Aberrant Activation of Androgen Receptor in a New Neuropeptide-Autocrine Model of Androgen-Insensitive Prostate Cancer

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Abstract

Treatment of advanced prostate cancer with androgen deprivation therapy inevitably renders the tumors castration-resistant and incurable. Under these conditions, neuroendocrine differentiation of prostate cancer (CaP) cells is often detected and neuropeptides released by these cells may facilitate the development of androgen independence. Exemplified by gastrin-releasing peptide (GRP), these neuropeptides transmit their signals through G protein–coupled receptors, which are often overexpressed in prostate cancer, and aberrantly activate androgen receptor (AR) in the absence of androgen. We developed an autocrine neuropeptide model by overexpressing GRP in LNCaP cells and the resultant cell line, LNCaP-GRP, exhibited androgen-independent growth with enhanced motility in vitro. When orthotopically implanted in castrated nude mice, LNCaP-GRP produced aggressive tumors, which express GRP, prostate-specific antigen, and nuclear-localized AR. Chromatin immunoprecipitation studies of LNCaP-GRP clones suggest that GRP activates and recruits AR to the cognate promoter in the absence of androgen. A Src family kinase (SFK) inhibitor, AZD0530, inhibits androgen-independent growth and migration of the GRP-expressing cell lines, and blocks the nuclear translocation of AR, indicating the involvement of SFK in the aberrant activation of AR and demonstrating the potential use of SFK inhibitor in the treatment of castration-resistant CaP. In vivo studies have shown that AZD0530 profoundly inhibits tumor metastasis in severe combined immunodeficient mice implanted with GRP-autocrine LNCaP cells. This xenograft model shows autocrine, neuropeptide- and Src kinase–mediated progression of androgen-independent CaP postcastration, and is potentially useful for testing novel therapeutic agents. [Cancer Res 2009;69(1):151–60]

Introduction

Prostate cancer is the most frequently diagnosed cancer in American men and is the second leading cause of cancer deaths (1). Androgen withdrawal initially induces apoptosis and cell cycle arrest in prostate cancer (CaP); however, CaP eventually loses its dependence on androgens and progresses to an androgen-independent state. Various mechanisms have been postulated to account for the conversion of CaP into a castration-resistant state, including the aberrant activation of androgen receptor (AR) by peptide growth factors and ligands for G protein–coupled receptors (GPCR; refs. 2–4). If true, these mediators and components of their signal pathways are potential targets for therapeutic intervention of castration-resistant CaP. It has been reported that androgen withdrawal from androgen-dependent CaP cells (5) or treatment with stimuli such as interleukin 6 and forskolin in vitro promotes the acquisition of the neuroendocrine phenotype through transdifferentiation (6). Cumulative evidence suggests that neuroendocrine differentiation of CaP may be a cofactor involved in tumor progression and androgen independence (7).

Neuroendocrine cells are identified by their neurosecretory granules and expression of neuron-specific markers including chromogranin A, neuron-specific enolase, and mitogenic neuropeptides such as bombesin/gastrin-releasing peptide (GRP), somatostatin, calcitonin, and parathyroid hormone-related peptides (7). Neuropeptides have been identified as potent paracrine and autocrine growth factors in human cancers including lung, gastrointestinal, pancreatic, brain, and prostate cancers (8–13). In prostate cancer, previous studies by others and by us have shown that neuropeptides promote cell growth (14), migration and protease expression in PC-3 cells (15), and androgen-independence in LNCaP cells (3, 5). Androgen independence in CaP patients is shown to correlate well with elevated serum levels of chromogranin A (16). Elevated expression of GRP receptors are often detected in CaP specimens (17, 18).

