cell debris was pelleted and supernatants were collected and diluted with chromatin immunoprecipitation assay (ChIP) dilution buffer. To preclear, diluted samples were incubated with salmon sperm DNA/protein A agarose slurry with rotation for 1 h at 4°C. The beads were then pelleted and the supernatants were collected. Immunoprecipitations were performed at 4°C overnight with 2 μg CCDC62/ERAP75 monoclonal antibody; immunoprecipitates were then sequentially washed with immune complex wash buffers containing low salt, high salt and lithium chloride followed by two washes with Tris–ethylendiaminetetraacetic acid buffer. DNA associated with immunoprecipitation was eluted with fresh elution buffer. The cross-linking was reversed by the addition of 1/25 volume of 5 M NaCl followed by incubation at 65°C for 6 h. The DNA was recovered with a QIAGEN PCR cleaning kit and 1 μl eluate was subjected to PCR and real-time PCR. The primers used for the region encompassing the activity protein-1 (AP-1) site were as described previously (29).

**siRNA and retrovirus infection experiment**

CCDC62/ERAP75 siRNA target was designed using OligoEngine siRNA design software. The targeted sequences were analyzed by BLAST search to eliminate any significant sequence homology with other genes. The Superior-retro.puro vector (OligoEngine, Seattle, WA) was used for the expression of siRNA in LNCaP cells. The specific CCDC62/ERAP75 siRNA insert was GTGAGCTATATCCATCCA. A scramble negative control siRNA was constructed using an insert: GTTGAACCCGTGACTAA, which contains nucleotide substitutions at the 19 nt-targeting sequence and with no significant homology to any mammalian gene sequence. CCDC62/ERAP75 siRNA or scrambled siRNA was transfected using SuperFect reagent (Qiagen, Chatsworth, CA) into Phoenix packaging cells (the Nolan Lab, Stanford University). LNCaP cells were then cultured in the presence of the viral supernatant and selected with 1 μg/ml puromycin (Sigma) for 5–7 days. After the selection, the cells were cultured in 10% CD-FBS media and treated with 10 nM E2 for 24 h before luciferase reporter assay or real-time PCR.

**Cell proliferation assay**

LNCaP cells were seeded into 24-well plates in medium containing 5% CD-FBS. After 24 h doxycycline (Dox) (2 μg/ml) treatment, 10 nM E2 was added to the cells. Medium was replenished and cell proliferation was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay at the indicated time points as described previously (27). Briefly, 0.5 ml 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (0.5 mg/ml) was added into each well. After 2–3 h incubation at 37°C, 1 ml 0.04 M HCl in isopropyl alcohol was added to solubilize the formazan product and the absorbance was recorded at a wavelength of 595 nm. The experiment was performed in triplicate.

**Statistical analysis**

The statistical analyses were done using the Student’s t-test. P values <0.05 were considered to be statistically significant.

**Results**

**Ligand-dependent interaction of ERs and CCDC62/ERAP75 in yeast**

In order to identify the novel proteins interacting with ERβ, the human ERβ-LBD (233–530 aa) was fused with the Gal4DBD as bait in yeast two-hybrid screening. A pretransformed normal human tests cDNA library was screened in the presence of 10 nM E2. A total of 26 individual yeast clones were first identified by nutrition deprivation and the interaction with ERβ-LBD confirmed by a clone lift assay (data not shown). Sequence analyses found that a few clones encode SRC-1 (data not shown). Two positive cDNA clones were identical and encoded 85 aa followed by a 1 kb 3′ untranslated region, which matched the CCDC62/ERAP75 sequence deposited in the DDBJ/EMBL/GenBank database (accession no.: NM_201435). This CCDC62/ERAP75 fragment (aa 600–684; CCDC62/ERAP75-C) from yeast lies in the C-terminus of CCDC62/ERAP75 and contains two conserved LXXLL motifs. Using 5′ rapid amplification of cDNA ends PCR method, we determined and cloned the full-length cDNA sequence of this transcript that contains 3044 nt and encodes an open reading frame of 684 aa with a predicted molecular weight of 75 kDa. Two putative coiled-coil domains exist in the N terminus of CCDC62/ERAP75 protein (aa 16–163 and aa 199–255). Cotransformation of the bait vector and the candidate clones or SRC-1 clones into the yeast strain AH109, followed by growth selection and α-galactosidase assay (X-α-gal as substrate), further confirmed the interaction between ERβ and CCDC62/ERAP75-C or SRC-1 (Figure 1A). Next, we applied the liquid β-galactosidase assays to quantify the interaction between ERβ and CCDC62/ERAP75-C. Constructs containing either CCDC62/ERAP75-C or SRC-1 showed a strong and similar interaction with the ERβ in a ligand-dependent manner (Figure 1B, lanes 5–8). There was no autologus transactivation activity detectable when transfecting GalAD-CCDC62/ERAP75-C with GalDBD (Figure 1B, lane 4 versus 3).

