RasGRP3 Contributes to Formation and Maintenance of the Prostate Cancer Phenotype

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Abstract

RasGRP3 mediates the activation of the Ras signaling pathway that is present in many human cancers. Here, we explored the involvement of RasGRP3 in the formation and maintenance of the prostate cancer phenotype. RasGRP3 expression was elevated in multiple human prostate tumor tissue samples and in the human androgen-independent prostate cancer cell lines PC-3 and DU 145 compared with the androgen-dependent prostate cancer cell line LNCaP. Downregulation of endogenous RasGRP3 in PC-3 and DU 145 cells reduced Ras-GTP formation, inhibited cell proliferation, impeded cell migration, and induced apoptosis. Anchorage-independent growth of the PC-3 cells and tumor formation in mouse xenografts of both cell lines were likewise inhibited. Inhibition of RasGRP3 expression reduced AKT and extracellular signal-regulated kinase 1/2 phosphorylation and sensitized the cells to killing by carboplatin. Conversely, exogenous RasGRP3 elevated Ras-GTP, stimulated proliferation, and provided resistance to phorbol 12-myristate 13-acetate–induced apoptosis in LNCaP cells. RasGRP3-overexpressing LNCaP cells displayed a markedly enhanced rate of xenograft tumor formation in both male and female mice compared with the parental line. Suppression of RasGRP3 expression in these cells inhibited downstream RasGRP3 responses, caused the cells to resume the LNCaP morphology, and suppressed growth, confirming the functional role of RasGRP3 in the altered behavior of these cells. We conclude that RasGRP3 contributes to the malignant phenotype of the prostate cancer cells and may constitute a novel therapeutic target for human prostate cancer.

Introduction

Recent investigations have highlighted the important role of Ras activity for cell growth and survival in prostate cancer cells. Blocking Ras activation results in growth arrest and cell death (1, 2). In the androgen-dependent prostate cancer cell line LNCaP, stable expression of an activated Ras mutant shifted the cells toward a more malignant phenotype with reduced androgen dependence; tumor formation was enhanced and the tumors failed to regress after androgen depletion (3). Conversely, expression of dominant-negative Ras restored androgen dependence to C4-2 cells (a hormone-refractory deriv-
describe the expression of RasGRP3 in two androgen-independent human prostate cancer cell lines: PC-3 and DU 145. Using several conditional expression approaches, we show that RasGRP3 contributes to proliferation, anchorage-independent growth, and tumor growth in mouse xenografts both in these tumor cell lines and in a line derived from the androgen-dependent LNCaP prostate tumor cells in which it was overexpressed. Considering that its C1 domain is a known molecular target for several natural products, RasGRP3 could be a novel target for prostate cancer therapy.

Materials and Methods

Cell line, reagents, and antibodies
LNCaP clone FGC, PC-3, 22Rv1, and DU 145 cell lines were obtained from the American Type Culture Collection. The extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2, Snail, and Slug antibodies were obtained from Cell Signaling. Ras antibody was purchased from Upstate. Anti-V5 antibody, the ViraPower Lentiviral expression system, CyQuant NF cell proliferation assay kit, YO-PRO-1, 7-amino-actinomycin D (7-AAD), Stealth RNAi duplexes, and Lipofectamine 2000 were from Invitrogen. Antibodies for androgen receptor (AR), phospho-AKT1/2/3, AKT1/2/3, vimentin, E-cadherin, Twist, and prostate-specific antigen (PSA) were obtained from Santa Cruz Biotechnology. Recombinant human hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and tumor necrosis factor-α (TNF-α) were from R&D Systems. Carboplatin was purchased from Sigma. The AKT inhibitor V was from EMD.

Small interfering RNA transfection
The sequences of RasGRP3 small interfering RNAs (siRNA) are provided in Supplementary Table S1. The Stealth RNAi Negative Control Duplex Pool (Invitrogen) and the ON-TARGETplus siCONTROL Non-targeting Pool (Dharmacon RNA Technologies) were used as negative controls. Lipofectamine 2000 and 80 nmol/L siRNA were applied. The cells were subjected to assays at times between 24 and 120 hours after transfection as indicated.

