Novel Interaction between the Co-chaperone Cdc37 and Rho GTPase Exchange Factor Vav3 Promotes Androgen Receptor Activity and Prostate Cancer Growth*

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Background: The Rho GTPase guanine nucleotide exchange factor, Vav3, is overexpressed in human prostate cancer and enhances androgen receptor transcriptional activity.

Results: Cdc37 is a novel Vav3 binding partner that enhances androgen receptor co-activation by Vav3 and increases prostate cancer cell proliferation.

Conclusion: Vav3-Cdc37 interaction is required for maximal androgen receptor function and prostate cancer growth.

Significance: Vav3-Cdc37 interaction is a potential therapeutic target for prostate cancer.

Elevated androgen receptor (AR) activity in castration-resistant prostate cancer may occur through increased levels of AR co-activator proteins. Vav3, a guanine nucleotide exchange factor, is up-regulated following progression to castration resistance in preclinical models and is overexpressed in a significant number of human prostate cancers. Vav3 is a novel co-activator of the AR. We sought to identify Vav3 binding partners in an effort to understand the molecular mechanisms underlying Vav3 enhancement of AR activity and to identify new therapeutic targets. The cell division cycle 37 homolog (Cdc37), a protein kinase-specific co-chaperone for Hsp90, was identified as a Vav3 interacting protein by yeast two-hybrid screening. Vav3-Cdc37 interaction was confirmed by GST pulldown and, for native proteins, by co-immunoprecipitation experiments in prostate cancer cells. Cdc37 potentiated Vav3 co-activation of AR transcriptional activity and Vav3 enhancement of AR N-terminal-C-terminal interaction, which is essential for optimal receptor transcriptional activity. Cdc37 increased prostate cancer cell proliferation selectively in Vav3-expressing cells. Cdc37 did not affect Vav3 nucleotide exchange activity, Vav3 protein levels, or subcellular localization. Disruption of Vav3-Cdc37 interaction inhibited Vav3 enhancement of AR transcriptional activity and AR N-C interaction. Diminished Vav3-Cdc37 interaction also caused decreased prostate cancer cell proliferation selectively in Vav3-expressing cells. Taken together, we identified a novel Vav3 interacting protein that enhances Vav3 co-activation of AR and prostate cancer cell proliferation. Vav3-Cdc37 interaction may provide a new therapeutic target in prostate cancer.

The androgen receptor (AR)4 has a critical role in prostate cancer development, growth, and progression. Androgen deprivation therapy is typically implemented in advanced prostate cancer and effectively halts tumor growth; however, this response is temporary. Recurrent disease is termed “castration-resistant” and involves the reactivation of AR signaling through several mechanisms including increased expression of AR co-activator proteins (1–3). We and others demonstrated that the Rho GTPase guanine nucleotide exchange factor (GEF) Vav3 is up-regulated in cell culture and mouse models of progression to castration-resistant prostate cancer (CRPC) (4–8). Vav3 is overexpressed in human prostate cancer samples versus benign tissue (5). Higher levels of Vav3 were recently demonstrated in metastatic human prostate cancer specimens, and Vav3 expression in primary disease was shown to predict earlier biochemical recurrence (9). Targeting a constitutively active Vav3 allele to prostate epithelium of transgenic mice results in prostate adenocarcinoma development (10). Consistent with a key role in CRPC, Vav3 enhances AR transcriptional activity and confers robust castration-resistant growth in a tumor xenograft model (4, 11).

Vav3 may also participate in other human cancers (12–15). Vav3 overexpression is correlated with poor differentiation of breast cancer and is a predictor of decreased survival in patients with glioblastoma (12). Vav3 also plays a role in the development of anaplastic large cell lymphomas (13). Vav3 is up-regulated in human gastric cancer, and Vav3 overexpression is inversely correlated with gastric cancer patient survival (14).

Vav3 and related family members, Vav1 and Vav2, form a subgroup of diffuse B-cell lymphoma (Dbl) GEF proteins. Vav3 activates Rho GTPases by catalyzing the exchange of GDP for GTP (16). Like other Dbl proteins, Vav3 contains a tandem

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arrangement of the Dbh homology (DH) domain and a pleckstrin homology (PH) domain. The DH domain interacts with Rho proteins and is responsible for catalytic activity. We previously found that GEF deficient Vav3 mutants retain the capacity to enhance androgen-inducible AR activity and AR N-C interaction, a requirement for optimal receptor transcriptional activity (17). However, mutation (W493L) or deletion of the Vav3 PH domain results in failure of Vav3 to co-activate AR. Further, the Vav3 W493L PH domain mutant is largely excluded from the nucleus. Nuclear localization of Vav3 is needed for AR co-activation, and Vav3 is present with AR on androgen response element-containing regions of chromatin (11).

