BEYOND CARRIER PROTEINS

IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms

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Abstract

Traditionally, binding proteins are known to regulate the activity of ligands by prolonging their half-life, and insulin-like growth factor (IGF)-binding proteins (IGFBPs) are no exception to this. The IGFBP family contains six high-affinity members with variable functions and mechanisms of actions. In addition to functioning as simple carrier proteins, IGFBPs in serum function to regulate the endocrine actions of IGFs by regulating the amount of IGF available to bind to signaling IGF-I receptors, whereas locally produced IGFBPs act as autocrine/paracrine regulators of IGF action. Furthermore, recent *in vitro* and *in vivo* findings that IGFBPs function independently of the IGFs as growth modulators are particularly exciting. Regarding the role of IGFBPs as ligand-independent growth modulators, our recent data that IGFBP-5 stimulates markers of bone formation in osteoblasts lacking functional IGFs provide evidence that IGFBP-5 itself is a growth factor that can act independently of IGFs to regulate bone formation. In

terms of the mechanism by which certain IGFBPs mediate their effects in a ligand-independent manner, the binding of IGFBP to its putative receptor on the cell membrane may stimulate the signaling pathway independent of an IGF receptor, to mediate the effects of IGFBPs in certain target cell types. IGFBPs may also exert IGF-independent effects by transcriptional activation of genes by IGFBPs transported into the nucleus via their nuclear localization signal. In conclusion, IGFBPs are unusually pleiotrophic molecules with functions ranging from the traditional role of prolonging the half-life of the IGFs to functioning as growth factors independent of the IGFs. In this regard, it was surprising to find that the human genome contains only about 35 000 genes. One mechanism to account for such complexity with a relatively small number of genes is strikingly illustrated by the multifunctional IGFBP class of proteins.

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Introduction

Insulin-like growth factors (IGFs)-I and -II are small peptides, approximately 7 kDa in size, that are structurally similar to insulin. First identified in 1957, IGFs were then known by several other names, including sulfation factors, non-suppressible insulin-like activity, multiplication stimulating activity and somatomedins. IGFs-I and -II are growth factors that have both mitogenic and metabolic actions that participate in the growth, survival and differentiation of a number of cell types and tissues (Cohick & Clemmons 1993, Stewart & Rotwein 1996). IGFs are unique among growth factors in that they can act both systemically, as a hormone, and locally, as autocrine/paracrine factors (Cohick & Clemmons 1993, Mohan & Baylink 1996, 1999, Stewart & Rotwein 1996, Butler & LeRoith 2001). Although liver-derived IGF-I contributes

to the main circulating source of IGF-I, it is now known that many tissues, including the brain, muscle and bone, produce IGF-I (Cohick & Clemmons 1993, Mohan & Baylink 1996, 1999, Stewart & Rotwein 1996, Butler & LeRoith 2001). The importance of IGFs in the growth and maintenance of various tissues is evident from studies using mice lacking functional IGFs or their signaling type-I IGF receptor (Liu *et al.* 1993, 1998; Mohan *et al.* 2000). It is currently known that the actions of IGFs, mediated via IGF receptors, are dependent on six high-affinity IGF-binding proteins (IGFBPs) (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000).

Several low-affinity IGF binders, called IGFBP-related peptides, have also been discovered that exhibit significant structural homology to the amino (N)-terminal region of

high-affinity IGFBPs encoded by the first exon in the IGFBP gene family (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000). The functional significance of these IGFBP-related peptides to the IGF system, if any, is not known at present. The actions of IGFBPs are, in turn, modulated by IGFBP proteases that are further dependent on activators and inhibitors of IGFBP proteases. In terms of the functions of IGFBPs, it is now well established that some of the IGFBPs inhibit IGF actions, whereas others potentiate IGF actions (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000). In addition, recent data also demonstrate evidence for IGF-independent actions of IGFBPs, which is unexpected.

In this paper, we will briefly review the descriptive features of the IGFBP system and then discuss experimental data to demonstrate that IGFBPs are multifunctional and act via both IGF-dependent and -independent mechanisms. Because it is impossible to provide a comprehensive coverage of all published papers dealing with the *in vitro* and *in vivo* effects of six high-affinity IGFBPs in various tissues within the limited space available, we have attempted to develop general concepts and perspectives on IGFBP actions, for the sake of clarity.

Descriptive features of the IGFBP system

Structural features

Six high-affinity IGFBPs cloned in mammals share a 50% homologous protein sequence overall, and up to 80% sequence homology of corresponding IGFBPs of different mammalian species (Rechler 1993, Kelley *et al.* 1996). The genes for mammalian IGFBPs show similar organization, possessing four exons, with exons 1 and 4 encoding the conserved N- and carboxy (C)-terminal regions of IGFBPs respectively. The six high-affinity IGFBPs share distinct structural and functional characteristics, including: 1) a conservation of gene organization; 2) conserved N- and C-terminal regions that are cysteine rich; and 3) an ability to bind IGF with high affinity (K_d approximately 10^{-10} M).

In terms of the region of IGFBP involved in binding to IGFs, mutagenesis studies reveal evidence for the importance of N-terminal IGFBP residues in interacting with IGFs (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, 2001, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000). Because disruption of C-terminal regions of IGFBPs significantly decreases the IGF binding affinity of IGFBPs, it is believed that both the N- and C-terminal domains of IGFBPs are likely to be involved in high-affinity IGF-IGFBP complexes (Qin *et al.* 1998, Clemmons 2001).

Post-translational modifications

There are at least three major post-translational modifications that have been shown to influence the IGF binding affinity of IGFBPs. These modifications are briefly discussed below.

