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Award Number: W81XWH-04-1-0184

TITLE: The Protein Kinase RSK Family - Roles in Prostate Cancer

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REPORT DATE: February 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2005	Annual (12 Jan 2004 - 11 Jan 2005)

4. TITLE AND SUBTITLE
The Protein Kinase RSK Family - Roles in Prostate Cancer

5. FUNDING NUMBERS
W81XWH-04-1-0184

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The Ser/Thr protein kinase p90-kDa ribosomal S6 kinase (RSK) is an important downstream effector of MAPK but its roles in prostate cancer have not previously been examined. We have now discovered that RSK2 regulates the expression of PSA, an important diagnostic marker for prostate cancer. Furthermore, RSK regulates the growth of some human prostate cancer cell lines. Taken together with our observations that RSK levels are higher in ~50% of human prostate tumors compared to normal prostate tissue suggest that RSK is an important drug target for prostate cancer. To further test this hypothesis we are developing prostate cell lines that will inducible overexpress RSK and transgenic mice that will overexpress RSK in the prostate. RSK-specific inhibitors, such as SL0101, which we recently discovered, should be useful as chemotherapeutic agents for the treatment of prostate cancer. The *in vivo* efficacy of SL0101 to inhibit the growth of prostate cancer is currently being evaluated using a PC-3 xenograft model in mice.

14. SUBJECT TERMS
Cell signaling, oncogenesis, drug efficacy

15. NUMBER OF PAGES
42

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

An increase in the activity of the mitogen-activated protein kinase (MAPK) has been correlated with the progression of prostate cancer to advanced disease in humans (1). The Ser/Thr protein kinase p90-kDa ribosomal S6 kinase (RSK) is an important downstream effector of MAPK (2) but its roles in prostate cancer have not previously been examined. Therefore, the overall objective of this research project is to evaluate the importance of RSK as a chemotherapeutic target in prostate cancer. As part of this evaluation we will determine the efficacy of the RSK-inhibitor, SL0101, to inhibit prostate cell growth. We identified this RSK-inhibitor by screening a collection of rare botanical extracts for their ability to inhibit RSK activity (3). SL0101 was isolated from the tropical plant *Forsteronia refracta* and is currently the only known, small-molecule inhibitor, which specifically inhibits RSK *in vitro* and *in vivo*.

Body

The research accomplishments associated with each Aim are described below:

Aim 1. Evaluate the efficacy of the RSK-specific inhibitor, SL0101, to inhibit the proliferation of prostate cancer cells.

Task 1. Test ability of SL0101 to inhibit growth in culture and soft agar.
A synthetic analog of SL0101, 3Ac-SL0101 is being used in these studies. This analog is more hydrophobic than SL0101, which makes it more potent in intact cell assays (4). The biological activity of SL0101 and 3Ac-SL0101 are identical (4).

Progress on this Task is detailed in the enclosed manuscript "The Ser/Thr protein kinase, RSK, is an important regulator of prostate cancer cell proliferation". In brief, we found that the RSK inhibitor, 3Ac-SL0101, inhibits the proliferation of LNCaP and PC-3 cells (Fig. 5, attached manuscript). Based on these results we determined the effect of 3Ac-SL0101 on the normal prostate line, RWPE-1. Remarkably, 3Ac-SL0101 did not affect the growth of RWPE-1 cells (Fig. 1). These results are very encouraging because they suggest that 3Ac-SL0101 may specifically inhibit prostate tumor growth without effecting normal cells, which would make it an ideal chemotherapeutic agent. Based on our observations that the proliferation of PC-3 cells is more sensitive to RSK inhibition than that of LNCaP cells (Fig. 5B, attached manuscript) we are using a PC-3 xenograft model. The PC-3 line is thought to represent an advanced stage of prostate cancer and currently, there are no targeted therapies for the treatment of advanced prostate cancer. Therefore, our results are particularly interesting because it is possible that RSK may be a good drug target for advanced prostate cancer. Therefore, rather than continue investigating the sensitivity of other prostate cancer lines to inhibition by 3Ac-SL0101 we accelerated the timetable for the *in vivo* evaluation of the RSK-inhibitor. We will complete the analysis of the remaining cell lines after completing the efficacy studies with the PC-3 xenografts.

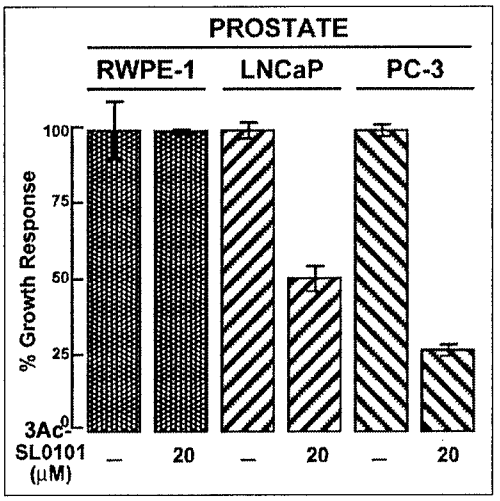

Figure 1 The RSK-inhibitor 3Ac-SL0101 inhibits the growth of prostate cancer cells. *The various cell lines were treated with vehicle [-] or 20 μM 3Ac-SL0101, and cell viability was measured after 48 hrs of treatment. Values are % of the growth observed in vehicle-treated cells. Columns, mean [n=2, in triplicate]; bars, SD.*

Task 2. Test ability of SL0101 to inhibit tumor formation in nude mice.

In order to evaluate the efficacy of 3Ac-SL0101 it is important to determine the optimum dose and scheduling of administration, which will maximize the probability of observing an effect of the inhibitor on the tumor while minimizing toxicity to the test animal. However, before we could start these studies it was necessary to solubilize 3Ac-SL0101 in a carrier that would be well tolerated in animals. This procedure proved to be much more difficult than anticipated and required considerable effort because the compound is quite hydrophobic. Various concentrations of Tween 80, polyethylene glycol 400, dextrose, hydroxypropyl-β-cyclodextrin and DMSO were tested without success. The only carrier that allows high concentrations of 3Ac-SL0101 to remain soluble is cremophor. Cremophor, the standard vehicle for clinically used Taxol, is necessary to prevent any precipitation of the compound from the solution and subsequent trapping in the capillary beds of the lungs following intravenous (iv) administration to the mice. To maintain solubility, 3Ac-SL0101 is initially solublized in cremophor: ethanol (1:1) to achieve a concentration of 336 mg/ml. The compound is then diluted with sterile saline to a final ethanol:cremophor:saline ratio of 1:1:10 to achieve a final concentration of 30 mM. We determined the maximum tolerated dosage (MTD) and maximum tolerated total dosage (MTTD) for 3Ac-SL0101 using the NCI's general instructions. The compound was administered by iv bolus. The MTD of 3Ac-SL0101 was a single dose of 100 mg/kg. This dose should result in peak plasma concentrations that are 125-fold higher than that needed to observe inhibition of proliferation in PC-3 cells in tissue culture. We also determined that 3Ac-SL0101 can be administered at the MTD every fourth day for three treatments (q4dx3) (Fig. 2). This schedule is based on the doubling time of the PC-3 tumors in xenograft models. The animals did not demonstrate pain or unacceptable levels of distress or exhibit body weight loss of ≥20% of their initial weight. Therefore, we have determined that using the MTD for the recommended dosing schedule does not exceed the MTTD.

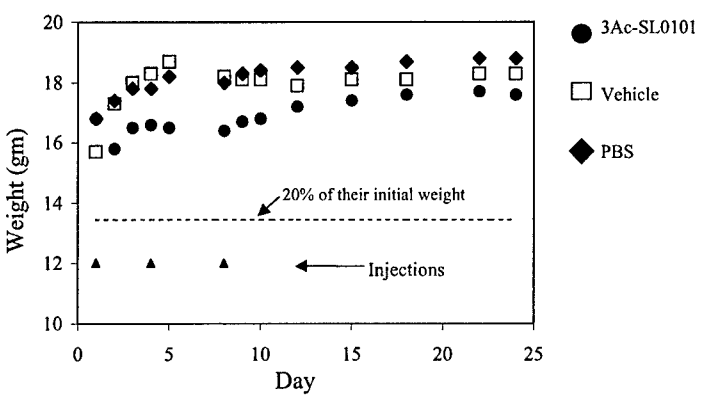

Figure 2. MTTD determination for 3Ac-SL0101. *SCID mice were administered the MTD of 3Ac-SL0101 every fourth day for three treatments (q4dx3). The mice were monitored for signs of distress and morbidity. The mice did not demonstrate unacceptable levels of distress nor body weight loss of ≥20% of their initial weight.*

Aim 2: Test whether enhanced RSK1 and RSK2 activity increases prostate cell growth.