To understand in greater detail Vav3 enhancement of AR transcriptional activity in prostate cancer, we searched for novel Vav3 interacting proteins. Because we found that the central region of Vav3 encompassing the DH-PH and cysteine-rich domains (CRD) was sufficient for co-activation of the AR, we used this portion of Vav3 in a yeast two-hybrid screen to identify Vav3 binding partners that might participate in AR co-activation. Interestingly, we identified the Hsp90 co-chaperone Cdc37 as a new Vav3 interacting protein. Cdc37 confers Hsp90 specificity for client protein kinases (18–20). In addition to serving as an Hsp90 co-chaperone, Cdc37 appears to also function as a chaperone independent of Hsp90 with client proteins ranging from protein kinases to steroid hormone receptors (21–27). Analysis of publicly available databases and published data reveals that Cdc37 is up-regulated in localized human prostate cancer compared with benign prostate tissues (28). We demonstrate here that Cdc37 interacts with Vav3 in human prostate cancer cells and selectively enhances Vav3 co-activation of AR, AR N-C interaction, and proliferation of Vav3-expressing prostate cancer cells.

EXPERIMENTAL PROCEDURES

Culture and Chemical Reagents—Cell culture media (RPMI 1640 and DMEM) were obtained from Life Science Technologies (Gaithersburg, MD). FBS was obtained from Hyclone Laboratories, Inc. (Logan, UT). The human prostate cancer cell lines LNCaP (ATCC, Manassas, VA, catalog no. CRL 1740; batch F-11701) and PC-3 (ATCC catalog no. CRL 1435; batch F-11154) were cultured in RPMI 1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (Life Science Technologies), and 10% FBS. The HEK293T (ATCC catalog no. CRL 11268) and COS1 (ATCC catalog no. CRL 1650) were cultured in DMEM supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (Life Science Technologies), and 10% FBS. The synthetic analog of androsten, R1881, was purchased from PerkinElmer Life Sciences. 5-Bromo-4-chloro-3-indoxyl-β-d-galactopyranoside was purchased from Gold BioTechnology, Inc. (St. Louis, MO).

Plasmids—The PSA luciferase (PSA-Luc) reporter plasmid pCMV AR, and 250 ng of pIRES2-EGFPVav3 was kindly provided by Dr. Michael Garabedian (New York University School of Medicine, New York, NY) (29, 30). Vav3DPC (amino acids 190–561) was amplified using Expand Hi Fidelity PCR system (Roche Applied Bioscience) and then subcloned into the EcoRI site of pG4-5. pIRES2-EGFP Vav3 was kindly provided by Dr. Michael McClelland (Sidney Kimmel Cancer Center, San Diego, CA). The fragment encoding Cdc37 amino acids 140–378 was subcloned into the BamHI and EcoRI sites of pKH3 (a mammalian expression vector in which three copies of HA are fused). Cdc37 and Cdc37 140–378 were subcloned into pGEX5X3 using BamHI and EcoRI. Cdc37 S13A was made using Stratagene QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Gal4DBD-ARLB, VP16AD-ARTAD, and Gal4-Tat-Luc (generously provided by Dr. Karen Knudsen, Thomas Jefferson University) were used for mammalian two-hybrid assays to evaluate AR N-C interaction.

Yeast Two-Hybrid Screening—Yeast two-hybrid screening was carried out using a LexA system based on the method of Field and Song (31) and variations thereon (32) as described in the Application Guide from OriGene Technologies. The LNCaP cDNA prey library (29) was cloned into the DNA-binding domain-containing vector, pEG202 (generously provided by Dr. Michael Garabedian). The bait was generated by subcloning the Vav3-DH-PH-CRD (DPC) domains (amino acids 190–561) in frame with the B42 activation domain in pJG4-5 to generate pG4-5-DPC (TRP1, 2u, GAL1:B42-HA–DPC). For the screen, the LNCaP cDNA library was transformed into yeast EGY48 (MATa trp1 his3 ura3 leu2:6LexAop–LEU2) containing the lacZ reporter vector, pSH18–34, and pG4-5-DPC. Approximately 3.6 × 10⁶ independent library transformants were selected on Úra–Trp–His plates and pooled. 2.6 × 10⁷ pooled plates were plated and selected on Gal/Raf–Úra–Trp–His–Leu +5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal). The 480 candidates that were able to activate both the LEU2 and lacZ reporters were further tested for dependence of the interaction on galactose, i.e., only when expressing B42-HA–DPC. Forty-eight plasmids showed activation only on galactose and were shuttled into and purified from bacteria for sequencing. Two overlapping candidates with sequences in frame with the DNA-binding protein LexA were further characterized for this study.

