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The Role of GGAP2 in Prostate Cancer

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14. ABSTRACT The major goal of this study is to characterize the functions and expression profile of GGAP2 in human PC (HPC). Our data show that GGAP2 protein expression is increased in HPC in both HPC cell lines and clinical patient samples. Biochemical studies indicate that GGAP2 activate both AKT and NF-kB pathways and can promote HPC growth at cellular level and in mouse model. Using PCR with > 100 clinical HPC tissue samples, cloning and direct sequencing, we found that multiple low-frequency mutations may exist in PC. Our data showed some of these mutations activate the GTPase domain of GGAP2 and enhance its effects on cancer growth. PC3 cells stably expressing wild-type GGAP2 form larger volume tumors in nude mice model when compared to control, while knockdown of GGAP2 or overexpressing the dominant negative form GGAP2 S629A dramatically inhibit tumor growth. These findings indicate that GGAP2 may function as an important modulator in HPC which can regulate both AKT and NF-kB pathways. This assists us to understand better the mechanism of HPC development. Detection methods and novel therapeutic tools targeting GGAP2 specifically may be successful in reducing HPC incidence and slow down cancer development.					
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Introduction

PC (PC) is the most common visceral malignancy in US men and the second leading cause of cancer deaths. The clinical behavior of PC is extremely heterogeneous, ranging from indolent disease to aggressive, metastatic cancer with rapid mortality. GGAP2 is a GTPase protein with a GTPase activating protein (GAP) domain at the C-terminus (Xia et al., 2003). It was reported that GGAP2, or PIKE-A, can activate AKT and inhibit neuronal cell apoptosis (Ahn et al., 2004). Our previous study indicates that GGAP2 expression is elevated in clinical PC patient samples, and wild-type GGAP2 may enhance PC growth via AKT and NF- κ B pathways. The main purpose of this training grant focuses on characterization of the functions of wild-type and mutant GGAP2 in PC progression and metastasis. Our results indicate that GGAP2 is highly expressed with multiple low-frequency mutations detected in human PC. Overexpression of wild-type GGAP2 along can promote PC growth, and some mutations found in clinical patient samples can further enhance its actions. Further studies showed that PC metastasis may be correlated with GGAP2 expression. Our study focused on the biochemical mechanism of GGAP2's functions in PC showed that GGAP2 may function as a rare modulator to regulate both AKT and NF- κ B pathways.

Body

Our research follows strictly the approved Statement of Work.

Task 1 Screen the mutations of GGAP2 in clinically localized cancer, non-PSA recurrent, late PSA recurrent and early PSA recurrent cancer tissue samples.

We performed RT-PCR, cloning and direct sequencing on >100 normal and clinical PC (PC) patient samples to examine the GGAP2 mutation rate in clinical PC patient samples. Two sets of primers were used to examine the N-terminal GTPase domain and the GAP domain of GGAP2, respectively. As shown in Table 1, although in very low frequency, multiple mutations were found in both domains in PC patient samples. All clones with mutations were resequenced to minimize the misreading errors in sequencing process. Most of these mutations were detected at a low frequency in 1-2 out of 10-15 clones examined. Mutations in both domains of GGAP2 were detected at a significant higher frequency in cancer patient samples compared to normal. We will use mutation specific probe and Taqman real-time PCR to further verify these mutations.

To investigate the role of these mutations in PC, we used site-directed mutagenesis to clone these mutations into eukaryotic (CMV-Tag2B with a G418 selection marker, Stratagene) and prokaryotic expression vectors (GEX4T-1 for GST fusion protein purification, Promega). Stable prostate cell lines expressing control vector, wild-type GGAP2 and these mutant GGAP2 were generated using stable selection with Geneticin at recommended concentration. As Fig. 1 shows, the GGAP2 mutants stimulate PNT1A and DU145 cell proliferation, indicating that these mutations may contribute to PC growth.

GGAP2 is a GTPase whose activation is determined by bound guanine nucleotide via GTP/GDP switching mechanism. We performed in vitro guanine nucleotide binding assay to examine the effects of these clinically identified mutations on GGAP2 GTP/GDP exchange process. Our data showed that

mutant GGAP2 binds to GTP faster than wild-type GGAP2 and empty GST control beads, indicating these mutations may enhance GGAP2 activation (Fig.2).

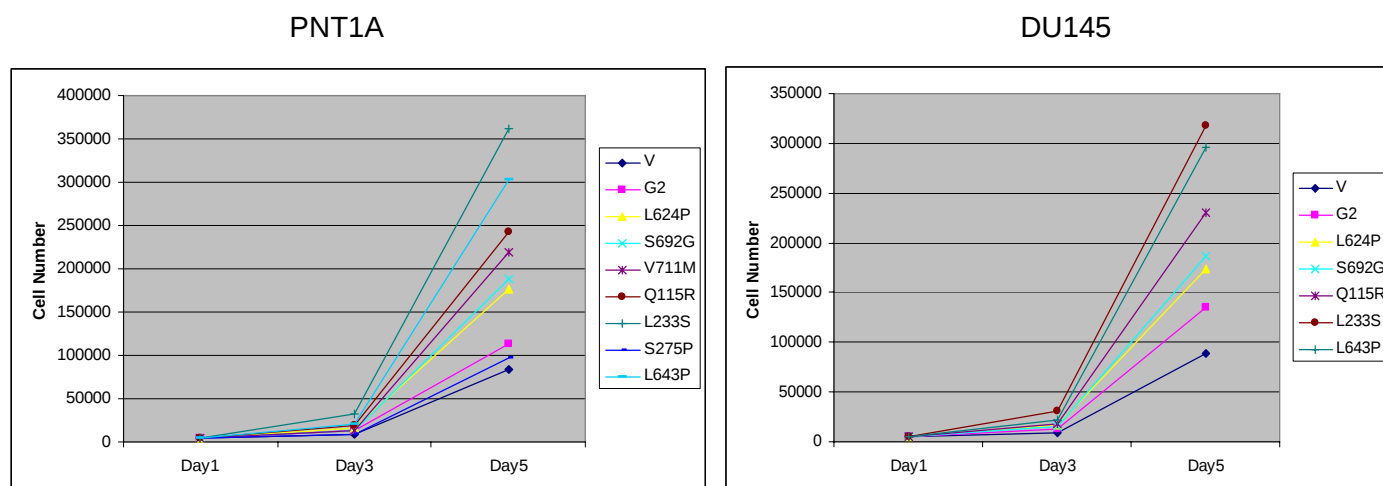
Overall, these data showed that GGAP2 is heterogeneously mutated in human clinical PC samples, and some of these mutations may lead to GGAP2 GTPase activation and therefore promote cancer development.

Table 1 Multiple mutations of GGAP2 were found in human PC patients.

	GTPase domain		
	Normal	Late PSA recurrent	Early PSA recurrent
total # of samples examined	10	10	10
total # of clones examined	55	55	55
total # of readable clones	40	37	39
total # of clones w/ mutations	1	18	12
total # of clones w/ silent mutations	2	0	0

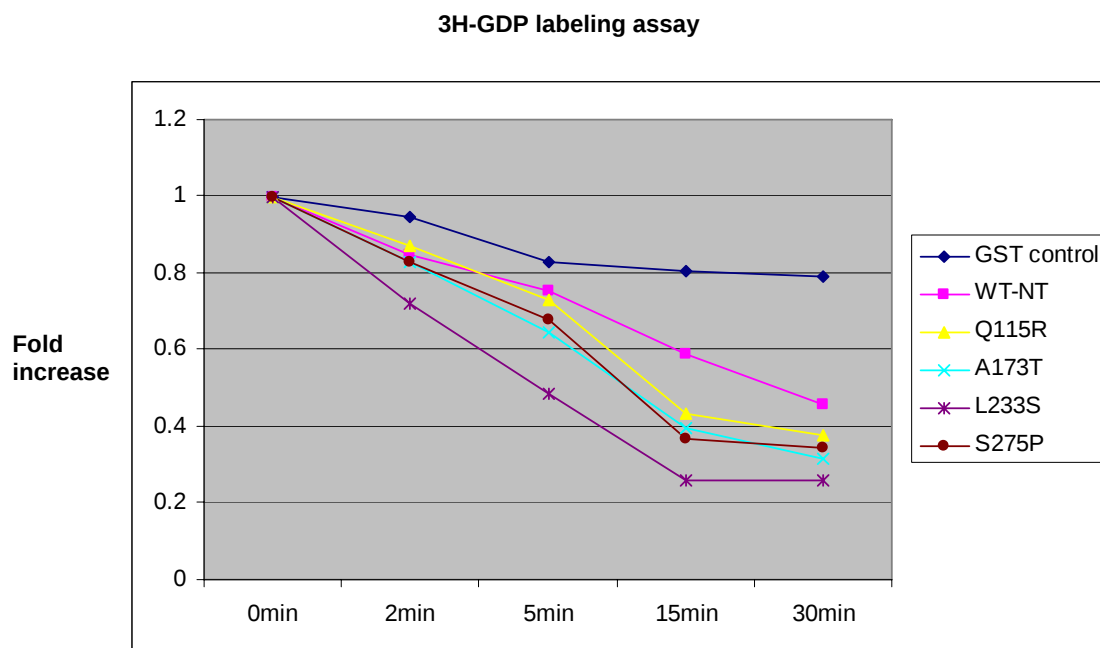
	GAP domain	
	Normal	Cancer
total # of samples examined	30	72
total # of clones examined	320	764
total # of readable clones	216	520
total # of clones w/ mutations	2	195
total # of clones w/ silent mutations	8	4

Figure 1 Mutations identified in PC patients enhance prostate cell proliferation.



V refers to Vector control and G2 to wild-type GGAP2. 5000 cells each well in a 6-cell plate were plated at day 1 All experiments were performed in triplicate. Averages were shown.

Figure 2 Mutations identified in PC patients stimulates GTP binding activity of GGAP2 leading to its activation.



