Direct Functional Interactions between Insulin-like Growth Factor-binding Protein-3 and Retinoid X Receptor-α Regulate Transcriptional Signaling and Apoptosis*

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Insulin-like growth factor-binding protein (IGFBP)-3 regulates apoptosis in an IGF-independent fashion and has been shown to localize to nuclei. We cloned the nuclear receptor retinoid X receptor-α (RXR-α) as an IGFBP-3 protein partner in a yeast two-hybrid screen. Multiple methodologies showed that IGFBP-3 and RXR-α bind each other within the nucleus. IGFBP-3-induced apoptosis was abolished in RXR-α-knockout cells. IGFBP-3 and RXR ligands were additive in inducing apoptosis in prostate cancer cells. IGFBP-3 enhanced RXR response element and inhibited RARE signaling. Thus, RXR-α-IGFBP-3 interaction leads to modulation of the transcriptional activity of RXR-α and is essential for mediating the effects of IGFBP-3 on apoptosis.

The insulin-like growth factor (IGF)-1-binding proteins (IGFBPs) are a family of proteins that bind IGFs with high affinity and specificity. They modulate IGF action by inhibiting or potentiating IGF binding to the IGF receptor. There are six high-affinity IGFBPs, of which IGFBP-3 is the most abundant in serum (1). Circulating IGFBP-3 is derived mainly from hepatic Kupffer cells, primarily under regulation by growth hormone, but IGFBP-3 is also produced locally in many tissues, where it serves important paracrine and autocrine roles in modulating cellular growth (2). In cells, IGFBP-3 is potently regulated by a number of factors including p53 (3), transforming growth factor-β (4), and hypoxia-induced factor-1 (5). IGFBP-3 has been shown to directly induce apoptosis in prostate cancer cells (6), breast cancer cells (7), and other cell types. In these instances, IGFBP-3 acts directly, independently of the IGF-IGF receptor system, by binding to its own receptor(s), the nature of which is currently being unraveled.

The ability of IGFBP-3 to bind other molecules in addition to the IGFs has been previously demonstrated. IGFBP-3 binds to ALS, which together with IGF forms a stable ternary complex in serum (8). IGFBP-3 can undergo post-translational modification by proteases and can form an intermediary complex with plasmin and related enzymes (9). IGFBP-3 is noted to have a heparin-binding domain in its mid-region and is known to interact with heparin-containing molecules in the extracellular matrix (10) and with fibrin (11). Several groups, including our own, have demonstrated specific binding of IGFBP-3 to other uncharacterized proteins in serum (12), cell lysates (13), and cellular membranes (6). It has also been proposed that IGFBP-3 may share a common receptor with transforming growth factor-β (14). It is of yet unclear what role these interactions play in mediating IGFBP-3 direct actions on cells.

IGFBP-3 has been observed in the nucleus of certain cells and contains a nuclear localization sequence that may facilitate shuttling into nuclei (15–17). The role of nuclear IGFBP-3 is currently unknown, but the cellular effects of IGFBP-3 appear to involve modulation of gene transcription, and thus, interactions of IGFBP-3 with transcription factors have been hypothesized.

The retinoid X receptor-α (RXR-α) serves a key role in the regulation of gene transcription mediated by a variety of factors (18). RXR-α is an obligate co-factor for the retinoic acid receptors, RARs (19), the peroxisome proliferator activating receptors (20), the thyroid receptors (21), and the vitamin D receptors (22). RXR-α can form heterodimers with these nuclear transcription factors and signal through specific DNA response elements such as the RARE, peroxisome proliferator receptor element, thyroid receptor element, and vitamin D receptor elements (23), or form homodimers and signal through RXRE (24). The activity of these transcriptional dimers is further regulated by a variety of transcriptional co-activators and co-inhibitors that modulate gene transcription in various states (25).