The bombesin/GRP family is among the most studied neuroendocrine growth factors in CaP. Bombesin/GRP transduces signals by engaging heterotrimeric GPCRs located on the cell surface (19). Upon binding to its receptors, bombesin/GRP elicits calcium mobilization in PC-3 and DU 145 cells (20, 21), and promotes growth and cell invasiveness via the proteolytic activities of matrix metalloproteinases in LNCaP and PC-3 cells (15). We have previously shown that exogenous bombesin/GRP activates AR and supports androgen-independent growth in LNCaP through signaling mediated by non–receptor tyrosine kinases such as Src, FAK, and Etk (3). In vivo androgen withdrawal following the establishment of LNCaP tumors results in an increased number of neuroendocrine cells (5). Together, these data suggest that castration-induced neuroendocrine differentiation may release soluble factors which sustain the growth and survival of androgen-deprived cells, contributing to tumor androgen-independence and metastasis.

In this article, we describe a neuropeptide xenograft model and use it to test the inhibition of the tyrosine kinase pathway implicated in the development of androgen-independence. We introduced the GRP-expressing vector into LNCaP cells to establish an autocrine neuroendocrine model. The GRP clones exhibited enhanced proliferation and migration properties under
androgen-depleted conditions, and developed significant tumors in castrated nude mice, providing evidence for GRP’s role in androgen-independent growth through modulation of AR. We tested the effect of a Src family kinase (SKF) inhibitor AZD0530 on re-cultured xenograft cells both in vitro and in vivo. Our results showed that AZD0530 effectively blocked the androgen-independent growth and migration of LNCaP cells mediated by autocrine GRP, through inhibiting activation of the Src/FAK/Etk complex. Severe combined immunodeficient (SCID) mice implanted with GRP-autocrine LNCaP cells and treated with AZD0530 showed a complete inhibition of tumor metastasis.

Materials and Methods

Cell culture. LNCaP cells (American Type Culture Collection, passages 38–43) were kept in RPMI 1640 with 10% regular fetal bovine serum. When stimulated, cells were switched to phenol red–free RPMI 1640 with 5% charcoal-stripped (CS) androgen-free serum.

Proliferation assays. Cells were grown in CS medium alone or supplemented with 100 nmol/L of bombesin, 1 μg/mL of bombesin/GRP-specific monoclonal antibody 2A11 (22), 5 μmol/L of GRP receptor antagonist RC3095 (23), or transfection of 100 μmol/L of small inhibitory RNA [siRNA, sense sequence GGAGAGCAUCAACACGUCAU, validated by reverse transcription-PCR (RT-PCR) for inhibition; Dharmacon] for GRP receptor. Cells were trypsinized and counted by trypan blue exclusion method after 48 h or over 6 days for siRNA transfection.

Chemotaxis migration assay. Migration assays were performed in a Boyden chamber with 8 μm Nuclepore membrane coated with human plasma fibronectin (50 μg/mL). LNCaP cells (2 × 10^6) were placed in the upper wells with testing agents in the lower wells, and incubated at 37°C for 4 h to allow cell migration. At the end of incubation, the membrane was stained by Diff-Quik Stain Kit and mounted on microscopic slides for counting. Each experiment was performed in triplicate. AZD0530 (500 nmol/L) or siSRC transfection was used for inhibitor studies.

GRP-expressing construct and transfection. GRP cDNA was amplified from the small cell lung carcinoma DMS53 cell line (American Type Culture Collection), which expresses GRP. The amplified cDNA was inserted into mammalian expression vector pcDNA3.1-Zeocin (Invitrogen). LNCaP cells were transfected with this GRP construct or the empty vector and stable transfectants were selected with Zeocin (100 μg/mL). LNCaP cells (2 × 10^6) were placed in the lower chamber of a Boyden chamber with 8 μm pore size, complexed with Lipofectamine 2000 (Invitrogen) and delivered to cells grown in CS medium at a final concentration of 100 nmol/L.

Chromatin immunoprecipitation. LNCaP-Zeo, GRP, and GRP-Pro cells grown to subconfluence were switched to CS medium for 3 days. Treatment with R1881 was performed 6 h before harvesting. Chromatin immunoprecipitation (ChIP) was performed as described (4, 25, 26) with 6 μg of anti-AR antibody (PG-21; Millipore). Standard PCR cycling protocol was performed with 58°C for annealing for 30 cycles. Primers for AR enhancer region were 5′ctcgatcattagtcacagtct5′ and 5′tcegctgctttctcttgac3′ and for the proximal ARE region, 5′tctcggctctcttcgac3′ and 5′aacccatcataaacttcttct5′; and for the intervening region, 5′ctcgatcattagtcacagtct5′ and 5′gcgagagaa-tagatgggaggac3′.

