Introduction
Prostate cancer (CaP) continues to be the most frequently occurring malignancy (aside from skin cancers) in American men. Large epidemiological studies have shown that serum insulin-like growth factor (IGF)-I levels are elevated and insulin-like growth factor-binding protein-3 (IGFBP-3) levels are reduced in patients with CaP, with differences in these serum markers becoming evident 5 years prior to the development of clinical CaP (1). Multiple lines of evidence point to IGFBP-3 as an antioxidant molecule (2). Indeed, in vivo demonstration of tumor suppression by IGFBP-3, either administered to cancer xenografts in combination with an RXR ligand via pDNR-mediated Creator™ technology (Clontech, Palo Alto, CA), LipofectAMINE and PLUS reagent were from Invitrogen (Carlsbad, CA). All other chemicals were from Sigma–Aldrich (St. Louis, MO).

Materials
Insmed (Glen Allen, VA) provided recombinant human IGFBP-3. Commercial antibodies included the following: anti-human IGFBP-3 from DSL (Webster, TX), anti-Nur77 and blocking peptide were from Geneka Biotechnology (Montreal, Canada) and control goat IgG from Santa Cruz Biotechnologies (Santa Cruz, CA). p-Akt, total Akt, p-Jun N-terminal kinase (JNK) and total JNK antibodies as well as Akt kinase assays were from Cell Signaling Technology (Danvers, MA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) reagents, Tween and fat-free milk were purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Bucks, UK). Full-length IGFBP-3 and Nur77 cDNAs were cloned into pL-P-IRESneo mammalian expression vector via pDNR-mediated Creator™ technology (Clontech, Palo Alto, CA). LipofectAMINE and PLUS reagent were from Invitrogen (Carlsbad, CA).

Subcellular fractionation procedures
Nu-CLEAR Protein Extraction kit™ was from Sigma–Aldrich. Subcellular fractions were isolated according to the manufacturer’s protocol.

Co-immunoprecipitation and western immunoblots
22RV1 nuclear or cytoplasmic extracts were immunoprecipitated with or anti-IGFBP-3 antibodies. Briefly, 250 μl of protein A-agarose was incubated overnight at 4°C with 5 μl of anti-human IGFBP-3 antibodies. One hundred and twenty-five microliters of each antibody-treated protein A-agarose were added to 10 μg of protein extract and incubated for 3 h at 4°C with shaking. Immunoprecipitated proteins were pelleted by centrifugation and washed three times with 100 μl of SACI buffer. Sample buffer (200 μl) was added to each sample and vortexed vigorously. Samples were boiled and vortexed again to release protein–antibody complexes from the protein A-agarose. The protein A-agarose was then separated from the immunoprecipitated complexes by centrifugation. The supernatants were saved, and the immunoprecipitated proteins were separated by non-reducing SDS–PAGE (8%) at constant voltage over-night, and then transferred to nitrocellulose for 4 h at 170 mA. The nitrocellulose was immersed in blocking solution [5% non-fat milk/Tris-buffered saline (TBS)] for 45 min, washed with TBS and 0.1% Tween and incubated with primary anti-human Nur77 or anti-human IGFBP-3 antibody (1:4000) for 2 h. After washing off any unbound antibodies, the nitrocellulose was incubated with a secondary antibody (1:10000) for 1 h. The membrane was developed with enhanced chemiluminescence reagents and the blots were developed using X-ray film.
washed four times with TBS. 0.1% Tween and TBS. Bands were visualized using the peroxidase-linked enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Experiments were repeated three times.

**Caspase assays**

The caspase assay was done using Apo-ONE™ homogenous caspase-3/7 assay (Promega) and performed according to the manufacturer's instructions. rhIGFBP-3 was used at final concentrations of 1–10 µg/ml. Two percent dimethylsulfoxide was used as a control in some experiments.

**Generation of Nur77−/− and wild-type primary mouse embryo fibroblast lines**

Nur77−/− and wild-type (WT) mice were generated as described previously (9). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% l-glutamine. All cells were used before passage 6.

