REVIEW

IGF-binding protein-5: flexible player in the IGF system and effector on its own

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Abstract

The multiple activities of IGF-I and -II are modulated by a family of IGF-binding proteins (IGFBP-1 to -6). Although structurally related, each IGFBP has unique properties and exerts specific functions. IGFBP-5 is the most conserved IGFBP across species and was identified as an essential regulator of physiological processes in bone, kidney and mammary gland. In addition, IGFBP-5 appears to play a decisive role in the control of proliferation of specific tumour cell types. In many situations IGFBP-5 exerts biological activities in the absence of IGFs, indicating the existence of IGF-independent actions. This concept was supported by the unexpected localisation of IGFBP-5 in the nucleus and the description of IGFBP-5-specific membrane-bound IGFBP-5 receptor(s). The scope of this review is to summarise the available information about the structure of IGFBP-5 and the regulation of its expression. Furthermore, the potential significance of IGFBP-5 in the regulation of physiological processes will be critically analysed in the light of recent experimental data.

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Introduction

The insulin-like growth factors (IGF-I and IGF-II) are the major growth-promoting factors present in the circulation. As true peptide growth factors they are secreted by a great variety of cells and have a multifunctional nature, stimulating the survival and promoting the proliferation and differentiation of many cell types (Stewart & Rotwein 1996). The A and B domains of these single-chain peptides are $\sim$50% identical to the A and B chains of insulin, explaining the origin of this nomenclature (Daughaday & Rotwein 1989). The IGFs elicit their effects through binding to the type I IGF receptor (IGF-I R), a heterotetrameric protein complex with a tyrosine kinase signal transduction pathway (De Meyts *et al.* 1994). The structurally distinct IGF-II R lacks tyrosine kinase activity and is actually identical to the cation-independent mannose 6-phosphate receptor (Kornfeld 1992). It is involved in the degradation of IGF-II and the sorting of lysosomal enzymes in the Golgi apparatus (Wang *et al.* 1994). The essential importance of the IGFs and their receptors for normal embryonic, fetal and postnatal development was highlighted by gene-targeting experiments (Efstratiadis 1998).

The observation that most of the IGFs present in serum migrate in higher molecular mass fractions, while the molecular mass of free IGFs is approximately 7·5 kDa, led investigators to propose the existence of carrier proteins. The initial interpretation of the function of such binding proteins was that they would prolong the half-life of the IGFs in the circulation and inhibit their metabolic effects by preventing them from binding to the receptors (Zapf *et al.* 1979), which would be an essential property as the IGFs are also able to interact with the insulin receptor and are present in serum at a concentration 1000 times higher than that of insulin (Daughaday & Rotwein 1989). Intense research in this area led to the sequential discovery of six different IGF-binding proteins (IGFBPs). They were isolated from different tissues in several species and are characterised at the molecular level. Data obtained from several *in vitro* and *in vivo* experiments, and more recently also from transgenic animal models (Schneider *et al.* 2000), indicate that the IGFBPs are far more than mere binding proteins. These highly conserved proteins are secreted in a

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tissue- and developmental stage-specific manner, they are present in different concentrations in different body compartments, they can modulate IGF bioactivity positively or negatively and they also exert IGF-independent effects (Kelley *et al.* 1996, Rajaram *et al.* 1997). Additionally, at least three mechanisms have been shown which alter the affinity of IGFBPs for the IGFs: proteolysis, phosphorylation and adherence to either cell-surface proteins or the extracellular matrix (ECM) (Clemmons 1997). Further proteins with structural and functional similarities to the IGFBPs have recently been discovered. This has led to the proposal of an IGFBP superfamily, comprising the six high-affinity IGFBPs and low-affinity IGF binders, termed IGFBP-related proteins (IGFBP-rPs) (Hwa *et al.* 1999).

Despite their structural similarity, each IGFBP has unique characteristics and functions. IGFBP-5, the subject of this review, is considered to be rather a stimulatory IGFBP that appears to counteract the inhibitory actions of IGFBP-4 in systems like bone (Mohan *et al.* 1995*b*) and cultured vascular smooth muscle cells (Duan & Clemmons 1998). Across species barriers IGFBP-5 is the most conserved IGFBP. Its amino acid sequence is 83% identical between human and chicken (Allander *et al.* 1997) and more than 97% identical between human, mouse and rat (James *et al.* 1993). It plays an important role in several biological processes including bone, ovary, mammary gland and kidney physiology (Kelley *et al.* 1996). Several unexpected and unique characteristics of this binding protein have been reported in the last years. In a dogma-breaking discovery it was recently identified as one component of a ternary complex with IGF-I or -II and the acid-labile subunit (ALS) in the human circulation (Twigg & Baxter 1998, Twigg *et al.* 1998, 2000). Also surprising was the recent discovery of IGFBP-5 being localised in the nuclear compartment of human breast cancer cells (Schedlich *et al.* 1998, 2000). Furthermore, a growing number of reports support the idea that IGFBP-5 is involved in the regulation of proliferation of cancer cells of different histogenetic origin (Higo *et al.* 1997, Gregory *et al.* 1999, Nickerson *et al.* 1999, Parisot *et al.* 1999, Rozen & Pollak 1999, Miyake *et al.* 2000*a*,*b*). IGFBP-5 is one of the IGFBPs that displays IGF-independent effects. Accordingly, potential signalling receptors for IGFBP-5 have been described (Andress 1995, 1998, Leal *et al.* 1999). The aim of this review is to present and critically discuss our current knowledge about the structural properties and biological functions of IGFBP-5.

Genomic organisation of the IGFBP-5 gene

In the mouse, the IGFBP-5 gene has a length of 17 kb and is localised on chromosome 1 (Kou *et al.* 1994). In humans it spans 33 kb and was identified on chromosome 2 (Allander *et al.* 1994). The IGFBP-5 gene is located on the same chromosome as the IGFBP-2 gene but orientated in a tail-to-tail fashion (opposite transcriptional direction). The distance between both genes comprises only 20 kb in humans (Allander *et al.* 1994) and 5 kb in mice (Kou *et al.* 1994). Similarly, the IGFBP-1 and -3 genes are tightly linked and are positioned in a tail-to-tail orientation on chromosome 7 in humans (Ehrenborg *et al.* 1992). The IGFBP-4 and -6 genes are located on separate chromosomes. The genomic distribution and the close relationship between certain IGFBPs suggest that these proteins have developed after duplication of an ancestral IGFBP. The resulting gene pair might then have been dispersed to different chromosomal locations (Allander *et al.* 1994). Since IGFBP-6 is the most divergent IGFBP it appears likely that it represents the binding protein which is most similar to the ancestral proto-IGFBP (Reinecke & Collet 1998).

The IGFBP genes are associated with the homeobox (HOX) genes, which are widely expressed in multicellular organisms and encode transcription factors that are crucial for early development. IGFBP-1 and -3 are localised on the same chromosome as the HOXA cluster, the IGFBP-2 and -5 genes map to the same chromosomal region as the HOXD cluster while the IGFBP-4 gene is found in the vicinity of the HOXB genes. Finally, IGFBP-6 and HOXC genes are found on the same chromosome. This suggests that IGFBPs and HOX genes were probably linked prior to the initial duplication event, implying that the IGFBPs are phylogenetically ancient molecules (Allander *et al.* 1994). Supporting this concept, several proteins that are able to bind to the IGFs were detected in the serum of reptiles, of the agnathan lamprey and in bony fish (reviewed in Reinecke & Collet 1998).