**Distribution of CCDC62/ERAP75 in human cell lines and mouse tissues**

To detect the expression of CCDC62/ERAP75 in different cell lines, western blotting was performed using a mouse monoclonal antibody against human CCDC62/ERAP75-C antigen (aa 338–684). We first validated CCDC62/ERAP75 antibody by immunoblotting CCDC62/ERAP75 protein in COS-1 cells transfected with CCDC62/ERAP75, LNCaP cells and COS-1 parental cells with or without neutralizing antigen. As shown in the Figure 2A, the specific band was abolished after antigen neutralization, which indicated the band identified is, in fact, CCDC62/ERAP75. The CCDC62/ERAP75 protein was detectable in most of the cell lines tested with the highest expression levels in DU145, PC3 and 22Rv1 cell lines, moderate expression in LNCaP,
merging, the colocalization of ERβ and CCDC62/ERAP75 can be revealed in the nuclei of LNCaP cells. We also examined CCDC62/ERAP75 expression in the normal human prostate tissue via immunohistochemistry (IHC) staining. CCDC62/ERAP75 was expressed in the nuclei of both prostatic epithelial cells (arrows, Figure 2E) and stromal cells (arrowheads, Figure 2E), but predominantly expressed in the prostatic epithelial cells. Overall, CCDC62/ERAP75 is expressed in the human normal prostate, PCa cell lines and the mouse prostate and is primarily present in the cell nucleus.

**Interaction of CCDC62/ERAP75 and ERβ in vitro and in vivo**

To further confirm the interaction between CCDC62/ERAP75 and ERβ from yeast two-hybrid screening, we examined the interaction using both in vitro and in vivo assays. In mammalian two-hybrid assays, the interaction between GalDBD-ERβ-LBD and VP16-AD-CCDC62/ERAP75-C or VP16-AD-DDC62/ERAP75 (Figure 3A, lanes 4 and 5) can be detected in the presence of E2. Furthermore, ICI182, 780, a pure antagonist of E2, abolished the E2-induced interaction between ERβ and CCDC62/ERAP75. Similar to the results from yeast two-hybrid assay, there was no autologous transactivation activity detectable when transfecting VP16-AD-DDC62/ERAP75-C or VP16-AD-DDC62/ERAP75 with GalBD (Figure 3A, lanes 2 and 3). The association of the CCDC62/ERAP75 protein with ERβ was also tested by in vitro coimmunoprecipitation. The [35S]methionine-labeled ERβ-LBD and HA-fused CCDC62/ERAP75 were transcribed and translated in vitro, separately. After interacting with HA-fused CCDC62/ERAP75, ERβ-LBD was precipitated by an anti-HA antibody in the presence of E2 (Figure 3B, lane 5). The interaction between ERβ and CCDC62/ERAP75 was also confirmed by coimmunoprecipitation from protein extracts prepared from LNCaP cells. We demonstrated that endogenous CCDC62/ERAP75 in LNCaP cells could be coimmunoprecipitated with endogenous ERβ using the anti-ERβ antibody (Figure 3C). Together, assays from mammalian two-hybrid assay as well as in vitro and in vivo coimmunoprecipitation assays demonstrated that CCDC62/ERAP75 could interact with ERβ in a ligand-dependent manner.