No Tasks were associated with this Aim in year one.

Although no specific Tasks were associated with this Aim in year one we were able to perform some initial studies, which will facilitate progress on this Aim in year two. Progress on this Aim is detailed in the enclosed

manuscript "The Ser/Thr protein kinase, RSK, is an important regulator of prostate cancer cell proliferation". In brief, we found that RSK levels are higher in ~50% of human prostate cancers compared to normal prostate (Fig. 1, attached manuscript). These increased RSK levels are likely to be biologically significant because RSK2 enhances prostate specific antigen (PSA) (Fig. 2, attached manuscript).

To successfully generate RWPE-1 cell lines that will inducible express RSK it is necessary to have >50% transfection efficiency. For reasons that are not clear most normal human cell lines are difficult to transfect. For example, the RWPE-1 is completely refractory to transfection by Lipofectamine 2000 whereas this method gives >70% transfection efficiency in LNCaP cells. We have now optimized a transfection method based on Nucleofection, a relatively new procedure. We are now able to obtain ~ 70% transfection efficiency in RWPE-1 cells as determined by immunofluoresence using green fluorescent protein as a marker. Therefore, we are now ready to begin the production of inducbile RWPE-1 cell lines.

Aim 3: Determine whether increased RSK1 or RSK2 activity induces prostate cancer in a transgenic mouse model.

Task 1: Produce and Characterize Transgenic Mice.
The cDNA for RSK1 and RSK2 is in the process of being subcloned into a vector that will allow expression of an epitope-tagged RSK1 and RSK2 in the prostate. Work on this Aim was slightly delayed because of the difficulties encountered in identifying a carrier for 3Ac-SL0101.

Key Research Accomplishments

- Discovered that RSK2 regulates AR-mediated transcription. The AR is known to play an important role in prostate cancer.
- Determined that RSK2 regulates PSA expression, an important diagnostic marker for prostate cancer.
- Discovered that a synthetic analog of SL0101, 3Ac-SL0101, preferentially inhibits the growth of some human prostate cancer lines without affecting the growth of a normal human prostate line. These results are extremely exciting and support the continued evaluation of SL0101 as chemotherapeutic agents for prostate cancer.
- Identified a carrier for 3Ac-SL0101 that is biologically compatible.
- Determined the MTD and MTTD for 3Ac-SL0101 in mice. These studies demonstrated that 3Ac-SL0101 appears to have low *in vivo* toxicity.
- Initiated *in vivo* studies with PC-3 xenografts in mice to determine whether 3Ac-SL0101 will inhibit RSK activity in the tumor. These studies are very important in providing the necessary preclinical data to determine whether 3Ac-SL0101 will eventually be evaluated in human clinical trials.

Reportable Outcomes

Manuscript: Clark, D.E., Errington, T.M., Smith, J.A., Frierson, Jr., H.J., Weber, M.J. and Lannigan, D.A. (in press) The Ser/Thr protein kinase, RSK, is an important regulator of prostate cancer cell proliferation. Cancer Res.

Conclusions

RSK2 regulates the expression of PSA, an important diagnostic marker for prostate cancer. The RSK-specific inhibitor, 3Ac-SL0101, inhibits proliferation of some human prostate cancer cells but does not prevent the growth of normal human prostate cells. Taken together with our observations that RSK levels are higher in ~50% of human prostate tumors compared to normal prostate tissue suggest that RSK is an important drug target for prostate cancer. Therefore, RSK-specific inhibitors, like 3Ac-SL0101, should be useful as

chemotherapeutic agents for the treatment of prostate cancer. These studies are also providing important preclinical data to aid in the development of 3Ac-SL0101 as an anti-cancer drug.

References

1. Weber, M.J. and Gioeli, D. (2004) Ras signaling in prostate cancer progression. J. Cell. Biochem. 91:13-25.
2. Frodin, M. and Gammeltoft, S. (1999) Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. Mol. Cell. Endocrinol. 151:65-77.
3. Smith, J.A., Poteet-Smith, C.E., Xu, Y., Errington, T.M., Hecht, S.M. and Lannigan, D.A. (2005) Identification of the first specific inhibitor of p90 Ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. Cancer Res 65:
4. Smith, J.A., Reddy, Y.K., Xu, Y., De, S., Holman, N.J., Hecht, S.M. and Lannigan, D.A. (submitted) Synthesis of the prodrug, kaempferol 3-O-(2",3",4"-tri-O-acetyl-α-L-rhamnopyranoside)(3Ac-SL0101), a novel inhibitor of the Ser/Thr protein kinase, RSK.

Appendices

Manuscript attached.

The Ser/Thr protein kinase, RSK, is an important regulator of prostate cancer cell proliferation.

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Running Title: RSK and prostate cancer

Keywords: RSK, signal transduction, prostate cancer, proliferation, PSA

Footnotes: Funding sources: Department of Defense #PC030511

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Abstract

An increase in the activity of mitogen-activated protein kinase (MAPK) has been correlated with the progression of prostate cancer to advanced disease in humans. The Ser/Thr protein kinase p90-kDa ribosomal S6 kinase (RSK) is an important downstream effector of MAPK but its role in prostate cancer has not previously been examined. Increasing RSK isoform 2 (RSK2) levels in the human prostate cancer line, LNCaP, enhanced prostate specific antigen (PSA) expression, an important diagnostic marker for prostate cancer, whereas inhibiting RSK activity using a RSK-specific inhibitor, 3Ac-SL0101, decreased PSA expression. The RSK2 regulation of PSA expression occurred via a mechanism involving both RSK2 kinase activity and its ability to associate with the co-activator, p300. RNA interference of the androgen receptor (AR) demonstrated that the AR was important in the RSK2-mediated increase in PSA expression. RSK levels are higher in ~ 50% of human prostate cancers compared to normal prostate tissue, which suggests that increased RSK levels may participate in the rise in PSA expression that occurs in prostate cancer. Furthermore, 3Ac-SL0101 inhibited proliferation of the LNCaP line and the androgen-independent human prostate cancer line, PC-3. These results suggest that proliferation of some prostate cancer cells is dependent on RSK activity and support the hypothesis that RSK may be an important chemotherapeutic target for prostate cancer.

Introduction

Prostate cancer progresses from a dependence on the hormone androgen, to androgen-independence (1). Nonetheless, the androgen receptor (AR) continues to play a key role in regulating prostate cancer cell proliferation even in advanced disease (2). The AR is a member of the superfamily of ligand-activated transcription factors (3), and like other superfamily members, the transcriptional activity of the AR can be further stimulated by second messenger signaling pathways (reviewed in (4)). There is substantial evidence that prostate cancer progression involves the activation of second messenger signaling pathways, in response to increased expression of growth factors and their receptors (reviewed in (4), (5)). It is possible that the activated signaling pathways enhance AR-mediated transcription to such an extent that normal levels of androgens become less important (6). However, the molecular mechanisms by which these changes result in prostate cancer progression remain unknown and there are no targeted therapies available for advanced prostate cancer.

An increase in mitogen-activated protein kinase (MAPK) activity has been correlated with the progression of prostate cancer to advanced disease in humans (7). These results, together with observations that Ras activity regulates the androgen requirement of prostate tumor growth in xenografts, suggest that the MAPK pathway plays a role in prostate cancer proliferation (6, 8). The family of Ser/Thr protein kinases, p90 ribosomal S6 kinase (RSK), function as downstream effectors of MAPK (9, 10). The RSK family consists of four isoforms, which are the products of separate genes (11). RSK isoform 4 (RSK4) is an outlier in the family and may be functionally distinct (12-14), but it is not known whether RSK1-3 have differing functions *in vivo*. RSKs play an important role in cell survival and proliferation in somatic cells through their ability to phosphorylate and regulate the activity of key substrates, including several transcription factors

and kinases, the cyclin-dependent kinase inhibitor, p27Kip1, the tumor suppressor, tuberin, and the pro-apoptotic protein, Bad (15-17). Additionally, a possible role for RSKs in tumorigenesis is suggested by their involvement in phosphorylation of filamin A, a regulator of cellular migration (18). We have also found that RSKs regulate the transcriptional activity of estrogen receptor α (ERα), a close relative of the AR (19, 20). These observations combined with the known importance of MAPK in prostate cancer, raised the possibility that RSKs may play a role in prostate cancer progression.

We now show that RSK2 regulates the expression of prostate-specific antigen (PSA), an important diagnostic marker for prostate cancer. Furthermore, the RSK-specific inhibitor, 3Ac-SL0101, inhibits proliferation of the human prostate cancer lines, LNCaP and PC-3. Taken together with our observations that RSK levels are higher in ~ 50% of human prostate tumors compared to normal prostate tissue, we suggest that RSK may be an important novel chemotherapeutic target for prostate cancer.

