Constitutive Activation of the Ras/Mitogen-activated Protein Kinase Signaling Pathway Promotes Androgen Hypersensitivity in LNCaP Prostate Cancer Cells

Robert E. Bakin, Daniel Gioeli, Robert A. Sikes, Eric A. Bissonette, and Michael J. Weber

ABSTRACT

Progression of prostate cancer ultimately results in a disease that is refractory to hormone ablation therapy but nevertheless continues to require the androgen receptor. Progression to hormone refractory disease is often correlated with overexpression of growth factors and receptors capable of establishing autocrine and/or paracrine growth-stimulatory loops. Many of these growth factor receptors engage the Ras/mitogen-activated protein (MAP) kinase pathway as part of their signaling activities. This raises the possibility that chronic activation of Ras/MAP kinase signaling could cause or contribute to the progression of prostate cancer. We have demonstrated previously that MAP kinase activation correlates with progression to advanced hormone refractory disease in patient samples. Here we demonstrate that stable expression of Ras effector-loop mutants that activate the Ras/MAP kinase pathway is sufficient to reduce the androgen requirement of LNCaP prostate cancer cells for growth, prostate-specific antigen expression, anchorage-dependent and -independent growth, and increased tumorigenicity. We propose that chronic activation of endogenous c-Ras by autocrine and paracrine growth factor stimulation sensitizes the androgen receptor transcriptional complex to subphysiological levels of androgen. This provides a common mechanism for prostate cancer progression driven by diverse agonists.

INTRODUCTION

Numerous reports have implicated up-regulated expression of diverse autocrine and paracrine growth factors with progression of prostate cancer (reviewed in Refs. 1–4). Such factors include ligands for the EGF receptor, fibroblast growth factor, basic fibroblast growth factor, keratinocyte growth factor, insulin-like growth factor binding protein-5, tumor necrosis factor α, hepatocyte growth factor, transforming growth factor β, the anti-inflammatory cytokines IL-4, IL-6, and IL-10, vascular endothelial growth factor, neuropetides, and neuroendocrine hormones. The growth factor receptor c-HER-2/neu is overexpressed in 16–69% of prostate cancers (5–12), and overexpression has been shown to promote steroid-independent growth in both prostate (13, 14) and breast cancer (15, 16). Conversely, inhibition of growth factor receptors with monoclonal antibodies or tyrosine kinase inhibitors inhibits growth in androgen-dependent (17, 18) and androgen-independent (19, 20) prostate cancer xenografts. A change from paracrine to autocrine regulation of the EGF receptor has been observed in the progression of prostate cancer to androgen-independence (21). Taken together, these results demonstrate that up-regulated activation of mitogenic signaling pathways by autocrine and paracrine growth factor loops is closely correlated with acquisition of hormone refractory disease.

Many of the growth factor pathways implicated in prostate cancer progression use the small GTPase c-Ras as a component of their signaling activities. Ras is the prototypical member of a class of low molecular weight GTP binding proteins involved in the regulation of many cellular functions including cell cycle progression (22), apoptosis (23), cytoskeletal organization (24), membrane trafficking (25), and differentiation. In response to a diverse array of extracellular stimuli, Ras recruits and activates downstream signaling cascades leading to the regulation of both nuclear and cytoplasmic processes (26). Whereas activating Ras mutations are infrequent in prostate cancer (27–29), overexpression of c-Ras is often found in breast cancer where it correlates positively with poor prognosis (30, 31). We propose that Ras is positioned uniquely to function as a common mediator of prostate cancer progression by coordinat- ing mitogenic signals of disparate origins.

Ras is a multifactorial signaling molecule that controls several signaling pathways (26). One of the best-characterized effector pathways triggered by Ras activation is the MAPK pathway. Our laboratory has previously used a phosphorylation state-specific antibody that reacts with the activated form of MAPK and has demonstrated a correlation between elevated levels of active phospho-MAPK and prostate tumors of advanced stage and grade (32). These findings provided the first evidence that a Ras effector, and presumably Ras itself, was chronically activated in at least some advanced prostate cancers.

Here we provide data showing that stable expression of activated Ras mutants promoting constitutive activation of the Ras/MAPK pathway is sufficient to sensitize hormone-responsive LNCaP prostate cancer cells to subphysiological levels of androgen with respect to endogenous PSA expression, anchorage-dependent and-independent growth, and increased tumorigenicity. We propose that chronic activation of c-Ras by autocrine and paracrine growth factor stimulation provides a common mechanism for prostate cancer progression driven by a broad array of mitogens.