**Recruitment of CCDC62/ERAP75 by ERE-ERβ in the presence of ligand**

ERs bind to ERE with high affinity and transactivate gene expression in response to estrogen. We examined whether CCDC62/ERAP75 can be recruited by the ERE–ER complex. Using the electrophoretic mobility shift assay (EMSA) assay as shown in Figure 3D, ERβ bound with ERE both in the absence or presence of E2 in vitro (Figure 3D, lanes 3, 7 and 8) and the ERE–ER complex was supershifted by ERβ antibody (Figure 3D, lane 4). The addition of a 100-fold molar excess unlabeled ERE could effectively eliminate ERE–ER complex formation (Figure 3D, lane 5). In contrast, the excess unlabeled mutant ERE did not affect the complex formation (Figure 3D, lane 6), which proved that the ERE–ER complex (arrow) has specific binding between ERβ and ERE probe. CCDC62/ERAP75 did not autologously bind with ERE (Figure 3D, lane 2). In the presence of E2, recruitment of CCDC62/ERAP75 resulted in the ERE-bound receptor with slowed migration (Figure 3D, lane 10). Addition of CCDC62/ERAP75 in the absence of E2 did not supershift the ERE–ERβ complex (Figure 3D, lane 9), confirming that the interaction between ERβ and CCDC62/ERAP75 is ligand dependent. The excess unlabeled ERE could effectively eliminate ERE–ERβ complex formation so that ERE–ERβ–CCDC62/ERAP75 complex was completely abolished (Figure 3D, lane 11), whereas ERE–ERβ–CCDC62/ERAP75 complex was not affected by the presence of excess mutant ERE (Figure 3D, lane 12). Together, CCDC62/ERAP75 can be recruited by the ERE–ERβ complex in vitro in the presence of ligand.

**CCDC62/ERAP75 is recruited to the AP-1 site of cyclin D1 promoter in the presence of estrogen**

ERs can also tether with other transcription factors to form the trans-activation complexes where ERs interact with another DNA-bound transcription factor to stabilize the DNA binding of that transcription factor and recruit coactivators to the complexes and regulate the target
Fig. 2. CCDC62/ERAP75 expression in different human cancer cell lines and various mouse tissues. (A) The validation of CCDC62/ERAP75 monoclonal antibody. The lysates from COS-1 transfected with CCDC62/ERAP75, LNCaP cells and COS-1 parental cells were analyzed by immunoblotting with CCDC62/ERAP75 monoclonal antibody without (lanes 1–3) or with (lanes 4–6) 10 μg His-CCDC62/ERAP75 antigen (aa 338–684) competing peptide. Note that after antigen neutralization, the 75 kDa specific bands were abolished. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to demonstrate equal loading of protein amounts. (B) Western blotting detection of CCDC62/ERAP75 and ERβ protein in different cell lines. The β-actin was used to demonstrate equal loading of protein amounts. (C) Semiquantitative reverse transcription–PCR for CCDC62/ERAP75 messenger RNA in different tissues of a 2-month-old C57BL/6 male mouse. We used 3 μg total RNA to generate first-strand cDNA and then used genespecific primers to amplify the gene CCDC62/ERAP75. The mouse CCDC62/ERAP75 shared 50% homology with human CCDC62/ERAP75. (D)
gene expression in the context of the native chromatin (30). To assess if CCDC62/ERAP75 is assembled into transactivation complexes bound to ERs, we used the ChIP assay to examine the estrogen-responsive cyclin D1 promoter in LNCaP cells. The estrogen-responsive cyclin D1 expression is mediated by ERs through the AP-1 promoter, not the ERE sequence (29,33). During the ChIP analyses, LNCaP was treated with or without 10 nM E2 for 3 h and native chromatin-associated protein complexes were cross-linked to the DNA. The ChIP analyses were carried out with IgG (negative control) or anti-CCDC62/ERAP75 antibody. As shown in Figure 3E, the presence of E2, anti-CCDC62/ERAP75, but not the preimmune ChIP, reveals a 7-fold recovery of cyclin D1 promoter amplification product (Figure 3E, lower panel, E2: EtOH = 13.4 versus 1.9, input as 100), indicating that CCDC62/ERAP75 becomes associated with the ERβ at the cyclin D1 promoter in a ligand-dependent manner. The results are specific because estrogen induces the occupancy of cyclin D1 promoter by ERβ at the same site (data not shown). There is no specific band amplified while using a distal promoter sequence of cyclin D1 as the control, which does not contain an AP-1 or ERE regulatory element (data not shown). Overall, these results demonstrate that CCDC62/ERAP75 can interact with ERβ and is one of the components of the ER transactivation complexes bound to ER native target genes.