Generation of tetracycline-inducible H1 lentiviral short hairpin RNA constructs
The sequences encoding the short hairpin RNAs (shRNA) for this study are listed in Supplementary Table S2. The pLenti4/BLOCK-it-DEST vectors containing specific shRNA were constructed according to the manufacturer’s instructions. All the constructs were verified by DNA sequencing. The lentiviral constructs were then produced and titered.

Establishment of tetracycline-regulated shRNA-expressing stable cell lines and the cell lines stably overexpressing wild-type RasGRP3 or its mutant
For detailed experimental procedures, see Supplementary Materials and Methods.

Cell proliferation assay
Cell proliferation was measured using the CyQuant NF cell proliferation assay as described by the manufacturer (Invitrogen). This assay quantifies binding of a fluorescent dye to cellular DNA.

Detection of apoptotic cells
Cells were harvested and incubated with 1 μmol/L YO-PRO-1 in Dulbecco’s PBS for 20 minutes at 4°C in the dark. 7-AAD was then added at a 5 μg/mL final concentration 10 minutes before analysis by the FACSCalibur system (Becton Dickinson). Data were analyzed with FlowJo 7 software (Tree Star, Inc.).

Reverse transcription-PCR and quantitative real-time PCR
Relative RasGRP3 mRNA levels were determined by reverse transcription-PCR (RT-PCR) and quantitative real-time PCR (Q-PCR) in human prostate cancer cell lines, normal prostate samples, prostate tumor samples, and prostate cDNA arrays (TissueScan Prostate qPCR Array Panel I and II, Origene). For detailed experimental procedures, see Supplementary Materials and Methods.

Analysis of Ras activation using the Raf1–RBD–glutathione S-transferase pull-down assay
Activation of Ras was evaluated as described by Lorenzo and coworkers (13).

Western blot assay
The samples containing 20 μg total protein were separated by electrophoresis and transferred onto Immobilon-P membranes (Millipore Corp.). After the membranes were blocked and labeled with the appropriate primary and secondary antibodies, the signal was developed by enhanced chemiluminescence (Amersham) and imaged on BioMax XAR or MR films (Kodak).

Growth of cell lines in mouse xenograft system
NOD.SCID/NCr male mice (NIH, Frederick, MD) were injected s.c. in the flanks with 5 × 10⁶ or 1 × 10⁷ cells per injection. In the case of those cell lines containing tetracycline-inducible expression systems, on the 8th day after injection, half the animals in each treatment group were shifted to food containing doxycycline. The animals were sacrificed after 5 weeks for mice injected with PC-3 cells, RasGRP3-LNCaP cells, or the LNCaP control cells, and after 8 weeks for mice injected with DU 145 cells.

Anchorage-independent growth assay in soft agar
The assay was performed as described previously (27) with minor modifications. For details, see Supplementary Materials and Methods.

Scratch wound assay
The cells plated at confluent density (∼1 × 10⁷ per well) on 24-well Essen ImageLock plates were transiently transfected with siRNAs the following day. After 12 hours, a single wound
per well was created using the Essen WoundMaker. The plates were scanned in the IncuCyte at 2-hour intervals for
24 hours. The data were analyzed by the IncuCyte scratch wound assay software.

**Growth inhibition by chemotherapeutic drugs**

After cells (1 × 10^4 per well) were seeded and cultured for 24 hours with or without 1 μg/mL tetracycline in 24-well
plates, chemotherapeutic drugs were added. After further in-
cubation for the indicated times, cells were analyzed by cell
proliferation assay.

**Statistical analysis**

The statistical analysis is described in Supplementary
Materials and Methods.

**Results**

**RasGRP3 is expressed in human prostate carcinoma
cell lines**

Both Yu and colleagues (21) and Tomlins and colleagues
(22) described that RasGRP3, a Ras activator, was overex-
pressed in metastatic prostate cancer. Using Q-PCR, we ex-
amined five normal human prostate samples and seven
human prostate tumor samples for RasGRP3 expression.
The levels of RasGRP3 in the tumors were significantly higher
than in the controls (P = 0.0303, Mann-Whitney test; Fig. 1A).
Additionally, we have determined RasGRP3 mRNA expres-
sion by Q-PCR in commercially available cDNAs prepared
from normal (n = 15) and tumor prostate tissue (stage
I–IV, n = 65). Using outlier robust t statistics (23), we observed
that 26% of tumor samples (n = 17), identified as outliers for
RasGRP3 overexpression, expressed significantly higher
RasGRP3 levels compared with normal prostate tissue (P =
0.0226; Fig. 1B). No outliers expressing significantly lower
RasGRP3 levels were identified (P = 0.5420).

