

Proline-, Glutamic Acid-, and Leucine-Rich Protein-1/Modulator of Nongenomic Activity of Estrogen Receptor Enhances Androgen Receptor Functions through LIM-Only Coactivator, Four-and-a-Half LIM-Only Protein 2

Sujit S. Nair, Zhiyong Guo, Judith M. Mueller, Shahriar Koochekpour, Yun Qiu, Rajeshwar Rao Tekmal, Roland Schüle, Hsing-Jien Kung, Rakesh Kumar, and Ratna K. Vadlamudi

Department of Obstetrics and Gynecology (S.S.N., R.R.T., R.K.V.), University of Texas Health Science Center, San Antonio, Texas 78229; Department of Microbiology and Immunology and Stanley S. Scott Cancer Center (S.K.), Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112; Universitaets-Frauenklinik und Zentrale Klinische Forschung (J.M.M., R.S.), D-79106 Freiburg, Germany; Department of Pharmacology and Experimental Therapeutics (Z.G., Y.Q.), University of Maryland School of Medicine, Baltimore, Maryland 21201; Department of Biological Chemistry (H.-J.K.), University of California at Davis, Sacramento, California 95817; and The University of Texas MD Anderson Cancer Center (R.K.), Houston, Texas 77030

Proline-, glutamic acid-, and leucine-rich protein-1 (PELP1) is a coregulator of multiple nuclear receptors. Molecular mechanisms of PELP1 function are not completely understood, but its expression is up-regulated in hormonal-dependent cancers. Using a yeast two-hybrid screen, we found that four-and-a-half LIM-only protein 2 (FHL2) interacted with PELP1. FHL2 is a transcriptional regulator that associates with nuclear cofactors, including androgen receptors (ARs), and contains an intrinsic activation domain. PELP1 and FHL2 interact *in vitro* and *in vivo* and colocalize in the nuclear compartment. PELP1 interacts with FHL2 via LIM domains 3 and 4 and synergistically enhances the transcriptional activity of FHL2. Src kinase is required for PELP1-mediated enhancement of FHL2

functions because knockdown of Src kinase expression or function abolished PELP1-mediated FHL2 activation functions. PELP1 interacted with AR and enhanced FHL2-mediated AR transactivation functions. PELP1 knockdown by small interfering RNA or PELP1 mutant, which lacks an activation domain, reduced FHL2-mediated AR transactivation. Biochemical analyses revealed a complex consisting of PELP1, FHL2, and AR in prostate cancer cells. PELP1/MNAR expression was elevated in high-grade prostate tumors. Our results suggest that PELP1 functions as a molecular adaptor, coupling FHL2 with nuclear receptors, and PELP1-FHL2 interactions may have a role in prostate cancer progression. (*Molecular Endocrinology* 21: 613–624, 2007)

PROLINE-, GLUTAMIC ACID-, and leucine-rich protein-1 (PELP1) [also termed modulator of nongenomic activity of estrogen receptor (MNAR)] is a nuclear receptor (NR) coregulator that contains 10 NR-interacting boxes (LXXLL motif) and acts as a coactivator of several NRs including estrogen receptors and

androgen receptors (ARs) (1, 2). PELP1/MNAR mediates NR genomic activity via histone interaction (3), and nongenomic activity by activating c-Src kinase and phosphatidylinositol 3 kinase pathways (1, 2, 4). PELP1/MNAR expression is up-regulated in hormone-responsive cancers (4, 5), but the role of PELP1/MNAR in cancer progression remains unclear. PELP1 appears to function as a scaffolding protein, coupling NRs with several proteins that are implicated in oncogenesis (6).

AR functions as a ligand-dependent transcription factor and plays a critical role in prostate cancer progression (7). AR controls transcriptional activation by recruiting coregulators (8–10). Coregulators facilitate AR-mediated transcription primarily through chromatin remodeling and histone modifications (10, 11). Coregulators play a role in androgen responsiveness, androgen independence, and tumor progression (10, 12). However, the complete repertoire of AR coregu-

First Published Online December 27, 2006

Abbreviations: aa, Amino acids; AR, androgen receptor; CREB, cAMP response element-binding protein; FHL2, four and a half lim domain protein 2; GFP, green fluorescent protein; GST, glutathione-S-transferase; HEK, human embryonic kidney; MMTV, murine mammary tumor virus; MNAR, modulator of nongenomic actions of estrogen receptor; NR, nuclear receptor; PELP1, proline-, glutamic acid-, and leucine-rich protein; PSA, prostate-specific antigen; shRNA, short hairpin RNA; siRNA, short interfering RNA.

***Molecular Endocrinology* is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.**

lators and the mechanisms by which AR coregulators promote tumorigenesis remain unknown.

Four-and-a-half Lim 2 (FHL2) is a member of the LIM-only transcriptional coactivator family, and contains four-and-a-half LIM domains (13, 14). A LIM domain is a cysteine- and histidine-rich domain composed of two tandem zinc-finger repeats that are important in protein-protein interactions and transcription (15). FHL2 acts as a coactivator of several transcription factors, including AR (16), cAMP response element binding protein (CREB) (17), β -catenin (18), Wilms' tumor (WT1) (19), and AP1 (20). FHL2 does not bind DNA, but it possesses an intrinsic transactivation domain, is expressed in the prostate and interacts with AR in a ligand-dependent manner (16), and its expression is deregulated in higher-grade prostate tumors (21). However, the molecular mechanism by which FHL2 modulates AR transactivation remains unclear.

In this study, using a yeast-based two-hybrid screen, we identified that PELP1/MNAR interacts with FHL2. Using various biochemical assays, we also have shown that this interaction has physiological and functional consequences. Our results provide evidence that PELP1 modulates FHL2 transactivation and enhances FHL2 interaction with NRs, and its deregulation may have a role in prostate cancer progression.

RESULTS

PELP1/MNAR Interacts with FHL2

We used a yeast two-hybrid screen to identify PELP1/MNAR-interacting proteins. We found that

the AR coactivator FHL2 interacted with PELP1/MNAR after screening of 10^6 expression library transformants with the PELP1/MNAR N-terminal region [amino acids (aa) 1–400] as the bait. The specificity of the interaction between FHL2 and PELP1/MNAR was confirmed using yeast cotransformation followed by a survival assay. Only yeast cells that were transformed with both PELP1/MNAR and FHL2, but not with either one alone, showed growth (Fig. 1A), confirming the interaction of FHL2 and PELP1/MNAR in yeast. To further verify the interaction between FHL2 and PELP1/MNAR, we transiently cotransfected human embryonic kidney (HEK)293 cells with T7-PELP1/MNAR and Flag-FHL2. The immunoprecipitation results showed that PELP1/MNAR interacted with Flag-FHL2 (Fig. 1B). An anti-PELP1/MNAR antibody could immunoprecipitate endogenous FHL2 from HeLa cell lysates, confirming the physiological association between endogenous PELP1/MNAR and FHL2 (Fig. 1C). Confocal analysis revealed that PELP1/MNAR colocalized with FHL2, predominantly in the nuclear compartment (Fig. 1D).

