

## Suppression of Androgen Receptor Transactivation by Pyk2 via Interaction and Phosphorylation of the ARA55 Coregulator\*

Received for publication, November 26, 2001, and in revised form, February 14, 2002  
Published, JBC Papers in Press, February 20, 2002, DOI 10.1074/jbc.M111218200

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**The proline-rich tyrosine kinase 2 (Pyk2) was first identified as a key kinase linked to the MAP kinase and JNK signaling pathways that play important roles in cell growth and adhesion. The linkage between Pyk2 and the androgen receptor (AR), an important transcription factor in prostate cancer progression, however, remains unclear. Here we report that using the full-length androgen receptor-associated protein, ARA55, coregulator as bait, we were able to isolate an ARA55-interacting protein, Pyk2, and demonstrated that Pyk2 could repress AR transactivation via inactivation of ARA55. This inactivation may result from the direct phosphorylation of ARA55 by Pyk2 at tyrosine 43, impairing the coactivator activity of ARA55 and/or sequestering ARA55 to reduce its interaction with AR. Our finding that Pyk2 can indirectly modulate AR function via interaction and/or phosphorylation of ARA55 not only expands the role of Pyk2 in AR-mediated prostate cancer growth but also strengthens the role of ARA55 as an AR coregulator.**

The androgen receptor (AR),<sup>1</sup> a transcription factor, requires coregulators to exert its optimal or proper function in the control of cell growth and death (1–4). Several AR coregulators including ARA24, ARA55, ARA70, ARA160, and ARA267 were isolated in our previous studies (5–9). Transactivation assays indicated that ARA55 can function as a coactivator to enhance AR function in a ligand-dependent manner in several prostate cancer cells (6). Thereafter, Yang *et al.* (10) found that Hic-5, a mouse homolog of human ARA55, could also function as a coregulator to increase the transactivation of AR or glucocorticoid receptor and induce cell senescence in fibroblasts (11). Tissue distribution studies suggest that ARA55 may be differentially expressed during various stages of prostate cancer (12). The detailed physiological role of ARA55 and its potential

regulation of prostate cancer progression, however, remain unclear.

Early studies showed that various kinase signaling pathways could modulate AR transactivation via phosphorylation of AR at various amino acids (13–15). For example, the HER2/Neu-mitogen-activated protein (MAP) kinase pathway can phosphorylate AR, increasing its ability to recruit coregulators and enhancing AR transactivation (14). In contrast, the PI3K/Akt pathway can phosphorylate AR, reducing its ability to recruit coregulators and decreasing AR transactivation (15). Similar results indicating cross-talk between kinase signaling pathways and other nuclear receptors (NR) to increase NR recruitment of coregulators have been reported including estrogen receptor (16) and steroidogenic factor 1 (SF-1) (17). Furthermore, several kinases have been reported to phosphorylate NR coregulators resulting in increased NR transactivation including SRC-1 and SRC-3 (18, 19). These findings lead us to hypothesize that some kinases may be able to modulate AR function via phosphorylation of AR coregulators. Using full-length ARA55 as bait in a yeast two-hybrid assay we found that proline-rich tyrosine kinase 2 (Pyk2) can interact with ARA55. We subsequently investigated if Pyk2 could modulate AR function via interaction/phosphorylation of ARA55.

Pyk2, a member of the focal adhesion kinase (FAK) family, is a mediator of G-protein-coupled receptors and may be involved in the regulation of the MAP kinase and JNK signal pathways (20–23). Early studies suggested that some upstream regulators such as integrins, platelet-derived growth factor (PDGF), stress signals, or interleukin-2 could induce Pyk2 activity by modulating the phosphorylation of Pyk2 (24–28). Pyk2 is detected in many cells such as neurons, bone marrow, smooth muscle, and prostate cells (29, 30). Tissue staining also indicates that Pyk2 expression is decreased with increasing malignancy of prostate cancer (30). The significance of Pyk2 interaction/phosphorylation of ARA55 in prostate cancer progression is currently unclear.

Here we demonstrate that Pyk2 is an ARA55-interacting protein that represses AR transactivation via phosphorylation of ARA55. This new signal pathway from Pyk2-ARA55-AR may represent a novel mechanism to modulate AR function in prostate cancer.

### EXPERIMENTAL PROCEDURES

**Materials and Plasmids**—5 $\alpha$ -dihydrotestosterone (DHT) and doxycycline were obtained from Sigma. PDGF-B-chain homodimer (PDGF-BB) and hygromycin B were purchased from Invitrogen. The anti-AR polyclonal antibody, NH27, was produced as described (31). A monoclonal antibody for ARA55 (anti-Hic-5) was purchased from Transduction Laboratories. Antibodies for Pyk2 (anti-Pyk2) and phosphotyrosine (anti-Tyr(P)) were purchased from Upstate Biotechnology. His probe (H-3) was purchased from Santa Cruz Biotechnology. The pKH3Pyk2 expres-

\* This work was supported by National Institutes of Health Grant CA71570. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: AR, androgen receptor; JNK, c-Jun NH<sub>2</sub>-terminal kinase; NR, nuclear receptors; DHT, 5 $\alpha$ -dihydrotestosterone; Pyk2, proline-rich tyrosine kinase 2; PKM, kinase-negative Pyk2; ARA, androgen receptor-associated protein; DBD, DNA-binding domain; LUC, luciferase; MMTV, mouse mammary tumor virus; RL, *Renilla* luciferase; PSA, prostate-specific antigen; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein; FCS, fetal calf serum; -UL, minus uronolactone.

sion plasmid was kindly provided by Dr. Jun-Lin Guan, Cancer Biology Laboratory, Dept. of Molecular Medicine, College of Veterinary Medicine, Cornell University. The pEF-PKM expression plasmid (kinase-negative Pyk2) was kindly provided by Dr. Aknori Takaoka, Dept. of Immunology, University of Tokyo, Japan and Dr. Joseph Schlessinger, Dept. of Pharmacology, New York University Medical Center. The PKM cDNA was subcloned into the *EcoRI* site of the pcDNA4A expression vector. The PC-3(AR)2 cell line was provided by T. J. Brown, University of Toronto, Ontario, Canada. We also constructed ARA55 into the pcDNA4-His, GAL4, and VP16 vectors using the *Bam*HI enzyme. The Pyk2 C terminus (675–1009) that was isolated from the yeast library was constructed into the GAL4 and VP16 vectors.