MATERIALS AND METHODS

Ras Effector-Loop Expression Plasmid Construction. HA-tagged Ras effector-loop mutants T35S, E37G, and Y40C (pDCR) were received as a gift from Channing Der (University of North Carolina, Chapel Hill, NC) and subcloned into pcDNA3.1 Neo (Invitrogen). Untagged c-Ras (pEXV-Amp) was received as a gift from Dr. Daniel Theodorescu, University of Virginia. FLAG epitope was added by PCR, and the tagged c-Ras was subcloned into pcDNA1.

Establishment of Ras Effector-Loop LNCaP Stable Cell Lines. LNCaP cells were transfected with 5 µg of Ras vector {N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate; Boehringer-Mannheim(Roche)}, after 8 h were rinsed, grown with phenol red-free RPMI 1640 supplemented with 10% FBS for 48 h, and then selected with 600 µg/ml active G418. Multiple colonies from five 10-cm dishes were trypsinized, pooled, and replated in G418 containing medium. The pooled cells were amplified and shown to be >95% positive by immunofluorescence of either HA or FLAG epitope, with predominant membrane staining. Western blotting with a pan-Ras antibody showed RasT35S and RasE37G expression approximately equal to that of endogenous Ras, whereas...
c-Ras and RasY40C expression levels were significantly higher (data not shown). Cell lines were subjected to reselection every 2 weeks with 300 μg/ml active G418 to maintain expression of Ras effector-loop mutants.

Reagents. Anti-phospho MAPK antibody was from Calbiochem, anti-PSA antibody from Bio Genex, and anti-MAPK antibody 1B3B9 from Upstate Biotechnology. Casodex (Bicalutamide) was a generous gift from Zeneca, Inc. (Wilmington, DE). The synthetic androgen R1881 was purchased from DuPont/NEN, U0126 from Calbiochem, charcoal/dextran-treated FBS from HyClone, Inc., and FBS (certified) and RPMI 1640 were from Life Technologies, Inc.

Data Quantitation. Relevant Western and Northern data were quantitated using Alpha Innotech AlphaEase FC analysis software.

Agarose Colony Assay. Cells (25,000) of each stable cell line were trypsinized and resuspended in 1 ml of 0.5% soft agarose, and layered on top of 0.5% agarose. Agarose assays were repeated via transient transfection of pCMV5/H11003 and pCMV5/H9262 expressing either HA-tagged or FLAG-tagged Ras proteins were characterized by addition of either biotin-conjugated polyclonal anti-HA (Santa Cruz) or biotin-conjugated monoclonal M5 anti-FLAG antibody (Sigma) followed by secondary horseradish peroxidase. The antibody specific for the dually phosphorylated MAPKs, ERK1 and ERK2, has been described previously (32, 33).

Northern Analysis. Total RNA was isolated using Qiagen RNeasy kit. Ten μg of total RNA was separated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and UV cross-linked (UV Crosslinker; Fisher Scientific). Membrane was stained with methylene blue to ensure equivalent sample loading as well as RNA integrity, prehybridized at 68 °C, hybridized with 32P-labeled GAPDH to reaffirm equivalent RNA loading. A 500-bp RI fragment of the PSA coding sequence and a 1-kb SacI/KpnI restriction fragment of GAPDH coding sequence were used in the hybridization buffers. After exposure to X-ray film, the blot was stripped in boiling 0.5% SDS solution for 15 min and subsequently reprobed with 32P-labeled GAPDH to reaffirm equivalent RNA loading. A 500-bp EcoRI fragment of the PSA coding sequence and a 1-kb SacI/KpnI restriction fragment of GAPDH coding sequence were used in the hybridization buffers. After exposure to X-ray film, the blot was stripped in boiling 0.5% SDS solution for 15 min and subsequently reprobed with 32P-labeled GAPDH to reaffirm equivalent RNA loading.

Animal Studies. Male BALB/cAnN-Crl-nuBR mice (5–6 weeks old; Charles River) were bilaterally inoculated s.c. with 2 × 106 cells in Matrigel (Becton Dickinson) at 50:50 volume for a total volume of 100 μl/injection site. Average tumor burden was calculated with calipers in millimeters as the mean tumor diameter measured in two dimensions. To minimize unnecessary animal use, included in the final data were a proportion of control mice harboring C4–2 xenografts from separate parallel experiments treated with the antibiotic doxycycline. Doxycycline administration was shown to have no observable effects on either in vivo or in vitro growth.

Bilateral castration was performed on Ketamine/Diazepam-treated anesthetized mice having tumor burdens at least 5 mm in diameter using standard surgical techniques. Mice were sacrificed using carbon dioxide asphyxiation, placed on ice, and tumors were excised. Tumor samples were fixed in 10% buffered formalin for 48 h then paraffin embedded for immunohistochemistry. Alternatively, tumor samples were snap frozen in liquid nitrogen, pulverized with mortar and pestle, resuspended in 0.1% SDS-PAGE lysis buffer containing protease and phosphatase inhibitors, then sonicated on ice with eight 1-s bursts. Samples were subsequently microcentrifuged at 4 °C for 1 min, lysate removed from residual precipitated tissue, and subjected to standard SDS-PAGE on a 10% polyacrylamide gel.