PELP1/MNAR-Interacting Regions in FHL2

To determine the PELP1/MNAR-interacting region in FHL2, we produced various deletions of FHL2. COS-7 cells were transfected with T7 epitope-tagged PELP1/MNAR, and total lysates were incubated with glutathione S-transferase (GST) or various GST-FHL2 fusion proteins (aa 1–279, 1–162, and 163–279). A Western blot analysis of eluates from GST pull-down assays showed that the

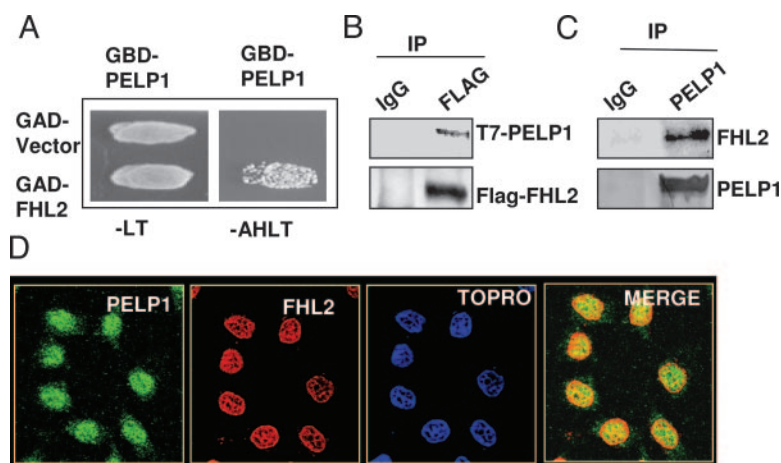


Fig. 1. PELP1/MNAR Interacts with FHL2

A, Yeast cells were transfected with a control Gal4-activation domain (GAD) vector or GAD-FHL2, along with a Gal4-DNA binding domain (GBD) vector or GBD-PELP1/MNAR. Growth was recorded after 72 h on selection plates lacking leucine and tryptophan (–LT) or adenosine, histidine, leucine, and tryptophan (–AHLT). B, HEK293 cells were transfected with T7-PELP1/MNAR and Flag-FHL2. Immunoprecipitation was performed with a Flag antibody, followed by Western blot analysis with a T7 antibody. C, Nuclear extracts from HeLa cells were subjected to immunoprecipitation using a control IgG or a PELP1/MNAR antibody, followed by Western blot analysis using an FHL2 antibody. D, HeLa cells grown in 10% serum were fixed and costained with antibodies against PELP1/MNAR (green) and FHL2 (red). DNA is stained blue by Topro-3 (TOPRO). The images were analyzed by confocal microscopy. Colocalization of PELP1/MNAR and FHL2 is shown in yellow. IP, Immunoprecipitation.

C-terminal region of FHL2 that contains LIM domains 3 and 4 constituted the PELP1/MNAR-interacting domain (Fig. 2A). We then identified the FHL2-binding domain in PELP1 using a series of PELP1 deletion constructs that spanned the entire coding region of PELP1. GST pull-down assays with ^{35}S -labeled PELP1-constructs showed that FHL2 interacts with two binding sites in the N-terminal region of PELP1, one in aa 1–400 and the other in aa 401–600.

PELP1/MNAR Modulates FHL2/MNAR Transactivation

PELP1/MNAR acts as a coactivator of several NRs (2), and we wanted to determine whether it was a coactivator of FHL2-dependent transcription. Because FHL2 does not bind DNA but possesses an intrinsic transactivation domain, we used a Gal_{5X}-E1bTATA-luciferase reporter (G5E1b-luc) and the Gal4-FHL2 system (22). As expected, cotransfection of Gal4-FHL2 led to

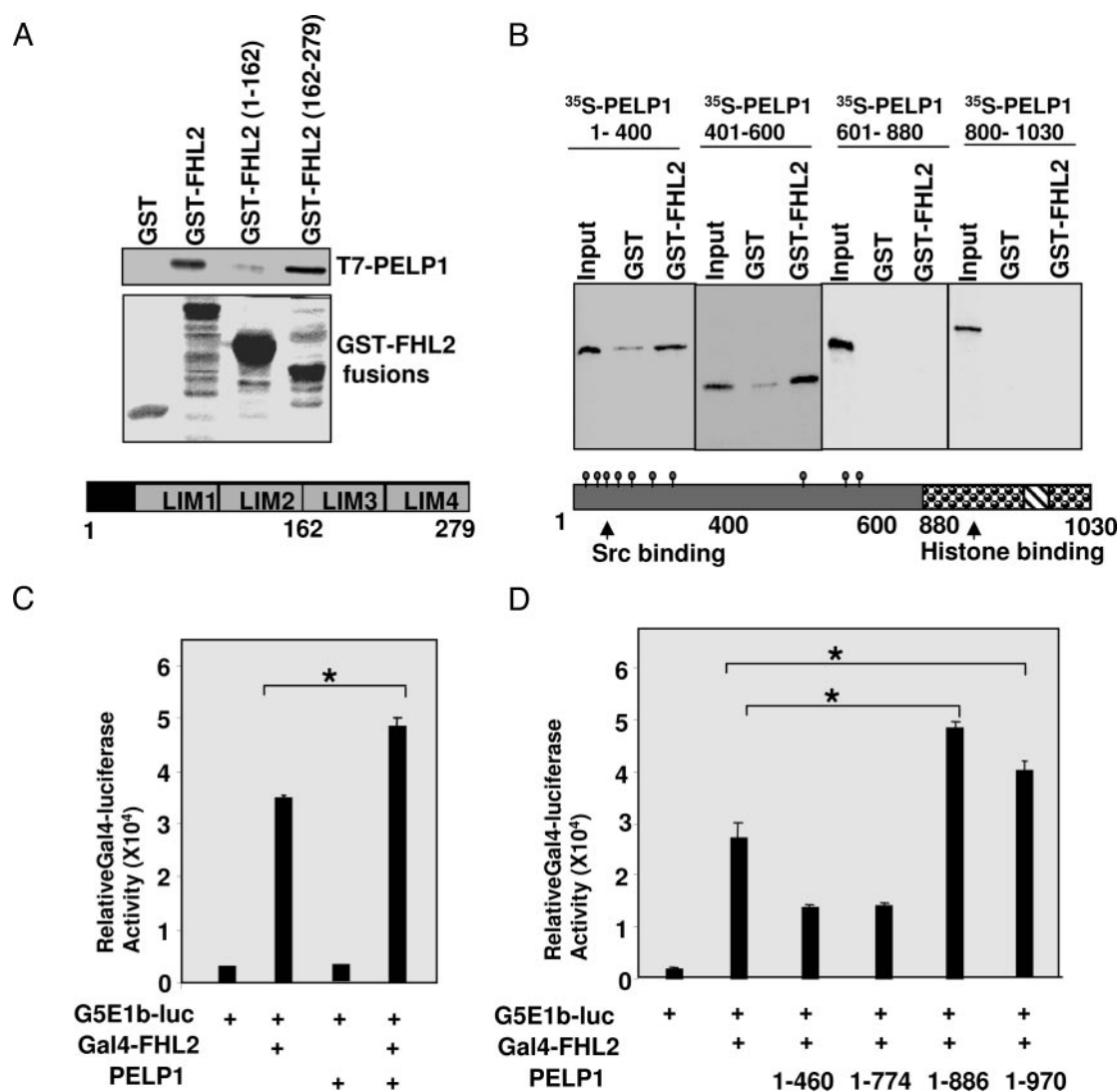


Fig. 2. PELP1/MNAR Is a Coactivator of FHL2-Dependent Transcription

A, Total cell lysates from COS-7 cells expressing T7-PELP1/MNAR were incubated with GST-FHL2 fusion proteins of various lengths, and the PELP1/MNAR binding region in FHL2 was analyzed with a GST pull-down assay. B, PELP1 peptides of indicated lengths were translated *in vitro* using the TNT system in the presence of [^{35}S]methionine, and their ability to bind GST-FHL2 was analyzed by GST pull-down analysis. C, PC3 cells were cotransfected with a Gal4-based Luc reporter (G5E1b-Luc), β -Gal reporter, Gal4-FHL2 with or without PELP1/MNAR. After 48 h, G5E1b-Luc reporter activity was measured. D, PC3 cells were cotransfected with G5E1b-Luc, β -Gal reporter and Gal4FHL2 with or without full-length or with serial deletions of PELP1/MNAR. After 48 h, G5E1b-Luc reporter activity was measured. In all the reporter gene assays parental vectors were used as controls and the total amount of the DNA in the transfections was kept constant by adding appropriate vectors. β -Gal values were used to normalize luciferase activity for transfection efficiency. Data shown are the means of \pm SE from three independent experiments performed in triplicate wells. *, $P < 0.05$.

a 7-fold increase in Gal-reporter gene activity. Co-transfection of PELP1/MNAR with Gal4-FHL2 led to a 10-fold increase in GAL4-reporter gene activity, suggesting that PELP1/MNAR augments FHL2 transactivation (Fig. 2C). PELP1/MNAR contains several domains, including a Src-binding domain, a LXXLL-rich domain, a proline-rich domain, a glu-rich domain, and a histone-binding domain (1–3). To identify the PELP1/MNAR domains involved in FHL2 transactivation, we performed Gal4-reporter gene assays using serial deletions of PELP1/MNAR (Fig. 2D). PELP1 peptides encoding aa 1–886 but not aa 1–460, aa 1–774 contributed to FHL2-mediated transactivation. Curiously, transfection of PELP1 N-terminal fragments showed an inhibitory effect on the FHL2-mediated transcriptional activation. Because the PELP1 N-terminal region contains FHL2 binding site, overexpression of these fragments probably led to inhibition of FHL2 activity in a dominant-negative manner. Ability of PELP1 peptides containing aa 1–886 to promote FHL2 transactivation functions also suggest that PELP1/MNAR C-terminal region that contained a proline-rich region (aa 774–886) plays an important role in PELP1/MNAR-mediated FHL2 transactivation functions.

