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Nuclear Actions of Insulin-like Growth Factor Binding Protein-3

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Abstract

In addition to its actions outside the cell, cellular uptake and nuclear import of insulin-like growth factor binding protein-3 (IGFBP-3) has been recognized for almost two decades, but knowledge of its nuclear actions has been slow to emerge. IGFBP-3 has a functional nuclear localization signal and interacts with the nuclear transport protein importin-β. Within the nucleus IGFBP-3 appears to have a role in transcriptional regulation. It can bind to the nuclear receptor, retinoid X receptor-α and several of its dimerization partners, including retinoic acid receptor, vitamin D receptor (VDR), and peroxisome proliferator-activated receptor-γ (PPARγ). These interactions modulate the functions of these receptors, for example inhibiting VDR-dependent transcription in osteoblasts and PPARγ-dependent transcription in adipocytes. Nuclear IGFBP-3 can be detected by immunohistochemistry in cancer and other tissues, and its presence in the nucleus has been shown in many cell culture studies to be necessary for its pro-apoptotic effect, which may also involve interaction with the nuclear receptor Nur77, and export from the nucleus. IGFBP-3 is p53-inducible and in response to DNA damage, forms a complex with the epidermal growth factor receptor (EGFR), translocating to the nucleus to interact with DNA-dependent protein kinase. Inhibition of EGFR kinase activity or downregulation of IGFBP-3 can inhibit DNA double strand-break repair by nonhomologous endjoining. IGFBP-3 thus has the ability to influence many cell functions through its interactions with intranuclear pathways, but the importance of these interactions *in vivo*, and their potential to be targeted for therapeutic benefit, require further investigation.

Keywords

apoptosis; DNA damage repair; IGFBP-3; nuclear receptor; transcriptional regulation

1. Key features of IGFBP-3

Insulin-like growth factor binding protein-3 (IGFBP-3) is a multifunctional protein with a strong evolutionary link to the five other members of the IGFBP family (Daza et al., 2011). These proteins share the properties of high-affinity IGF binding and a high degree of

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structural conservation in their cysteine-rich amino- and carboxyterminal domains (Forbes et al., 2012). The terminal domains each comprise about one-third of the mature 28.7-kDa IGFBP-3 protein (Baxter, 2000). The central or linker domain shows little or no structural similarity among the six IGFBPs.

Like the other IGFBPs, IGFBP-3 is secreted by many cell types and is found in the circulation where, in adults, it is by far the most abundant IGFBP, with typical levels of 3–5 mg/L (Baxter, 1993; Friedrich et al., 2014). IGFBP-3 is the main circulating transport protein for IGF-I and IGF-II, which compete for a single binding site with similar affinities around 10^{10} L/mol (Martin and Baxter, 1986). Circulating IGF-IGFBP-3 complexes are found almost entirely bound to another protein, the acid-labile subunit (ALS), to form ternary complexes (Baxter et al., 1989). Among the other IGFBPs, only IGFBP-5 forms similar complexes with ALS (Twigg and Baxter, 1998). The site of interaction of IGFBP-3 with ALS includes a basic motif in the carboxyterminal domain (Figure 1); mutation of residues 228–232 to the corresponding residues of IGFBP-1 decreased ALS affinity by >90% while having little effect on IGF-binding affinity (Firth et al., 1998). Note: in this review, amino acid residues are numbered for mature IGFBP-3 containing 264 amino acids, excluding the 27-residue signal peptide.

By binding the anabolic and mitogenic peptides IGF-I and IGF-II with high affinity and restricting their access to their shared receptor, the type 1 IGF receptor (IGF1R), IGFBP-3 is growth-inhibitory in many systems *in vitro* and *in vivo* (Firth and Baxter, 2002). High-affinity binding of the IGFs appears to be achieved by their interaction with hydrophobic residues in the aminoterminal domain (Figure 1), as well as carboxyterminal residues (Payet et al., 2003; Forbes et al., 2012), which may act cooperatively to maintain IGF binding even if the protein is partially proteolyzed (Yan et al., 2009), as observed in pregnancy and some other conditions (Hughes et al., 1995). Although isolated IGFBP-3 fragments have greatly reduced IGF-binding affinity, there are reports that they retain growth-inhibitory activity in a variety of cell systems (Lalou et al., 1997; Booth et al., 1999).

1.1. Post-translational modification

IGFBP-3 has three potential sites of N-linked glycosylation (Figure 1), of which either two (Asn89, Asn109) or all three are normally occupied by glycans, resulting in a doublet of approximately 40 kDa when analyzed by SDS-PAGE (Martin and Baxter, 1986; Firth and Baxter, 1999). Decreased glycosylation has little effect on IGF or ALS binding by IGFBP-3 but enhances its cell-surface binding (Firth and Baxter, 1999), as well as its interaction with glucose-regulated protein 78 (GRP78) (Grkovic et al., 2013). Since this interaction has been implicated in the induction of autophagy by IGFBP-3, it has been proposed that IGFBP-3 hypoglycosylation under conditions of nutritional deprivation might be a signal for enhanced autophagy (Grkovic et al., 2013).

