Bombesin attenuates pre-mRNA splicing of glucocorticoid receptor by regulating the expression of serine-arginine protein p30c (SRp30c) in prostate cancer cells

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Abstract

Although glucocorticoids are frequently administered to patients with hormone refractory prostate cancer, their therapeutic effectiveness is limited by the development of glucocorticoid resistance. The molecular mechanisms of glucocorticoid resistance are unknown but are believed to involve neuropeptide growth factors and cytokines. We examined the functional interaction between bombesin and dexamethasone in PC-3 cells and found that bombesin could act as a survival factor by interfering with dexamethasone-mediated growth inhibition. Because glucocorticoids exert their effects through glucocorticoid receptors (GRs), we measured the expression of GRα and GRβ isoforms in the presence of bombesin. Western blotting and real time PCR revealed bombesin induced expression of GRβ, but not GRα. Because GR isoforms are generated by alternative splicing of a common GR gene, we examined the expression of serine-arginine (SR) proteins involved in alternative splicing, and found that the expression of SRp30 was induced by bombesin in PC-3 cells. To characterize the role of SRp30 in splicing of GR isoforms, siRNAs specific to various SRp30 isoforms were transfected into PC-3 cells. We found that suppression of SRp30c expression by siRNA specifically antagonized bombesin’s effect on glucocorticoid-mediated inhibition of PC cells, suggesting that bombesin-induced expression of SRp30c affects GR pre-mRNA splicing, leading to increased GRβ expression and contributing to glucocorticoid resistance in PC cells.

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1. Introduction

Glucocorticoid (GC) monotherapy in patients with hormone refractory prostate cancer is of limited utility, resulting in response rates of approximately 20% [1–3]. Such responses, characterized by decreases in PSA, tumor size, and/or pain are typically of short duration [4–8] due to the development of GC resistance. The molecular mechanisms governing GC resistance are unknown.

Glucocorticoids bind glucocorticoid receptors (GR) and subsequently exert their anti-tumor effects by facilitating signaling pathways leading to apoptosis or necrosis [9–12]. Human glucocorticoid receptors (hGR), members of the nuclear receptor superfamily, undergo pre-mRNA alternative splicing in exon 9 resulting in two highly homologous receptor isoforms, hGRα and hGRβ. These isoforms are identical through amino acid 727 but differences at their carboxyl-termini account for their differing functions. While GRα functions as a ligand-dependent transcription factor, hGRβ inhibits the transcriptional activity of hGRα in a dose dependent manner [13,14].

Splicing in eukaryotes occurs in the spliceosome, comprising serine-arginine-rich proteins (SR proteins) and small nuclear
2. Materials and methods

2.1. Materials and chemicals

Bombesin and dexamethasone (DEX) were purchased from Sigma Biotechnology Inc. Small interfering (si) RNA of non-targeting siRNA and siRNAs against the a, b, and c subspecies of SRp30 proteins were purchased from Dharmacon Inc. (Chicago, IL). Anti-GR α antibody was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA); and anti-GR β from Affinity Bioreagents (Golden, CO). Anti-actin antibody was obtained from Sigma-Aldrich Inc. (Saint Louis, MO). Antibodies against SR proteins were purchased from ATCC, Inc. The plasmid pSRp30c-c2 (expressing a fusion protein GFP-SRp30c) was kindly provided by Dr. S. Stamm, (Institute of Biochemistry, Erlangen, Germany).

2.2. Cell culture and growth inhibitory assays

PC-3 cells were maintained in RPMI1640 supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml streptomycin and penicillin, and 10% FCS. This media was replaced with RPMI 1640 containing 5% charcoal stripped serum (CS) prior to various treatments. Growth assays were performed as described [23,24]. All growth assays were performed in triplicate on three separate occasions with similar results.

2.3. Western blotting

Cells were lysed in RIPA buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1.2% aprotinin, 5 µM leupeptin, 4 µM antipain, 1 mM phenylmethylsulfonyl fluoride and 0.1 mM Na3VO4]. Twenty to fifty micrograms of protein from each sample was suspended in 2× laemml sample buffer, resolved on a 7 or 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Western blotting was performed as described previously [23,24] using diluted primary and secondary antibodies. All Western blot experiments were performed at least twice using different cell lysates with similar results.