**Cell Culture**—DU145, PC-3, H1299, and MCF-7 human cancer cells, as well as COS-1 monkey kidney cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (25 units/ml), streptomycin (25  $\mu$ g/ml), and 10% fetal calf serum (FCS). PC-3(AR)2 human cancer cells were maintained in RPMI enzyme 1640 containing penicillin (25 units/ml), streptomycin (25  $\mu$ g/ml), and 10% charcoal-stripped FCS. LNCaP human prostate cells were maintained in RPMI 1640 containing penicillin (25 units/ml), streptomycin (25  $\mu$ g/ml), and 10% FCS.

**Screening of Human Prostate cDNA Library by Yeast Two-hybrid Assay**—A CytoTrap yeast two-hybrid system (Stratagene) and pMyr plasmid library (Stratagene) consisting of a DNA sequence encoding a myristylation membrane localization signal fused with human prostate and testis cDNAs were used for yeast two-hybrid screening. The pSos vector containing the *hSos* gene fused with the full-length ARA55 cDNA using the *Bam*HI cloning site served as bait. Expression of the myristylation sequence-tagged target protein is induced by galactose but not glucose and is anchored to the cell membrane. The library was screened by co-transformation of the pSos-ARA55 bait construct into a temperature-sensitive *cdc25H* yeast strain that cannot grow at 37 °C. Once the bait protein physically interacts with the target protein, the *hSos* protein is recruited to the membrane activating the Ras signaling pathway and allowing the temperature-sensitive mutant yeast strain to grow at 37 °C.

**Phosphorylation Site-directed Mutagenesis of Wild Type ARA55**—The potential tyrosine kinase target site on wild type ARA55 was mutated by site-directed mutagenesis using two primers 5'-GGACCACCTGTTTCAGCACGGTATG-3' and 5'-CATACCGTGTGAACAGGTGTGCC-3', substituting phenylalanine for tyrosine at amino acid 43 (Y43F). The mutant ARA55 PCR product was constructed into the pcDNA4A expression vector for the AR transactivation assay and constructed into the GAL4 and VP16 expression vectors for mammalian two-hybrid assay.

**Stable Transfection of DU145 Cells**—DU145 cells, an ARA55-negative human prostate cancer cell line, was transfected with pBig or pBig-ARA55 for 24 h using SuperFect (Qiagen, Chatsworth, CA). The cells were then selected using 100  $\mu$ g/ml hygromycin B (32). A single colony was chosen, amplified, and confirmed by reporter gene and Western blotting assay.

**Transfection and Report Gene Assay**—DU145, H1299, MCF-7, LNCaP, PC-3(AR2), and COS-1 cells were grown in appropriate medium. Transfection was performed by modified calcium phosphate precipitation as previously described (33) or by using SuperFect according to the manufacturer's procedure. After incubation for 24 h with charcoal-stripped medium, the medium was changed, and cells were treated with ethanol or DHT for another 24 h and then harvested for the luciferase assay. The MMTV-LUC or PSA-LUC plasmids were used as the reporter genes, and SV40-pRL (Promega) was used as an internal control. The Dual-luciferase reporter 1000 assay system (Promega) was employed to measure LUC activity.

**Mammalian Two-hybrid Assay**—Transfections in DU145, H1299, or COS-1 cells were performed using the calcium phosphate precipitation method. The cells were transiently co-transfected with the pG5-LUC reporter plasmid and 3.5  $\mu$ g of both the GAL4 and pVP16-hybrid expression plasmids. The SV40-pRL plasmid was used as an internal control. For the interaction between ARA55 and AR the cells were treated with 10 nM DHT for another 24 h and then harvested for luciferase assays as previously described.

**Co-immunoprecipitation and Western Blotting**—H1299 cells were co-transfected with Pyk2 and His-ARA55 expression plasmids by SuperFect. Cells were lysed using radioimmune precipitation buffer (RIPA) following the protocol from Santa Cruz Biotechnology and supplemented with protease inhibitor mixture tablet (Roche Molecular Biochemicals) and 1 mM phenylmethylsulfonyl fluoride. Anti-His probe antibody (Santa Cruz) was used to immunoprecipitate the complex from the whole cell lysate. The complex was then resolved on a 10% SDS-

polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane and then blotted with anti-Pyk2. The Pyk2 bands were resolved by an alkaline phosphatase detection kit (Bio-Rad). In the PC-3 whole cell lysate, an anti-Hic-5 antibody was used to immunoprecipitate the endogenous ARA55 and Pyk2 complex followed by the previously described procedure. After transfection with various combinations of pcDNA4A-ARA55, pSG5AR, Pyk2, and PKM by SuperFect, H1299 cells were treated with 10 nM DHT for another 24 h and then starved in DMEM with 0.1% FCS and 10 nM DHT for 16 h. The cells were treated with 1.5 nM PDGF-BB and then harvested. His probe was used to precipitate the His-ARA55 and AR complex that was resolved by Western blotting using anti-AR NH27 and anti-Hic-5 antibodies, respectively. For the ARA55 phosphorylation experiment, RIPA was supplemented with 1 mM pyrophosphate, 50 mM sodium fluoride, and 2 mM sodium vanadate. The anti-Hic-5 antibody was used to immunoprecipitate ARA55, and then the anti-phosphotyrosine antibody was applied for Western blotting.