The other well-characterized post-translational modification of IGFBP-3 is phosphorylation. The IGFBP-3 sequence contains multiple consensus sites for Ser/Thr phosphorylation (Coverley and Baxter, 1997), and appears to be constitutively phosphorylated at the protein kinase CK2 sites, Ser111 and Ser113 (Hoeck and Mukku, 1994). CK2 phosphorylation of IGFBP-3 has no effect on IGF binding but decreases its binding to ALS and the cell surface,

and makes it relatively resistant to proteolysis (Coverley et al., 2000). In contrast to the lack of effect of CK2, and several other Ser/Thr kinases, on IGF binding, phosphorylation of IGFBP-3 by DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) abolishes IGF binding (Schedlich et al., 2003). The role of this phosphorylation is further discussed below. IGFBP-3 also contains several predicted tyrosine kinase consensus sites (http://www.hprd.org/PhosphoMotif_finder), including sites for Src kinase and EGF receptor (EGFR) kinase. Phosphotyrosine residues identified by mass spectrometry include the putative EGFR kinase sites Tyr163 and Tyr183 (http://www.phosphosite.org/).

This review will focus on nuclear actions of IGFBP-3, and takes many examples from cancer cell biology, where most of the relevant discoveries have been made. For a broader view of IGFBP-3 physiology, genetics, and signaling, the reader is referred to other recent reviews (Jogie-Brahim et al., 2009; Yamada and Lee, 2009; Baxter, 2014; Johnson and Firth, 2014).

2. Nuclear import and export of IGFBP-3

2.1. Cell uptake and nuclear import

Radulescu (Radulescu, 1994) first identified a putative nuclear localization signal (NLS) in the IGFBP-3 sequence, although its activity was not tested. It was postulated that IGFBP-3 might associate in the nucleus with IGF-I, which was shown in an earlier electron microscopy study to translocate to the nucleus of chicken lens epithelial cells (Soler et al., 1990). Subsequently it was shown that cells at the growing edge of a monolayer could internalize both IGF-I and IGFBP-3 to the nucleus, where they co-localized (Li et al., 1997). In contrast, in resting cells the two proteins co-localized in endosome-like structures. IGF-I nuclear transport, demonstrated in digitonin-permeabilized Chinese hamster ovary cells, was dependent on IGFBP binding, since LR3-IGF-I, an IGF-I analog with greatly reduced IGFBP interaction, showed little nuclear translocation (Schedlich et al., 2003). The presence of nuclear IGFBP-3 is well-recognized in clinical histopathology (Hunziker et al., 2008; Seligson et al., 2013) and in cell lines (Jaques et al., 1997; Wraight et al., 1998), and extensive cell biology studies suggest important biological roles for IGFBP-3 in the nucleus.

Extracellular IGFBP-3 has been shown to enter the cell through a variety of endocytic mechanisms that may involve both caveolin 1 and clathrin-coated pits (Lee et al., 2004; Micutkova et al., 2012). Its nuclear import appears to share a common pathway with IGFBP-5 (Schedlich et al., 1998), both mediated by binding to importin-β (also known as karyopherin-β) (Schedlich et al., 2000; Micutkova et al., 2012), part of the importin-α/β nuclear transport complex. This contrasts with IGFBP-6, which interacts more strongly with importin-α (Iosef et al., 2008). The different specificities for importin binding suggest the possibility of different nuclear localization and/or function (Marfori et al., 2011). All three IGFBPs interact with the nuclear transport complex through a basic 18-residue bipartite NLS domain in their carboxyterminus (Figure 1), concordant with the NLS originally proposed by Radulescu. This basic domain corresponds to the ALS-binding domain described above (Firth et al., 1998). Mutation of key basic residues within the NLS domains of all three IGFBPs greatly inhibited their nuclear localization (Schedlich et al., 2000; Iosef et al., 2008). In contrast to the basic domain bipartite NLS common to IGFBP-3, IGFBP-5,

and IGFBP-6, a short (monopartite) NLS motif has been characterized in the central domain of IGFBP-2 (Azar et al., 2014).

Using digitonin-permeabilized cells it was shown that both the kinetics of IGFBP-3 nuclear import, and its retention within the nucleus, were enhanced by IGFBP-3 phosphorylation by the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (Schedlich et al., 2003). This phosphorylation was also inhibitory to IGF binding and prevented the IGFBP-3-mediated nuclear import of IGF-I. DNA-PKcs has been shown by co-immunoprecipitation and proximity ligation assay to form a nuclear complex with IGFBP-3 in breast cancer cells exposed to etoposide, but it is not known whether IGFBP-3 phosphorylation by DNA-PKcs is necessary for this interaction (Lin et al., 2014). However, this phosphorylation is reported to be an essential step in the induction of apoptosis by IGFBP-3 transfected into prostate cancer cells (Cobb et al., 2006).

DNA-PKcs preferentially phosphorylates Ser/Thr residues followed by Gln, and preceded by Gln, Glu or Asp (Lees-Miller and Anderson, 1991). By mutating the three consensus IGFBP-3 sites for DNA-PKcs (Ser156, Ser165, and Thr170, numbered excluding the signal peptide), Ser156 was shown to be essential for IGFBP-3 mediated caspase activation, which did not occur in cells expressing the S156A-IGFBP-3 mutant. NU7026, a specific DNA-PKcs inhibitor, also blocked apoptosis (Cobb et al., 2006). In contrast, a study in retinal endothelial cells found that cells expressing S156A-IGFBP-3 had *increased* apoptosis, and concluded that DNA-PKcs phosphorylation of Ser156 was a critical step in the ability of IGFBP-3 to *prevent* apoptosis (Zhang and Steinle, 2013). A mechanistic explanation for this discrepancy is currently lacking.