2.4. Transfection, CAT assays and application of siRNAs

Transfection of CAT reporter vectors was conducted using Lipofectamine (Invitrogen Inc.) as previously described [27,30]. The experiments were performed in duplicate or triplicate on at least three separate occasions and CAT activities expressed relative to untreated controls.

2.5. Real-time quantitative RT-PCR analysis

PCR was performed using the ABI 7000 system and SYBR green I dye (ABI). Thermocycling was carried out in a final volume of 20 µl, containing 2 µl of cDNA sample, 3.5 mM MgCl2, 0.1 µM primers and 10 µl SYBR green I master mix. After a 10 min initial denaturation at 95 °C, the 50-cycle run consisted of a 15-s denaturation step at 95 °C and an annealing and extension step at 60 °C for 1 min. To avoid amplification of genomic DNA, the forward and reverse primers were designed spanning different exons. The mean of three repeated PCR values was used in the statistical analysis. Results were normalized to β-actin by dividing the individual RT-PCR values by the mean of three repeated β-actin test values of the representative samples to reduce variability between RNA amounts introduced into the RT-PCR reactions. To distinguish the specific PCR products from non-specific products and primer dimers, the melting curve was determined as described previously [31]. Samples were also analyzed by agarose gel electrophoresis to verify that the amplified products exhibited their calculated molecular weights.

2.6. Statistics

The student unpaired t-test (Prism 4 for Windows, GraphPad Software Inc. CA) was used for calculation of P values.

3. Results

3.1. Bombesin interferes with GC induced growth inhibition in PC-3 cells

In vitro studies demonstrate an antiproliferative effect of GCs on human PC cells [32]. To examine whether bombesin, a neuropeptide acting as a survival factor, interferes with GC-induced PC cell growth inhibition, we first performed cell growth assays in the presence of bombesin and DEX in PC-3 cells. Fig. 1A demonstrates that treatment of PC-3 cells with DEX concentrations of 0.1, 1, 10 nM and 100 nM led to growth inhibition of 60%, 66%, 60% and 52%, respectively, compared to untreated controls. Treatment with 50 nM bombesin+10 nM DEX (shaded bar vs. open bar in lane 10, P=0.011) and 50 nM bombesin+100 nM DEX (shaded bar vs. open bar, P=0.013) significantly reduced GC-induced growth inhibition.
result from altered splicing of GR, we measured the effect of bombesin on GRα and GRβ mRNA and protein levels. Fig. 2B showed that following bombesin treatment, GRβ mRNA increased 3.2 (lanes 4h vs. 0h, \( P=0.009 \)) and 1.7 fold relative to control (lanes 18h vs. 0h, \( P=0.008 \)) at 4 and 18 h, respectively, whereas little change was detected in GRα mRNAs (1.3 and 0.8 fold relative to the control, Lanes 4h vs. 0h, \( P=0.52 \), and lanes 18 h vs. 0 h, \( P=0.62 \), respectively). The increase in GRβ mRNA paralleled an increase in GRβ protein at 18 h (Fig. 2C). The increased expression of GRβ mRNA and protein suggested that bombesin treatment specifically altered the splicing of the GRβ gene.

3.3. Induction of SRp30 proteins by bombesin

SR proteins are major components of the spliceosome and play a key role in pre-mRNA splicing of steroid receptors and other proteins [34]. We therefore assessed whether bombesin altered SR protein expression in PC-3 cells. Western blot analysis using the antibody (16H3, ATCC, CRL-2385) which recognizes SRp20, SRp30, SRp40, SRp55 and SRp75 proteins [34] revealed that SRp30 protein expression but not that of other SR proteins was augmented at 18 h following treatment with 50 nM bombesin (Fig. 3, lanes bombesin vs. control). A kinetic analysis showed that the expression of SRp30 protein was induced by bombesin at 18 h. These data indicate that SRp30, like GRβ, is induced by bombesin treatment of PC-3 cells.