PELP1/MNAR Enhances FHL2-Mediated Induction of Target Genes

FHL2 is a serum-inducible factor that plays a role in serum-mediated induction of genes including cyclin D1 (18, 22, 23). We therefore examined whether PELP1 could enhance FHL2-mediated cyclin D1 promoter activity. Cotransfection of PELP1/MNAR with FHL2 synergistically increased cyclin D1 reporter gene activity (Fig. 3A). PELP1 interacts with and activates c-Src kinase, which influences PELP1/MNAR modulation of NR signaling (2). We therefore analyzed the role of c-Src kinase in PELP1/MNAR-mediated FHL2 functions using c-Src-specific inhibitor PP2 and also by c-Src-specific shRNA. Treatment of cells with c-Src-shRNA reduced the expression of c-Src to 60–70% of the levels seen in control cells (Fig. 3C). Inhibition of c-Src kinase by inhibitor PP2 (Fig. 3B) or down-regulation of c-Src expression by short hairpin RNA (shRNA) (Fig. 3, C and D) reduced the ability of PELP1/MNAR to enhance FHL2 transactivation. Src inhibition by PP2 showed more significant effect of PELP1/MNAR-mediated transcriptional activation than c-Src-shRNA, and this could be due to incomplete inhibition of c-Src expression by shRNA. To further examine the role of PELP1/MNAR-FHL2 interactions in cyclin D1 promoter induction, we have repeated these experiments with NIH 3T3 cells. Reporter gene assays indicated that PELP1 and FHL2 act synergistically to up-regulate cyclin D1 promoter activity (Fig. 3E). To confirm PELP1-FHL2 regulation of cyclin D1 protein expression, we have transiently co-transfected NIH 3T3 cells with green fluorescent

protein (GFP) or GFP-tagged PELP1 along with or without FHL2. Western analysis results showed that PELP1 and FHL2 synergistically act to enhance serum-mediated cyclin D1 induction compared with FHL2 or PELP1 overexpression alone (Fig. 3F). From these results we conclude that PELP1/MNAR plays an important role in serum-mediated induction of FHL2 target gene such as cyclin D1 and that c-Src plays an important role in PELP1/MNAR-mediated FHL2 transactivation functions.

PELP1/MNAR Enhances FHL2-Mediated AR Transactivation

FHL2 is a coactivator of AR (16), and the PELP1/MNAR-FHL2 interaction may have a synergistic effect on AR-dependent transcription. We used a murine mammary tumor virus (MMTV)-Luc construct with AR response elements as a reporter of AR activation, and the synthetic androgen, R1881, as a ligand. Cotransfection of the MMTV-Luc reporter and AR with either FHL2 or PELP1/MNAR into PC3 cells enhanced AR-mediated transactivation of MMTV-Luc (Fig. 4A). No activation of MMTV reporter was observed by PELP1 or FHL2 when AR expression plasmid was not included in the transfection (Fig. 4B), suggesting PELP1, FHL2-mediated MMTV activation requires functional AR. Cotransfection of PELP1/MNAR and FHL2 further potentiated AR transactivation, reaching synergistic levels. We then determined whether the truncated PELP1/MNAR mutant (aa 1–600), which contains the FHL2-binding domain but lacks the activation domain, could act as an inhibitor of FHL2-mediated AR transactivation. Cotransfection of the PELP1/MNAR-1–600 mutant inhibited FHL2-mediated MMTV-Luc activity (Fig. 4C), whereas cotransfection with PELP1/MNAR-800–1130, which lacks the FHL2-binding domain, had no effect on FHL2-mediated transactivation. Similarly, down-regulation of PELP1/MNAR expression by short interfering RNA (siRNA) also reduced the magnitude of AR transactivation by FHL2 (Fig. 4D). PELP1-mediated up-regulation of FHL2 activity required functional c-Src kinase and could be inhibited by inhibitor PP2 (Fig. 4E).

PELP1 Interacts with AR in the Nuclear Compartment

We next examined whether PELP1/MNAR modulate prostate-specific antigen (PSA) gene expression, an endogenous AR target gene, using LNCaP prostate model cell line, which expresses endogenous AR. PELP1/MNAR and FHL2 synergistically enhanced AR-mediated transactivation of PSA promoter activity (Fig. 5A). Localization of PELP1 varies depending on the cell type (4); therefore, we determined the localization of PELP1/MNAR by subcellular fractionation in PC3(AR)2 cells (24). PELP1/MNAR was predominantly localized in the nuclear compartment (Fig. 5B), but some PELP1/MNAR was also seen in

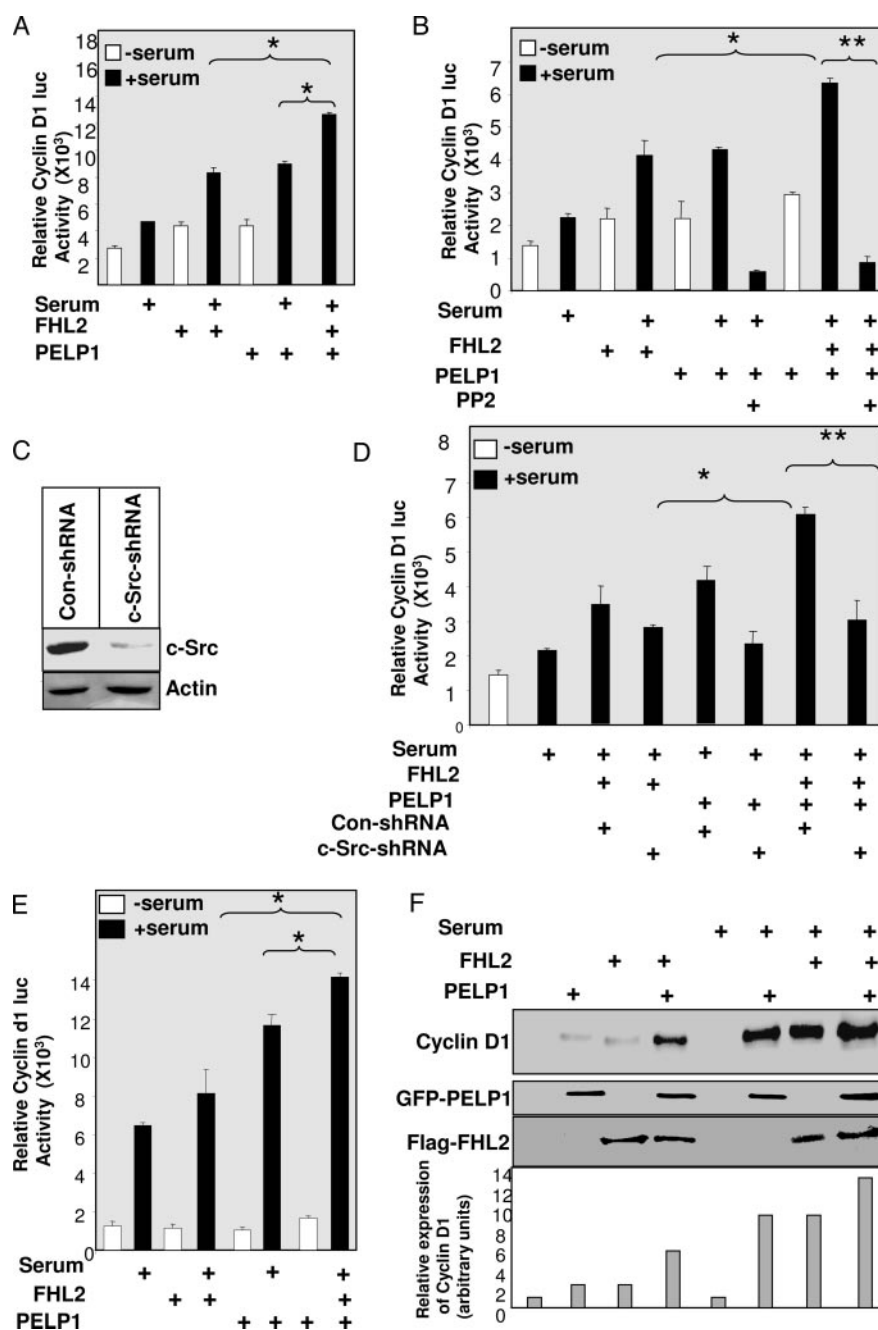


Fig. 3. PELP1 Modulates FHL2-Mediated Target Gene Activation in an Src-Dependent Manner

