Glucocorticoid Receptor β Stimulates Akt1 Growth Pathway by Attenuation of PTEN*

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Background: The glucocorticoid receptor β (GRβ) is a positive regulator of growth.
Results: GRβ suppression of PTEN resulted in enhanced phosphorylation of Akt and growth.
Conclusion: GRβ enhances insulin-induced proliferation by suppressing PTEN and activating Akt1.
Significance: GRβ suppression of PTEN indicates that it has an important role in growth factor signaling and potentially cancer.

Glucocorticoids (GCs) are known inhibitors of proliferation and are commonly prescribed to cancer patients to inhibit tumor growth and induce apoptosis via the glucocorticoid receptor (GR). Because of alternative splicing, the GR exists as two isoforms, GRα and GRβ. The growth inhibitory actions of GCs are mediated via GRα, a hormone-induced transcription factor. The GRβ isoform, however, lacks helix 12 of the ligand-binding domain and cannot bind GCs. While we have previously shown that GRβ mRNA is responsive to insulin, the role of GRβ in insulin signaling and growth pathways is unknown. In the present study, we show that GRβ suppresses PTEN expression, leading to enhanced insulin-stimulated growth. These characteristics were independent of the inhibitory qualities that have been reported for GRβ on GRα. Additionally, we found that GRβ increased phosphorylation of Akt basally, which was further amplified following insulin treatment. In particular, GRβ specifically targets Akt1 in growth pathways. Our results demonstrate that the GRβ/Akt1 axis is a major player in insulin-stimulated growth.

Regulation of growth pathways is an important component of cellular physiology that maintains division, nutrient uptake, and cell fate. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is essential for growth regulation, and its inactivation or deletion has been shown in a variety of cancers (1). A prominent feature of PTEN signaling is inhibition of the phosphoinositide 3-kinase (PI(3)-kinase) pathway, which results in reduced 3-phosphoinositide (PIP3) levels and suppressed growth (2). A plethora of growth factors and cytokines activate the PI3-kinase lipid enzyme, resulting in the conversion of 2-phosphoinositide (PIP2) to 3-phosphoinositide (PIP3) (3). PIP3 binds to the PH domain of Akt resulting in phosphorylation and activation, which increases cell survival, growth, and metabolism (2, 3). Interestingly, glucocorticoids (GCs), which inhibit growth (4) and induce apoptosis in a variety of cells (5), have been shown to increase expression of PTEN in A549 human lung carcinoma cells (6). This suggests that one aspect of the anti-growth properties of GCs may be mediated through the up-regulation of PTEN. However, the roles of the glucocorticoid receptors (GRs), especially the separation of GRα and GRβ isoforms, in the regulation of PTEN-mediated effects on growth pathways, have not been investigated.

As a result of alternative splicing of exon 9 of a single gene, there exists two GR isoforms, α and β, which vary at their C terminus. There also exist a GRγ isoform from the use of an alternative splice donor site in which three base pairs are retained between exons 3 and 4 (7). As a consequence, an additional arginine is located in the DNA binding domain, resulting in decreased binding (8). The GR α and β isoforms have been the most studied. The GRα is a hormone-activated transcription factor that controls many physiological processes, extending from apoptosis, glucose metabolism to lung development (9, 10). The GRβ isoform, however, lacks helix 12 of the C terminus in the ligand-binding domain and cannot bind GCs (11). The anti-proliferative properties of GCs are mediated by GRα, which have been attributed to elevation of cell cycle arrest proteins, p27 and p21 (12, 13). A reduction in GRα in murine macrophages results in enhanced growth and decreased expression of p27 (14). This implies that resistance to GC-induced apoptosis may occur by reducing GRα levels. In humans, GC resistance can occur by two major mechanisms: loss-of-function mutations in GRα (15), or by increased expression of GRβ, which acts as a dominant-negative inhibitor to GRα (11). Although GRα mutations can result in a type of GC resistance that is both systemic and severe, these mutations are rare. In contrast, the evolving evidence suggests that GC resistance based on GRβ is much more common and likely to be tissue-specific in nature.