Reporter Gene Assays and Transfections—The transfection for reporter gene assays was done using the cationic lipid Lipofectamine (Invitrogen) according to the manufacturer’s instructions. PC3 cells were plated at a density of 3.0 × 10⁵ well of 6-well dishes or 7 × 10⁶/60-mm plates, 24 h before transfection, and the media were changed to unsupplemented DMEM prior to the transfection. The following plasmids were transfected per 60-mm plates: 5 μg of reporter plasmid PSA-luc, 250 ng of pCMV AR, and 250 ng of pIRES2-EGFP Vav3 or control vector. Half the amounts of plasmids were used per well of 6-well plates. After 4–5 h, the transfection mixture was removed, and the cells were refed with RPMI 1640 medium supplemented with 2% charcoal-stripped serum containing

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either vehicle or 1 nM synthetic androgen R1881 (PerkinElmer Life Sciences). Relative luciferase units were normalized to protein concentration.

The following plasmids were transfected per 35-mm plates for mammalian two-hybrid experiments (to assess AR N-C interaction): 250 ng of Gal4DBD-AR-LBD, 250 ng of VP16AD-ARTAD, and 250 ng of Gal4-Tata-Luc, as well as a combination of 500 ng of Vav3, 500 ng of Cdc37 (or 500 ng of Cdc37 140–378), or 500 ng of the corresponding empty vector controls. The cells were plated in 2% charcoal-stripped serum in phenol red-free medium for 24 h prior to an 18-h R1881 (1 nM) or vehicle treatment.

Virus Production and Transduction—Cdc37 shRNA in pLKO.1 was purchased from Open Biosystems (TRCN0000116633), and the control vector pLKO.1 shGFP was provided by Dr. Priyamvada Rai (University of Miami). For virus production, pLKO.1 plasmids, the packaging plasmids pCMV delta 8.2 and pCMV VSV-G were transfected into HEK 293T cells using a CalPhos mammalian transfection kit (Clontech). Eight hours after transfection, the transfection mixture was replaced with DMEM containing 10% FBS. Forty-eight hours after transfection, virus-containing media were collected, filtered through 0.45-μm cellulose acetate filters, and frozen at −80 °C.

Virus-containing media were added to cell monolayers, and after overnight incubation, the virus-containing media were replaced with fresh growth medium. After another 8 h, puromycin was added at a final concentration of 500 ng/ml for selection.

RNA Extraction and Quantitative RT-PCR—Total RNA was extracted from cells using TRIzol according to the manufacturer’s protocol (Invitrogen). Five hundred ng of total RNA was reverse transcribed using the cDNA archive kit (Applied Biosystems). Real time PCR was performed on an ABI Prism 7700 machine. TaqMan probes for PSA and 18 S RNA were purchased from Applied Biosystems. One hundred ng of cDNA was used for quantitative PCR for PSA determination and 1 ng for 18 S RNA. Relative mRNA levels were determined as described (33).

GST Protein Purification and GST Pulldown—A colony of BL21 cells containing either pGEX5x3 or pGEX5x3 Cdc37 was inoculated into 20 ml of LB and grown at 37 °C overnight. The overnight culture was seeded into fresh LB containing 100 μg/ml ampicillin. The culture was grown at 37 °C until an A600 of 1.0, and the expression of GST-tagged protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h. The cells were centrifuged at 6000 × g for 10 min. The bacteria were resuspended in 1 × PBS containing 1% Triton X-100 and 1 mM PMSF and lysed using sonication for 10 s with 30-s intervals 5 times. The cell debris was clarified by centrifugation at 15,000 × g for 10 min. The supernatant was incubated with GST-Sepharose for 30 min. The beads were then washed four times with 1 × PBS.

GST-tagged Cdc37 or Cdc37 fragments were expressed in BL21 cells. GST-tagged proteins were purified by affinity chromatography using glutathione-Sepharose 4B (Amersham Biosciences). Lysates from LNCaP cells stably expressing Vav3-FLAG expressing GFP or Cdc37 140–378 were plated in RPMI 1640 medium supplemented with 10% FBS in 6-well dishes. Cell number was determined by counting using a hemocytometer on days 2, 5, and 7 after plating. The doubling time was calculated as described (34).

Rac1 GTPase Activity Pulldown Assay—The assay was done as described with modification (35). Briefly, pQClxin Rac1 and pIRE2-EGFP CaVav3 or pIRE2-EGFP were transfected into HEK293T cells using the CalPhos mammalian transfection kit (Clontech). Forty-eight hours later, the cells were harvested into Rac assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 10 mM MgCl2, 10% glycerol, and protease inhibitor mixture) containing GST-PBD (PBD is the Rac/Cdc42-binding domain of p21-activated kinase), and the cell debris was removed by centrifugation. The cell lysates were then incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 30 min followed by four washes with Rac assay buffer. Bound proteins were solubilized in Laemmli and resolved on 12% SDS-PAGE.