HeLa cells were cotransfected with a cyclin D1-Luc reporter with or without PELP1/MNAR and with or without FHL2. A, After serum starvation for 48 h, cells were stimulated with serum (10%) for 12 h, and luciferase activity was measured. B, After 48 h, cells were pretreated with an Src inhibitor PP2 (10 μ M, for 1 h) and then stimulated with serum for 12 h. C, Down-regulation of c-Src expression by shRNA was analyzed by Western analysis. D, At 24 h after transfection, indicated wells were infected with control or c-Src-shRNA-specific lentivirus. After 72 h of virus infection, cells were treated with or without serum, and luciferase activity was measured. In all the reporter gene assays parental vectors were used as controls, and the total amount of the DNA in the transfection was kept constant by adding appropriate empty vectors. β -Gal values were used to normalize luciferase activity for transfection efficiency. Data shown are the means \pm SE from three independent experiments performed in triplicate wells. *, $P < 0.05$ comparing PELP1+FHL2 activity vs. PELP1 or FHL2. **, $P < 0.001$ comparing PELP1 vs. PP2 or c-Src-shRNA. E, NIH 3T3 cells were cotransfected with a cyclin D1-Luc reporter, β -Gal reporter gene, with or without PELP1/MNAR and with or without FHL2. After serum starvation for 48 h, cells were stimulated with serum (10%) for 12 h, and luciferase activity was measured. F, NIH 3T3 cells were transfected with GFP or GFP-PELP1 vector along with or without flag-FHL2 vector. After 48 h cells were subjected to fluorescence-activated cell sorting for GFP expression. Equal number of GFP or GFP PELP1 sorted cells were cultured in duplicate plates. After 48 h, cells were treated with or without serum for 12 h and cyclin D1 levels were analyzed by Western blotting. Con-shRNA, Control shRNA.

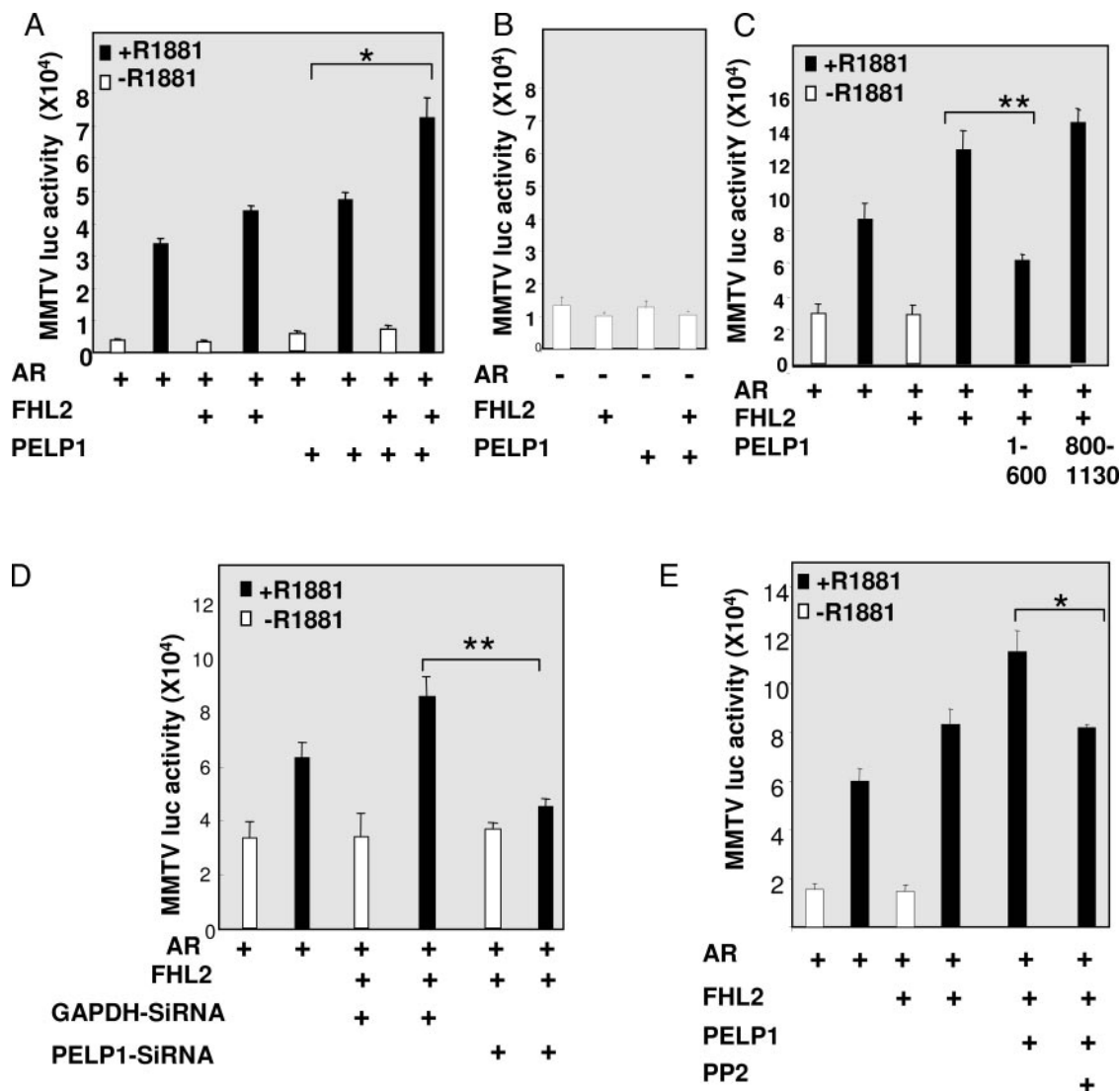


Fig. 4. PELP1/MNAR Enhances FHL2-Mediated AR Transactivation

A, PC3 cells were cotransfected with MMTV-Luc, β -Gal reporter gene, and AR, with or without PELP1/MNAR and with or without FHL2. After 48 h, cells were treated with R1881, and MMTV-Luc reporter activity was measured. B, PC3 cells were cotransfected with MMTV-Luc, β -Gal reporter gene, with or without PELP1/MNAR and with or without FHL2, and after 48 h, reporter gene activation was measured. C, PC3 cells were cotransfected with MMTV-Luc, β -Gal reporter gene, AR, and FHL2 with PELP1/MNAR-1–600 or PELP1/MNAR-800–1130. After 48 h, cells were treated with R1881 for 12 h, and luciferase activity was measured. D, PC3 cells were transfected with control or PELP1/MNAR-specific siRNA. After 24 h, they were transfected with MMTV-Luc, AR, and FHL2. After 48 h, cells were treated with R1881 for 12 h, and luciferase activity was measured. E, PC3 cells were cotransfected with MMTV-Luc, β -Gal reporter gene, and AR, with or without FHL2. After 48 h, cells were pretreated with the c-Src inhibitor, PP2 ($10 \mu\text{M}$ for 1 h), before ligand treatment. After 48 h, cells were treated with R1881 for 12 h, and luciferase activity was measured. β -Gal values were used to normalize luciferase activity for transfection efficiency. Data shown are the means \pm SE from three independent experiments performed in triplicate wells. *, $P < 0.05$ comparing PELP1+FHL2 activity vs. PELP1 or FHL2. **, $P < 0.001$ comparing FHL2 activation in the presence or absence of PELP1/MNAR siRNA. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

the cytoplasmic compartment. Ligand treatment did not change the localization of PELP1, but increased the distribution of AR in the nuclear compartment. To determine whether PELP1/MNAR interacts with AR in the cytoplasmic or nuclear compartments, we performed immunoprecipitation with an AR anti-

body. PELP1 interacted with AR in the nuclear compartment, and the interaction was enhanced by ligand treatment (Fig. 5C). Immunoprecipitation of nuclear extracts from another AR-positive cell line (LNCaP) also confirmed that AR interacts with PELP1 in the nuclear compartment (Fig. 5D).