2.2. Nuclear export and stability

Hydrophobic nuclear export signals (NES) that interact with the export protein CRM1 (exportin 1) typically have the sequence $\Phi1\text{-}X_{2,3}\text{-}\Phi2\text{-}X_{2,3}\text{-}\Phi3\text{-}X\text{-}\Phi4$, where Φ represents Leu, Val, Ile, Phe, or Met and X can be any amino acid (Xu et al., 2012). The IGFBP-3 sequence includes several putative NES motifs in its central and C-terminal domains. One such motif, starting at Met190 (excluding the signal peptide) has been reported to serve as a functional NES (Paharkova-Vatchkova and Lee, 2010). In fact this sequence overlaps another putative NES starting at Leu194 (Figure 1). Mutation of Leu197 and Leu200, which are included in both of the overlapping sequences, increased the nuclear retention of IGFBP-3, and abolished the apoptotic effect of IGFBP-3 in 22RV1 prostate cancer cells (Paharkova-Vatchkova and Lee, 2010). Since nuclear entry of IGFBP-3 is believed to precede, and have a role in, its induction of apoptosis (Lee and Cohen, 2002; Santer et al., 2006; Leibowitz et al., 2013), this result suggests that IGFBP-3 may first enter, and then leave, the nucleus for its pro-apoptotic activity. A possible mechanism for this nucleocytoplasmic shuttling, involving the orphan nuclear receptor Nur77, is discussed below. IGFBP-3 in the nucleus has been reported to be quite unstable, with a shorter half-life than cytoplasmic IGFBP-3. It is stabilized by proteasome inhibitors, and its degradation follows polyubiquitination at multiple sites in the carboxyterminal domain [39].

In contrast to the proposed obligatory nuclear entry of IGFBP-3 as part of its apoptotic effect, a mutant form of IGFBP-3 in which a five-residue motif in the NLS domain was

altered to the corresponding IGFBP-1 residues (Schedlich et al., 2000) was still able to induce apoptosis in T47D breast cancer cells despite being excluded from the nucleus (Butt et al., 2002), suggesting that an alternative pathway of apoptosis induction by IGFBP-3, independent of nuclear entry, may also exist.

3. IGFBP-3 and nuclear receptors

3.1. Interactions with retinoid receptors

In a landmark study, retinoid X receptor-α (RXRα, NR2B1) was identified by yeast 2-hybrid screen as an IGFBP-3 binding partner, and confirmed as a functional interacting protein by a variety of biochemical methods (Liu et al., 2000). RXRα is a ligand-dependent transcription factor that is a member of the nuclear receptor (NR) family (Evans and Mangelsdorf, 2014), specifically the type II NR family. It functions by dimerizing with itself or several other NR family members including retinoic acid receptor-α (RARα, NR1B1), vitamin D receptor (VDR, NR1I1), peroxisome proliferator-activated receptor-γ (PPARγ, NR1C3), thyroid hormone receptor-α (NR1A1), and Nur77 (NR4A1). In a luciferase reporter assay, IGFBP-3 was shown to enhance transcriptional activity stimulated by the RXR ligand SR11235 (RXR homodimer signaling), and to increase the apoptotic activity of another RXRα agonist, LG1069, in prostate cancer cell lines (Liu et al., 2000). The IGFBP-3-RXRα interaction involves residues in the basic carboxyterminal domain of IGFBP-3 Figure 1), but residues Thr58 and Arg60 in the aminoterminal domain are also involved (Schedlich et al., 2007a). Important RXRα residues involved in the interaction include RXRα Gln49 and Arg52 in the second Zn-finger loop of the DNA-binding domain. IGF-I binding to IGFBP-3, but not 9-*cis*-RA binding to RXRα, inhibited IGFBP-3-RXRα interaction (Schedlich et al., 2007a).

In contrast to the stimulatory effect of IGFBP-3 on RXRα homodimer signaling, IGFBP-3 was inhibitory to RXR-RAR heterodimer signaling stimulated by all-*trans* retinoic acid (atRA) (Liu et al., 2000). IGFBP-3 inhibits atRA-stimulated transcription through direct binding to RARα as well as RXRα (Schedlich et al., 2004), and immunoneutralization of IGFBP-3 in breast cancer cells enhanced the atRA transcriptional response in a reporter assay. Similarly, immunoneutralizing IGFBP-3 was able to sensitize the relatively atRA-resistant triple-negative breast cancer cell lines, MDA-MB-231 and Hs578T, to growth inhibition by atRA, suggesting that endogenous IGFBP-3 produced by these cell lines was limiting their responsiveness to atRA (Schedlich et al., 2004). IGFBP-3 interaction with RARα has been shown to involve the same amino- and carboxyterminal IGFBP-3 residues as the IGFBP-3-RXRα interaction (Schedlich et al., 2007a).