3.4. SRp30c is responsible for aberrant GRβ splicing induced by bombesin

Xu, et al. reported that SRp30 protein directs alternative splicing of GR pre-mRNA to GRβ in neutrophils [20]. The expression of SRp30 induced by bombesin suggests SRp30 may be responsible for the aberrant splicing of GR isoforms by bombesin. SRp30 comprises three subspecies: SRp30a (SF2/ASF), SRp30b (SC35) and SRp30c. To identify the subspecies potentially responsible for the altered splicing of GRβ, we conducted quantitative real time reverse transcriptase PCR, quantifying their relative expression in PC-3 cells. All subspecies were detected with SRp30c being the most abundant (Fig. 4A). This expression pattern differed markedly from that observed in neutrophils, where SRp30c was the only detectable subspecies [20].

To assess the relative contribution of the various SRp30 subspecies in modulating GR splicing in PC-3 cells, we introduced siRNAs of SRp30a, SRp30b and SRp30c into PC-3 cells to suppress their expression (Fig. 4B–E), and assessed GR splicing following bombesin treatment. Alternative splicing of GR pre-mRNA to GRβ was not affected with treatment of non-targeting siRNA, nor was it affected with siRNAs specifically targeting SRp30a and b proteins (panels GRβ, Fig. 4B–D). However, targeting of SRp30c with a specific siRNA resulted in a significant reduction in GRβ production at 18 h after transfection (panel GRβ, Fig. 4E), whereas GRα production was not affected (panels GRα, Fig. 4B–E). These data suggest that in the presence of bombesin, SRp30c mediates
the alternative splicing of GR pre-mRNA, leading to the increased generation of GRβ mRNA.

3.5. SRp30c affects bombesin-mediated interference of GC action in MMTV-CAT

To confirm the functional significance of SRp30c expression in the GR pathway, we next tested the SRp30 isoform siRNAs on expression of the hormone-responsive MMTV promoter using PC-3 cells transfected with a plasmid containing an MMTV promoter upstream of a CAT reporter gene. As shown in Fig. 5A, DEX-induced CAT activities were reduced to 42% in the presence of 50 nM bombesin compared to treatment with DEX alone (lanes 4 vs. 3) similar to that observed for the GRE-tk-CAT vector (as described in Fig. 1A). While siRNAs to SRp30a and b did not affect CAT activities (38% and 45%, Fig. 5A, lanes 5 and 6 vs. 3), transfection of SRp30c siRNA effectively abolished bombesin inhibition of MMTV-CAT activity induced by DEX (Fig. 5A, lanes 7 vs. 3). Prostate cancer cells transfected with the vector pSRp30c-c2 over-expressed SRp30c as a GFP fusion protein (Fig. 5B) and exhibited lower CAT activities in both GC and bombesin + GC treated cells (Fig. 5C), when compared to that of control vector expressing GFP (45%, lane 7 vs. lane 3, \( P=0.009 \), and 44%, lane 8 vs. lane 4, \( P=0.008 \)). These results suggest that the overexpression of SRp30c led to elevated GRβ expression and resulted in decreased GR reporter activity. In contrast, no SRP30c dependent changes in CAT activity were detected between treated and untreated cells (Fig. 5C, lanes 5 vs. 1 and lanes 6 vs. 2). Taken together, the data demonstrate that elimination (or enhancement) of SRp30c expression can regulate the splicing of GRβ to antagonize (or augment) the effect of bombesin on GC action.

3.6. Suppression of SRp30c expression eliminates bombesin's antagonism of GC action on PC-3 cell growth

The above results demonstrate that SRp30c plays a key role on bombesin-mediated interference of GC induced CAT activities in vitro. To verify whether this is the case in vivo, we examined the effects of siRNA of SRp30c on growth of PC-3 cells in the presence of GC and bombesin. As shown in Fig. 6,
after transfection of control siRNA (non-targeting siRNA) into PC-3 cells (lanes 1–4), inclusion of 50 nM bombesin together with 100 nM DEX resulted in a 22% increase in cell number (lanes 4 vs. 3, \( P=0.0135 \)) relative to treatment with 100 nM DEX alone. When siRNA directed against SRp30c was transfected, the increase in cell number following bombesin treatment was significantly reduced to 16% (lanes 5 vs. 4, \( P=0.0374 \)), showing that suppression of SRp30c expression using a specific siRNA attenuates bombesin’s antagonism of GC action in PC cells. Together these data indicate that SRp30c subspecies plays an important role in bombesin-mediated survival effect in the presence of GC in PC cells.