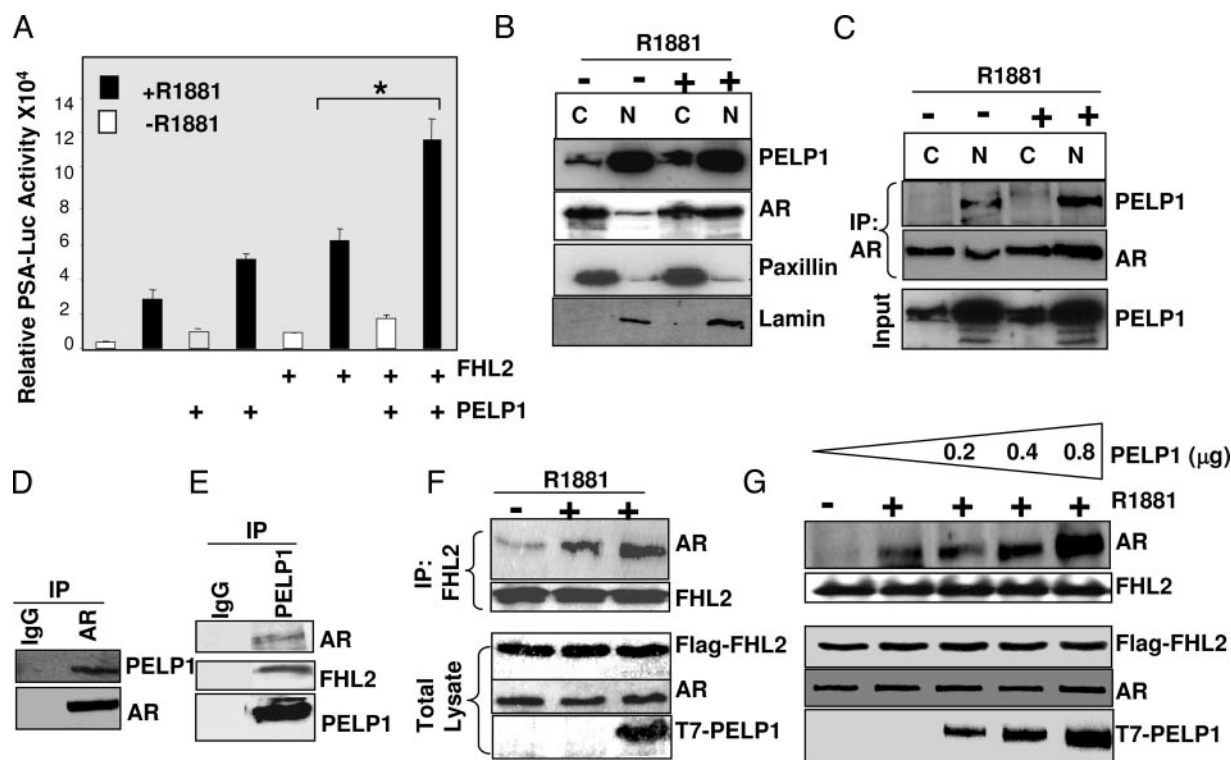


Fig. 5. PELP1/MNAR, AR, and FHL2 Form Functional Complexes *in Vivo*

A, LNCaP cells were cotransfected with a PSA-Luc reporter, with or without PELP1/MNAR and with or without FHL2. After 48 h, cells were treated with R1881 for 12 h, and luciferase activity was measured. *, $P < 0.05$. B, PC3(AR)2 cells were biochemically fractionated into nuclear and cytoplasmic extracts, and the total lysates were analyzed for PELP1/MNAR by Western analysis. Lamin B1 and paxillin were used as markers of the nucleus and cytoplasm, respectively. C, AR was immunoprecipitated from PC3(AR)2 cytoplasmic and nuclear lysates, and the presence of PELP1/MNAR in the immunoprecipitates was analyzed by Western blotting. D, LNCaP cells were treated with R1881, and nuclear extracts were prepared and subjected to immunoprecipitation with control IgG or AR antibody. The presence of PELP1/MNAR in the immunoprecipitates was analyzed by Western analysis. E, HEK293 cells coexpressing T7-PELP1/MNAR, Flag-FHL2, and AR were treated with 1% formaldehyde to stabilize the complexes, and cellular lysates were immunoprecipitated with T7 antibody. The presence of AR and FHL2 in the immunoprecipitates was analyzed by Western analysis. F, HEK293 cells were transfected with AR and Flag-FHL2 and with or without PELP1/MNAR. After 72 h, cells were treated with R1881 for 1 h, and the cell lysates were immunoprecipitated with a Flag antibody. The presence of AR in the immunoprecipitates was analyzed by Western blotting. G, HEK293 cells were transfected with AR and Flag-FHL2 with increasing amounts of PELP1/MNAR. After 72 h, cells were treated with R1881 for 1 h, and the cell lysates were immunoprecipitated with a Flag antibody. The presence of AR in the immunoprecipitates was analyzed by Western blotting. IP, Immunoprecipitation.

PELP1/MNAR, AR, and FHL2 Form Functional Complexes *in Vivo*

We then examined whether AR, FHL2, and PELP1/MNAR form a complex under physiological conditions. Our initial experiments with traditional immunoprecipitation techniques did not pull down this complex, indicating the complexes were transient or weak in nature. Therefore, we used formaldehyde-based cross-linking to stabilize the complexes, followed by immunoprecipitation as described in *Materials and Methods*. A similar approach has been used to demonstrate the formation of complexes by transcriptional coactivators (25). Western blot analysis of the immunoprecipitates showed the presence of FHL2 and AR in the complexes (Fig. 5E).

Because PELP1/MNAR interacts with AR using LXXLL motifs (2) and acts as an adaptor protein, coupling several proteins to NRs, including Src, phosphatidylinositol 3 kinase, and signal transducer and activator of transcription 3 (2, 4, 26), we wanted to determine whether PELP1/MNAR enhances the FHL2/AR interaction by serving as a molecular adaptor. To analyze this possibility, HEK293 cells were cotransfected with Flag-FHL2, AR, and with or without PELP1. FHL2-AR interaction was analyzed by immunoprecipitation. Ligand treatment promoted AR-FHL2 interaction (Fig. 5F, lane 2). Interestingly, PELP1/MNAR overexpression further enhanced immunoprecipitation of AR with FHL2 (Fig. 5F, lane 3). To validate the significance of these findings, we performed another FHL2 immunopre-

precipitation assay using HEK293 cells that express varying levels of PELP1/MNAR. Increased expression of PELP1/MNAR enhanced the amount of FHL2 associated with AR in a dose-dependent manner (Fig. 5G). However, overexpression of PELP1-600-1130 that lacks FHL2 binding site did not affect the interaction of AR and FHL2 (data not shown). Collectively, these results suggest that AR, PELP1/MNAR, and FHL2 form functional complexes *in vivo*.

PELP1 Expression Is Deregulated in Prostate Tumors

To examine whether the expression of PELP1/MNAR is deregulated in prostate cancer, we determined the expression of PELP1/MNAR in human prostate cancer tissues using a commercially available AccuMax prostate tissue array with an affinity-purified PELP1/MNAR antibody (27). Immunohistochemical analysis revealed that all the tumors analyzed had PELP1/MNAR-positive staining ($n = 40$). PELP1/MNAR staining was predominantly localized in the nuclear compartment (Fig. 6, A–C). However, there was a 2- to 3-fold increase in staining intensity in the PELP1/MNAR high-grade tumors (Gleason grade 7–9) compared with the nonneoplastic or low-grade tumors (Gleason grade 3–6) (Fig. 6D).

DISCUSSION

In this study, we provide evidence, for the first time, that the NR coregulator, PELP1/MNAR, functionally interacts with FHL2. Our conclusions are based on the following observations: 1) FHL2 interacts with PELP1 *in vitro* and *in vivo*, and both proteins colocalize in the nuclear compartment; 2) PELP1 enhances FHL2-mediated transcriptional activity in Gal4-luciferase assays; 3) PELP1 and FHL2 synergistically enhance activity of multiple FHL2 target reporter genes; 4) knockdown of PELP1 or overexpression of the PELP1 mutant reduces FHL2-mediated transactivation; and 5) PELP1 enhances the interaction of FHL2 with nuclear receptors.