Materials and Methods

Materials

ICI 182,780 was a gift from Zeneca Pharmaceuticals, Cheshire, U.K. Monoclonal antibodies anti-HA (12CA5) and anti-myc (9E10) were from the Lymphocyte Culture Center (Univ. Virginia). Monoclonals anti-AR (441) and anti-RSK2 (E-1) and the polyclonal antibody anti-RSK1 (C-21) were from Santa Cruz Biotechnology, Inc. Monoclonals anti-Ran (20) and anti-MAPK (16) were from BD Biosciences. Polyclonals anti-pPDK1 (Ser-241), anti-active MAPK and anti-RSK2 were from Cell Signaling Technology, Promega Corporation and Upstate Cell Signaling Solutions, respectively. Immunoblots were performed using anti-mouse or anti-rabbit secondary IgGs conjugated to horseradish peroxidase (Jackson Laboratory). The siGENOME™ SMARTpool® AR siRNA and siCONTROL non-targeting siRNA #1 were from Dharmacon.

Cell Culture

LNCaP cells were obtained from Lelund Chung (Emory University) and maintained as described by American Type Culture Collection (ATCC). The PC-3 line was obtained from the ATCC and maintained in the recommended media.

Expression Vectors and Receptor Mutants

HA-tagged RSK2, RSK2(Y707A) and RSK2(K100A/Y707A) and myc-tagged RSK2 were described previously (20). E1A cDNA, the pKH3 vector and the vector encoding ERα (HEGO) were provided by Dan Engel (Univ. Virginia), Ian Macara (Univ. Virginia), and Pierre Chambon (Univ. Louis Pasteur), respectively. HA-AR and HA-E1A were produced by amplification of the open reading frame and inserted in-frame with the HA-tag of the eukaryotic expression vector, pKH3. Sequences were verified by the Biomolecular Research Core (University of Virginia).

Tissue Sample Analysis

These studies were done with approval from the Human Investigation Committee at the Univ. Virginia. Samples of prostate biopsies that had been performed on patients with elevated PSA levels were obtained from the Tissue Procurement Facility (Univ. Virgina). The samples were T2a (n=3), T2c (n=3), T3a (n=5), T3b (n=1) and N (n=1). The Gleason scores were 6 (n=3), 7 (n=6), 8 (n=2) and 9 (n=1). The samples were obtained from Caucasians ranging from 48 to 67 yr. The frozen tissue blocks were examined microscopically and prostate cancer tissue was excised from areas of the block that were predominantly tumor. Normal and benign prostatic tissues were obtained from areas of the block containing abundant epithelium. Tissues were finely ground in the presence of liquid N_2 and added to boiling 2-X SDS loading buffer and then heated at 100°C for 3 min. The total protein concentration in the denatured lysates was determined and equivalent protein amounts were electrophoresed and immunoblotted with anti-RAN. The immunoblots were analyzed and quantitated by densitometry as described (21) and the samples normalized to each other based on RAN levels. Normalized samples were electrophoresed, immunoblotted with anti-RSK1 and anti-RSK2 and the immunoblots analyzed and quantitated by densitometry.

Proliferation Assay

Proliferation assays were performed as described (22). In the washout experiments each well was coated with 0.25 μg fibronectin (Invitrogen). It was not possible to perform washout experiments with the LNCaP line because these cells are only weakly attached even when plated onto dishes with fibronectin, and are lost from the surface during replacement of the medium.

In vitro Kinase Assay

Production and purification of glutathione-S-transferase (GST) fusion proteins was performed according to the manufacturer's protocol (Amersham Biosciences).

The ability of purified, recombinant RSK2(Y707A) (5 nM) (22) to phosphorylate GST-AR-tide was determined in kinase buffer (23) with 100 μM ATP, γ-^{32}P-ATP (~6000 cpm/pmol), and 20 mg/ml of the appropriate substrate at 30°C with shaking at 1000 rpm. At 10 and 30 min an aliquot of the supernatant was removed, added to 2X-SDS loading buffer and boiled. Samples were electrophoresed and the amount of incorporated ^{32}P determined by Phosphorimager.

Endogenous RSK2 activity in LNCaP and PC-3 cells was determined according to the following protocol. Cells (150 mm dishes) were pre-treated for 4 hr with vehicle or U0126 (25 μM), and then 100 ng/ml EGF or vehicle was added for 8 min. (An 8 min EGF treatment of LNCaP and PC-3 cells results in maximum RSK2 activity (data not shown). After lysis an aliquot was removed and RSK2 was immunoprecipitated with anti-RSK2 (4 μg) and GammaBind plus Sepharose beads. The beads were washed twice with 500 μl of kinase wash buffer (23). Pelleted beads were incubated with kinase buffer and 20 mg/ml of the appropriate substrate at 30°C with shaking at 1000 rpm. Aliquots were removed and analyzed as for GST-AR-tide. The relative amount of RSK2 on the beads was determined by immunoblotting and the signal quantified by densitometry.

Transcriptional Analysis

LNCaP cells were transfected, treated and assayed for luciferase and β-galactosidase as described previously except that Lipofectamine™ 2000 (Invitrogen) was used and the cells were treated for 15 hr before lysis (21). In siRNA experiments the appropriate siRNA (20 nM) was transfected using Trans IT-TKO® (Mirus) 6 hr after the first transfection with the reporter constructs and either the RSK2(Y707A) construct or vector control. The cells were treated for 15 hr before lysis. PSA expression was determined in supernatants of transfected LNCaP cells using the ACTIVE® PSA ELISA kit (Diagnostic Systems Lab., Inc.).

Results

RSK is overexpressed in human prostate cancers.

Malignant transformation and progression in human cancers are frequently associated with over-abundance or increased activity of proteins that are involved in normal cellular processes (24). Therefore, we investigated whether the levels of RSK protein expression were increased in human prostate samples. We found that the average RSK1 and RSK2 levels were ~ 3.5-fold and ~ 2.5-fold higher, respectively, in the prostate cancer (PC) samples than in normal prostate (N) and benign prostatic hyperplasia samples (BPH) (Fig. 1). Approximately 50% of the PC samples had RSK1 and RSK2 levels higher than the median value for the N samples. Both the RSK1 and RSK2 levels in the PC samples were statistically higher than the N and BPH samples with a confidence level >0.95 using an unpaired t-test with unequal variances.

RSK2 enhances PSA expression.

RSK2 is the best characterized of the isoforms and is important in regulating transcription through its ability to enhance the activity of transcription factors by phosphorylation and to associate with the transcriptional co-activator, p300, and its close relative, CREB binding protein (CBP) (25). Therefore, we focused our studies on RSK2. To determine whether the elevated RSK2 levels observed in the prostate cancer tissue are physiologically significant we used the human prostate cancer line, LNCaP. These cells are a good model system for these studies because they have RSK2 levels intermediate between those of normal and tumorigenic prostate tissues. Specifically, LNCaP cells express ~ 2-fold higher levels of RSK2 compared to normal

human prostate tissue, but the level of RSK2 is similar to the average RSK2 level observed in human prostate cancer tissues (Fig. 1).

Increased PSA levels are correlated with prostate cancer and therefore, we determined whether increasing RSK2 levels stimulated transcription from the PSA promoter. LNCaP cells were co-transfected with reporter constructs and a control vector, plus a vector encoding epitope-tagged wild type RSK2 ((HA)-RSK2) or a constitutively active RSK2 mutant (HA-RSK2(Y707A)). The mutant RSK2(Y707A) is a constitutively active kinase, and thus eliminates the requirement of RSK2 to be activated by MAPK (23). To minimize active MAPK levels, the cells were serum-starved during the various treatments. RSK2(Y707A) enhanced expression from the PSA promoter by ~ 15% in the absence of the synthetic androgen, R1881, compared with the vector control (Fig. 2A). However, in the presence of R1881, transcription was increased ~ 35% and ~60% by RSK2 and RSK2(Y707A), respectively, compared to the vector control. Interestingly, in the presence of EGF both the wild-type and mutant RSK2 dramatically increased transcription ~ 30-fold above the control. Upon EGF activation there was no statistical difference in the ability of RSK2 and RSK2(Y707A) to stimulate transcription. These observations are in agreement with results from *in vitro* kinase assays in which RSK2(Y707A) has a higher basal activity than RSK2 but has a similar activity upon activation by EGF (23).