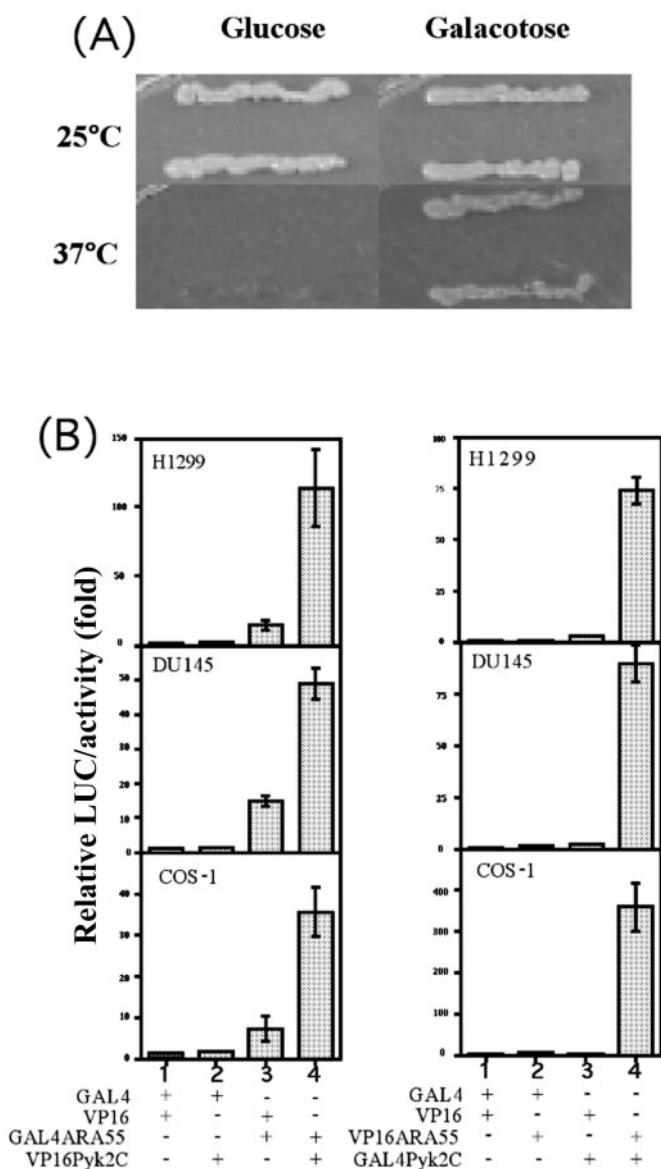
## RESULTS

**ARA55 Interacts with Pyk2 in Yeast and Mammalian Cells**—Full-length ARA55 was used as bait to screen prostate and testis libraries using the CytoTrap yeast two-hybrid system. The Pyk2 C-terminal sequence (amino acids 675–1009) was isolated, and its interaction with ARA55 was reconfirmed in a yeast growth assay. When we co-transfected the pSos-ARA55 and pMyr-Pyk2 C terminus plasmids into the temperature-sensitive mutant yeast, cell colonies appeared on both SD/glucose (-UL) agar and SD/galactose (-UL) agar plates at 25 °C and also on SD/(-UL)/galactose agar plates at 37 °C (Fig. 1A). Glucose represses the expression of target proteins, preventing yeast growth at 37 °C. Then we constructed ARA55 and the Pyk2 C terminus into both the GAL4 and VP16 vectors and tested their interaction via the mammalian two-hybrid system in various human cell lines. As shown in Fig. 1B, GAL4-ARA55 and VP16-Pyk2 C terminus (*left panel*) and GAL4-Pyk2 C terminus and VP16-ARA55 (*right panel*) can interact strongly in H1299, DU145, and COS-1 cells.

ARA55 also interacts with Pyk2 *in vivo* by co-immunoprecipitation. As shown in Fig. 2A, exogenous Pyk2 can be co-immunoprecipitated with His-ARA55 from H1299 whole cell extracts using an anti-His antibody. In addition, endogenous ARA55 and Pyk2 can be co-immunoprecipitated in PC-3 cells using an anti-Hic-5 antibody, confirming the *in vivo* interaction of the two proteins (Fig. 2B). Together, results from Figs. 1 and 2 demonstrate that ARA55 can interact with Pyk2 using various *in vitro* and *in vivo* systems in several yeast and mammalian cells. Interestingly, whereas Pyk2 can interact with ARA55, an AR-interacting protein, Pyk2 cannot interact with AR in the same mammalian two-hybrid assay (Fig. 3) or co-immunoprecipitation assays (data not shown) suggesting that any effect of Pyk2 on AR activity may require interaction with ARA55.

**Expression of Pyk2 and ARA55 in Various Cell Lines**—50  $\mu$ g of whole cell lysate of each cell line were separated on a SDS-PAGE gel and blotted by anti-Pyk2 and anti-ARA55 antibodies. Pyk2 is almost ubiquitously expressed among these cell lines with the exception of H1299 (Fig. 4). In contrast, ARA55 was only expressed in PC-3, PC-3(AR2), and COS-1 cells (Fig. 4). H1299 cells were then used for further study because of relatively lower expression of both Pyk2 and ARA55. Results from Fig. 4 also offered us the opportunity to study Pyk2 function in ARA55-positive versus -negative cells.

