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TITLE: Role of GGAP/PIKE-A in prostate cancer progression

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Role of GGAP/Pike-A In Prostate Cancer Progression

The clinical behavior of prostate cancer 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. It was reported that GGAP2, or PIKE-A, can activate Akt and inhibit apoptosis. The goal of this proposal is to characterize the role of GGAP2 in prostate cancer progression.

Our results indicate that GGAP2 expression is increased in prostate cancer. In addition, GGAP2 is mutated in human prostate cancer. The multiplicity of GGAP2 mutations imply that these mutations may emerge in an environment of the multifocal heterogeneity of prostate cancer tissues. According to our results, these mutations can stimulate cancer cell proliferation and enhance GGAP2 activation, indicating that they may contribute to increased activity of GGAP2 in prostate cancer. In summary, GGAP2 may promote prostate cancer growth and progression via overexpression and/or mutation and as such is an important therapeutic target.

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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. GGAP2 is a GTPase protein with a GTPase activating protein (GAP) domain at the C-terminus (Xia et al., 2003). It has been reported that GGAP2, or PIKE-A, can activate Akt and inhibit apoptosis (Ahn et al., 2004). The goal of this proposal is characterize the role of GGAP2 in prostate cancer progression.

BODY

As outlined in our Statement of Work, we will seek to accomplish four major tasks in our three years of funding. We have accomplished or made substantial progress on all of these tasks.

Task 1: (Months 1-12) Immunohistochemical studies of GGAP2 in prostate cancer

We have carried out studies of GGAP2 expression in prostate cancer. To examine whether GGAP2 was overexpressed in prostate cancer we performed Western blots of 8 prostate cancers and 8 normal peripheral zone tissues, which revealed expression of GGAP2 in 3 of 8 cancers but in none of the benign tissues (see our recently published manuscript, Cai et al 2009, attached, and Fig 1A, below). The antibody used also detects PIKE-L since 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 90kDa GGAP2 protein was present in the Western blot. We have 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 prostate cancer tissues within the cancer epithelial cells. Staining was primarily cytoplasmic in those cases with staining. Nuclear staining was also identified in some cases (Fig 1B). Similar staining was observed in high grade prostatic intraepithelial neoplasia (Fig. 1B). Absent or weak staining was seen in the more than 95% of the normal epithelial tissues (Fig. 1B). Weak staining of stromal tissues was also noted. We then quantified cytoplasmic expression of GGAP2 using a visual quantitation as described previously (Agoulnik et al 2006). 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). Results are summarized in Figure 1C. The mean score for normal epithelium was 0.86 +/- 0.18 (SEM, n=72), the mean for PIN was 3.6 +/- 0.9 (n=65) and 5.8 +/- 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). Western blot of the cancer cell lines with anti-GGAP2 antibody reveals easily detectable protein expression in all six prostate cell lines tested with higher expression of GGAP2 in prostate cancer cell lines than in immortalized normal prostate epithelial (PNT1A) cells (Fig. 1D). Thus GGAP2 is expressed at increased levels in human prostate cancers.
We have now extended these studies to examine GGAP2 expression by IHC using a total of 567 prostate cancers using a large tissue microarray. We then compared expression of GGAP2 with expression of a large number of other markers previously examined using this array. We found 30 statistically significant correlations of these markers with cytoplasmic or nuclear GGAP2 using quantitation as described above followed by analysis using Spearman’s nonparametic
correlation analysis. Of note, we found a highly statistically significant correlation of phosphorylated AKT (r=0.27, p<0.0001) and nuclear NFKB (r=0.19, p<0.001) with cytoplasmic GGAP2, confirming our in vitro data that GGAP2 increases AKT and NFKB activity (see attached manuscript, Cai et al, 2009). Interestingly, we found evidence that GGAP2 may alter interactions with host microenvironment since GGAP2 was significantly associated both reactive stroma grade 3 and perineural invasion.