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‡ The abbreviations used are: PTEN, phosphatase and tensin homolog; GC, glucocorticoid; GR, glucocorticoid receptor; PNA, peptide nucleic acid; BPS, branch point sequence; CPP, cell-penetrating peptide.

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GRβ Increases Growth by Suppression of PTEN

The best-studied apoptotic roles of GCs have been in cancers of the immune system such as lymphoma and leukemia. Immune system homeostasis is balanced by GCs, which regulate immune cell turnover by suppressing cytokine production and promoting apoptosis. GC insensitivity due to elevated human GRβ (hGRβ) expression results in higher levels of pro-inflammatory cytokines, leading to escalated cell growth and reduced cell death (16). As well, pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), increase expression of GRβ via the NF-κB pathway (17). Ascending levels of GRβ in asthma patients cause many disease complications, including GC resistance that can verge to a complete loss of drug response (18). Importantly, both leukemia (19) and systemic lupus erythematosus (20) have been linked to high levels of GRβ, which may underlie the exacerbated immune cell growth observed in these patients. How GRβ regulates growth is unknown, but the mechanism may involve inhibition of GRα-induced apoptosis or direct GRβ epigenetic signaling. A previous study reported gene-specific activity for GRβ that is independent of GRα (21), a known inhibitor of growth. Therefore, in this study, we investigated the effect of GRβ on the insulin signaling pathways guiding proliferation. Our findings demonstrate that GRβ plays an important role in the control of insulin signaling by suppressing PTEN, resulting in the enhancement of growth.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The mouse 3T3-L1, C2C12, RAW 264.7, S49 and embryonic fibroblast (MEF) were routinely cultured and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% bovine calf serum or FBS with 1% penicillin-streptomycin. The scramble and GRβ MEF cell lines were grown as previously described (11).

Whole Cell Extraction—Cells were washed and collected in 1× PBS followed by centrifugation at 1500 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in 1× PBS. After a short spin at 20,800 × g for 5 min at 4 °C, the pellet was rapidly frozen on a dry ice ethanol mix and stored at −80 °C for 30 min. The frozen pellet was then resuspended in 3 volumes of cold whole cell extract buffer (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, pH 7.4) with protease inhibitors and incubated on ice for 10 min. The samples were centrifuged at 100,000 g for 5 min at 4 °C. Protein levels were measured spectrophotometrically by a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE). The supernatants were either stored at −80 °C or used immediately for Western analysis to determine protein expression levels.

Quantitative Real-Time PCR Analysis—Total RNA was extracted from mouse tissues using 5-Prime PerfectPure RNA Cell Kit (Fisher Scientific Company, LLC). Total RNA was read on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR amplification of the cDNA was performed by quantitative real-time PCR using TrueAmp SYBR Green qPCR SuperMix (Smart Bioscience). The thermocycling protocol consisted of 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C and finished with a melting curve ranging from 60–95 °C to allow distinction of specific products. Primer sequences were downloaded from a primer database. Normalization was performed in separate reactions with primers to GAPDH.

Generation of Lentiviral Constructs—To establish a 3T3-L1 cell line that has mGRβ stably overexpressed, mGRβ cDNA was ligated into the XbaI/HinfI sites of the FG12 vector that has an independent GFP marker and transformed in DH5α cells (Invitrogen). The construct was co-transfected together with vectors expressing gag-pol, REV, and VSV-G into 293FT cells (Invitrogen) to generate a third generation lentiviral construct. Transfection was achieved using GeneFect (Alkali Scientific, Inc.) using 100 ng of total DNA per cm² of the growth plate or well. The supernatants were harvested, and the cell debris was removed by centrifugation at 2000 × g. The supernatant was used to infect 3T3-L1 cells after addition of polybrene (5 ng/ml, Sigma) to establish cell lines with stable overexpression of mGRβ mRNA (3T3-GRβ) or expressing empty vector (3T3-V). After 72 h, the cells were sorted by flow cytometry for GFP by the Flow Cytometry Core Facility at the University of Toledo Health Science Campus. GFP-positive cells were used for all experiments.