3.2. Interaction with PPARγ

The interaction between IGFBP-3 and RXRα prompted an investigation of its possible effects on PPARγ activity. PPARγ has often been described as the master regulator of adipogenesis, impacting on the complex cascade of events that lead to the formation of mature adipocytes (Lefterova et al., 2014). RXRα-PPARγ heterodimers are known to bind to thousands of sites in the adipocyte genome (Nielsen et al., 2008). IGFBP-3 was found to bind directly to PPARγ and to co-immunoprecipitate with PPARγ from lysates of mouse

3T3-L1 adipocytes (Chan et al., 2009). It also inhibited the formation of RXRα-PPARγ dimers and inhibited PPARγ transcriptional activity stimulated by the thiazolidinedione PPARγ agonist rosiglitazone in 3T3-L1 cells (Chan et al., 2009). These results suggested that IGFBP-3 was likely to affect adipogenic differentiation, and it was found that IGFBP-3 could inhibit 3T3-L1 preadipocyte maturation as indicated by decreased induction of the adipocyte markers resistin and adiponectin (de Silva et al., 2012), reversal of the loss of the preadipocyte marker plasminogen activator inhibitor-1, and a decrease in the generation of triglycerides detected by Nile red staining. Further, treatment of mature adipocytes with IGFBP-3 caused a partial reversion to a less differentiated phenotype (Chan et al., 2009). The inhibitory effect of IGFBP-3 on adipogenesis was not simply due to sequestration of stimulatory IGF-I, since IGFBP-2, another high-affinity IGFBP, had no inhibitory effect on 3T3-L1 differentiation. Finally, a basic-domain mutant of IGFBP-3 that retains IGF-binding activity was also without inhibitory activity (Chan et al., 2009). Endogenous IGFBP-3 expression increases through the course of adipogenesis (de Silva et al., 2012), and it may be speculated that this serves as a termination signal once differentiation has been achieved.

PPARγ agonists have been extensively investigated as possible anticancer agents in either mono-or combination therapies, but evidence of clinical utility is limited (Robbins and Nie, 2012). PPARγ agonists are typically growth-inhibitory when tested in cancer cell lines *in vitro*, but in at least some cases their effects may not be mediated by activation of PPARγ-dependent transcription (Wei et al., 2009; Robbins and Nie, 2012). Since IGFBP-3 affects PPARγ function in preadipocytes, its role in PPARγ-dependent anti-cancer activity was investigated in breast cancer cell lines. Surprisingly in view of its PPARγ-antagonistic role in adipogenesis, IGFBP-3 was not only growth-inhibitory itself, but potentiated growth inhibition caused by the synthetic and natural PPARγ ligands, rosiglitazone and 15-deoxy$\Delta^{12,14}$prostaglandin J_2 (15dPG), both of which were acting in a PPARγ-dependent manner as indicated by blockade of their effect by the PPARγ antagonist GW9662 (Pon et al., 2015). Growth inhibition by rosiglitazone or 15dPG was found to depend on the presence of IGFBP-3, since the effect of either agent was significantly attenuated in three breast cancer cell lines when IGFBP-3 was downregulated by siRNA. Further, two mutant forms of IGFBP-3, with reduced PPARγ binding, had no inhibitory effect when tested alone, and entirely blocked the inhibitory effect of rosiglitazone (Pon et al., 2015). These results indicate that breast cancer cell growth inhibition by PPARγ requires its interaction with endogenous IGFBP-3, and non-binding IGFBP-3 mutants can exert a dominant-negative blocking effect on the inhibition. Since endogenous IGFBP-3 is required for PPARγ ligands to exert anti-tumor effect, this study suggests that tumor IGFBP-3 expression may serve as a biomarker for the efficacy of these agents.

3.3. Interaction with Nur77

As alluded to above, nuclear export of IGFBP-3 may involve its functional interaction with the nuclear receptor Nur77, a RXRα dimerization partner. The induction of apoptosis by IGFBP-3 in prostate cancer cells was reported to be initiated by IGFBP-3-dependent translocation of Nur77 from the nucleus to the mitochondria, an effect requiring RXRα as it was not seen in RXRα-deficient cells (Lee et al., 2005). In contrast to the inhibitory effect of IGFBP-3 on the RXRα interaction with RARα and PPARγ, described above, IGFBP-3 was

found to enhance RXRα-Nur77 interaction, and mitochondrial RXRα accumulation (Lee et al., 2005). Although a direct IGFBP-3-Nur77 interaction was not demonstrated, IGFBP-3 could be co-immunoprecipitated with Nur77 in the cytoplasm of prostate cancer cells, perhaps associated through their common binding partner RXRα (Lee et al., 2007). The importance of these interactions was suggested by the loss of IGFBP-3-induced apoptosis in Nur77-deficient cells. As noted earlier, mutation of a putative CRM1-binding NES motif on IGFBP-3 also prevented IGFBP-3-induced apoptosis, and also resulted in increased nuclear RXRα-Nur77 retention (Paharkova-Vatchkova and Lee, 2010). Together these date indicate a role for nuclear IGFBP-3 in the CRM1-mediated export of RXRα-Nur77 complexes during IGFBP-3-mediated apoptosis in prostate cancer cells.