Serum PSA levels were quantified using an automated Abbott Laboratories IMX MEIA clinical assay machine.

Statistical Methods. Profiles of tumor diameter were modeled using random coefficient regression models, which incorporated all of the available post-treatment data for each animal (34). Baseline was set at the first measurement before the animals in the treatment group were castrated. Models were fit separately within each cell line. When multiple tumors were available for an animal, both contributed curves to the analyses. The curves were estimated as restricted cubic spline functions of time since castration (in weeks) with three knots, which was deemed sufficient by Akaike’s information criterion. The random coefficient regression model, in essence, estimates and averages curves calculated for each tumor within each group, accounting for the correlation among the repeated measurements over time. The models also included a term for precastration tumor diameter, which served as a baseline adjustment. The model was fit with no main effects for treatment group and treatment period. Curves shapes were not found to be dependent on size of tumor at baseline, although we had very limited power to test for this effect. Type 3 F tests were used to test for differences between the castrated and noncastrated curves. The models were fit using the MIXED procedure in SAS Version 8.2 (SAS Institute, Cary, NC).

AUC was calculated using the Trapezoid rule and is presented as a graphical summary (Fig. 6) of the growth curve analyses (Table 1). The last value was carried forward to the end of study for animals sacrificed more than halfway through the experiment. Two tumors from the C4–2 group were excluded from the AUC summary because of an animal death resulting in only two measurements (of a possible five). Confidence intervals (95%) for tumor takes and tumors remaining were generated using an exact binomial distribution.

RESULTS

MAPK Activation in Ras Effector-Loop Stable Cell Lines. Ras is a common downstream focal point of many growth factor signaling pathways reported to play a role in prostate cancer progression. To determine which Ras effector pathways might be responsible for acquisition of hormone refractory growth, we established LNCaP stable cell lines expressing mutationally activated (V12) Ras with secondary effector-loop mutations, RasT355, RasE37G, and RasY40C. These mutants preferentially activate some effector arms better than others (43–47), with different selectivity.

Table 1 Summary of xenograft studies

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>C4-2</th>
<th>c-Ras</th>
<th>T35S</th>
<th>E3TG</th>
<th>Y40C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor take</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8/32</td>
<td>22/24</td>
<td>4/40</td>
<td>25/32</td>
<td>16/32</td>
<td>10/32</td>
</tr>
<tr>
<td>(25%)</td>
<td>(0.11–0.43)</td>
<td>(0.92–0.99)</td>
<td>(0.73–0.99)</td>
<td>(0.40–0.29)</td>
<td>(0.60–0.91)</td>
<td>(0.32–0.68)</td>
</tr>
<tr>
<td>Average time to tumor take</td>
<td>39 days</td>
<td>25 days</td>
<td>40 days</td>
<td>21 days</td>
<td>29 days</td>
<td>39 days</td>
</tr>
<tr>
<td>Tumors postcastration</td>
<td>0/4</td>
<td>11/11</td>
<td>2/2</td>
<td>8/13</td>
<td>3/6</td>
<td>4/5</td>
</tr>
<tr>
<td>(0%)</td>
<td>(0.0–0.60)</td>
<td>(0.72–1.0)</td>
<td>(0.16–1.0)</td>
<td>(0.32–0.86)</td>
<td>(0.12–0.88)</td>
<td>(0.28–0.99)</td>
</tr>
<tr>
<td>Invasive tumors</td>
<td>0/4</td>
<td>0/22</td>
<td>0/11</td>
<td>0/25</td>
<td>0/16</td>
<td>5/12</td>
</tr>
<tr>
<td>Constitutive in vitro p-MAPK</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Constitutive in vivo p-MAPK postcastration</td>
<td>n/a</td>
<td></td>
<td></td>
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* n/a, not applicable.
T35S preferentially activating Raf, E37G activating Ral-GDS, and Y40C activating phosphatidylinositol 3'-kinase. Cells transfected with empty vector or c-Ras were established similarly and used as controls.