Proteolysis of IGFBPs IGFBP proteases are capable of cleaving IGFBPs into forms that have either significantly reduced or no affinity for IGFs. One of the first identified proteases was a pregnancy-associated IGFBP-3-specific protease that is active from the first trimester of pregnancy in humans (Giudice *et al.* 1990, Hossenlopp *et al.* 1990). Subsequently, IGFBP proteases have been demonstrated in a number of body fluids and cell culture conditioned media (Conover 1995, Rajaram *et al.* 1997, Maile & Holly 1999). Some of these IGFBP proteases have been shown to be relatively specific to a given IGFBP, in that they degrade one IGFBP at a greater potency than they do other IGFBPs. It has been shown by Conover and her collaborators (Lawrence *et al.* 1999) that the pregnancy-associated plasma protein-A (PAPP-A) is relatively specific to IGFBP-4. PAPP-A is produced by a variety of cell types, including fibroblasts, granulosa cells and osteoblasts, and is present in both normal and pregnancy serum (Lawrence *et al.* 1999, Byun *et al.* 2001, Conover *et al.* 2001). Clemmons and his collaborators (Busby *et al.* 2000) have shown that complement C1s produced by smooth muscle cells is relatively specific to IGFBP-5. We have recently found that ADAM-9 (a disintegrin and metalloprotease) produced by human osteoblasts is also relatively specific to IGFBP-5 (Mohan *et al.* 2002). In addition to these specific proteases, body fluids and culture media are also known to contain a number of proteases that degrade several IGFBPs in addition to other proteins. For example, plasmin, cathepsin D and prostate specific antigen (PSA) degrade IGFBPs and other proteins (Conover 1995, Fowlkes *et al.* 1995, Rajah *et al.* 1995, Rajaram *et al.* 1997, Maile & Holly 1999).

The issue of whether or not any of the above proteases exerts a role to regulate the effective concentration of a given IGFBP in local body fluid depends on a number of variables. These including the abundance of the protease, relative potency with which the protease degrades a given IGFBP, presence or absence of other potential substrates, presence or absence of activators and inhibitors of the protease, and so on. In this regard, among the important modulators of IGFBP protease activity are the IGFs themselves (Rajaram *et al.* 1997). IGFs, for example, stimulate IGFBP-4 proteolysis by binding to IGFBP-4 and exposing the cleavage site for PAPP-A (Qin *et al.* 2000). In contrast, proform of eosinophil major basic protein (proMBP) binds to PAPP-A, thereby blocking IGFBP-4 proteolysis (Giudice *et al.* 2002). Thus the IGFBP protease component system provides a mechanism for the acute regulation of IGFBP degradation and alteration of the IGF binding affinity of IGFBPs.

Figure 1 Model of local and endocrine actions of IGFBPs. The majority of the IGFs in the circulation exist as IGF-IGFBP complexes (BP+IGF). Regarding the source of IGFs and IGFBPs in the circulation, the liver is the major source of circulating IGF-I, whereas IGF-II and IGFBPs in serum are likely to be contributed by a multitude of tissues including brain, bone, muscle and kidney. About 80% of IGF is present as a 150 kDa ALS-IGFBP-3/IGFBP-5-IGF complex (shown here as ALS+BP-3+IGF) and cannot cross the vascular endothelium unless this complex is broken down. The remaining 20% of IGF is present as BP+IGF complex or free IGF-I (<1%), which can easily cross the vascular endothelium and therefore is bioavailable to target tissues. Therefore, the IGFBPs in the serum could act to regulate the endocrine actions of IGFs by regulating the amount of bioavailable IGF (i.e. the 50 kDa IGF form and free IGF). In addition, the locally produced IGFBPs could act as autocrine/paracrine regulators of IGF actions in various tissues.

Phosphorylation of IGFBPs Some of the IGFBPs are secreted as phosphoproteins in certain cell types (Jones *et al.* 1991, Hoeck & Mukku 1994, Coverley & Baxter 1997). In the case of human IGFBP-1, phosphorylation has been shown to influence the binding of IGF-I, such that the binding affinity of phosphorylated human IGF-I is sixfold greater than that of non-phosphorylated IGFBP-1 (Jones *et al.* 1991). Conversely, phosphorylation does not appear to influence the IGF binding affinity of IGFBP-3 (Coverley & Baxter 1997). Thus the issue of how alterations in the phosphorylation of some IGFBPs could influence certain functional features remains to be established.

Binding of IGFBPs to extracellular matrix proteins

Several of the IGFBPs, notably IGFBPs-3 and -5, have been reported to bind to the cell surface or extracellular matrix (Rajaram *et al.* 1997, Clemmons 1998, Baxter 2000). IGF binding studies have generally shown less affinity when IGFBPs are associated with the cell surface or extracellular matrix. IGF-I affinity of IGFBP-3 bound to the human fibroblast cell line was reduced by 40-fold compared with IGFBP-3 in solution (Conover & Powell 1991). Similarly, IGFBP-5 binding to the extracellular matrix resulted in an eightfold reduction in IGF binding affinity compared with IGFBP-5 in solution (Jones *et al.* 1993a). The reduced IGF binding affinity of the extra-

cellular matrix or cell surface bound IGFBPs has been implicated in IGFBP modulation of IGF actions (see below).

Regulation of IGFBP production

In terms of IGFBP expression, it is known that several tissues produce more than one IGFBP, although a given tissue may express one or two IGFBPs more abundantly than others. Studies on regulation of IGFBP expression in various cell types have provided evidence that IGFBP production is under the control of both systemic hormones and local regulators (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000, Clemmons 2001). For example, expression of both IGFBP-4 and IGFBP-5 in osteoblasts is known to be regulated by a variety of systemic hormones, including growth hormone, parathyroid hormone, glucocorticoid, 1,25 dihydroxyvitamin D₃ and local growth factors, including IGFs, bone morphogenetic proteins, transforming growth factor- β (TGF- β) and interleukins (Mohan & Baylink 1999). Studies on the molecular mechanisms by which hormones and growth factors regulate expression of IGFBPs provide evidence for complex regulation involving both transcriptional and post-transcriptional mechanisms. It has been

speculated that the complexity of IGFBP regulation may provide the required flexibility for modulating IGF actions by a multitude of systemic and local effectors in various tissues.

