Targeting Gβγ Signaling to Inhibit Prostate Tumor Formation and Growth*

Angela L. Bookout‡, Amanda E. Finney‡, Rishu Guo‡, Karsten Peppel§, Walter J. Koch‡, and Yehia Daaka‡¶
From the Departments of ‡Surgery, §Medicine, and ¶Pharmacology/Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

Prostate cancer starts as androgen-dependent malignancy and responds initially to androgen ablative therapy. Beneficial effects of androgen ablation, however, are often temporary and the cancer reappears as androgen-independent tumor, suggesting the existence of additional factors responsible for progression of the disease. Attention has focused on receptor tyrosine kinases as the growth mediators of androgen-independent prostate cancer; overexpression of epidermal growth factor receptors or their ligand heparin-binding epidermal growth factor, for example, promotes transition to androgen independence. Emerging data demonstrate involvement of another class of cell membrane-anchored receptors, the heterotrimeric guanine-binding (G) protein-coupled receptors (GPCRs) in prostate cancer. In vitro, stimulation of many endogenous GPCRs induces mitogenic signaling and growth of prostate cancer cells. The GPCRs transduce mitogenic signals via activated G proteins in the form of Ga-GTP and Gβγ subunits. Here, we show that expression of a Gβγ inhibitor peptide derived from carboxy terminus of G protein-coupled receptor kinase 2 obliterates serum-regulated prostate cancer cell growth in vitro and prevents prostate tumor formation in vivo. We also demonstrate that inhibition of Gβγ signaling retards growth of existing prostate tumors by inducing cell death. These data establish a central role for heterotrimeric G proteins in prostate cancer and suggest targeted inhibition of Gβγ signaling may serve as specific molecular therapy tool to limit pathologic growth of advanced prostate cancer.

The GPCRs relay signals mostly via heterotrimeric G proteins in the form of Ga-GTP and Gβγ subunits (19, 20). In particular, the Gβγ subunits of several G proteins promote mitogenic Ras-ERK signals via activation of multiple effectors, including phosphatidylinositol 3-OH kinase, non-receptor tyro-

* This work was supported by Grants AG17952 and DK69017 from the National Institutes of Health (to Y. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked 
**advertisement** in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Surgery, Duke University Medical Center, Box 2607, Durham, NC 27710. Tel.: 919-684-8440; Fax: 919-684-9990; E-mail: daaka001@mc.duke.edu.

The abbreviations used are: AD, androgen-dependent; AI, androgen-independent; AR, androgen receptor; GPCR, G protein-coupled receptor; GRK2, GPCR kinase 2; GRK2ct, carboxy terminus 194 amino acid of GRK2; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; Ad-β-Gal, adenoviruses encoding β-galactosidase; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; 7-AAD, 7-amino-actinomycin D; pfu, plaque-forming units.
sine kinases, and phospholipases (21), which participate in regulation of cell growth and survival. Involvement of the Gβγ subunits in the GPCR-regulated ERK activation was determined by the use of a specific Gβγ-binding peptide derived from the carboxyl terminus of G protein-coupled receptor kinase 2 (GRK2ct) (22). The GRK2ct binds free Gβγ subunits and effectively prevents them from activating their downstream effector (23).

Recently, we demonstrated that stimulation of endogenous GPCRs for lysophosphatidic acid in 150 mM NaCl, 50 mM Tris-HCl (pH 8), 5 mM EDTA, 10 mM NaF, 10 mM NaPi, 1% Nonidet P-40, 0.5% deoxycholate, protease inhibitor tablets (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, and 100 μM Na3VO4, and protein concentrations were determined using Bradford assay. Equal amounts of protein from each sample were separated on SDS-polyacrylamide gels and transferred to nitrocellulose filters. GRK2ct expression was determined using a 1.5,000 dilution of C5/1 anti-GRK2/3 antibody (30). Blots were developed with a 1:7,000 dilution of horseradish peroxidase-conjugated secondary antibody, and proteins were visualized with enzyme-linked chemiluminescence (Amersham Biosciences).

Cell Survival Assay—Cells were immunostained with Annexin V and 7-amino-actinomycin D (7-AAD) antibodies and examined by FACS, according to the manufacturer's instructions (Pharmingen). Cells that are at an early stage of apoptosis are Annexin V-positive and 7-AAD-negative, whereas dead cells express both proteins.