The IGFBP-5 gene has a conserved structure of four exons separated by three introns in human, mouse and rat. The first intron has a length of several kilobases, encompassing more than 50% of the gene. The promoter region has a simple structure, typical for regulated eukaryotic genes. Conserved TATAA and CAAT consensus sequences are present upstream of the transcription start in human (Allander *et al.* 1994), mouse (Kou *et al.* 1995) and rat (Zhu *et al.* 1993). The essential promoter activity seems to reside in the proximal 200 bp (Kou *et al.* 1995). In addition, an AP-2 recognition sequence was identified 5′ of the TATA box, which explains at least in part the responsiveness of this gene to agents that increase intracellular cAMP levels (Duan & Clemmons 1995). The stimulation of IGFBP-5 transcription by progesterone was demonstrated to be mediated by a CACCC sequence in the proximal promoter (Boonyaratanakornkit *et al.* 1999).

IGFBP-5: a protein harbouring multiple functional domains

Although it is almost impossible to define exactly when IGFBP-5 was detected for the first time, there is a

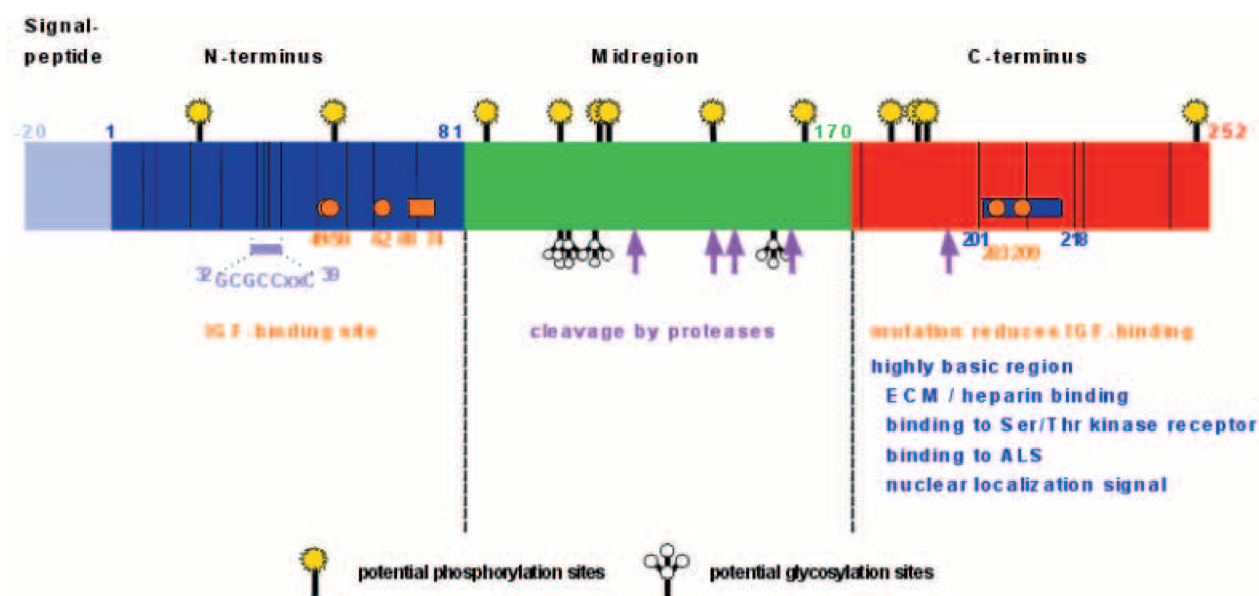

Figure 1 Schematic representation of the insulin-like growth factor binding protein (IGFBP)-5. Vertical lines represent Cys residues. Potential phosphorylation and glycosylation sites are indicated according to Coverley & Baxter (1997), Conover (1999) and Ständker *et al.* (1998). For further details refer to the text.

consensus in the literature that it was first purified and correctly identified almost at the same time in adult rat serum (Shimasaki *et al.* 1991), human bone extract (Bautista *et al.* 1991) and conditioned medium of human osteoblast-like cells (Andress & Birnbaum 1991). In the same year, full-length cDNA clones for rat and human IGFBP-5 were isolated (Kiefer *et al.* 1991, Shimasaki *et al.* 1991).

After cleavage of a 20 amino acid signal peptide, the mature IGFBP-5 consisting of 252 amino acids and with a molecular mass of approximately 29 kDa is secreted in humans and mice. The most important functional elements of human IGFBP-5 are depicted schematically in Fig. 1. Comparison of the amino acid sequence of all mammalian IGFBPs reveals that they share a common organisation with three distinct domains of similar size. While the N-terminal and the C-terminal domains are conserved, the midregion is highly variable. The positions of 18 cysteines in IGFBP-1, -2, -3 and -5 are highly conserved, 12 being located in the N-terminal region and the remaining six in the C-terminal domain. IGFBP-4 contains two additional cysteines in the midregion while in IGFBP-6 only 16 cysteines are found.

The N-terminal domain

The clustering of an even number of cysteines in this region of the IGFBP molecules suggests a complex structure with six possible intradomain disulphide bonds rather than a formation of interdomain disulphide bridges with the cysteines in the C-terminal domain (Hwa *et al.* 1999). Indeed, it was demonstrated that the last four cysteines of the N-terminal domain form overlapping disulphide linkages in human IGFBP-5. Nuclear magnetic resonance spectroscopy of this subdomain revealed a rigid, globular structure that consists of a centrally located three-stranded

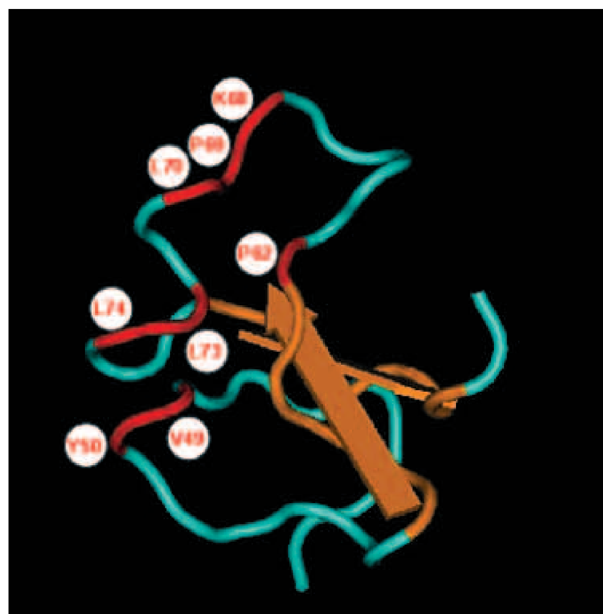

Figure 2 Three-dimensional structure of the IGF-binding region of IGFBP-5. Amino acids critical for IGF binding are indicated. The structure (1BOE) was downloaded from the NCBI homepage (http://www.ncbi.nlm.nih.gov).