Gene expression array analysis of the NCI 60 cell line panel
likewise indicated the expression of RasGRP3 in the PC-3 and
DU 145 prostate cancer cell lines (http://dtp.nci.nih.gov/
mtweb/hugosearch?genecard=RASGRP3). We confirmed by
RT-PCR (Fig. 1C) that RasGRP3 was expressed in these two
androgen-independent human prostate carcinoma cell lines,
with a barely detectable level of expression in the androgen-
dependent LNCaP cells and the 22Rv1 cells. The phenotype of
LNCaP clone FGC, PC-3, 22Rv1, and DU 145 cell lines was
well described previously (24–26). PC-3 harbors mutations
in PTEN and P53; DU 145 has mutations in RB, P53, CDKN2A,
and STK11 (30). For comparison, we show expression of
RasGRP3 in the Ramos B-cell leukemia cell line. The RT-
PCR results were confirmed by Q-PCR (Fig. 1C).

**Effects of suppressing the level of endogenous RasGRP3
in PC-3 and DU 145 cells on Ras activation, apoptosis,
and morphology**

The biological function of RasGRP3 was investigated by
knocking down endogenous RasGRP3 expression in the
PC-3 and DU 145 cells. In preliminary studies, we tested
the ability of multiple RasGRP3-siRNAs to knock down the
exogenous RasGRP3-V5 in RasGRP3-LNCaP cells (Supple-
mentary Fig. S1A). To minimize possible off-target effects
of the siRNAs, we also created a RasGRP3-siRNA pool con-
taining equal amounts of RasGRP3-siRNA 126, 774, and 1260.

Both the RasGRP3-siRNA pool (Fig. 2A) and the individual
RasGRP3-siRNAs (Supplementary Fig. S1C) inhibited the endo-
genous RasGRP3 expression in both the PC-3 and DU 145
cells. Inhibition of RasGRP3 expression was accompanied by
a reduced level of Ras-GTP in both cell lines (Fig. 2A), show-
ing that RasGRP3 was indeed an important contributor to the
state of Ras activation in these cells.

Inhibition of RasGRP3 expression caused marked apopto-
sis in both the PC-3 and DU 145 cells (Fig. 2B; Supplementary
Fig. S2A). Likewise, the morphology of the two cell lines (Sup-
plementary Fig. S2B) was shifted from a flattened morphol-
ogy to a more dendritic or spindle-shaped one (resembling
LNCaP), with many rounded cells.

**Inhibition of endogenous RasGRP3 expression
inhibited migration of the PC-3 and DU 145 cells**

Transient downregulation of endogenous RasGRP3 inhibited
cell migration, as detected by the wound scratch assay,
in both the PC-3 and DU 145 cell lines (see Fig. 2C and Sup-
plementary Fig. S3 for images).

**Downregulation of RasGRP3 suppressed proliferation
of the PC-3 and DU 145 cells**

To achieve long-term suppression of RasGRP3 expression,
we established cell lines derived from PC-3 or DU 145 express-
ing tet-on-inducible RasGRP3 shRNA. Because of promoter
leakage with the tet-on system, the level of endogenous
RasGRP3 expression in the absence of tetracycline was al-
ready somewhat reduced in the PC-3 derivative sh854 cell
line and in both of the DU 145 derivative cell lines, sh236
and sh854 (Fig. 3A). To confirm the specificity of the effects
of the RasGRP3 shRNAs, we created a mutated variant of
RasGRP3, retaining its amino acid sequence but altering its
coding sequence to diminish the homology with sh854.
Stable cell lines were prepared by introducing this RasGRP3
mutant into the PC-3 derivative sh854 and DU 145 derivative
sh854 cell lines. RT-PCR results showed that the RasGRP3
mutant was resistant to sh854 (Fig. 3A).