The Raf/MEK/ERK pathway is a major effector arm of Ras, and its activation has been correlated with disease progression in several cancer models (32, 35–39). Western blotting with a phospho-specific MAPK antibody demonstrated constitutive phospho-MAPK activity in both RasT35S and RasE37G stable cell lines under all of the cell culture conditions tested (Fig. 1A). Importantly, constitutive MAPK activity was observed under serum-free culture conditions where media were changed frequently to minimize confounding autocrine effects. Both EGF and serum were capable of elevating MAPK levels in Ras T35S and RasE37G cell lines, indicating MAPK activity is still regulated in these cell lines. Alterations in levels of steroid did not affect phospho-MAPK profiles, as similar results were obtained using steroid depleted csFBS with or without R1881 supplementation (data not shown). Addition of either RasT35S or RasE37G conditioned medium to serum-starved parental LNCaP cells did not promote MAPK activation (data not shown) suggesting that activation of MAPK was not because of autocrine signaling.

Consistent with results published in other systems, RasY40C-expressing cells did not display significant constitutive activation of MAPK. Interestingly, serum stimulation of prestarved cells promoted robust phospho-MAPK activity suggesting whereas RasY40C is alone unable to promote constitutive phosphorylation of MAPK, its overexpression can sensitize the MAPK pathway to transient activation by serum. Parental LNCaP, empty vector pcDNA3.1 (data not shown), and c-Ras stable cell lines all behaved similarly in demonstrating robust phospho-MAPK activity after a 5-min addition of 10 ng/ml EGF. EGF stimulation resulted in noticeably higher phospho-MAPK activity in the c-Ras-overexpressing cell line relative to parental LNCaP, demonstrating that c-Ras overexpression renders these cells hypersensitive to growth factor stimulation. Inhibition of phospho-MAPK in both RasT35S and RasE37G stable cell lines was observed after a 30-min addition of the MEK inhibitor U0126, demonstrating the requirement for MEK activity (Fig. 1B).

RasT35S and RasE37G Induce Androgen Hypersensitivity. The correlation of hormone refractory prostate cancer with up-regulated growth factor signaling pathways, including MAPK, led us to ask whether LNCaP cell lines demonstrating constitutive phospho-MAPK activity would have a growth advantage under steroid-depleted culture conditions. MTT growth assays revealed that whereas all of the cell lines exhibited similar growth curves both in 10% nFBS supplemented medium as well as steroid depleted, csFBS back-supplemented with 1 nm R1881, only RasT35S and RasE37G cells retained the ability to proliferate under reduced levels of androgen (Fig. 2). In particular, significant cell growth was seen in RasT35S and RasE37G stable cell lines growing in charcoal-stripped serum back-supplemented with as little as 10 pM R1881, a steroid concentration 2 orders of magnitude lower than normal physiological levels (~1 nm). Importantly, RasT35S and RasE37G cell lines did not display significant growth when steroid levels fell below 10 pM, demonstrating that the cells had become hypersensitive to androgen rather than completely androgen independent.

Western analysis of cells growing under these conditions confirmed constitutive phospho-MAPK activity in RasT35S and RasE37G cells (data not shown). No detectable alterations in AR protein level or electrophoretic mobility were observed (data not shown). Attempts to directly attribute enhanced growth in steroid depleted medium to constitutive MAPK activity were hampered by toxicity of long-term repeated administration of the MEK inhibitor U0126. These results demonstrate a correlation between constitutive activation of the Ras/MAPK pathway by Ras effector loop mutants and hypersensitive growth responses to subphysiologic levels of androgen.

RasT35S and RasE37G Expression Is Sufficient for Androgen Hypersensitivity of Anchorage-independent Growth. Cell anchorage is required for efficient signal propagation between Ras and downstream effectors such as MAPK, and for growth of fibroblasts and epithelial cells (40). Anchorage-independent growth is a hallmark of malignant transformation of these cell types. Therefore, we asked whether Ras expression in LNCaP cells would alter the steroid dependence of anchorage-independent growth. Parental androgen-dependent LNCaP cells readily formed colonies in agarose medium supplemented with 5% nFBS containing endogenous levels of androgen (data not shown). However, LNCaP cells were unable to form colonies in agarose supplemented with steroid-depleted charcoal-

![Fig. 2. RasT35S and RasE37G expression hypersensitizes adherent growth of LNCaP cells to low levels of androgen. Parental LNCaP and Ras effector-loop stable cell lines were plated in 24-well dishes at a concentration of 30,000 cells/well. After 24-h growth in 10% nFBS, media were changed to 5% csFBS with or without R1881. Ethanol vehicle control was kept constant at 0.1%. Cell number was determined every 3 days by MTT. Data are presented as cell number at day 12. Results are representative of three independent experiments; bars, ±SD.](image-url)
RasT35S and RasE37G Effector-Loop Stable Cell Lines Express High Levels of PSA at Low Levels of Androgen. Serum PSA levels are used routinely as a clinical marker of tumor burden and disease progression after androgen ablation therapy. Because Ras/MAPK signaling can reduce the androgen requirement of prostate cancer cell growth, one would predict that this signaling might similarly hypersensitize androgen-responsive gene expression to low levels of androgen. We have measured endogenous PSA expression as an indicator of androgen regulated gene expression.