Immunohistochemical Staining—Tumors were surgically dissected and photographed using a Coolpix 995 digital camera (Nikon) attached to a dissection microscope (Bio-Rad). Paraffin-embedded tumor sections (5-μm thick) were stained by the avidin-biotin complex method, as described previously (16). Briefly, endogenous peroxidase activity was inhibited by exposure to 0.3% H2O2/methanol, and antigen retrieval was performed by incubation for 15 min in 10 mM citrate buffer, pH 6.5, at 94 °C for 15 min. Nonspecific binding was blocked by incubating tissue sections in 10% normal goat serum for 30 min at ambient temperature, followed by overnight incubation at 4 °C with 1:200 dilution of rabbit polyclonal anti-CD-31 antibody. Tumor cell death was determined by immunostaining tissue sections to detect fragmented DNA using commercially available kits (FragEL DNA Fragmentation, YWR Scientific Products) according to the manufacturer's instructions. Sections were washed twice with PBS, incubated with 5 mg/ml goat anti-rabbit biotinylated antibodies in PBS containing 1% bovine serum albumin for 30 min at ambient temperature, and then incubated with avidin-biotin complex reagent for 30 min. Specific immunostaining was visualized with 3,3'-diaminobenzidine. Slides were dehydrated and counterstained with hematoxylin. Specific staining was visualized using a Zeiss LSM 510 laser scanning confocal microscope equipped with a Zeiss 63×1.4 numerical aperture water immersion lens.

RESULTS

In Vitro Adenoviral GRK2ct Gene Transfer—Protein immunoblotting of lysates obtained from androgen-independent prostate cancer PC3 cells infected with the GRK2ct adenovirus revealed the dose- and time-dependent expression of the 30 kDa GRK2ct peptide, which was not present in cells infected with empty vector (Fig. 1, A and B). Maximal expression of the GRK2ct peptide occurred 3 days post-infection (Fig. 1B). Importantly, we observed that expression of endogenous GRK2 decreased on day four post-infection (Fig. 1B), perhaps due to decreased viability of the prostate cancer cells.

In Vivo Effects of GRK2ct Expression on Cell Proliferation and Survival—Inhibition of Gβγ signaling attenuates GPCR and serum-mediated activation of ERK (24, 28), which is required for prostate cell growth (26). We determined the effect of GRK2ct expression on growth of cells cultured in medium containing 10% serum. Cells were infected with adenovirus that encodes individual GRK2ct peptide and green fluorescent protein (Ad-GFP-GRK2ct). Control cells were infected with adenovirus that encodes the GFP alone. Exposure of cells to adenovirus at a 10 multiplicity of infection resulted in nearly 100% infection efficiency as determined by fluorescence signal of cells infected with the GFP virus (data not shown), consistent with previous data obtained using vascular smooth muscle cells (28) and ventricular myocytes (31). Infected cells that express GFP (Fig. 1C) were selected using FACS and were allowed to grow in 10% serum-containing medium. Growth rate was determined by counting cells (26) using a hemocytometer. As shown in Fig. 1E, expression of the GRK2ct peptide limited the serum-induced PC3 cell proliferation, compared with wild type uninfected and Ad-GFP-infected cells. The observed decrease in growth rate of PC3 cells expressing the GRK2ct is consistent with the finding that Gβγ subunits regulate ERK activation (13, 24), which control growth factor-regulated prostate cancer cell growth (26).

The reduced number of viable GRK2ct-expressing prostate cells may result from inhibition of cell growth or induction of cell death, apoptosis. Cell survival was determined by immunostaining for Annexin V and 7-AAD and analyzed by FACS. Transient expression of GRK2ct for 2 days promoted the dose-dependent increase in apoptotic PC3 cells, reaching 50% at a
viral titer of 50 plaque-forming units (pfu) (Fig. 2). Infection with similar concentrations of control Ad-GFP showed no effect on the PC3 cell survival. Transient expression of the GRK2ct peptide for 2 days did not modulate the number of dead cells (Fig. 2), in agreement with cell proliferation data shown in Fig. 1. However, infection for longer periods promoted the time-dependent death of cells and no viable cells were detected after 1 week of infection. Importantly, these data were collected using cells cultured in serum, which demonstrates that Gβγ subunits are critical regulators of prostate cancer cell growth and survival under physiologically relevant conditions.