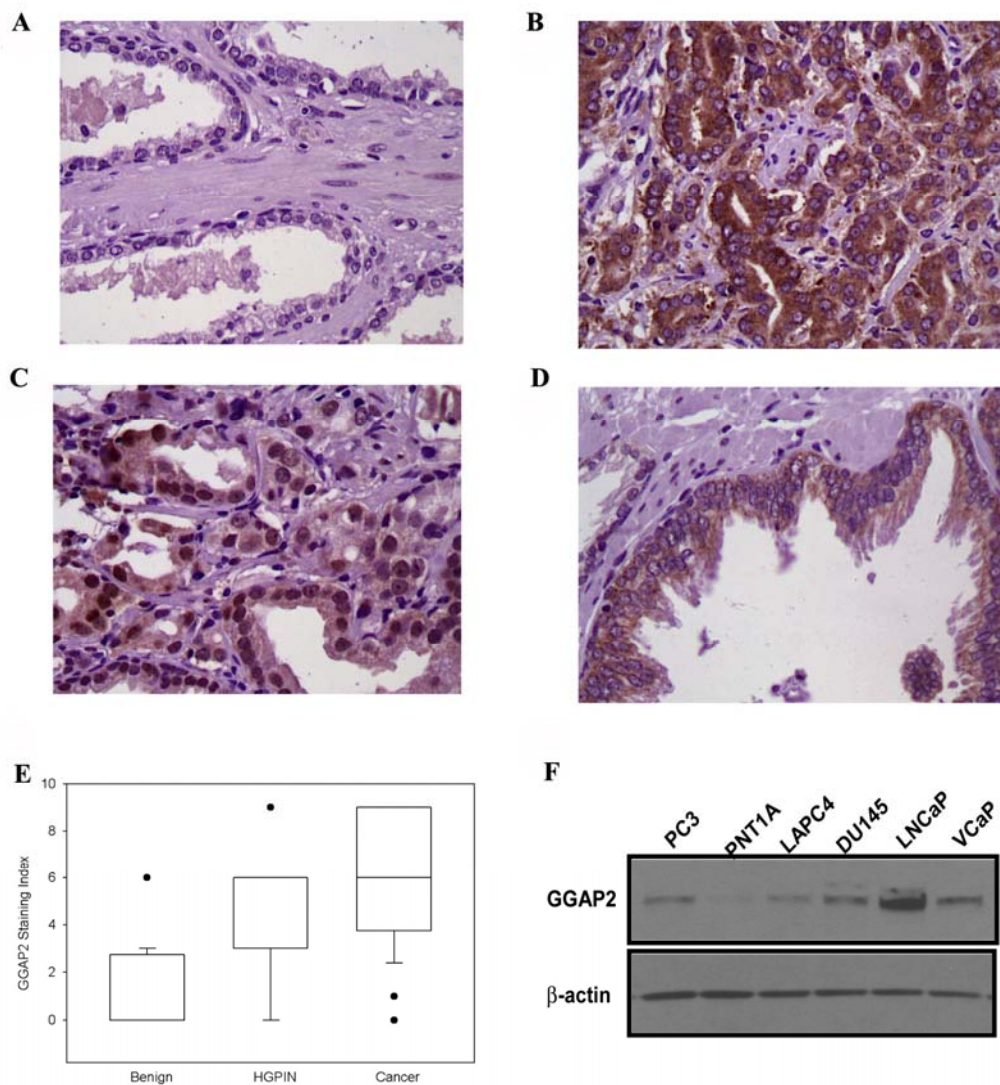
Purified GST-wild type or GST-mutant N-terminal GGAP2 GTPase domain fusion proteins on glutathione-agarose beads were used to quantify the GTP binding and GDP dissociation abilities of GGAP2. GST control refers to GST protein along without fusing with other peptides and WT-NT to wild-type N-terminal GGAP2 GTPase domain. Briefly, 1 μ M GST-wild type or mutant GTPases was incubated with 1 μ M [³H]GDP at 25°C in the buffer B containing 50mM HEPES (pH 7.6), 100mM NaCl, and 1mM dithiothreitol. To stabilize the [³H]GDP-bound GTPases, the reaction mixtures were supplemented with 20mM MgCl₂. After a binding equilibrium was reached (~60 min), the GDP/GTP exchange reactions were initiated by the addition of excess free 400 μ M GTP (final concentration). At different time points, the reactions were terminated by filtration of 20 μ l of the mixtures through nitrocellulose filters. And the filters were washed twice with the ice-cold buffer B. The amount of the radionucleotides remaining bound to the GTPase were quantified by scintillation counting, and normalized as the percentage of [³H]GDP bound at time 0. For each time point, the samples were assayed in triplicate. Averages were shown.

Task 2 Characterize the function of GGAP2 in PC occurrence and progression.

We carried out immunohistochemistry with anti-GGAP2 antibody using tissue microarrays containing matched normal, cancer and high-grade prostatic intraepithelial neoplasia (PIN) tissues. Moderate to high expression of GGAP2 protein was seen in 75% of PC tissues within the cancer epithelial cells (Fig. 3A-D). Staining was primarily cytoplasmic in those cases with staining. Nuclear staining was also identified in some cases. Similar staining was observed in high grade prostatic intraepithelial neoplasia. Absent or weak staining was seen in the more than 95% of the normal epithelial tissues. Weak staining of stromal tissues was also noted. We then quantified cytoplasmic expression of GGAP2 using a visual quantitation. This quantitation is based on a multiplicative score based the extent (scored 1-3) and intensity (scored 0-

3) of staining and results in scores ranging from 0 (absent) to 9 (strong, diffuse staining). The mean score for normal epithelium was 0.86 $\pm$ 0.18 (SEM, n=72), the mean for PIN was 3.6 $\pm$ 0.9 (n=65) and 5.8 $\pm$ 0.4 (n=56). The difference in staining score between normal and both PIN and cancer was highly statistically significant ($P < 0.001$, Mann-Whitney, Rank Sum test) (Fig. 3E). It's noted that GGAP2 expression is also increased in human prostate cancer cell lines compared to the immortalized normal prostate epithelial cell line PNT1A (Fig. 3F). For further information, please refer to the published manuscript in the Appendices section. We also analyzed the expression level of GGAP2 and metastasis using tissue microarrays. However, no statistically significant correlation was found (data not shown).

Figure 3 GGAP2 expression is increased in human prostate cancer.

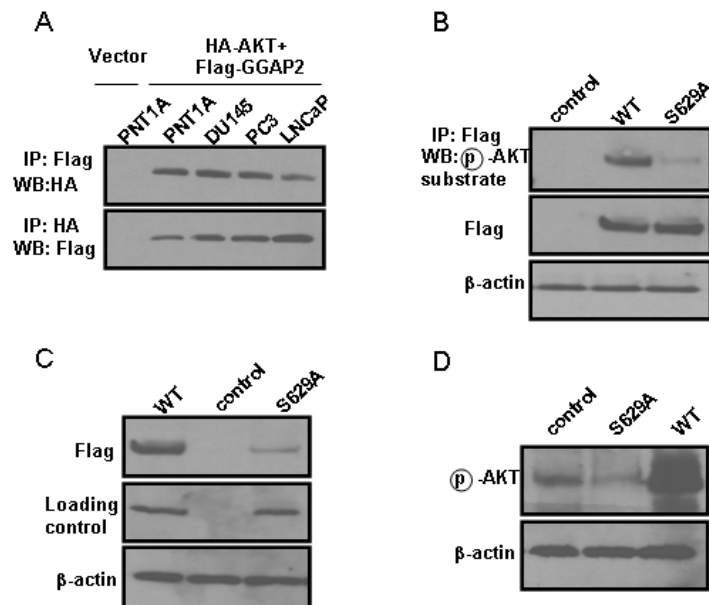


(A)-(D) Immunohistochemistry demonstrating an increased GGAP2 level in human prostate cancer tissues. (A): normal prostate tissue showing very weak epithelial staining; (B): cancer tissue with strong cytoplasmic staining; (C): nuclear staining in prostate cancer tissues; (D): high grade prostatic intraepithelial neoplasia with moderate cytoplasmic staining. (E) Boxplot of staining indices of

benign, high grade prostatic intraepithelial neoplasia (HGPIN), and cancer tissues. (F) GGAP2 expression in prostate cancer cell lines. 20ug of total cell protein were used in each lane. GGAP2 was barely detectable in the immortalized prostate epithelial cell line PNT1A but was easily detectable in the prostate cancer cell lines, especially LNCaP cancer cells.

Next we seek to unveil the underlying mechanisms through which GGAP2 promotes cell growth in vitro and in vivo. . It is known that GGAP2/PIKE-A can enhance Akt kinase activity via direct interaction (Ahn et al., 2004), but it is also possible that Akt can also modulate the activity of GGAP2. As a matter of fact, we identified a serine at amino acid 629 that is a potential Akt phosphorylation site using simulated motif scan program (<http://scansite.mit.edu>) Flag-tagged GGAP2 was transiently transfected and immunoprecipitated (with anti-Flag Ab) followed by Western blotting with antibody to Akt consensus phosphoserine. This study showed phosphorylation of GGAP2 by Akt that is abolished by mutation of serine 629 to alanine (Fig. 4B). This was confirmed by an *in vitro* kinase assay (data not shown). To determine if Akt can modulate the GTPase activity of GGAP2, we carried out immunoprecipitation with lysates from 293T cells overexpressing Flag tagged vector, wild-type and S629A GGAP2 incubated with GTP-conjugated agarose beads followed by Western blot using anti-Flag antibody. The mutation at position 629 of GGAP2 from serine to alanine dramatically decreased the GTP binding ability (Fig. 4C), indicating that Akt phosphorylation of GGAP2 enhances GTP binding, presumably by downregulating the GTPase activity of the GAP domain. Finally, when transfected into DU145 prostate cancer cells, GGAP2 markedly increases Akt phosphorylation (Fig. 4D, lane 3), while transfection of the S629A mutant actually decreases AKT phosphorylation (Fig. 4D, lane 2), suggesting that the mutant GGAP2 (S629A) acts as a dominant negative mutant in decreasing AKT activation.

Figure 4 Akt binds to and modulates GGAP2 activity.