It is conceivable that RSK2 might increase transcription by enhancing AR levels. To test this possibility HA-tagged AR and myc-tagged RSK2(Y707A) were co-transfected into LNCaP cells. We determined the levels of HA-tagged AR rather than endogenous AR because the transfection efficiency in LNCaP cells is only ~20% and we wanted to determine AR levels in those cells that also contained RSK2(Y707A). We have previously determined that after co-transfection $\geq$ 90% of the transfected cells express both constructs (data not shown). Because

the transfection efficiencies were similar for the different conditions the lysates were normalized to each other based on total MAPK levels. In the vector control we observed that R1881 increased AR levels, which is consistent with the literature (26), but also found that EGF increased AR levels (Fig. 2B). However, the levels of AR were similar in the presence of RSK2(Y707A) compared to the vector control. Therefore, there does not appear to be a correlation between the level of AR and the ability of RSK2(Y707A) to enhance transcription.

RSK2 activates AR-mediated transcription.

RSK2 is known to stimulate the transcriptional activity of ERα (20), a close relative of the AR. Therefore, we determined whether RSK2 also enhances PSA expression by increasing the transcriptional activity of the AR. To test this possibility we reduced AR expression levels using short interfering RNA (siRNA). The AR siRNA dramatically decreased AR expression in both the RSK2(Y707A) and vector control transfected cells (Fig. 3A-inset). Our transfection efficiency for siRNA is ~ 90% and the knockdown for AR is among the most effective that we have performed (data not shown). The transcriptional response in the absence of ligand was < 1/2 of that observed with RSK2(Y707A) (Fig. 3A). In the presence of R1881 the AR siRNA essentially abolished transcription in the vector control, and decreased transcription by ~ 90% in the RSK2(Y707A) transfected cells. These effects were not due to nonspecific effects on transcription because β-galactosidase expression was unaffected by the AR siRNA (data not shown). Thus, RSK2(Y707A) appears to work predominately through an AR-dependent mechanism in both the absence and presence of R1881. However, in response to EGF, the AR siRNA decreased transcription by only ~ 30%, which suggests that in this case PSA expression is being regulated only partly through the AR.

RSK2 kinase activity regulates PSA expression.

To determine the importance of RSK2 kinase activity in enhancing PSA expression we tested whether the kinase-inactive mutant RSK2(K100A/Y707A) could stimulate transcription. The kinase-inactive mutant has the essential ATP-binding Lys-100 mutated to Ala in the N-terminal kinase domain of RSK2. In the absence or presence of R1881 the kinase-inactive mutant produced the same transcriptional response as the vector control (Fig. 3B). Surprisingly, however, in the presence of EGF the kinase-inactive mutant still stimulated transcription to a level ~1/2 that observed with RSK2(Y707A). These results suggest that RSK2 kinase activity regulates the transcriptional activity of the AR in the absence and presence of R1881 but that RSK2 kinase activity is only partly responsible for the increased PSA expression observed in response to EGF.

Because RSK2 enhances ERα-mediated transcription (20) and it has been reported that LNCaP cells contain ERα, albeit at very low levels (27) we investigated whether inhibition of ERα would influence the ability of RSK2 to stimulate PSA expression. In agreement with the literature (28) we observed a modest 20% increase in estrogen-dependent transcription using an ERE-luciferase reporter system in LNCaP cells (data not shown). Ectopic expression of ERα led to an ~10-fold increase in estrogen-responsiveness (data not shown), thus ERα-mediated transcription is most likely limited by ERα levels in LNCaP cells. To ensure that we could observe an effect of inhibiting ERα activity on RSK2 stimulation of PSA expression we measured transcription from the PSA promoter with or without ectopically expressed ERα. However, regardless of ERα levels, the inhibition of ERα activity with the antiestrogens, 4-hydroxytamoxifen and ICI 182,780, did not influence the ability of RSK2(Y707A) to stimulate

PSA levels (Fig. 3C). Thus inhibition of ERα does not affect the role of RSK2 in regulating PSA expression.

It is possible that RSK2 kinase activity enhances AR-mediated transcription through phosphorylation of the AR, or through associated co-activators or via both mechanisms. To distinguish between these possibilities we asked whether RSK2 could phosphorylate the AR. RSK2 phosphorylates Ser or Thr residues that lie within the motif, RXRXXS/T, where X is any amino acid (29). Based on this motif there is a putative RSK2 phosphorylation site, RAREAS (residues 203 to 208 in the human AR accession number M34233), which is contained in the activation function 1 (AF-1) region. It is possible that RSK2 phosphorylation of Ser-208 enhances the activity of AF-1, which is the main transactivation domain of the AR. This putative site is highly but not completely conserved across various species. To test whether Ser-208 could be phosphorylated by RSK2 we measured the ability of RSK2 to phosphorylate *in vitro* a protein consisting of GST fused to the sequence from 203 to 213 of the human AR, which we call GST-AR-tide. As a positive control we compared the ability of RSK2 to phosphorylate GST-AR-tide versus GST-ER-tide. GST-ER-tide contains Ser-167 in human ERα, a site phosphorylated *in vivo* by RSK2 (19, 20). RSK2 phosphorylated GST-ER-tide ~ 150-fold over background compared to 2-fold for GST-AR-tide (Fig. 3D). Thus, *in vitro*, GST-AR-tide can be phosphorylated by RSK2, but it is not a particularly good substrate compared to GST-ER-tide.

There is another putative RSK2 phosphorylation site, RMRHLS (residues 773 to 778), which is contained within the ligand-binding domain. The sequence RMRHLS is contained within helix 7 of the ligand-binding domain and is completely conserved across all species. Mutations within this region are known to influence ligand-binding (30) and therefore, it is possible that RSK2 phosphorylation of Ser-778 enhances the affinity of the AR for its ligand. But according to

the crystal structure of the AR ligand-binding domain, Ser-778 is located within the binding pocket and is buried in the interior of the protein (31). Therefore, it seems unlikely that a kinase would have access to this residue. Nonetheless, to test whether RSK2 could phosphorylate Ser-778, we determined whether RSK2 altered the affinity of the AR for ligand by measuring PSA expression at various doses of R1881. At each dose the transcriptional response was higher in the RSK2(Y707A) transfected cells than in the vector control transfected cells (Fig. 3E). However, the R1881 EC_{50} for the RSK2(Y707A) and the vector control was ~0.3 nM, which suggests that RSK2(Y707A) does not alter the affinity of the AR for R1881. Therefore, it seems unlikely that RSK2 phosphorylates the RMRHLS motif. Taken together, these results argue that RSK2 protein kinase activity stimulates AR-mediated transcription by phosphorylation of an AR co-activator rather than by direct AR phosphorylation.

The RSK2/p300 complex regulates PSA expression.

RSK2 is known to interact with the co-activator p300/CBP. This interaction is independent of RSK2 catalytic activity and is important in Ras-mediated gene activation (25). Furthermore, p300 has been shown to regulate prostate cancer growth (32, 33). Therefore, we hypothesized that the interaction between RSK2 and p300 might play a role in the regulation of PSA expression. To test this possibility, we determined whether ectopic co-expression of p300 and RSK2 could enhance PSA expression over that obtained with each protein expressed individually. Suboptimal levels of the constitutively active RSK2 mutant (RSK2(Y707A) were used in these experiments, to ensure that an enhancement in PSA expression could be observed when p300 and RSK2(Y707A) were co-expressed. In the vehicle-treated cells co-expression of p300 and RSK2(Y707A) increased PSA expression ~2-fold over that obtained with each separately (Fig. 4A). EGF-treatment did not further enhance the response obtained

with co-expression of RSK2(Y707A) and p300 compared to vehicle-treatment. It could be that the increased expression of both RSK2(Y707A) and p300 bypasses the requirement for activation of the MAPK pathway. The additive transcriptional response of RSK2(Y707A) and p300 in the vehicle-treated cells suggests that both molecules are influencing the same transcriptional event, which is consistent with our hypothesis that the interaction between RSK2 and p300 is important in regulating PSA expression.

To further test our hypothesis, we asked whether the viral oncoprotein E1A would inhibit the ability of RSK2 to activate transcription. E1A is known to associate with p300 and the binding of RSK2 and E1A to p300 are mutually exclusive. Therefore, E1A can compete for the binding of RSK2 to p300 (25). The ectopic expression of E1A increased PSA expression in both the EGF-treated and vehicle-treated cells, for reasons that are unclear (Fig. 4B). Importantly, however, E1A inhibited the ability of RSK2(Y707A) to activate transcription ~ 2-fold and 3-fold in the absence and presence of EGF, respectively. The effects of p300 and E1A on RSK2-stimulated PSA expression were not due to differences in the RSK2(Y707A) levels because RSK2(Y707A) expression levels were similar with or without p300 and E1A (Fig. 4B-inset).

RSK regulates prostate cancer cell proliferation.