Domains involved in the interaction between ERβ and CCDC62/ERAP75

Since the initially isolated CCDC62/ERAP75 clones only encoded the C-terminus of CCDC62/ERAP75, we examined whether the C-terminus in the CCDC62/ERAP75 was responsible for interacting with ERβ and determined which domains of ERβ might be responsible for this interaction. To dissect the CCDC62/ERAP75 interaction domain on the ERβ, four ER mutants fused with GST were tested in GST pull-down assays. As shown in Figure 4A, GST-ERβ-LBD, but not GST-ERβ-N, GST-ERβ-DBD or GST-ERβ-AF2 domain can interact with CCDC62/ERAP75 in the presence of E2. This indicates that ERβ-LBD in the ERβ protein is responsible for the interaction between ERβ and CCDC62/ERAP75. On the other hand, three GST-fused CCDC62/ERAP75 mutants: GST-CCDC62/ERAP75-N (aa 1–180, containing the first coiled coil), GST-CCDC62/ERAP75-M (aa 180–498, containing the second coiled coil) and GST-CCDC62/ERAP75-C (aa 498–684, containing the LXXLL motif) were used to determine which domain of CCDC62/ERAP75 interacted with the ERβ protein. As shown in Figure 4B, GST-CCDC62/ERAP75-C, but not GST-CCDC62/ERAP75-N or -M, is responsible for binding to ERβ in the presence of E2. Together, those results are consistent with the studies from yeast two-hybrid screening.

The LXXLL motif is required for the interaction between ER and CCDC62/ERAP75

LXXLL is a conserved motif present in the NRs coactivators and is necessary and sufficient to mediate binding of the coactivators to liganded NRs (9). The LXXLL motifs individually are able and sufficient to bind to hormone receptors, but display preferences for certain receptors (32). However, in other coactivators, such as PRIP, not all the LXXLL motifs are necessary to mediate the interaction between coactivators and their receptors (33). Since the CCDC62/ERAP75-C contains two LXXLL motifs, we investigated whether both LXXLL motifs are required for the interaction between ERβ and CCDC62/ERAP75. CCDC62/ERAP75 mutants of the LXXLL motifs were tested in a GST pull-down assay (Figure 4C). The results showed that if the mutation occurred in the first LXXLL motif, the interaction between ERβ and CCDC62/ERAP75 was abolished. In contrast, when the second LXXLL motif of CCDC62/ERAP75 was mutated, there was no alteration on its interaction with ERβ (Figure 4C). We also tested the interaction between CCDC62/ERAP75 mutants and ERβ in a mammalian two-hybrid assay. Similarly, the interaction between ERβ and CCDC62/ERAP75 was reduced around 70% when the first LXXLL motif was mutated in the two-hybrid assay (Figure 4D, lane 7 versus 4). There is no change on the interaction between ERβ and CCDC62/ERAP75 when the second LXXLL motif is mutated (Figure 4D, lane 10 versus 4). These results suggested that the first LXXLL motif is critical to mediate the interaction between CCDC62/ERAP75 and ERβ.

CCDC62/ERAP75 enhances the ER transactivation activity

To determine whether the interaction between CCDC62/ERAP75 and ERβ affects the ERβ transactivation activity, we used luciferase assays with ERE-luc reporter genes. In the COS-1 cell line, which has a barely detectable CCDC62/ERAP75 expression (Figure 2A), E2 treatment led to a 4-fold increase in the ERβ transactivation (Figure 5A, lane 3 versus 1). Importantly, addition of CCDC62/ERAP75 led to a substantial, CCDC62/ERAP75 dose-dependent increase in E2-induced ERβ transactivation in COS-1 cells (Figure 5A, lanes 4–7 versus 3). For a comparison, we also tested the influence of CCDC62/ERAP75 on ERα transactivation. As shown in Figure 5A, CCDC62/ERAP75 was also able to enhance ERα-mediated transactivation (Figure 5A, lane 13 versus 10). Similar activation was also observed when we replaced COS-1 cells with H1299 and LNCaP cells (data not shown). These results demonstrate that CCDC62/ERAP75 can function as a coactivator to mediate ERs transcriptional activation.

A comparison of CCDC62/ERAP75 effect on different NRs transactivation was performed in the COS-1 cells. When transfected with 0.7 μg coactivator, CCDC62/ERAP75 showed the most significant activation of ERβ transactivation (Figure 5A, lane 7 versus 3), compared with the moderate activation of progesterone receptor, glucocorticoid receptor and androgen receptor (AR) (Figure 5B, lane 4 versus 3; lane 8 versus 7 and lane 12 versus 11, respectively), and little effect on vitamin D receptor (VDR) transactivation (Figure 5B, lane 16 versus 15). These results suggested that the CCDC62/ERAP75 has a preference in transactivation of various NRs.