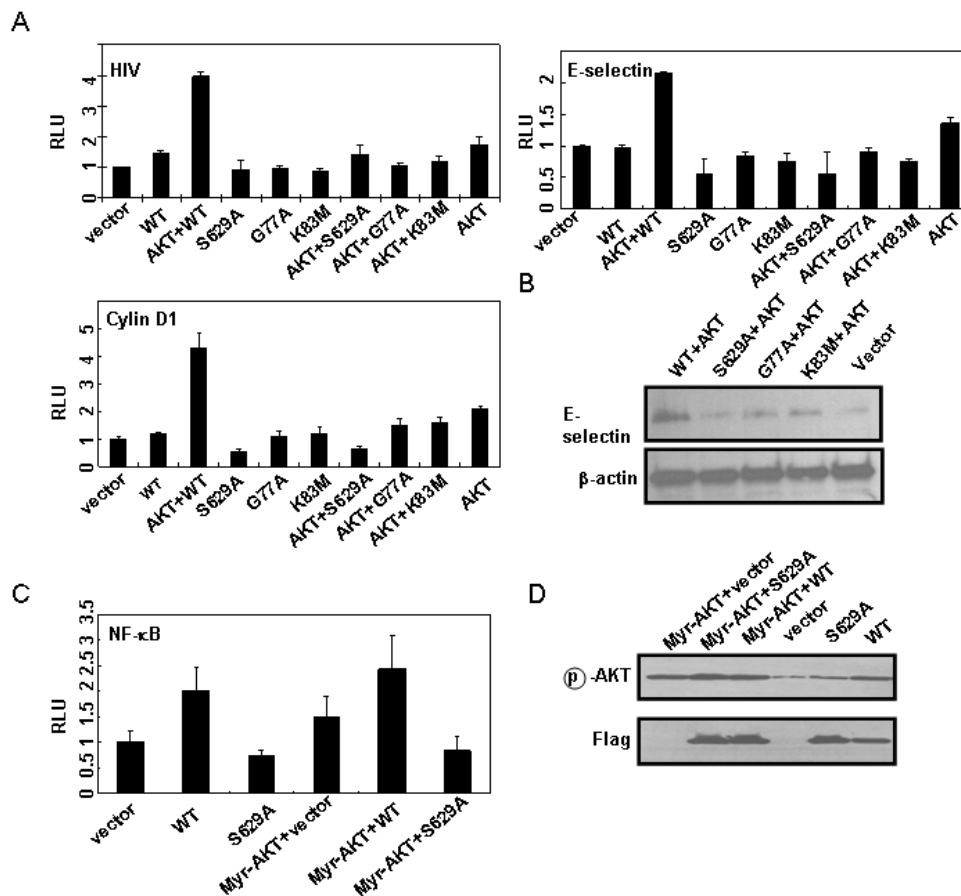


(A) Transiently transfected HA-Akt1 and Flag-GGAP2 coimmunoprecipitate in prostate cell lines. PNT1A cells transfected with empty vector were used as a control. (B) Mutation of GGAP2 at S629, a potential Akt phosphorylation site, significantly reduces its interaction with Akt. PNT1A cells were transiently transfected with Flag-tagged wild type (WT) or S629A mutant of GGAP2 and immunoprecipitated with anti-Flag antibody followed by Western blot (WB) with a phospho-(Ser/Thr) Akt substrate antibody which recognizes potential Akt phosphorylation substrate peptides containing phospho-Ser/Thr preceded by Lys/Arg at positions -5 and -3. (C) Akt phosphorylation of GGAP2 is essential for GGAP2 GTP-binding activity. Cell lysates prepared as in (B) expressing control vector, wild-type and S629A mutant GGAP2 were incubated with GTP-agarose conjugate beads. Anti-Flag antibody was used to detect wild-type and S629A GGAP2 bound to GTP beads. Anti-flag antibody was used to control protein expression in cell lysate direct loading. (D) DU145 cells were transiently transfected with WT or S629A mutant GGAP2 and phospho-Akt (S473) levels determined by Western blotting.

NF- κ B is a transcription factor and the promoter regions of many genes contain NF- κ B binding sites. To determine the effect of Akt and GGAP2 on the transcriptional activation of NF- κ B, we utilized three luciferase-reporter vectors which contain NF- κ B binding sites from three different target genes (HIV, E-selectin, Cyclin D1). Co-transfection of AKT and wild-type GGAP2 augmented the luciferase activities of all three promoters (Fig. 6A), indicating that the combination of Akt and GGAP2 stimulates NF- κ B transcriptional activity. In contrast, GGAP2 mutants, including the Akt phosphorylation site mutant S629A and the two guanine nucleotide binding site mutants, G77A and K83M, did not enhance the luciferase activity even in the presence of Akt (Fig. 6A), suggesting that GGAP2 activation is required for Akt-GGAP2-induced NF- κ B transcriptional activation. Several studies indicated that E-selectin is highly expressed in prostate cancer cells and the expression level of E-selectin has been reported to be regulated by NF- κ B. Our data suggest that the combination of Akt and GGAP2 may regulate E-selectin expression in prostate cancer cells through regulating NF- κ B. To determine if GGAP2 played a role in E-selectin expression from its endogenous promoter, we performed Western blots to detect E-selectin protein expression in LNCaP cells stably transfected with empty vector, wild-type GGAP2, S629A, G77A and K83M mutants, and together with Akt plasmid (Fig. 6B). Compared to cells transfected with the control vector and mutant GGAP2, wild-type GGAP2 and Akt enhanced the expression of E-selectin at the protein level.

While we have demonstrated that GGAP2 can interact directly with NF- κ B, it is possible that NF- κ B activation is due to increased Akt activity induced by GGAP2. To address this question, we cotransfected DU145 cells with myristylated Akt, empty vector, wild-type GGAP2 or S629A, respectively. Wild-type GGAP2 transfection still enhanced NF- κ B transcriptional activity, despite the fact that there was no further increase in activated AKT in these cells (Fig. 6C and 6D). Of note, transfection of the S629A mutant decreased the luciferase activity below vector control level in this study, suggesting that this mutant, which affects GGAP2- NF- κ B interaction, may have a dominant negative effect on NF- κ B activity in DU145 cells. These studies are consistent with a direct effect of GGAP2 on NF- κ B transcriptional activity. For more detailed information, please refer to the published manuscript in the Appendices section.

Figure 5 Akt-GGAP2 activates transcriptional activities of NF-kB.



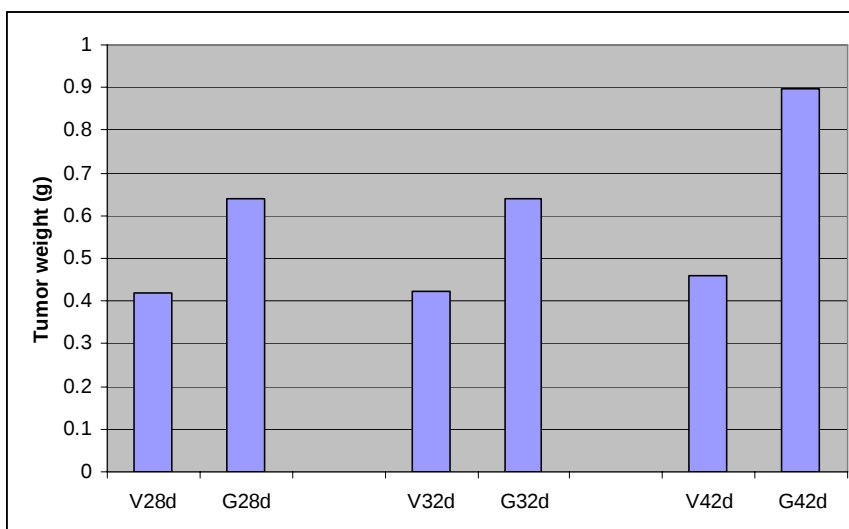
(A) Co-expression of Akt and GGAP2 activates transcription of a reporter gene under the control of native HIV, E selectin and Cyclin D1 promoter regions, while mutations of GGAP2 decreased the activation of NF-kB activation with or without Akt overexpression. Luciferase gene was fused with the promoters from native HIV, E-selectin, and Cyclin D1 genes, respectively, which contain the NF-kB binding sites. Luciferase activities were measured to determine the transcriptional activation of the promoter regions. Luciferase activity of each group were divided by the control vector to produce relative luciferase activities and shown as fold increase. 293T cells were used for transfection. (B) Endogenous E-selectin protein expression level is increased in LNCaP cells transiently expressing GGAP2 and Akt. (C-D) GGAP2 activates NF-kB transcriptional activity independent of Akt overexpression in DU145 cells. Stable DU145 cell lines expressing myristylated Akt or vector controls were used for transient transfection with plasmids as indicated. All luciferase activity assays were performed in triplicates. Levels of phospho-Akt were simultaneously measured by Western blotting with anti-phospho-Akt antibody (S473).

Task 3 Investigate the role of GGAP2 in cancer progression and metastasis using orthotopic models of human PC.

We generated PC3 and LNCAP cell lines stably expressing control vector, wild-type GGAP2, S629A (AKT phosphorylation site) mutant GGAP2 and mutant GGAP2 identified from clinical PC patients. To examine the effects of GGAP2 on in vivo cancer growth, we carried out athymic nude mice orthotopic injection using PC3 cells stably transfected with control vector or wild-type GGAP2 plasmid with 20 mice in each group. Briefly, athymic male nude mice (nu/nu; 8–10 weeks of age) were obtained from NCI-Frederick (Frederick, MD). Animal handling and experimental procedures were approved by the Baylor College of Medicine animal experiments committee. On the day of surgery, after total body anesthesia with intraperitoneal injection of Nembutal (50mg/kg), an incision was made in the lower abdomen to expose the prostate, the urinary bladder and the semiovules. A suspension of PC3 (1×10^6) in 30 μ l cell culture media (RPMI1640 without antibiotics) was injected into a lateral lobe of the prostate and the wound was closed with surgical metal clips, which were removed 7 days after the surgery. This cell concentration was necessary to achieve consistent local tumor growth within 7 days of implantation. Mice were sacrificed 28, 32 and 42 days after the injection respectively. The primary tumor growth and sites of metastasis were determined by visual inspection. We then excised, measured and weighed the primary tumors. Specimens were fixed in formalin and embedded in paraffin for H&E staining or stored in liquid nitrogen immediately for further protein and/or RNA analysis.

Our results showed that PC3 cells stably transfected with GGAP2 plasmid formed larger primary tumor than vector control at all three time points (Fig. 6). Lymph node metastasis in GGAP2 group was increased compared to the control group although not to a statistically significant level (P value=0.0583, χ^2 test).

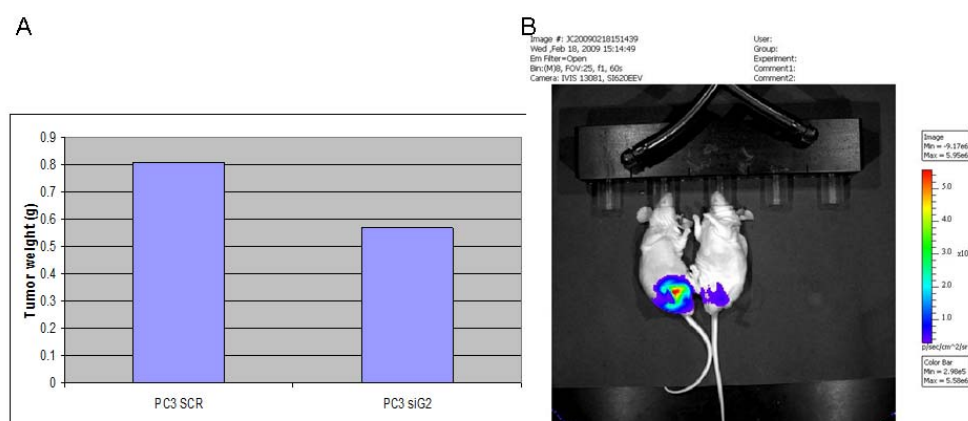
Figure 6 GGAP2 promotes in vivo PC3 cell tumor growth in orthotopic nude mice model.



Student T-test was performed to validate the data. P value<0.05 at all three time points. All 40 mice were injected with PC3 cells on the same day. At 28, 32 and 42 days after the injection, 5, 5, and 10 mice from each group were sacrificed, respectively. Averages were shown.