We report here the identification of a novel partner for IGFBP-3 in the form of the nuclear receptor RXR-α, confirm the validity of RXR-α/IGFBP-3 binding through multiple independent in vitro methods, and demonstrate physiologically significant consequences of RXR-α/IGFBP-3 binding on transcrip-
tional signaling and cellular apoptosis. This unexpected interaction between the IGF/BP growth factor cascade and nuclear receptors represents a novel paradigm shift in our understanding of these systems.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protigene (Mountain View, CA) provided recombinant human IGFBP-3 and the NLS mutant IGFBP-3. Amersham Pharmacia Biotech (Sweden) provided recombinant human IGF-I. Lizand (San Diego, CA) and SRI International (Menlo Park, CA) provided the RXR-specific ligands LG1069 and SR11235, respectively. These ligands bind the retinoid receptor X exclusively with maximal binding achieved at 1 μM (26). 125I-IGFBP-3 and anti-human IGFBP-3 antibodies, which were affinity purified on an IGFBP-3 column, were purchased from DLSL (Webster, TX). Anti-human RXR-α antibodies, RAR antibodies, and HeLa nuclear extracts were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). IGFBP-3 blocking peptides were purchased from Genemed Synthesis (South San Francisco, CA). Retinoic acid, dimethyl sulfoxide, and Igepal CA-630 were purchased from Sigma. Tris (crystalized free base) was purchased from Fisher (Fair Lawn, NJ). SDS-polyacrylamide gel electrophoresis (PAGE) reagents, Tween, and fat-free milk were purchased from Bio-Rad. Yeast two-hybrid screening kits were purchased from CLONTECH (Palo Alto, CA). The HeLa cDNA library from Stratagene (La Jolla, CA), and SRI International (Menlo Park, CA) provided the RXR-α and RXR-β cDNAs, respectively. The RXR-α cDNA was isolated by plasmid recovery, amplified using polymerase chain reaction, sequenced using the manufacturer’s instructions (CLONTECH Matchmaker protocol handbook). The RXR-β cDNA was purchased from CLONTECH. The fusion gene IGFBP3/3-AD was constructed by splicing cDNA encoding the human IGFBP-3 gene into the plasmid pGBT9 directly 5’ to and in-phase with the gene encoding the binding domain (BD) of the yeast transcriptional activator GALA (CLONTECH). A HeLa cDNA library with the activation domain (AD) of the GALA gene was purchased from Stratagene and screened by co-transforming yeast with both plasmids. Yeast colonies were made competent for transformations according to the manufacturer’s instructions (CLONTECH Matchmaker protocol handbook). Positive co-transformants were selected by growth on histidine-deficient agar media and assayed for β-galactosidase activity according to the manufacturer’s instructions (CLONTECH Matchmaker protocol handbook). All results were reproducible in at least two independent assays. Genes encoding IGFBP-3-binding proteins identified through this method were isolated by plasmid recovery, amplified using polymerase chain reaction, sequenced using the manufacturer’s instructions (CLONTECH Matchmaker protocol handbook). The full-length RXR-α cDNA was provided by Dr. D. J. Mangelsdorf and has been previously described (27). GST-RXR-α fusion protein was produced in Escherichia coli DH5α, transformed with a GST-RRX-α construct, which were lysed and loaded on glutathione-Sepharose 4B beads from Sigma. 10 μg of purified GST-RXR-α bound to beads were incubated with 5 μg of recombinant IGFBP-3 protein or IGFBP-3 mutants and then separated by centrifugation. The bound proteins were analyzed by nonreducing SDS-PAGE gel and autoradiography. Experiments were repeated three times.

**Immunoprecipitation and Autoradiography**—125I-IGFBP-3 was incubated with 10 μg of HeLa nuclear extracts for 3 h at room temperature, and then with 5 μl of IGFBP-3, RRX-α, or RAR antibodies overnight. Complexes were then immunoprecipitated with protein A-agarose as above. Samples were subjected to separation on 12.5% nonreducing SDS-PAGE gel and autoradiography. Experiments were repeated three times.

**Gel Mobility Shift Assays**—γ-32PATP-labeled RXRE or RARE were incubated with HeLa nuclear extracts in 25 μl of binding buffer containing 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 0.2% Nonidet P-40, 20 μg of bovine serum albumin, 36 μg of salmon sperm DNA, and 10% glycerol at 25 °C for 20 min. Mixtures were incubated with or without IGFBP-3 or RAR-α antibodies for an additional 30 min. Incubations were carried out with or without unlabeled competitors as indicated. Protein-DNA complexes were separated from free probe on a 4.5% polyacrylamide gel in 1× TGE at 12 V/cm for 3 h, and visualized by autoradiography. Experiments were repeated three times.

**Tissue Culture**—COS-7 cells, F9 embryonal carcinoma cells from ATCC, and F9 RXR-α−/− cells (28) were routinely maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc.), 100 units of penicillin/ml, and 100 units of streptomycin/ml in a humidified environment with 5% CO2. PC-3 cells from ATCC were cultured in F12K medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units of penicillin/streptomycin per ml in a humidified environment with 5% CO2. LAPC-4 cells (29) were cultured in Iscove’s modified Dulbecco’s medium containing 10% fetal bovine serum (Quimexa), 1% l-glutamine, 1% R1181 (NEN Life Science Products Inc.).