Table 1 IGFBP-5-specific proteolytic activities

	Molecular weight (kDa)	Cleavage site	Protease(s)	Reference
Origin of the fragment				
Human pregnancy serum	22 and 15	ND	Gelatinase	Kübler *et al.* (1998)
Rat granulosa cells	19·5 and 17·5	ND	Non-MMP Zn^{2+} metalloproteinase	Resnick *et al.* (1998)
Human seminal plasma	20 and 14	ND	PSA and unknown protease	Lee *et al.* (1994)
Human fibroblasts	22, 20 and 17	ND	?	Nam *et al.* (1994)
	23	ND	?	Camacho-Hubner *et al.* (1992)
	22	ND	Complement C1s	Busby *et al.* (2000)
Human osteoblasts	18–20	ND	67 and 167 kDa proteases	Kanzaki *et al.* (1994)
	23	ND	?	Andress & Birnbaum (1992)
Ovine follicular fluid	19	ND	Metallo- and serine proteases	Besnard *et al.* (1996*a*)
Ovine chondrocytes	22 and 16	ND	Serine protease	Sunic *et al.* (1998*b*)
Porcine smooth muscle cells	22	Arg^{138}-Arg^{139}	?	Imai *et al.* (1997)
Mouse osteoblasts	22 and 14	ND	57–72 kDa MMPs and 97 kDa non-MMP	Thrailkill *et al.* (1995)
Human amniotic fluid	22 and 15	ND	>100 kDa serine protease	Claussen *et al.* (1994)
Incubation with thrombin	24, 23 and 20	Lys^{120}-His^{121}, Arg^{156}-Ile^{157}, Arg^{192}-Ala^{193}	Thrombin	Zheng *et al.* (1998)
Incubation with PAPP-A2	ND	Ser^{143}-Lys^{144}	PAPP-A2	Overgaard *et al.* (2001)

ND=not determined; PSA=prostate-specific antigen; MMP=matrix metalloproteinase; PAPP-A2=pregnancy-associated plasma protein-A2.

anti-parallel β-sheet whose scaffold is further stabilised by these disulphide bonds (Kalus *et al.* 1998). Using the IGFBP-5 fragment Ala^{40}-Ile^{92} these authors also demonstrated that this region contains the primary binding site for IGFs (Fig. 2) comprising Val^{49}, Tyr^{50}, Pro^{62} and Lys^{68}–Leu^{75}, which form a hydrophobic patch on the surface of the protein. *In vitro* mutagenesis of the intact IGFBP-5 confirmed residues 68, 69, 70, 73 and 74 as being essential for high-affinity binding to IGF-I (Imai *et al.* 2000). However, the complete N-terminus has substantially lower affinity than the intact protein and sequences in the C-terminus, although not physically interacting with the IGFs, are indispensable for stable and high-affinity binding of the ligands.

Also located in the N-terminal domain of the IGFBPs (except IGFBP-6) is a GCGCCxxC motif (Fig. 1). It is also highly conserved in the IGFBP-rPs and can be found in several other unrelated proteins. Its significance, however, remains unknown at present (Hwa *et al.* 1999).

The midregion

Since the amino acid sequence is almost unique for each IGFBP this region was believed to function only as a structural hinge between the other two highly conserved domains. However, exactly the opposite is true. The midregion has an important physiological role since most post-translational modifications of the IGFBPs take place in this domain. It is this unique domain that makes each IGFBP structurally different and consequently distinct in its function, although a direct correlation between an individual sequence and divergent properties still awaits definite proof.

Proteolysis All IGFBPs can be cleaved by specific proteases, a phenomenon that results in reduction or loss of IGF-binding activity. Most proteolytic sites identified so far are located in this non-conserved region (Fig. 1), suggesting a potential mechanism by which IGF activities can be regulated in a tissue-specific manner (Clemmons 1997). The cleavage site of a serine protease secreted by porcine smooth muscle cells was identified as the di-basic Lys^{138}/Lys^{139} motif in IGFBP-5 (Imai *et al.* 1997). IGFBP-5 proteolytic activity was identified in biological fluids and in conditioned media of cell cultures. In some cases specific proteases have been characterised (Conover 1999). Table 1 presents some of the IGFBP-5-specific proteolytic activities described so far. In some instances the responsible proteases have been identified; however, it remains to be elucidated whether all of the other described activities represent distinct molecules.

Glycosylation Analysis of U-2 human osteosarcoma conditioned medium by Western ligand blot showed IGFBP-5 as an IGF-binding triplet of 29, 32 and 34 kDa. Sequential treatment with neuraminidase and O-glycanase, but not with N-glycanase or endoglycosidase F, reduced all bands to the 29 kDa core protein, suggesting that IGFBP-5 was O-glycosylated (Conover & Kiefer 1993). Similar 29–34 kDa forms were identified in conditioned media of human fibroblasts and osteoblasts. O-glycosylation of Thr^{152} was detected in IGFBP-5 fragments isolated from human serum (Ständker *et al.* 1998). Thus, it appears that all predicted glycosylation sites (Thr^{103}, Thr^{104} and Thr^{111}) (Conover 1999) are used in IGFBP-5, although this modification might happen in a tissue-specific manner. In IGFBP-6, the other

O-glycosylated IGFBP, the glycosylation sites are also clustered in the midregion, and this modification makes the molecule less susceptible to proteolysis (Bach 1999).

Phosphorylation The IGFBP-5 molecule has 12 potential phosphorylation sites (Fig. 1). Such sites are also present in all other IGFBPs but only phosphoisoforms of IGFBP-1, -3 and -5 have been reported so far (Coverley & Baxter 1997). IGFBP-5 secreted by human fibroblasts can be highly phosphorylated (Jones *et al.* 1992). Phosphorylation of IGFBP-1 increases its affinity for IGF-I substantially (Jones *et al.* 1993). Therefore, this post-translational modification might also affect the property of IGFBP-5 to modulate IGF bioactivity. In addition, phosphorylation might alter the susceptibility for proteolysis. Conover (1999) suggested that phosphorylation might be important for the affiliation of IGFBP-5 with hydroxyapatite in bone. Nevertheless, phosphorylation is a common mechanism to modulate the activity of intracellular proteins and it may be important for the interaction of IGFBP-5 with cellular proteins on its way to the nucleus (see below).

The C-terminal domain

Six cysteines are found in this region of the IGFBPs at strictly conserved positions (Fig. 1). Consistent with the formation of intradomain disulphide bridges in the N-terminal region, there is evidence that these cysteines also interact with each other (Hwa *et al.* 1999). Although it is not the primary IGF-binding site, this domain was demonstrated to be essential for high-affinity and stable IGF binding. Site-specific mutagenesis of the strictly conserved amino acids Gly^{203} or Gln^{209} dramatically reduced the IGF binding affinity of rat IGFBP-5 (Bramani *et al.* 1999, Song *et al.* 2000). In addition, it was demonstrated that N-terminal fragments of IGFBP-5 have a 10- to 200-fold lower affinity for the IGFs compared with the full-length protein (Kalus *et al.* 1998), consistent with the reduced IGF affinity observed in C-terminally truncated fragments (Andress & Birnbaum 1992, Andress *et al.* 1993). The region comprising the last five cysteines of IGFBP-5 shares 37% similarity with the thyroglobulin-type I domain. The function of this domain, also found in several other unrelated proteins, is unknown (Hwa *et al.* 1999). Finally, these basic regions found in the C-terminal domain of IGFBP-3, -5 and -6 were shown to be able to inhibit IGFBP-4 degradation (Fowlkes *et al.* 1997).