Task 2: (Months 1-18) Identification of GGAP2 mutants in prostate cancers and correlation with clinical and pathological parameters of outcome
To determine if GGAP2 is mutated in prostate cancer we initially focused on the GAP domain, since this region is an important negative regulator of GGAP2 activity. We initially analyzed cDNAs from 15 cancers and 9 benign prostate tissues from radical prostatectomy specimens. The GAP domain was amplified and individual clones were isolated and sequenced. Results are shown in Table 1. Twelve of fifteen cancer cases had at least one clone with a GGAP2 missense and/or stop mutation while only 2 of 9 benign cases had such mutation. The benign cases had only a single mutant clone each while up to 42 percent of clones in the cancer cases were mutated. Overall 38 of 206 clones from the cancer tissues were mutant versus 2 of 137 in benign. This difference was highly statistically significant (p<0.001, Chi square). To rule out an artifact due to reverse transcription or the possibility that mutant transcripts may be transcribed preferentially or have increased stability we directly analyzed the GAP domain in genomic DNAs from 46 cancers and 22 benign tissues. As shown in Table 1, 20 of 46 cancer tissues contained at least one mutant clone and in 12 of 46 cancer tissues more than 30% of clones contained missense or stop mutations. Only a single mutant clone was identified from the benign tissues. Overall, 52 of 334 clones from the cancer tissues were mutant versus 1 of 167 from the benign tissues (p<0.001). Combining cDNA and genomic analysis, 32 of 61 cancer cases contained clones with GAP domain mutations and in 14 cases 30% or more of the clones were mutant.

Surprisingly we found that the missense mutations were highly heterogeneous. There were no recurrent missense mutations and even in tissues with multiple mutant clones there was only one case with two identical mutant clones. There was variability in the distribution of the missense mutations with the regions between amino acids 640-660 and 700-710 having relatively more frequent mutations while mutations were uncommon from amino acids 540-570 but there was no statistically significant “hot spots”. In several clones we found 2 mutations in the same clone. This is similar to the observation of Hu et al (2005), who found multiple mutations in several mutant GGAP2 cDNAs isolated from sarcoma and glioblastoma cell lines. In addition to the multiple missense mutations, we observed 3 stop mutations, all at the carboxy terminal portion of the GAP domain (aa 703-709). Of note, Hu et al (2005) found a truncation at amino acid 756 in the GGAP2 cDNA from CRL-2098 osteosarcoma cells.

Given this surprising heterogeneity we considered the possibility that this may represent a PCR misincorporation artifact. However, we found only 3 silent mutations among 540 clones from cancer tissues (versus 90 missense or stop) while in the benign tissues we found 2 silent mutations among 304 clones (versus 3 missense mutations). The proportion of missense and stop versus silent mutation was much higher in the cancer tissue than in the benign tissue and the difference was statistically significant (p=0.02, Fisher exact). This is inconsistent with a random misincorporation. To further examine this point, we systematically determined the consequences
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**Table 1. Missense and stop mutations in the GAP and GTPase domain of GGAP2 in prostate cancer and benign prostate tissue**
of transition mutations on amino acid sequence for all nucleotides in the GAP domain. We only examined transitions since 82% of the observed mutations were transition mutations (data not shown). Systematic transition mutation of each nucleotide in the GAP domain would yield 273 missense, 15 stop mutations and 162 silent mutations. The difference in the proportions of missense and stop versus silent mutations we observed (90 and 3) compared the predicted distribution (288 and 167) was highly statistically significant (p<0.001, Chi sq). Finally, we considered the possibility that the cancer tissues had an increase rate of mutation targeting the first and second bases of each codon resulting in random missense mutation in all genes. We therefore examined 5 cancer and 5 benign tissues for mutations in β-actin. We found no mutations in 32 clones from cancer tissue and 36 clones from benign tissues. The proportion of missense and stop clones in GGAP2 was statistically significantly higher than in β-actin (p=0.02, chi sq). Thus the observed heterogeneous mutations in the GAP domain of GGAP2 are indeed genuine.

The GTPase domain is also a key regulator of GGAP2 activity. We therefore examined cDNAs from 23 cancer and 12 benign tissues for GTPase domain mutations. The results were very similar to those observed with GAP domain. Fifteen of 23 cancers contained missense mutations versus 1 of 12 benign tissues. In four cancer cases had 40% or more of clones were mutant, while only a single mutant clone was observed in the benign tissue. Overall, 28 of 188 clones from the cancer tissues were mutated versus 1 of 88 in benign tissue (p<0.001, chi sq). The overall pattern of mutations in the cancer tissue was similar to the GAP domain in that mutations were highly heterogeneous, both within a single tissue and between tissues. We found one double mutant clone, similar to the GAP domain. Of note, no stop mutations were observed. Given the amino terminal location of the GTPase domain, any stop mutations would almost certainly yield inactive protein since it would lack the PH domain. We found only 2 silent mutations, one in a cancer and one from benign tissue. We did detect 9 tissues with multiple clones containing the described polymorphism at L246, which occurs in approximately 28% of populations previously studied, which is similar to our finding.