**In Vivo Effects of Gβγ Inhibition on Prostate Tumor Growth**—To further implicate the Gβγ subunits in prostate cancer cell growth, we established a xenograft model to assess effect of GRK2ct expression on PC3 tumor formation in mice. PC3 cells were infected with either Ad-GFP or Ad-GFP-GRK2ct at a multiplicity of infection of 10, and cells expressing the GFP protein were sorted by FACS. Selected cells were suspended in Matrigel and injected subcutaneously into the flanks of athymic male mice. Tumors from wild type and Ad-GFP-expressing PC3 cells were detectable 6–10 days after implantation (Fig. 3A) and were highly angiogenic (Fig. 3, B and C). On the other hand, PC3 cells expressing the Gβγ inhibitor GRK2ct showed a delayed tumor formation response; tumors were not visible until thirty days after injection (Fig. 3A). Moreover, whereas most animals injected with wild type or Ad-GFP-expressing PC3 cells formed tumors (27 tumors out of 30 injected animals), only 6 out of 30 mice injected with the Ad-GFP-GRK2ct-infected PC3 cells formed tumors. These tumors were retrieved and tested for expression of the GRK2ct by immunoblotting. No tested tumor showed expression of the GRK2ct peptide, suggesting they most likely originated from cells that did not express the Gβγ inhibitor.

**Effects of GRK2ct Expression on Growth of Existing Prostate Tumors**—To begin to assess the role of Gβγ subunits as potential effective targets to limit growth of existing prostate tumors, we established PC3 tumors in mice and tested the effect of expressing the GRK2ct peptide on the rate of tumor growth. The mice were randomly divided into two groups when tumor volume reached about 250 mm³. In the first group, animals were injected intratumorally with Ad-GRK2ct and in the second group equal number of animals were similarly injected with control adenovirus that expresses Ad-β-Gal. Both groups received two injections that were 5 days apart. Results show that tumors injected with the Ad-GRK2ct displayed a retarded growth rate, compared with tumors injected with Ad-β-Gal (Fig. 4A). Interestingly, significant differences in tumor volume between the two groups were not noticeable until about 1 week after the second treatment. Moreover, there was no actual reduction in tumor size; rather expression of the Gβγ inhibitor suppressed the further tumor growth. These data mirror the in vitro results showing that expression of the GRK2ct peptide prevented growth of the prostate cancer cells (Fig. 1E).
Expression of the GRK2ct peptide caused tumor cytostasis (Fig. 4A), suggesting the inhibition of Gβγ signaling may promote tumor cell death. To verify this possibility, tumors were excised and their content of fragmented DNA, an index of apoptosis, determined. Tumors that were harvested from animals injected with the Ad-GRK2ct showed clear positive staining for fragmented DNA (Fig. 4B), whereas those obtained from control animals did not (Fig. 4C). The reason for the circular appearance of apoptotic centers likely reflects the limited diffusion of injected virus and inability of the adenovirus to further replicate and infect adjacent tumor cells. Thus, only initially infected cells express the GRK2ct, which induces apoptosis. We also observed that the apoptotic regions displayed reduced blood vessel density as determined by decreased expression of the angiogenic marker CD-31 (data not shown). Together, these data demonstrate that expression of the Gβγ inhibitor regulates prostate tumor size by promoting cell death.

**DISCUSSION**

Prostate cancer is a heterogeneous disease, and it is commonly observed the cancerous gland contains multiple independent tumors (32), suggesting the existence of distinct mechanisms involved in the initiation and progression of the disease. The major finding of this study is that Gβγ subunits exert a critical role in prostate cancer cell growth and survival, and that targeted inhibition of Gβγ signaling results in significant reduction in rate of tumor growth in vivo. These observations provide an added dimension to the diversity of signals involved in prostate tumorigenesis and cellular responses controlled by Gβγ subunits of heterotrimeric G proteins. The inhibition of Gβγ signaling was achieved by expression of a peptide derived from the carboxyl terminus of GRK2, which contains specific Gβγ-binding domain (22, 23). Our results provide a link between Gβγ signaling and pathological growth of the prostate and have implications for the pathogenesis and potential treatment of prostate cancer.