Chromatin Immunoprecipitation—ChIP assays were done as previously described (11). Briefly, LNCaP cells stably expressing HA-tagged Cdc37 were grown in phenol red-free RPMI 1640 containing 2% charcoal stripped serum for 3 days. The cells were treated with 1 nM R1881 or vehicle for 16 h and then
fixed with 1% formaldehyde. Nuclear lysates containing soluble sheared chromatin were immunoprecipitated with anti-AR (Millipore), anti-HA (Sigma), or IgG control (Millipore) and protein A-Sepharose beads (Millipore). Cross-linking was reversed, and DNA fragments were purified with a Qiagen PCR purification kit. Real time PCR was performed using iQ SyberGreen supermix (Bio-Rad) to amplify the distal enhancer of the PSA gene.

### Statistical Analysis

Significance was determined using a two-tailed Student’s *t* test. *p* values less than 0.05 or 0.01 were designated with one or two asterisks, respectively.

## RESULTS

### Identification of the Co-chaperone Cdc37 as a Vav3-interacting Protein

To understand in greater detail the mechanisms of Vav3 co-activation of AR, we wanted to identify novel Vav3-interacting proteins. To define those Vav3-interacting proteins that were likely to be most relevant to AR co-activation, we determined the minimal portion of Vav3 that was sufficient to enhance AR activity. We found that the DPC truncated form of Vav3 (Fig. 1A) retained the capacity to co-activate AR (Fig. 1B). Thus, we carried out yeast two-hybrid screens using Vav3-DPC and a cDNA library from the human prostate cancer cell line, LNCaP (29) as described under “Experimental Procedures.” Two candidates with overlapping regions of the co-chaperone Cdc37 (amino acids 230–378 and 244–378) were identified. Because of the relevance of Cdc37 to AR and prostate cancer, we decided to pursue this protein further. To confirm Cdc37-Vav3 interaction in yeast, the DPC “bait” and each of the two Cdc37 clones identified in the screen were co-transformed into yeast containing the *lacZ* reporter vector pSH18-34. Galactose induced the expression of B42 activation domain in pJG4-5 (Ev) or its fusion protein, B42-HA-Vav3DPC (DPC) or B42-HA-Vav3DPCW493L (DPCW493L). The larger dark patches are positive controls containing pBait and pTarget plasmid. The white patches are negative controls containing pJG4-5-DPC and pEG202. Right panel, Western blot of B42-HA-Vav3DPC and B42-HA-Vav3DPCW493L expressed in yeast is shown.

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### Endogenous Vav3 and Cdc37 Interact in Human Prostate Cancer Cells

To determine whether full-length Vav3 and Cdc37 interact in mammalian cells, we performed GST pull-down assays using lysates from LNCaP cells stably expressing Vav3-FLAG. In these experiments, GST-tagged Cdc37 interacted specifically with Vav3 (Fig. 2A). The previously characterized interaction of AR with Cdc37 (25) was used as a positive control (Fig. 2A). Cdc37-Vav3 interaction was also observed in co-immunoprecipitation assays using HEK293T cells overexpressing Myc-tagged Vav3 and HA-tagged Cdc37 (Fig. 2B). Lastly, we showed that endogenous Cdc37 and Vav3 interacted in the CRPC human cell line, 22R1, which was derived from a xenograft model of recurrent disease (Fig. 2C) (36).
Cdc37 Selectively Enhances Vav3 Co-activation of Androgen Receptors—Because Cdc37 interacted with Vav3, we next examined the possible role of Cdc37 in Vav3 co-activation of AR. Reporter gene assays were conducted in the human prostate cancer cell line PC3 transfected with AR, Vav3, Cdc37, and the reporter plasmid PSA-luciferase. In this reporter construct, luciferase expression is under the control of the ARE-containing enhancer and the promoter of the PSA gene (4). Vav3 was found to enhance AR activity as shown previously (Fig. 3A), whereas Cdc37 alone had minimal effects (Fig. 3A). Similarly, depletion of Cdc37 using an shRNA-targeted Cdc37 lentiviral construct had no effect on AR activity in PC3 cells (Fig. 3B). Coexpression of Vav3 and Cdc37 significantly increased AR transcriptional activity compared with the effects of Vav3 alone (Fig. 3A). Thus, Cdc37 itself did not co-activate AR but instead potentiated effects of Vav3 on AR.

The phosphorylation of Cdc37 at serine 13 is involved in the regulation of Cdc37 co-chaperone activity for protein kinases (37–40). To determine whether Cdc37 potentiation of Vav3 co-activation of AR requires Cdc37 serine 13 phosphorylation, we examined a Cdc37 (S13A) mutant. Mutation of Ser-13 did not affect Cdc37 enhancement of Vav3 co-activation (Fig. 3A).