Based on our observations that increasing RSK2 stimulates PSA expression, we tested directly whether RSK was important in regulating prostate cancer cell proliferation. We recently discovered a cell-permeable, RSK-specific inhibitor, 3Ac-SL0101, which functions as a competitive inhibitor for ATP binding (22, 34). 3Ac-SL0101 inhibits the proliferation of the human breast cancer cell line, MCF-7, but does not alter the growth of a normal human breast cell line, MCF-10A (Fig. 5A). These data demonstrate that RSK activity is not essential for the

growth of all cell lines. However, the growth of the LNCaP line was substantially inhibited by 3Ac-SL0101, with an IC_{50} of ~ 30 μM (Fig. 5B). Based on these results we determined whether the growth of PC-3 cells, another human prostate cancer line, was also dependent on RSK activity. The PC-3 line does not express the AR and therefore represents a more aggressive prostate cancer than the LNCaP line (35, 36). 3Ac-SL0101 inhibited the growth of PC-3 cells with an IC_{50} of ~ 10 μM (Fig. 5B). Removal of 3Ac-SL0101 from PC-3 cells after 72 hr resulted in renewed growth of the cells, which indicates that the inhibitor at 20 μM is cytostatic (Fig. 5C). These data argue that the proliferation of both prostate lines is dependent on RSK. Furthermore, we found that inhibition of RSK activity in LNCaP cells by 3Ac-SL0101 also decreased the endogenous expression of PSA (Fig. 5D). These results are consistent with our previous observations that increased RSK activity in LNCaP cells stimulates PSA expression. Only prostate lines with AR express PSA, so PC-3 cells could not be used for these studies (36).

To understand why the proliferation of the PC-3 line was more sensitive to inhibition of RSK activity than the LNCaP line we analyzed the endogenous RSK2 activity in these lines. RSK2 is increased 2-fold in LNCaP cells and by 6-fold in PC-3 cells, compared to the average RSK2 level for normal human tissue (Fig. 1 and Fig. 6A). Basal RSK2 specific activity was ~3.5-fold higher in PC-3 than in LNCaP cells (Fig. 6B). Upon activation by EGF the specific activity of RSK2 was ~ 10-fold higher in PC-3 than in LNCaP cells. MAPK and PDK1 are the only known regulators of RSK activity (37, 38). Therefore, the differences in RSK2 specific activity are surprising because the levels of active MAPK and active PDK1 in the EGF-treated cells were less in PC-3 than in LNCaP cells (Fig. 6A). Taking into account the differences in RSK2 expression levels, the total RSK2 activity under basal and EGF-stimulated conditions is ~10-fold and ~35-fold higher, respectively, in PC-3 than in LNCaP cells. It has been

hypothesized that during the process of tumorigenesis there is an increased dependency on compromised signaling pathways and concomitantly that alternative signaling pathways become dormant (39, 40). We speculate that the higher levels of RSK2 activity in PC-3 compared to LNCaP cells have made the growth of PC-3 cells more dependent on the RSK pathway and thus more sensitive to the RSK-specific inhibitor, 3Ac-SL0101.

Discussion

We propose that RSK is an important regulator of proliferation in some prostate cancer cells. We base this hypothesis on the observations that in LNCaP cells an increase in RSK2 levels stimulates the expression of PSA. Additionally, we have found that the RSK-specific inhibitor, 3Ac-SL0101, reduced PSA expression in LNCaP cells and inhibited the growth of both LNCaP and PC-3 cells. These results are likely to be physiologically significant because we found that RSK levels were increased in ~50% of human prostate cancer tissues compared to normal prostate tissue.

We have found that RSK2 regulates AR-mediated transcription. The AR is known to be a phosphoprotein but the importance of this phosphorylation on AR transcriptional activity is not clearly understood. AKT/protein kinase B (PKB) has an identical phosphorylation consensus motif as RSK (41). Ser-210 and Ser-778, located in the putative RSK2 consensus phosphorylation sites, are phosphorylated in cells engineered to overexpress AKT/PKB (42). However, in other studies in which mass spectroscopy was used to map phosphorylated residues in the AR, Ser-210 was not found to be phosphorylated in LNCaP cells stimulated with EGF (43). These latter observations are in agreement with our *in vitro* phosphorylation studies, which demonstrate that Ser-210 is an extremely poor RSK2 substrate. Furthermore, RSK2 did not affect the affinity of the AR for ligand, which strongly suggests that RSK2 does not phosphorylate Ser-778, which is located in a region known to be important in ligand binding. Therefore, it is more likely that RSK2 phosphorylation of an AR coactivator accounts for the ability of RSK2 to increase PSA expression rather than by direct phosphorylation of the AR. However, it is of interest to note that under conditions in which RSK2 kinase activity is extremely high, less than ideal substrates, such as the AR, may be phosphorylated.

Increasing RSK2 levels dramatically enhanced PSA expression in response to EGF treatment. This stimulation is partly dependent on the kinase activity of RSK2 to enhance AR-mediated transcription. However, in addition to its kinase activity, it appears that the association of RSK2 with the co-activator p300/CBP is important. The association between RSK2 and p300/CBP has been shown to be independent of the catalytic activity of RSK2 (25), which is consistent with our observations that both constitutively active and the kinase-inactive RSK2 mutants stimulate PSA expression. Furthermore, E1A, through its ability to compete with RSK2 for p300, inhibits the ability of RSK2 to stimulate PSA expression. It has been proposed that RSK2 kinase activity and p300 histone acetyltransferase (HAT) activity are regulated by the interaction of RSK2 with p300 (44). Thus the EGF-stimulation in p300 HAT activity may enhance PSA expression through a mechanism involving acetylation of histone and transcription factors such as the AR (45).

The Gleason scores of the prostate cancer tissues we analyzed were >6, which indicates that our samples were all of advanced cancers. These studies support the analysis of RSK levels in a larger clinical study to determine whether there is a correlation between RSK levels and prostate cancer grade. We found that there is a positive linear relationship between RSK1 and RSK2 levels in the prostate samples ($P < 0.01$;data not shown). RSK1 and RSK2 are on different chromosomes and thus it seems unlikely that the increased levels of both isoforms are both due to gene amplification. Therefore, we propose that alterations in signal transduction are responsible for the increase in RSK levels observed in some prostate cancers. Although all cancer tissues contain active MAPK, we did not find a linear relationship between RSK and active MAPK levels (data not shown) and thus the increased RSK levels are not simply a reflection of MAPK activity. The major RSK regulatory inputs are thought to be MAPK and

PDK1 (9, 15). Interestingly, we found that RSK2 activity in response to EGF is 35-fold greater in PC-3 than LNCaP cells, even though their active MAPK and PDK1 levels were similar, which suggests that not all RSK regulatory inputs have been identified. It may be that the identification of these additional inputs will elucidate why RSK levels are increased in some prostate cancers and why RSK has become important in the growth of some prostate cancers.

References

1. Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. J Cell Biochem 2004;*91:* 483-90.
2. Suzuki H, Ueda T, Ichikawa T, Ito H. Androgen receptor involvement in the progression of prostate cancer. Endocr Relat Cancer 2003;*10:* 209-16.
3. Chang C, Saltzman A, Yeh S, Young W, Keller E, Lee HJ, Wang C, Mizokami A. Androgen receptor: an overview. Crit Rev Eukaryot Gene Expr 1995;*5:* 97-125.
4. Heinlein CA, Chang, C. Androgen receptor in prostate cancer. Endocr Rev 2004;*25:* 276-308.
5. Weber MJ, Gioeli D. Ras signaling in prostate cancer progression. J Cell Biochem 2004;*91:* 13-25.
6. Bakin RE, Gioeli D, Sikes RA, Bissonette EA, Weber MJ. Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res 2003;*63:* 1981-9.
7. Gioeli D, Mandell JW, Petroni GR, Frierson HF Jr, Weber MJ. Activation of mitogen-activated protein kinase associated with prostate cancer progression. Cancer Res 1999;*59:* 279-84.
8. Bakin RE, Gioeli D, Bissonette EA, Weber MJ. Attenuation of Ras signaling restores androgen sensitivity to hormone-refractory C4-2 prostate cancer cells. Cancer Res 2003;*63:* 1975-80.
9. Frodin M., Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. Mol Cell Endocrinol 1999;*151:* 65-77.
10. Nebreda AR, Gavin AC. Perspectives: signal transduction. Cell survival demands some Rsk. Science 1999;*286:*1309-10.

11. Yntema HG, van den Helm B, Kissing, J., van Duijnhoven G, et al. A novel ribosomal S6-kinase (RSK4; RPS6KA6) is commonly deleted in patients with complex X-linked mental retardation. Genomics 1999;*62:* 332-43.