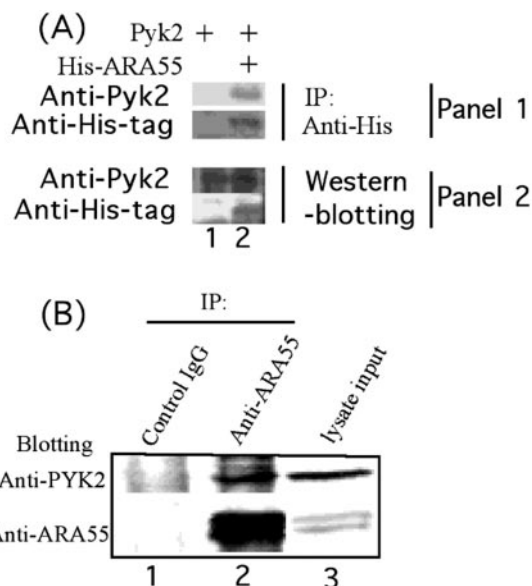
**Suppression of ARA55-induced AR Transactivation by Pyk2**—To study the potential influence of Pyk2 on AR function via interaction with ARA55, we compared the effect of Pyk2 on AR transactivation in ARA55-negative versus ARA55-positive cells. As shown in Fig. 5, whereas Pyk2 can significantly repress AR transactivation in ARA55-positive PC-3(AR)2 cells, Pyk2 has only a marginal effect in LNCaP, MCF-7, and DU145



**FIG. 1. ARA55 interacts with Pyk2 in yeast and mammalian cells.** *A*, the interaction between ARA55 and Pyk2 C terminus in yeast. 0.3  $\mu$ g of pSos-ARA55 and pMyr-Pyk2C plasmids was co-transformed into *cdc25H* yeast cells. The entire transformation reaction was plated onto a 100 mm SD/glucose (-UL) agar plate that was then incubated at 25 °C until colonies appeared. Colonies were transferred to two SD/galactose (-UL) and two SD/glucose (-UL) agar plates that were then cultured at 25 °C (upper panel) and 37 °C (lower panel). At the selective temperature of 37 °C colonies only appeared on SD/(-UL)/galactose agar plate because glucose represses the expression of target proteins. *B*, ARA55 interacts with the Pyk2 C terminus in the mammalian two-hybrid assay. DU145, COS-1, and H1299 cells cultured in 60-mm dishes were transiently transfected with 3  $\mu$ g of the pG5-LUC reporter plasmid and 10 ng of the SV40-pRL internal control plasmid as well as 3.5  $\mu$ g of GAL4DBD, VP16, GAL4DBD-ARA55, VP16-ARA55, GAL4DBD-Pyk2 C terminus, or VP16-Pyk2 C terminus as indicated. LUC assay was performed 24 h after transfection. The LUC activity of the interaction between GAL4DBD and VP16 was set as 1-fold. All values represent the mean  $\pm$  S.D. of three independent experiments.

cells that lack endogenous ARA55. These results further suggest that Pyk2 may suppress AR transactivation through the interaction with ARA55.

We then used PC-3(AR2), an ARA55-positive cell line stably transfected with AR to further characterize the effect of Pyk2 on AR transactivation. As shown in Fig. 6, Pyk2 can suppress AR transactivation in a dose-dependent manner using the PSA or MMTV promoters linked to the LUC (Fig. 6A) or chloram-



**FIG. 2. In vivo co-immunoprecipitation of ARA55 and Pyk2.** *A*, 5  $\mu$ g of pcDNA4A-His, or pcDNA4A-His-ARA55, and 5  $\mu$ g of pKH3-Pyk2 were co-transfected into H1299 cells for 24 h as indicated. Anti-His antibody was added to 500  $\mu$ g of protein lysate for 2 h to immunoprecipitate the His-ARA55 and Pyk2 complex. After SDS-PAGE, the protein complexes were transferred to a polyvinylidene difluoride membrane that was Western blotted with anti-Pyk2 and anti-His antibodies (*A*, panel 1). The relative expression of Pyk2 in the protein lysate was shown by Western blotting (*A*, panel 2). *B*, normal IgG (lane 1) and anti-Hic-5 (lane 2) for ARA55 were added to PC-3 whole cell lysates for 2 h to immunoprecipitate the ARA55 and Pyk2 complex. The samples were then separated as described above and Western blotted with anti-Pyk2 and anti-Hic-5 antibodies. PC-3 lysate input was loaded to show the relative expression of Pyk2 and ARA55 (lane 3).

phenolic acetyltransferase (CAT) reporter systems (data not shown). In contrast, PKM with a lysine to alanine substitution at amino acid 475 of Pyk2 (20) only slightly affected AR transactivation (Fig. 6A).

To verify that ARA55 is required for Pyk2 to suppress AR activity, we then stably transfected DU145 with ARA55 using a doxycycline-inducible system. As with PC-3(AR2), transfection of Pyk2, ARA55, and AR into parent ARA55-negative DU145 cells resulted in suppression of AR transactivation in a dose-dependent manner (data not shown). Treatment of the stably transfected pBIG-ARA55 DU145 cells with doxycycline to induce ARA55 expression resulted in enhanced AR transactivation that could be suppressed by the exogenous Pyk2. In contrast, DU145 cells stably transfected with the pBIG vector demonstrated no increased AR activity upon doxycycline treatment and were unaffected by exogenous Pyk2. (Fig. 6B). These results support the essential role of ARA55 in suppression of AR transactivation by Pyk2.

