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 RXR α), 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. RXR α 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.

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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 (Gallicchio *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

Figure 1 A schematic representation of the IGF-IGFBP axis and its interacting molecules. See text for details.

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 (Krywicky *et al.* 1993), testes (Drescher *et al.* 1997), leukemia (Wex *et al.* 1998), adrenal (Boulle *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.* 1991, Giudice *et al.* 1993, Rosenfeld *et al.* 1994, Nickerson *et al.* 1997) depending upon the cell types and culture conditions used (De Mellow & Baxter 1988, Blum

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 cell lines 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

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).

Further evidence supporting IGF-independent effects of IGFBP-3 includes induction of IGFBP-3 mRNA and protein expression by multiple antiproliferative and pro-apoptotic agents, including transforming growth factor- β (TGF- β) (Martin & Baxter 1991, Oh *et al.* 1995, Gucev *et al.* 1996, Hwa *et al.* 1997, Rajah *et al.* 1997), tumour necrosis factor- α (Rozen *et al.* 1998), retinoids (Adamo *et al.* 1992, Andreatta-Van Leyen *et al.* 1994, Martin *et al.* 1995, Gucev *et al.* 1996, Han *et al.* 1997, Hwa *et al.* 1997), antiestrogens (Huynh *et al.* 1996), p53 (Buckbinder *et al.* 1995) and 1,25 dihydroxyvitamin D₃ (Velez-Yanguas *et al.* 1996, Huynh *et al.* 1998, Boyle *et al.* 2001), and additional data utilizing antisense oligonucleotides and neutralizing antibodies that suggest that IGFBP-3 mediates these effects in part.

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, Knudtson *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 (Wraight *et al.* 1998). Nuclear transport of IGFBP-3 was not unexpected, because both IGFBPs-3 and -5 possess basic C-terminal nuclear localization signals (Radulescu 1994). Recombinant IGFBPs-3 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 (RXR α) 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.

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

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 1995).

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 (Luisi *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, Kliewer *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).

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 RXR α 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 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 . This was further verified via glutathione S-transferase (GST) pull-down experiments using RXR α linked to GST and various forms of natural and recombinant IGFBP-3 proteins. Co-immunoprecipitation and fluorescence immunocytochemistry indicated that RXR α 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

Figure 3 RXR is required for IGFBP-3-induced apoptosis. F9 teratocarcinoma cells were derived to have targeted deletions of RXR (F9 RXR (-/-)). Survival was assessed after IGFBP-3 (BP3) treatment. AU, arbitrary units; WT, wild-type. * $P < 0.01$ relative to control.

envelopes were permeabilized, IGFBP-3 is retained within the nucleus (Schedlich *et al.* 2000), suggesting interaction with nuclear components such as RXR α .

Interactions of IGFBP-3 with the DNA-transcription factor complex involving RXR α 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 RXR α -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 transcriptional 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 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 (Fig. 3). IGFBP-3 treatment dramatically reduced cell viability in the F9 cell line, but IGFBP-3 had no discernible effects in the RXR α knockout line, indicating that RXR α 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 RXR α 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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