Transient Transfection—For transient transfection cells were plated on a 6-well dish in DMEM containing 10% calf serum prior to transfection and allowed to grow to 85–90% confluency. Cells were washed with OPTI-MEM and transfected using GeneFect (Alkali Scientific, Inc.), according to the manufacturer’s protocol. OPTI-MEM was removed after 5 h and DMEM containing 10% calf serum was added. All insulin treatments were done 24 h post-transfection for 30 min.

Targeting of Mouse GRβ mRNA—Targeting of mouse GRβ was designed and purchased using the Integrated DNA Technologies (iDT) sciTools RNAi Design website. Two siRNA targets were designed: Seq 1 (gatgtagatcacaataaca) and Seq 2 (gtcagagatcagagatccta), as well as scramble control. Transient transfection of the siRNAs was performed using GeneFect (Alkali Scientific, Inc.) for 24 h.

Targeting of the Mouse GR Gene—A peptide nucleic acid (PNA) targeting the branch point sequences (BPS) in intron 8 of the mouse GR gene (previously identified in Ref. 11) was designed using PANAGENE. Two different PNAS were designed: CPP-PNA-i8BPS (gactgattggtatat) and CPP-PNA-i8BPS (aatatcactagatcactag). All PNAS were attached to an O Linker and a modified TAT protein (VQRKQKLMYP) for delivery into the cell (cell penetrating peptide - CPP). Treatment with CPP-PNAS was performed for 24 h.

Promoter Reporter Assays—Expression vector for mGRβ (pMGβ-H57) was constructed as previously described (11). PTEN promoter (full-length, truncations and mutants) activity was measured by luciferase, and these constructs were made as defined in Ref. 22, and pRL-CMV Renilla reporter for normalization to transfection efficiency. Transient transfection was achieved using GeneFect (Alkali Scientific, Inc.). 24-h post-transfected cells were lysed, and the luciferase assay was performed using the Promega luciferase assay system (Promega).

Gel Electrophoresis and Western Blotting—Whole cell extracts (WCE) were prepared by freezing the cell pellet overnight at −80 °C. The pellet was then resuspended in 3 volumes
Results of WCE buffer (20 mM HEPES, 0.42 mM NaCl, 0.2 mM EDTA, 25% glycerol, pH 7.4) plus protease inhibitor mixture and incubated on ice for 10 min followed by 100,000 × g centrifugation at 4 °C. Protein samples were resolved by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon-FL membranes. Membranes were blocked at room temperature for 1 h in TBS (TBS; 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl) containing 3% BSA. Subsequently, the membrane was incubated overnight at 4 °C with FiGR antibody for mGRα (Santa Cruz Biotechnology, Dallas, Texas) or mGRβ antibody for mGRβ (described in Ref. 11) at a dilution of 1:1000 in TBS. Antibodies that recognize HSP90, Akt1, Akt2, pAkt, and total Akt were purchased from Santa Cruz Biotechnology and added at a dilution of 1:1000 in TBS. After three washes in TBST (TBS plus 0.1% Tween 20), the membrane was incubated with an infrared anti-rabbit (IRDye 800, green) or anti-mouse (IRDye 680, red) secondary antibody labeled with IRDye infrared dye (LI-COR Biosciences) (1:15,000 dilution in TBS) for 2 h at 4 °C. Immunoreactivity was visualized and quantified by infrared scanning in the Odyssey system (LI-COR Biosciences) (1:15,000 dilution in TBS) for 2 h at 4 °C.