**Molecular Mechanisms of Pyk2 Suppression of ARA55-induced AR Transactivation**—Since PKM, a kinase-negative Pyk2 mutant (20), failed to repress AR transactivation, we suspected that Pyk2 may need to phosphorylate ARA55 to suppress AR function. Sequence analysis revealed a potential Pyk2 tyrosine kinase phosphorylation site, HLYST, on ARA55 at amino acids 41–45. We therefore mutated this potential Pyk2 target site to HLFST and tested its effect on the AR transactivation. Results in Fig. 7A demonstrate that PDGF-induced Pyk2 can enhance the tyrosine phosphorylation of ARA55 (lane 3 versus lane 2) *in vivo*. In contrast, PDGF-induced Pyk2 only marginally increased the phosphorylation of mutant ARA55 (lane 4 versus lane 3). As shown in Fig. 7B wild type ARA55 alone, but not Pyk2, enhances AR transactivation (lanes 4 versus 2 and lanes 3 versus 2, respectively), but addi-

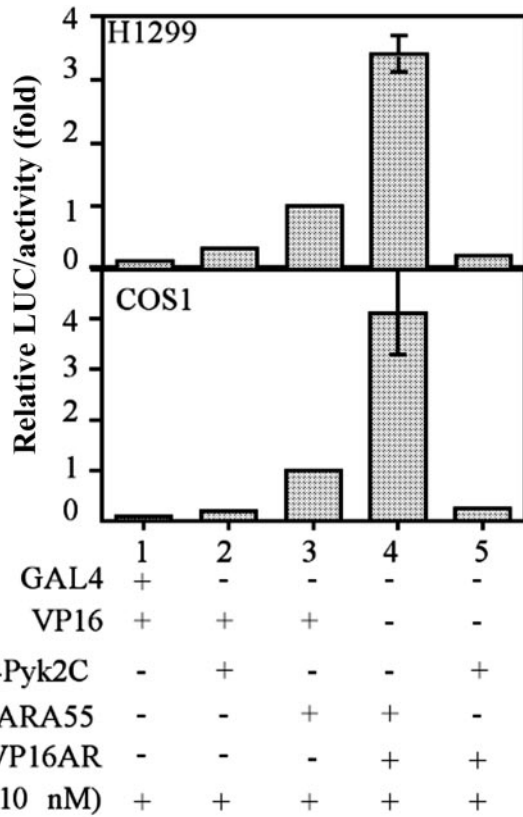


FIG. 3. **Pyk2 interacts with ARA55 but not AR in the mammalian two-hybrid assay.** COS-1 and H1299 cells were transiently co-transfected with 3  $\mu$ g of pG5-LUC reporter plasmid, 10 ng of SV40-pRL internal control plasmid, as well as 3.5  $\mu$ g of GAL4DBD, VP16, GAL4-ARA55, GAL4-Pyk2C terminus, or VP16-AR as indicated. 24 h after transfection the cells were treated with 10 nM DHT for another 24 h. The LUC activity of the sample transfected with GAL4DBD and VP16 was set as 1-fold. All values represent the mean  $\pm$  S.D. of three independent experiments.

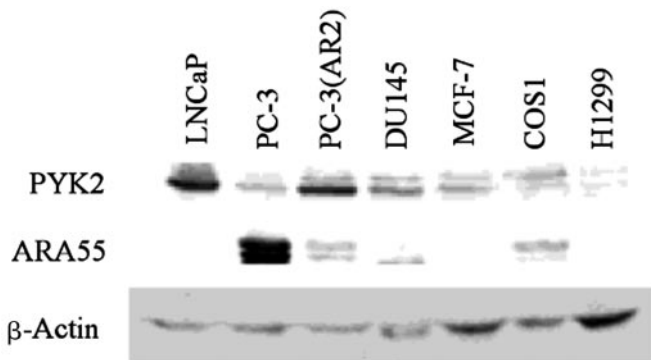


FIG. 4. **The expression of ARA55 and Pyk2 in various cell lines.** LNCaP, PC-3, PC-3(AR2), DU145, MCF-7, COS-1, and H1299 cells were cultured and then harvested for Western blotting. 50  $\mu$ g of whole cell lysate from each cell line were loaded and separated on a 10% SDS-PAGE gel. Anti-Pyk2 and anti-Hic-5 (ARA55) antibodies were used to Western blot the polyvinylidene difluoride membrane to detect the expression of Pyk2 and ARA55, respectively. Anti- $\beta$ -actin antibody was used to detect  $\beta$ -actin expression as a loading control.

tion of Pyk2 suppresses the ARA55-enhanced AR transactivation (lane 5 versus lane 4). Interestingly, the mutant ARA55, like wild type ARA55, enhances AR transactivation (lane 6 versus lane 2), but addition of Pyk2 only marginally suppresses mutant ARA55-induced AR transactivation (lane 6 versus lane 7). The data from Fig. 7, A and B demonstrate that Pyk2 enhances the phosphorylation of ARA55 at residue 43 to suppress AR transactivation.