**Kinase assay methods**

22RV1 CaP cells were incubated for 24 h in serum-free media in the presence or absence of 1 µg/ml recombinant human IGFBP-3. Akt kinase assay was assessed using a non-radioactive Akt kinase assay kit (Cell Signaling Technology) following the manufacturer's instructions. Briefly, Akt was immunoprecipitated from 100 µg cell lysate overnight using an immobilized Akt antibody. Immunoprecipitated protein was then incubated with 1 µg GSK-3 fusion protein in the presence of ATP for 30 min at 30°C. Proteins were separated by SDS–PAGE and analyzed by autoradiography for phospho and total GSK-3.

**Tumor xenografts**

The LAPC-4 human CaP cell line expresses a WT androgen receptor and was used for counterstaining. Sections were incubated over-night according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen and commercial hematoxylin was used for counterstaining.

**Immunohistochemical staining for Nur77**

Paraffin-embedded, 4 µm thick tissue sections of LAPC-4 xenografts were stained for the Nur77 protein. The sections were deparaffinized in a series of xylene baths and then rehydrated using a graded alcohol series. The sections were then immersed in methanol containing 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity and then incubated in 2.5% blocking serum to reduce non-specific binding. Sections were incubated over-night at 4°C with primary anti-Nur77 antibody (1:100). The sections were then processed using standard avidin–biotin immunohistochemistry techniques according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen and commercial hematoxylin was used for counterstaining.

**TUNEL staining**

Paraffin-embedded sections were prepared from LAPC-4 tumors harvested on day 21. After deparaffinization of tissue section, apoptotic DNA fragments were labeled by terminal deoxynucleotidyl transferase and detected by anti-digoxigenin antibody conjugated to fluorescein (ApopTag Fluorescein In Situ Apoptosis Detection kit, Chemicon, Temecula, CA). Cells were examined at ×20 using an inverted fluorescence microscope (Axiovert 135M, Carl Zeiss, New York, NY). Apoptotic staining was quantified by pixel histogram (Adobe Photoshop, Mountain View, CA) and confirmed by manual counting (r = 0.98).

**Statistical analysis**

All experiments were repeated at least three times. Means ± SDs are shown. Statistical analyses were performed using analysis of variance utilizing InStat (GraphPad, San Diego, CA). Differences were considered statistically significant when P < 0.005, denoted by **. 

**Results**

**Subcellular association of IGFBP-3 and Nur77**

We and others have described the physical interaction between IGFBP-3 and the nuclear receptors RXRα and RARα (10,11). We investigated the ability of IGFBP-3 to associate with Nur77 in nuclear and cytoplasmic compartments utilizing co-immunoprecipitation techniques. 22RV1 CaP cells were taken serum free overnight. Protein A-agarose was used to immunoprecipitate-bound complexes and these were resolved by SDS–PAGE.

Western immunoblotting (Figure 1) showed that endogenous IGFBP-3 associates with Nur77 in cytoplasmic fractions only and complexes were not detected in nuclear fractions. Controls of whole-cell lysate show presence of endogenous Nur77 and IGFBP-3 in the immunoprecipitation input. CCRF-CEM is a T lymphoblastoid cell line (ATCC), and nuclear extract was used as a positive control for Nur77. The major band of endogenous IGFBP-3 was detected in the nucleus, consistent with our previous observations (12). Similarly, Nur77 is typically a nuclear transcription factor. This observation is indicative of distinctive subcellular co-distribution whereby unassociated Nur77 and IGFBP-3 are prevalent in the nucleus, but their complexes are uniquely cytoplasmic.

**Contribution of Nur77 to the apoptotic effects of IGFBP-3**

To further study the functional interface of IGFBP-3 and Nur77, we derived Nur77 null (Nur77−/−) embryonic fibroblast cells (MEFs) from the Nur77−/− knockout (KO) mouse. Using these Nur77 null MEFs, we tested the ability of IGFBP-3 to induce apoptosis (Figure 2A). Overnight treatment with IGFBP-3 resulted in a 60% increase in apoptosis as measured by fluorometric measurement of caspase-3/7 activation in fibroblasts derived from the WT animal, but was not able to induce further caspase activation in the line derived from the Nur77−/− KO animal. The phenomenon was specific to IGFBP-3-induced apoptosis as caspase activation induced by 2% dimethylsulfoxide was not different in WT versus KO MEFs (Figure 2B). Thus, Nur77 significantly contributes to the pro-apoptotic effect of IGFBP-3 at this dose.