Immunofluorescent staining of AR. Cells (2,500) were plated in four-well chamber slides in CS medium a day before fixing with 2% paraformaldehyde for staining. Anti-AR (N-20; Santa Cruz) and anti-rabbit AlexaFluor 647 (Invitrogen) were used as the primary and secondary antibodies for bodies staining, respectively. Immunofluorescent cells were visualized using an Olympus BX61 motorized reflected fluorescence microscope system with an AMCA filter for 4′,6-diamidino-2-phenylindole and a Cy5 filter for AlexaFluor647 using the SlideBook4.1 software (Intelligent Imaging Innovations).

Immunoprecipitation and Western blot. LNCaP-Zeo, GRP, and GRP-Pro cells were subjected to androgen withdrawal for 3 days with or without exposure to AZD0530 (1 μmol/L). Cell lysates were collected in immunoprecipitation buffers containing proteinase and phosphatase inhibitors, incubated with anti-FAK, and subsequently, protein G agarose beads for immunoprecipitation. Phosphorylation of the respective protein was detected by anti-phospho family (Cell Signaling), p-FAK (Invitrogen), and p-Etk (Cell Signaling) antibodies after Western blotting analysis. Signals were detected by enhanced chemiluminescence system (Amersham) followed by exposure to X-ray film.

Statistics. All in vitro data were from at least three independent experiments and subjected to paired t tests using Statview program.

Results

It has been shown that bombesin confers androgen-independent growth of LNCaP cells (3). We validated that bombesin signals through the GRP receptor with specific inhibitors such as bombesin/GRP-specific monoclonal antibody 2A11 and GRP receptor antagonist RC3940-II. Bombesin also stimulated cell migration as seen by RT-PCR (Fig. 1B for inhibition; Dharmacon). For inhibitor studies, 14 castrated SCID mice were orthotopically implanted with 4 × 10^6 re-cultured GRP-Pro cells. SCID mice were used to better study tumor metastasis. Two weeks after surgery, mice were divided into two groups, seven were treated with 50 mg/kg/d via esophageal gavage (AZD0530-treated) and seven with buffer only (control). The study was terminated when one of the control mice succumbed to tumor burden. All the mice were euthanized, their primary tumors excised for weighing and immunohistochemical staining with p-Src (Cell Signaling), p-FAK (ABR), or AR antibodies and lymph nodes examined for metastasis.

Transient transfection assays. Zeo, GRP4-9, and GRP Pro cells were seeded in 24-well plates, transfected with 0.2 μg of PSA-Luc (promoter region, 630 bp) with the internal control pTK-RL using Effectene (Qiagen). Transactivation was examined by the dual-luciferase assay (Promega). For RNA interference, standard siCONTROL (D-001210-02; SC) and on-target plus SMART pool human Src (L-003175-00, SiSrc; Dharmacron) were complexed with Lipofectamine 2000 (Invitrogen) and delivered to cells grown in CS medium at a final concentration of 100 nmol/L.