12. Berns K, Hijmans EM, Mullenders J, et al. large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature 2004;*428:* 431-7.

13. Myers AP, Corson LB, Rossant J, Baker JC. Characterization of mouse Rsk4 as an inhibitor of fibroblast growth factor-RAS-extracellular signal-regulated kinase signaling. Mol Cell Biol 2004;*24:* 4255-66.

14. Dummler BA, Hauge C, Silber J, et al. Functional characterization of RSK4, a new 90 kDa ribosomal S6 kinase, reveals constitutive activation in most cell types. J Biol Chem 2005.

15. Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 2004;*68:* 320-44.

16. Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci U S A 2004;*101:* 13489-94.

17. Yang X, Matsuda K, Bialek P, et al. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. Cell 2004;*117:* 387-98.

18. Woo MS, Ohta Y, Rabinovitz I, Stossel TP, Blenis J. Ribosomal S6 kinase (RSK) regulates phosphorylation of filamin A on an important regulatory site. Mol Cell Biol 2004;*24:* 3025-35.

19. Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J, Lannigan DA. pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. Mol Cell Biol 1998;*18:* 1978-84.

20. Clark DE, Poteet-Smith CE, Smith JA, Lannigan, DA. Rsk2 allosterically activates estrogen receptor alpha by docking to the hormone-binding domain. EMBO J 2001;*20:* 3484-94.

21. Joel PB, Traish AM, Lannigan DA. Estradiol-induced phosphorylation of Serine 118 In the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. J Biol Chem *273:* 13317-23, 1998.

22. Smith JA, Poteet-Smith, CE, Xu Y, Errington TM, Hecht SM, Lannigan DA. Identification of the first specific inhibitor of p90 Ribosomal S6 Kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. Cancer Res In Press;*65.*

23. Poteet-Smith CE, Smith JA, Lannigan DA, Freed TA, Sturgill TW. Generation of constitutively active p90 ribosomal S6 kinase in vivo. Implications for the mitogen-activated protein kinase-activated protein kinase family. J Biol Chem 1999;*274:* 22135-38.

24. Fernandez PL, Hernandez L, Farre X, Campo E, Cardesa, A. Alterations of cell cycle-regulatory genes in prostate cancer. Pathobiology 2002;*70:* 1-10.

25. Nakajima T, Fukamizu A, Takahashi J, et al. The signal-dependent coactivator CBP is a nuclear target for pp90RSK. Cell 1996;*86:* 465-74.

26. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 1995;*9:* 208-18.

27. Linja MJ, Savinainen KJ, Tammela TL, Isola JJ, Visakorpi T. Expression of ERalpha and ERbeta in prostate cancer. Prostate 2003;*55:* 180-6.

28. Maggiolini M, Recchia AG, Carpino A, et al. Oestrogen receptor beta is required for androgen-stimulated proliferation of LNCaP prostate cancer cells. J Mol Endocrinol 2004;*32:* 777-91.

29. Leighton IA, Dalby KN, Caudwell FB, Cohen PT, Cohen P. Comparison of the specificities of p70 S6 kinase and MAPKAP kinase-1 identifies a relatively specific substrate for p70 S6 kinase: the N- terminal kinase domain of MAPKAP kinase-1 is essential for peptide phosphorylation. FEBS Lett 1995;*375:* 289-93.

30. Poujol N, Wurtz JM, Tahiri B, et al. Specific recognition of androgens by their nuclear receptor. A structure-function study. J Biol Chem 2000;*275:* 24022-31.

31. Matias PM, Donner P, Coelho R, et al. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 2000;*275:* 26164-71.

32. Debes JD, Sebo TJ, Lohse CM, Murphy LM, Haugen de AL, Tindall DJ. p300 in prostate cancer proliferation and progression. Cancer Res 2003;*63:* 7638-40.

33. Debes JD, Schmidt LJ, Huang H, Tindall DJ. p300 mediates androgen-independent transactivation of the androgen receptor by interleukin 6. Cancer Res 2002;*62:* 5632-6.

34. Smith JA, Yalamareddy K, Xu Y, Holman NJ, Hecht SM, Lannigan DA. Synthesis of the prodrug, kaempferol 3-O-(2", 3", 4"-tri-O-acetyl-a-L-rhamnopyranoside) (3Ac-SL0101), a novel inhibitor of the Ser/Thr protein kinase, RSK., Submitted.

35. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol 1979;*17:* 16-23.

36. van Bokhoven A, Varella-Garcia M, Korch C,et al. Molecular characterization of human prostate carcinoma cell lines. Prostate 2003;*57:* 205-25.

37. Frodin M, Jensen CJ, Merienne K, Gammeltoft S. A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1. EMBO J 2000;*19:* 2924-34.

38. Smith JA, Poteet-Smith CE, Malarkey K, Sturgill TW. Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo. J Biol Chem 1999;*274:* 2893-8.

39. Mills GB, Lu Y, Kohn EC. Linking molecular therapeutics to molecular diagnositics: Inhibition of the FRAP/RAFT/TOR component of the PI3K pathway preferentially blocks PTEN mutant cells in vitro and in vivo. Proc Natl Acad Sci U S A 2001;*98:* 10031-3.

40. Neshat MS, Mellinghoff IK, Tran C, et al. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc Natl Acad Sci U S A 2001;*98:* 103144-9.

41. Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. FEBS Lett 1996;*399:* 333-8.

42. Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci U S A 2001;*98:* 7200-5.

43. Gioeli D, Ficarro SB, Kwiek JJ, et al. Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J Biol Chem 2002;*277:* 29304-14.

44. Merienne K, Pannetier S, Harel-Bellan A, Sassone-Corsi P. Mitogen-regulated RSK2-CBP interaction controls their kinase and acetylase activities. Mol Cell Biol 2001;*21:* 7089-96.

45. Fu M, Wang C, Reutens AT, et al. p300 and p300/cAMP-response element-binding protein-associated factor acetylate the androgen receptor at sites governing hormone-dependent transactivation. J Biol Chem 2000;*275:* 20853-60.

46. Tukey J. W. Box-and-Whisker Plots. In:Tukey, JW, editor. Explanatory Data Analysis. Reading, MA: Addison-Wesley; 1977. p. 39-43.

47. Cleutjens KB, van der Korput HA, Ehren-van Eekelen CC,et al. A 6-kb promoter fragment mimics in transgenic mice the prostate-specific and androgen-regulated expression of the endogenous prostate-specific antigen gene in humans. Mol Endocrinol 1997;*11:* 1256-65.

Figure Legends

Fig.1 RSK levels are higher in human prostate cancer tissue. (A) Denatured tissue lysates were normalized to RAN and the normalized aliquots immunoblotted. A subset of the PC samples is presented. (B) Immunoblots were quantitated by densitometry (21) and the intensities plotted as a box-and-whisker plot (46). Bar in the box is at the statistical median and ends of the box are at the upper and lower quartiles. N indicates normal tissue (n=7), BPH indicates benign hyperplastic prostate tissue (n=4), PC indicates prostate cancer tissue (n=13) and L indicates the LNCaP cell line.

Fig. 2 RSK2 enhances PSA expression in prostate cancer cells. (A) LNCaP cells were co-transfected with luciferase and β-galactosidase reporter vectors. Luciferase expression was either under the control of the 6 kb region upstream of the start of the PSA gene (PSA-LUC) (47) or the control of a minimal promoter (MIN-LUC). Additionally, the cells were transfected with a control vector (V), a vector encoding RSK2 or RSK2(Y707A). The cells were serum-starved and treated with vehicle (-), 5 nM R1881 or 100 ng/ml EGF for 15 hrs. The luciferase data were divided by the β-galactosidase data to control for differences in transfection efficiency. The data obtained from the MIN-LUC reporter were subtracted from that obtained with the PSA-LUC reporter to control for non-specific transcription effects. The data were normalized so that, in the vector control, the response to vehicle addition was zero and the response to R1881 was 100. ($n \geq 5$, performed in triplicate.) The values are means $\pm$ SEM. *$P<0.05$ and **$P<0.01$ obtained by comparing vector control to RSK2 or RSK2(Y707A). (B) LNCaP cells were co-transfected with expression vectors encoding HA-AR with or without myc-RSK2(Y707A) and treated as in (A). Lysates of the transfected cells were immunoblotted. Equal loading of lysate is shown by the anti-MAPK immunoblot.