**Transient Transfection Analysis**—Cells grown in 24-well culture plates were transfected with the appropriate combination of expression plasmids using LipofectAMINE (Life Technologies, Inc.). The total amount of plasmid DNA was adjusted to 500 ng/plate. The transfection solution was removed after 6 h of transfection, and the cells were cultured for 24 h. Cells were then incubated with or without 1 μM retinoic acid or RXR ligand overnight. Cells were subjected to luciferase assays as described previously (30). Luciferase activities were expressed as the means and standard deviations of five identical wells. Luciferase reporter plasmid constructs contained either the DR1 RXRE (AGGTCA), or the DR5 RARE (AGTTCA) in a direct repeat separated by 12 bp and five nucleotides in the context of a thymidine kinase promoter, or a control plasmid containing the thymidine kinase promoter but no response element (TK-LUC) (31). The full-length IGFBP3 cDNA was constructed in the expression vector (pGK3226) and co-transfected when indicated. Experiments were repeated three times.

**Cell Viability Assays**—Cells were plated at a starting density of 250 cells/well in 96-well plates and treated with varying doses of IGFBP-3 for 3 days. Cell number was determined as follows: the fluorescent dye...
calf AM (1 ml) was added to the medium for 30 min prior to termination of the assay. The plate was then read on a Biolumin 960 fluorescence plate reader (Molecular Dynamics, Inc.). The excitation and emission wavelengths are 485 and 530 nm, respectively, for calf AM. The intensity of fluorescence of enzymatically cleaved calcein AM is a positive measure of cell number. Values are expressed as mean ± S.D.

**RESULTS**

**Cloning of RXR-α as an IGFBP-3 Partner—**A yeast two-hybrid system was used to identify novel IGFBP-3 partners. We utilized the yeast strain HT107 (which is unable to grow in histidine, leucine, or tryptophan-deficient media) with a histidine-selection marker and β-galactosidase marker under control of the GAL1 promoter. We performed co-transformations with: 1) pGBT9 plasmid containing the fusion gene IGFBP-3/GAL1 promoter, and a histidine-selection marker; 2) pGAD424 plasmid containing a HeLa cell cDNA library/GAL4 AD fusion gene, a tryptophan-selection marker. Positive co-transformations were isolated by growth on tryptophan-, leucine- and histidine-deficient media, and colonies with β-galactosidase activity were harvested to recover library plasmids. Library fragments were amplified using PCR and sequenced. We isolated a 1200-base pair cDNA fragment encoding the C-terminal portion of the human RXR-α gene followed by the 3’-untranslated region of the human RXR-α cDNA. Yeast mating experiments confirmed the interaction between IGFBP-3 and RXR-α and co-transfected colonies displayed potent β-galactosidase activity, which was completely absent in single transformants or in co-transformations of either hybrid with an empty vector. The RXR clone was one of 5 positive clones that reproducibly bound IGFBP-3 in the yeast two-hybrid screen. It was cloned out in four separate experiments.

**Verification and Characterization of IGFBP-3-RXR-α Binding—**In order to investigate the specificity of RXR-α binding to IGFBP-3, we performed GST pull-down experiments using RXR-α linked to GST and various forms of IGFBP-3 proteins. GST-RXR-α was able to “pull-down” various forms of natural and recombinant IGFBP-3. As shown in **Fig. 1**, GST-RXR-α bound recombinant IGFBP-3 as well as the NLS mutant IGFBP-3, which has 2 amino acids mutated in the NLS region of IGFBP-3 (Table I). However, the IGFBP-3-HBD-BP1 mutant, which has 2 amino acids mutated in the NLS region of IGFBP-3, we performed GST pull-down experiments using IGFBP-3-HBD-BP1. GST pull-down experiments confirmed IGFBP-3 binding to RXR-α.

