BEYOND CARRIER PROTEINS

Nuclear effects: unexpected intracellular actions of insulin-like growth factor binding protein-3

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Abstract
Insulin-like growth factor (IGF) binding protein (IGFBP)-3 has been shown to be a growth inhibitory, apoptosis-inducing molecule by virtue of its ability to bind IGFs, in addition to previously demonstrated IGF-independent effects. The recent discovery of the interaction between nuclear IGFBP-3 and 9-cis retinoic acid receptor-α (retinoid X receptor α RXRα), a nuclear receptor, and its involvement in the regulation of transcriptional signaling and apoptosis represents an important paradigm shift in the understanding of IGFBP function. RXRα is required for the apoptosis-inducing effects of IGFBP-3. IGFBP-3 and RXR ligands are additive in inducing apoptosis in cancer cells. IGFBP-3 has direct effects on gene transcription, as RXR response element reporter signaling was enhanced and the all-trans retinoic acid receptor response element reporter signaling was inhibited. Accumulating evidence further confirms IGF-independent functions of this multifunction binding protein. Other binding proteins, in addition to other members of the IGF axis, have now been described in the nucleus and are postulated to have effects on transcriptional events. Investigation into these new interactions will expose new protein partners in the interface between the nuclear receptor and growth factor pathways and reveal new targets to be exploited in the treatment of cancer and other diseases.

Introduction
The growth-promoting and metabolic activities of the insulin-like growth factors (IGFs)-I and -II are modulated by a family of six high-affinity IGF-binding proteins (IGFBPs) and two IGF receptors (IGF-IR and IGF-IIR), most of the biological functions of the IGFs being mediated via the type-I receptor (LeRoith 1996, Wetterau et al. 1999). The actions of IGFs may be modulated by the IGFBPs in either a positive or a negative way, depending on tissue type and physiological/pathological status (Rajaram et al. 1997).

The majority of IGFs-I and -II in serum are found in a 150 kDa ternary complex formed by an IGF, IGFBP-3/IGFBP-5, and a glycoprotein known as the acid-labile subunit (ALS) (Baxter 1994). This ternary complex increases the half-life of IGF-I or IGFBP-3 an order of magnitude over those of the free forms (Hasegawa et al. 1995). Most tissue IGFs are bound to IGFBPs as binary complexes, leaving only small amounts of local free IGF. The liver produces most of the circulating IGFs, although physiologically important autocrine and paracrine production occurs within other tissues (Cohen & Rosenfeld 1995).

The first five IGFBPs demonstrate high affinity for both IGFs-I and -II, share at least 50% homology among themselves, and share 80% homology between different species (Lamson et al. 1991, Shimasaki & Ling 1991). Homology is most conserved at the amino (N)- and carboxy (C)-terminal regions, which are involved in IGF binding (Bramani et al. 1999, Imai et al. 2000, Song et al. 2000). IGFBP-6 has 100-fold greater affinity for IGF-II than for IGF-I (Baxter & Saunders 1992). Recent experiments in neuroblastoma (Grellier et al. 2002), colon cancer (Kim et al. 2002), and rhabdomyosarcoma (Galicchio et al. 2001) cells suggest that growth inhibition may result, at least in part, from IGFBP-6-mediated disruption of the IGF-II autocrine loop in these cancer cells.

IGFBP genes lie in close proximity to homeobox gene clusters HoxA to HoxD, which produce DNA-binding proteins that are widely expressed in multicellular...
organisms and encode transcription factors that are crucial for early development. This has been suggested to be a result of linkage before an initial duplication event, implying conservation of their important regulatory processes (Allander et al. 1993). The IGF–IGFBP axis is summarized in Fig. 1.

**IGFBP-3 – a biological mediator of cancer cell apoptosis**

Growth factors in general, and the IGF system in particular, have crucial roles in normal cell proliferation and malignant transformation (Aaronson 1991, Baserga 1995, Werner & LeRoith 1996). Dysregulation at several levels of the axis occur in human cancer (LeRoith et al. 1995).

This axis has been characterized in many human cancer models, including breast (Sachdev & Yee 2001), hepatocellular (Hanafusa et al. 2002), lung (Wegmann et al. 1993), colon (Giovannucci 2001), ovary (Krywicki et al. 1993), testes (Drescher et al. 1997), leukemia (Wex et al. 1998), adrenal (Bouille et al. 2001), brain (Zumkeller & Westphal 2001) and prostate (Djavan et al. 2001).