FHL2 interacts with the N-terminal region of PELP1 (aa 1–600), which also has binding sites for Src kinase and other nuclear receptors, including signal transducer and activator of transcription 3, estrogen receptor, and AR, suggesting that PELP1 may facilitate a complex formation of FHL2 with other nuclear receptors or signal transducers. Further, FHL2 contains an autologous transactivation domain that functions as an activator in Gal-4-based luciferase assays, where PELP1 enhanced the transactivation potential of FHL2. The C-terminal proline-rich region (aa 774–886) in PELP1 is essential for PELP1-mediated enhance-

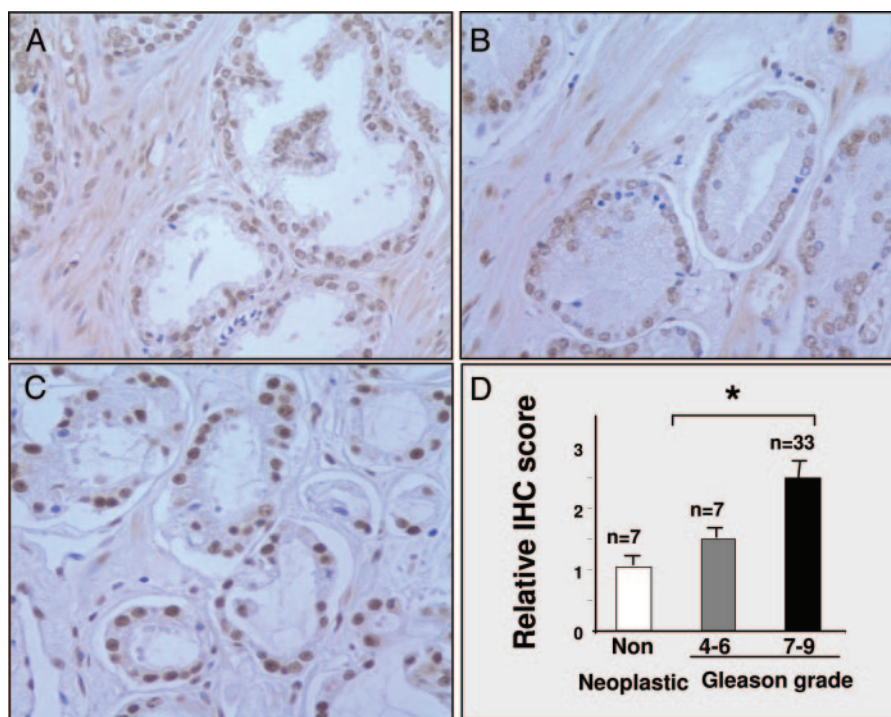


Fig. 6. Expression of PELP1/MNAR in Prostate Cancer

Immunohistological analysis of PELP1/MNAR in a prostate tumor using an AccuMax tissue array and an affinity-purified PELP1 antibody (Bethyl Laboratories). A, Nonneoplastic; B, Gleason grade 5; C, Gleason grade 8; D, quantitation of the PELP1/MNAR expression in benign, low-grade (Gleason 4–6) and high-grade tumors (Gleason 7–9). Scoring was done as follows: weak/focal (1+), moderate (+2), and strong (+3). *, $P < 0.05$. Non, Nonneoplastic.

ment of FHL2 transactivation. Because this 112-amino acid region contains 19 SH3 motifs, four PDZ motifs, and three class IV WW domain motifs (which facilitates phosphorylation-dependent interactions), the PELP1-mediated FHL2 activation may also involve other cofactors via these protein-interaction motifs.

Initial studies suggested that FHL2 expression was tissue specific, with abundant expression in heart tissue (28). Subsequent studies, however, have reported FHL2 expression in other tissues including the ovary, placenta, uterus, mammary gland, and adrenal gland (29). FHL2 is highly expressed in prostate cancer tissues, and nuclear localization of FHL2 is associated with progression to a more malignant phenotype (16, 21). Breast tumors express FHL2, and there is a significant correlation between FHL2 expression levels and the patient's survival. FHL2 interacts with estrogen receptors (30) and breast cancer susceptibility gene 1 (BRCA1) (31). Similarly, PELP1 expression is deregulated in breast, endometrial, and ovarian tumors (6), and the results from this study suggest that PELP1 expression may be up-regulated in higher-grade prostate cancers.

Specific FHL2-binding proteins, such as PELP1, in a given cell may play a role in FHL2 function (29). PELP1 is shown to play a role in chromatin remodeling (3) via its interactions with histones and histone-modifying complexes, including CREB-binding protein/P300 (1, 3) and histone deacetylase 2 (32). The predominant nuclear localization of PELP1, along with its interaction with FHL2, its colocalization with FHL2 in the nuclear compartment, and the immunoprecipitation of AR-PELP1 complexes from nuclear extracts strongly suggest that PELP1-mediated genomic functions might regulate FHL2 transactivation. Even though FHL2 interacts with AR in a ligand-dependent manner (16), it does not contain the NR interaction motif (LXXLL) often used by AF2 domain-interacting proteins. Because PELP1 contains 10 LXXLL motifs, PELP1 may stabilize the FHL2-AR interaction. PELP1 is widely expressed in hormonally responsive tissues, including testis, prostate, brain, mammary gland, and ovary (1). Because FHL2 is also expressed in these tissues, the PELP1-FHL2 interaction may have a functional role in these tissues.

FHL2 is present in multiple cellular compartments, including the cytoplasm, focal adhesions, and nucleus (33). Serum and mitotic stimulation strongly increase FHL2 expression and induce its nuclear localization (20, 34). FHL2 possesses an intrinsic activation domain but can be either an activator or a repressor of transcription. For example, FHL2 enhances transcription of AP1 (23), CREB (17), and AR (16) but represses transcription of E4F1 (35) and FOXO1 (36). The mechanism by which FHL2 activates/represses transcription of nuclear receptors is largely unknown. Because PELP1/MNAR is a coregulator of multiple nuclear transcription factors and interacts with both acetylases and deacetylases (6), the availability and localization of

PELP1/MNAR in a given cell type may have implications for FHL2-mediated coactivation or repression.

AR signaling plays an important role in the progression of prostate cancer. Src kinases are required for androgen-independent growth and AR activation (37). PELP1/MNAR was initially identified as an Src SH2 domain-binding protein (38), and PELP1/MNAR can stabilize estrogen receptor interactions with Src kinase, leading to activation of the Src-MAPK pathway (2). AR, PELP1/MNAR, and Src are constitutively complexed in prostate cancer model cells that exhibit androgen independence, and constitutive activation of the Src-MEK-1/2-ERK-1/2-CREB pathway is associated with the androgen independence phenotype (39). Our results suggest that PELP1/MNAR-mediated FHL2 transactivation also requires functional Src kinase. Reduction of Src kinase expression using shRNA or inhibition with the Src kinase-specific inhibitor, PP2, reduced both PELP1/MNAR-mediated transactivation and its synergistic effect on FHL2 activity. Because PELP1/MNAR interacts with Src kinase and activates Src-MAPK pathways (2, 39), the Src-MAPK-mediated regulation/modification of PELP1 or other associated cofactors may be required for PELP1 to function as a coactivator of FHL2. PELP1/MNAR also could function as a molecular adaptor, coupling FHL2, AR, and Src kinase, thus modulating prostate cancer progression and AR cross-talk with non-genomic pathways.

In summary, our results suggest that PELP1/MNAR interacts with and promotes FHL2-mediated transactivation functions, and that PELP1/MNAR expression is elevated in high-grade prostate tumors. Because PELP1 functions as a molecular adaptor, coupling FHL2 with AR, our findings suggest that deregulation of PELP1/MNAR in prostate cancer may alter AR-FHL2 coactivator complex formation. We speculate that increased AR-FHL2 complex formation by PELP1 overexpression may lead to enhanced AR signaling or sensitize cells to low levels of androgen, and thus, may facilitate the progression of hormone-refractory prostate cancer.