To further verify the effects of GGAP2 in PC initiation and development, we also generated PC3 cells stably transfected with a lentiviral vector expressing either irrelevant scrambled RNA sequence or shRNA specifically designed to knock down GGAP2. These cells were further infected with lentiviral particles encoding the luciferase gene before being orthotopically injected into the lateral lobe of nude mice prostate as described above. By using IVIS live imaging system, we were able to monitor the real-time PC3-origin tumor growth. We found knock down of GGAP2 dramatically reduced tumor volume which is consistent with the GGAP2 overexpression orthotopic injection described in the previous paragraph (Fig.7)

Figure 7 Knock down of GGAP2 decreased PC3 tumor growth in vivo.



40 athymic nude mice were separated into two groups as shown. Mice were sacrificed and tumors collected 7 weeks after the PC3 orthotopic injection. (A) Knock down of GGAP2 leads to reduced tumor growth in orthotopic nude mice model. Student T-test was performed to validate the data. P value<0.01. All 40 mice were injected with PC3 cells on the same day. At 28, 32 and 42 days after the injection, 5, 5, and 10 mice from each group were sacrificed, respectively. SCR refers to irrelevant scrambled RNA group. Averages were shown. (B) A representative picture is shown for luciferase detection in live mice using IVIS system. The mice on the left side is from PC3 SCR group and the right from PC3 siG2 group.

Task 4 Interim analyses.

We performed Interim statistical analyses of data obtained from the experiments as described in Task 2. Annual report was submitted and approved as required by the program.

Task 5 Final analyses and report writing.

The final analyses of data from all experiments and the final report are completed before the due date required by this training fellowship program. One manuscript based on the research supported by this

fellowship was published by Cancer Research (2009 Feb 1;69(3):819-27. Epub 2009 Jan 27.). Another article reporting the data regarding GGAP2 mutations in human prostate cancer is in preparation.

Key Research Accomplishments

- multiple low-frequency mutations of GGAP2 were identified in clinical PC patients
- the clinically identified mutations of GGAP2 stimulate prostate cell proliferation
- mutations of GGAP2 may enhance GGAP2 GTP binding activity
- GGAP2 expression is increased in PC

Reportable Outcomes

- One manuscript accepted by Cancer Research (2009 Feb 1;69(3):819-27. Epub 2009 Jan 27.)
- Second manuscript in preparation.
- Poster presentation of findings of this project in Special Conference titled Advances in PC Research (January 21–24, 2009)

Conclusion

GGAP2 expression is increased in PC. Our results indicate that GGAP2 may be mutated in human PC in a low frequency. The multiplicity of GGAP2 mutations imply that these mutations may emerge in an environment of the multifocal heterogeneity of PC tissues. According to our results these mutations can stimulate cancer cell proliferation and enhance GGAP2 activation, indicating that they may contribute to the functions of GGAP2 in PC. In summary, GGAP2 may promote PC growth and progression via overexpression and/or mutation.

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Appendices

GGAP2/PIKE-A Directly Activates Both the Akt and Nuclear Factor- κ B Pathways and Promotes Prostate Cancer Progression

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Abstract

GGAP2/PIKE-A is a GTP-binding protein that can enhance Akt activity. Increased activation of the AKT and nuclear factor- κ B (NF- κ B) pathways have been identified as critical steps in cancer initiation and progression in a variety of human cancers. We have found significantly increased expression GGAP2 in the majority of human prostate cancers and GGAP2 expression increases Akt activation in prostate cancer cells. Thus, increased GGAP2 expression is a common mechanism for enhancing the activity of the Akt pathway in prostate cancers. In addition, we have found that activated Akt can bind and phosphorylate GGAP2 at serine 629, which enhances GTP binding by GGAP2. Phosphorylated GGAP2 can bind the p50 subunit of NF- κ B and enhances NF- κ B transcriptional activity. When expressed in prostate cancer cells, GGAP2 enhances proliferation, foci formation, and tumor progression *in vivo*. Thus, increased GGAP2 expression, which is present in three quarters of human prostate cancers, can activate two critical pathways that have been linked to prostate cancer initiation and progression. [Cancer Res 2009;69(3):819–27]

Introduction

Prostate cancer is the most common visceral malignancy in US men and the second leading cause of cancer deaths. The clinical behavior of prostate cancer is extremely heterogeneous, ranging from indolent disease to aggressive, metastatic cancer with rapid mortality. Although many advances have been made in our understanding of prostate cancer biology, there are still significant gaps in our knowledge regarding the regulation of critical pathways regulating prostate cancer progression.

Activation of phosphatidylinositol-3 kinase (PI3K) pathway and Akt serine-threonine kinases play a central role in prostate cancer initiation and progression (for review, see ref. 1). After the activation of PI3K by tyrosine kinase receptors or other cell surface receptors, the resulting lipid second messenger product phospholipids phosphatidylinositol 3, 4, 5-trisphosphate or phosphatidylinositol 3, 4-bisphosphate, recruit Akt to the plasma membrane and bind to its pleckstrin homology (PH) domain. This binding leads to the conformational change in Akt, resulting in phosphorylation

at Ser-473 in the regulatory domain. Phosphorylated Akt can then phosphorylate and regulate the function of many cellular proteins involved in cell proliferation, survival, and mobility—processes that are critical for tumorigenesis and metastasis (1). The net result of Akt activation include enhanced cell proliferation (1, 2), decreased apoptosis (1–3), and increased tumor angiogenesis (1, 4, 5), all of which can promote prostate cancer progression.

The nuclear factor- κ B (NF- κ B) pathway also plays a critical role in prostate cancer progression (6–12). NF- κ B is present in cells as a heterodimer of two subunits, p50 and p65. This complex is retained in the cytoplasm of unstimulated cells by its interaction with I κ B α . After stimulation of cells by cytokines and/or growth factors, I κ B α is phosphorylated by the IKK complex, leading to degradation of I κ B α by the 26S proteasome. This allows translocation of the NF- κ B complex to the nucleus where it can activate transcription of a number of genes that can promote neoplastic progression in prostate cancer (6, 13–16). These include Bcl-2, *c-myc*, interleukin (IL)-6, IL-8, vascular endothelial growth factor, matrix metalloproteinase 9, urokinase-type plasminogen activator, and uPA receptor, all of them may play a role in prostate cancer initiation and progression. Consistent with these biological activities, immunohistochemical studies have shown that increased nuclear NF- κ B staining is a strong independent predictor of biochemical recurrence after radical prostatectomy (10–12).

Given the critical role of the PI3-K pathway in many cellular processes, it is not surprising that it is regulated by both positive and negative regulators. The PTEN tumor suppressor gene encodes a lipid phosphatase and is inactivated in a wide variety of malignant neoplasms, including prostate carcinoma (1, 17, 18). The tumor suppressor activity of the PTEN tumor suppressor gene is primarily due to its ability to dephosphorylate phosphatidylinositol (3–5) phosphate at the 3-position and negatively regulate the activity of the PI3-K pathway (1). A novel group of positive regulators of the PI3-K pathway are the PIKE/GGAP2 proteins (19–26). These proteins are all encoded by a single gene on chromosome 12q13.3 (21, 22). PIKE-L and PIKE-S are alternatively spliced variants of the same transcript (21). The PIKE-A/GGAP2 transcript arises from an alternative promoter within the *PIKE* gene (22), which was first identified as PIKE-S. It contains NH₂-terminal proline-rich domains, a Ras homology domain (G domain), and a PH domain. PIKE-L is an alternatively spliced isoform which also contains a COOH-terminal Arf-GAP domain and two ankyrin repeats. Both of these proteins can bind PI3-K via their proline-rich domains and modulate its activity in the central nervous system and may play an important role in central nervous system biology (19–21). The third form, known as GGAP2 or PIKE-A (we will use the designation GGAP2), is expressed in cancer (27).

The GGAP2 protein is similar to PIKE-L except the NH₂-terminal proline-rich domains due to an alternative promoter. This protein was originally characterized in 2003 by Xia and colleagues (23) as

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Y. Cai and J. Wang contributed equally to this project.

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GGAP2. It was shown that GGAP2 has a GTPase activity, as expected from its RAS homology domain, and could play critical roles in modulating many normal cellular processes as well as in neoplastic transformation and tumor progression. The GAP domain can activate the GTPase activity via either intramolecular or intermolecular interaction. Further studies of the PIKE-A/GGAP2 protein (22, 24, 25) showed that the protein binds to activated Akt and this binding is promoted by GTP binding. In addition, the GTPase domain of GGAP2 is responsible for binding activated Akt. This binding enhances Akt activation, whereas a dominant negative form of GGAP2 decreases Akt activity. Increased GGAP2 protein results in increased invasion and resistance to apoptosis in glioblastoma cell lines and Akt is necessary for these changes in the phenotype (22). In summary, GGAP2 can promote Akt activity via direct binding with the protein, which can be modulated by GTP binding.

There is evidence linking alterations of GGAP2 activity to neoplastic transformation. The GGAP2 locus at 12q13.3 is amplified in glioblastoma cell lines, primary glioma cultures, and in glioblastoma tumors from patients (22, 24, 26). More than 90% of glioblastomas overexpress GGAP2 (26), indicating that it is probably a key target in this amplicon. GGAP2 is also amplified in sarcoma and neuroblastoma cell lines (22, 24). Dot blot hybridization by Liu and colleagues (27) showed the increased expression of GGAP2/PIKE-A mRNA in a wide variety of human malignancies, including two of four prostate cancers. Thus, increased GGAP2 activity is seen in a number of human malignancies secondary to amplification and/or overexpression. A number of comparative genomic hybridization studies of prostate cancer have shown gain of the 12q13.3 region where the GGAP2 gene is located (28, 29). We therefore undertook studies to examine the potential role of GGAP2 in human prostate cancer.