*In vitro*, IGFBP-3 both enhances and inhibits IGF-I actions, which was originally believed to be solely through regulation of free IGF bioavailability via high-affinity binding and thereby IGF mitogenic activity (Cohen et al. 1989, Gopinath et al. 1989, Conover et al. 1990, 2000, Schmid et al. 1991, Ramagnolo et al. 1994, Martin et al. 1995). *In vivo*, IGFBP-3 transgenic mice exhibit significant reduction in both birth weight and litter size, with a reduction in some organ weights (Modric et al. 2001), and non-small-cell lung cancer tumors stably transfected with IGFBP-3 exhibited a reduction in size compared with controls when transplanted into nude mice (Hochscheid et al. 2000).

This critical role of IGFBP-3 as a gene that may be inactivated on the cellular path to immortalization is supported by the fact that IGFBP-3 gene expression is uniformly lost in human prostate cancer cells and xenografts, and it was detected in DNA microarray analysis of cancerous compared with non-cancerous cells (Schwarze et al. 2002). Decreased IGFBP-3 expression is associated with prostate cancer progression, demonstrating more frequent loss of expression in advanced disease, in both human and mouse models (Hampel et al. 1998, Grimberg & Cohen 1999, Kaplan et al. 1999). In addition, in a recent experiment that sought to identify genes induced by the inhibitory all-trans-retinoic acid in lung epithelial carcinoma cells, IGFBP-3 was identified as one of 20 upregulated genes in synchronized SCC-25 cells (Le et al. 2002). Indeed, IGFBP-3 is the target of the E7 protein encoded by human papillomavirus type 16, one of the few viral genes that can immortalize primary human cells and thereby override cellular senescence. IGFBP-3-mediated apoptosis is inhibited by E7, which binds to IGFBP-3 and triggers its proteolytic cleavage. Two

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**Figure 1** A schematic representation of the IGF–IGFBP axis and its interacting molecules. See text for details.
transformation-deficient mutants of E7 failed to inactivate IGFBP-3, suggesting that inactivation of IGFBP-3 may contribute to cellular transformation (Mannhardt et al. 2000).

New paradigms of IGFBP actions – IGF-independent effects

Transfection of the IGFBP-3 gene into murine fibroblasts inhibited cell growth by a mechanism that was not reversible by the addition of excess insulin, even though insulin has mitogenic activity in these cells, does not bind IGFBP-3, and would presumably saturate the IGF-IR (Cohen et al. 1993). Subsequently, it was shown that a proteolyzed fragment of IGFBP-3, with negligible binding affinity for IGF-I and presumably none for insulin, inhibited insulin- and IGF-I-stimulated DNA synthesis in chick embryo fibroblasts (Lalou et al. 1996). Additional experiments with this fragment showed inhibition of mitogenesis in murine fibroblasts with a defective IGF-IR that could respond to basic fibroblast growth factor but not IGF, epidermal growth factor, or platelet-derived growth factor (Zadeh & Binoux 1997).

In 1993, our laboratory provided the first direct evidence for IGF-independent apoptotic effects of IGFBP-3 using a mouse fibroblast cell line that lacked IGF-I receptors. The IGFBP-3 gene was transfected into a cell line derived from an IGF receptor ‘knockout’ mouse that is not IGF-responsive. Cell growth decreased significantly in this model, showing that IGFBP-3 inhibits cell growth independently of the type-I IGF receptor (Cohen et al. 1993). Previously, IGF-independent effects of IGFBP-3 had been suggested in a breast carcinoma line, utilizing IGF analogs with decreased affinity for IGFBP-3 (Oh et al. 1993). Since then, several laboratories have confirmed this observation in a variety of cellular systems (Gill et al. 1997, Wu et al. 2000, Spagnoli et al. 2001), culminating recently in the creation of IGFBP-3 mutants that minimally bind IGFs and still induce apoptosis in prostate cancer cells (Buckway et al. 2001, Hong et al. 2002).


The search for IGFBP-3 binding partners

At the cellular level, we and others have described several putative IGFBP-3 binding partners on the cell surface, and in the cytoplasm (Oh et al. 1993, Rajah et al. 1997, Wu et al. 2000, Knudston et al. 2001) and extracellular matrix (Booth et al. 1996). The suggestion that the type V TGF-β receptor is the putative IGFBP-3 receptor (Leal et al. 1997, 1999) has not been reproduced and probably represents an extracellular matrix protein association. Recently, IGFBP-3 has been shown to modulate the Stat-1 (Spagnoli et al. 2002) and Smad (Fanayan et al. 2002) signaling cascades, although the precise mechanism by which it interacts with these pathways remains to be established.