To examine the effect of suppressing endogenous RasGRP3
expression on growth of the PC-3 and DU 145 derivative
lines, the cells were cultured with or without 1 μg/mL tetracy-
cline for 120 hours. RasGRP3 expression was detected by
RT-PCR (Fig. 3A). Inhibition of cell proliferation mirrored
the suppression of RasGRP3 expression (Fig. 3B). A reduction
in cell growth was detected in the cells that already ex-
pressed a reduced amount of RasGRP3 as a result of promot-
er leakage of the shRNA compared with the cell lines
expressing a normal level of RasGRP3 (Fig. 3A and B). A fur-
ther reduction in cell proliferation accompanied the further
reduction in RasGRP3 expression on induction of the
RasGRP3 shRNAs by tetracycline treatment. Induction of the
control shRNAs by tetracycline had little effect (Fig. 3A
and B). As a control for shRNA specificity, expression of
Figure 1. RasGRP3 expression in prostate tumors and prostate cancer cell lines. A, Q-PCR analysis of RasGRP3 gene expression in human normal prostate (N) and prostate tumor (Tu). Values represent mean ± SEM (mean ± SD for #2335). B, RasGRP3 expression in normal prostate and prostate tumor cDNAs. The log2 of normalized (to actin) expression values relative to the mean expression of the normal is presented. The line in the middle of the box represents the median, with the lower and upper edges showing the first and third quartile, respectively. The whiskers in the normal group to the 1.1*IQR (interquartile range) indicate the cancer outlying expression as defined by the outlier robust t statistics (23). The red circles indicate outliers for RasGRP3 overexpression. C, RT-PCR and Q-PCR analysis of RasGRP3 gene expression in prostate cancer cells. Results are representative of three independent experiments for RT-PCR and two independent experiments for Q-PCR. Columns, average; bars, SD.
Figure 2. Blocking endogenous RasGRP3 expression caused reduction of Ras activation, cell apoptosis, cell migration, and inhibition of cell proliferation in PC-3 and DU 145 cells. The cells were treated with RasGRP3-siRNA pool or control siRNA pools 1 and 2 for 96 h unless stated otherwise.

A, endogenous RasGRP3 expression was determined by RT-PCR. Ras-GTP levels were detected by pull-down assay and immunoblotting. B, the siRNA-treated cells were stained with YO-PRO-1 and 7-AAD and analyzed by flow cytometry. C, scratch wound assay. D, the proliferation of siRNA-treated cells was determined using the CyQuant NF cell proliferation assay, with values normalized to the levels of nontreated cells. All results are representative of three experiments. Columns, mean of three independent experiments; bars, SE.
Figure 3. Inhibition of endogenous RasGRP3 expression retarded cell proliferation, inhibited xenograft tumor growth of both PC-3 and DU 145 cells, and impeded colony formation of PC-3 cells in soft agar. A, the PC-3 and DU 145 tet-on stable cell lines were treated with/without tetracycline for 120 h. Endogenous RasGRP3 expression was determined by RT-PCR. Results are representative of three experiments. B, the proliferation of PC-3– and DU 145–derived cells treated as above was determined using the CyQuant NF cell proliferation assay. The values were normalized to the nontreated scrambled shRNA 1 control. Columns, mean of four independent experiments; bars, SE. Addition of tetracycline caused a statistically significant (P < 0.002) decrease in proliferation relative to the control in both the sh236- and sh854-derived lines. C, the NOD.SCID/NCr male mice were injected s.c. with 1 × 10⁷ cells of the indicated PC-3– and DU 145–derived lines. On the 8th day, half the animals in each group were shifted to food containing deoxytetracycline. The animals were sacrificed 5 wk (PC-3) or 8 wk (DU 145) after injection. D, colony formation of the PC-3–derived cells in soft agar. Columns, mean of four independent experiments; bars, SE.
the sh854-resistant RasGRP3 mutant maintained RasGRP3 expression and rendered the cell growth rate insensitive to expression of sh854. Using the siRNA pool to transiently downregulate endogenous RasGRP3, we likewise could show inhibition of cell proliferation of the PC-3 and DU 145 cells (Fig. 2D).

**Inhibition of endogenous RasGRP3 expression inhibited anchorage-independent growth of the PC-3 cells**

For PC-3 cells, the induction of the RasGRP3 shRNA expression with tetracycline treatment decreased both the total number and size of colonies in soft agar (Fig. 3D; Supplementary Fig. S4). No inhibition was observed for either of the scrambled shRNA controls. The inhibition of the growth in soft agar caused by the sh854 RNA was blocked by introducing the sh854-resistant mutant RasGRP3 back into the sh854 derivative PC-3 cell line. Because of the very weak growth of DU 145 cells in soft agar (27), the corresponding studies could not be done reliably in that cell line.