Several investigations using amino acid substitution and peptide competition experiments have identified a highly basic region between amino acids Arg^{201} and Arg^{218} to which several important functions were ascribed. These functions include binding to the ECM and binding to ALS and cell membranes and they are described in the sections below.

Binding to ECM Early experiments have shown that IGFBP-5 has the ability of binding to the ECM and that this interaction results in reduced affinity for the IGFs and stimulation of IGF activities (Andress & Birnbaum 1992, Jones *et al.* 1993). This was confirmed by the observation that co-incubation of IGFBP-5 with glycosaminoglycans (GAGs) reduced the IGF affinity 17-fold (Arai *et al.* 1994). In addition, GAGs inhibited proteolysis of IGFBP-5 (Arai *et al.* 1994). The basic region between residues 201 and 218 was identified as being important for the binding to heparin and to other components of the ECM (Arai *et al.* 1994, 1996, Parker *et al.* 1996, 1998, Campbell & Andress 1997, Nam *et al.* 1997, 2000, Rees & Clemmons 1998, Song *et al.* 2000). It was hypothesised that binding of heparin to this domain would produce a conformational change that might reduce the affinity for the IGFs (Arai *et al.* 1994, 1996). A systematic mutational analysis of this region identified Arg^{207} and Arg^{214} as the most critical amino acids for ECM binding (Parker *et al.* 1998). A helical wheel prediction places these residues adjacent to Gly^{203}, which was demonstrated to be critical for IGF binding, while Glu^{209}, another amino acid essential for IGF binding, is flanked by two other basic residues of the wheel (Parker *et al.* 1998, Bramani *et al.* 1999). In addition, mutation of the basic residues 201, 202, 206 and 214 resulted in attenuated heparin binding but only in a small reduction of the affinity for the IGFs (Song *et al.* 2000). This strongly suggests that ECM and IGF binding sites are located in close proximity to each other and may even overlap, providing an alternative explanation for the reduced IGF affinity of ECM-bound IGFBP-5.

Binding to ALS Until recently IGFBP-3 was believed to be the only IGFBP able to participate in the formation of a ternary complex with ALS and IGF-I or -II. For IGFBP-3 the C-terminal region is essential for the interaction with the ALS. This is of interest for the study of IGFBP-5 because it was recently demonstrated that this IGFBP can also form a ternary complex of about 130 kDa with ALS and one IGF molecule (Twigg & Baxter 1998). IGFBP-3 and -5 share a high degree of similarity in the C-terminal region (54%) and the sequence 201–218 appears to be the primary binding site of IGFBP-5 for ALS (Twigg *et al.* 1998) and Lys^{211}, Arg^{214}, Lys^{217} and Arg^{218} were identified as the key residues for ALS binding (Firth *et al.* 2001). In addition, mutation of specific residues in the midregion resulted in a small decrease in the binding affinity for ALS (Firth *et al.* 2001).

Binding to cell membrane proteins In 1995 it was demonstrated that IGFBP-5 binds to and is internalised by a 420 kDa membrane protein of mouse osteoblastic cells which is probably not a proteoglycan (Andress 1995). The highly basic region 201–218 appears to be important for

Table 2 Major sites of IGFBP-5 expression in different species

	Expression site	Reference
Species		
Human	Testis	Zhou & Bondy (1993*b*), Drescher *et al.* (1997)
	Trabecular meshwork	Wirtz *et al.* (1998)
	Bone	Bautista *et al.* (1991)
	Lung	Allen *et al.* (2000)
	Uterus and placenta	Zhou *et al.* (1994), Han & Carter (2000), Zygmunt *et al.* (2000)
	Ovary	Zhou & Bondy (1993)*a*)
Mouse	Limbs (e)	Van Kleffens *et al.* (1998)
	Kidney (e)	Lindenbergh-Kortleve *et al.* (1997)
	Lung (e)	Schuller *et al.* (1995)
	Ovary	Adashi *et al.* (1997), Wandji *et al.* (1998)
	Spinal cord	Arnold *et al.* (2000)
	Skeletal tissues	Wang *et al.* (1995)
	Mammary gland	Wood *et al.* (2000)
Rat	Pancreas (e)	Hill *et al.* (1999)
	Kidney (e)	Matsell *et al.* (1994)
	Ovary (e)	Erickson *et al.* (1992)
	Lung (e)	Van de Wetering *et al.* (1997), Wallen *et al.* (1997)
	Kidney	Price *et al.* (1995)
	Forebrain	Stenvers *et al.* (1994)
	Eyes	Burren *et al.* (1997)
	Liver	Zimmerman *et al.* (2000)
	Pituitary	Bach & Bondy (1992), Gonzalez-Parra *et al.* (2001)

e=embryonic stages.

this binding, which can be modulated by GAGs. The fact that this protein is rapidly downregulated by IGFBP-5 suggested that it may function as a receptor. Further analysis revealed that intact IGFBP-5 as well as IGFBP-$5^{1–169}$ and IGFBP-$5^{201–218}$ were able to stimulate the phosphorylation of this protein (Andress 1998), a clear example of an IGF-independent action. It remains to be elucidated whether this protein is identical to the 400 kDa type V transforming growth factor-β receptor which has been shown to interact with IGFBP-3, -4 and -5, although the effect of these molecules on DNA synthesis remained rather minor (Leal *et al.* 1999). The IGFBP-$5^{1–169}$ fragment does not contain a known heparin-binding domain, but the existence of a cluster of basic amino acids between residues 133 and 143 may explain the ability of the IGFBP-$5^{1–169}$ fragment to bind to the membrane protein (Andress 1995). A similar membrane protein was identified in mesangial cells (Abrass *et al.* 1997) and it was demonstrated that IGFBP-$5^{201–218}$ was able to stimulate Cdc42 GAP aggregation and filopodia formation in migrating mesangial cells by binding to a serine/threonine kinase receptor (Berfield *et al.* 2000).

Nuclear localisation Potential nuclear localisation signals are present in IGFBP-3 and -5 (Radulescu 1994). Consistent with this was the finding that recombinant IGFBP-3 and -5, but not IGFBP-1 and -2 are translocated to the nucleus of human breast cancer cells (Schedlich *et al.* 1998). Further analysis showed that this occurs by a nuclear localisation signal-dependent pathway and is mediated mainly by the importin-β nuclear transport factor (Schedlich *et al.* 2000). The significance of the intranuclear localisation of these IGFBPs is not clear at present, but may be linked to IGF-independent activities.