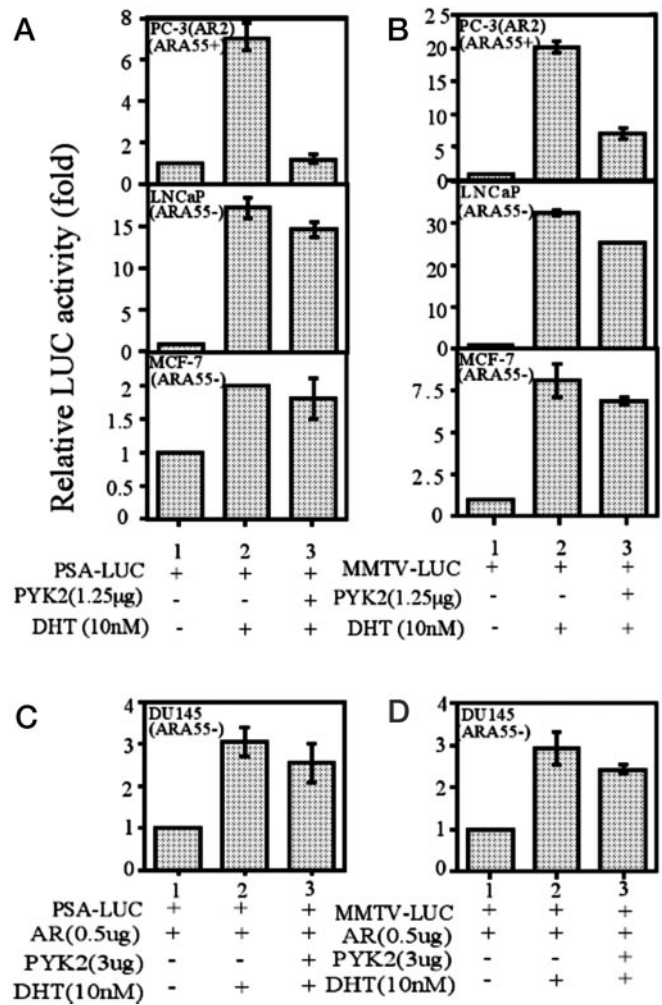
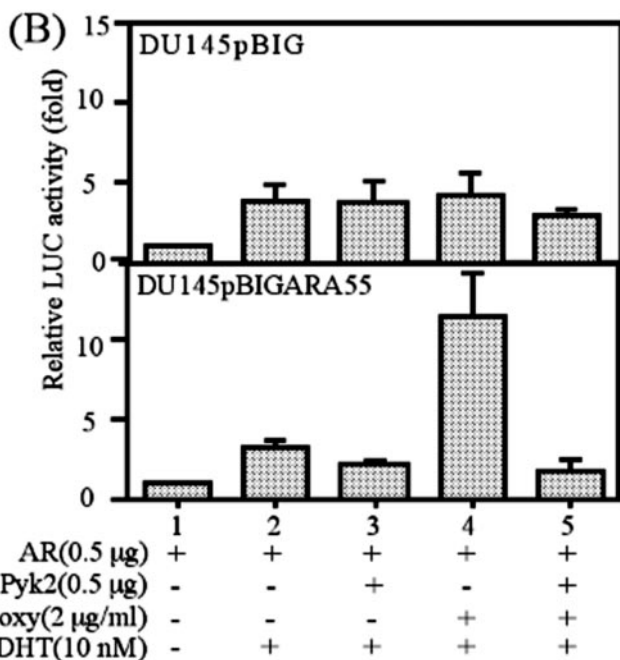
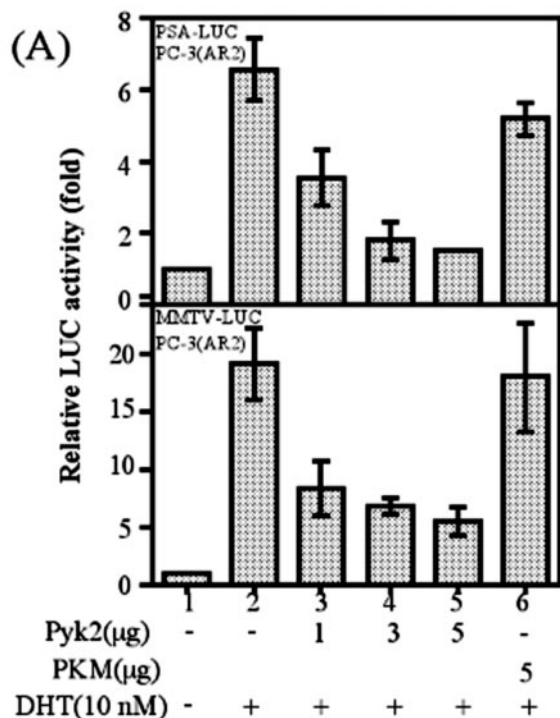