For inhibitor studies, 14 castrated SCID mice were orthotopically implanted with 4 × 10^6 re-cultured GRP-Pro cells. SCID mice were used to better study tumor metastasis. Two weeks after surgery, mice were divided into two groups, seven were treated with 50 mg/kg/d via esophageal gavage (AZD0530-treated) and seven with buffer only (control). The study was terminated when one of the control mice succumbed to tumor burden. All the mice were euthanized, their primary tumors excised for weighing and immunohistochemical staining with p-Src (Cell Signaling), p-FAK (ABR), or AR antibodies and lymph nodes examined for metastasis.
enzyme immunoassay confirmed GRP expression in the two GRP clones (Fig. 1B). GRP1-1 and GRP4-9 cells produce almost 5-fold more GRP than the control lines, but comparable to DMS53 cells. Antibody 2A11, GRP receptor antagonist RC3905, and siRNA for the GRP receptor effectively inhibited the androgen-independent growth of GRP1-1 and GRP4-9 to 20% to 60% of the control (Fig. 1C). Negative control using siRNA targeting green fluorescence protein showed no effect on growth (data not shown). These data support the notion that GRP/bombesin is able to confer androgen-independent growth of LNCaP through binding to its membrane receptor. If the androgen-independent growth is due to the autocrine release of GRP into the medium, we would expect a chemotactic effect from GRP CM. As expected, LNCaP-Zeo migration was stimulated by bombesin (Fig. 1D). GRP CM stimulated LNCaP-Zeo migration by more than 3-fold and this migration was significantly reduced by 2A11 (P ≤ 0.001), suggesting GRP’s involvement. Migration of GRP1-1 and GRP4-9 towards Ctl CM was 2-fold greater than that of LNCaP-Zeo, and could be further stimulated by GRP CM, and significantly inhibited by 2A11 (P ≤ 0.001). These data showed that LNCaP-GRP cells release GRP, which confers androgen-independent growth and migration through the autocrine loop.

**GRP promotes in vitro and in vivo tumorigenesis in androgen-free environments.** Soft agar assay was performed to assess in vitro tumorigenicity. GRP1-1 and GRP4-9 produced significantly more colonies than LNCaP-Zeo in CS medium, suggesting that the autocrine GRP induces both androgen- and anchorage-independent growth (Fig. 2A). 2A11 significantly inhibited colony formation of both GRP1-1 and GRP4-9 (P ≤ 0.05 and P ≤ 0.0005). We then used the GRP clones for in vivo tumor study. Orthotopic prostatic implantation of GRP4-9 cells into prostates of castrated nude mice resulted in tumor growth in 8 of 12 mice. In contrast, 0 of 20 castrated mice implanted with LNCaP-Zeo cells displayed any tumor growth. To generalize this finding, GRP1-1 was also orthotopically implanted and four of five mice produced tumors. H&E staining of the tumors showed characteristic human CaP tumors adjacent to normal mouse prostate tissue (Fig. 2B). Immunohistochemical staining (Fig. 2C) showed that GRP was evident throughout the cytoplasm of the tumor regions,
yet was minimally detected in the normal mouse prostate epithelium of the tumor. Staining with anti-AR antibody showed its nuclear translocalization in tumor cells, indicative of GRP ligand activation. PSA expression was extensive in the tumor specimens, again supporting GRP-mediated AR activation. Mean serum PSA levels in castrated LNCaP-GRP tumor mice was $208.9 \pm 24.6$ ng/mL serum, as compared with $6.13 \times 10^{-5}$ ng/mL in castrated LNCaP-Zeo mice.

Tumors harvested from GRP-implanted mice were re-cultured in vitro to establish a xenograft cell line, labeled GRP-Pro. Expression of PSA, AR, and GRP in GRP-Pro cells was analyzed by RT-PCR analysis for the authenticity of the clones (Supplementary Fig. S2). Equal endogenous levels of PSA mRNA for all clones was also verified. Soft agar assay using GRP-Pro cells showed their aggressive nature as manifested by their androgen- and anchorage-independent growth in 2 weeks (Fig. 3A). This growth was partially inhibited by 2A11 and the androgen inhibitor, bicalutamide, individually or in combination (with significant difference $P \leq 0.05$), suggesting that growth is dependent on both GRP and AR.