Both RasT35S and RasE37G cell lines growing in 10% nFBS displayed significantly elevated endogenous steady-state cellular PSA transcription relative to parental LNCaP, c-Ras, and RasY40C stable cell lines (Fig. 3A). Quantitation of PSA mRNA levels from RasT35S and RasE37G stable cell lines revealed an increase over parental LNCaP of 3-fold and 2-fold, respectively. RasT35S and RasE37G PSA protein levels demonstrated an enhancement of 8-fold and 6-fold, respectively (Fig. 4B). The antiandrogen Casodex significantly attenuated PSA protein levels in all of the stable cell lines, including RasT35S and RasE37G, confirming a necessary role of the AR in the enhanced expression of endogenous PSA in response to elevated Ras/MAPK signaling (Fig. 4B). Daily administration of a subtoxic 20-μM dose of U0126 over a 3-day period significantly reduced PSA protein levels, but not ERK2 levels in both the RasT35S and RasE37G cell lines, confirming the requirement of MAPK signaling in the elevation of endogenous PSA expression (Fig. 4C). In sum, constitutive phospho-MAPK activity is sensitive to MEK inhibition. The MAPK pathway was inhibited with 20-μM dose of U0126 administered once daily over a 3-day period. DMSO was used as a vehicle control. Quantifications of band intensities are displayed as fold increase over parental LNCaP.

If MAPK activation was driving anchorage-independent growth, pharmacological inhibition of the MAPK pathway should inhibit RasT35S- and RasE37G-mediated colony formation. We found that addition of a single 20-μM dose of the MEK inhibitor U1026 at the time of plating inhibited RasT35S and RasE37G growth in steroid-depleted agarose, even when back-supplemented with optimal levels of androgen (Fig. 3B). Anchorage-independent growth of C4–2 cells, treated similarly, was not affected by short-term MEK inhibition. This makes it unlikely that the growth inhibition was because of generalized toxicity of transient MEK inhibition, and suggests that constitutive phospho-MAPK activity is likely necessary for (at least) the early phases of anchorage-independent growth induced by Ras effector loop mutants.

In sum, expression of the mutants RasT35S and RasE37G served to bypass the anchorage requirement for efficient MAPK activation, rendering LNCaP cells hypersensitive to low androgen under anchorage-independent conditions. Qualitatively similar results were obtained using transient transfection assays (data not shown).

Fig. 3. RasT35S and RasE37G expression hypersensitize anchorage-independent growth of LNCaP cells to low levels of androgen. A, 25,000 cells of each cell line were plated in 5% csFBS supplemented agarose reconstituted with R1881 or ethanol vehicle. Cells were grown for 3 weeks at 37°C and 5% CO₂. Plates were scanned on a flatbed scanner and colony number counted via computer with a constant minimum cutoff value for colony size. B, similar experiment as in A, except addition of a one-time 20-μM dose of U0126 at the time of plating. Results are representative of three separate experiments; bars, ±SD.

RasT35S and RasE37G cell lines growing in 10% nFBS displayed significantly elevated endogenous steady-state cellular PSA transcription relative to parental LNCaP, c-Ras, and RasY40C stable cell lines (Fig. 4A). Quantitation of PSA mRNA levels from RasT35S and RasE37G stable cell lines revealed an increase over parental LNCaP of 3-fold and 2-fold, respectively. RasT35S and RasE37G PSA protein levels demonstrated an enhancement of 8-fold and 6-fold, respectively (Fig. 4B). The antiandrogen Casodex significantly attenuated PSA protein levels in all of the stable cell lines, including RasT35S and RasE37G, confirming a necessary role of the AR in the enhanced expression of endogenous PSA in response to elevated Ras/MAPK signaling (Fig. 4B). Daily administration of a subtoxic 20-μM dose of U0126 over a 3-day period significantly reduced PSA protein levels, but not ERK2 levels in both the RasT35S and RasE37G cell lines, confirming the requirement of MAPK signaling in the elevation of endogenous PSA expression (Fig. 4C). In sum, constitutive phospho-MAPK activity is sensitive to MEK inhibition. The MAPK pathway was inhibited with 20-μM dose of U0126 administered once daily over a 3-day period. DMSO was used as a vehicle control. Quantifications of band intensities are displayed as fold increase over parental LNCaP.