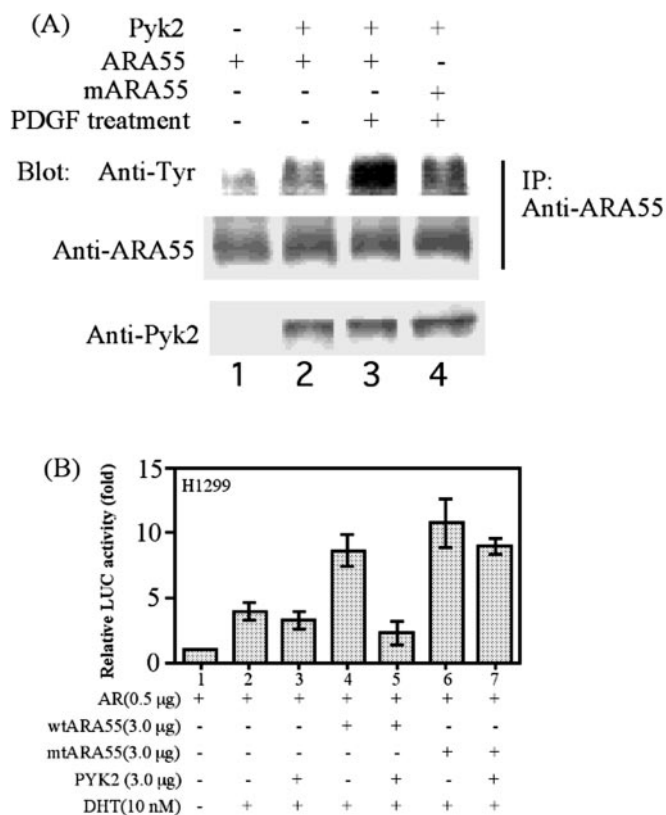
FIG. 5. **The effect of Pyk2 on AR transactivation in ARA55-positive and -negative cells.** PC-3(AR)2, LNCaP, and MCF-7 cells were seeded in 35-mm dishes. Cells were transiently co-transfected with 0.75  $\mu$ g of MMTV-LUC or PSA-LUC reporter plasmids, 5 ng of SV40-pRL internal control plasmid, as well as 1.25  $\mu$ g of Pyk2 using the SuperFect reagent. DU145 cells were seeded in 60-mm dishes and co-transfected with 3  $\mu$ g of each reporter plasmid, 10 ng of SV40-pRL internal control plasmid, 0.5  $\mu$ g of AR, and 3  $\mu$ g of Pyk2 using calcium phosphate precipitation. The total amount of DNA in all experiments was adjusted by addition of the backbone vectors. Cells were treated with ethanol or 10 nM DHT for 24 h after transfection as indicated. The LUC activity of AR with ethanol treatment was set as 1-fold. All values represent the mean  $\pm$  S.D. of three independent experiments.

Pyk2 may also inhibit ARA55-induced AR transactivation by blocking the interaction between AR and ARA55 or by sequestering ARA55 away from AR. Results from the mammalian two-hybrid assay in H1299 cells show that wild type Pyk2, but not kinase-negative PKM, can block the interaction between AR and ARA55 (Fig. 8A). Interestingly, the interaction between AR and the Y43F ARA55 mutant can only be partially blocked by either Pyk2 or PKM.

We then used co-immunoprecipitation to verify the ability of Pyk2 to block the interaction between AR and ARA55 in H1299 cells. As shown in Fig. 8B, anti-His antibody precipitates the complex containing AR and wild type or mutated ARA55. Addition of Pyk2 in the presence of PDGF, a growth factor that activates Pyk2, can then block the interaction between AR and wild type ARA55 but not the interaction between AR and mutated ARA55 (lanes 3 and 4 versus lanes 7 and 8). PKM only slightly blocks the interaction between AR and ARA55 (lanes 5 and 6). Results from both the mammalian two-hybrid and co-immunoprecipitation assays demonstrate that Pyk2, but not



**FIG. 6. Pyk2 suppresses AR transactivation in ARA55-positive PC-3(AR2) and ARA55 stably transfected DU145 cells.** *A*, PC-3(AR2) cells were transiently co-transfected with 3 μg of PSA-LUC or MMTV-LUC reporter plasmids, 10 ng of SV40-pRL internal control plasmid, increasing amounts of full-length Pyk2, and 3 μg of PKM for 16 h as indicated. The total amount of DNA in each transfection was adjusted by addition of backbone vectors. Cells were treated with ethanol or 10 nM DHT for 24 h and then harvested for the LUC assay. *B*, DU145(pBIGARA55) and DU145(pBIG) stably transfected cells were transiently co-transfected with 1 μg of MMTV-LUC reporter plasmid, 5 ng of SV40-pRL internal control plasmid, and 0.5 μg of AR and Pyk2 expression plasmids using SuperFect for 16 h as indicated. The cells were then treated with ethanol or 10 nM DHT and 2 μg/ml doxycycline for another 24 h as indicated. The total amount of DNA in each transfection was adjusted by addition of backbone vectors. The LUC activity of AR with ethanol treatment was set as 1-fold. All values represent the mean ± S.D. of three independent experiments.



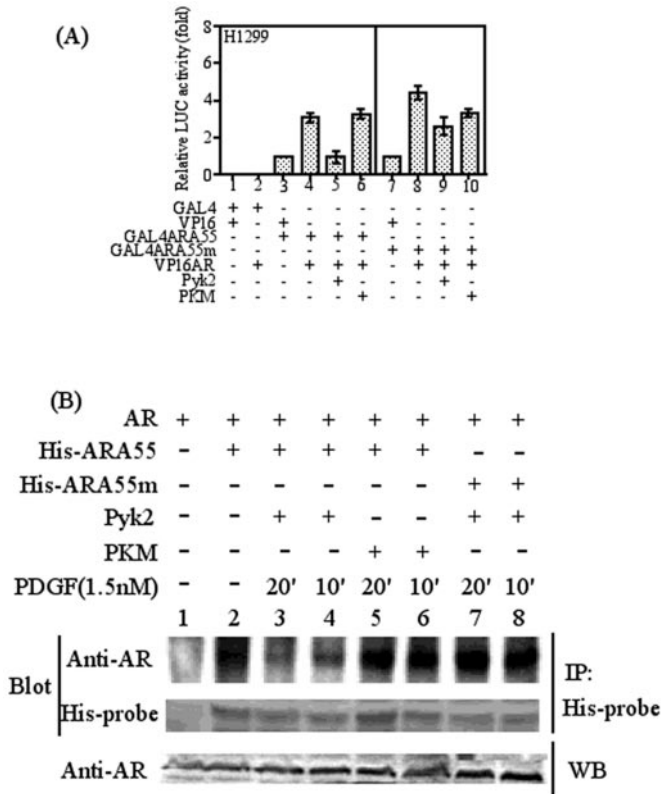
**FIG. 7. Pyk2 suppresses AR transactivation by phosphorylation of ARA55.** *A*, H1299 cells were transfected with Pyk2 and ARA55 or mutant ARA55-Y43F for 24 h and then starved in 0.1% serum medium for another 16 h. Before harvesting, Pyk2 activity was induced by 1.5 nM PDGF for 10 min. The phosphorylation of ARA55 was analyzed by immunoprecipitation with anti-Hic-5(ARA55) antibody and Western blotting with anti-phosphotyrosine antibody (*Anti-Tyr*). Anti-Hic-5 antibody was also used to monitor the expression of ARA55. Pyk2 expression was detected by Western blotting with anti-Pyk2. *B*, H1299 cells were transiently co-transfected with 3 μg of MMTV-LUC reporter plasmid, AR, ARA55, mtARA55-Y43F, or Pyk2 expression plasmids, as indicated for 16 h. The total amount of DNA in each transfection was adjusted by addition of backbone vectors. Cells were treated with 10 nM DHT for another 24 h as indicated, and then the LUC assay was performed. The LUC activity of AR with ethanol treatment was set as 1-fold. All values represent the mean ± S.D. of three independent experiments.