We then examined the correlation of missense and stop mutations with clinical and pathological parameters of aggressive disease. We compared the proportion of clones with missense or stop mutations in cases with early PSA recurrence (<2 years after radical prostatectomy, which is associated with death from disease) versus cases with late or no PSA recurrence. The proportion of mutant clones was significantly higher in cases with early PSA recurrence (P<0.001, Chi square). Similar analysis showed a significant increase in the proportion of mutant clones in cases with either pelvic lymph node metastasis (p=0.027) and seminal vesicle invasion (p=0.07) compared to cases without these adverse pathological parameters. Thus, mutant is GGAP2 is strongly associated with aggressive disease.

Our finding of common but heterogeneous missense mutations in GGAP2 is highly novel and may indicate that there is unexpected complexity in the mutational spectrum of prostate cancer.

Task 3. (Months 6-30) Biochemical and biological characterization of GGAP2 mutants

To evaluate the impact of the observed mutations in the GTPase domain on the biochemical properties of GGAP2 we cloned wild type and GTPase mutant GGAP2 into a GST fusion vector. Partially purified fusion protein was then loaded with 3H-GDP, incubated with excess unlabelled GTP and the residual bound 3H-GDP determined at 2, 5 and 15 minutes. As can be seen in Fig 2,
all of the mutants showed increased rates of GDP loss compared to wild type and at 15 min all mutant GGAP2 proteins retained significantly less labeled GDP (all <p=0.04, t-test). These findings indicate that the mutants all have enhanced G-protein activity.

Figure 2. GDP disassociation from wild-type and mutant GGAP2 proteins.

To evaluate the biological effect of the GAP and GTPase domain mutants, we cloned wild type and mutant GGAP2 proteins into mammalian expression vectors and selected stable cell lines expressing the wild type and mutant GGAP2 proteins in immortalized normal prostate epithelial cells (PNT1A) or DU145 prostate cancer cells. We then compared the proliferation rate of these cell lines to vector controls. As can be seen in Fig 3, wild type GGAP2 enhanced proliferation over vector controls, consistent with our prior data. Four of the five mutant GGAP2 proteins further enhanced proliferation (p <0.05, t-test). The L624P mutant enhanced proliferation in both cell lines, but this was not statistically significant (both p=0.08). Thus the missense mutants show biochemical and biological properties consistent with promotion of tumor progression relative to wild type GGAP2.
Figure 3. Proliferation of immortalized prostate epithelial cells (PNT1A) and DU145 prostate cancer cells containing wild-type (WT) or mutant GGAP2 or vector only. Cell number at day 6 is shown. Statistically significant differences between WT and mutant GGAP2 are indicated (*).

Task 4: Months (24-36) Study the effect of GGAP2 on progression and metastasis in orthotopic models of prostate cancer

We have generated PC3 and LNCAP cell lines stably expressing control vector, wild-type GGAP2, S629A (Akt phosphorylation site) mutant GGAP2 and mutant GGAP2 identified from prostate cancer tissue samples. These cells are now ready for orthotopic injections and mice are available. These experiments should be completed before fall 2009.

KEY RESEARCH ACCOMPLISHMENTS
- We have established that GGAP2 is expressed at increased levels in human prostate cancer. In addition we have established that GGAP2 is directly phosphorylated by Akt and when phosphorylated activates NKB signaling. These results have been reported in our recently published manuscript in Cancer Research (Cai et al, 2009, attached)
- We have shown that GGAP2 mutations are common in human prostate cancer and that they can promote biological processes that are associated with cancer progression. Such mutations are associated with clinical and pathological parameters of aggressive disease.
This implies intratumoral heterogeneity of GGAP2 mutation. This finding has important general implications for the study of mutations in prostate cancer. We are currently preparing a manuscript for submission to Cancer Cell, which will submit in the summer of 2009.

- We have established key reagents and a baseline protocol for future studies of the role of GGAP2 in prostate cancer progression in vivo

REPORTABLE OUTCOMES

- Published manuscript in Cancer Research: Cai et al 2009
- GGAP2 is mutated at high rates in human prostate cancer. We plan to submit these finding for publication in the summer of 2009

CONCLUSION

GGAP2 expression is increased in prostate cancer. Our results indicate that GGAP2 is also mutated in human prostate cancer. The multiplicity of GGAP2 mutations imply that these mutations may emerge in an environment of the multifocal heterogeneity of prostate cancer 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 prostate cancer. In summary, GGAP2 may promote prostate cancer growth and progression via overexpression and/or mutation and as such is an important therapeutic target.