Fig. 4. RasT35S and RasE37G LNCaP cells display elevated endogenous PSA. A, Ras T35S and E37G up-regulate PSA mRNA levels. Northern analysis of PSA mRNA was analyzed under identical conditions as in part B. Equivalent mRNA loading was confirmed via GAPDH blotting, as well as ethidium bromide staining of 28S and 18S RNase (data not shown). B, RasT35S and RasE37G expression sensitizes endogenous PSA expression to normal levels of androgen. Cell lines were grown in 10% nFBS with a one-time 30-μM dose of the antiandrogen Casodex or DMSO control vehicle for 5 days. Cells were lysed and soluble fractions Western blotted with polyclonal anti-PSA antibody. Equal protein loading was confirmed by blotting for MAPK. C, RasT35S and E37G up-regulated PSA protein expression is sensitive to MEK inhibition. The MAPK pathway was inhibited with 20-μM of U0126 administered once daily over a 3-day period. DMSO was used as a vehicle control. Quantifications of band intensities are displayed as fold increase over parental LNCaP.
Constitutive MAPK activation can hyperinduce PSA expression in LNCaP cells under normal levels of androgen. Because constitutive MAPK activity can enhance PSA expression under normal androgen levels, we asked whether activation of the Ras/MAPK pathway could hypersensitize endogenous PSA expression to low levels of androgen. Western analysis revealed intense PSA expression in the RasT35S and RasE37G cell lines growing in 10% csFBS supplemented with as little as 10 pM R1881 (Fig. 5). Similar PSA expression in parental LNCaP cells required an R1881 concentration 2 orders of magnitude higher (1 nM). No alterations in immunoreactive AR content were observed (data not shown). These data show that MAPK activity can enhance endogenous PSA expression under normal and reduced levels of steroid, but not in the absence of steroid, indicating coregulation of the PSA promoter by the Ras/MAPK pathway and the AR.

RasT35S and RasE37G cell lines demonstrated hormone refractory in vivo growth. Elevated levels of phospho-MAPK have been correlated with disease progression in several in vivo cancer models. Here we show that the LNCaP-stable Ras effector-loop cell lines RasT35S and RasE37G when injected s.c. into athymic nude mice demonstrated enhanced tumor take, elevated rate of tumor growth, and significant hormone refractory proliferation. Whereas empty vector control LNCaP, c-Ras, and Y40C stable cell lines demonstrated modest tumor take (25%, 13%, and 31%, respectively), both RasT35S (78%) and RasE37G (50%) showed significantly enhanced tumor takes in intact athymic male nude mice (Table 1). Moreover, the rate of tumor development was accelerated in both RasT35S and RasE37G cell lines, demonstrating measurable tumors within 21 and 29 days, respectively. The fully hormone-refractory LNCaP derivative cell line, C4–2, injected similarly, had a >90% tumor take occurring within 25 days.

The ability of prostate cancer to proliferate under castrate levels of androgen is a hallmark of disease progression. We asked whether tumors expressing RasT35S and RasE37G would demonstrate continued survival and growth in a reduced androgen environment. Tumor-bearing mice were either castrated or sham-castrated when tumor burdens achieved a minimal average diameter of 5 mm. As expected, castration of mice harboring androgen-dependent LNCaP parental tumors resulted in rapid regression of all of the tumors within 3 weeks (Fig. 6; Table 1). No evidence of tumor rebound or PSA in peripheral blood was observed in the time period after castration (~16 weeks). The hormone-refractory derivative, C4–2, maintained 100% tumor burden postcastration with no observable tumor regression and significant serum PSA levels proportional to their volume.

In a manner paralleling their in vitro phospho-MAPK activity, RasT35S and RasE37G tumors exhibited a significant degree of hormone refractory growth and survival after castration. Here, 62% of all of the T35S tumors and 50% of all of the E37G tumors either maintained tumor volume or rapidly rebounded to original tumor volume within 3 weeks (Fig. 6; Table 1). To our surprise, two of two c-Ras and four of five RasY40C tumors either maintained size or continued to grow and secrete PSA after castration. As expected, all of the RasT35S and RasE37G tumors that survived androgen ablation displayed significant in vivo phospho-MAPK ac-

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**Fig. 5.** Ras effector-loop cell lines RasT35S and RasE37G mediate PSA hypersensitization to low levels of androgen. Parental LNCaP, RasT35S, and RasE37G cell lines were grown for 5 days in either 10% nFBS or 10% csFBS with decreasing amounts of R1881. Whole cell lysates were subjected to Western analysis with anti-PSA antibody. Equal protein loading was confirmed via Western blotting with anti-MAPK antibody.