kinase-negative PKM, can block the interaction between AR and wild type ARA55.

#### DISCUSSION

Cross-talk between various kinase signals and the androgen/AR pathway has been well documented. The phosphorylation of AR co-regulators to modulate AR transactivation, however, is currently unclear. Pyk2 is an important tyrosine kinase that can be induced by various extracellular stimuli (24–28) resulting in the activation of the MAPK and JNK kinase pathways (20, 23). The linkage from the Pyk2 pathway to NR transactivation, however, remains largely unknown. Here we provide evidence demonstrating that Pyk2 can interact with and phosphorylate ARA55 to suppress AR transactivation. This finding may represent a new mechanism to modulate AR function and supports the role of ARA55 as an AR co-regulator (6).

Tissue distribution analysis indicates that ARA55 is differentially expressed in various stages of prostate cancer (6, 12). Stanzione *et al.* (30) also reported that Pyk2 expression declines with increasing prostate cancer grade. These findings, in addition to our data showing that ARA55 and Pyk2 modulate



**FIG. 8. Pyk2 blocks the interaction between ARA55 and AR.** A, H1299 cells were transiently co-transfected with 3  $\mu$ g of pG5-LUC reporter plasmid, 3  $\mu$ g of GAL4-ARA55, GAL4-mtARA55 Y43F, VP16-AR, Pyk2, or PKM as indicated. The total amount of DNA in each transfection was adjusted by addition of backbone vectors. Cells were treated with 10 nM DHT for another 24 h, and then the LUC assay was performed. The LUC activity of the sample transfected with GAL4DBD and VP16 was set as 1-fold. All values represent the mean  $\pm$  S.D. of three independent experiments. B, 3.5  $\mu$ g of His-ARA55, His-mtARA55-Y43F, AR, Pyk2, or PKM in various combinations were transfected into H1299 cells using Superfect. Cells were treated with 10 nM DHT for 24 h and then starved in 0.1% serum medium containing 10 nM DHT for another 16 h. Before harvesting, Pyk2 activity was further induced with 1.5 nM PDGF as indicated. Anti-His antibody was added to immunoprecipitate the His-ARA55 or His-mtARA55-Y43F and AR complex. 10% SDS-PAGE was used to separate the complexes. NH27 and anti-His probe antibodies were used to detect AR and ARA55 or mtARA55, respectively (B, upper). The expression of AR in the cell lysate was determined by Western blotting with NH27 (B, lower).

AR function, suggest that the regulation of AR function may be altered in prostate cancer by changes in Pyk2 and ARA55 expression. The significance of these alterations in the progression of prostate cancer from androgen dependence to androgen independence, however, has yet to be determined.

Results from Figs. 5 and 6 clearly demonstrate that Pyk2 needs ARA55 to suppress AR function. Pyk2, a tyrosine kinase linked to the MAPK and JNK signaling pathways and thereby the regulation of cell growth and adhesion, may also utilize non-ARA55-mediated pathways to exert its physiological functions. The discovery of new pathways that cross-talk with Pyk2 $\rightarrow$ ARA55 $\rightarrow$ AR signaling may therefore expand the importance of Pyk2 in the control of prostate cancer growth.

The inability of PKM to suppress AR function via wild type ARA55 and the inability of Pyk2 to suppress AR function via mutated ARA55 suggests that the Pyk2 phosphorylation of ARA55 at residue 43 is critical in the suppression of AR function. Since this phosphorylation site is not located in the AR interaction domain of ARA55, amino acids (251–444) (6), it is

possible that other mechanisms may also be involved in the suppression of AR function by Pyk2-ARA55. Nevertheless, our mammalian two-hybrid and co-immunoprecipitation assays indicate that Pyk2 blocks the interaction between AR and ARA55. Therefore, it is possible that Pyk2 may have multiple ways to communicate with ARA55 to suppress AR transactivation.

In summary, Pyk2 can dramatically repress AR transactivation by inhibiting the coregulatory activity of ARA55. This interruption may entail both the direct phosphorylation of ARA55 to impair its coregulatory activity and/or sequestering ARA55 to reduce its interaction with AR. Our findings not only expand the role of Pyk2 in AR-mediated prostate cancer growth but also support the importance of ARA55 in the control of AR function.

**Acknowledgments**—We thank Drs. Jun-Lin Guan, Aknori Takaoka, and Joseph Schlessinger for valuable plasmids and Dr. T. J. Brown for the PC-3(AR2) cell line. We also thank Karen Wolf for manuscript preparation.

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