To more closely examine the role of Nur77 in IGFBP-3-induced apoptosis, we treated Nur77 KO and WT MEFs with increasing doses of recombinant IGFBP-3 that ranged from 1 to 10 µg/ml >12 h (Figure 2C). As expected from the former experiment, IGFBP-3 significantly induced apoptosis in WT MEFs in a dose-dependent manner. Surprisingly, IGFBP-3 also significantly induced caspase activation in the KO line in a dose-dependent manner, although this was minimal when compared with the WT line. However, this does suggest that a small portion of IGFBP-3-induced caspase activation may be Nur77 independent, although the functional relevance of this is currently unknown.

**Reintroduction of Nur77 in Nur77 KO MEFs restores responsiveness to IGFBP-3**

To validate our findings of Nur77 as a mediator of IGFBP-3 action, we reintroduced Nur77 into Nur77 KO MEFs by transient transfection, with and without co-expression of IGFBP-3. Control transfection was with empty expression vectors and was not significantly different from that of transfection with empty Nur77 expression vector and IGFBP-3 over-expression. Over-expression of Nur77 in combination with empty IGFBP-3 expression vector did induce apoptosis activation consistent with previous reports (13,14). Reintroduction of Nur77 via transient transfection restored responsiveness to IGFBP-3
over-expression in the Nur77 KO line (Figure 3). Thus, rescue of IGFBP-3-induced apoptosis was associated with restoration of Nur77 expression.

**IGFBP-3 induces phosphorylation of c-JNK and suppression of Akt phosphorylation and activity**

Nur77 is phosphorylated by JNK and by Akt (15–17). This phosphorylation is required for its nuclear export and involves JNK activation (phosphorylation) and inhibition of Akt phosphorylation in lung cancer and HEK cells (18). We investigated the effect of IGFBP-3 treatment of cancer cells on JNK phosphorylation and Akt phosphorylation and activation. Treatment with 1 μg/ml of IGFBP-3 for 24 h induced phosphorylation of JNK in 22RV1 CaP cells although the expression levels of total JNK were not changed (Figure 4C). Associated with this is a significant reduction in phosphorylated Akt and Akt activity as evidenced by an Akt kinase assay (Figure 4A and B). Thus, the apoptotic action of IGFBP-3, which we have described previously to involve RXR/Nur77 translocation via an IGF-independent mechanism (5), involves inhibition of Akt and phosphorylation of JNK.

**IGFBP-3 induces apoptosis and translocates Nur77 in vivo**

To determine the effect of IGFBP-3 on Nur77 translocation and apoptosis in vivo, we utilized LAPC-4 cells in Matrigel to create human CaP xenografts on SCID mice. Accordingly, cells were injected and tumors established for 2 weeks. At that time, IGFBP-3 or saline was given as a daily injection at a dose of 4 mg/kg/day intra-peritoneally for 21 days, after which mice were euthanized. Tumor sections were stained with a Nur77 antibody to assess subcellular localization in response to IGFBP-3 as well as subjected to TUNEL staining as a marker for apoptosis. Consistent with our in vitro data, Nur77 exhibited a predominantly nuclear staining pattern in the rapidly growing control tumors (Figure 5A, upper panels). Treatment with IGFBP-3 resulted in predominantly cytoplasmic staining of Nur77 with hematoxylin-counterstained nuclei prominent (Figure 5A, lower panels). Associated with this was a marked significant increase in staining for TUNEL as a marker of apoptosis (Figure 5B). In concert, these data support a novel extranuclear mechanistic role for the orphan receptor Nur77 in mediating the apoptotic actions of IGFBP-3 in vitro and in vivo.