**Fig. 6.** Ras expression induces xenograft growth refractory to castration. AUC was calculated for each xenograft with time rescaled between 0 and 1 for visual comparability among cell lines. represent animals that were surgically castrated. Dashed horizontal lines are medians whereas solid lines are means.
Interestingly, although c-Ras and RasY40C cell lines did not display detectable constitutive phospho-MAPK activity in vitro, significant in vivo phospho-MAPK activity was observed in all of the c-Ras and RasY40C tumors after castration as illustrated by a representative Western analysis (Fig. 7A). Complete regression of parental LNCaP tumors prevented analysis of phospho-MAPK levels after castration. Curiously, a small percentage of all of the Ras-expressing tumors spontaneously regressed in both castrated and noncastrated animals for unknown reasons. Like parental LNCaP tumors, complete tumor regression prevented the determination of the phospho-MAPK status of these tumors. Immunohistochemistry of tumor samples confirmed significant nuclear and cytoplasmic phospho-MAPK staining in the tumor epithelial cell population as well as membrane localized exogenous Ras expression (Fig. 7, B and C). We observed no significant alterations in expression or electrophoretic mobility of AR in the various cell lines either in vitro or in vivo (data not shown).

In sum, LNCaP cell lines demonstrating constitutive in vitro activation of the Ras/MAPK pathway displayed elevated tumor take, rate of tumor growth, and significant hormone refractory proliferation in castrated athymic nude mice.

**DISCUSSION**

**LNCaP Cells Expressing Activated Ras Mutants.** We have reported previously that elevated MAPK activation in patient specimens was associated with prostate cancer progression (32). This observation led us to hypothesize that chronic activation of the Ras to MAPK pathway by growth factors overexpressed in advanced prostate cancer
may contribute to the acquisition of hormone refractory growth. To determine whether activation of this pathway could be causal in reducing dependence on androgen, we established stable LNCaP prostate cancer cell lines expressing activated RasV12 with effector-loop mutations at T35S, E37G, and Y40C. These effector loop mutants are reported to preferentially engage some effectors better than others, thus allowing analysis of which effector arms of Ras are important for a biological effect (43–47). We observed constitutive phospho-MAPK activation in the RasT35S LNCaP cell line in accordance with its reported ability to directly activate the Raf/MAPK pathway (42). Unexpectedly, we observed a similar MAPK activation profile for the RasE37G stable cell line. This Ras mutant has been reported not to activate the MAPK pathway in fibroblasts (43) or to physically interact with c-Raf-1 (42, 44). Nevertheless, it has been shown that protein kinase A activity stimulates MAPK phosphorylation in thyroid epithelial cells stably expressing RasE37G (45). These findings demonstrate the importance of cellular context when using and interpreting results obtained with these molecular reagents. It is possible that the high levels of expression of B-Raf in LNCaP cells (48) contributes to the unexpected effects of RasE37G in this context.

We were unable to isolate stable cell lines expressing either mutationally activated MEK (S218/222D) or activated RasV12 without secondary effector loop mutations. In light of the observations that activated RasV12 is apoptotic in fibroblasts (46) whereas activated Raf-1 promotes cell cycle arrest in LNCaP cells (47), we suspect that stable expression of either RasV12 or MEK 218/222D is incompatible with long-term survival of LNCaP cells. It is likely that stable expression of either of these two molecules can occur only with the appropriate balance of growth stimulatory and antipapoptotic signaling. This may help explain why fully activating Ras mutations are rarely encountered in clinical prostate cancer specimens.

Activation of the Ras/MAPK Pathway Correlates with Hormone Refractory Tumor Growth. We found that the LNCaP cell lines with enhanced in vitro Ras/MAPK activity (e.g., RasT35S and RasE37G) had significantly higher tumor takes and decreased time to tumor establishment, in athymic nude mice. Indeed, the tumor take of the Ras T35S derivative approached the highly tumorigenic and hormone refractory LNCaP derivative C4–2.

To assess hormone dependence for the growth of these tumors, we initiated tumor formation in intact male mice followed by castration after tumors were established. This paradigm parallels the course of human prostate cancer initiation, progression, and subsequent clinical intervention. No tumor rebound was observed in parental LNCaP tumors during the course of this study (16 weeks after castration). In contrast, a significant proportion of both RasT35S- and E37G-expressing tumors demonstrated minimal regression, and marked hormone refractory tumor growth in castrated mice.