3.4. Interaction with other nuclear receptors

The *IGFBP3* gene is a transcriptional target of the VDR (Krishnan et al., 2003), its induction by $1,25(OH)_2D_3$ leading to growth inhibition through cell cycle arrest in prostate cancer cells. IGFBP-3 also binds directly to VDR, and the two proteins have been shown to co-localize in the cell nucleus (Li et al., 2013). IGFBP-3 appears to compete for the binding of IGFBP-5, another VDR binding partner (Schedlich et al., 2007b). IGFBP-3 has been shown to inhibit VDR-dependent transcriptional activity (Li et al., 2013) and $1,25(OH)_2D_3$-stimulated CD11b induction in HL-60 human promyelocytic leukemia cells (Ikezoe et al., 2004). In osteoblasts, $1,25(OH)_2D_3$-dependent differentiation is inhibited by overexpression of IGFBP-3 (Li et al., 2013), similar to the inhibitory effect of IGFBP-5 (Schedlich et al., 2007b). The thyroid hormone receptor-α, another RXRα heterodimerization partner, is also reported to bind to IGFBP-3 *in vitro* and co-localize with it in the nucleus of HEK-293 cells (Qiu et al., 2011). IGFBP-3 was inhibitory to T_3-mediated gene transcription. Whether other type II NRs will be shown to bind to IGFBP-3, and their transcriptional activity to be regulated by it, remains to be determined.

3.5. Interaction with histone-DNA complex

A study of the domains of IGFBP-5 with transcriptional regulatory activity reported direct interaction of IGFBP-5 with the nuclear histone-DNA complex, and highlighted amino-terminal residues that contribute to this interaction (Zhao et al., 2006). Notably, the N-terminal domain of IGFBP-3 showed transcriptional activity as strong as that of the corresponding domain of IGFBP-5. Among the IGFBP-5 residues shown by mutagenesis to be involved in transcriptional activation, residues corresponding to Glu8, Asp11, Glu30, Pro31, Glu43, and Glu52 of IGFBP-5 are all conserved in IGFBP-3 (Figure 1), strongly suggesting that the N-terminal domain of IGFBP-3 is also involved in histone-DNA interaction. As discussed earlier, it is also notable that Thr58 and Arg60, that flank Glu59 of IGFBP-3 (equivalent to Glu52 of IGFBP-5), influence the IGFBP-3 interaction with both RXRα and RARα (Schedlich et al., 2007a), suggesting overlap between residues involved in nuclear hormone receptor binding by IGFBP-3, and those involved in histone-DNA binding – or indeed that IGFBP-3 binds to DNA as part of a nuclear hormone receptor complex.

In addition to direct IGFBP-3 protein interactions with the histone-DNA complex, chromosomal interactions of the *IGFBP3* gene have also been examined. Long-range chromosomal interactions of *IGFBP3* have been shown to differ between normal and

cancerous mammary epithelial cell lines (Zeitz et al., 2013). Among a multitude of *IGFBP3*-interacting genes, an interaction with *EGFR* was notable, suggesting a possible coordination of these genes at the genomic level (Zeitz et al., 2013), reinforcing evidence for the functional interaction of their encoded proteins in cancer (Martin et al., 2014).

4. Role of IGFBP-3 in the DNA damage response

4.1. IGFBP-3 and p53

In response to genotoxic stress, the tumor suppressor p53 is activated and stabilized by the primary DNA damage sensor, ataxia-telangiectasia mutated (ATM) (Shiloh and Ziv, 2013). IGFBP-3 is a transcriptional target of p53 (Buckbinder et al., 1995), and its expression is induced by the topoisomerase II poison, doxorubicin, and other DNA-damaging chemotherapy drugs in many cell lines. The *IGFBP3* gene contains putative p53-responsive sites both within introns 1 and 2 (Buckbinder et al., 1995), and in the upstream promoter region (Bourdon et al., 1997). Hypermethylation of p53 regulatory regions in the upstream promoter can suppress p53-dependent IGFBP-3 induction (Hanafusa et al., 2005) and could conceivably contribute to radio- or chemoresistance of some tumors, since p53-dependent IGFBP-3 induction appears to account for at least some of the pro-apoptotic activity of p53 (Hollowood et al., 2000; Grimberg et al., 2002).

The C-terminal basic domain of p53 has been reported to be inhibitory to IGFBP-3 induction, such that a p53 form lacking both this domain and the N-terminal activation domain was able to induce IGFBP-3 expression under conditions where full-length p53 did not (Harms and Chen, 2005). However, this effect appears to depend on histone deacetylase (HDAC) activity, since in the presence of an HDAC inhibitor the ability of full-length p53 to induce IGFBP-3 was restored (Harms and Chen, 2005). Some point mutations in the p53 DNA binding domain also impair its ability to induce IGFBP-3, even while remaining transcriptionally active as determined by p21 induction (Ludwig et al., 1996). Indeed, some p53 “hot-spot” mutations, such as Arg175, Arg273 and Arg282, that appear to be associated with gain-of-function activity (Freed-Pastor and Prives, 2012), can suppress *IGFBP3* promoter activity below its basal level, an effect also reversible by HDAC inhibition (Vikhanskaya et al., 2007).