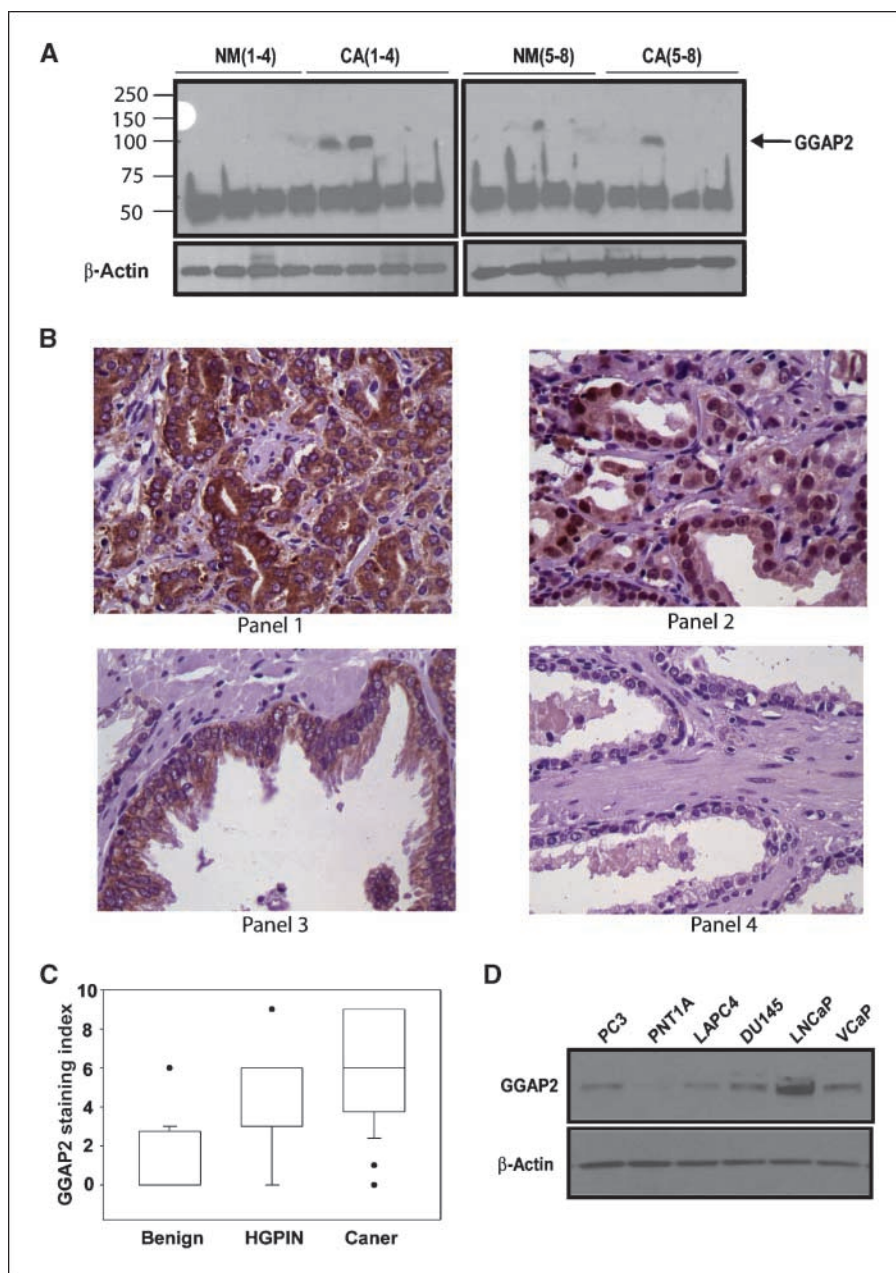


Figure 1. GGAP2 expression is increased in human prostate cancer. **A**, GGAP2 in patient prostate cancer tissue lysates. Protein (1.5 mg of total) was incubated with anti-GGAP2 rabbit antibody and protein A agarose beads for 4 h before the immunoprecipitates were immunoblotted with the same antibody. Molecular weight marker sizes (in kDa) are shown. Arrow, location of GGAP2 protein bands. The lower bands in each lane are antibody heavy chain. **B**, immunohistochemistry demonstrating an increased GGAP2 level in human prostate cancer tissues. 1, cancer tissue with strong cytoplasmic staining; 2, nuclear staining in prostate cancer tissues; 3, high-grade PIN (HGPIN) with moderate cytoplasmic staining; 4, normal prostate tissue showing very weak epithelial staining; **C**, boxplot of staining indices of benign, high-grade PIN, and cancer tissues. **D**, GGAP2 expression in prostate cancer cell lines. Twenty micrograms of total cell protein were used in each lane. GGAP2 was barely detectable in the immortalized prostate epithelial cell line PNT1A but was easily detectable in the prostate cancer cell lines, especially LNCaP cancer cells.

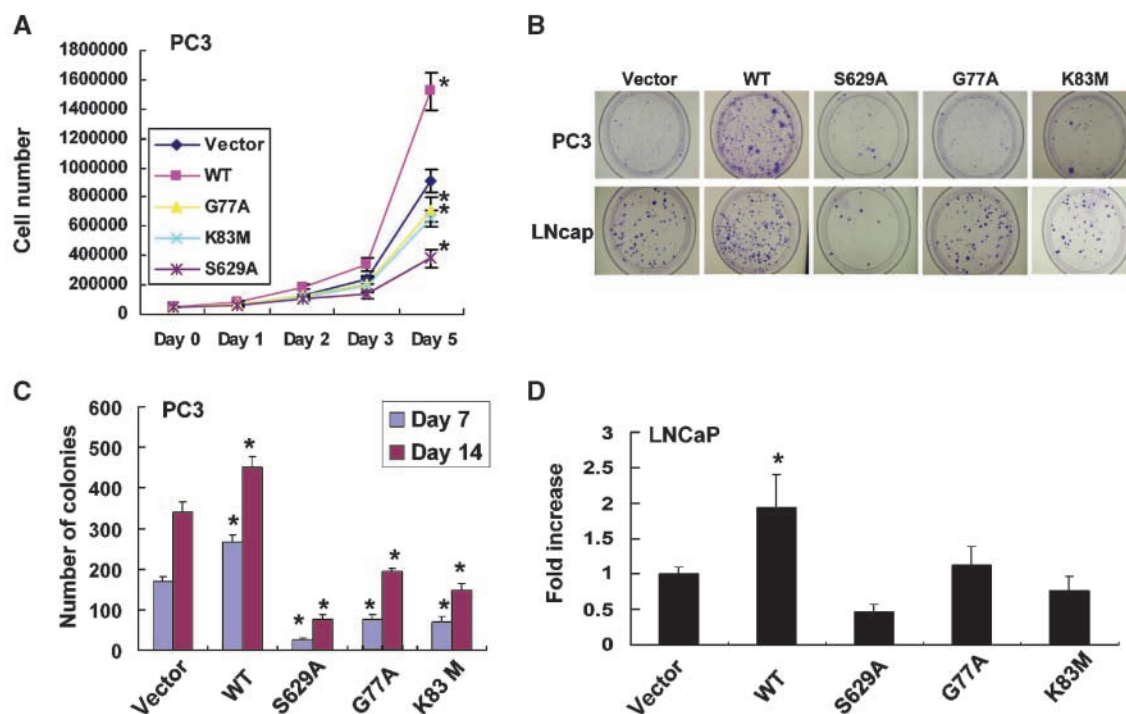


Figure 2. GGAP2 promotes prostate cell proliferation and cancer growth *in vitro*. **A**, prostate cell lines PC3 were stably transfected with various plasmids stated in the figure. Fifty thousand cells were plated in triplicate and collected for counting at day 1, 2, 3, and 5. **B** to **C**, GGAP2 enhanced colony formation in PC3 cells. **D**, stable LNCaP cell lines as expressing wild-type (WT) or mutant GGAP2 or vector controls used for soft agar assays. Foci number of each group was compared with the control cells (vector) and shown as fold increase. Representative data from one cell line were shown. Similar results were observed in all prostate cell lines tested (see text). ANOVA was used to examine the significance of the data using Sigmapstat software. Asterisks were used to identify groups with statistically significant changes with a *P* value of <0.05 compared with the vector control group at the same time point.

Materials and Methods

Reagents. Mouse monoclonal horseradish peroxidase-conjugated anti-p50, anti-p65, anti-RelB, anti-RelC, anti-Myc, anti-HA, and anti-E-selectin antibodies were obtained from Santa Cruz Biotechnology, Inc. Mouse monoclonal Flag antibody was from Stratagene. Rabbit polyclonal anti-GGAP2 (PIKE) was from Prosci, Inc. Rabbit anti-phospho-(Ser/Thr) Akt substrate antibody (#9611) and phospho-Akt (Ser 473) antibody (#4058) were from Cell Signaling Technology. Rabbit polyclonal anti-Akt1 was from Abgent (AP7028b). GTP bound agarose beads were from Sigma. The Matrigel Basement Membrane Matrix for PC3 xenograft inoculation was from BD Biosciences. The p50, p65, RelB, and RelC expression plasmids were kindly provided by Dr. Paul Chiao from University of Texas M.D. Anderson Cancer Center, Houston.

Immunohistochemistry. Immunohistochemistry was performed on tissue array slides with anti-GGAP2 antibody using standard procedures (ABC-Elite; Vector Laboratories). Briefly, array slides were preincubated with normal goat serum and blocking avidin for 20 min then rinsed in PBS. Epitope retrieval was performed by incubating the slides in a pressure cooker for 30 min in 0.01 mol/L citrate buffer. Anti-GGAP2 antibody was applied onto slides at a 1:1,000 dilution. Slides were incubated with the secondary antibody (biotinylated anti-rabbit IgG made in goat diluted 1:350 in normal goat serum; Vector laboratories) for 1 h. Solutions A and B (ABC-Elite) were added onto the slides for 30 min. Diaminobenzidine was used as a chromogen. Arrays were visualized and photographed using standard light microscopy and analyzed manually. Staining was evaluated in the epithelial cytoplasm in normal luminal epithelium and prostate cancer as described previously (30–35). Staining intensity was graded as absent (0), weak (1+), intermediate (2+), or strong (3+). The extent of staining was estimated and scored as follows: no staining (0), 1% to 33% of cells stained (1+), 34% to 66% of cells stained (2+), or 67% to 100% of cells stained (3+). The staining index

for each case was then calculated by multiplying the average intensity score for the three cores by the average percentage score for the three cores, yielding a 10-point tumor staining index ranging from 0 (no staining) to 9 (extensive, strong staining) for each case. Control slides incubated with secondary antibody only showed no staining.

Transfection, immunoprecipitation, immunoblotting, and luciferase assay. Immunoprecipitation (IP), Western blot, and luciferase reporter assay were carried out in 293T and prostate cell lines as described (23, 36). For patient tissue samples, frozen patient tissues in liquid nitrogen were ground using a mortar, and lysed in 1 mL radioimmunoprecipitation assay buffer supplemented with proteinase inhibitor cocktail containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL leupeptin, 10 µg/mL pepstatin, and 10 µg/mL aprotinin. Protein concentrations were measured before usage. Native HIV and E-selectin promoter driving luciferase reporter plasmids were gifts from Dr. Jing Ke from University of Texas Houston. The synthetic NF-κB promoter luciferase plasmid was obtained from Stratagene.

Site-directed mutagenesis. Single nucleotide mutagenesis was carried out according to the manufacturer's protocol (Stratagene). Briefly, primers with the target mutations were used in PCR to generate Akt phosphorylation site mutation S629A and GTPase mutations G77A and K83M. DpnI enzyme was added to PCR products for 1 h in 37°C to digest template plasmid DNA before the transformation. Clones were sequenced to verify the mutations.

GTP-agarose pull-down assay. Purified proteins or cell lysates expressing wild-type and mutant GGAP2 were equilibrated in GTP binding buffer [20 mmol/L Tris-HCl (pH 7.0), 150 mmol/L NaCl, 5 mmol/L MgCl₂, and 0.1% Triton X-100, supplemented with proteinase inhibitor cocktail containing 1 mmol/L PMSF, 10 µg/mL leupeptin, 10 µg/mL pepstatin, and 10 µg/mL aprotinin] and incubated with 100 µL of GTP-agarose beads (Sigma-Aldrich) for 4 h at 4°C. Agarose beads were then centrifuged and washed thrice with GTP binding buffer. Bound proteins were eluted by

boiling in immunoblot loading buffer before separated by SDS-PAGE electrophoresis and detected by either Coomassie blue staining or Western blot using specific antibody against the proteins.