**Discussion**

In the prostate, IGFs and IGFBPs play an important role in the proliferative processes that lead to benign prostatic hyperplasia and CaP. IGFBP-3 is secreted and reuptaken by endocytic pathways (specifically caveolin and transferrin receptor-mediated) for apoptosis induced by transforming growth factor-β (12). After internalization, IGFBP-3 rapidly localizes to the nucleus where it interacts with RXRα, RAR and potentially RNA Polymerase II binding subunit as recently described (10,11,19), implicating IGFBP-3 as a potential direct modulator of gene transcription. Indeed, IGFBP-3 modulates signaling of nuclear receptor ligands and alters their action (20). Nuclear import of IGFBP-3 is a nuclear localization signal-dependent process and mediated by binding to importin-β (21). The first in vivo validation for IGF-independent actions of IGFBP-3 on tumor suppression has recently been published in a mouse model of CaP (4). Important findings in this study include the
Following: (i) dramatic tumor suppressive effects by a non-IGF-binding mutant of IGFBP-3, (ii) observation that autocrine/paracrine IGFBP-3 expression correlated with tumor suppression, rather than circulating serum levels, and (iii) no obvious effect of IGFBP-3 on non-cancerous tissues. IGFBP-3 transgenic mice have been described to be modestly growth restricted and glucose intolerant, in mechanisms that may be largely related to IGF inhibition (22). However, in vitro evidence would suggest that this too may also involve IGF-independent effects (23). It is probable that growth inhibiting and apoptosis-promoting effects by IGFBP-3 in the whole animal involve IGF-dependent and -independent mechanisms and both are major contributors to tumor suppression.

Although we have demonstrated using the Nur77 KO MEFs that a significant portion of IGFBP-3-induced caspase activation is Nur77 dependent, we were able to show a small but significant contribution that is Nur77 independent. This may be a cell type-specific phenomena or may involve activation of other various pathways that have been described for IGFBP-3 including the following: (i) activation of caspase 8 and 9 (24), (ii) cell-surface receptors (25), (iii) Stat1 activation (26), (iv) SMAD inhibition (27), (v) phosphatase activation (28) and (vi) calcium flux regulation (29).

Fig. 4. IGFBP-3 induces phosphorylation of c-JNK and suppression of Akt phosphorylation. 22RV1 CaP cells were incubated in serum-free media for 24 h in the presence or absence of 1 μg/ml IGFBP-3. Experiments were repeated three times. (A) Western immunoblot for phospho- and total Akt. (B) Akt kinase assay as assessed by phosphorylation of a GSK-3 fusion protein after immunoprecipitation of Akt. (C) Western immunoblot of phospho- and total JNK. H2O2 was used as a positive control for p-JNK activation. Experiments were repeated three times.

Fig. 5. IGFBP-3-induced apoptosis involves translocation of Nur77 in CaP xenografts in vivo. (A) Cross-sectional LAPC-4 tumors stained with anti-Nur77 (×100, oil immersion). Diaminobenzidine was used as a chromogen (dark brown) and commercial hematoxylin was used for counterstaining (blue nuclei). Note predominant nuclear Nur77 staining in control tumor versus empty blue nuclei in the IGFBP-3-treated tumor. Top panel is ×40 magnification; lower panel is ×100 magnification (oil immersion). Arrows indicate nuclei in which Nur77 is intra-nuclearly stained or translocated to the mitochondria. Control immunohistochemistry shows competition of signal by blocking peptide, as well as lack of binding of secondary antibody. (See Supplementary material, available at Carcinogenesis Online, for coloured figure.) (B) Quantitation of TUNEL staining was used as a measure of apoptosis. TUNEL staining was quantitated by pixel histogram as indicated in bar graph. *P < 0.005 relative to control treatment.
have additionally identified DNA protein kinase to phosphorylate IGFBP-3 in vivo and that this phosphorylation regulates nuclear localization (38,39). Additionally, casein-kinase-2 has been shown to phosphorylate and negatively regulate IGFBP-3 (40). Complex kinase networks that control phosphorylation of both Nur77 and IGFBP-3 underscore the importance of multiple regulatory levels to determine activity of both these molecules and their subcellular localization. In summary, the current work and previous work done by our group shows that IGFBP-3 is secreted and reuptaken by endocytic mechanisms to the nucleus (12). In the nucleus it interacts with the nuclear receptor RXR, where binding may expose a nuclear export sequence described previously in the DNA-binding domain of RXR (41) and indirectly enhance Nur77 binding to RXR (5). Conformation of this complex may further be changed after export to the cytoplasm where IGFBP-3 directly interacts with Nur77 as described in the current study. Immunohistochemical demonstration of the ability of IGFBP-3 to translocate Nur77 in vivo offers an output measure to assess IGFBP-3 efficacy in the clinical arena. Further work, including mitochondrial targeting of this complex, is needed to unravel the molecular mechanisms of IGFBP-3 intracellular activity and its potential clinical application.

Supplementary material

Supplementary figure can be found at http://carcin.oxfordjournals.org/.

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References


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