IGFBP-3 is also strongly downregulated by ΔNp63α (Barbieri et al., 2005), a variant of the p63α splicing isoform that lacks the N-terminal transactivation domain (Deyoung and Ellisen, 2007) and has been shown to oppose p53-dependent transcriptional activity (Yang et al., 1998). ΔNp63α is induced by doxorubicin (Petitjean et al., 2008), so in cells where this isoform predominates, it might contribute to IGFBP-3 downregulation rather than induction of IGFBP-3 in response to similar chemotherapy drugs.

4.2. IGFBP-3 and DNA damage-induced apoptosis or survival

When subjected to ionizing radiation, T47D breast cancer cells transfected to express IGFBP-3 show significantly worse survival over 14 days than cells expressing empty vector (Butt et al., 2000). Cell cycle distribution between the two cell lines does not differ, but the IGFBP-3-expressing cells show a marked increase in apoptosis. Other studies have similarly shown an enhanced apoptotic response to radiation in the presence of increased IGFBP-3

(Hollowood et al., 2000; Yoshino et al., 2011). IGFBP-3 is reported to be more highly expressed in radiosensitive than radioresistant cell lines (Achary et al., 2000; Zhao et al., 2012), and IGFBP-3 downregulation can reduce radiosensitivity (Yoshino et al., 2011). Similar effects of IGFBP-3 are reported in modulating tumor or cell line chemosensitivity (Granata et al., 2003; Ibanez de Caceres et al., 2010).

A dual role for IGFBP-3 has been described in human umbilical vein endothelial cells, in which IGFBP-3 potentiated doxorubicin-induced apoptosis but also enhanced survival during serum starvation (Granata et al., 2004). The proapototic response to doxorubicin was accompanied by an increase in the cell content of ceramide, a sphingolipid that has also been shown in other studies to act with IGFBP-3 in enhancing apoptosis (Gill et al., 1997). The survival effect of IGFBP-3 was associated with activation of sphingosine kinase 1 (SphK1), which generates the pro-survival lipid, sphingosine-1-phosphate (S1P), a product of ceramide metabolism (Granata et al., 2004). Activation of SphK1 by IGFBP-3, leading to S1P-dependent transactivation of EGFR, has also been described in normal mammary and breast cancer cell lines (Martin et al., 2009; Martin et al., 2014). SphK1 downregulation by shRNA has been shown to increase doxorubicin-induced DNA damage as indicated by enhanced phosphorylation of histone H2AX (Huwiler et al., 2011); conversely SphK1 activation by IGFBP-3 may be predicted to attenuate chemotherapy-induced DNA damage.

IGFBP-3 has recently been shown to have an active role in DNA repair in response to chemotherapy (Figure 2), perhaps surprising in view of its well-established pro-apoptotic role. Analyzed by co-immunoprecipitation and proximity ligation assay, IGFBP-3 was found to form nuclear complexes with EGFR and DNA-PKcs in response to chemotherapy, an effect blocked by EGFR kinase inhibition (Lin et al., 2014). When IGFBP-3 was downregulated by siRNA, DNA-PKcs autophosphorylation and nuclear EGFR-DNA-PKcs complex formation were inhibited. Non-homologous end-joining activity in nuclear extracts was also attenuated when IGFBP-3 was downregulated, indicating that the repair of chemotherapy-induced DNA double strand breaks requires the formation of nuclear complexes between IGFBP-3, EGFR and DNA-PKcs (Lin et al., 2014). It may be speculated that this effect of IGFBP-3 also involves SphK1 activation, but this was not demonstrated.

5. Summary and Conclusions

There is clearly a great deal remaining to be understood about nuclear actions of IGFBP-3. In the two decades since IGFBP-3 was first recognized as having the potential for nuclear translocation, there has been only a gradual advance in revealing how this translocation occurs and what nuclear functions IGFBP-3 is involved in. This relatively slow progress parallels a growing realization that many other regulatory proteins that were initially identified as extranuclear (e.g. tyrosine kinase receptors like EGFR and IGF1R), also have significant functions within the nucleus, and indeed that some other IGFBPs (IGFBP-2, IGFBP-5, and IGFBP-6) are also active intranuclearly.

The most significant advance in understanding the nuclear role of IGFBP-3 has resulted from the recognition that it interacts with RXRα and several of its nuclear receptor heterodimerization partners. This has revealed the potential for IGFBP-3 to be directly

involved in transcriptional regulatory complexes, in one case (RXRα-dependent transcription) exerting a stimulatory effect, but in others (e.g. RARα- and VDR-dependent transcription) being inhibitory, apparently by disrupting heterodimeric RXRα complexes. In its apoptosis-inducing complex with the orphan receptor Nur77, its major role may be extranuclear, and in its complex with PPARγ, current evidence suggests that PPARγ activity is opposed by IGFBP-3 during adipogenesis, but requires IGFBP-3 in tumor suppression.

Involvement of IGFBP-3 in the DNA damage response may be another key intranuclear role, but once again, some of the observations are contradictory and the relevant mechanisms have not been fully elicidated. There is evidence to support roles for IGFBP-3 both in mediating, or at least supporting, cell cycle arrest and induction of apoptosis in response to DNA damage, while at the same time participating in DNA repair through interaction with EGFR and DNA-PKcs. These opposing actions have been termed, respectively, the gatekeeper and caretaker responses (van Heemst et al., 2007), and the involvement of IGFBP-3 in both suggests that it may have a fundamental role in supporting genomic integrity.