**TABLE I**

| Wild type IGFBP-3 | 215 KKGFYKKKQCRPSKGKKR | 220 CRR  |
| NLS mutant IGFBP-3 | 215 KKGFYKKKQCRPSGCKR | 220 CRR  |
| IGFBP-3-HBD-BP1 | 215 KNQFTSHRQCEITESMDGEA | 220 CRR  |

**FIG. 1.** GST-RXR-α pull-down IGFBP-3. Recombinant E. coli-derived 29-kDa IGFBP-3 protein, E. coli-derived 29-kDa IGFBP-3 NLS mutant, and baculovirus-derived 35-kDa IGFBP-3-HBD-BP1 mutant incubated with or without GST-RXR-α fusion protein, which was loaded on glucose-Sepharose 4B beads. Proteins were analyzed by Western immunoblotting using anti-IGFBP-3 antibodies. RXR-α bound the wild type and the NLS mutant, but not the HBD-BP1 mutant.
IGFBP-3 (data not shown). As shown in Fig. 2A, there was no appreciable binding of IGFBP-3 to either human or bovine albumin nor to insulin. The N terminus peptide of IGFBP-3 blocked IGF-I binding, but had no effect on RXR-α binding (Fig. 2B), whereas the HBD peptide blocked RXR-α binding, but had no effect on IGF-I binding to IGFBP-3 (Fig. 2B). This indicates that RXR-α binds the N terminus domain of IGFBP-3, while IGF-I binding involves the N-terminal domain. In additional blots (data not shown) insulin, human albumin, and bovine albumin were dot blotted and probed with radiolabeled IGFBP-3 and showed no binding, indicating that RXR-IGFBP-3 binding is specific.

In order to further investigate the specificity of RXR-α binding to IGFBP-3 in vivo, we performed co-immunoprecipitation experiments. In Fig. 3A, we used anti-IGFBP-3 and anti-RXR-α antibodies to immunoprecipitate these proteins and any interacting molecules from HeLa nuclear extracts then immunoblotted the samples with protein A-agarose and separated on 12.5% SDS-PAGE. Both the IGFBP-3 antibodies immunoprecipitated all of the IGFBP-3 in the nuclear extracts samples and 29 ± 11% of the RXR-α available in the nuclear extracts. RXR-α antibodies successfully precipitated all the RXR-α in nuclear extracts and more than 84 ± 17% of the IGFBP-3 in the nuclear extract samples.

In Fig. 3B, 125I-IGFBP-3 was incubated with HeLa nuclear extracts. Antibodies for IGFBP-3, RXR-α, or RAR were bound with protein A-agarose and then further incubated with the nuclear extract mixtures. After precipitation, proteins were separated on 12.5% SDS-PAGE and autoradiographed. Both the IGFBP-3 antibody and the RXR-α antibody precipitated a complex of 125I-IGFBP-3 and RXR-α, whereas an RAR antibody did not precipitate IGFBP-3. This indicates that the IGFBP-3-RXR-α binding is specific and that IGFBP-3 only binds RXR-RAR heterodimers.

Cellular Co-localization of RXR-α and IGFBP-3—Using fluorescence immunocytochemistry with antibodies for IGFBP-3 and RXR-α, confocal microscopy revealed that IGFBP-3 and RXR-α were present in both the cytoplasm and nucleus of LAPC-4 cells (Fig. 4) and PC-3 cells (data not shown). Moreover, IGFBP-3 and RXR-α co-localized to a high degree in both cellular compartments. Furthermore, after the cells were treated with the RXR ligand LG1069 for 24 h, cytoplasmic IGFBP-3 and RXR-α both translocated to the nucleus as shown in the lower panel of Fig. 4. Similar results were seen upon treatment with 9-cis-RA (data not shown).

Interactions of IGFBP-3 with the RXR-α/RXRE Complex—Fig. 5 demonstrates the interactions of IGFBP-3 with the DNA-transcription factor complex involving RXR-α and the RXR response element (RXRE) in electromobility shift assays. HeLa nuclear extracts were incubated with 32P-labeled DR-1 RXRE, then separated by 4.5% polyacrylamide gel. Specific binding of RXR-α in HeLa nuclear extracts to the DR-1 RXRE was demonstrated in lanes 1–3. As expected, the addition of an RXR-α antibody in lane 8 supershifts the complex. The addition of an IGFBP-3 antibody in lane 4 also supershifts the complex, indi-
cating that IGFBP-3 is bound to the RXR-RXR-α complex. Similarly labeled DR-5 RARE binds RAR in HeLa nuclear extracts (lane 5), but this complex did not supershift with an IGFBP-3 antibody, suggesting that IGFBP-3 only forms a complex with the RXR-RXR homodimer, not with RXR-RAR heterodimers. Conducting these experiments in the presence of RXR-specific ligands had no effect on the ability of IGFBP-3 antibodies to supershift the complex, suggesting that RXR-α binds IGFBP-3 at a site different than the ligand-binding domain of the RXR-α molecule.