IGF-IGFBP complexes in biological fluids

In serum, most of the IGFs (about 75%) circulate as a 150–200 kDa complex, which consists of 7.5 kDa IGF-I/IGF-II plus a 38–43 kDa IGFBP-3 and an 80–90 kDa acid-labile subunit (ALS) (Mohan & Baylink 1996, Rajaram *et al.* 1997, Clemmons 1998, Baxter 2000). Recent studies also demonstrated that a complex of IGFBP-5 plus IGF binds to an ALS (Twigg *et al.* 1998). However, the relative contribution of IGFBP-5 to the circulating 150 kDa complex appears to be smaller compared with that of IGFBP-3 (Baxter *et al.* 2002). The large molecular mass IGF complex acts as a reservoir because of its prolonged half-life (15–20 h) and because it cannot cross the vascular endothelial barrier (Rajaram *et al.* 1997, Clemmons 1998, Baxter 2000). About 20–25% of IGFs exist as a 40–50 kDa complex by binding to one of the remaining five high-affinity IGFBPs. These small molecular mass complexes can cross the vascular endothelium, making this pool bioavailable to the local tissues (Rajaram *et al.* 1997, Clemmons 1998, Baxter 2000). In addition to their presence in serum, IGFBPs have been identified in a variety of biological fluids, including amniotic fluid, follicular fluid, cerebrospinal fluid, seminal fluid and milk (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000, Clemmons 2001). Characterization of the types and amounts of different IGFBPs present in various body fluids reveals evidence that extracellular fluids of certain tissues are enriched with specific IGFBPs. For example, IGFBP-1 is present in amniotic fluid in concentrations a thousand-fold greater than that found in serum. IGFBPs-2 and -6 are present in high concentrations in cerebrospinal fluid, whereas IGFBP-3 is the most abundant IGFBP in follicular fluid. IGFBP-5 is present in high concentrations in bone matrix.

Functional features of the IGFBP system

The IGFBPs in serum act to regulate the endocrine actions of IGFs by regulating the amount of bioavailable IGF (i.e. 50 kDa IGF form and free IGF). In addition, the locally produced IGFBPs act as autocrine/paracrine regulators of IGF actions (Rechler 1993, Kelley *et al.* 1996, Mohan *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000, Clemmons 2001) (Fig. 1). Furthermore, some of the IGFBPs also act by a mechanism independent of IGFs.

IGF-dependent actions of IGFBPs

IGFBPs have been proposed to play a part in modulating the actions of IGFs by regulating their availability to target tissues. Because IGFBPs can modulate the biological activity of IGFs in both a positive and a negative manner, and because IGFBP production is regulated by a variety of systemic and local regulators in various tissues, IGFBPs may have a central role in the local regulation of IGF actions. Locally produced IGFBPs may also serve to fix IGFs in the extracellular matrices for future actions. Furthermore, serum concentrations of IGFBPs may play an important part in regulating the endocrine actions of IGFs.

Modulation of IGF actions by inhibitory IGFBPs

Studies in a number of laboratories have shown that IGFBPs are capable of modulating IGF-induced cell proliferation in both a positive and a negative manner (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000, Clemmons 2001). Several IGFBPs (e.g. IGFBP-4) inhibit IGF actions by binding to IGFs and preventing the binding of IGFs to the IGF receptors (Mohan *et al.* 1995b, Qin *et al.* 1998). Because the affinity of IGFBPs for IGFs is an order of magnitude greater than the affinity of IGF receptors towards IGFs, very little IGF binds to receptors in the presence of an equimolar concentration of receptor and binding protein (Fig. 2). In this regard, it is known that the concentration of IGFBPs is generally in excess over the concentration of IGFs on a molar basis in a variety of body fluids (Malpe *et al.* 1997, Rajaram *et al.* 1997). The conclusion that the inhibitory effect of certain IGFBPs is mediated primarily via an IGF-dependent mechanism is supported by studies using analogs of IGFs with reduced affinity for the IGFBPs, but normal affinity for the IGF-I receptor. In this regard, we and others have found that inhibitory IGFBP-4 was much less potent in blocking the biological activity of IGF analogs that exhibited >100-fold reduced affinity for binding to IGFBP-4 (Canalis *et al.* 1991, Mohan *et al.* 1995b). Similarly, long arginine (LR3) IGF-I was demonstrated to be threefold more potent than wild-type IGF-I in stimulating muscle protein synthesis in diabetic rats, indicating that the effect of IGF-I on muscle protein synthesis was limited because of the presence of excess of inhibitory IGFBPs in diabetic rats (Tomas *et al.* 1992).

Consistent with the *in vitro* data, recent *in vivo* studies have shown that locally produced IGFBP-4 blocks the effects of IGF (Miyakoshi *et al.* 1999). In this regard, we found that concomitant administration of intact IGFBP-4, along with IGF-I, completely blocked IGF-I-induced increases in markers of bone formation in mice. This effect of IGFBP-4 to block the effects of IGF-I was IGF-I-

dependent because: 1) the IGFBP-4 analog with reduced affinity for IGF did not inhibit an IGF-induced increase in markers of bone formation (Miyakoshi *et al.* 1999); and 2) IGFBP-4 was not active in mice lacking functional IGF-I (Miyakoshi *et al.* 2001b). Consistent with the above data, Wang *et al.* (1998) have shown that overexpression of IGFBP-4 in the smooth muscle cells of transgenic mice through a smooth muscle α -actin promoter caused a smooth muscle hypoplasia – a phenotype reciprocal to that of transgenic mice overexpressing IGF-I. Furthermore, double transgenic mice derived from mating IGFBP-4 transgenic and IGF-I transgenic lines showed a decrease in wet weight of selected smooth muscle tissues, thus suggesting that these effects of IGFBP-4 may be IGF-I-dependent. Thus a number of *in vitro* and *in vivo* findings suggest that the inhibitory effect of IGFBP-4 is mediated via a mechanism involving blocking the binding of IGF to IGF receptor.