Nuclear localization of IGFBP-3

While we were investigating the IGF-independent effects of IGFBP-3 and observing ¹²⁵I-IGFBP-3 binding in nuclear extracts, a report emerged detailing the presence of IGFBP-3 in the nucleus in opossum kidney cells (Li et al. 1997); within the same year, endogenous IGFBP-3 was detected in the nuclei of A549 human lung cancer cells (Jaques et al. 1997), and soon thereafter it was found in the nuclei during cell division in human keratinocytes (Wright et al. 1998). Nuclear transport of IGFBP-3 was not unexpected, because both IGFBP-3 and -5 possess basic C-terminal nuclear localization signals (Radulescu 1994). Recombinant IGFBP-3s and -5 are actively translocated to the nucleus of human breast cancer cells (Schedlich et al. 1998) and this occurs via a nuclear localization signal–dependent pathway, mediated by the importin-β (Schedlich et al. 2000) nuclear transport factor. A new mechanism of IGF-independent IGFBP-3 action emerged when we cloned the nuclear retinoid X receptor α (RXRα) as an IGFBP-3 protein partner in a yeast two-hybrid screen (Liu et al. 2000).

The nuclear receptor superfamily

Lipophilic hormones exert genetic control of differentiation and homeostasis via a superfamily of highly related transcription factors known as the nuclear receptors (Mangelsdorf et al. 1995). Transcriptional regulation depends on specific and ordered interactions with DNA and on subsequent binding of co-repressor or co-activator proteins, or both (Kurokawa et al. 1995, Glass et al. 1997, Zamir et al. 1997). Small lipophilic molecules that serve as ligands tightly control the transcriptional activity of these receptors. Included in this family are many additional factors, known as orphan receptors, which have the characteristics of hormone receptors, but for which the bioactive ligands have not been identified at this time.
The DNA targets of nuclear receptors, known as hormone response elements (HREs), are the sequences through which receptors mediate the control of ligand-responsive genes. Most non-steroid and orphan receptors recognize the consensus sequence 5′-AGGTCA-3′ in DNA that contains one or two copies of this sequence. This group includes nearly all known non-steroid receptors, including the 9-cis retinoic acid receptor (the retinoid X receptor, RXR), the all-trans retinoic acid receptor (RAR), the thyroid hormone receptor (TR), the vitamin D receptor (VDR), the peroxisome proliferator-activated receptor (PPAR), and nerve growth factor induced-B (NGFI-B). These HREs directly reflect the mode of receptor binding, which can be as heterodimers, homodimers or monomers. In contrast, the steroid hormone receptors bind exclusively as homodimers to palindromes separated by three nucleotides (Mangelsdorf & Evans 1990).

The general architecture of nuclear receptor proteins is shown in Fig. 2. The nuclear receptor DNA-binding domain (DBD) is one of the most prevalent DNA-interacting regions known. It is composed of a highly conserved 66 amino acid core domain located centrally in each nuclear receptor, together with a short, non-conserved extension into the hinge region of the receptor (Fig. 2) (Wilson et al. 1992, Rastinejad 1998). Its modular design is composed of two zinc-binding loops and a pair of α-helices (Luise et al. 1991). One of these helices mediates sequence-specific recognition of the AGGTCA sequence via major groove contacts. In some cases, additional sequences in the hinge region contribute to the DNA-binding specificity, nuclear localization or transactivation. The ligand-binding domain (LBD) may also contain sequences important for dimerization, nuclear localization, transactivation, silencing and repression. The major dimerization domain of receptors has been localized in the C-terminal half of the LBD (Fawell et al. 1990, Forman & Samuels 1990).

RXR is a combinatorial partner in the nuclear receptor family, able to form heterodimers with a variety of hormone and orphan receptors (Yu et al. 1991, Kliwerer et al. 1992a, b, Forman et al. 1995). The dimerization partners of RXR include itself, RAR, VDR, TR, PPAR, and other receptors. The formation of heterodimers between DBDs is induced by specific repertoires of high-affinity response elements containing direct repeats (DRs) with characteristic inter-half-site spacings. These spacings can vary from one to five base pairs (DR1–DR5).

Figure 2 A schematic model of nuclear receptors. See text for details.

The pattern of site selectivity based on the spacing of DRs is known as the 1–5 rule (Umesono et al. 1991). In addition, RXR can homodimerize, and signals through the RXR response element (RXRE) (Lehmann et al. 1992).