To examine further the effect of Cdc37 on Vav3 co-activation of AR, we stably depleted Cdc37 in PC3 using shCdc37 and examined Vav3 effects on AR activity. Cdc37 knockdown decreased Vav3 co-activation of AR (Fig. 4A). Although shCdc37 effectively depleted Cdc37 levels, this knockdown of Cdc37 did not affect Vav3 levels in the presence or absence of androgen (Fig. 4B). We generated a knockdown-resistant version of Cdc37 and found that Cdc37 re-expression in PC3 cells stably expressing shCdc37 restored Vav3 co-activation of AR, indicating specificity of the Cdc37 shRNA construct (Fig. 4C).

To assess the effect of Cdc37 depletion on AR regulation of the PSA target gene, we introduced the shCdc37 construct into LNCaP cells stably expressing Vav3-FLAG or GFP and measured PSA mRNA. Cdc37 knockdown decreased Vav3 co-activation of AR as determined by target gene (PSA) regulation (Fig. 4D).

To determine whether Cdc37 enhancement of Vav3 co-activation of AR was selective, we examined whether Cdc37 influenced the ability of the well characterized p160 co-activator,
TIF2, to potentiate AR activity (41, 42). Although, as expected, TIF2 increased AR activity in PC3 cells, knockdown of Cdc37 did not affect this enhancement of AR activity, suggesting that Cdc37 displayed selectivity for Vav3 (Fig. 4E).

To test the importance of Vav3-Cdc37 interaction on Vav3 co-activation of AR, we disrupted Vav3-Cdc37 interaction by overexpressing the Vav3-binding region of Cdc37 in PC3 cells. For this purpose, we made a truncated construct of Cdc37 encompassing amino acids 140–378, which lacks the N-terminal protein kinase-binding domain (39, 43) but retains the Vav3-interacting region as determined by GST pulldown (Fig. 5A). Cdc37 140–378 had no effect on AR transcriptional activity in the absence of Vav3 expression. In contrast, disruption of Vav3-Cdc37 interaction decreased Vav3-co-activation of AR (Fig. 5B; compare Vav3 with Vav3+Cdc37 140–378). This finding is consistent with a requirement for Vav3-Cdc37 interaction in enhancing AR transcriptional activity.

Cdc37 Does Not Affect Vav3 Subcellular Localization—We previously showed that Vav3 partially localizes to nuclei and that this nuclear localization is important for Vav3 co-activation of AR (11). Therefore, we tested whether Cdc37 might influence Vav3 subcellular localization. We examined transfected Vav3 levels in nuclear and cytosolic fractions of PC3 cells

**FIGURE 4. Knockdown of Cdc37 selectively decreases Vav3 co-activation of AR.** A, PC3 cells stably expressing shCdc37 or shGFP were transfected with AR, PSA-luciferase, and either Vav3 or empty vector. The means (±S.E.) of triplicate determinations from 8–10 independent experiments are plotted as fold induction (hormone/vehicle-treated) in Vav3-expressing cells compared with empty vector controls. B, cell lysates from A were subjected to SDS-PAGE and immunoblotted with indicated antibodies. C, PC3 cells with stable knockdown of Cdc37 were transfected with AR, PSA-luciferase, Vav3, and either knockdown-resistant Cdc37 (KDResCdc37) or empty vector (Ev). The means (±S.D.) of triplicate determinations from one representative experiment are plotted as fold induction (hormone/vehicle-treated). D, left panel, LNCaP cells stably expressing Vav3-FLAG or GFP were transduced with lentivirus encoding shGFP or shCdc37. The cells were treated 3 days later with either vehicle or 0.1 nM R1881 for 16 h. PSA mRNA was determined using reverse transcriptase real time PCR. The means (±S.E.) of triplicate determinations from three independent experiments are plotted as fold induction (hormone/vehicle-treated). Right panel, LNCaP cells were transduced with shGFP or shCdc37 lentiviral constructs and after 3 days were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. E, PC3 cells stably expressing shCdc37 or shGFP were transfected with AR, PSA-luciferase, and either TIF2 or empty vector. The means (±S.E.) of triplicate determinations from three independent experiments are plotted as fold induction (hormone/vehicle-treated) in TIF2-expressing cells compared with empty vector controls.

**FIGURE 5. Disruption of Vav3-Cdc37 interaction reduces Vav3 co-activation of AR.** A, lysates from LNCaP cells stably expressing Vav3-FLAG were incubated with glutathione bead-purified GST, GST-Cdc37, or GST-Cdc37 140–378. Bound proteins were immunoblotted with indicated antibodies (FLAG or GST). B, PC3 cells were transfected with AR, PSA-luciferase, and either Vav3, Cdc37 140–378, Vav3 plus Cdc37 140–378, or empty vectors (Ev). The cells were treated with vehicle or 1 nM R1881, and luciferase activity was determined 48 h later. The means (±S.E.) of triplicate determinations from three independent experiments are presented as fold induction (hormone/vehicle-treated).
that expressed shCdc37 or control (shGFP). Histone (nuclear marker) and superoxide dismutase (cytoplasmic marker) were used to estimate the purity of the nuclear and cytosolic fractions. Depletion of Cdc37 did not affect total levels of Vav3 or Vav3 subcellular localization (Fig. 6, A and B). To confirm the above finding, we disrupted Vav3-Cdc37 interaction by overexpressing a fragment of Cdc37 (140–378) and found that there was no change in Vav3 subcellular localization (data not shown). We also examined GFP-Vav3 localization by fluorescence microscopy and found no difference in Vav3 localization.