Expression of IGFBP-5 *in vivo*

Tissue-specific and developmental expression

Preimplantation mouse embryos transcribe IGFBP-1, -2, -3, -4 and -6 but not IGFBP-5 mRNA (Hahnel & Schultz 1994, Liu *et al.* 1997), whose expression is detectable as early as day 10·5 of gestation in the rat embryo (Green *et al.* 1994) and at day 11 of gestation in the mouse (Schuller *et al.* 1993). In contrast, IGFBP-5 mRNA expression was detected by RT-PCR in bovine blastocysts (Winger *et al.* 1997) and shown to be upregulated by exogenous IGF peptides (Prelle *et al.* 2001). Initial studies identified the meninges, vertebrae, lung, kidneys and intestine as the major sites of IGFBP-5 expression in the late gestation mouse conceptus (Schuller *et al.* 1993) and kidney, lung, heart, brain, muscle, ovary and testes in the adult animal (James *et al.* 1993, Schuller *et al.* 1994). Table 2 summarises the major sites of IGFBP-5 expression in embryonic and adult tissues of different species.

IGFBP-5 is present at different levels in slow and fast rat muscles, and denervation dramatically upregulates transcription of its mRNA (Bayol *et al.* 2000). In agreement,

overloading mouse skeletal muscle decreased IGFBP-5 expression to one-third of the control value, while unloading by hind limb suspension doubled this value (Awede *et al.* 1999). In contrast, the expression of the IGFs and IGFBP-5 was not altered acutely by nutrients or insulin in human skeletal muscle (Schimke *et al.* 1999). This suggests that IGFBP-5 may play a role in muscle long-term adaptation to changes in loading. In the involuting rat mammary gland (Tonner *et al.* 1997), prostate (Thomas *et al.* 1998) and thyroid gland (Phillips *et al.* 1994) IGFBP-5 expression was demonstrated to be upregulated, suggesting that it may be involved in the regulation of apoptosis-mediated involution of these organs.

IGFBP-5 in serum and hormonal regulation

The most important regulator of IGFBP-5 expression *in vivo* is IGF-I. This is underlined by the upregulation of IGFBP-5 in the brain of IGF-I-transgenic mice (Ye & D'Ercole 1998) and consistent with the observation that both proteins are spatially and temporally co-expressed during brain development (Bondy & Lee 1993). In normal adult human serum, IGFBP-5 levels are positively correlated with IGF-I concentrations (Mohan *et al.* 1996). As mentioned previously, IGFBP-5 in human serum is capable of forming a ternary complex of 130 kDa with ALS and IGF-I or -II. The mean percentage of IGFBP-5 in ternary complexes was 58·2% in normal human serum (Baxter *et al.* 2000). However, since IGFBP-5 is a minor IGF-carrier, the physiological significance for this phenomenon remains to be determined. The expression of IGFBP-3, ALS and IGF-I is strictly regulated by growth hormone (GH). This is also true for IGFBP-5 under some circumstances (Mohan *et al.* 1995*a*, Ono *et al.* 1996, Thoren *et al.* 1998, Ulinski *et al.* 2000). It is plausible that for IGFBP-5 the ALS binding is a characteristic conserved during evolution after the gene duplication event that originated IGFBP-3 and -5, but not of outstanding physiological significance at the systemic level. Liver is the source of serum ALS, but extrahepatic expression was detected in bone and renal cortex (Chin *et al.* 1994), also sites of high IGFBP-5 expression.

IGFBP-5 serum levels increase in puberty and decrease with ageing (Rajaram *et al.* 1997). The skeletal content of IGFBP-5 was also demonstrated to decrease by 28% between the age of 20–29 years and 54–64 years, which correlates positively with both IGF-I and -II levels (Mohan *et al.* 1995*a*). The report that the intrinsic capacity of human bone cells to produce these IGF components is largely preserved with age illustrates the complex interplay of molecules in the bone environment (Pfeilschifter *et al.* 2000). However, since Kveiborg *et al.* (2000) observed an age-related impairment in the production of components of the IGF system by osteoblasts *in vitro*, the reduction observed *in vivo* can either reflect their secretion levels by osteoblasts or the serum level.

Expression of IGFBP-5 *in vitro*

There is some information on signals which modulate IGFBP-5 expression *in vivo*, e.g. prolactin (PRL) in the mammary gland (Tonner *et al.* 1997). However, most of our knowledge about regulation of IGFBP-5 expression is derived from *in vitro* studies. Many cell types express IGFBP-5 mRNA and serve as biological systems to identify molecules which alter IGFBP-5 expression.

Similar to the *in vivo* conditions, IGF-I is the most important regulator of the expression of IGFBP-5 in a large number of cell types in different species *in vitro*. Depending on the cell type this can occur in two different ways. The first is direct stimulation of the transcription of the gene (Conover & Kiefer 1993, Matsumoto *et al.* 1996) (Table 3). The second occurs post-translationally by interaction with the secreted protein; when bound to IGF-I, IGFBP-5 is protected from proteolysis (Camacho-Hubner *et al.* 1992, Matsumoto *et al.* 1996). IGF-I-mediated transcription of the IGFBP-5 gene is generally believed to occur after the activation of the IGF-I R, but how these two phenomena are linked together in detail is poorly defined. In porcine vascular smooth muscle cells this activation of the IGFBP-5 gene was demonstrated to be dependent on the activation of the PI 3-kinase-PKB/Akt-p70^{s6k} signalling cascade but not on the MAPK pathway (Duan *et al.* 1999). Likewise, IGF-II and insulin can stimulate transcription of the IGFBP-5 gene (see Table 3). Interestingly, the expression of IGFBP-5 is also affected by cell density in porcine aortic smooth muscle cells (Duan & Clemmons 1998) and ovine granulosa cells (Monget *et al.* 1998). Apart from these components of the IGF-system, IGFBP-5 can be regulated by hormones, cytokines and other molecules in a cell-specific manner. Table 3 gives an overview of these molecules.

Physiological role of IGFBP-5

Since neither the overexpression nor the genetic ablation of IGFBP-5 in animal models has been published our knowledge about the function of IGFBP-5 *in vivo* remains restricted. In that situation *in vitro* assays are the almost exclusive source to gather information about the physiological activities of IGFBP-5 and the use of cellular systems has enabled us to link IGFBP-5 activity with IGF-mediated cell proliferation, differentiation and motility. In addition, cell lines have clearly indicated that IGFBP-5 also triggers events which occur independently of IGFs. Some of these activities are listed in Table 4. However, specific effects of IGFBP-5 *in vivo* which correspond with *in vitro* data suggest that IGFBP-5 plays a significant role in the regulation of organ function, including the development of the central nervous system (Lee *et al.* 1995, Ye & D'Ercole 1998), involution of the mammary gland (Tonner *et al.* 1997) and bone physiology (Richman *et al.* 1999, Miyakoshi *et al.* 2001).