**RESULTS**

**Detection of GRβ Expression in Tissues and Cells from Different Marine Lineages**—The recent discovery of the mouse GRβ (mGRβ) has opened new avenues for studying glucocorticoids and the actions of glucocorticoid receptors (11): whether mouse GRβ (mGRβ) is expressed by various cell types and lineages are largely unknown. To detect mGRβ and mGRα in different tissues of murine origin, we analyzed lysates from muscle, liver, spleen, kidney, and heart (Fig. 1A). We found in Hepa1c1c7 hepatocytes that mouse GRβ (mGRβ) is expressed by various cell types and lineages are largely unknown. To detect mGRβ and mGRα in different tissues of murine origin, we analyzed lysates from muscle, liver, spleen, kidney, and heart (Fig. 1A). The mGRα was detected at 97 kDa in muscle, liver, spleen, and to a lesser extent in kidney and heart. We detected the GRβ protein in muscle and liver at 92 and 102 kDa. In spleen, kidney, and heart only the 102-kDa GRβ band was present. The shift in molecular weight was also noticed for human GRβ in heart and skeletal muscle tissues (24). We therefore wanted to analyze the expression of mGRβ in murine cells from hepatic and muscle lineages (Fig. 1B). We found in Hepa1c1c7 hepatocytes that mouse GRβ is detectable at the 92 and 102 kDa bands, and C2C12 myocytes had a single band at 102 kDa. The mGRα levels were lower in C2C12 myocytes compared with Hepa1c1c7 hepatocytes. These results suggest that cells from mesenchymal and endodermal origins may have a differential GRβ and GRα expression. To compare murine cells of mesenchymal origin we used mouse embryonic fibroblast (MEF), 3T3-L1 and C2C12 cells (Fig. 1C). Western blotting analysis showed that MEF and C2C12 have similar detectable levels of mGRβ (p = 0.23). C2C12 cells have reduced mGRα levels compared with MEF (p = 0.007). Interestingly, the 3T3-L1 cell line has very low to undetectable levels of mGRβ and mGRα. Furthermore, we determined mGRβ and mGRα expression in two murine.
immune cell lines, RAW 264.7 leukemic monocyte/macrophage, and S49 lymphoma cells (Fig. 1D). Interestingly, S49 lymphocytes have significantly higher expression of mGRβ (p = 0.0036) compared with RAW 264.7 cells, and lower expression of mGRα (p < 0.001).

Mouse GRβ Sensitizes to Insulin Signaling—We previously shown that mGRβ mRNA was increased in MEF cells and in livers of mice that were fasted and refed (11). Here, we treated MEF cells with 100 nM insulin for 24 h and determined mGRβ expression. Confirming our previous mRNA results, mGRβ protein expression was significantly increased following insulin treatment and no change was observed in mGRα (Fig. 2A). The capacity of GRβ in insulin signaling is unknown. Therefore, we determined the impact of mGRβ on the phosphorylation of Akt with and without insulin treatments for 30 min in MEF cells overexpressing mGRβ. To our surprise, overexpression of mGRβ basally increased pAkt, which was further elevated upon insulin treatment compared with vector controls (Fig. 2B).

Mouse GRβ Enhances Growth by Suppression of PTEN—Our studies in Fig. 1C showed that 3T3-L1 cells have undetectable levels of mGRβ and mGRα protein. Therefore, we created a cell line that stably overexpressed only mGRβ (3T3-GRβ) or vector control (3T3-V) to elucidate the actions of mGRβ outside of its known GRα inhibitory role (11, 25). In Fig. 3A, we show that the 3T3-GRβ cell line highly expressed mGRβ protein. Interestingly, this cell line had significantly reduced PTEN expression.
suggesting a positive role of mGR in insulin signaling. Therefore, we measured protein expression of Akt1 and Akt2. Unexpectedly, the 3T3-GR cells had significantly higher Akt1 (p = 0.0060) with no change in Akt2 (p = 0.1867) expression (Fig. 3A). To confirm that mGR regulates phosphorylation of Akt, we treated 3T3-GR and 3T3-V cells with insulin for 30 min. Basally, mGR significantly increased pAkt, which was further enhanced upon insulin treatments (Fig. 3B). Because mGR inhibited expression of PTEN and enhanced Akt1, which has been shown to regulate cellular proliferation (26), we investigated proliferation in the 3T3-GR cells (Fig. 3C). Growth of 3T3-GR cells was significantly higher (ANOVA, p = 0.0001) than the 3T3-V cells (Fig. 3D), specifically at days 4 (p < 0.001) and 5 (p < 0.001) of growth. Additionally, 24 h of insulin treatment significantly increased growth of 3T3-GR cells (p = 0.0405).