Fig. 3 RSK2 regulates AR-mediated transcription. (A) LNCaP cells were transfected and analyzed as in Fig. 2(A) except that only the PSA-LUC reporter was used. Additionally, a second transfection was done six hours after the first using control or AR siRNA (n=2, performed in triplicate). *P<0.05 and **P<0.01 obtained by comparing the control siRNA to the AR siRNA. Aliquots of the transfected cells were also immunoblotted. Equal loading of lysate is shown by the anti-RAN immunoblot. (B) LNCaP cells were transfected and analyzed as in Fig. 2(A) except that a vector encoding RSK2(K100A/707A) was also used (n=2, performed in triplicate). **P<0.01 obtained by comparing the vector control to either RSK2(Y707A) or RSK2(K100A/Y707A). (C) LNCaP cells were transfected as analyzed as in Fig. 2(A) except that a vector encoding ERα was also used. Additionally, either 0.5 μM 4-hydroxytamoxifen (4-OHT) or 1 μM ICI 182,780 was added with R1881 (n=2, performed in triplicate). (D) Recombinant, purified RSK2(Y707A) was used in an *in vitro* kinase assay and the amount of ^{32}P incorporated into the fusion proteins determined by Phosphorimager. (E) LNCaP cells were transfected and analyzed as in Fig. 2(A) except that varying concentrations of R1881 were added (n=2, performed in triplicate). The maximum responses and the concentrations at half the maximum response (EC_{50}) were determined by performing a best-fit analysis of the data (GraphPad Prism®).

Fig. 4 The RSK2/p300 complex regulates PSA expression. LNCaP cells were transfected and analyzed as in Fig. 2(A) except that only the PSA-LUC reporter was used and a vector encoding either p300 (A) or E1A (B) was also used (n=2, performed in triplicate). **P<0.01 obtained by comparing the response obtained in (A) to vector control or in (B) to RSK2(Y707A). Aliquots of the transfected LNCaP cells were lysed and immunoblotted.

Fig.5 RSK activity regulates prostate cancer cell proliferation. (A) MCF-10A and MCF-7 cells were treated with vehicle or 20 μM 3Ac-SL0101 for 72 hr. A proliferation assay was performed and the data expressed relative to time 0 (n=2, performed in triplicate). **P<0.01 obtained by comparing the vehicle to 3Ac-SL0101. The values are means ± SEM. (B) Cells were treated with vehicle or varying concentrations of 3Ac-SL0101 for 72 hrs. A proliferation assay was performed and analyzed as in 5(A) (n=2, performed in triplicate). **P<0.01obtained by comparing vehicle with 3Ac-SL0101. (C) PC-3 cells were treated with vehicle or 20 μM 3Ac-SL0101 for 72 hr. The cells treated with drug were washed and incubated with or without 3Ac-SL0101 for an additional 72 hr (n=2, performed in triplicate). A proliferation assay was performed and analyzed as in 5(A) **P<0.01 obtained by comparing vehicle with 3Ac-SL0101. (D) LNCaP cells were treated with vehicle or 30 μM 3Ac-SL0101. Proliferation (n=2, performed in triplicate) and PSA (n=2, performed in duplicate) assays were performed. The data was expressed relative to time 0. **P<0.01 obtained by comparing vehicle with 3Ac-SL0101.

Fig. 6 Characterization of RSK in prostate cancer cells. Serum-starved LNCaP and PC-3 cells were treated with either 25 μM U0126 or 100 ng/ml EGF and lysed. (A) An aliquot of the lysate was immunoblotted. (B) RSK2 was immunoprecipitated and used in an *in vitro* kinase assay. Specifically incorporated ^{32}P was obtained by subtracting the values obtained from GST-S6-tide from those obtained with GST. The relative level of RSK2 was determined by densitometry of immunoblots and the data normalized to the RSK2 level in U0126-treated LNCaP cells. Relative specific activity was determined by dividing the specifically incorporated ^{32}P by the normalized RSK2 level (n=2, performed in duplicate). **P<0.01 obtained by comparing LNCaP with PC-3 cells.

FIGURE 1 CLARK ET AL.

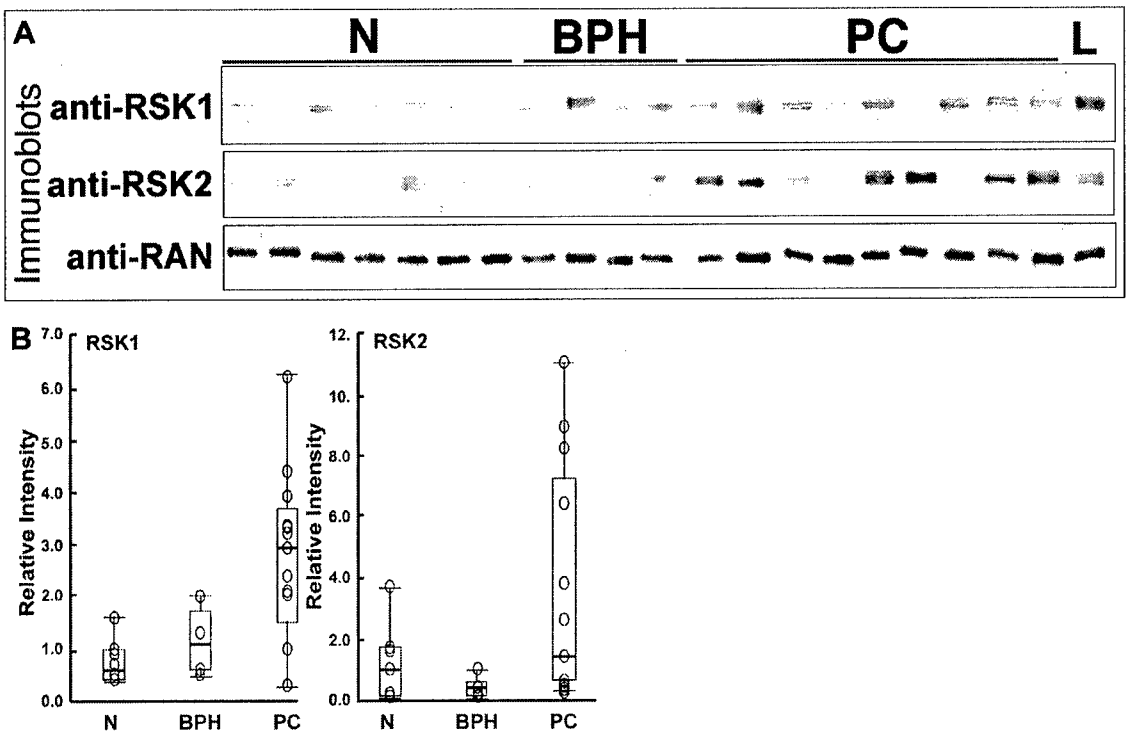

FIGURE 2 CLARK ET AL.

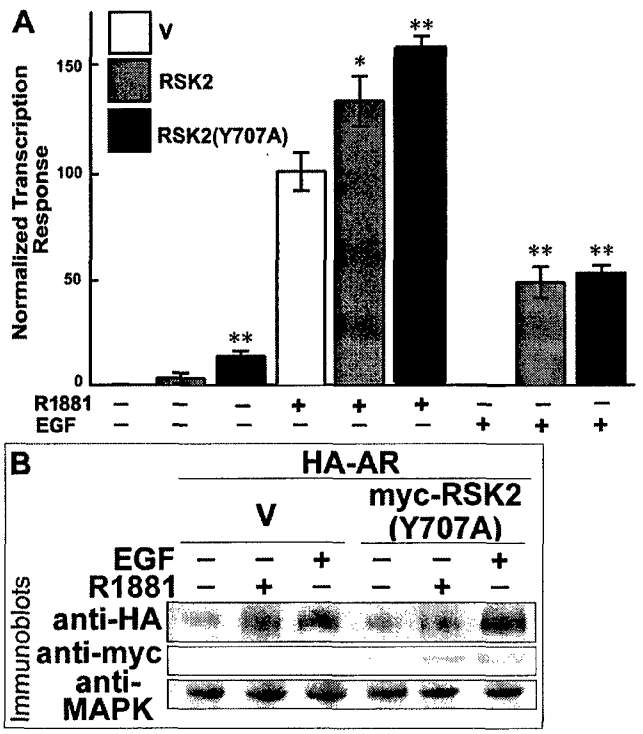

FIGURE 3 CLARK ET AL.