**GRP modulates the activation of AR.** We further sought to illustrate GRP-mediated AR activation at the molecular level. Transactivation assay was performed with LNCaP-Zeo, GRP4-9, and GRP-Pro cells in CS medium using promoter PSA-Luc as the reporter. The expression of PSA-Luc in GRP4-9 and GRP-Pro was 1.8-fold and 4.5-fold higher than in LNCaP-Zeo cells (Fig. 3B). This suggests that GRP secreted from GRP cells is driving the expression. The addition of synthetic androgen R1881 induced PSA-Luc expression in LNCaP-Zeo cells more than 6-fold, but much less in GRP4-9 and GRP-Pro cells, probably because the GRP-activated AR, through posttranslational modification, already adopted an active conformation and may not be further stimulated by R1881. If GRP activates AR in GRP-Pro cells, AR should be recruited to ARE sites in the PSA promoter. We therefore performed the ChIP assay on LNCaP-Zeo, GRP4-9, and GRP-Pro cells in CS or CS+R1881 conditions. AR binding was analyzed by PCR using respective primers against enhancer (E) and proximal (P) ARE regions, and an intervening (I) region void of any ARE sites. Figure 3C shows that AR binds to PSA P region in GRP4-9 and GRP-Pro even in the absence of androgen. When treated with R1881, AR
binds preferentially to the E site in LNCaP-Zeo; whereas in GRP4-9 and GRP-Pro, AR binding was detected evenly at both P and E sites. Src and FAK tyrosine kinases play important roles in GRP-mediated androgen-independent growth and migration. Exogenous bombesin induces AR nuclear translocation, and this induction is inhibited by Src inhibitor PP2 (25). In our LNCaP GRP mouse model, AR is localized to the nuclei as shown in the tumor immunohistochemistry staining (Fig. 2C). We further compared the GRP cells with mock controls by immunofluorescent staining to confirm AR nuclear localization in GRP cells through autocrine GRP-mediated activation (Fig. 4). Staining of AR is limited to the cytoplasm in Zeo cells grown in CS medium but was concentrated to the nuclei of GRP cells (counted 65% nuclei with AR). This localization was inhibited by AZD0530, a selective SFK inhibitor demonstrating significant effects on prostate cancer cells (27). Almost half of the GRP cells (35% nuclei with AR remaining) lost nuclear staining of AR when Src activity was inhibited. These data confirm that GRP activates AR through Src and promotes its nuclear translocation, consistent with recent data that Src directly phosphorylates AR at Y534, resulting in nuclear translocation (28).

Among all the tyrosine kinases expressed in LNCaP cells, we previously showed that Src and FAK are most prominently activated by bombesin (3). Activated Src and FAK engage Etk, a tyrosine kinase shown to be involved in prostate carcinogenesis (3, 29). Src and FAK form a complex through binding between the phosphorylated Y397 in FAK and the SH2 domain in Src (30), whereas FAK associates with Etk via the FERM domain of FAK and the PH domain of Etk (31). These three kinases cross-activate one another with increased tyrosine phosphorylation of the complex. Using AZD0530, we examined whether the androgen-independent growth and migration stimulation observed in our autocrine model was mediated through the Src/FAK signaling pathway. In LNCaP cells, in addition to Src, another member of SFK, Lyn, is also significantly expressed. We thus examined the phosphorylation status of Src, Lyn, and FAK kinases in all cell lines grown in CS medium. We immunoprecipitated Src and Lyn proteins with their respective antibody then probed with anti–p-Src or anti–p-Y antibodies. For FAK, we used anti–p-FAKY861, residue phosphorylated by Src as another indicator of SFK activity. All the GRP and GRP-Pro lines displayed higher levels of kinase phosphorylations compared with Zeo cells after exposure to CS serum for 3 days, and the phosphorylations were inhibited by AZD0530 (Fig. 5A). The data showed that (a) autocrine-GRP indeed activates the SFKs; and (b) AZD0530, a pan-Src inhibitor, effectively blocks the activity of Src family members. Thus, whereas in the ensuing studies we will focus on the molecular characterizations of Src, the biological...
effects observed are likely due to the combined inhibition of all SFKs expressed in LNCaP cells. We previously reported that when activated, Src forms a complex with FAK and Etk, and that these kinases cross-activate one another. Coimmunoprecipitation of Src, FAK, and Etk kinases with the anti-FAK antibody confirms complex formation and showed elevated activation of the three kinases in GRP and GRP-Pro cells compared with Zeo cells. Treatment with AZD0530 significantly reduced the degree of tyrosine phosphorylation of all three kinases, and to a much lesser extent, the association between FAK and Src (Fig. 5A).