Targeting of Mouse GR Gene Enhances mGR Expression—We have previously identified two putative branch point sequences (BPS) within intron 8 of the mouse GR gene (11). We wanted to determine if specific targeting of the BPS utilizing a PNA conjugated to a cell-penetrating peptide (CPP) (modified TAT protein) can bind to intron 8 of the mouse GR gene to regulate alternative splicing of GR or GRα. A dose-dependence treatment with CPP-PNA-i8BPS significantly enhanced mGR expression at 100 nM and 500 nM, and decreased expression of mGRα (Fig. 5A). Treatment with the control CPP-PNA-i8C resulted in no change in either mGRβ or mGRα mRNA expression. We have shown in Fig. 3A that increasing mGRβ expression decreased PTEN. Therefore, we measured mRNA expression of PTEN with 100 nM BPS1-CPP-i8BPS treatment for 24 h. Increasing mGRβ expression via CPP-PNA-i8BPS significantly decreased expression of PTEN (p = 0.0405), enhanced mGRβ (p = 0.0358), and no change in GRα (p = 0.3716).

Mouse GRβ Regulates Promoter Activity of PTEN—To determine if mGRβ inhibits the PTEN promoter we utilized the PTEN-luciferase construct. Overexpression of mGRβ in MEF cells resulted in a significant (p = 0.0136) reduction of PTEN.

**FIGURE 4. Knockdown of GRβ increases PTEN expression.** A, targeting of siRNA knockdown of mGRβ. B, Western blot and densitometry of GRβ, GRα, PTEN, and HSP90 in MEF cells. ***, p < 0.001 (versus scramble); **, p < 0.05 (versus scramble) (± S.E.; n = 6).
FIGURE 5. Increasing GRβ expression by PNA decreases PTEN expression. A, real-time PCR of MEF cells treated with CPP-PNA-i8BPS (targeting GRβ) or CPP-PNA-i8C (control) for 24 h to enhance mouse GRβ expression-dose dependence (0, 25, 50, 100, and 500 nM PNA) in dialyzed serum (± S.E.; n = 6). B, real-time PCR expression of PTEN in MEF cells treated with 100 nM CPP-PNA-i8C or CPP-PNA-i8BPS for 24 h. ***, p < 0.001 (versus control) (± S.E.; n = 3). C, Western blot and densitometry GRβ and α MEFs with 100 nM CPP-PNA-i8BPS or CPP-PNA-i8C treatment for 24 h in dialyzed serum. ***, p < 0.01 (versus control); *, p < 0.01 (versus control); (± S.E.; n = 3).