Knockdown of the endogenous CCDC62/ERAP75 expression by siRNA-suppressed ERβ transactivation and target gene expression

In order to rule out the artificial effect linked with luciferase reporter assays and demonstrate that the endogenous CCDC62/ERAP75 can function as an ERβ coactivator, we established LNCaP cells stably expressing CCDC62/ERAP75 or scramble siRNA. As shown in Figure 5C and D, endogenous CCDC62/ERAP75 expression was reduced >80% by CCDC62/ERAP75 siRNA as demonstrated in both real-time reverse transcription-PCR and western blot assay. Notably, ER transactivation was decreased when the endogenous CCDC62/ERAP75 was suppressed (Figure 5E). To further confirm the roles of endogenous CCDC62/ERAP75 in ERβ function, we examined the messenger RNA expression of ER target genes (pS2, cathepsin D, cyclin D1 and TERT) in LNCaP cells upon knockdown of endogenous CCDC62/ERAP75 gene expression by real-time PCR. As shown in Figure 5F, E2 cannot regulate the expression of pS2 and cathepsin D in LNCaP cells, but can induce the expression of cyclin D1 and TERT expression. Significantly, the induction of the messenger RNA expression of cyclin D1 and TERT was reduced upon transfection of CCDC62/ERAP75 siRNA2 (Figure 5F, lane 12 versus 10; lane 16 versus 14, P < 0.05). These results confirmed the activator role of endogenous CCDC62/ERAP75 in E2-mediated transactivation and target gene expression in LNCaP cells.

Immunofluorescence localization of CCDC62/ERAP75 in the LNCaP cells. LNCaP cells were treated with 10 nM E2 or solvent (0.01% ethanol) for 24 h. Immunofluorescence staining was performed as described in Materials and Methods. (E) IHC analyses of CCDC62/ERAP75 protein in the non-malignant human prostate tissues. CCDC62/ERAP75 was expressed in the nuclei of both prostatic epithelial (arrows) and stromal cells (arrowheads), with predominant expression in the prostatic epithelial cells.
Increase of ERβ target gene expression and estrogen-mediated cell growth in LNCaP cells with Dox-induced CCDC62/ERAP75

We studied the consequence of CCDC62/ERAP75 as a coactivator to enhance the ERβ transactivation in the cell proliferation assay. We constructed a Dox-inducible CCDC62/ERAP75 stable LNCaP cell line (LNCaP-flag-CCDC62/ERAP75). As shown in Figure 6A, addition of 2 μg/ml Dox induced flag-CCDC62/ERAP75 expression in LNCaP-CCDC62/ERAP75 cells as detected by anti-flag antibody. The induction of CCDC62/ERAP75 could then enhance the expression of ERβ endogenous target gene, cyclin D1, after 24 h E2 treatment in LNCaP cells (Figure 6B, lane 8 versus 4 and 7), but had no influence on the β-actin gene expression. In contrast, addition of Dox in control LNCaP-vector cells, stably transfected with parental vector pRev-TRE, showed little or no influence on the cyclin D1 expression (Figure 6B, lane 4 versus 3). We then studied the consequence of activation of ERβ transactivation and target genes expression by CCDC62/ERAP75 via the cell proliferation assay. LNCaP cell growth was significantly stimulated by E2 treatment (Figure 6C).
Dox-induced CCDC62/ERAP75 expression, E2-stimulated LNCaP cell growth was further enhanced (Figure 6C). Although the cell proliferation slowed down at day 7, our data suggested that higher expression of CCDC62/ERAP75 could potentiate cell growth and survival. Together, these results extended our in vitro studies and demonstrated that the consequence of the protein–protein interaction between ERβ and CCDC62/ERAP75 can result in the ERβ target gene expression and enhance E2/ERβ-stimulated cell growth in LNCaP PCa cells.