**FIGURE 6.** Vav3-Cdc37 interaction does not affect Vav3 or AR subcellular localization. A, PC3 cells stably expressing shGFP or shCdc37 were transfected with Vav3-Myc. Forty-eight hours later, cell lysates were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. Densitometry was performed on blots from three independent experiments to assess Cdc37 and Vav3 levels. B, cells from A were fractionated as described under “Experimental Procedures.” Total, cytosolic, and nuclear proteins were immunoblotted with indicated antibodies. C, total, cytosolic, and nuclear proteins from LNCaP cells stably expressing Vav3 and Cdc37 or GFP (treated with R1881 (1 nM) or vehicle for 1 h) were resolved and blotted with indicated antibodies.

**FIGURE 7.** Vav3-Cdc37 interaction does not depend on AR or Hsp90. A, left panel, cell lysates of LNCaP/Vav3 depleted of AR (shAR) or scrambled control shRNA (Scr) were subjected to SDS-PAGE and immunoblotted with indicated antibodies. Right panel, GST pulldown was performed as described above with cell lysates from LNCaP/Vav3 cells depleted of AR (shAR) or scrambled control shRNA (Scr). B, 22Rv1 cells were pretreated with 3 μM geldanamycin (GA) or vehicle control for 45 min and cell lysates were immunoprecipitated (IP) with anti-AR antibody or control IgG. The precipitated proteins were immunoblotted using indicated antibodies. Hc signifies the antibody heavy chain. C, GST pulldown assays were done as described under “Experimental Procedures” with lysates of LNCaP/Vav3-FLAG cells treated with 3 μM geldanamycin (GA) or vehicle control. D, co-immunoprecipitation assays were performed with lysates of 22Rv1 cells treated with 3 μM geldanamycin (GA) or vehicle control. Immunoprecipitation with Vav3 antibody compared with nonspecific IgG resulted in greater amounts of Cdc37 in the immunocomplexes. Geldanamycin did not decrease amounts of Cdc37 in the co-immunoprecipitates.
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in PC3 cells depleted of Cdc37 compared with controls (data not shown).

Vav3-Cdc37 Interaction Does Not Affect AR Subcellular Localization—We next tested whether Vav3-Cdc37 interaction increases ligand-dependent AR nuclear translocation and thereby increases AR transcriptional activity. We examined AR subcellular localization in LNCaP cells stably expressing Vav3 and Cdc37 (or GFP). Overexpression of Cdc37 did not affect AR subcellular localization in either the presence or absence of androgen (Fig. 6C). We confirmed these findings by examining the effect of disrupting Vav3-Cdc37 interaction by overexpressing Cdc37 140–378 in LNCaP/Vav3 cells; AR subcellular localization also was not changed by the disruption of Vav3-Cdc37 interaction (Fig. 6D). This finding suggests that Vav3 enhancement of AR co-activation by Cdc37 is not through increasing AR nuclear localization.

Vav3-Cdc37 Interaction Does Not Require Functional AR or Hsp90—Because Vav3-Cdc37 interaction was important for Vav3 co-activation of AR and AR binds to Cdc37 (25), we tested whether AR affects Vav3-Cdc37 interaction. Knockdown of AR in LNCaP/Vav3 cells did not decrease Vav3-Cdc37 binding; rather binding was somewhat increased in these in vitro GST pulldown assays (Fig. 7A). Using the Hsp90 inhibitor geldanamycin to disrupt AR-Cdc37 and hsp90-AR interactions (Fig. 7B) did not affect Vav3-Cdc37 interaction as determined by GST pulldown assays in LNCaP/Vav3 cells (Fig. 7C). Geldanamycin treatment of 22Rv1 cells also had no effect on the interaction of endogenously expressed Vav3 and Cdc37 (Fig. 7D).

Together these data indicate that neither AR nor functional Hsp90 is needed for Vav3-Cdc37 interaction.
Cdc37 Does Not Bind to PSA Enhancer AREs in Chromatin—Because Vav3 is recruited with AR to AREs in the PSA enhancer (11), we examined whether Cdc37 might also reside in transcriptional complexes. We overexpressed HA-tagged Cdc37 in LNCaP/Vav3 cells and performed chromatin immunoprecipitation assays. Although we could detect AR in the PSA enhancer region following androgen treatment, we did not detect HA-tagged Cdc37 in this region (Fig. 8). This result indicates that Cdc37 did not enhance Vav3 co-activation of AR through modulation of the AR transcriptional complex.