Because the affinity of IGFBP towards IGF is an important determinant in mediating the inhibitory effect of an IGFBP, a number of conditions that regulate IGF binding affinity of a given IGFBP, including phosphorylation, proteolysis and binding to extracellular matrix proteins, could influence the potency of an inhibitory IGFBP, as discussed in the previous section (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000, Clemmons 2001). Of the various control mechanisms that could release IGFs bound to inhibitory IGFBPs, IGFBP proteases have received a great deal of attention during the past few years. In this regard, it is now known that IGF bound to an inhibitory IGFBP could subsequently be released by an IGFBP protease capable of degrading IGFBP into a form that has a considerably lower affinity for IGF (100-fold or more reduced affinity) compared with that of intact IGFBP (Conover 1995, Fowlkes *et al.* 1995, Rajah *et al.* 1995, Rajaram *et al.* 1997, Maile & Holly 1999). The finding that IGFBP proteases are altered under certain physiological and pathological conditions in various tissues provides support to the concept that degradation of IGFBPs by proteases could have a key regulatory role in the regulation of IGF bioavailability. The activity of IGFBP proteases may, in turn, depend on the activators and inhibitors of proteases. In this regard, it has recently been shown that proMBP is an inhibitor of PAPP-A, an IGFBP-4 protease produced by osteoblasts and other cell types (Conover *et al.* 2001). Thus regulation of IGFBP-4 proteolysis by its protease could provide a mechanism of acutely changing IGFBP-4 concentrations and thereby influence the effect of IGF in local areas of bone and other tissues.

Modulation of IGF actions by stimulatory IGFBPs

Turning to stimulatory IGFBPs, *in vitro* studies have also shown that a number of IGFBPs (e.g. IGFBPs-1, -3 and

-5) stimulate IGF actions in a variety of cell types. The same IGFBP could act to potentiate or inhibit IGF actions, depending on a number of variables, including culture conditions, cell type, IGFBP dose and post-translational modifications (Rechler 1993, Kelley *et al.* 1996, Rajaram *et al.* 1997, Clemmons 1998, Ferry *et al.* 1999, Hwa *et al.* 1999, Poretsky *et al.* 1999, Baxter 2000, Collett-Solberg & Cohen 2000, Clemmons 2001). In this regard, non-phosphorylated IGFBP-1 has been shown to increase the action of IGF-I, whereas phosphorylated IGFBP-1 decreases IGF-I actions in cultured smooth muscle cells or fibroblasts (Busby *et al.* 1988). The potentiating effect of IGFBP-1 was not seen with insulin, which does not bind to IGFBP-1, thus suggesting that the effect of IGFBP-1 is mediated via an IGF-dependent mechanism.

On the basis of certain experimental data, the following mechanisms have been postulated to explain the potentiating effect of certain IGFBPs (Fig. 3).

Cell association of IGFBPs Pre-incubation of human fibroblasts with IGFBP-3, before the addition of IGF-I, has been shown to potentiate the effect of IGF-I (De Mellow & Baxter 1988, Conover 1991). The potentiation of IGF action after pre-incubation with low concentrations of IGFBP-3 is believed to require localization to the cell surface to concentrate IGFs and to facilitate their presentation to IGF-I receptors. The reduction in the affinity of IGFBP-3 for IGF-I (10-fold or more) caused by cell association would facilitate a slow exchange of IGF-I between the receptor and IGFBP-3, and avoid downregulation of the IGF-I receptor by excess IGF-I (De Mellow & Baxter 1988). IGFBP-5 has also been shown to bind to the osteoblast cell surface. In addition, IGFBP-5 treatment has been shown to increase IGF-I binding in monolayer cultures of osteoblasts (Andress & Birnbaum 1992, Mohan *et al.* 1995b). On the basis of these findings, it has been proposed that the association of IGFBP-5 with proteins on the cell surface could increase the local concentration of IGFs in the vicinity of IGF receptors, thereby allowing IGF to bind to IGF receptors (Mohan *et al.* 1995b).

Extracellular matrix association of IGFBPs The potentiation of the actions of IGF by IGFBP-5 in smooth muscle cells has been shown to involve the binding of IGFBP-5 to the extracellular matrix (Clemmons 1998). Interestingly, the binding of IGFBP-5 to an extracellular matrix reduces its affinity for IGF-I by several-fold (Jones *et al.* 1993a). Furthermore, disruption of an extracellular matrix binding domain found in a C-terminal region of IGFBP-5 (Clemmons 2001) abolishes extracellular matrix binding and the potentiating action of IGFBP-5 in smooth muscle cells. On the basis of these findings, it has been postulated that IGFBP-5 binding to the extracellular matrix localizes IGF in a low-affinity complex, from which it can be released slowly to stimulate signaling IGF-I receptors (Jones *et al.* 1993a, Clemmons 1998).

Figure 2 Model of inhibition of IGF actions by IGFBP-4 (BP-4) in osteoblasts. It is known that free IGF-I or -II binds to signaling type-I IGF receptor to stimulate its biological effects. If IGF-I is bound to an inhibitory IGFBP (e.g. IGFBP-4), this complex is not able to bind to IGF-I receptor. Because the affinity of IGFBP-4 for IGF is an order of magnitude greater than that of the receptor, it is likely that very little IGF is available to bind to receptor in the presence of excess of IGFBP-4 (IGFBP-4 is produced in excess compared with IGFs on a molar basis by serum-free cultures of osteoblasts). This inhibitory effect of IGFBP-4 is in turn modulated by IGFBP-4-specific protease/s capable of degrading IGFBP-4 into fragments, which bind IGFs with low affinity, thus releasing IGF from IGFBP-4 for subsequent biological action. The activity of IGFBP-4 protease is in turn subject to regulation by activators and inhibitors of IGFBP-4 protease. Thus IGFBP-4 and its protease provide a mechanism by which the actions of IGF are regulated through regulation of the effective concentration of IGFBP-4 in local areas of bone.