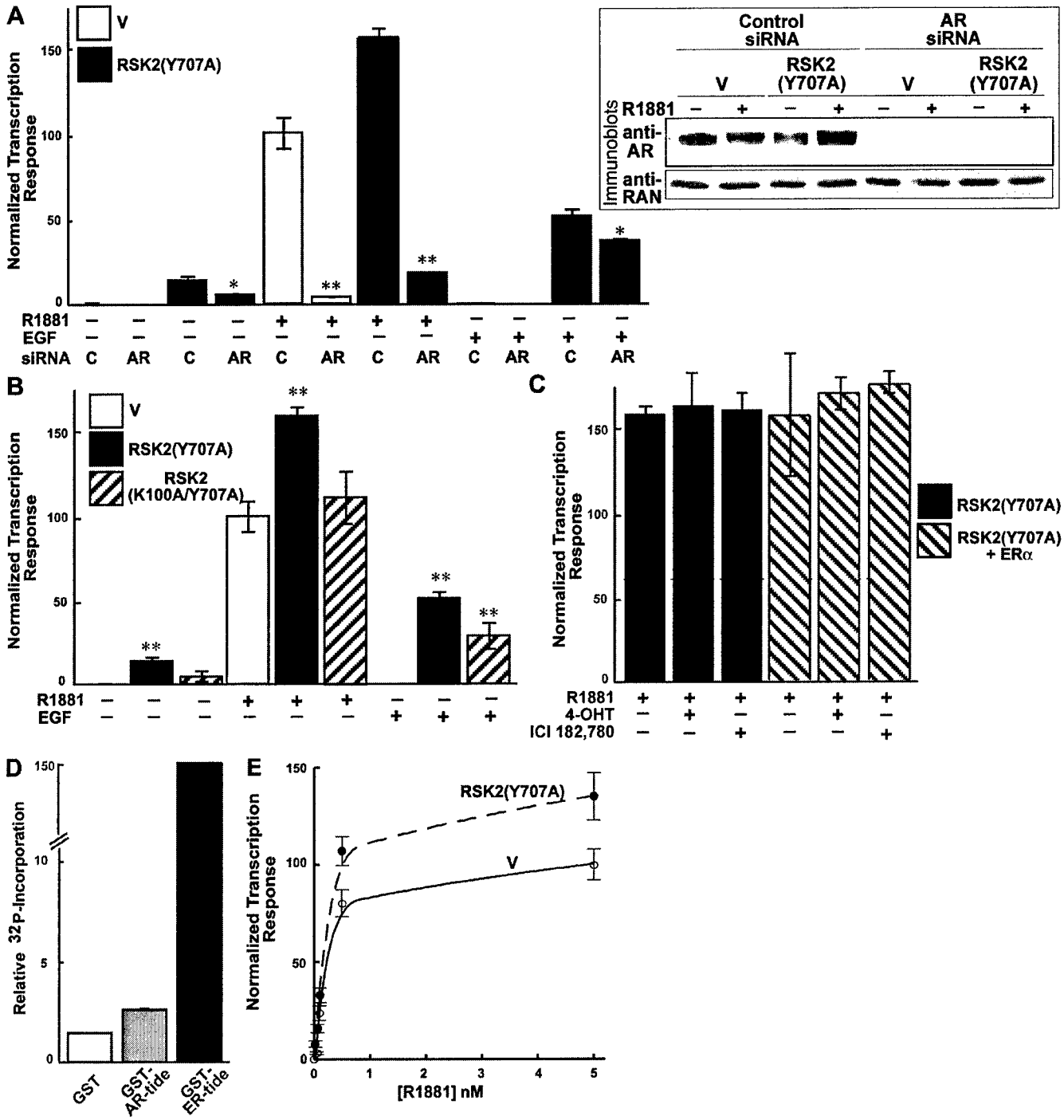

FIGURE 4 CLARK ET AL.

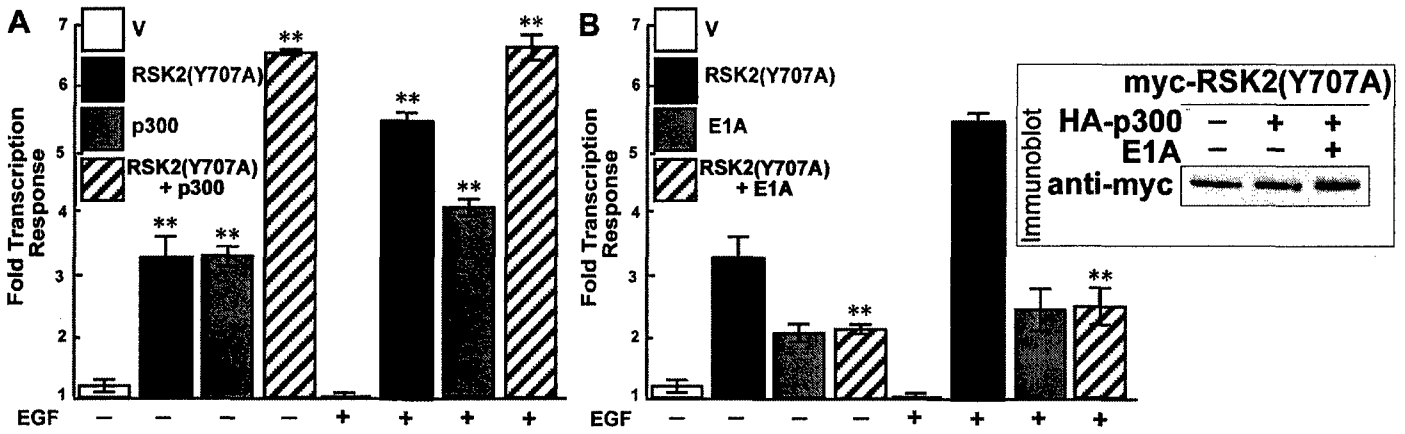

FIGURE 5 CLARK ET AL.

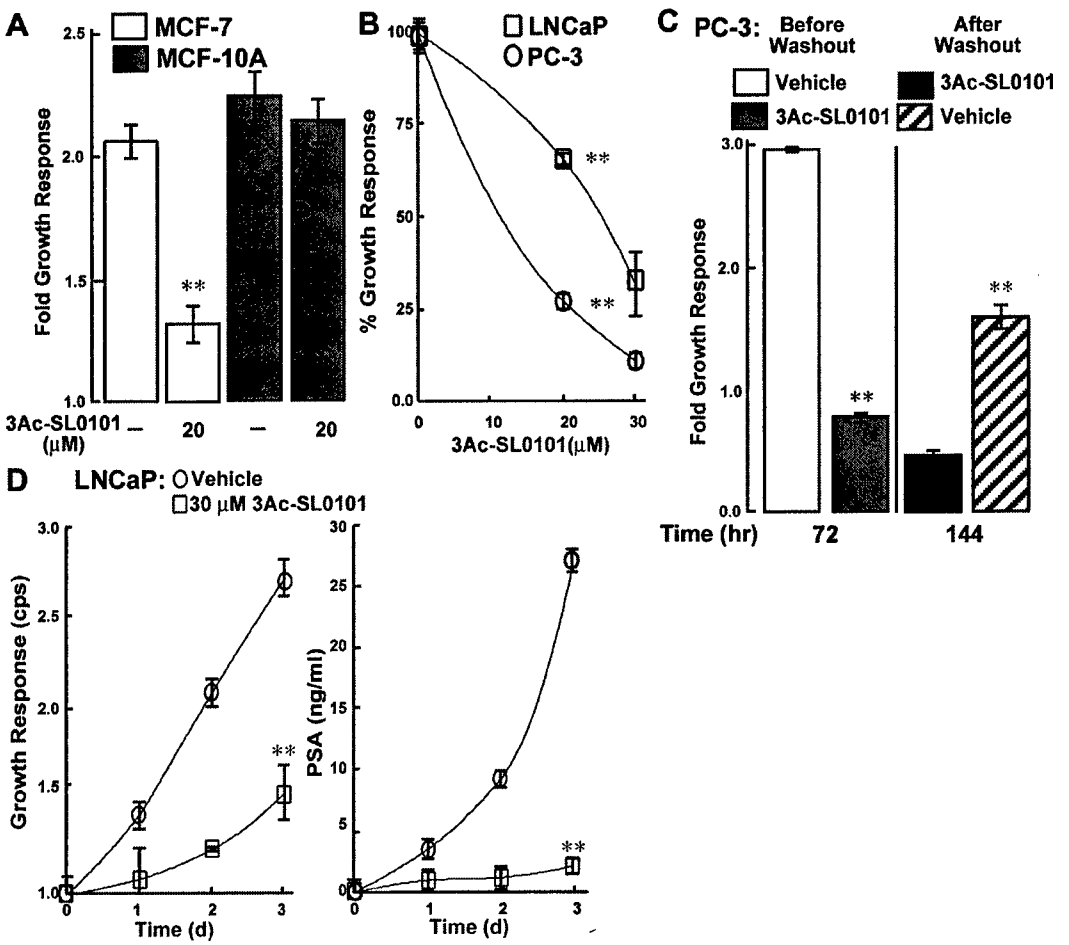

FIGURE 6 CLARK ET AL.