To date, all of the above observations have been restricted to a variety of cell culture systems, and it is unclear how important they will turn out to be *in vivo*, notwithstanding clear evidence that IGFBP-3 can be seen intranuclearly on histological examination of cancer and other tissues. Continuing investigation of these phenomena *in vitro*, and the development of suitable *in vivo* models, should be an important research goal, since a better understanding of IGFBP-3 actions in the nucleus may open new opportunities for therapeutic intervention in cancer and other diseases.

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Abbreviations

IGFBP-3	insulin-like growth factor binding protein-3
ALS	acid-labile subunit
IGF1R	type 1 IGF receptor
GRP78	glucose-regulated protein 78
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
EGFR	epidermal growth factor receptor
NLS	nuclear localization signal
NES	nuclear export signal
RXRα	retinoid X receptor-α

NR	nuclear receptor
RARα	retinoic acid receptor-α
VDR	vitamin D receptor
PPARγ	peroxisome proliferator-activated receptor-γ
atRA	all-*trans* retinoic acid
15dPG	15-deoxy$\Delta^{12,14}$prostaglandin J_2
ATM	ataxia-telangiectasia mutated
HDAC	histone deacetylase
SphK1	sphingosine kinase 1
S1P	sphingosine-1-phosphate

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Highlights

- IGFBP-3 is a multifunctional protein with roles both outside and inside the cell.
- IGFBP-3 binds to nuclear hormone receptors, regulating transcriptional activity.
- Nuclear transport of IGFBP-3 may be required for its pro-apoptotic activity.
- Nuclear IGFBP-3 also influences cell survival by promoting DNA damage repair.

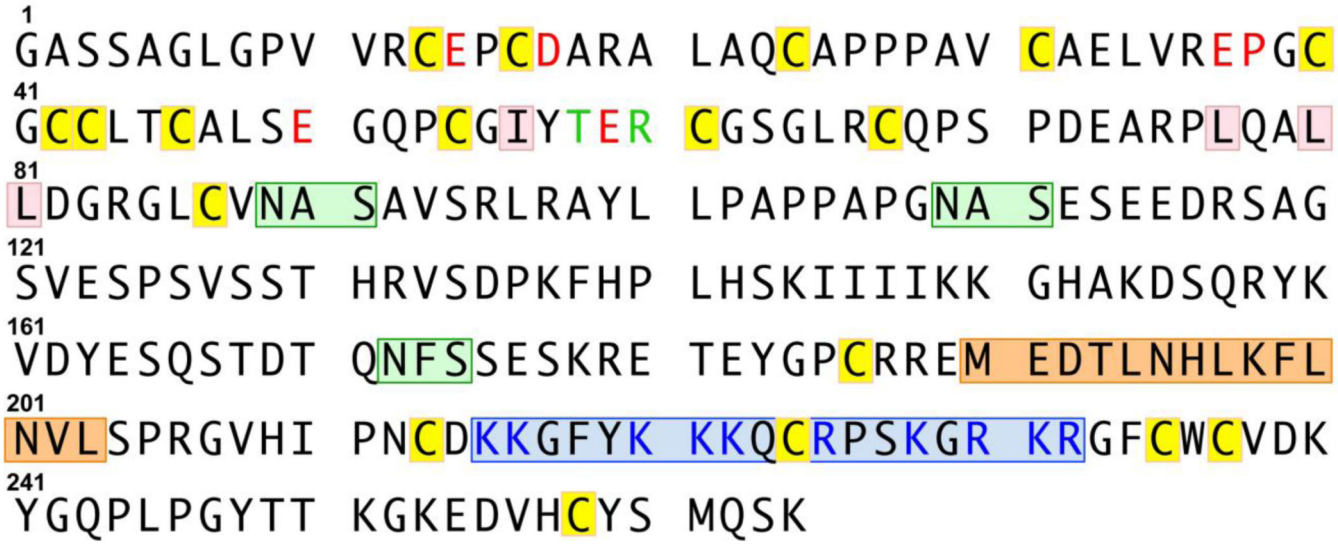

Figure 1.

Aminoacid sequence of mature human IGFBP-3 (residues 1–264), omitting the 27-residue signal peptide. Conserved cysteine residues are boxed in yellow, and hydrophobic residues involved in IGF binding, in pink. Residues assumed (by comparison with IGFBP-5) to be involved in transcriptional regulation (Zhao et al., 2006), are in red font; residues shown by mutagenesis to be involved in nuclear receptor binding (Schedlich et al., 2007a), in green font. The bipartite nuclear localization signal that includes residues that bind ALS and RXRα is boxed in blue, and its basic residues are shown in blue font. The three N-glycosylation motifs are boxed in green. Putative overlapping hydrophobic nuclear export sequences are boxed in orange. The canonical 264-residue mature sequence shown above has been termed *isoform 1* or *isoform b* (NCBI Reference Sequence: NP_000589.2, UniProt: P17936-1). The 270-residue mature product of an alternatively spliced transcript, termed *isoform 2* or *isoform a* (NCBI Reference Sequence: NP_001013416.1, UniProt: P17936-2) has a 6-residue insertion after Pro107.

