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GGAP2/PIKE-A directly activates both the Akt and NF- κ B pathways and promotes prostate cancer progression

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Abstract

GGAP2/PIKE-A is a GTP-binding protein which can enhance Akt activity. Increased activation of the AKT and 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.

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. While 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 (1)). Following 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 (PI-3,4,5-P₃) or phosphatidylinositol 3, 4-bisphosphate (PI-3,4-P₂), 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

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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 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, IL-6, IL-8, VEGF, MMP9, μ PA and μ PAR, 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 following 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 (3,4,5) 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 N-terminal proline-rich domains, a Ras homology domain (G domain), and a pleckstrin homology domain. PIKE-L is an alternatively spliced isoform which also contains a C-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 N-terminal proline-rich domains due to an alternative promoter. This protein was originally characterized in 2003 by Xia et al as GGAP2 (23). 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) demonstrated 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 while 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 et al (27) showed the increased expression of GGAP2/PIKE-A mRNA in a wide variety of human malignancies, including 2 of 4 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.

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, Burlingame, CA). Briefly, array slides were pre-incubated with normal goat serum and blocking avidin for 20 minutes then rinsed in PBS. Epitope retrieval was performed by incubating the slides in a pressure cooker for 30 min in 0.01 M 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 hour. Solutions A and B (ABC-Elite) were added onto the slides for 30 minutes. 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), 1-33% of cells stained (1+), 34-66% of cells stained (2+) or 67-100% 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, 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 1ml RIPA buffer supplemented with proteinase inhibitor cocktail containing 1 mM 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 hour 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 mM Tris-HCl (pH 7.0), 150 mM NaCl, 5 mM MgCl₂, and 0.1% Triton X-100, supplemented with proteinase inhibitor cocktail containing 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml aprotinin) and incubated with 100ul of GTP-agarose beads (Sigma-Aldrich) for 4 hours at 4°C. Agarose beads were then centrifuged and washed 3 times 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: Forward: 5'-GCGAATTCATGGGGAGCAGCAAGA-3'; Reverse: 5'-GCGGATCCTCAATGGTGATGGTGAT-3'. DU145 cells were transfected with the myristylated Akt1-pCDH1 plasmid and selected with puromycin for stable expression.

In vitro proliferation assays

50,000 cells of each stable cell type were plated in 10-cm dishes. Cells were incubated in triplicate at 37°C with RPMI 1640 media 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 et al (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 days. 2µg 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

5000 LNCaP cells stably transfected with various plasmids were mixed with the 0.7% agarose (top agar) and warm 2XRPMI 1640 + 20% FBS 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 days before the foci were stained with 0.005% Crystal Violet and counted.

RNA interference (shRNA)

For stable GGAP2 shRNA expression, we designed the primers as follows: Top 5'-CACCGCATTAACGGGCTCGTCAATTTCGAAAATTGACGAGCCCGTTAATGC-3' Bot 5'-AAAAGCATTAACGGGCTCGTCAATTTTCGAATTGACGAGCCCGTTAATGC-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.

Subcutaneous PC3 injection

A total of 15 SCID mice were separated into 3 experimental groups (5 mice/group) and were subcutaneously injected with PC3 cells expressing control vector, wild-type and S629A plasmids, respectively. 50 μ l cell suspensions containing 1×10^6 PC3 Cells in PBS were mixed with 50 μ l Matrigel Basement Membrane Matrix (BD Biosciences) and subcutaneously implanted to each SCID mice using no anaesthesia in the lumbar postero-lateral woulds bilaterally as a duplicate. Mice were sacrificed and tumors were collected and weighed 4 weeks after the inoculation. Tumor size was measured using a caliber. Animals were euthanized according to tumor size and by cervical dislocation. Tumors were harvested and fixed in 10% formalin for histological analysis.