4. Discussion

GCs have been used in clinical oncology for more than three decades and have been an integral part of endocrine treatment for advanced PC. However the molecular mechanisms underlying the development of GC resistance are poorly understood. In this study we report that the survival factor bombesin can reverse GC action (Fig. 7). We demonstrate that: (1) bombesin interferes with the inhibitory effects of GCs on proliferation of PC cells; (2) bombesin causes an up-regulation of protein and...
mRNA levels of GRβ; (3) bombesin augments the expression of SRp30 protein; and 4) bombesin-induced alternative splicing of GR can be reversed by suppressing the SRp30c subspecies.

Our data confirm and extend the finding that neuropeptides can antagonize the action of glucocorticoids on PC cells. Hormones or cytokines either employed in prostate cancer endocrine therapy or elevated in prostate cancer have been reported to function as GR antagonists. Krishnan, et al. demonstrated that estrogen, E2 had an anti-GC action in human breast cancer cells [35]. Autocrine or paracrine secretion of lymphokines with subsequent activation of survival pathways has been implicated in GC resistance in numerous systems [36]. Recently, the proinflammatory cytokine, IL-1α, was shown to antagonize DEX action in mouse L929 cells [37]. Our results suggest that bombesin modulates GR-mediated
transactivation by altering expression of GRs. GRs are reported to be present in several human prostate cancer cell lines. GC activity is lacking in GC-negative LNCaP cells, but clearly present in GR-positive PC-3 and DU-145 cells [38].

Several studies report GRβ protein is capable of exhibiting a dominant negative function [32,39]. The preferential increase in the beta isoform of the human GR has also been seen in several disease states resistant to GC therapy. Recently, increased GRβ levels have been reported in human T cells localized to airways, peripheral blood mononuclear cells, and in tuberculinduced inflammatory lesions in GC-insensitive asthmatics [40]. In another report, higher levels of GRβ were found in 10 of 12 patients with GC-resistant colitis [41]. The cytokines TNF-α and IL-1 were reported to interfere with GC-induced apoptosis by changing the GRα and GRβ ratio [42,43]. Additionally, isolated peripheral blood mononuclear cells, when stimulated with various superantigens, became insensitive to GC, and this insensitivity is believed to be the result of increased GRβ [44]. All these studies underscore the strong correlation between the expression of GRβ and generalized resistance to GC therapy in human disease states. Our studies are consistent with those previous observations and also reveal a molecular mechanism by which GC resistance may be achieved. The mechanisms could potentially impact multiple GC target genes and interact with downstream effectors of bombesin as well.

To investigate the aberrant splicing of GRβ, we studied the expression of splicing factors SR proteins. Specifically, the SRp30c subspecies leads to increased alternative splicing of GRβ mRNA and expression in the presence of bombesin. SRp30c is a repressor of 3′ splice site utilization [19] and has been found to stimulate alternative splice site selection in CD45 pre-mRNA in leukocytes [45]. A bioinformatics search of the region covering the sequences responsible for splicing of GR gene revealed the presence of several exonic splicing enhancers (ESEs) for SRp30c interaction (data not shown), suggesting that the augmented expression of SRp30c by bombesin might lead to the repression of the splicing of exon 9α (or enhancement of the splicing of exon 9β) to generate a higher level of GRβ.

Our studies demonstrated that SRp30c played the primary function in regulation of GR splicing both in vitro and in vivo. Nevertheless, the antagonizing effects of SRp30c siRNA on cell growth was relatively weak when compared to that in vitro. Other splicing factors, such as hnRNPs which antagonize the effects of SR proteins, may play a role in bombesin-mediated GR splicing in vivo. Recently it has been reported that transcription may couple with the splicing process. All these warrant our further studies when considering the mechanisms underlying bombesin-induced GC resistance.

This study is first to demonstrate neuropeptide antagonism of GC function by altering GR splicing. Anti-survival factor therapy aims at neutralizing the protective effect conferred by the survival factors derived from the local microenvironment. Since GRβ is intimately involved in GC insensitivity, and GRβ is dependent on the presence of SRp30c, the pathway involving SRp30c protein may be an attractive therapeutic target for restoring GC sensitivity to hormone refractory PC and may account for patient differences in GC responsiveness. Moreover, the ability of neuropeptides to induce GC resistance may play an important role in other clinical settings such as bone turnover and the neuroendocrine response to stress.

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