Cdc37 Does Not Affect Vav3 GEF Activity—Because Vav3 is a Rho GTPase family GEF protein, we examined the possibility that Vav3 GEF activity might be affected by Cdc37. We previously showed that Vav3 stimulates Rac1 activity in prostate cancer cells (35). To facilitate these experiments, we used a constitutively active Vav3 mutant that lacks the N-terminal autoinhibitory loop but retains the domains that interact with Rac1 (Fig. 9A) (44). In Rac1 activity pulldown assays, Cdc37 depletion (Fig. 9, B and C) did not affect GEF activity of constitutively active Vav3 (Fig. 9, D and E). This finding is consistent with our demonstration that Vav3 GEF activity is not required for Vav3 co-activation of AR in the presence of hormone (4).

Cdc37 Increases Vav3 Potentiation of AR N-C Interaction—We demonstrated previously that Vav3 increases the interaction between the N and C termini of AR (11), which is required for robust AR transcriptional activity (17). To determine whether Cdc37 participates in the increased AR N-C interaction seen with Vav3, we performed mammalian two-hybrid assays. This assay examines the interaction of two AR fusion proteins consisting of the AR N-terminal region (1–565, TAD) linked to the transcriptional activation domain of VP16 (VP16AD-ARTAD) and the AR ligand-binding domain (amino acids 614–919) fused to the Gal4 DNA-binding domain (Gal4DBD-ARLBD). When the two fusion proteins containing the AR N and C termini interact, transcription of the reporter plasmid Gal4-Tata-Luc occurs. As shown previously, expression of Vav3 greatly increases AR N-C interaction in transfected PC3 cells (Fig. 10). Expression of Cdc37 significantly increases AR N-C interaction in cells expressing Vav3 (Fig. 10A). Conversely, disruption of Cdc37-Vav3 interaction with Cdc37 140–378 decreases Vav3 enhancement of AR N-C interaction (Fig. 10B). These data suggest that the mechanism of Cdc37 enhancement of Vav3 co-activation of AR is through promoting the critical AR N-C interaction.

Cdc37 Selectively Promotes Proliferation of Vav3-expressing Prostate Cancer Cells—To test the biological importance of Vav3-Cdc37 interaction, we examined prostate cancer cell proliferation in cells stably expressing either Cdc37 140–378 (to block Vav3 interaction with full-length Cdc37) or GFP in LNCaP or LNCaP/Vav3 cells. Interestingly, Cdc37 140–378 selectively decreased the growth (increased doubling time) of Vav3 expressing LNCaP cells but not of parental LNCaP cells (Fig. 11, A and B). Conversely, full-length Cdc37 increased the proliferation (decreased doubling time) of Vav3 expressing LNCaP cells but not of parental LNCaP cells (Fig. 11, C and D). These findings are in line with our observations that Cdc37 140–378 decreased and full-length Cdc37 increased Vav3 co-activation of AR but did not affect AR transcriptional activity in the absence of Vav3 expression.

DISCUSSION

AR transcriptional activity is critical during prostate cancer development and remains indispensable for the emergence and growth of CRPC. Vav3 enhances AR transcriptional activity in the setting of androgen-dependent growth, as well as in castration-resistant disease (4, 5, 11, and 35 and unpublished data). Because of the critical nature of Vav3 action in prostate cancer,
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we sought to identify novel Vav3 interacting proteins that selectively affect Vav3 augmentation of AR action. We identified Cdc37, a co-chaperone of Hsp90, as a novel binding partner of Vav3 that is required for maximal Vav3 co-activation of AR and Vav3 promotion of AR N-C interaction. Further, the interaction between Vav3 and Cdc37 promotes the proliferation of prostate cancer cells. These data support the importance of Vav3-Cdc37 interaction for elevated AR activity and prostate cancer growth.

Other Vav3 binding partners include the Rho GTPases (Rac1, Cdc42, RhoA, and RhoG), as well as signaling proteins such as PI3K, Shc, Grb2, phospholipase C_2y, and adaptor protein APS (45, 46). Using a series of Vav3 GEF deficient mutants, we previously showed that GEF activity is not needed for Vav3 enhancement of hormone-induced AR activity (4). Thus, Rho GTPase interaction with Vav3 is unlikely to be involved in Vav3 co-activation of AR. Other binding proteins such as EGF receptor, insulin receptor, and insulin-like growth factor receptor interact with the C-terminal SH3-SH2-SH3 domains of Vav3, which are also dispensable for AR co-activation (4). In this study, we sought to identify proteins that interact with Vav3 and may play a role in AR co-activation. We first defined the minimal Vav3 region that retains the capacity to modulate AR. Based on our earlier work showing that neither the N- nor C-terminal Vav3 domains are needed, we tested the central regions DH-PH-CRD (DPC) and found that this truncated Vav3 protein was fully able to enhance AR activity.