REFERENCES


GGAP2/PIKE-A Directly Activates Both the Akt and Nuclear Factor-κB 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-κB (NF-κB) pathways have been identified as critical steps in cancer initiation and progression in a variety of human cancers. We have found significantly increased expression of 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-κB and enhances NF-κB 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-κB (NF-κB) pathway also plays a critical role in prostate cancer progression (6–12). NF-κB 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 IκBα. After stimulation of cells by cytokines and/or growth factors, IκBα is phosphorylated by the IKK complex, leading to degradation of IκBα by the 26S proteasome. This allows translocation of the NF-κB 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-κB 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- (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 Sigmastat software. Asterisks were used to identify groups with statistically significant differences 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 GGAP2, p50, p65, RelB, and RelC expression plasmids were from Cell Signaling Technology. Rabbit polyclonal anti-rabbit IgG antibody (H+L) was from Abcam. Rabbit anti–phospho-(Ser/Thr) Akt antibody was from Cell Signaling Technology. GGAP2 (PIKE) was from Prosci, Inc. Rabbit anti–phospho-(Ser/Thr) Akt antibody was from Cell Signaling Technology. Rabbit polyclonal anti-Akt1 was from Abgent. Anti-RelB, anti-RelC, anti-Myc, anti-HA, and anti–E-selectin antibodies were obtained from Santa Cruz Biotechnology, Inc. 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 GGAP2, 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 1:1000 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. Slides were visualized and photographed using standard light microscopy and analyzed manually. Staining was evaluated on 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 protease 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. Dpn1 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 Akt cDNA in pU6/Eaamp vector was purchased from Upstate Biotechnology. The cDNA sequence was then cloned using PCR, digested with EcoRI and BamH1, and ligated into a lentiviral vector pCDH1-MSC1-EF1-Puro and sequenced. Primers used were as follows: Forward, 5′-GCCGATTCATGGGGAGCGAGCAG-CGAAG-3′; Reverse, 5′-GCCGATTCCTAAATGGTGATGGTGATG-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 cell lines 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 RPMI 1640 supplemented with corresponding antibiotics. Cells were then plated in 6-well plates and incubated at 37°C for 14 d before the foci were stained with 0.005% Crystal Violet and counted.

RNA interference. To examine the effect of GGAP2 shRNA expression, we designed the primers as follows:

Top 5′-CACCAGCTAAACGGGCTCGTCAATTGCAAAATTGAC-GAGCCCGTATAAGC-3′
Bot 5′-AAAAGCATTAACGGGCTCGTCAATTTCGAATTGAC-GAGCCCGTAAATGC-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 Blastidin 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 groups with 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 × 106 PC3 Cells in PBS were mixed with 50 μL Matrigel Basement Membrane Matrix (BD Biosciences) and s.c. implanted into each SCID mouse using no anesthesia in the lumber 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
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 led to growth that was even lower than the control levels (Fig. 2A). Experiments using immortalized normal prostate 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
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 (THLSRVRSLDLDDWP) using simulated motif scan.

Flag-tagged GGAP2 was transiently transfected and immunoprecipitated (with anti-Flag Ab) followed by Western blotting with antibody to Akt consensus phospho-serine. This study showed phosphorylation of GGAP2 by Akt that is abolished by mutation of serine 629 to alanine (Fig. 4B), 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-κB. To investigate whether there is a direct connection between GGAP2 and NF-κB, we overexpressed Flag-GGAP2 and subunits of NF-κB into 293T cells and performed reciprocal IP and Western blot using specific antibodies against Flag-GGAP2 and NF-κB 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
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 K83 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-κB.** NF-κB is a transcription factor and the promoter regions of many genes contain NF-κB binding sites. To determine the effect of Akt and GGAP2 on the transcriptional activation of NF-κB, we used three luciferase-reporter vectors, which contain NF-κB 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-κB 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-κB 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-κB (39). Our data suggest that the combination of Akt and GGAP2 may regulate E-selectin expression in prostate cancer cells through regulating NF-κB. 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.
Although we have shown that GGAP2 can interact directly with NF-κB, it is possible that NF-κB 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-κB 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-κB interaction, may have a dominant negative effect on NF-κB activity in DU145 cells. These studies are consistent with a direct effect of GGAP2 on NF-κB 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-κB. In the presence of S629 phosphorylated GGAP2, the transcriptional activity of NK-κB is significantly increased, perhaps by direct interaction with the protein in the nucleus, or by enhancing the nuclear translocation of NF-κB or both. Further mechanistic studies are needed to establish the exact mechanism by which GGAP2 promotes NF-κB 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-κB pathways in human cancer and clinical progression of disease.

Both the Akt and NF-κB 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-κB 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-κB 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-κB transcriptional activity within prostate cancer cells. By activating NF-κB 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.

Acknowledgments

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