**Downregulation of endogenous RasGRP3 suppressed xenograft tumor formation by the PC-3 and DU 145 cells**

The effect of endogenous expression of RasGRP3 on tumor formation by the PC-3 and DU 145 cells was assessed in a mouse xenograft model using the cell lines expressing either control shRNA, sh854, or sh854m cells that further expressed the mutated RasGRP3 resistant to sh854 (Fig. 3C). Expression of sh854 caused a marked reduction in the size and the number of tumors for both of the PC-3 and DU 145 cell lines (Table 1). In the animals treated with doxytetracycline, a further reduction was observed, leading to no detectable tumors at the time when the experiment was terminated. In the case of control cells expressing scrambled shRNA, robust growth was observed and doxytetracycline had no effect. Both for the PC-3–and DU 145–derived cells, expression of the mutated RasGRP3 resulted in resistance to the effect of sh854 on tumor growth and, in the case of DU 145, fully restored growth to the level of the cells expressing the scrambled control.

**Endogenous RasGRP3 contributes to AKT and ERK1/2 activation in both DU 145 and PC-3 cells**

AKT and ERK1/2 represent primary downstream effectors of Ras signaling. Modulation of RasGRP3, upstream of Ras, would therefore be expected to contribute to AKT and ERK1/2 activation. HGF represents an important signaling molecule in prostate cancer (28, 29), leading to activation of phospholipase C (30), an upstream effector of PKC and RasGRP. We have evaluated the ability of HGF and other growth factors (EGF, VEGF, and TNF-α) to modulate phosphorylation of ERK or AKT in both PC-3 and DU 145 cells and the involvement of RasGRP3 in this activity. In the case of the PC-3 cells, the basal level of AKT phosphorylation was already high, reflecting the PTEN mutation in this cell line (31). Although AKT phosphorylation was not significantly enhanced by the addition of HGF or EGF in the PC-3 cells (Supplementary Fig. S5A), AKT phosphorylation was nonetheless decreased by the suppression of the endogenous RasGRP3 expression by shRNA (Fig. 4A) and by siRNA (Fig. 4C). Additionally, HGF and EGF treatment increased phosphorylation of ERK1/2 in the PC-3 cells (Supplementary Fig. S5A). We confirmed that downregulation of RasGRP3 reduced the HGF-induced ERK phosphorylation (Fig. 4C). In the DU 145 cells, HGF treatment significantly increased phosphorylation of both AKT and ERK1/2 (Supplementary Fig. S5B). Conversely, downregulation of RasGRP3 with either the shRNAs (Fig. 4B) or siRNA pool (Fig. 4D) reduced the HGF-induced AKT phosphorylation in these cells and variably reduced the basal level of AKT phosphorylation. ERK1/2 activation and HGF-induced ERK1/2 activation were also inhibited (Fig. 4D). Once again, introducing the exogenous RasGRP3 mutant resistant to sh854 restored the level of phospho-AKT in both cell lines (Fig. 4A and B). Consistent with the central role of AKT pathway in the survival of PC-3 and DU 145 cells, decreasing the level of phospho-AKT using an AKT inhibitor resulted in significant inhibition of cell proliferation in both cell lines (Supplementary Fig. S5C and D).

**Table 1. Inhibition of endogenous RasGRP3 expression inhibited xenograft tumor growth of both PC-3 and DU 145 cells**

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<thead>
<tr>
<th>Cell line</th>
<th>Total tumor/total injection</th>
<th>Tumor weight (mg)</th>
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<tr>
<td></td>
<td>Deoxytetracycline</td>
<td>-</td>
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<td></td>
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<td>-</td>
</tr>
<tr>
<td>PC-3</td>
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<tr>
<td></td>
<td>sh854</td>
<td>5/12</td>
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<td></td>
<td>sh854m</td>
<td>9/12</td>
</tr>
<tr>
<td>DU 145</td>
<td>Control</td>
<td>10/12</td>
</tr>
<tr>
<td></td>
<td>sh854</td>
<td>8/12</td>
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<tr>
<td></td>
<td>sh854m</td>
<td>9/12</td>
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Endogenous RasGRP3 expression contributes to resistance to carboplatin in the PC-3 and DU 145 cells