Interestingly, growth of all of the c-Ras and 80% of RasY40C-overexpressing tumors was refractory to hormone ablation. This was surprising, because these cell lines (in contrast to the T35S and E37G lines) did not display elevated basal MAPK activity in cell culture. However, because we observed that activation of MAPK in these cell lines was hyper-responsive to serum or EGF addition (Fig. 1A), we wondered whether comparable paracrine stimulation could induce MAPK activation in vivo. When we examined these tumors, either by Western blots or histochemistry, we found substantial phospho-MAPK. We speculate that in the c-Ras- and Y40C-overexpressing tumors, paracrine signaling from stroma is responsible for triggering in vivo Ras signaling, phospho-MAPK activity, and subsequent hormone refractory growth. Moreover, both c-Ras and RasY40C cells showed levels of Ras expression much higher than endogenous, as has also been observed in advanced breast cancer (30). Western blotting of tumors formed from LNCaP cells overexpressing c-Ras failed to detect the mobility shift commonly associated with activating Ras mutations (49), suggesting that mutational activation of c-Ras was not responsible for the acquired tumorigenicity of these cells (data not shown). Thus, it is plausible that chronic paracrine signaling may mediate activation of MAPK in vivo in cells overexpressing c-Ras.

Acquisition of Androgen Hypersensitivity. We found that activation of Ras/MAPK signaling diminished but did not replace the requirement of LNCaP cells for androgen. LNCaP cells expressing Ras T35S or E37G were able to grow robustly at concentrations of androgen that were 1–2 orders of magnitude lower than the $10^{-9}$ m that were required by the parental LNCaP cells. However, reduction of androgen to $10^{-12}$ m resulted in growth arrest even for the Ras-expressing cells. Comparable results were obtained whether measuring adherent or nonadherent growth. Thus, these cells have become hormone hypersensitive rather than hormone independent.

A similar phenomenon was observed when measuring PSA production, consistent with previous results showing that the PSA promoter can be dually regulated by signal transduction pathways in addition to androgens (50–54). As expected, we found that steady state endogenous cellular PSA was dramatically elevated in the RasT35S and RasE37G stable cell lines. Northern analysis confirmed up-regulated PSA mRNA expression in both of these cell lines. However, as with the growth response, PSA expression diminished when androgen levels were reduced to $10^{-12}$ m or less. We additionally demonstrated that the antiandrogen Casodex and the MEK inhibitor U0126 were each able to attenuate the elevated endogenous PSA levels in RasT35S and RasE37G cell lines, highlighting the cross-talk between Ras directed signaling pathways and AR-dependent gene expression. Thus, Ras signaling did not bypass the requirement for steroid of this androgen-dependent gene expression. Rather, Ras-mediated signaling cascades apparently converted the normal regulatory machinery to an androgen hypersensitive state.

The Role of the AR in Ras-expressing Cells. The fact that Ras signaling did not eliminate the steroid requirement of LNCaP cells is consistent with the concept that hormone refractory prostate cancer still requires the AR (55, 56). This is reflected in the fact that retention, overexpression, and mutation of ARs are common in tumors that are unresponsive to hormone ablation. More recent reports have demonstrated that knockdown of the AR by hammerhead ribozyme, antibody (57), or antisense (58, 59) blocks prostate cancer cell growth.

How could signal transduction sensitize the AR to steroids? In the case of the estrogen receptor, several reports demonstrate that signal-transducing kinases can directly phosphorylate the steroid receptor and decrease or eliminate the requirement for estrogen (60). However, we recently have mapped the major phosphorylation sites on the AR (61), and suspect that none of them is sufficient for regulating the transcriptional activation of the AR.

On the other hand, several transcriptional coregulators have been shown to be targets of Ras-related signal transduction. In addition, recent reports point to overexpressed transcriptional coactivators as regulators of androgen sensitivity in advanced disease (62–65). We suspect that these coactivators may be major targets of signaling pathways that regulate androgen dependence.

Clinical androgen ablation therapy reduces but does not eliminate androgen from the circulation (66). Residual steroid creates conditions favorable for selection of cancer cells having the ability to proliferate under reduced levels of androgen. Compensatory mechanisms that allow growth in castrate levels of androgen include overexpression or mutation of the AR (55, 56), overexpression of transcriptional coregulators (62, 65), and, as shown by us (32), activation of Ras signaling. These mechanisms are not mutually exclusive but are likely to be mutually reinforcing.

Taken together, our findings show that chronic activation of Ras by
mutation, overexpression, or growth factor signaling can reduce the androgen requirement of prostate cancer cells with respect to proliferation, tumorinitigencity, and gene expression. These findings provide a common mechanism for prostate cancer progression driven by diverse primary alterations, and thus identify the Ras to MAPK pathway as an attractive target for therapeutic intervention.

ACKNOWLEDGMENTS

We thank Dr. Daniel Theodorescu for provision of plasmids and advice and the Paul Mellon Prostate Cancer Research Institute group for advice; Sharon Birdsell for immunochemistry work; and all of the other members of the Weber laboratory for helpful discussions.

REFERENCES


Constitutive Activation of the Ras/Mitogen-activated Protein Kinase Signaling Pathway Promotes Androgen Hypersensitivity in LNCaP Prostate Cancer Cells