Myristylated Akt plasmid construction. Myristylated Akt1 cDNA in pUSEamp vector was purchased from Upstate Biotechnology. The cDNA sequence was then cloned using PCR, digested with EcoRI and BamHI, and ligated into a lentiviral vector pCDH1-MSC1-EF1-Puro and sequenced. Primers used were as follows: Forward, 5'-GCGAATTCATGGGAGCAGCAAGA-3'; Reverse, 5'-GCGGATCCTCAATGGTGTGGTGTAT-3'. DU145 cells were transfected with the myristylated Akt1-pCDH1 plasmid and selected with puromycin for stable expression.

In vitro proliferation assays. Fifty thousand cells of each stable cell type were plated in 10-cm dishes. Cells were incubated in triplicate at 37°C with RPMI 1640 supplemented with corresponding antibiotics. Cells were trypsinized and counted using a Coulter Counter.

Clonogenic assay. The clonogenic ability of prostate cells were examined as described by Franken and colleagues (37) with minor revision. Prostate cell lines plated in 6-well plates were transfected with various plasmids in Fig. 2C before being selected with G418 antibiotics for up to 14 d. Two micrograms DNA were used to transfect 20,000 cells each well. Cell colonies were then stained with 0.005% Crystal Violet before visualization and quantitation.

Soft agar assays. Five thousand LNCaP cells stably transfected with various plasmids were mixed with the 0.7% agarose (top agar) and warm 2×RPMI 1640 + 20% fetal bovine serum and plated in each well of a 6-well plate on top of the prepared 1% base agar. Incubate assay at 37°C for 14 d before the foci were stained with 0.005% Crystal Violet and counted.

RNA interference. For stable GGAP2 shRNA expression, we designed the primers as follows:

Top 5'-CACCGCATTAACGGGCTCGTCAATTCGAAAATTGAC-GAGCCCGTTAATGC-3'.

Bot 5'-AAAAGCATTAAACGGGCTCGTCAATTTTCGAATTGAC-GAGCCCGTTAATGC-3'.

Oligonucleotides were annealed to generate a double stranded oligonucleotide, which was cloned into pENTR/U6 vector (Invitrogen) that contains U6 promoter, RNA polymerase III-dependent promoter, and Pol III terminator. The DNA sequences of a U6 RNAi cassette containing U6 promoter, double-stranded oligo encoding the shRNA against GGAP2, and Pol III terminator from pENTR/U6 vector was transferred to plenti6/BLOCKit-DEST vector during LR recombination process as instructed by the manufacturer's protocol. The plenti6/BLOCKit-DEST vector contains a Blasticidin selection marker, which can be used to generate cell lines stably expressing GGAP2 specific shRNA.

S.c. PC3 injection. A total of 15 severe combined immunodeficient (SCID) mice were separated into 3 experimental groups (5 mice per group) and were s.c. injected with PC3 cells expressing control vector, wild-type, and S629A plasmids, respectively. Fifty-microliter cell suspensions containing 1×10^6 PC3 Cells in PBS were mixed with 50 μ L Matrigel Basement Membrane Matrix (BD Biosciences) and s.c. implanted into each SCID mouse using no anesthesia in the lumbar posterolateral region bilaterally as a duplicate. Mice were sacrificed and tumors were collected and weighed 4 wk after the inoculation. Tumor size was measured using a caliper. Animals were euthanized according to tumor size and by cervical dislocation. Tumors were harvested and fixed in 10% formalin for histologic analysis.

Results

Expression of GGAP2 in prostate cancer. To examine whether GGAP2 was overexpressed in prostate cancer, we performed Western blots of eight prostate cancers and eight normal peripheral zone tissues, which revealed detectable expression of GGAP2 in three of eight cancers but in none of the benign tissues (Fig. 1A). The antibody used also detects PIKE-L because it recognizes a carboxy-terminal epitope, but no evidence of PIKE-L expression, which would show a 125 kDa protein band, was seen and only the 90 kDa GGAP2 protein was present in the Western blot (Fig. 1A). We then carried out immunohistochemistry with specific anti-GGAP2 antibody using tissue microarrays containing matched normal, cancer, and high-grade prostatic intraepithelial neoplasia (PIN) tissues (Fig. 1B). Moderate to high levels of expression of GGAP2 protein was seen in 75% of prostate cancer tissues within the cancer epithelial cells. Staining was primarily cytoplasmic (Fig. 1B, 1) in those cases with staining. Nuclear staining was also identified in some cases (Fig. 1B, 2). Similar staining was observed in high grade PIN (Fig. 1B, 3). Absent or weak staining was seen in >95% of the normal epithelial tissues (Fig. 1B, 4). Weak staining of stromal tissues was also noted. Tissues stained with secondary antibody only were completely negative (data not shown). We then quantified cytoplasmic expression of GGAP2 using a visual quantitation as described in numerous prior reports (30–35). This quantitation is based on a multiplicative score in the extent (scored 1–3) and intensity (scored 0–3) of staining, and results in scores ranging from 0 (absent) to 9 (strong, diffuse staining). The mean score for normal epithelium was 0.86 ± 0.18 (SE, $n = 72$), the mean for PIN was 3.6 ± 0.9 ($n = 65$), whereas the mean for cancer was 5.8 ± 0.4 ($n = 56$). The

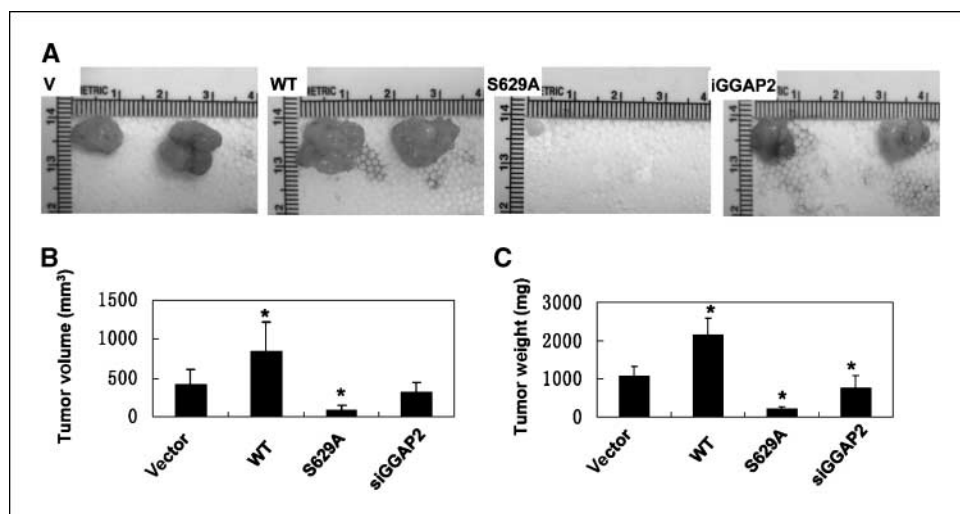
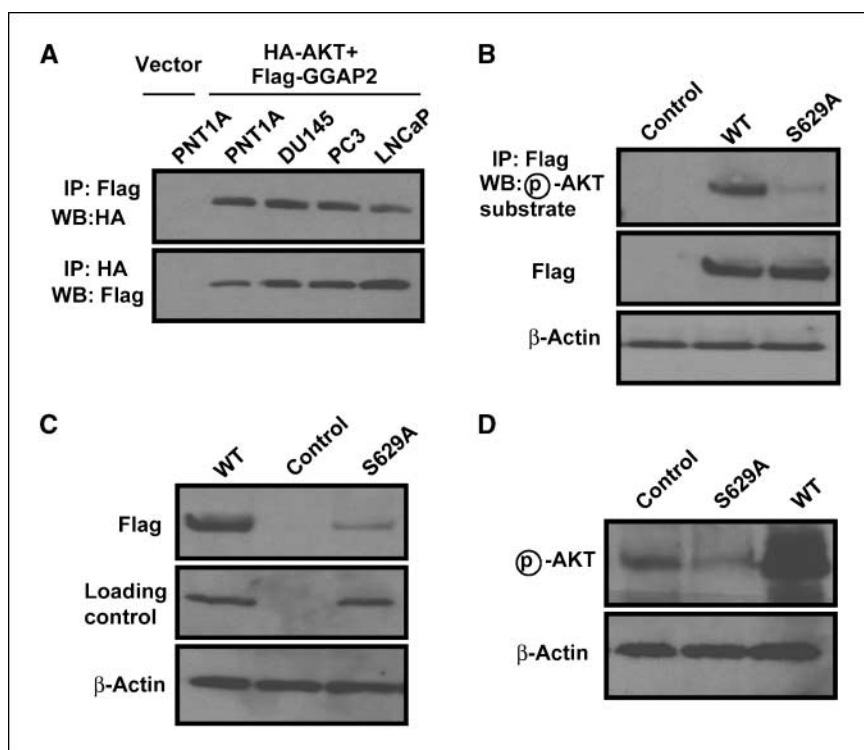


Figure 3. GGAP2 promotes cancer cell growth *in vivo*. A to C, GGAP2 promotes cancer growth *in vivo* using PC3 cell s.c. injection SCID mice model. Average tumor size and weight are shown, and do not include cases with no visible tumor. Tumor volume was calculated by multiplying the length, width, and height of the tumor tissues. Columns, mean; bars, SD. ANOVA was used to examine the significance of the data using Sigmaplot software. Asterisks were used to identify groups with statistically significant changes with a P value of <0.05 compared with the vector control group.

Figure 4. Akt binds to and modulates GGAP2 activity. **A**, transiently transfected HA-Akt1 and Flag-GGAP2 co-IP in prostate cell lines. PNT1A cells transfected with empty vector were used as a control. **B**, mutation of GGAP2 at S629, a potential Akt phosphorylation site, significantly reduces its interaction with Akt. PNT1A cells were transiently transfected with Flag-tagged wild-type or S629A mutant of GGAP2 and immunoprecipitated with anti-Flag antibody followed by Western blot (WB) with a phospho-(Ser/Thr) Akt substrate antibody, which recognizes potential Akt phosphorylation substrate peptides containing phospho-Ser/Thr preceded by Lys/Arg at positions -5 and -3. **C**, Akt phosphorylation of GGAP2 is essential for GGAP2 GTP-binding activity. Cell lysates prepared as in **B** expressing control vector, wild-type, and S629A mutant GGAP2 were incubated with GTP-agarose conjugate beads. Anti-Flag antibody was used to detect wild-type and S629A GGAP2 bound to GTP beads. Anti-flag antibody was used to control protein expression in cell lysate direct loading. **D**, DU145 cells were transiently transfected with WT or S629A mutant GGAP2 and phospho-Akt (S473) levels determined by Western blotting.



difference in staining scores between normal, PIN, and cancer was highly statistically significant ($P < 0.001$, Mann-Whitney Rank Sum test; Fig. 1C).