IGFBP binding to putative receptors Putative IGFBP receptors have been demonstrated for certain IGFBPs, including IGFBPs-3 and -5 (Oh *et al.* 1993, Mohan *et al.* 1995b, Andress 1998). The binding of IGFBP-5 to its receptor, for example, may stimulate an IGFBP-5 signaling pathway that may interact with the IGF-I signaling pathway at one or more points to amplify the IGF-I signaling (Mohan *et al.* 1995b).

Facilitation of the storage of IGFs in extracellular matrices for future action The ability of skeletal tissues to regenerate after injury has been attributed to the presence of large amounts of growth factors in bone (Mohan & Baylink 1991). Studies on the relative distribution of growth factors in humans revealed that bone contains many more IGFs than any of the growth factor systems tested to date. Our studies on the mechanism by which IGFs are fixed in such large quantities led to the finding that they are fixed in bone via IGFBP-5 (Bautista *et al.* 1991, Nicolas *et al.* 1995). On the basis of the findings that the complex of IGFBP-5 and IGFs, but not

IGFs alone, binds to hydroxyapatite, we have proposed that the amount of IGFs stored in bone may be largely determined by the amount of IGFBP-5 produced by osteoblasts. In terms of the functional significance of IGFs stored in bone, we and others have proposed that IGFs fixed in bone via IGFBP-5 are eventually released during bone resorption in a bioactive form, to participate in the site-specific replacement of bone lost during bone resorption (Bautista *et al.* 1991, Nicolas *et al.* 1995). This process of site-specific replacement of bone lost during resorption is important, because otherwise the bone architecture would not provide efficient mechanical support. If IGFs are stored in bone to act in a delayed paracrine manner, then one would expect the concentration of IGFs to change during certain physiological and pathological conditions. Accordingly, it is now known that the skeletal concentration of IGFs, in addition to that of IGFBP-5, is altered during conditions known to affect bone metabolism (Mohan *et al.* 1995a). Similar to the role for stored IGFs via IGFBP-5 in bone, discussed above, IGFs stored in the extracellular matrices of soft tissue via IGFBP-5 binding to extracellular matrix proteins could also play a part in wound healing.

Modulation of the endocrine actions of IGF In addition to modulating the local actions of IGFs, IGFBPs could modulate their endocrine actions. Since the discovery of the sulfation factor by William Daughaday almost four decades ago, it has been well known that IGFs could act as endocrine hormones (Daughaday 2000). In this regard, it is interesting that the total concentration of IGFs-I and -II in blood is about 800 µg per liter or 100 nmol per liter, which is about 1000 times greater than that of circulating insulin (Mohan & Baylink 1996, Rajaram *et al.* 1997, Baxter 2000). Because IGFs are known to be involved in promoting general growth by stimulating the growth and differentiation of a number of cell types, a large, readily available reserve of IGFs may be necessary in order for IGFs to serve as endocrine hormones. IGFs circulate in such high abundance in blood because of the presence of IGFs as IGF-IGFBP complexes in serum. Furthermore, the presence of IGFs as these complexes also provides a mechanism for the effective regulation of the endocrine actions of IGFs. These functions of IGFBPs are discussed below.

IGFBPs prolong the half-life of IGFs As discussed earlier, much of the IGF in the blood exists as a 150 kDa ALS-IGFBP-3-IGF complex. Because of its size, the 150 kDa complex is retained in the vascular compartment, prolonging the half-life of IGFs in the blood to approximately 15–20 h (Zapf 1995). The importance of the 150 kDa complex in increasing the concentration of IGFs in the circulation is evident from studies involving mice with a disruption of the ALS gene, in which the circulating concentration of IGF is reduced by 80% compared

Figure 3 Model of potentiation of the actions of IGF by IGFBP-5 in osteoblasts. Three mechanisms are proposed. 1) IGFBP-5 (BP-5) has been shown to bind to a number of extracellular matrix (ECM) proteins produced by osteoblasts. ECM-bound IGFBP-5 could bind IGF and increase local concentration of IGF by preventing escape of IGF into extracellular fluid or by protecting IGF from proteolysis, or both. ECM-bound IGF could be slowly released for binding to IGF receptor. 2) IGFBP-5 binds to the osteoblast cell surface independent of IGFs (putative IGFBP-5 receptor). The putative IGFBP-5 receptors may recruit IGFs at the surface of IGF-responsive cells, enabling the ligand to be easily captured by IGF receptors. 3) IGFBP-5 binding to its putative receptor could stimulate a signaling pathway that could interact with the IGF-I signaling pathway to amplify the IGF-I signaling pathway.

with control mice (Boisclair *et al.* 2001). Because the half-lives of free IGFs and the small molecular mass (40–50 kDa) IGF–IGFBP complex are in the order of 10 and 30 min respectively, it is not surprising that the amount of IGFs in the circulation is decreased considerably in the absence of the 150 kDa IGF complex in the ALS knockout mice.