The importance of our results is the unexpected finding that prostate cancer cell growth is mediated by Gβγ-dependent signals. In *vitro*, expression of the GRK2ct peptide significantly attenuated serum-induced PC3 cell growth, suggesting that the majority of the mitogenic activity present in serum is mediated by factors that signal through Gβγ. The inhibition of Gβγ signaling was also effective in limiting growth rate of prostate tumors in animals. These findings demonstrate that Gβγ subunits play a prominent role in the growth of prostate cancer cells under physiologically relevant conditions.

The finding that Gβγ controls prostate cell growth indicates receptors that couple to G proteins are important mitogenic signal transducers. Indeed, prostate cancer tissues express elevated levels of GPCRs, such as bradykinin 1 and endothelin 1A receptors (16, 17), and GPCR ligands, such as follicle-stimulating hormone and endothelin-1 (17, 18), compared with benign specimens. Thus, it is likely the cancerous gland contains activated GPCRs that could contribute, via the Go-GTP and Gβγ subunits, to initiation or progression of the disease. Our
results support the notion that Gβγ subunits control prostate tumor growth in vivo. Many studies have addressed the contribution of peptide growth factors and their cognate receptor tyrosine kinases to prostate tumorigenesis (6, 7, 13). In vitro growth factors and their cognate receptor tyrosine kinases to in vivo expression levels of EGFRs and progression to AI prostate cancer in humans. Signoretti et al. (34) reported that expression of HER2/neu protein was higher in tumors that were treated with androgen ablative therapy, compared with pre-treatment levels, and that the incidence of HER2/neu-positive tumors increased after androgen deprivation. However, Calvo et al. (35) showed no correlation between HER2/neu expression and progression to androgen insensitive prostate cancer. It is likely that activation stage, rather than expression level of HER2/neu, is the important factor for transition to AI prostate cancer. In support of this conclusion are the recent findings that inhibition of HER2/neu signaling attenuates growth of xenografted breast and prostate cancer tumors that do not overexpress the receptor (36).

Existing data suggest the likely contribution of EGFRs to progression to AI prostate cancer. Less clear, however, are the mechanisms responsible for EGFR activation in the prostate cancer cells. EGFRs could become activated in the prostate due to overexpression of the receptors themselves (11, 12) or their ligands (33). Importantly, we recently reported that stimulation of endogenous GPCRs in prostate cancer cells induces activation of EGFRs (25, 26) in the absence of exogenously added EGFR ligands. For example, stimulation with lysophosphatidic acid promoted activation of matrix metalloproteinases that cleave plasma membrane-anchored proEGFR ligands leading to their secretion. The released EGFR ligands bind their cognate receptors in a paracrine and autocrine fashion and activate them. Thus, GPCRs act as master regulators able to control EGFR activity in prostate cells. The significant finding of our present studies is that targeted inhibition of Gβγ signaling with GRK2ct suppresses growth of xenografted prostate tumors that originate from cells that do not overexpress EGFRs.

The results of the in vitro cell growth in serum and in vivo tumor growth in mice showing GRK2ct regulates cell proliferation suggest that signals from GPCRs are critical for prostate cell growth. Gβγ have recently been shown to also transduce signals from bona fide receptor tyrosine kinases, such as ERK activation by insulin-like growth factor 1 (37) and smooth muscle cell proliferation by the platelet-derived growth factor (29) receptors. The latter observation is of particular interest because PDGF is a known proangiogenic factor, and angiogenesis is critical for tumor growth. We observed that expression of the GRK2ct peptide in existing prostate tumors reduced the staining intensity for the angiogenic marker CD-31. Thus, Gβγ may act as convergence loci of mitogenic signals initiated by not only GPCRs, but also certain receptor tyrosine kinases.

In summary, the results of these preclinical studies show that Gβγ subunits regulate prostate cancer cell growth and survival by the different factors present in serum. Inhibition of Gβγ signaling represents a potentially effective therapeutic strategy to treat advanced prostate cancer patients. Blockade of Gβγ signaling can be achieved by genetic transfer of peptides such as the GRK2ct or small pharmaceutical molecules.

Acknowledgments—We thank Dr. R. J. Lifkowitz for the C5/1 anti-GRK2/5 antibody, L. Pascal for adenosine purification, and J. House for excellent secretarial assistance.

REFERENCES