Effects of IGFBP-3 on RXR-α-mediated Signaling—In luciferase-based transcriptional assays, we used the DR1-RXRE and the DR5-RARE reporter systems in COS7 cells (data not shown). In both cases, luciferase signaling was enhanced by co-treatment with the appropriate ligand (SR11235 or RA). However, IGFBP-3 co-transfections potently and dose-dependently inhibited RA signaling via RARE, but enhanced RXR-specific ligand signaling via the RXRE, indicating that IGFBP-3 enhances RXR-RXR homodimer-mediated signaling via the RXRE, but blocks RAR-RXR heterodimer-mediated signaling via the RARE.

Requirement of RXR-α for IGFBP-3 Actions—To further study the functional interface of IGFBP-3 and RXR-α in the nucleus, we performed viability assays utilizing the F9 embryonic carcinoma cell line and a sister cell line, in which RXR-α has been knocked out. IGFBP-3 treatment dramatically reduced cell viability in the F9 cell line, but IGFBP-3 had no discernible effects in the RXR-α knock out line, indicating that RXR-α is required for IGFBP-3 induced apoptosis. IGFBP-3...
In this report, we describe for the first time the identification of RXR-α as an IGFBP-3-binding protein/receptor. After cloning RXR-α in a two-hybrid screen, we have demonstrated specific RXR-α to IGFBP-3 binding through several in vitro methods, including GST-pull down, co-immunoprecipitation, Western blot techniques, and confocal microscopy. These studies demonstrated not only that RXR-α associates with IGFBP-3 in nuclei, but that this binding occurs at a specific region of IGFBP-3, near the NLS/HBD domain.

We have further demonstrated physiologically significant ramifications of RXR-α/IGFBP-3 interactions on the modulation of cell proliferation and apoptosis in several mammalian cellular systems. RXR-α agonists and IGFBP-3 are both growth inhibitory in many cancer cells (38), and the co-incubation of these molecules in the LAPC-4 model resulted in an additive effect on apoptosis. This phenomenon is consistent with IGFBP-3 binding RXR dimers, and with the ability of IGFBP-3 to enhance RXRE-mediated signaling. This observation suggests that retinoids may be more effective as anti-cancer agents in the presence of high IGFBP-3 levels. This is compatible with reports that show that high IGFBP-3 levels in serum protect from the risk of colon (39), breast (40), and prostate cancers (41).

The effects of IGFBP-3 on cell growth and apoptosis appear to require an intact RXR-signaling pathway, as RXR knockout cells were unresponsive to IGFBP-3-induced apoptosis. In mice, the targeted disruption of the RXR-α gene is embryonic lethal (42), however, cell-specific effects of RXR-α signaling has been unraveled with the use of the F9 cell system in which the essential role of RXR-α in mediating retinoid signaling has been established (43). The cell-regulatory effects of RXR-related transcriptional systems are very diverse. RXR-α is required for the actions of multiple natural ligands including thyroid hormone, vitamin D, and retinoic acid. RXR-α is also critical for the effects of several classes of novel drugs such as peroxisome proliferator activating receptor agonists, and synthetic retinoids, which are being developed as cancer therapies as well as agents for the treatment of diabetes and osteoporosis. The relationship between RXR-α and the IGF-IGFBP-3 axis is poorly understood. Retinoids enhance the expression of IGFBPs, including IGFBP-3, but retinoids do not (44). Since we observed that IGFBP-3 blocks RA-mediatedRAR signaling, this raises the possibility that a negative feedback loop which involves IGFBP-3 limits the extent of retinoid signaling through induction of IGFBP-3 which then blocks further RAR-mediated transcription. A further component of this loop may be related to the observation that IGF-I induces RAR-β expression (36) (which would also be blocked by IGFBP-3).

The discovery of RXR-α as an IGFBP-3 interacting protein adds a further level of complexity to the modulation of cellular growth. In addition to the modulation of IGF activity at the cell membrane and direct interactions with its own specific receptors, IGFBP-3 may also affect cell growth through several RXR-α-dependent mechanisms. Namely, IGFBP-3 could enhance retinoid action but block signaling mediated by ligands of other RXR-α partners. This represents a new paradigm in our understanding of the actions of peptide growth factors. As such, the IGFBP-3/RXR-α interaction represents an interface of two previously unrelated signaling pathways and opens new directions in studying cross-talk between growth factors and nuclear receptor ligands in cancer and other diseases.

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