Regarding proliferation, AZD0530 reduced GRP-Pro cell growth in a dose-dependent manner and inhibited the anchorage- and androgen-free growth of GRP-Pro cells (Supplementary Fig. S3). To ensure that AZD0530 targets Src through which GRP mediates AR activation, RNA interference experiments for Src (siSrc) were performed. Transfection of siSrc into GRP4-9 and GRP-Pro cells greatly impaired their ability to grow in CS medium compared with their respective non-target controls (SC, scramble RNA). Whereas the LNCaP-Zeo cells did not grow well in androgen-deprived conditions with or without siSrc (Fig. 5B). These data support Src as a major target in neuropeptide-mediated AR activation, possibly through its downstream kinases such as FAK and Etk. Both FAK and Etk function in cell adhesion and migration, and inhibition of Src would reduce LNCaP-GRP and GRP-Pro cell migration. As a result, the motility of GRP4-9 (P ≤ 0.05) and GRP-Pro (P ≤ 0.0005) cells was significantly inhibited by AZD0530 (500 nmol/L; Fig. 5C).

Knocking down Src with siSrc transfection into GRP4-9 and GRP-Pro cells also reduced cell migration to comparable levels as Zeo cells. These data support the notion that the GRP-mediated androgen-independent growth and migration is principally through SFKs, especially Src kinase.

**SFK inhibitor AZD0530 prevents tumor metastasis in SCID mice.** With the encouraging results of AZD0530 inhibition in vitro, we evaluated it in our orthotopic GRP mouse model. Fourteen castrated SCID mice implanted with GRP-Pro cells; half of them were administered 50 mg/kg/d of AZD0530 (treatment) beginning 2 weeks after surgery (to permit tumor establishment) and half with buffer only (control) for 8 weeks. All control animals grew tumors with lymph node metastasis (Fig. 6A). H&E staining (inset) of the lymph node validated its human prostate cancer origin. Five of seven treated animals produced primary tumors, but none had metastasis. Immunohistochemical staining using anti-p-Src and anti-p-FAK antibodies showed reduced phosphorylation levels in the treatment samples (Fig. 6B), confirming the effect of AZD0530 in tumors. When probed with anti-AR antibody, the control tumor showed AR nuclear localization as in Fig. 2C. AR staining became undetectable in AZD0530-treated tumors because castrated animals were used. As a result, PSA levels from the sera of AZD0530-treated mice showed significant reduction (P = 0.02) compared with controls (Fig. 6C). Primary tumor sizes in the treated animals were smaller, although not statistically significant (P = 0.104) when compared with control animals. AZD0530,
however, completely blocks tumor metastasis possibly through inhibiting SFK and FAK.

Discussion

In this study, we report the development of a neuropeptide-autocrine model for androgen-insensitive CaP. This model was not designed to study neuroendocrine tumors of the prostate, which are relatively rare, but to study the effect of neuropeptides released from neuroendocrine prostate cells on CaP progression following androgen ablation. There is abundant literature documenting the correlation of increased numbers of postmitotic neuroendocrine cells with the development of castration-resistant CaP and reports showing the overexpression of neuropeptides and neuropeptide receptors in advanced CaP (16–18). Yet, the biological effect of neuropeptides on CaP has not been clearly shown. We present in vitro and in vivo data that the GRP autocrine loop is sufficient to establish androgen independence in LNCaP cells by inappropriate activation of AR. We also show that GRP activates Src, Lyn, FAK, and Etk tyrosine kinases, which confer motility and invasiveness to CaP. Our in vivo inhibitor study shows that administration of Src inhibitor AZD0530 completely blocks tumor metastasis in an androgen-independent environment.