IGFBPs prevent the insulin-like activity of IGFs If all IGFs in the circulation were bioavailable to act on target tissues, the results would be quite destructive as a result of the anticipated hypoglycemia produced by the insulin-like activity of the IGFs. Such an event does not occur for two reasons: 1) formation of 150 kDa (ALS–IGFBP-3/IGFBP-5–IGF) complexes that cannot cross the vascular endothelial cell barrier allow high concentrations of IGFs to accumulate in the blood without risk of hypoglycemia; 2) much of the IGF in the remaining 40–50 kDa complex is bound to inhibitory IGFBPs, and the IGFs in this complex are not bioavailable unless this complex is broken down (Rajaram *et al.* 1997). The importance of IGFBPs in preventing the insulin-like effects of IGFs is evident from

studies involving non-islet cell tumor hypoglycemia (NICTH), in which hypoglycemia is seen in the absence of detectable insulin because of the overproduction of partially processed biologically active IGF-II by mesenchymal tumors (Daughaday *et al.* 1993, Zapf 1994). The state of hypoglycemia found in patients with NICTH is associated with an increase in the serum concentration of pro IGF-II, a decrease in the circulating concentration of the 150 kDa complex, and a corresponding increase in the circulating concentration of 50 kDa complex (Daughaday *et al.* 1993, Zapf 1994, Rajaram *et al.* 1997). Because pro IGF-II does not bind effectively to IGFBP-3, much of the pro IGF-II exists as 50 kDa or free form, which can easily cross the endothelial cell barrier, bind to insulin receptors and elicit insulin-like action to induce hypoglycemia.

IGFBPs control the bioavailability of IGFs The transportation of serum IGFs from the vasculature into extracellular fluid is necessary for IGFs to elicit a growth-stimulating response in the target tissues. In contrast to the 150 kDa complex, the 50 kDa IGFBP–IGF complex (approximately 20%) and the free IGF (<1%) can easily cross the

vascular endothelium (Rajaram *et al.* 1997, Baxter 2000). This ability of the 40–50 kDa IGFBP–IGF complexes to cross the vascular endothelial barriers intact makes them important as transporters of IGFs in target tissues. Subsequent proteolysis of IGFBP in the 50 kDa IGFBP–IGF complex in the target tissues may lead to an increase in the amount of IGFs that exhibit growth-promoting effects. In addition, IGFBP proteases are also present in the circulation, which may degrade the IGFBP–IGF complex and release IGFs for transport and subsequent biological action. Thus the biological activity of circulating IGFs to the tissues is determined by mechanisms involving shifting of IGF from the 150 kDa complex to the 50 kDa complex and subsequent proteolysis of IGFBP in the 50 kDa complex by protease to release IGF in the circulation or in the local body fluid, or both (Rajaram *et al.* 1997, Baxter 2000). If IGFBP proteases are important in modulating IGF bioavailability, we would expect the activities of proteases responsible for limited proteolysis of various IGFBPs to change during a variety of physiological and pathological situations. This is indeed true, as a number of conditions, including protein deprivation, pregnancy, diabetes, chronic renal failure, acute medical illness and surgery (Rajaram *et al.* 1997, Maile & Holly 1999), are known to influence IGFBP proteolysis in serum and other body fluids.

It is postulated that the existence of different forms of IGFs and the IGFBP proteases provides mechanisms for the effective regulation of endocrine actions of IGFs. The role of IGFBPs in regulating the endocrine actions of IGFs is illustrated by our experimental data involving systemic administration of IGFBP–4 on bone formation parameters in mice. In our *in vivo* studies on the effects of IGFBP–4 on bone formation parameters, we found that local administration of IGFBP–4 inhibited IGF–I-induced bone formation parameters, as expected. However, to our surprise, we found that systemic administration of IGFBP–4 alone at pharmacological doses increased markers of bone formation and did not inhibit the effect of IGF–I (Miyakoshi *et al.* 1999). Because systemic administration of IGFBP–4 caused a shift in IGF–I from the 150 kDa to the 50 kDa complex, it is possible that a systemic administration of IGFBP–4 increases IGF bioavailability via an IGFBP–4 protease-dependent mechanism. Accordingly, we found that systemic administration of wild-type, but not protease resistant, IGFBP–4 increased the concentration of free IGF–I in serum and markers of bone formation in mice (Miyakoshi *et al.* 2001a). Furthermore, IGFBP–4 alone did not increase bone formation parameters in IGF–I midi mice, which have severely depleted circulating concentrations of IGF–I. On the basis of these findings, we have proposed a model in which systemically administered IGFBP–4 causes an acute increase in serum IGFBP–4 concentrations, which increases IGF–I in the IGFBP–4 complex. Subsequent proteolysis of IGFBP–4 leads to an increase in free IGF–I, thus increasing bone for-

mation. Our data are consistent with the concept that the observed mechanism by which IGFBP–4 increases the concentration of free IGF–I in serum may have physiological relevance with respect to regulating free IGF–I concentrations in serum. Other IGFBPs (e.g. IGFBPs–1, –2 and –5) may act similarly to increase free IGF–I in serum (Rajaram *et al.* 1997). Accordingly, physiologic regulation of the IGF–I in the 50 kDa pool may influence the concentrations of free IGF–I in the serum and, thereby, the endocrine actions of IGF–I.

IGF-independent actions of IGFBPs

The discovery of IGF-independent modulation of growth by IGFBPs in various cell types has added a further layer of complexity and importance to the IGF axis. IGF-independent actions of IGFBPs include effects on cell migration, cell growth and apoptosis. The following section briefly describes the known IGF-independent effects of IGFBPs and the potential mechanisms that could explain these ligand-independent effects of IGFBPs.