Table 3 Effect of different agents on the expression of IGFBP-5 *in vitro*

Agent	Cell type	Effect	References
IGF-I	Porcine aortic smooth muscle cells	s	Duan *et al.* (1996, 1999), Duan & Clemmons (1998)
	Human chondrocytes	s	Matsumoto *et al.* (2000)
	Rat chondrocytes	s	Matsumoto *et al.* (1996), Koedam *et al.* (2000)
	Ovine chondrocytes	s	Sunic *et al.* (1998*a*,*b*)
	Human breast cancer cells	s	Sheikh *et al.* (1993), Shemer *et al.* (1993)
	Human fibroblasts	s	Camacho-Hubner *et al.* (1992), Conover *et al.* (1995), Yoshizawa & Clemmons (2000)
	Rat intestinal smooth muscle	s	Zimmerman *et al.* (1997), Hou *et al.* (2000)
	Mouse myoblasts	s	Rousse *et al.* (1998)
	Rat osteoblasts	s	McCarthy *et al.* (1994), Dong & Canalis (1995), Gabbitas & Canalis (1998)
	Human osteosarcoma	s	Conover & Kiefer (1993)
	Mouse pituitary cells	s	Fielder *et al.* (1993)
	Rat Schwann cells	s	Cheng *et al.* (1999)
	Rat thyroid follicular cells	s	Backeljauw *et al.* (1993)
IGF-II	Human aortic smooth muscle	s	Duan *et al.* (1996)
	Rat chondrocytes	s	Matsumoto *et al.* (1996), Koedam *et al.* (2000)
	Rat osteoblasts	s	McCarthy *et al.* (1994)
	Mouse pituitary cells	s	Fielder *et al.* (1993)
	Rat thyroid follicular cells	s	Backeljauw *et al.* (1993)
Insulin	Human aortic smooth muscle cells	s	Duan *et al.* (1996)
	Rat chondrocytes	s	Koedam *et al.* (2000)
	Mouse myoblasts	s	Rousse *et al.* (1998)
	Mouse pituitary cells	s	Fielder *et al.* (1993)
	Rat thyroid follicular cells	s	Backeljauw *et al.* (1993)
Dexamethasone	Rat chondrocytes	i	Koedam *et al.* (2000)
	Human fibroblasts	i	Conover *et al.* (1995)
	Mouse pituitary cells	i	Fielder *et al.* (1993)
FSH	Rat granulosa cells	i	Adashi *et al.* (1991, 1993), Liu *et al.* (1993)
GH	Rat osteoblasts	s	McCarthy *et al.* (1994)
Interleukin-1α	Ovine chondrocytes	s	Sunic *et al.* (1998*a*)
Interleukin-6	Rat osteoblasts	s	Franchimont *et al.* (1997)
Osteogenic protein-1	Rat fetal calvarial cells	i	Yeh *et al.* (1998), Yeh & Lee (2000)
Parathyroid hormone	Rat osteosarcoma cells	s	Torring *et al.* (1991), Conover *et al.* (1993), Nasu *et al.* (1997, 1998)
	Rat osteoblasts	s	Schmid *et al.* (1996)
Prostaglandin E_2	Rat osteoblasts	s	McCarthy *et al.* (1994, 1996), Pash & Canalis (1996), Ji *et al.* (1999)
Retinoic acid	Rat osteoblasts	s	Dong & Canalis (1995)
	Human breast carcinoma	i	Shemer *et al.* (1993)
TSH	Rat thyroid follicular cells	i	Backeljauw *et al.* (1993)
Vitamin D3	Rat osteosarcoma cells	s	Nasu *et al.* (1997)
	Rat osteoblasts	s	Schmid *et al.* (1996)

i=inhibition; s=stimulation; TSH=thyroid-stimulating hormone.

Bone physiology and pathology

Soon after its molecular characterisation IGFBP-5 was identified as an essential regulator of IGF activities in bone cells. As a consequence, this property became a target of extensive studies. Expression of IGFBP-5 in bone cells is tightly regulated by the IGFs and several other hormones and growth factors (Table 3). In addition, not only the expression, but also the effect and the fate of IGFBP-5 are different in distinct bone cell lines (Schmid *et al.* 1995).

In contrast to IGFBP-4, which exerts exclusively inhibitory actions on bone cells both *in vitro* (Mohan *et al.* 1989, 1995*b*) and *in vivo* (Miyakoshi *et al.* 1999), IGFBP-5 was identified as a stimulator of osteoblast mitogenesis (Andress & Birnbaum 1991, 1992, Bautista *et al.* 1991, Andress *et al.* 1993, Mohan *et al.* 1995*b*). The stimulatory

Table 4 Examples of IGF-stimulatory, -inhibitory and -independent actions postulated for IGFBP-5 in cultured cells

Action	Example	Reference
Stimulatory	Mouse skeletal muscle cell survival and differentiation	James *et al.* (1993), Stewart *et al.* (1996), Meadows *et al.* (2000)
	Rat intestinal smooth muscle cell fibrogenesis	Zimmerman *et al.* (1997)
	Mouse osteoblast-like cell mitogenesis	Andress & Birnbaum (1992)
	Human fibroblast growth	Jones *et al.* (1993)
	Human prostate tumour cell proliferation	Miyake *et al.* (2000*a*,*b*)
	Schwann cell differentiation	Cheng *et al.* (1999)
	Mouse mature osteoclast activation	Kanatani *et al.* (2000)
	Mouse osteoblast proliferation	Bautista *et al.* (1991)
	Myoblast differentiation	Ewton *et al.* (1998)
Inhibitory	Myoblast proliferation and differentiation	Ewton *et al.* (1998)
	Porcine smooth muscle cell migration, DNA and protein synthesis (protease-resistant form)	Imai *et al.* (1997)
	C2 myoblast differentiation	James *et al.* (1996)
	Human osteosarcoma proliferation	Kiefer *et al.* (1991, 1993)
	DNA and glycogen synthesis	Conover & Kiefer (1993)
	SV-40-transformed human fibroblast growth	Reeve *et al.* (1995)
	Rat mesangial cell migration	Abrass *et al.* (1997)
	Human cervical cancer cell growth	Higo *et al.* (1997)
	Central nervous system development	Roschier *et al.* (2001)
Independent	Stimulation of bone cell growth	Andress & Birnbaum (1992), Mohan *et al.* (1995*b*)
	Stimulation of rat mesangial cell migration	Abrass *et al.* (1997), Berfield *et al.* (2000)
	Mouse osteoclast-like cell formation	Kanatani *et al.* (2000)
	Mouse bone formation parameters	Richman *et al.* (1999)

properties of IGFBP-5 were postulated to be mediated by its ability to bind to the cell membrane or ECM (Andress & Birnbaum 1992). Bautista *et al.* (1991) have previously reported that among the IGFBPs IGFBP-5 has the unique property of binding to hydroxyapatite, a component of the mineralised ECM of bone. The authors proposed an IGF-dependent mechanism by which IGFs are sequestered and concentrated in bone. Release of IGFs during bone remodelling or after injury would then stimulate proliferation of neighbouring osteoblasts (Fig. 3A). The age-related decline in the skeletal concentration of IGF-I and IGFBP-5 supports this model and provides an explanation for the age-related impairment of bone formation vs resorption (Mohan *et al.* 1995*a*). In addition, bone is the only tissue where IGF-II is stored in higher concentrations than in serum (Mohan *et al.* 1988).