MATERIALS AND METHODS

Materials

HeLa, LNCaP, PC3, HEK293, Cos-1, and NIH 3T3 cells were obtained from the American Type Culture Collection and maintained in DMEM-F12 (1:1) supplemented with 10% fetal calf serum. The PC3 cells stably expressing wild-type AR [PC3(AR)²] were described previously (24). PC3 (AR)² cells were maintained in 5% dextran-coated charcoal-stripped serum. Antibodies against Flag (M2), vinculin, and actin were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies for the T7 epitope were obtained from Novagen (Madison, WI). Antibodies against AR were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies against FHL2 were purchased from Medical & Biological Laboratories Co. Ltd. (MBL) (Woburn, MA), and

polyclonal antibodies for FHL2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for PELP1 were purchased from Bethyl Laboratories, Inc. (Montgomery, TX). Src kinase inhibitor was purchased from Calbiochem (La Jolla, CA) and was dissolved in dimethylsulfoxide.

Two-Hybrid Library Screening and Plasmid Construction

PELP1 bait was constructed by amplifying DNA corresponding to aa 1–400 via PCR and subcloning into Gal4-binding domain vectors (pGBD vector; CLONTECH, Mountain View, CA). pGBD-PELP1 (1–400) was used as a bait to screen a cDNA library fused to a Gal4 activation domain per manufacturer's instructions (CLONTECH). A total of 2×10^6 clones were screened, and positive clones were isolated and sequenced. Positive clones also were verified by cotransformation using purified plasmids and by selection on agar plates lacking adenine, histidine, tryptophan, and leucine.

GST Pull-Down Assays

The GST pull-down assays were performed by incubating equal amounts of GST or GST-FHL2 deletions immobilized on glutathione-sepharose beads (Amersham Biosciences Corp., Piscataway, NJ) with ^{35}S -labeled, *in vitro* synthesized PELP1/MNAR peptides from various protein regions. The mixtures were incubated for 2 h at 4 C and washed six times with Nonidet P-40 lysis buffer. Bound proteins were eluted with $2 \times$ sodium dodecyl sulfate buffer, separated by SDS-PAGE, and visualized by autoradiography.

Reporter Gene Assays

PC3 or HeLa cells were transiently transfected with 100 ng of T7-PELP1/MNAR, 100 ng of FHL2, and 300 ng of cyclin D1-luc using the FuGENE6 method (Roche Diagnostics Corp., Indianapolis, IN). After 16 h of transfection, cells were serum starved for 48 h by culturing them in medium containing 0% serum and then treated with 10% serum for either 5 or 10 min. For MMTV- and PSA-Luc arrays, cells were cultured in medium containing 5% dextran-coated charcoal-stripped serum and, 48 h after transfection, cells were stimulated with the synthetic androgen R1881. The Gal₅X-E1bTATA-luciferase reporter (G5E1b-luc) and the Gal4-FHL2 system were constructed using HeLa cells as described elsewhere (16). Cyclin D1-luciferase reporter (-1745-CD1-Luc) used in the study was described earlier (41, 42). PSA-Luc vector used in the study contains 5.8-kb promoter region (-5834 to +12 relative to transcriptional start site) in PGL3 vector (43). Empty parental vectors were used in the reporter gene assays as controls, and the total amount of the DNA in the transfections was kept constant by adding appropriate empty vectors. A small aliquot of β -Gal reporter gene was also cotransfected along with all the reporter genes used in the study, and β -Gal values were used to normalize luciferase activity for transfection efficiency. Each transfection was performed in triplicate and repeated at least three times. At the end of the experiment, cells were lysed in passive-lysis buffer, and the luciferase assay was performed using a luciferase reporter assay kit (Promega Corp., Madison, WI). PELP1 siRNA and control glyceraldehyde-3-phosphate dehydrogenase siRNA were purchased from Dharmacon (Lafayette, CO). This PELP1 siRNA was used under conditions as described previously (26). The shRNA vectors for c-Src were constructed as described previously (40). The oligonucleotides encoding Src siRNA were 5'-TGGTGTCT TA TACTGTCTTTCAAGAGAAGGACAGTATTAAGACACCTT-

TTTTTC-3' and 5'-TCGAG AAAAAAGGTGTCTTAATACTGTC-CTTCTCTTGAAAGGA CAGTATTAAGACACCA-3'.

Immunoprecipitation and Cell Fractionation Studies

Cells were washed two times with PBS buffer, and cytoplasmic and nuclear extracts were prepared according to a previously described procedure (11). Equal amounts of cytoplasmic and nuclear extracts were incubated with the AR antibody for 4 h at 4 C, and immunocomplexes were precipitated with protein A beads. The precipitated proteins were eluted with $2 \times$ sodium dodecyl sulfate buffer and analyzed by Western analysis. For immunoprecipitation of AR, FHL2, PELP1/MNAR complexes, HEK293 cells were transfected with T7-tagged PELP1/MNAR, Flag-tagged FHL2, and AR expression plasmids. Cells were treated with R1881 for 1 h and cross-linked with formaldehyde. Sonicated lysates were subjected to immunoprecipitation with an anti-T7 antibody, de-cross-linked, and analyzed by Western blotting.

Immunofluorescence and Immunohistochemistry Studies

The cellular colocalization of PELP1/MNAR and FHL2 was determined by indirect immunofluorescence as described using HeLa cells (26). A confocal scanning analysis was performed with a Zeiss laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY) and immunohistochemical detection of PELP1/MNAR was performed as described elsewhere (41). An AccuMax prostate cancer tissue array was purchased from Petagen (<http://tissuearray.petagen.com>). The array slide was incubated with PELP1 antibody (1:500, Bethyl Laboratories). The sections were washed three times with 0.05% Tween in PBS for 10 min, incubated with secondary antibody, developed with 3,3'-diaminobenzidine- H_2O_2 , and then counterstained with Mayer's hematoxylin. Negative controls were performed by replacing the primary antibody with corresponding IgG. The sections were scored by three evaluators blinded to the clinical status and were verified by a trained pathologist, Dr. Adel El-Naggar at University of Texas MD Anderson Cancer Center. The following web site has the list of all clinical parameters related to the samples used in the array http://tissuearray.petagen.com/main/item_file/03.09.08_09.58.13_d41d8cd98f00b2/info.pdf.

Acknowledgments

We thank Dr. Adel El-Naggar and Dr. Balasenthil Seetharaman at University of Texas MD Anderson Cancer Center for their help in staining/scoring the tumor specimens.

Received June 29, 2006. Accepted December 19, 2006.

Address all correspondence and requests for reprints to: Ratna K. Vadlamudi, Department of Obstetrics and Gynecology, University of Texas Health Science Center, Floyd Curl Drive, San Antonio, Texas 78229-3900. E-mail: vadlamudi@uthscsa.edu.

This work was supported by National Institutes of Health/National Cancer Institute Grant CA095681 (to R.K.V.).

Disclosure Statement: The authors have nothing to disclose.

REFERENCES

- Vadlamudi RK, Wang RA, Mazumdar A, Kim Y, Shin J, Sahin A, Kumar R 2001 Molecular cloning and charac-