The effect of RasGRP3 on the sensitivities of both the PC-3 and DU 145 cells to the chemotherapeutic drugs carboplatin, etoposide, and doxorubicin was evaluated. In the PC-3 cell line, induction of either the sh236 or sh854 cells with tetracycline sensitized the cells to carboplatin, shifting the dose-response curve to the left, representing a 3- to 4-fold decrease in IC50 (Fig. 5A). Introducing the mutated RasGRP3 resistant to sh854 blocked the increase in sensitivity of the cells. Likewise, no change in sensitivity on tetracycline addition was observed for the cells containing the control shRNA. Unlike sensitivity to carboplatin, sensitivity to etoposide and doxorubicin was unaffected by the suppression of endogenous RasGRP3 (data not shown). For the DU 145 cells, the uninduced sh236 and sh854 cell lines already showed increased sensitivity to carboplatin relative to the control or sh854m cells, consistent with the reduced RasGRP3 levels caused by leakage of the promoter (Fig. 5B). There was no additional increase of sensitivity to carboplatin on the further reduction of RasGRP3 induced by tetracycline treatment (data not shown). As was the case for the PC-3 cells, suppression of RasGRP3 did not affect the sensitivity of the DU 145 cells to etoposide or doxorubicin (data not shown).

Transformation of LNCaP cells overexpressing RasGRP3

To further explore the potential effects of RasGRP3 overexpression in prostate cells, we established a LNCaP cell subline expressing V5-tagged human RasGRP3 (RasGRP3-LNCaP) as described before (32). RasGRP3 expression was confirmed by Western blotting (Fig. 6A) and was accompanied by the activation of Ras, reflected in the elevated level of Ras-GTP and an elevated level of phospho-ERK1/2 (Fig. 6A). Like the PC-3 and DU 145 cells, the RasGRP3-LNCaP cells lost or showed greatly diminished expression of PSA and AR (Fig. 6A). Like the PC-3 and DU 145 cells, they had highly elevated expression of vimentin (Fig. 6A) and loss of E-cadherin (Supplementary Fig. S6), both markers of the epithelial-mesenchymal transition (EMT). Like the DU 145 cells and unlike the PC-3 cells, the RasGRP3-LNCaP cells did not differ appreciably from the LNCaP cells in their expression of Slug and Snail, transcription factors contributing to EMT (Supplementary Fig. S6). Relative to the LNCaP cells, the RasGRP3-overexpressing LNCaP cells also showed a flattened, cuboidal shape with pseudopodia (Supplementary Fig. S7A). These morphologic properties resemble those of PC-3 and DU 145 cells (Supplementary Fig. S7A).
Figure 5. Downregulation of RasGRP3 increased the sensitivity to carboplatin of both the PC-3 and DU 145 cell lines. The indicated PC-3–derived (A) and DU 145–derived (B) cell lines were treated with/without tetracycline (T) for 24 h, followed by carboplatin treatment (PC-3 cells: 100–30,000 nmol/L; DU 145 cells: 100–10,000 nmol/L). After 72 h, cell proliferation was determined using the CyQuant NF cell proliferation assay. The results were normalized to no treatment with carboplatin. All results are representative of three independent experiments.
Figure 6. Phenotypic alterations and effects of inhibiting RasGRP3 expression in the RasGRP3-LNCaP cells. A, overexpression of V5-tagged RasGRP3 and its effects on the Ras pathway. V5-tagged RasGRP3, phospho-ERK1/2 (p-ERK1/2), ERK1/2, AR, vimentin, and PSA levels were determined by immunoblotting. Ras-GTP levels were determined by pull-down assay followed by immunoblotting. Levels of total Ras were used as control. Results are representative of three to four independent experiments. B, LNCaP and RasGRP3-LNCaP cells were cultured in 10% fetal bovine serum (FBS) containing medium to ∼30% confluency. The medium was then replaced with either normal medium or steroid-depleted medium containing 10% charcoal-stripped FBS. Cell proliferation was determined daily using the CyQuant NF cell proliferation assay, with values normalized to the levels on day 0. Results are representative of three independent experiments. C, NOD.SCID/NCr male and female mice were injected s.c. with 5 × 10⁶ RasGRP3-LNCaP or LNCaP cells. Five weeks later, the mice were sacrificed and the weight of the tumors was determined. D, RasGRP3-LNCaP cells were transiently transfected with RasGRP3-siRNA pool or control siRNA pools. Ninety-six hours later, V5-tagged RasGRP3, phospho-ERK1/2, vimentin, and Ras were detected by immunoblotting. GTP-bound Ras was analyzed by pull-down assay. RasGRP3-LNCaP cells were transiently transfected with RasGRP3-siRNA pool and control siRNA pool 1. The cell culture medium was changed to normal medium or steroid-depleted medium, and cell proliferation was determined 96 h after transfection using the CyQuant NF cell proliferation assay. The values are normalized to that of the “nontreated + FBS” group. All results are representative of three independent experiments.
In the presence of normal medium, the RasGRP3-LNCaP cells showed a markedly enhanced rate of growth compared with the LNCaP cells (Fig. 6B). Likewise, the RasGRP3-LNCaP cells grew in the presence of charcoal-stripped serum at a rate similar to that under usual culture conditions (Fig. 6B). In contrast, the parental LNCaP cells were sensitive to culture in the presence of charcoal-stripped serum, which is used to deplete the serum of steroids.