Inhibitory actions of IGFBP-5 on bone cells were reported by Kiefer *et al.* (1992, 1993) and Conover & Kiefer (1993). IGFBP-5 also inhibited IGF activity on osteosarcoma cells (Schmid *et al.* 1995). The important factor responsible for this apparent divergence might be the localisation of IGFBP-5; stimulatory actions were associated with binding to the cell membrane (Bautista *et al.* 1991, Andress & Birnbaum 1992). In contrast, inhibitory activities were correlated with IGFBP-5, which was present exclusively in the culture medium (Conover & Kiefer 1993). Similarly, stimulation of growth of human fibroblasts was associated with IGFBP-5 located in the ECM. When present only in the medium, IGFBP-5 became rapidly degraded and did not affect proliferation (Jones *et al.* 1993).

An IGF-independent effect of IGFBP-5 on bone cells, firstly reported by Andress & Birnbaum (1992) was confirmed by Mohan *et al.* (1995*b*) and demonstrated to occur also *in vivo* (Richman *et al.* 1999, Miyakoshi *et al.* 2001). The identification of putative IGFBP-5 receptors in the membrane of osteoblasts (Andress 1995, 1998) and mesangial cells (Abrass *et al.* 1997) and the detection of IGFBP-5 in the nucleus (Schedlich *et al.* 1998, 2000) support the idea that IGFBP-5 functions also as an independent growth factor (Fig. 3A). From all these data it appears evident that IGFBP-5 is an essential molecule for the maintenance of a complex network of factors which guarantee normal bone physiology. Disturbance of this equilibrium may lead to pathological situations like osteoporosis (Rosen *et al.* 1994, Rosen & Donahue 1998, Rosen 2000) or renal osteodystrophy (Jehle *et al.* 2000).

Mammary gland involution

Travers *et al.* (1996) reported that the involution of the mammary gland of rats induced by the combined deficiency of PRL and GH could not be overcome by IGF-I treatment, a rather unexpected finding if we

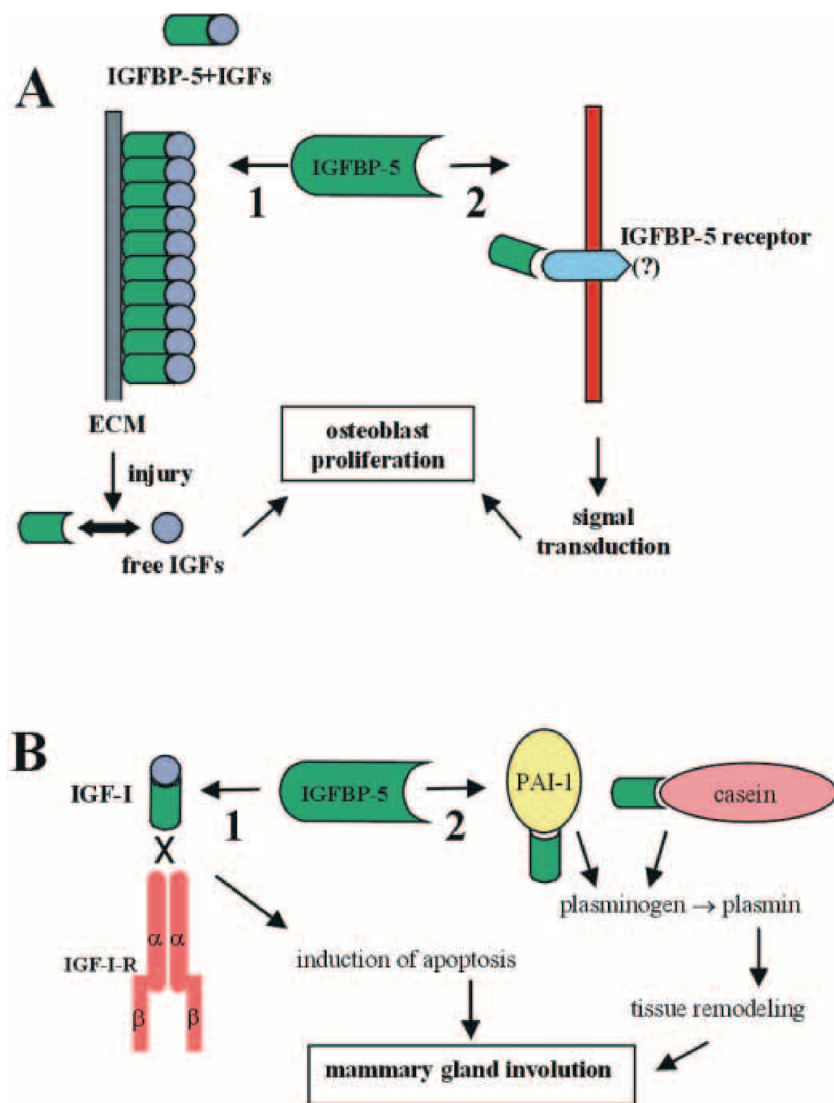

Figure 3 Physiological activity of IGFBP-5 in different tissues. (A) Regulation of osteoblast proliferation. IGFBP-5 allows the storage of IGFs in bone ECM, which can be mobilised later for stimulating repair processes after injury (1). IGFBP-5 (or proteolytic fragments) can also stimulate cell proliferation directly by binding to a membrane receptor and activating specific signalling pathways (2). (B) Mammary gland involution. IGFBP-5 inhibits the binding of IGF-I to its receptor thereby promoting increased apoptosis (1). Through an IGF-independent action IGFBP-5 interacts with casein and/or plasminogen-activator inhibitor-1 (PAI-1). As a consequence, plasmin is generated from plasminogen and extensive tissue remodelling (a key event of mammary gland involution) occurs (2).

consider that GH actions are often mediated by IGF-I. The authors suggested that this may be due to an inhibitory IGFBP, which was present in increasing amounts in the involuting mammary gland. This inhibitory IGFBP increased 50-fold in the milk after 2 days of mammary gland involution and was shown to be IGFBP-5 (Tonner *et al.* 1997). PRL inhibits apoptosis and involution of the mammary gland and also the increase in IGFBP-5. Furthermore, IGFBP-5 interacted with the casein micelles (which possess calcium phosphate), similar to its interaction with hydroxyapatite in bone (Bautista *et al.* 1991). Therefore, Flint *et al.* (2000) proposed that IGFBP-5 functions as an inhibitor of cell proliferation and survival during mammary gland involution, preventing the interaction of IGF-I with its receptor on epithelial cells (Fig. 3B). In contrast, GH and PRL cooperatively inhibit involution; GH increases IGF-I synthesis and PRL inhibits IGFBP-5 production. This model of IGFBP-5-induced

apoptosis through an IGF-dependent mechanism is supported by further experimental data; in conditional knockout mice lacking Stat3 in the mammary gland, delayed involution of the organ is associated with a lack of increase in IGFBP-5 expression (Chapman *et al.* 1999). Increased IGFBP-5 levels were also associated with apoptosis and involution in the rat ovary (Liu *et al.* 1993), prostate (Guenette & Tenniswood 1994, Thomas *et al.* 1998) and thyroid (Phillips *et al.* 1994). In addition, IGFBP-5 colocalises with areas of apoptosis during mouse development (Van Kleffens *et al.* 1999, Allan *et al.* 2000).