DISCUSSION

This is the first study to demonstrate that GRβ can enhance proliferation by the suppression of PTEN. Here we report that GRβ protein expression is elevated by insulin treatment, which enhances growth through modulating PTEN promoter activity. Increasing levels of PTEN has been shown to be essential for the suppression of PI3-kinase induced tumor growth in mice (27). Insulin increased PTEN promoter activity over a 24-h period, which implies a negative feedback regulation. Interestingly, GRβ suppressed the negative feedback regulation decreasing PTEN expression, leading to exacerbated Akt phosphorylation and growth. This suggests that long term increases in GRβ expression may enhance growth by suppressing PTEN leading to over stimulation of PI3-kinase and Akt. Inhibitors of the PI3-kinase pathway enhanced GC induced apoptosis in human lymphoma cells (28), which indicate a positive feedback between glucocorticoids/GRα and PTEN in the suppression of growth. The interplay between GRα and PTEN was demonstrated in T-cells extracted from human leukemia patients, which had increased PTEN expression following GC treatment (29). However, patients that relapsed had a complete loss of PTEN expression. The recruitment of GRβ to the PTEN promoter uncovers an essential pathway for understanding the mechanism of the inhibition of GC induced apoptosis and sensitization to Akt stimulated growth. The GRβ inhibition of GC-induced growth has been shown in peripheral mononuclear cells, in which cytokine stimulation increased GRβ expression and prevented apoptosis (30). Interestingly, we show that protein expression of GRβ is more abundant in mouse lymphoma cells compared with GRα. Similarly, high expression levels of GRβ have been reported in immune cells of lupus (20) and leukemia (19) patients, suggesting that a leading target for inhibiting growth in these cells would be GRβ. Here we show that siRNA targeting of GRβ in MEF cells concomitantly increased PTEN expression.

We have also discovered that GRβ selectively increases Akt1 expression over that of Akt2. Our finding of GRβ in mice (11) and the recent report by Dubois et al. on GRβ in the rat (31), suggest that rodent GRβ has a possible role in metabolic signaling (11). Therefore, we determined the impact of elevated GRβ on the insulin signaling pathway, showing that it leads to exacerbated Akt phosphorylation and growth. Akt exists as three structurally similar, yet different, isoforms: Akt1, Akt2, and Akt3. The functionality of these isoforms has been shown to be divergent, even though their structures are similar. The Akt1
isoform has been shown to be essential in the regulation of development and growth (26, 32). In contrast, the Akt2 isoform is mostly involved in glucose uptake (33) and adipogenesis (34). These actions have lead several investigators to study the involvement of Akt2 in metabolic diseases, such as diabetes and obesity (33). Akt3 has been the least studied of the isoforms, but investigations show that Akt3 may have a possible role in brain development (35). The growth regulation properties of Akt1 have made it a major target in cancer studies (32, 36, 37), especially PTEN-mediated inhibition of Akt1 in tumor development (38). The selectively of GRβ/H9252 indicates a potential capacity of this nuclear receptor in growth pathways. This was demonstrated by lentiviral overexpression of GRβ/H9252 in 3T3-L1 cells, which resulted in elevated Akt1 expression and enhanced insulin-stimulated proliferation. Akt1 is a direct target of the insulin/PI3-kinase induced growth pathway, and deletions have been beneficial in cancer, especially in lung tumorigenesis by mutant K-ras (36). Additionally, the suppression of the PI3-kinase/Akt1 growth pathway by gene targeting or by the PI3-kinase inhibition has been shown to decrease tumor mass size (37). Interestingly, Piovan et al. identify Akt1 as a negative regulator of GRα/H9251 by phosphorylating serine 134, which resulted in GC resistance in T cell acute lymphoblastic leukemia (39). They also show that suppression of PTEN inhibits GRα/H9251 expression and activity, enhances Akt phosphorylation, resulting in GC-resistance. To date, most studies have only considered GRβ as an inhibitor of GC action, primarily due to its ability to heterodimerize with GRα (18, 40) and to recruit histone deacetylases inhibiting gene activity (41). The impedimentary action of GRβ on GRα was recently demonstrated by Varricchio et al. in polycythemia vera (PV) patients, which have increased erythroid cell growth (42). This study found that PV patients have a polymorphism in the human GR gene that leads to the stabilization of the GRβ mRNA, resulting in higher protein levels. Interestingly, these patients have exacerbated GRβ/GRα heterodimerization and expansion of erythroblast. GRα is known to heterodimerize with other members of the steroid receptor family, such as the
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A