We further examined the expression level of GGAP2 in different human prostate cell lines. mRNA is expressed in the immortalized normal prostatic epithelial cell line (PNT1A), and in all four cancer cell lines tested (PC3, DU145, LNCaP, and LAPC4; data not shown). Western blot of the cancer cell lines with anti-GGAP2 antibody reveals easily detectable protein expression in all six prostate cancer cell lines tested, with higher expression of GGAP2 in cancer cell lines than in immortalized normal prostate cell line PNT1A cells (Fig. 1D). Thus, GGAP2 is expressed at increased levels in human prostate cancers.

Biological activities of GGAP2 *in vitro*. To investigate the biological role of GGAP2 in prostate cancer development, we first carried out cell proliferation assays by direct cell counting to determine if GGAP2 promotes cell growth. Prostate cancer cell line PC3 were stably transfected with wild-type and mutant (S629A, G77A, K83M) GGAP2 and selected with G418. The G77A and K83M mutations inactivate the GTPase activity of GGAP2 (see below). As will be shown below, the S629A mutation blocks phosphorylation of GGAP2 by Akt and also significantly down-regulates GTPase activity. Compared with vector-transfected cells, wild-type GGAP2 promotes prostate cancer cell proliferation, whereas S629A, K83M, or G77A mutations had no effect or lead to growth that was even lower than the control levels (Fig. 2A). Experiments using immortalized normal prostatic epithelial (PNT1A), LNCaP, and DU145 cell lines showed similar results (data not shown).

We next tested the ability of GGAP2 to promote proliferation at low densities by carrying out *in vitro* clonogenic assays using the same cell lines (Fig. 2B–C). GGAP2 strongly enhanced colony formation, whereas the mutant GGAP2-transfected cells showed colony formation that was significantly below vector controls (PC3

data were shown). Finally, we carried out soft agar foci formation assay using LNCaP cells stably expressing wild-type, S629A, G77A, and K83M. As shown in Fig. 2D, wild-type GGAP2 overexpressing cells, compared with the control, had increased numbers of foci that was not seen in the mutant GGAP2 expressing cells. Indeed, the decreased foci formation seen in the cells expressing the S629A GGAP2 mutant implies that this form of GGAP2 may be acting as a dominant negative in these cells.

GGAP2 promotes cancer growth in SCID mice. To further explore the potential role of GGAP2 in prostate cancer progression, we performed s.c. xenograft studies in SCID mice using PC3 cells stably transfected with wild-type GGAP2, the S629A mutant form, siGGAP2, or vector controls. Tumors were seen in five of five mice injected with PC3 wild-type GGAP2 or vector controls, but the tumor weight and volume were significantly increased in the GGAP2 expressing cells. In contrast, only two of five mice injected with cells expressing the S629A mutant developed tumors and the tumors were significantly smaller. This is consistent with the *in vitro* data and suggests that the S629A is acting as a dominant negative in this context as well. Knockdown of GGAP2 with stable expression of siGGAP2 decreased the tumor size compared with the vector control (Fig. 3A–C). Thus, GGAP2 strongly enhance proliferation, colony formation, focus formation, and tumor progression in a SCID mouse model.

Interaction of GGAP2 and AKT. Based on the observations described above (Figs. 2 and 3), we then sought the underlying mechanisms through which GGAP2 promotes cell growth *in vitro* and *in vivo*. It is known that GGAP2/PIKE-A can enhance Akt kinase activity via direct interaction (22, 24, 25). Co-IP experiments with HA-tagged Akt and Flag-tagged GGAP2 after transient transfection confirm that Akt and GGAP2 physically interact in immortalized prostate epithelial cells and prostate cancer cell lines (Fig. 4A). We then analyzed four prostate cancer cell lines for

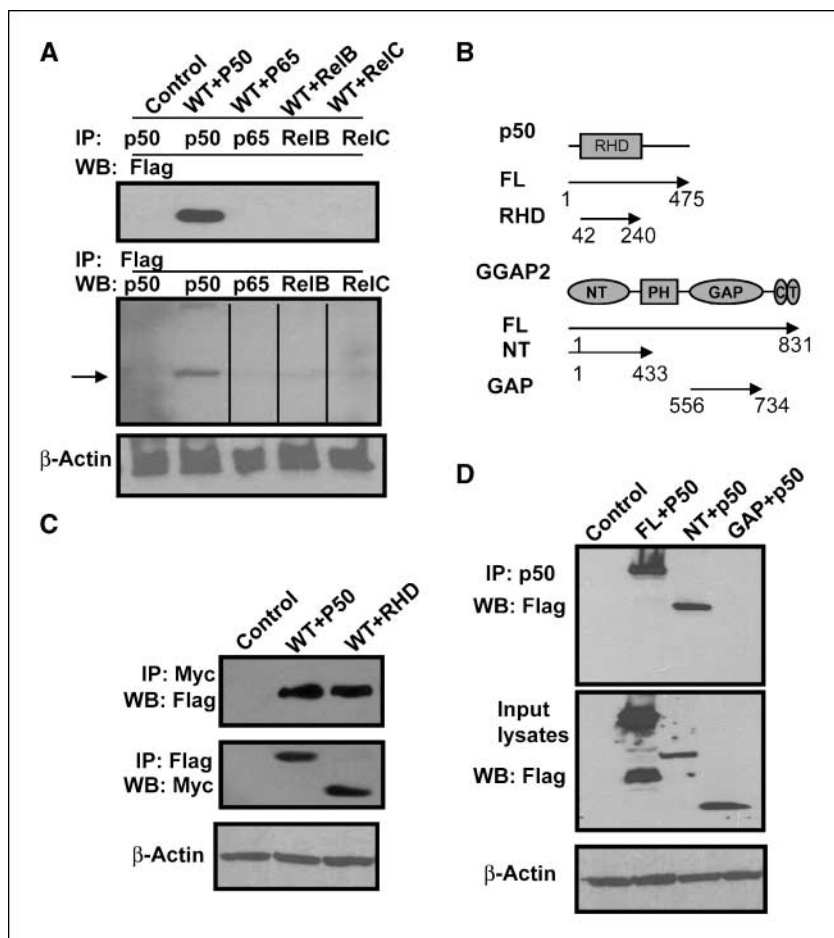


Figure 5. GGAP2 interacts with p50 subunit of NF- κ B transcription factor. **A**, GGAP2 coimmunoprecipitated with p50 specifically in transiently transfected 293T cells. Reciprocal IP and WB were performed using the respective antibodies. Anti-Flag antibody was used to detect Flag-GGAP2 fusion protein. **Middle**, IPs were pulled down with anti-Flag antibody and proteins were separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Each lane of the membrane was cut and incubated with individual antibodies as shown in the figure and then juxtaposed for exposure. **B**, domain structure and cloning of GGAP2 and p50 proteins, respectively. *Numbers*, the amino acid sequence number. *FL*, full length protein; *NT*, NH₂-terminal fragment; *RHD*, RHD domain; *CT*, COOH-terminal fragment. **C** to **D**, the NH₂-terminal GTPase domain of GGAP2 interacts with the RHD domain of p50. Reciprocal IP and WB were performed using respective antibodies stated in the figure. Western blot with anti- β -actin with equal protein amounts from input lysates are shown.

interaction of endogenous Akt and GGAP2 by IP with anti-Akt antibody followed by Western blot with anti-GGAP2 antibody. LNCaP showed a detectable band, whereas the other cell lines were negative (Supplementary Fig. S1A). LNCaP cells express the highest levels of GGAP2 (Fig. 1D) of the tested cell lines and contain high levels of phosphorylated Akt, at least in part due to mutation of the PTEN tumor suppressor gene. The absence of detectable bands in the other cell lines is presumably due to lower levels of expression of GGAP2 and/or phosphorylated Akt. It is known that GGAP2 can modulate Akt activity, but it is possible that Akt can also modulate the activity of GGAP2. We identified a serine at amino acid 629 that is a potential Akt phosphorylation site (THLSRVRLDLDWDP) using simulated motif scan.⁴ Flag-tagged GGAP2 was transiently transfected and immunoprecipitated (with anti-Flag Ab) followed by Western blotting with antibody to Akt consensus phosphoserine. This study showed phosphorylation of GGAP2 by Akt that is abolished by mutation of serine 629 to alanine (Fig. 4B). This was confirmed by an *in vitro* kinase assay (data not shown). To determine if Akt can modulate the GTPase activity of GGAP2, we carried out IP with lysates from 293T cells overexpressing Flag tagged vector, wild-type, and S629A GGAP2 incubated with GTP-conjugated agarose beads followed by Western blot using anti-Flag antibody. The mutation at position 629 of GGAP2 from serine to alanine dramatically decreased the GTP binding ability (Fig. 4C),

indicating that Akt phosphorylation of GGAP2 enhances GTP binding, presumably by down-regulating the GTPase activity of the GAP domain. Finally, when transfected into DU145 prostate cancer cells, GGAP2 markedly increases Akt phosphorylation (Fig. 4D, lane 3), whereas transfection of the S629A mutant actually decreases Akt phosphorylation (Fig. 4D, lane 2), suggesting that the mutant GGAP2 (S629A) acts as a dominant negative mutant in decreasing Akt activation.