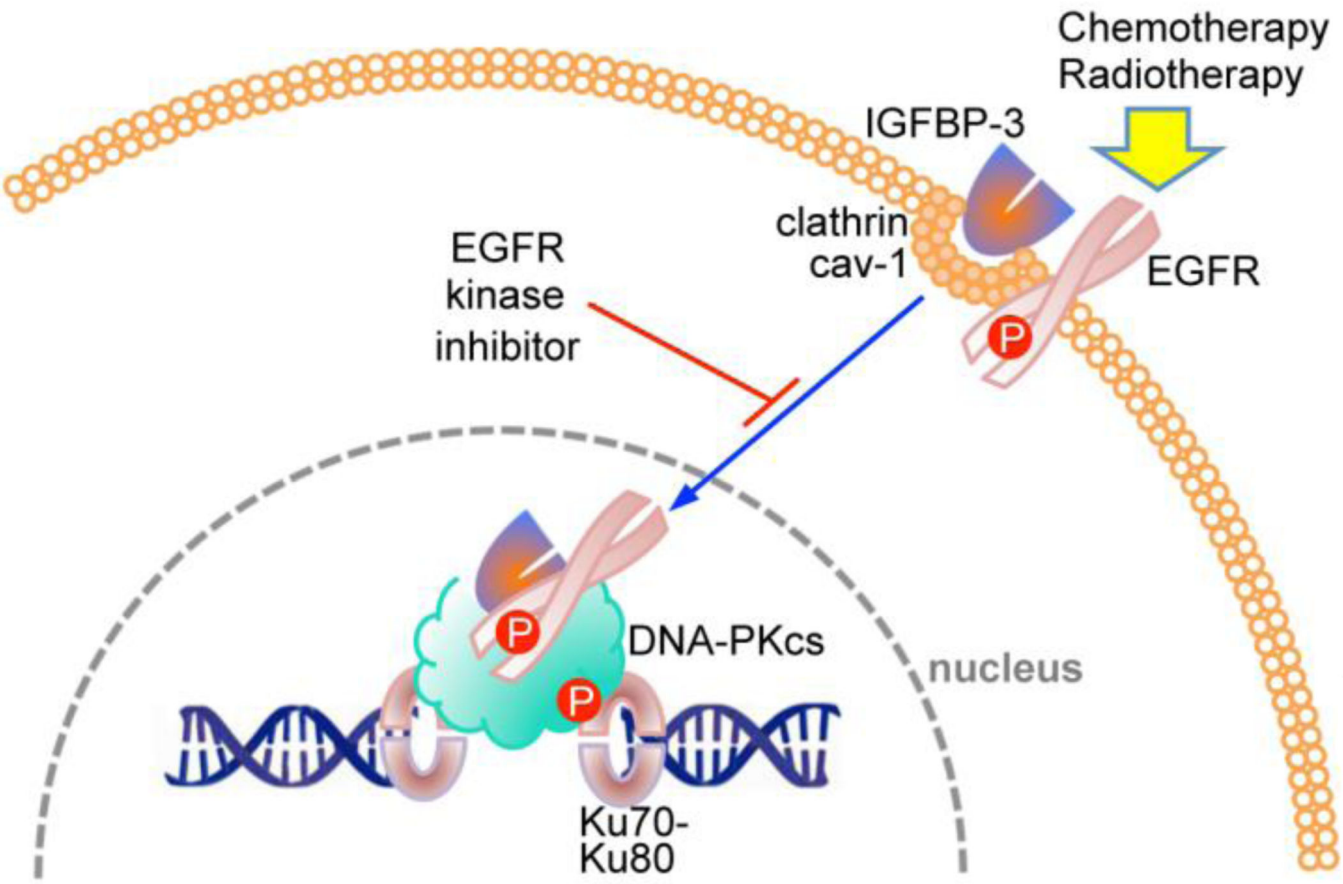

Figure 2.

Involvement of IGFBP-3 in DNA damage repair. In response to DNA damaging therapy, IGFBP-3 interacts with EGFR at the plasma membrane, and is transported to the nucleus where it binds to DNA-PKcs (complexed with Ku70/Ku80 at DNA double-strand breaks), leading to DNA-PKcs autophosphorylation (Lin et al., 2014). EGFR kinase inhibition prevents the nuclear complex from forming.

Table 1

Nuclear receptors known to interact with IGFBP-3

Nuclear Receptor	Abbreviation	Systematic Name*	Ligand	References
Retinoid X receptor-α	RXRα	NR2B1	9-*cis*-retinoic acid	(Liu et al., 2000; Schedlich et al., 2007a)
Retinoic acid receptor-α	RARα	NR1B1	All-*trans*-retinoic acid	(Liu et al., 2000; Schedlich et al., 2004)
Peroxisome proliferator-activated receptor-γ	PPARγ	NR1C3	Fatty acids, prostaglandin J_2, thiazolidinediones	(Chan et al., 2009; Pon et al., 2015)
Vitamin D receptor	VDR	NR1I1	1,25-dihydroxy-vitamin D_3	(Schedlich et al., 2007b; Li et al., 2013)
Nuclear receptor-77/Nerve growth factor-inducible receptor B	Nur77/NGFI-B	NR4A1	Orphan	(Lee et al., 2007)
Thyroid receptor-α	TRα	NR1A1	Thyroid hormones	(Qiu et al., 2011)

* (Germain et al., 2006)