Discussion

Using the yeast two-hybrid assay with ERβ1-LBD as bait to screen a human testis cDNA library, we identified CCDC62/ERAP75 as a new ER coactivator that reveals a strong ligand and LBD-dependent interaction with ERβ (Figures 3 and 4). Analysis of the genome sequence showed that CCDC62/ERAP75 gene consists of 13 exons and transcribes different alternative splicing transcripts. There are two similar transcripts, CCDC62/ERAP75 transcript 1 (NM_032573) and transcript 2 (NM_201435). The difference between transcript 1 and transcript 2 lies in the C-terminal exon 12, which encodes the last 15 aa for the transcript 2 and the last 12 aa for the transcript 1. All the other coding regions are identical. The CCDC62/ERAP75 identified in our yeast two-hybrid screening was the C-terminus of transcript 2 (NM_201435), but not transcript 1 (NM_032573). We acknowledge that transcript 1 could also potentially interact with ER bait. The reason that we did not identify the transcript 1 as the ER interaction protein could be due to the different abundance of these splice variants in the library.

Our studies also showed that CCDC62/ERAP75 protein is mainly present in the nucleus and widely expressed in many PCa cell lines (Figure 2). Coactivators are generally expressed in a variety of tissues and important for modulating the NRs transactivation and physiological function (34); however, individual coactivators have a tissue-specific expression pattern (35). The higher expression of CCDC62/ERAP75 in the PCa cells might suggest that it is important for the ERβ-signaling regulation in PCa cells. Therefore, we focused on characterization of CCDC62/ERAP75 function in PCa cells.

In the human tumor specimens, the amplification of coactivators is frequently observed, which may contribute to the pathogenesis of cancer. For example, SRC3/RAC3/AIB1 is often overexpressed in steroid-regulated tumors, especially in breast, ovarian and PCa (7). We examined the expression of CCDC62/ERAP75 in several commonly used PCa cell lines. CCDC62/ERAP75 is expressed more abundantly in the PCa cells (PC3, DU145, LNCaP, 22Rv1) than in the normal prostate epithelial cells (BPH-1). Recent studies have shown re-expression of high level ERβ in the majority of cases of PCa metastasized to bone and lymph node with little or no ERα expression (21). Whether the expression of CCDC62/ERAP75 correlates with the ERβ expression in the PCa tissue is currently unknown. Further studies are needed to determine the functional role of CCDC62/ERAP75.
CCDC62/ERAP75 contains two LXXLL motifs, which have been shown to be necessary and sufficient to mediate binding of the coactivators to liganded NRs (9). However, we found that only the first LXXLL motif, located at residues 634–638, is necessary for the interaction of CCDC62/ERAP75 with the ERβ. A mutation in the second LXXLL motif does not affect the interaction with or influence the coactivation of ERβ (see supplementary data and Figure S-1 available at Carcinogenesis Online). Detailed studies on residues flanking the second LXXLL motif and its secondary structure in this context are needed to elucidate the mechanisms by which this motif is unable to interact with ERβ. In CCDC62/ERAP75, we found that there are two potential coiled-coil domains at the N terminus, which have been known to fulfill a function in protein–protein interactions, DNA–protein interactions and dimerization (25). A coiled-coil domain has also been detected in CoCoA and AINT, two other NR coactivators (36,37). Interestingly, this coiled-coil domain has been suggested to mediate the interaction between the bHLH-PAS domain of p160 coactivator and its coactivators, such as CoCoA. Although our studies have shown that the coiled-coil domain in the CCDC62/ERAP75 is not necessary to mediate the interaction with ERβ, it is possible that the coiled-coil domain might facilitate the interaction between CCDC62/ERAP75 and p160 coactivators.
Tet-inducible overexpression of CCDC62/ERAP75 increases E2-regulated cyclin D1 expression and LNCaP cell growth and viability. (A) Flag-CCDC62/ERAP75 expression was induced by Dox in stably transfected LNCaP-Flag-CCDC62/ERAP75 cells. Cell extracts from LNCaP-Flag-CCDC62/ERAP75 cells were isolated in the absence or presence of 2 µg/ml Dox and analyzed by western blotting using flag antibodies. (B) Cell extracts were isolated from LNCaP-flag-CCDC62/ERAP75 and LNCaP-vector cells treated with or without 2 µg/ml Dox for 24 h followed by treatment with 10 nM E2 or ethanol for another 24 h. Western blotting was performed to determine the expression level of cyclin D1. The β-actin was used to demonstrate equal loading of protein amounts. (C) 3-(4,5-Dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay was done to measure the LNCaP-vector and LNCaP-Flag-CCDC62/ERAP75 cell growth. LNCaP cells were seeded in 5% CD-FBS media for 24 h. After treating with Dox to induce the expression of CCDC62/ERAP75 for 24 h, cells were treated with ethanol, 10 nM E2 or 10 nM E2 + 2 µg/ml Dox for 7 days. Media and treatments were renewed every day. The control of LNCaP-vector (ethanol treatment) was set at 100%. Each indicated time represents the mean ± SD of three independent experiments performed in triplicate. **P < 0.01 compared with ethanol treatment; *P < 0.05 compared with E2 treatment.