Evidence for ligand-independent actions of IGFBPs

IGFBP–1 Studies by Jones *et al.* (1993b) revealed that Chinese hamster ovary cells transfected with IGFBP–1 caused an increase in cell migration, an effect mediated via IGFBP–1 binding to $\alpha 5 \beta 1$ integrin (fibronectin receptor). This effect was shown to be IGF–I independent, as these cells do not produce IGFs and showed no increase in migration in response to exogenously added IGFs. Consistent with these data, it has recently been shown that IGFBP–1 stimulation of human trophoblast migration occurs by binding of its RGD domain to the $\alpha 5 \beta 1$ integrin, leading to an activation of focal adhesion kinase and stimulation of the mitogen-activated protein kinase pathway (Gleeson *et al.* 2001). In contrast to stimulatory effects of IGFBP–1 on cell migration, IGFBP–1 treatment caused an increase in apoptosis in breast cancer cells by a mechanism involving IGFBP–1 binding to integrin and dephosphorylation of focal adhesion kinase (Perks *et al.* 1999). Thus IGFBP–1 binding to integrin may elicit different biological effects in various cell types.

IGFBP–3 An accumulating body of evidence has revealed that IGFBP–3 has important IGF-independent effects *in vitro* in various cell types. Harel's group (Villaudy *et al.* 1991) first suggested that mouse IGFBP–3 inhibited fibroblast growth factor-stimulated DNA synthesis in chick embryo fibroblasts by a mechanism that did not involve IGFs. Oh and colleagues (Oh *et al.* 1993, Oh 1998) demonstrated that IGFBP–3, but not IGFBP–1, inhibited Hs578T human breast cancer cell growth by a mechanism independent of IGFs. IGF-independent growth inhibition by IGFBP–3 was also demonstrated by studies involving transfection of the IGFBP–3 gene into murine fibroblasts, which inhibited cell growth by a mechanism that was not

reversible by the addition of excess of insulin (which, when in excess, acts via the IGF receptor) (Cohen *et al.* 1993). The lack of involvement of a IGF-I receptor in growth inhibition by IGFBP-3 has been shown more definitively by the use of a mouse fibroblast cell line with a disrupted IGF-I receptor gene (Valentinis *et al.* 1995). Furthermore, Zadeh & Binoux (1997) demonstrated that a 16 kDa IGFBP-3 fragment, without measurable IGF binding activity, inhibited insulin- and IGF-I stimulated DNA synthesis in chick embryo fibroblasts. Thus several lines of evidence suggest that IGFBP-3 causes inhibition of cell growth via a mechanism independent of IGF signaling.

In addition to its effect on growth inhibition, IGFBP-3 has also been shown to induce apoptosis via an IGF-independent mechanism. Rajah *et al.* (1997) have shown that addition of exogenous IGFBP-3 to a prostate cancer cell line, PC-3, resulted in a dose-dependent increase in the apoptotic index, which was only partially attenuated by the addition of IGF-I and unchanged by the addition of IGF analogs with reduced affinity for IGFBP-3. Three additional findings demonstrated that the effects of IGFBP-3 on apoptosis might be mediated via an IGF-independent mechanism. Firstly, IGFBP-3 was shown to induce apoptosis in mouse fibroblasts lacking any functional IGF-I receptor (Zadeh & Binoux 1997). Secondly, Gill *et al.* (1997) have shown that IGFBP-3 enhanced the effect of ceramide analog C2 on apoptosis in the IGF-unresponsive breast cancer cell line, Hs578T. Thirdly, Hong *et al.* (2002) have recently demonstrated that a mutant of IGFBP-3, which does not bind to IGF, is as effective as wild-type IGFBP-3 in stimulating apoptosis in PC-3 cells. Thus IGFBP-3 inhibits both cell proliferation and apoptosis.

IGFBP-5 There is now compelling evidence that the effects of IGFBP-5 on osteoblasts are, in part, mediated via a mechanism independent of IGFs. During the course of our studies on the biological actions of IGFBP-5 in osteoblasts, we found that IGFBP-5 treatment increased differentiation in MG63 human osteosarcoma cells, which did not produce detectable concentrations of either IGF-I or IGF-II (Richman *et al.* 1999). In subsequent studies, we found that systemic administration of IGFBP-5 increased bone formation parameters but had no effect on circulating IGF-I concentrations in serum. Furthermore, Andress *et al.* (1993) showed that a C-terminal-truncated form of IGFBP-5, which binds IGFs with reduced affinity, increased osteoblast cell proliferation in the absence of IGF-I. Although these studies provided indirect evidence for an IGF-independent effect of IGFBP-5, it was only recently that direct evidence for an IGF-independent effect of IGFBP-5 was obtained using osteoblasts from IGF-I knockout mice. In mutant osteoblasts that produce neither of the two IGFs, we found that IGFBP-5 promoted cell growth, alkaline phosphatase activity and

osteocalcin expression as robustly as did wild-type cells (Miyakoshi *et al.* 2001b). Furthermore, IGFBP-5, when administered locally to the outer periosteum of the parietal bone of IGF-I knockout mice, increased markers of bone formation to concentrations comparable to those seen in wild-type mice (Miyakoshi *et al.* 2001b). Thus these data provide direct evidence that IGFBP-5 could function as a growth factor that mediates its actions, in part, via an IGF-independent mechanism.

Mechanisms for ligand-independent actions of IGFBPs In terms of the mechanism by which IGFBPs mediate their effects via IGF-independent pathways, a number of possibilities exist.