Phorbol 12-myristate 13-acetate (PMA) has been reported to induce apoptosis in the LNCaP cells but not in the PC-3 and DU 145 cells (33, 34). Like the PC-3 and DU 145 cells, the RasGRP3-LNCaP cells were resistant to PMA-induced apoptosis (Supplementary Fig. S7B). The more aggressive behavior of the RasGRP3-LNCaP cells in culture was reflected in their behavior in a tumor xenograft system. The RasGRP3-LNCaP cells were able to form tumors in both male and female immunodeficient mice within 5 weeks, when \(5 \times 10^6\) cells were injected s.c., whereas little growth was evident for the control LNCaP cells at this time (Fig. 6C; Table 2).

### Table 2. Overexpression of RasGRP3 enhances tumorigenicity of LNCaP cells

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<th>Cell line</th>
<th>Total tumor/total injection</th>
<th>Tumor weight (mg)</th>
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<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>LNCaP control</td>
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<td>0/7</td>
</tr>
<tr>
<td>RasGRP3-LNCaP</td>
<td>7/7</td>
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Reduction of RasGRP3 expression by siRNA treatment partially reverses the phenotype of the RasGRP3-LNCaP cells

To confirm that RasGRP3 expression caused phenotypic transformation, we knocked down the exogenous RasGRP3 expression with siRNA. We found that changes in RasGRP3 expression were linked to changes in Ras-GTP levels. This was the case both with the individual RasGRP3-siRNAs (Supplementary Fig. S1B) and with the RasGRP3-siRNA pool (Fig. 6D).

Reduction in the level of RasGRP3 expression in the RasGRP3-LNCaP cells reduced the phospho-ERK1/2 (Fig. 6D; Supplementary Fig. S1B). Similarly, the morphology of the RasGRP3-LNCaP cells on treatment with RasGRP3-siRNA assumed a spindle shape with a smaller nucleus, resembling the parental LNCaP cells (Supplementary Fig. S7C). In addition, the vimentin expression was reduced on the RasGRP3-siRNA treatment (Fig. 6D). Finally, cell proliferation was reduced in response to treatment with the pooled RasGRP3-siRNA and the cells became more sensitive to culture in the presence of charcoal-stripped medium (Fig. 6D).

Our results argue that the overexpression of RasGRP3 was partially responsible for the enhanced activity of Ras-ERK pathway and shifting the morphology and growth properties of the RasGRP3-LNCaP cells to more resemble the PC-3 and DU 145 cells.

### Discussion

Activation of Ras signaling has long been recognized to be important for carcinogenesis and progression of prostate cancer cells with respect to growth, androgen dependency, and tumorigenicity. Nonetheless, activating gene mutations of Ras alleles are infrequent in the development or progression of carcinoma of the prostate (35, 36). Weber and Gioeli (37) have thus suggested that wild-type Ras at its physiologic expression level can be chronically activated by upstream autocrine and paracrine growth factors. We show that RasGRP3, a Ras activator, is an important signaling element upstream of Ras, at least in two metastatic human androgen-independent prostate cancer cells.