GGAP2 interacts with p50 subunit of NF- κ B. To investigate whether there is a direct connection between GGAP2 and NF- κ B, we overexpressed Flag-GGAP2 and subunits of NF- κ B into 293T cells and performed reciprocal IP and Western blot using specific antibodies against Flag-GGAP2 and NF- κ B family members. GGAP2 was found to interact with p50 specifically (Fig. 5A). To investigate whether GGAP2 and p50 can interact in prostate cells, we transfected prostate cell lines with plasmids encoding Flag-GGAP2 and p50 subunit, and then examined the interaction between the two proteins, GGAP2-p50. Our data indicate that GGAP2 can interact with p50 in all four prostate cell lines (Supplementary Fig. S1B, top). To further examine the endogenous protein interaction, we performed similar experiments and found that endogenous GGAP2 was in the same IP complex with p50 (Supplementary Fig. S1B, bottom) in the prostate cancer cell lines tested. The specificity of this interaction was confirmed by knockdown of endogenous GGAP2 with a shRNA lentivirus (Supplementary Fig. S1B, bottom, lane siGGAP2). To further examine what specific regions of GGAP2 and p50 that mediate

⁴ <http://scansite.mit.edu>

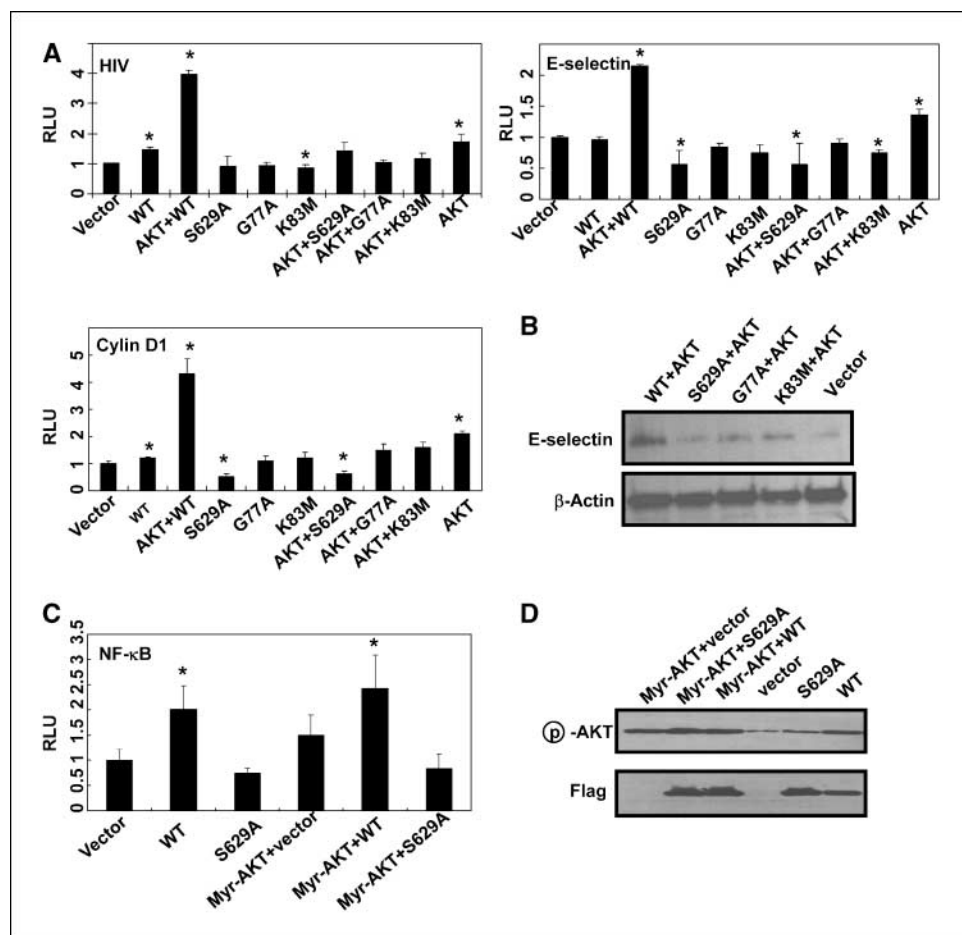
their interaction, we cloned the full-length p50 and its RHD domain into a eukaryotic expression vector XF-3HM with a Myc-tag, and full-length and distinct portions of GGAP2 into CMV-Tag2B vector with a Flag-tag (Fig. 5B). As shown in Fig. 5C and D, the NH₂-terminal GTPase domain of GGAP2 interacts with the RHD domain of p50. Of note, we have not been able to show interaction with the p105 precursor of p50 (data not shown).

Activation or inactivation of GTPases is determined by the guanine nucleotide binding status of the protein, which is controlled by guanine exchange factors, GTPase activating proteins (GAP), and other regulatory proteins. Activated or GTP bound form of GTPases function to regulate various signaling pathways in the cell. To understand the effects of activation of GGAP2 in prostate cancer, we generated two mutants in the NH₂-terminal GTPase domain of GGAP2, G77A, and K83M, which impair the ability of GGAP2 binding to GTP (Supplementary Fig. S1C), leading to GGAP2 GTPase inactivation. To explore the effects of the phosphorylation and activation status of GGAP2 on the GGAP2-p50 association, we transfected 293T cells with plasmids encoding p50 and the wild-type GGAP2, AKT phosphorylation site mutant GGAP2 (S629A), and the two GTPase domain mutants (G77A and K83M GGAP2), respectively. Reciprocal IP and Western blot were carried out to detect the GGAP2-p50 interaction. All three mutant GGAP2 showed a decrease in the association of GGAP2 with p50 (Supplementary Fig. S1D), indicating that Akt phosphorylation and GTP-binding/activation of GGAP2 play an important role in mediating the GGAP2-p50 interaction.

AKT-GGAP2 stimulates transcriptional activation of NF- κ B.

NF- κ B is a transcription factor and the promoter regions of many genes contain NF- κ B binding sites. To determine the effect of Akt and GGAP2 on the transcriptional activation of NF- κ B, we used three luciferase-reporter vectors, which contain NF- κ B binding sites from three different target genes (HIV, E-selectin, and Cyclin D1). Cotransfection of AKT and wild-type GGAP2 augmented the luciferase activities of all three promoters (Fig. 6A), indicating that the combination of Akt and GGAP2 stimulates NF- κ B transcriptional activity. In contrast, GGAP2 mutants, including the Akt phosphorylation site mutant S629A and the two guanine nucleotide binding site mutants, G77A and K83M, did not enhance the luciferase activity even in the presence of Akt (Fig. 6A), suggesting that GGAP2 activation is required for Akt-GGAP2-induced NF- κ B transcriptional activation. Several studies indicated that E-selectin is highly expressed in prostate cancer cells (38, 39) and the expression level of E-selectin has been reported to be regulated by NF- κ B (39). Our data suggest that the combination of Akt and GGAP2 may regulate E-selectin expression in prostate cancer cells through regulating NF- κ B. To determine if GGAP2 played a role in E-selectin expression from its endogenous promoter, we performed Western blots to detect E-selectin protein expression in LNCaP cells stably transfected with empty vector, wild-type GGAP2, S629A, G77A, and K83M mutants, and together with Akt plasmid (Fig. 6B). Compared with cells transfected with the control vector and mutant GGAP2, wild-type GGAP2, and Akt enhanced the expression of E-selectin at the protein level.

Figure 6. Akt-GGAP2 activates transcriptional activities of NF- κ B. **A**, coexpression of Akt and GGAP2 activates transcription of a reporter gene under the control of native HIV, E selectin, and Cyclin D1 promoter regions, whereas mutations of GGAP2 decreased the activation of NF- κ B activation with or without Akt overexpression. Luciferase gene was fused with the promoters from native HIV, Eselectin, and Cyclin D1 genes, respectively, which contain the NF- κ B binding sites. Luciferase activities were measured to determine the transcriptional activation of the promoter regions. Luciferase activity of each group were divided by the control vector to produce relative luciferase activities and shown as fold increase. 293T cells were used for transfection. **B**, endogenous Eselectin protein expression level is increased in LNCaP cells transiently expressing GGAP2 and Akt. **C** to **D**, GGAP2 activates NF- κ B transcriptional activity independent of Akt overexpression in DU145 cells. Stable DU145 cell lines expressing myristylated Akt or vector controls were used for transient transfection with plasmids as indicated. All luciferase activity assays were performed in triplicates. Levels of phospho-Akt were simultaneously measured by Western blotting with anti-phospho-Akt antibody (S473). ANOVA was used to examine the significance of the data using Sigmapstat software. Asterisks were used to identify groups with statistically significant changes with a *P* value of <0.05 compared with the vector control group.



Although we have shown that GGAP2 can interact directly with NF- κ B, it is possible that NF- κ B activation is due to increased Akt activity induced by GGAP2 (6). To address this question, we cotransfected DU145 cells with myristylated Akt, empty vector, wild-type GGAP2, or S629A, respectively. Wild-type GGAP2 transfection still enhanced NF- κ B transcriptional activity, despite the fact that there was no further increase in activated AKT in these cells (Fig. 6C and D). Of note, transfection of the S629A mutant decreased the luciferase activity below vector control level in this study, suggesting that this mutant, which affects GGAP2-NF- κ B interaction, may have a dominant negative effect on NF- κ B activity in DU145 cells. These studies are consistent with a direct effect of GGAP2 on NF- κ B transcriptional activity.

Discussion

It is well-established that activation of the PI-3K/Akt pathway plays an important role in prostate cancer initiation and progression (1). After activation of this pathway, there is both positive and negative regulation of its activity. Loss of the negative regulator PTEN in prostate cancer is well-known. We now show that there is also significant up-regulation of the positive regulator GGAP2 in prostate cancer. GGAP2 can interact and activate Akt in prostate cancer cells. In addition, we show that Akt can phosphorylate GGAP2 at serine 629, which is essential for both Akt activation and the interaction of GGAP2 with the p50 subunit of NF- κ B. In the presence of S629 phosphorylated GGAP2, the transcriptional activity of NF- κ B is significantly increased, perhaps by direct interaction with the protein in the nucleus, or by enhancing the nuclear translocation of NF- κ B or both. Further mechanistic studies are needed to establish the exact mechanism by which GGAP2 promotes NF- κ B activity. Thus, GGAP2 is a novel regulator for two of the most critical regulatory pathways in prostate cancer progression and our studies indicate a new mode of cross talk between these two key signaling pathways.

Consistent with its ability to activate two critical pathways in prostate cancer, we have shown that GGAP2 can promote proliferation, focus formation, and tumor formation in prostate cancer cells. Further studies are needed to determine whether there is a correlation between GGAP2 expression and activation of the Akt and NF- κ B pathways in human cancer and clinical progression of disease.

Both the Akt and NF- κ B pathways can be activated by multiple genetic alterations in human prostate cancer. For example,

increased expression of cytokines and/or growth factors can activate both of these pathways. Increased expression of the SRC-3 coactivator (40) or loss of PTEN (1) also plays important roles in increasing Akt activity in prostate cancer. We have found that GGAP2 can also increase Akt activation in prostate cancer. It has been known for some time that activated Akt can increase NF- κ B activity in cells (6, 41), although the exact mechanism for such activation may be cell type specific (6, 41, 42). We have shown that GGAP2 can significantly enhance NF- κ B activity in prostate cancer. It has been shown that Akt may directly interact with IKK to enhance its activity (41). It remains to be determined if GGAP2 is enhancing this activity or has other roles in promoting NF- κ B transcriptional activity within prostate cancer cells. By activating NF- κ B pathway, GGAP2 further amplifies the pleiotrophic tumor promoting effects of Akt activation in prostate cancer.

GGAP2/PIKE-A may also play an important role in many other common malignancies. Using dot blot hybridization, Liu and colleagues (27) have shown that GGAP2 mRNA is overexpressed in many common epithelial cancers including breast, colon, ovary, kidney, bladder, and lung cancers. Based on its common over-expression in human cancers and its ability to activate two critical pathways, GGAP2/PIKE-A is an important potential target for cancer therapy. The fact that many drugs target G-protein coupled receptors and signaling pathways make the development of inhibitors of GGAP-2 feasible. Such inhibitors should have clinical efficacy in prostate cancer and many other common epithelial malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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