- terization of PELP1, a novel human coregulator of estrogen receptor α . *J Biol Chem* 276:38272–38279
- Wong CW, McNally C, Nickbarg E, Komm BS, Cheskis BJ 2002 Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci USA* 99:14783–14788
 - Nair SS, Mishra SK, Yang Z, Balasenthil S, Kumar R, Vadlamudi RK 2004 Potential role of a novel transcriptional coactivator PELP1 in histone H1 displacement in cancer cells. *Cancer Res* 64:6416–6423
 - Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, Sahin AA, Kumar R 2005 Functional implications of altered subcellular localization of PELP1 in breast cancer cells. *Cancer Res* 65:7724–7732
 - Vadlamudi RK, Balasenthil S, Broaddus RR, Gustafsson JA, Kumar R 2004 Deregulation of estrogen receptor coactivator proline-, glutamic acid-, and leucine-rich protein-1/modulator of nongenomic activity of estrogen receptor in human endometrial tumors. *J Clin Endocrinol Metab* 89:6130–6138
 - Rajhans R, Vadlamudi RK 2006 Comprehensive analysis of recent biochemical and biologic findings regarding a newly discovered NR coregulator PELP1/MNAR. *J Clin Exp Metastasis* 23:1–7
 - Tsai MJ, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63:451–486
 - Culig Z, Comuzzi B, Steiner H, Bartsch G, Hobisch A 2004 Expression and function of androgen receptor coactivators in prostate cancer. *J Steroid Biochem Mol Biol* 92:265–271
 - Wang L, Hsu CL, Chang C 2005 Androgen receptor corepressors: an overview. *Prostate* 63:117–130
 - Shang Y, Myers M, Brown M 2002 Formation of the androgen receptor transcription complex. *Mol Cell* 9:601–610
 - Heinlein CA, Chang C 2002 Androgen receptor (AR) coregulators: an overview. *Endocr Rev* 23:175–200
 - Chang CY, McDonnell DP 2005 Androgen receptor-cofactor interactions as targets for new drug discovery. *Trends Pharmacol Sci* 26:225–228
 - Morgan ML, Madgwick AJ 1996 Slim defines a novel family of LIM-proteins expressed in skeletal muscle. *Biochem Biophys Res Commun* 225:632–638
 - Genini M, Schwalbe P, Scholl FA, Remppis A, Mattei MG, Schafer BW 1997 Subtractive cloning and characterization of DRAL, a novel LIM-domain protein down-regulated in rhabdomyosarcoma. *DNA Cell Biol* 16:433–442
 - Kadmas JL, Beckerle MC 2004 The LIM domain: from the cytoskeleton to the nucleus. *Nat Rev Mol Cell Biol* 5:920–931
 - Kahl P, Gullotti L, Heukamp LC, Wolf S, Friedrichs N, Vorreuther R, Solleder G, Bastian PJ, Ellinger J, Metzger E, Schule R, Buettner R 2006 Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. *Cancer Res* 66:11341–11347
 - Fimia GM, De CD, Sassone-Corsi P 2000 A family of LIM-only transcriptional coactivators: tissue-specific expression and selective activation of CREB and CREM. *Mol Cell Biol* 20:8613–8622
 - Wei Y, Renard CA, Labalette C, Wu Y, Levy L, Neuveut C, Prieur X, Flajolet M, Prigent S, Buendia MA 2003 Identification of the LIM protein FHL2 as a coactivator of β -catenin. *J Biol Chem* 278:5188–5194
 - Du X, Hublitz P, Gunther T, Wilhelm D, Englert C, Schule R 2002 The LIM-only coactivator FHL2 modulates WT1 transcriptional activity during gonadal differentiation. *Biochim Biophys Acta* 1577:93–101
 - Morlon A, Sassone-Corsi P 2003 The LIM-only protein FHL2 is a serum-inducible transcriptional coactivator of AP-1. *Proc Natl Acad Sci USA* 100:3977–3982
 - Muller JM, Metzger E, Greschik H, Bosserhoff AK, Mercep L, Buettner R, Schule R 2002 The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus. *EMBO J* 21:736–748
 - Muller JM, Isele U, Metzger E, Rempel A, Moser M, Pscherer A, Breyer T, Holubarsch C, Buettner R, Schule R 2000 FHL2, a novel tissue-specific coactivator of the androgen receptor. *EMBO J* 19:359–369
 - Johannessen M, Olsen PA, Johansen B, Seternes OM, Moens U 2003 Activation of the coactivator four-and-a-half-LIM-only protein FHL2 and the c-fos promoter through inhibition of protein phosphatase 2A. *Biochem Pharmacol* 65:1317–1328
 - Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ 1997 Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. *Mol Cell Endocrinol* 126:59–73
 - Endoh M, Zhu W, Hasegawa J, Watanabe H, Kim DK, Aida M, Inukai N, Narita T, Yamada T, Furuya A, Sato H, Yamaguchi Y, Mandal SS, Reinberg D, Wada T, Handa H 2004 Human Spt6 stimulates transcription elongation by RNA polymerase II in vitro. *Mol Cell Biol* 24:3324–3336
 - Manavathi B, Nair SS, Wang RA, Kumar R, Vadlamudi RK 2005 Proline-, glutamic acid-, and leucine-rich protein-1 is essential in growth factor regulation of signal transducers and activators of transcription 3 activation. *Cancer Res* 65:5571–5577
 - Khan MM, Hadman M, Wakade C, De Sevilla LM, Dhandapani KM, Mahesh VB, Vadlamudi RK, Brann DW 2005 Cloning, expression, and localization of MNAR/PELP1 in rodent brain: colocalization in estrogen receptor- α - but not in gonadotropin-releasing hormone-positive neurons. *Endocrinology* 146:5215–5227
 - Chan KK, Tsui SK, Lee SM, Luk SC, Liew CC, Fung KP, Wayne MM, Lee CY 1998 Molecular cloning and characterization of FHL2, a novel LIM domain protein preferentially expressed in human heart. *Gene* 210:345–350
 - Johannessen M, Moller S, Hansen T, Moens U, Van GM 2006 The multifunctional roles of the four-and-a-half-LIM only protein FHL2. *Cell Mol Life Sci* 63:268–284
 - Kobayashi S, Shibata H, Yokota K, Suda N, Murai A, Kurihara I, Saito I, Saruta T 2004 FHL2, UBC9, and PIAS1 are novel estrogen receptor α -interacting proteins. *Endocr Res* 30:617–621
 - Yan J, Zhu J, Zhong H, Lu Q, Huang C, Ye Q 2003 BRCA1 interacts with FHL2 and enhances FHL2 transactivation function. *FEBS Lett* 553:183–189
 - Choi YB, Ko JK, Shin J 2004 The transcriptional corepressor, PELP1, recruits HDAC2 and masks histones using two separate domains. *J Biol Chem* 279:50930–50941
 - Scholl FA, McLoughlin P, Ehler E, de Giovanni C, Schafer BW 2000 DRAL is a p53-responsive gene whose four and a half LIM domain protein product induces apoptosis. *J Cell Biol* 151:495–506
 - Philippart U, Schratz G, Dieterich C, Muller JM, Galgoczy P, Engel FB, Keating MT, Gertler F, Schule R, Vingron M, Nordheim A 2004 The SRF target gene Fhl2 antagonizes RhoA/MAL-dependent activation of SRF. *Mol Cell* 16:867–880
 - Paul C, Lacroix M, Iankova I, Julien E, Schafer BW, Labalette C, Wei Y, Cam AL, Cam LL, Sardet C 2006 The LIM-only protein FHL2 is a negative regulator of E4F1. *Oncogene* 25:5475–5484
 - Yang Y, Hou H, Haller EM, Nicosia SV, Bai W 2005 Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. *EMBO J* 24:1021–1032

37. Lee LF, Guan J, Qiu Y, Kung HJ 2001 Neuropeptide-induced androgen independence in prostate cancer cells: roles of nonreceptor tyrosine kinases Etk/Bmx, Src, and focal adhesion kinase. *Mol Cell Biol* 21:8385–8397
38. Joung I, Strominger JL, Shin J 1996 Molecular cloning of a phosphotyrosine-independent ligand of the p56lck SH2 domain. *Proc Natl Acad Sci USA* 93:5991–5995
39. Unni E, Sun S, Nan B, McPhaul MJ, Cheskis B, Mancini MA, Marcelli M 2004 Changes in androgen receptor non-genotropic signaling correlate with transition of LNCaP cells to androgen independence. *Cancer Res* 64:7156–7168
40. Jiang T, Guo Z, Dai B, Kang M, Ann DK, Kung HJ, Qiu Y 2004 Bi-directional regulation between tyrosine kinase Etk/BMX and tumor suppressor p53 in response to DNA damage. *J Biol Chem* 279:50181–50189
41. Balasenthil S, Sahin AA, Barnes CJ, Wang RA, Pestell RG, Vadlamudi RK, Kumar R 2004 p21-Activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells. *J Biol Chem* 279:1422–1428
42. Joyce D, Bouzahzah B, Fu M, Albanese C, D'Amico M, Steer J, Klein J, Lee RJ, Segall JE, Westwick JK, Der CJ, Pestell RG 1999 Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor- κ B-dependent pathway. *J Biol Chem* 274:25245–25249
43. Schuur ER, Henderson GA, Kmetec LA, Miller, JD, Lamparski HG, Henderson DR 1996 Prostate-specific antigen expression is regulated by an upstream enhancer. *J Biol Chem* 271:7043–7051



Molecular Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.