Activation of plasminogen to generate plasmin is a key event for the extensive ECM remodelling that occurs during mammary gland involution. The observation that IGFBP-5 interacts with both casein (Tonner *et al.* 1997) and plasminogen activator inhibitor-1 (Nam *et al.* 1997) suggests that IGFBP-5 may be also involved in the regulation of tissue remodelling. Tonner *et al.* (2000) proposed that IGFBP-5 may coordinate both apoptosis and tissue remodelling during mammary gland involution through IGF-dependent and -independent mechanisms respectively (Fig. 3B).

Ovarian physiology

The expression pattern of IGFBP-5 and its regulation were extensively studied in the rat ovary. Most studies concentrated on the expression of IGFBP-5 and its regulation by follicle-stimulating hormone (FSH) *in vitro* in granulosa cells (Adashi *et al.* 1990, 1991 1992, 1993, Liu *et al.* 1993). *In situ* hybridisation revealed that IGFBP-5 is expressed in a cell-specific manner: predominantly in granulosa cells of atretic follicles, to a lesser extent in interstitial cells, in corpora lutea and surface epithelium. This pattern is strongly affected by the stage of the oestrous cycle (Erickson *et al.* 1992).

The expression pattern of the IGF system in the mouse ovary and its hormonal regulation were found to be quite different from those of the rat (Adashi *et al.* 1997). While IGFBP-5 expression was associated with atretic follicles in the rat, it was linked to the survival of slowly growing and immature preantral follicles in the mouse (Wandji *et al.* 1998).

The regulation of expression and the potential function of IGFBP-5 and other components of the IGF system during follicular development and corpus luteum function were also investigated in large farm animals (Besnard *et al.* 1996*a*,*b*, Schams *et al.* 1999) and avian species (Onagbesan *et al.* 1999).

Kidney physiology and pathology

IGFBP-5 is abundantly expressed in the kidney. The specific expression pattern described in human (Matsell *et al.* 1994), mouse (Lindenbergh-Kortleve *et al.* 1997) and rat (Price *et al.* 1995) kidneys suggests specific roles in the development and physiology of this organ. Upregulation of IGFBP-5 expression was associated with the inhibited renal growth that follows hypophysectomy in rats (Hise *et al.* 1994) while reduced IGFBP-5 expression was reported after the onset of streptozotocin-induced diabetes (Landau *et al.* 1995). In fact, the whole renal IGF system undergoes massive alterations during the renal complications of diabetes (Flyvbjerg 1997). A stimulatory role was postulated for IGFBP-5 in children with chronic renal failure where the serum level of this IGFBP correlated with patient growth (Ulinski *et al.* 2000).

Muscle cell differentiation

A relationship between the secretion of specific IGFBPs and the differentiation of myoblasts was first reported by McCusker & Clemmons (1988). Tollefsen *et al.* (1989) observed that cultured mouse myoblasts secreted a single 29 kDa IGFBP during their terminal differentiation. This protein was later identified as IGFBP-5 and its expression was demonstrated to increase during the early stages of cell differentiation (James *et al.* 1993, Rotwein *et al.* 1995). Increased IGFBP-5 secretion was also observed in differentiating myoblasts that overexpress IGF-II (Stewart *et al.* 1996) or after treatment with insulin and IGFs (Ewton & Florini 1995). Direct evidence for a role of IGFBP-5 in myoblast differentiation came from transfection experiments; cells which overexpressed the construct in sense orientation failed to differentiate normally but had higher survival rates, while antisense RNA expression caused premature differentiation (James *et al.* 1996). These effects could be neutralised by the addition of exogenous IGFs. Another piece of evidence in this direction came from studies using L6A1 muscle cells; IGFBP-5 inhibited IGF-mediated cell proliferation and IGF-II-mediated differentiation, but potentiated IGF-I-stimulated differentiation under some circumstances (Ewton *et al.* 1998). The explanation for this dual role was proposed to be associated with the ability of IGFBP-5 to bind to the cell membrane. Finally, IGFBP-5 acts as a survival factor for differentiating myoblasts; IGFBP-5-overexpressing cells were protected from apoptosis induced by tumour necrosis factor-α (Meadows *et al.* 2000).

Even if it remains to be explained how the IGFs stimulate two almost mutually exclusive processes like proliferation and differentiation, it is clear that IGFBP-5 plays a major role in modulating these effects.

IGFBP-5 and cancer

The biological activity of IGFBP-5 on growth of neoplastic cells appears to be ambiguous even when assessed on a single cell line (Ewton *et al.* 1998). There is accumulating evidence that IGFBP-5 appears to be a protein with rather growth stimulatory functions also supported by the recent observation that it acts as a growth factor on bone cells

(Miyakoshi *et al.* 2001). IGFBP-5 stimulates growth of prostate cancer cells via IGF-dependent and -independent pathways *in vitro* (Gregory *et al.* 1999, Miyake *et al.* 2000*a*) and it promotes tumour growth in an animal model (Miyake *et al.* 2000*b*). Similarly, IGFBP-5 improves the survival of breast cancer cells. This function appears to be IGF-independent and to rely on an apoptosis-inhibiting effect triggered via a pathway which does not involve the mitochondria (Perks *et al.* 1999, 2000). However, breast carcinoma cells apparently are also susceptible to growth inhibitory signals of IGFBP-5 at certain stages if triggered by appropriate signals such as vitamin D (Rozen & Pollak 1999), anti-oestrogens (Parisot *et al.* 1999) or androgen deprivation (Nickerson *et al.* 1999). In addition, IGFBP-5 inhibits the proliferation of cervical carcinoma cells (Higo *et al.* 1997) and osteosarcoma cells (Kiefer *et al.* 1992, Conover & Kiefer 1993). In osteosarcoma cells it appears that at least some growth inhibition is mediated through IGF-independent mechanisms such as stimulation of differentiation which is accompanied by increased osteocalcin levels (Schneider *et al.* 2001). Thus, the effect of IGFBP-5 seems to be cell type-specific and the status of the cells of a certain tissue may also strongly influence and determine its sensitivity towards IGFBP-5. Future research has to unravel the molecular mechanisms of these biological pathways and may reveal potential therapeutic applications of IGFBP-5.

Conclusion and future perspectives

The molecular structure of IGFBP-5 was identified a decade ago and since then an enormous amount of data has been accumulated about the biological activity of this protein. Despite this progress, a number of central questions remain unanswered. Although cellular receptors have unambiguously been shown to exist, it remains completely unknown what happens after this interaction and to date no cellular partners of an IGFBP-5-specific signalling pathway have been identified. The localisation of IGFBP-5 in the nucleus suggests a role in the regulation of gene expression. Whether this happens through direct action of IGFBP-5 or whether it requires association with other proteins is also completely unsolved. Another major deficiency is the physiological function of IGFBP-5. Our present knowledge is mainly derived from cellular systems as no appropriate animal models exist. Although the knockout approach may be poorly informative due to the functional compensation assured by other members of the IGFBP superfamily, the overexpression of IGFBP-5 in selected tissues of transgenic models constitutes a promising strategy to reveal the role of this IGFBP in different systems. The establishment of such models and the unravelling of the molecular events which take place after ligand–receptor interaction are the most important challenges for researchers in this field in the near future.

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