RESULTS

Expression of GGAP2 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 detectable expression of GGAP2 in 3 of 8 cancers but in none of the benign tissues (Fig. 1A). 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 (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, panel one) in those cases with staining. Nuclear staining was also identified in some cases (Fig. 1B, panel two). Similar staining was observed in high grade prostatic intraepithelial neoplasia (Fig. 1B panel 3). Absent or weak staining was seen in more than 95% of the normal epithelial tissues (Fig. 1B, panel 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 (SEM, $n=72$), the mean for PIN was 3.6 ± 0.9 ($n=65$), while 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 downregulates GTPase activity. Compared to vector transfected cells, wild-type GGAP2 promotes prostate cancer cell proliferation, while 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 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 while 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 subcutaneous 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 5 of 5 mice injected with PC3 WT 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. Knock-down of GGAP2 with stable expression of siGGAP2 decreased the tumor size compared to the vector control (Fig. 3A -3C). 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 (Fig. 2 and Fig. 3), we then seek 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-immunoprecipitation (IP) experiments with HA-tagged Akt and Flag-tagged GGAP2 following transient transfection confirm that Akt and GGAP2 physically interact in immortalized prostate epithelial cells and prostate cancer cell lines (Fig. 4A). We then analyzed 4 prostate cancer cell lines for interaction of endogenous Akt and GGAP2 by immunoprecipitation with anti-Akt antibody followed by western blot with anti-GGAP2 antibody. LNCaP showed a detectable band while the other cell lines were negative (Supplemental Figure 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 (THLSRVRS¹LDLDDWP) using simulated motif scan¹. 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.

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 (Supp Fig S1B, upper panel). To further examine the endogenous protein interaction, we performed similar experiments and found that endogenous GGAP2 was in the same immunoprecipitation complex with p50 (Supp Fig 1B, lower panel) 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, lower panel, 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 5D, the N-terminal GTPase domain of GGAP2 interacts with the RHD domain of p50. Of note, we have not been able to demonstrate interaction with the p105 precursor of p50 (data not shown).

Activation or inactivation of GTPases is determined by the protein's guanine nucleotide binding status, which is controlled by guanine exchange factors (GEFs), GTPase activating proteins (GAPs) 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 N-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.

¹(<http://scansite.mit.edu>).

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 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 (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 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 (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 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.

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 upregulation of the positive regulator GGAP2 in prostate cancer. GGAP2 can interact and activate Akt in prostate cancer cells. In addition, we demonstrate 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 et al (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 overexpression 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. GGAP2 expression is increased in human prostate cancer

(A) GGAP2 in patient prostate cancer tissue lysates. 1.5 mg of total protein was incubated with anti-GGAP2 rabbit antibody and protein A agarose beads for 4 hours before the immunoprecipitates were immunoblotted with the same antibody. Molecular weight marker sizes (in kDa) are shown. The arrow refers to the 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. Panel 1: cancer tissue with strong cytoplasmic staining; Panel 2: nuclear staining in prostate cancer tissues; Panel 3: high grade prostatic intraepithelial neoplasia with moderate cytoplasmic staining; Panel 4: normal prostate tissue showing very weak epithelial staining; (C) Boxplot of staining indices of benign, high

grade prostatic intraepithelial neoplasia (HGPIN), and cancer tissues. (D) GGAP2 expression in prostate cancer cell lines. 20 μ g 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. 50,000 cells were plated in triplicate and collected for counting at day 1, 2, 3 and 5. (B)-(C) GGAP2 enhanced colony formation in PC3 cells. (D) Stable LNCaP cell lines as expressing WT or mutant GGAP2 or vector controls used for soft agar assays. Foci number of each group was compared to 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 <0.05 compared to the vector control group at the same timepoint.

Figure 3. GGAP2 promotes cancer cell growth *in vivo*

(A-C) GGAP2 promotes cancer growth *in vivo* using PC3 cell subcutaneous 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. The results were shown as average $\pm$ SD. ANOVA was used to examine the significance of the data using Sigmastat software. Asterisks were used to identify groups with statistically significant changes with a p value <0.05 compared to the vector control group.

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.

Figure 5. GGAP2 interacts with p50 subunit of NF-κB 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. In the middle panel, immunoprecipitations were pulled down with anti-Flag antibody and proteins were separated using SDS-PAGE and transferred to a PVDF 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 represent the amino acid sequence number. FL refers to full length protein, NT to N-terminal fragment, RHD to RHD domain and CT to C-terminal fragment. (C-D) The N-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.

Figure 6. Akt-GGAP2 activates transcriptional activities of NF- κ B

(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- κ B 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- 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 E-selectin protein expression level is increased in LNCaP cells transiently expressing GGAP2 and Akt. (C-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 Sigmastat software. Asterisks were used to identify groups with statistically significant changes with a p value <0.05 compared to the vector control group.