Using ERE-luc reporter assay, we found that CCDC62/ERAP75 can enhance ERs transactivation to a level similar to that of ERβ transactivation, which suggests that the interaction between CCDC62/ERAP75 and ERs is highly conserved in the context of the ERE promoter. Although certain coactivators have shown a preference when transactivating ERα versus ERβ (38), we did not observe any differential effect of CCDC62/ERAP75 on ERs transactivation; however, CCDC62/ERAP75 can moderately enhance progesterone receptor, glucocorticoid receptor and AR transactivation, but has very little effect on VDR transactivation. The selective effect of CCDC62/ERAP75 on NRs transactivation might implicate the relative importance of CCDC62/ERAP75 in mediating different NRs-signaling pathways.

Using the ChiP assay, we also showed that CCDC62/ERAP75 could be recruited to the AP-1 site within the promoter of the estrogen-responsive gene cyclin D1 to form a component of ERβ transactivation complexes. By real-time PCR, we also found that E2 regulated the ER target genes differentially in LNCaP cells and could induce the expression of cyclin D1 and TERT, but not the expression of pS2 and cathepsin D (Figure 5F). Cyclin D1 and TERT have been shown to promote cell proliferation. The ability of estrogen to modulate those genes expression may directly contribute to the estrogen mitogenic effect on LNCaP cells. We have determined whether overexpression of CCDC62/ERAP75 allows increased estrogen-dependent proliferation. Our studies showed that inducible overexpression of CCDC62/ERAP75 will stimulate E2-mediated cyclin D1 expression and E2-mediated LNCaP cell growth (Figure 6B and C). Previous studies by Castagnetta et al. (39) have shown that E2-stimulated LNCaP cell growth is mediated by its own receptor. Amplification of coactivators during PCa development may confer a selective growth advantage to ER-positive PCa cells. CCDC62/ERAP75 can potentiate the estrogen-mediated mitogenic effect on the LNCaP cells, which might suggest that CCDC62/ERAP75 is a potential regulator of cell proliferation by enhancing the ER transactivation and driving the cell cycle-related ER target gene expression.

An early study suggested that E2 might activate the mutant AR transactivation in LNCaP cells to activate the gene expression (40). The estrogen-dependent effects of CCDC62/ERAP75 on cyclin D1 upregulation could be mediated through AR. However, we found that the E2 induction of cyclin D1 and telomerase (TERT) did not change via knockdown of the AR expression in the LNCaP cells (see supplementary data and Figure S-2 available at Carcinogenesis Online); therefore, the E2 upregulation of cyclin D1 and TERT in LNCaP is mediated via ERβ, not AR. Importantly, we showed that by modulating endogenous CCDC62/ERAP75 protein expression via siRNA techniques, the downregulation of CCDC62/ERAP75 inhibited the ERβ-mediated transactivation and reduced the E2 induction of ER target genes (Figure 5F), which suggested an involvement of CCDC62/ERAP75 in the ERβ transactivation.

In the past 10 years, at least 40 different ER coactivators have been identified (2). The reasons for the existence of a variety of coactivators are unclear. It is possible that ERs might use a different subset of coactivators for maximal transcriptional activity in a cell-type and promoter-dependent manner and that such a subset of coactivators is preferentially expressed in the cells and complexed with other transcription factors for maximal cross-talk of transcriptional regulation. In the present study, we found that CCDC62/ERAP75 is preferentially expressed in PCa cells, enhances the ERβ transactivation, target genes expression and E2-mediated LNCaP cell growth. These findings raise the possibility for the role of CCDC62/ERAP75 as an ERβ coactivator to enhance its transactivation and receptor functions in PCa cells. Further studies may help us to better understand the roles of ERβ and CCDC62/ERAP75 in the PCa.

Supplementary material

Supplementary data and Figures S-1 and S-2 can be found at http://carcin.oxfordjournals.org/

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References


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850