There are numerous reports on growth factors (32), cytokines, chemokines (2, 4), and neuropeptides (3, 25) promoting the androgen-independent growth of LNCaP cells. Although the ligands inducing AR activation are different, many of them transmit signals through the SFK (3, 4, 25). In the present model, we focused on neuropeptides which are coupled to GPCRs and as we showed previously, activate the tyrosine kinase complex Src/FAK/Etk (3). We hypothesized that induced expression of GRP in LNCaP cells may facilitate a more aggressive phenotype via autocrine stimulation. Our engineered LNCaP GRP cells showed androgen- and anchorage-independent growth and superior migration compared with control LNCaP-Zeo cells, and the bombesin/GRP-specific antibody 2A11 partially inhibited the increased growth and migration. This incomplete inhibition by 2A11 may be due to the secretion of other neuropeptides such as neurotensin from the GRP clones (data not shown). These other factors also activate GPCRs, thus there is greater inhibition with GRP receptor inhibition compared with 2A11. Consistent with the in vitro properties, autocrine GRP activity supports androgen-independent tumorigenesis.
of LNCaP-GRP clones in castrated mice. Immunohistochemical staining showed nuclear localization of AR and PSA expression in tumor cells, supporting GRP stimulation of AR in the absence of testicular androgens, which is the sole source of androgen in mice. These observations build on those reported by Burchardt and colleagues, who showed that androgen withdrawal of established in vivo LNCaP tumors resulted in the enrichment of neuroendocrine cells (5). Herein, we show that Src mediates the nuclear translocation and target recruitment of AR induced by GRP based on in vitro (ChIP assay) and in vivo (tumor immunohistochemistry) analyses. A related report using a neuroendocrine mouse prostate allograft also showed that neuroendocrine secretions were sufficient to support androgen-independent growth of LNCaP and PSA expression in vivo (33). These data together firmly establish the potential of neuropeptides secreted by neuroendocrine-differentiated cells to induce androgen independence, and this process involves Src activation.

Elevated tyrosine phosphorylations, especially Src activation, were shown in hormone-refractory prostate cancer xenografts derived from castrated animals (28). In this study, we showed that Src (and likewise, Lyn) is activated both in the free form as well as in the Src/FAK/Etk complex form. As expected, FAK and Etk are also activated as indicated by their heightened phosphorylation status. Impressively, AZD0530 treatment completely blocked these activations. The exact mechanism of how bombesin/GRP activates AR to induce androgen-independent growth of LNCaP is not fully understood. Although GRP has been reported to mediate mitogen-activated protein kinase and Src activation through epidermal growth factor receptor in some human malignancies (34), we observed no increased tyrosine phosphorylation of epidermal growth factor receptors in LNCaP cells upon bombesin stimulation (data not shown). Despite reports implicating Src kinase in the development, growth, progression, and metastasis of human cancers, only one report correlates elevated Src activation and AR phosphorylation to hormone-refractory CaP (28). This report elegantly showed that tyrosine residue Y534 of AR is the direct target of Src phosphorylation, which effectively translocates AR into the nucleus for gene transcription in the absence of androgen. Another report relates the expression of a truncated c-kit tyrosine kinase, which is a strong activator of Src, to advanced stages of CaP (35), suggesting the importance of Src activity in CaP progression. Here, we show that reversion of androgen-independent growth of GRP lines by knocking out Src with siRNA supports a significant role for Src in GRP-mediated cell proliferation. It is speculated that modification of AR or its coactivators by phosphorylation (36) or acetylation (37) mimics the conformational change caused by androgen binding to activate AR in the absence of its cognate ligand. Src may potentially phosphorylate AR directly or through an