Hsp90 family members are molecular chaperones that are required for maturation and proper folding of many cell signaling molecules, including steroid hormone receptors and a variety of protein kinases (47). Cdc37 is an Hsp90 co-chaperone that confers Hsp90 specificity for protein kinases (18–20). Cdc37 binding to Hsp90 inhibits the intrinsic ATPase activity of Hsp90 and facilitates the loading of client proteins (48–50). The two Vav3-interacting Cdc37 clones that we identified in the yeast two-hybrid screens are C-terminal to the Hsp90 interacting region, and therefore Hsp90 and Vav3 are independently affect Vav3 augmentation of AR action. Cdc37 S13A was not compromised in promotion of Vav3 co-activation of AR. This conclusion is in agreement with previous work by Rao et al. (25), who showed that although Cdc37 interacts with AR, overexpression of Cdc37 did not enhance AR transcriptional activity. These authors did show that Cdc37 1–173 partially decreased AR activity in CAT assays conducted in transfected CV1 cells; however, the molecular mechanism of this relatively small effect is unknown and could be due to indirect/secondary effects of blocking kinase chaperone activity of Cdc37 (25). Together these data support a specific role of the Cdc37-Vav3 interaction in regulating AR activity.

We found that Cdc37 enhances Vav3 co-activation of AR through increasing the critical AR N-C interaction. Several other possible mechanisms were also explored. Although Cdc37 can affect client protein levels and subcellular localization (27, 52), for example, Cdc37 stabilizes and promotes nuclear localization of an active cleaved Ryk (52), we did not observe Cdc37 effects on Vav3 protein levels or subcellular localization. Vav3-Cdc37 interaction also did not increase AR nuclear translocation as occurs with type II AR co-regulators (53). We also ruled out the possibility that Vav3-Cdc37 increases AR transcriptional activity through direct modulation of the AR transcriptional complex in chromatin. Although Vav3 resides with AR in transcriptional complexes of the PSA enhancer (11), Cdc37 did not. Mutation of Cdc37 at Ser-13 disrupts Cdc37 co-chaperone activity (37–40). However, Cdc37 S13A was not compromised in promotion of Vav3 co-activation of AR. These results suggest that client kinases that depend on Ser-13 phosphorylation are not involved in Cdc37 enhancement of Vav3 co-activation of AR. However, we cannot rule out the involvement of Cdc37 client kinases that do not require Cdc37 serine 13 phosphorylation or N-terminal interaction with Cdc37. The Cdc37 N terminus was indispensable for enhancement of Vav3 co-activation of AR, although the N-terminal truncation mutants retained the capacity to bind to Vav3.

In contrast to Cdc37 interaction with its client kinases, Cdc37 binds to Hsp90 through its central and C-terminal segments (50). The two Vav3-interacting Cdc37 clones that we identified in the yeast two-hybrid screens are C-terminal to the Hsp90 interacting region, and therefore Hsp90 and Vav3 are unlikely to compete for binding to Cdc37. Further, functional Hsp90 was not required for Vav3-Cdc37 interaction because geldanamycin, an Hsp90 inhibitor, had no effect on this interaction.

Although 13 Hsp90 inhibitors are undergoing clinical evaluation and there are 23 active Hsp90 inhibitors in ongoing oncology trials, no Hsp90-targeted drugs have been approved, mostly because of toxicity (54). Additionally, 17-N-Allylamino-17-deoxygeldanamycin (17-AAG); retaspimycin hydrochloride (IPI-504), two Hsp90 inhibitors, provided no benefit to CRPC patients (55, 56). The poor therapeutic effect of Hsp90 inhibitors may be the consequence of inhibiting widespread client proteins and may thereby result in activation of oncogenic signaling pathways such as Src and co-chaperone Hsp27 up-regulation (57, 58). Inhibition of Cdc37, a co-chaperone of Hsp90 that is overexpressed in cancer cells, may represent a better drug target in cancers (59). However, Cdc37 also has a relatively wide range of clients. Given the importance of Cdc37 in
enhancing AR transcriptional activity via Vav3, disrupting Vav3-Cdc37 interaction may represent another therapeutic strategy. This approach could theoretically be accomplished through small molecule interference of the Cdc37-Vav3 interacting region, which might be a treatment option with potentially limited side effects. In conclusion, our data suggest that Cdc37 plays a unique role in regulating Vav3 enhancement of AR activity and prostate cancer growth. Targeting the interaction between Vav3 and Cdc37 may represent an opportunity to selectively inhibit AR activity and prostate cancer growth.

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