**PTEN Promoter Truncations**

| PTEN Promoter Truncations | Lucerase / Renilla |
|---------------------------|--------------------|
| 1344 - 0                  | 1.0                |
| 1344 - 1001              | 0.5                |
| 893 - 0                  | 0.0                |
| 601 - 0                  | 0.0                |
| 453 - 0                  | 0.0                |
| 203 - 0                  | 0.0                |

**Figure 7. Target area that GRβ inhibits the PTEN promoter.** A, 3T3-L1 cells were transiently transfected with the PTEN promoter truncated luciferase plasmid constructs for 48 h with vector and mGRβ overexpressed. ***, p < 0.001 (versus vector 1344–0); ***, p < 0.01 (versus vector 1344–1001); (**, p < 0.01 (versus vector 1344–0); ***, p < 0.001 (versus vector 1344–0); ***, p < 0.01 (versus vector SubA); #, p < 0.01 (versus vector SubD); (± S.E.; n = 12). B, 3T3-L1 cells were transiently transfected with the PTEN promoter mutant luciferase plasmid constructs for 48 h with vector and mGRβ overexpressed. ***, p < 0.001 (versus vector 1344–0); ***, p < 0.01 (versus vector SubA); #, p < 0.01 (versus vector SubD); (± S.E.; n = 6).

B

**PTEN Promoter Mutants**

| PTEN Promoter Mutants | Lucerase / Renilla |
|-----------------------|--------------------|
| 1344-0                | 1.0                |
| SubA                  | 0.5                |
| SubB                  | 0.0                |
| SubC                  | 0.0                |
| SubD                  | 0.0                |

**Figure 8. GRβ growth diagram.** GRβ suppresses PTEN expression, which leads to activation of Akt1, resulting in enhanced growth.

mineralocorticoid (MR) and androgen (AR) receptors (43, 44). It is also possible that GRβ may regulate steroid receptor activity other than GRα. The GRβ/Akt1 growth axis may have an important function in the regulation of AR activity. Knockdown of hGRβ by siRNA reduced androgen induced growth in LNCaP prostate cancer cells (45). However, Akt1 and PTEN were never investigated, and it is only now coming to light that GRβ has a consummate role in these pathways. Furthermore, bombesin treatment attenuated GC-induced apoptosis in PC-3 human prostate cancer cells by increasing hGRβ expression (46), albeit, through GRβ inhibition of PTEN. Importantly, GRβ may have a major function in the development of several cancers. The HT-29 colon cancer and MCF-7 breast carcinoma (47), as well as, Hut-78 T- and Raji B-lymphoma cell lines (48) have shown high expression of GRβ. Interestingly, treatment with growth inhibitors 5-aza-2’-deoxycytidine (5-dAzaC), sodium butyrate (NaBu), and trichostatin A (TSA) reduced GRβ and increased GRα expression in all of these cancer cell lines (47, 48). Unfortunately, sensitivity to GC-induced apoptosis or expression of PTEN was never tested in these studies. Likewise, hepatocarcinoma (HepG2) and osteosarcoma cells (SaOS-2) have elevated expression of GRβ, which was found to be mostly located within the nucleoli (49, 50).

In conclusion, the predominant aspects studied for GRβ have been on the inhibition of GRα. Our studies clearly show that GRβ has a preeminent role in insulin signaling and growth. These properties have not been previously known for GRβ, especially the epigenetic Akt1/PTEN growth signaling that is independent of GRα (Fig. 8). Given that Akt1 regulates embryonic and fetal growth (26), this work suggests that GRβ may have a paramount role in development and proliferation. Thus, the GRβ/Akt1 axis is emerging as a major signaling paradigm regulating growth, which may also lead to GC-resistance in cancer. Therapeutics inhibiting GRβ may increase sensitivity to GCs, as well as increase PTEN expression allowing for the regulation of growth.

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