Figure 6. In vivo inhibition study in SCID mice. A, primary prostate tumor with lymph node metastasis in an animal from the control group. H&E staining of the lymph node sample validates its prostate cancer origin. B, immunohistochemical staining of the control and AZD0530-treated tumor samples with anti–p-Src (Y419), anti–p-FAK (Y861), and anti-AR (PG-21) antibodies. C, means of PSA levels in sera, primary tumor weight, and metastasis incidents were plotted between the control and AZD0530 treatment groups.
intermediate molecule (28). Yet, because no tyrosine-phosphorylated AR was detected in bombesin-treated LNCaP cells (25), the exact mechanism of how Src is involved still remains to be elucidated. In the p38 assay, GRP mediated AR recruitment preferentially to the proximal ARE site in the PSA promoter, rather than to the enhancer ARE. This observation may reflect conformational modification of AR by Src or a downstream kinase, which facilitates AR activation by assembling a different coactivator complex to elicit gene transactivation in the absence of its natural ligand. Similarly, the reason why the addition of R1881 to GRP clone did not increase the reporter activity further may be that GRP-activated AR is already in active conformation and may not be further stimulated by androgen. Our studies also revealed that posttranslational activated AR may be conformationally different from ligand-bound AR, a finding supported by previous studies (4, 25). Further structural analysis will be required to substantiate this notion.

LNCaP cells are usually not very migratory, but overexpression of GRP under androgen-free conditions enhances LNCaP-GRP cell migration. Another reported mechanism is that bombesin activates RhoA and Rho-associated coiled-coil forming protein kinase to promote Ca²⁺ cell migration and invasion (38). Because RhoA can be activated by Etk (39), which is activated by Src (40), our data are consistent with their findings. FAK phosphorylation in bombesin-stimulated PC-3 cells is linked to cell motility and invasion (41). In collaboration with FAK, Etk is also involved in integrin signaling and promotes PC-3M migration (31). Knocking down Etk expression with its specific siRNA inhibits LNCaP cell proliferation (29, 42), and prostates from Etk transgenic mice exhibit pathologic changes resembling human prostate intraepithelial neoplasia (29). Complexing of these three kinases results in synergistic activation and may transduce GRP-modulated signaling in Ca²⁺ cell proliferation, migration, and survival.

Targeting the bombesin/GRP receptor for cancer therapy is undergoing early clinical trials (43). Other clinical trials have reported promising results using tyrosine kinase inhibitors in cancer therapy; for instance, imatinib (Gleevec, STI571) for chronic myelogenous leukemia and gastrointestinal stromal tumors (44, 45) and trastuzumab (Herceptin, Her-2 antibody) for breast cancer (46). Our approach suggests using a SFK inhibitor to target the activation of non–receptor tyrosine kinases. Through inhibiting Src, AZD0530 prevents the Src-specific activation of FAK, AR, and possibly, Etk and effectively blocks tumor metastasis in our GRP autocrine model. Complex growth factors available in tumor microenvironments and the compensatory pathways involving cell proliferation downstream to Src may be factors why AZD0530 alone could not halt primary tumor growth. IC₅₀’s for inhibiting FAK, paxillin, and P130Cas responsible for migration were 4-fold to 64-fold lower than those for cyclin D1 and c-Myc for proliferation (27). AZD0530 has been tested in tamoxifen-resistant breast cancer cells to suppress tumor cell migration through modulating FAK (47). Treating A549 lung carcinoma cells with AZD0530 results in the down-regulation of Id1 gene expression, possibly through BMP-Smad-Id pathway involved angiogenesis and metastasis (48). The other small molecule Src inhibitor, dasatinib (49), displays an inhibitory mechanism similar to AZD0530, with more inhibition on metastasis than tumor growth in vivo (50). Lyn, a member of SFK, found to play a role in PC-3 tumor progression, was also inhibited by AZD0530 (data not shown).

In addition to neuropeptides, we have previously shown that Src kinase activation is central to interleukin 8–induced androgen-independent prostate cell growth (4). Importantly, interleukin 8 is also a ligand for GPCRs. As such, inhibition of signaling transduction through Src kinase as a downstream target may block oncogenic stimulation for more than one ligand. The specific mechanisms activating AR remain to be elucidated, but the pathways identified suggest that Src kinase inhibition may prove useful in the treatment of androgen-independent CaP.

Disclosure of Potential Conflicts of Interest

C.P. Evans: Consultant, AstraZeneca. The other authors disclosed no potential conflicts of interest.

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References

17. Bartholdi MF, Wu JM, Pu H, Troncoso P, Eden PA,


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