The involvement of RasGRP family members in cancer development and progression is proving to be ever more extensive (9, 19, 38, 39). Our findings of RasGRP3 expression in the prostate tumor cell lines PC-3 and DU 145 further broaden the scope of tissue types, where RasGRP family members are of importance. Our demonstration of an elevated level of RasGRP3 miRNA in several of a small collection of prostate tumor samples and in 26% of a larger panel of tumor cDNAs supports the relevance of these findings in the two prostate cell lines, as do the cDNA microarray gene expression profiling studies of Yu and colleagues (21) and Tomlins and colleagues (22). Their data showed that RasGRP3 was overexpressed in metastatic prostate cancer compared with normal prostate.

Our finding that endogenous RasGRP3 expression makes an important contribution to the proliferation and tumorigenicity of the PC-3 and DU 145 cells not only shows its role in prostate but also implies that this level of expression of RasGRP3 can be physiologically important. The PC-3 and DU 145 cell lines are members of the NCI 60 tumor cell line panel. Comparable or higher levels of RasGRP3 expression are reported for several cell lines from multiple tumor types in the NCI 60 tumor cell line panel (http://dtp.nci.nih.gov/mtweb/browse.jsp). Although the functional potential of RasGRP3 will necessarily depend on the cellular context, our results suggest that RasGRP3 is of relevance in an appreciably broader range of tissue types than had previously been anticipated. This is particularly significant in the context of chemotherapy resistance, as our data show that reduction of RasGRP3 in both PC-3 and DU 145 cells enhanced sensitivity to carboplatin by 3- to 4-fold.

Although RasGRP3, when present in meaningful levels, should contribute to the signaling of those many receptors in cells that activate phospholipase C, the focus of studies thus far has been on its role in B- and T-cell receptor signaling. In the case of the prostate, serum levels of HGF have been reported to be elevated in patients with advanced-stage prostate cancer (40); c-Met, a HGF receptor, has been detected in androgen-independent PC-3 and DU 145 human prostate cells but not in the androgen-dependent LNCaP cells (41). HGF has been shown to induce cellular proliferation and promote the invasiveness of PC-3 and DU 145 prostate cancer cells (42–45), and is coupled to phospholipase C activation (46). Here, we showed that RasGRP3 contributed to signaling downstream of HGF, as reflected in the DU 145 cells by its role...
in the level of HGF-induced phosphorylation of AKT and ERK1/2. In the PC-3 cells, HGF did not enhance the level of AKT phosphorylation, perhaps reflecting the already high basal level of AKT phosphorylation caused by the loss of function PTEN mutation in these cells. Nonetheless, downregulation of RasGRP3 still decreased the level of phospho-AKT, suggesting that RasGRP3 might be involved in alternative drivers of AKT phosphorylation in the PC-3 cell lines. Additionally, HGF treatment induced ERK1/2 activation in both PC-3 and DU 145 cells. Downregulation of RasGRP3 partially blocked this activation. AKT and ERK1/2, downstream targets of Ras, play a critical role in activation of both cell proliferation and anti-apoptotic signaling (47, 48) and enhance tumor progression by promoting cell invasiveness and angiogenesis (49, 50). The effects of RasGRP3 on AKT and ERK1/2 phosphorylation in both the DU 145 and PC-3 cells are consistent with our demonstration that RasGRP3 is contributing to Ras activation in these cells, and likewise support the effects we observe on cell proliferation, chemoresistance, and tumorgenesis.

The identification of RasGRP3 as an additional important signaling element contributing to the cancer phenotype in the PC-3 and DU 145 prostate cancer cells, together with the evidence for its elevated expression in a subgroup of prostate tumors, reveals an additional potential target for pathway-directed chemotherapy in prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

The research was supported in part by the Intramural Research Program of the NCI, National Cancer Institute, Center for Cancer Research and in part by OTKA K63153 (T. Bíró). T. Bíró is a recipient of the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

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Received 01/04/2010; revised 07/12/2010; accepted 07/27/2010; published OnlineFirst 09/28/2010.

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Cancer Res 2010;70:7905-7917. Published OnlineFirst September 28, 2010.