Ligand binding to a nuclear receptor is now recognized to induce a switch in the complex of transcriptional co-factors with which it interacts. In the absence of ligands, receptors such as the RAR–RXR heterodimer are bound to a repression complex that contains histone deacetylases (HDACs), Sin3 and the co-repressors SMRT or NCo-R (Chen & Evans 1995, Horlein et al. 1995). Ligand binding triggers release of this complex and promotes interaction with activator complexes containing histone acetylases, including SRC co-activators and CBP/p300, that form a bridge to the basic transcription machinery (Chambon 1996, Perlmann & Evans 1997).

As ligand-activated transcription factors, nuclear receptors make ideal therapeutic targets. The ability to modulate receptor activity with synthetic ligands has led to the development of drugs for a number of diseases, including cancer and diabetes. With the discovery of the co-activator and co-repressor complexes has come the realization that receptor-co-factor interactions may represent another point at which nuclear receptor signaling pathways could be modulated pharmacologically.

An interface between the nuclear receptor and IGF axis – the nuclear association of RXRα and IGFBP-3

A yeast two-hybrid system was used to identify novel IGFBP-3 partners (Liu et al. 2000). We isolated a 1200-base pair cDNA fragment encoding the C-terminal portion of the human RXRα gene followed by the 3′-untranslated region of the human RXRα cDNA. Yeast mating experiments confirmed the interaction between IGFBP-3 and RXRα. This was further verified via glutathione S-transferase (GST) pull-down experiments using RXRα linked to GST and various forms of natural and recombinant IGFBP-3 proteins. Co-immunoprecipitation and fluorescence immunocytochemistry indicated that RXRα and IGFBP-3 co-localize in both the cytoplasm and nucleus of prostate cancer cells. In addition, after incubation with an RXR-specific ligand, both proteins were more evident in the nucleus, suggesting a ligand-dependent co-transport. This is consistent with previous data that showed that, when nuclear
envelopes were permeabilized, IGFBP-3 is retained within the nucleus (Schedlich et al. 2000), suggesting interaction with nuclear components such as RXRα.

Interactions of IGFBP-3 with the DNA–transcription factor complex involving RXRα and the RXRE were confirmed in electromobility shift assays. The addition of an IGFBP-3 antibody supershifts the complex, indicating that IGFBP-3 is bound to the RXRα–RXRE complex. Similarly, labeled RAR response element (RARE) binds RAR in HeLa extracts, but this complex does not nuclear supershift with an IGFBP-3 antibody, suggesting that IGFBP-3 forms a complex only with the RXR–RXR homodimer, not with RXR–RAR heterodimers.

In luciferase-based transscriptional assays, we used the DR1–RXRE and the DR5–RARE reporter systems in COS7 cells. In both cases, luciferase signaling was enhanced by co-treatment with the appropriate ligand. 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.

To study further the functional interface of IGFBP-3 and RXRα in the nucleus, we performed viability assays utilizing the F9 embryonic carcinoma cell line and a sister cell line, in which RXRα has been knocked out (Fig. 3). IGFBP-3 treatment dramatically reduced cell viability in the F9 cell line, but IGFBP-3 had no discernible effects in the RXRα knockout line, indicating that RXRα is required for IGFBP-3-induced apoptosis.

Natural and synthetic retinoids have also been used with some success in prostate cancer models (Lewis & Hochadel 1999, Sharp et al. 2001). We found that RXRs are expressed at high levels in normal human prostate tissue, in addition to prostate tumor cell lines and primary human tumors (P Cohen unpublished data). Moreover, we have shown that growth of LNCaP, LAPC-4, PC3, and DU-145 prostate cancer cells can be inhibited by the RXR-specific activator, LG 268. These observations illustrate the potential for several nuclear receptor signaling pathways to regulate growth of prostate cells.

In the described cloning and functional studies of RXRα and IGFBP-3, we discovered that IGFBP-3 and the specific rexinoid, LG1069, had additive effects in killing LAPC-4 cells (Liu et al. 2000). This is an important observation, as it suggests that IGFBP-3 and RXR ligands co-operate in inducing gene transcription that leads to apoptosis. A recent report described the four and a half LIM domain gene 2, FHL2, a transcriptional co-activator, as a specific IGFBP-5 nuclear binding partner cloned from a human osteosarcoma library (Amaar et al. 2002). This further establishes the IGFBPs as important modulatory factors involved in DNA transcription and extends the IGF axis beyond the serum and cellular surface. With recent advances in both protein partner discovery and modulation of cellular signaling, we are confident that investigation into the interaction between nuclear receptors, DNA transcription and the IGF axis will reveal several new targets for co-adjuvant treatment in the treatment of malignancy.

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