Signaling via IGFBP specific putative cell surface receptor The first evidence for the existence of cell-surface IGFBP-3 association proteins/receptors came from the studies of Oh *et al.* (1993), who demonstrated specific binding of IGFBP-3 to breast cancer cell membrane proteins of 20–50 kDa in size. Subsequently, TGF- β receptor type V (400 kDa) was also shown to bind to IGFBP-3 in mink lung epithelial cells (Leal *et al.* 1997). We and others have shown that, consistent with the presence of putative receptors for IGFBP-3 in certain cell types, osteoblasts contain a putative receptor for IGFBP-5 (Andress 1998, Mohan *et al.* 1995b). It is possible that binding of IGFBP to its putative receptor may stimulate a signaling pathway independent of an IGF receptor to mediate the effects of IGFBPs in certain target cell types. An IGFBP-5 putative receptor, a 420 kDa protein, was purified from membrane preparations of mouse osteoblasts using an IGFBP-5^{201–218} affinity column, on the basis of data that IGFBP-5^{201–218} bound directly to the osteoblast surface (Andress 1998). Co-incubation of the affinity-purified 420 kDa protein with ³²P-ATP resulted in autophosphorylation at serine residues, which was enhanced both by intact IGFBP-5 and by IGFBP-5 fragments (Andress 1998), thus suggesting that serine/threonine kinase activation may be important in mediating some of the IGF-independent effects of IGFBP-5 (Fig. 3). On the basis of the findings that certain cell types contain putative receptors for IGFBPs-3 and -5, and that one or more of these putative receptors exhibit serine/threonine kinase activity, it has been predicted that the binding of IGFBP to its putative receptors on the cell surface may stimulate signaling pathways to mediate some of its effects independent of a IGF-receptor-mediated signaling pathway (Mohan *et al.* 1995b, Andress 1998).

Nuclear localization and interaction with transcriptional modulators Another mechanism by which IGFBPs could exert IGF-independent effects is by the transcriptional activation of genes by IGFBPs transported into the nucleus via their nuclear localization signal. In this regard, Radulescu (1994) brought to attention the presence of a

Figure 4 Model of IGF-independent action of IGFBP-5 (BP-5) in osteoblasts. In the proposed IGF-independent mechanism, IGFBP-5 from cytoplasm or an extracellular source enters the nucleus via its nuclear localization sequence and by binding to the nuclear transporter, importin- β . IGFBP-5 may bind to FHL2, a transcription modulator, in the cytoplasm and shuttle FHL2 into the nucleus or bind FHL2 in the nucleus to stimulate transcription of target genes.

bipartite nuclear localization signal in the C-terminal domain of IGFBP-3. A similar sequence is also present in the C-terminal region of IGFBP-5. Subsequently, several studies have provided evidence for nuclear localization of IGFBPs-3 and -5 in several cell types (Radulescu 1994, Amaar *et al.* 2002). It has also been demonstrated that the nuclear localization of IGFBPs-3 and -5 is mediated via a nuclear transporter protein, importin- β , in breast cancer cells (Radulescu 1994). More recently, Liu *et al.* (2000) have shown that IGFBP-3 interacts with retinoic acid receptor α (RXR α) and this interaction results in the modulation of the transcriptional activity of RXR α , which is essential for mediating the effects of IGFBP-3 on apoptosis in prostate cancer cells.

We have recently found evidence for a nuclear uptake of IGFBP-5 by human osteoblasts (Amaar *et al.* 2002). In order to identify cellular proteins that could be IGFBP-5 receptors in addition to nuclear proteins that regulate transcription, we recently undertook studies to identify proteins that bind to IGFBP-5, using IGFBP-5 as bait in a yeast two-hybrid screen of a U2 human osteosarcoma cell cDNA library. Using this approach, we have identified a zinc finger protein, FHL2 (four and a half LIM domain protein), as a binding partner for IGFBP-5 in osteoblasts (Amaar *et al.* 2002). On the basis of the findings that FHL2 is strongly expressed in a variety of human osteoblast cell types and found to be localized in the nucleus as a complex with IGFBP-5, and that FHL2 can function as transcription factor or coactivator in other cell types (Muller *et al.* 2000), it can be speculated that FHL2 is an intracellular mediator of the action of IGFBP-5 in osteoblasts (Fig. 4). In the proposed IGF-independent mechanism, IGFBP-5 from an extracellular source or

cytoplasm enters the nucleus. The question of whether or not the putative receptor on the cell surface is involved in mediating the intracellular localization of extracellular sources of IGFBP-5 remains to be studied. In any case, IGFBP-5 from the cytoplasm may be transported into the nucleus through binding to nuclear transporters such as importin- β (Radulescu 1994, Schedlich *et al.* 2002). As FHL2 does not contain a nuclear localization sequence, it is possible that IGFBP-5 may bind to FHL2 or other transcriptional modulators and shuttle them into the nucleus to stimulate transcription of putative target genes that may be involved in the regulation of osteoblast proliferation and differentiation. The role of FHL2 as an intracellular mediator of the IGF-independent effects of IGFBP-5 needs to be verified in future experimental studies.

Conclusions

Since the elucidation of the identity of the first IGFBP in 1986 (Koistinen *et al.* 1986), we now know that the IGFBP system is extremely complex, involving several IGFBPs, IGFBP proteases, inhibitors and activators of IGFBP proteases. Furthermore, we have made considerable progress towards understanding the functions of IGFBPs, revealing that they are unusually pleiotrophic molecules, with functions ranging from traditional carrier proteins to growth factors independent of IGFs. In terms of the question why IGFBPs are so pleiotrophic, it is now known that the number of genes in the human genome (35 000) is much smaller than was predicted on the basis of the complex physiologic nature of humans. One

mechanism to account for this complexity with a relatively low number of genes is based on the diversification of genes, in terms of products, regulation and function. The small number of genes also implies that proteins may have additional roles than previously anticipated, that the same gene may function differently in one tissue compared with another, and that members of the same gene family may have different roles in different tissues. The IGFBP family provides an example of such diversification in terms of regulation and function. Although many advances have been made in terms of our understanding of the potential role of IGFBPs, much remains to be learned about the molecular pathways involved in regulating the various actions of IGFBPs. In particular, we know little about intracellular signaling pathways by which IGFBPs regulate proliferation, differentiation and apoptosis of various cell types in an IGF-independent manner. With recent developments in terms of completion of the human genome sequence and new molecular technologies for elucidation of gene expression and function, it is very likely that there will be tremendous progress in the next few years towards our understanding of molecular pathways by which IGFBPs exert their pleiotrophic effects and the role of IGFBPs during physiological and pathological conditions.

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