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Cellular Actions of the Insulin-Like Growth Factor Binding Proteins

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In addition to their roles in IGF transport, the six IGF-binding proteins (IGFBPs) regulate cell activity in various ways. By sequestering IGFs away from the type I IGF receptor, they may inhibit mitogenesis, differentiation, survival, and other IGF-stimulated events. IGFBP proteolysis can reverse this inhibition or generate IGFBP fragments with novel bioactivity. Alternatively, IGFBP interaction with cell or matrix components may concentrate IGFs near their receptor, enhancing IGF activity. IGF receptor-independent IGFBP actions are also increasingly recognized. IGFBP-1 interacts with $\alpha_5\beta_1$ integrin, influencing cell adhesion and migration. IGFBP-2, -3, -5, and -6 have heparin-binding domains and can bind glycosaminoglycans. IGFBP-3 and -5 have carboxyl-terminal basic motifs incorporating heparin-binding and additional basic residues that interact with the cell surface and matrix, the nuclear transporter importin-β, and other proteins. Serine/threonine kinase receptors are proposed for IGFBP-3 and -5, but their signaling functions are poorly understood. Other cell surface IGFBP-interacting proteins are uncharacterized as functional receptors. However, IGFBP-3 binds and modulates the retinoid X receptor-α, interacts with TGFβ signaling through Smad proteins, and influences other signaling pathways. These interactions can modulate cell cycle and apoptosis. Because IGFBPs regulate cell functions by diverse mechanisms, manipulation of IGFBP-regulated pathways is speculated to offer therapeutic opportunities in cancer and other diseases. (*Endocrine Reviews* 23: 824–854, 2002)

Abbreviations: ALS, Acid-labile subunit; CHO, Chinese hamster ovary; ECM, extracellular matrix; FAK, focal adhesion kinase; HBD, heparin-binding domain; IGFBP, IGF binding protein; IGFRI and IGFRII, type I and II IGF receptors; NSCLC, non-small-cell lung carcinoma; PAPP-A, pregnancy-associated plasma protein A; PI, phosphatidylinositol; PKB, protein kinase B; RA, retinoic acid; RAR, RA receptor; RGD, Arg-Gly-Asp; RGE, Arg-Gly-Glu; RXR, retinoid X receptor; STAT1, signal transducer and activator of transcription-1; TβRI, TβRII, and TβRV, type I, II, and V receptor for TGFβ; vitD, 1,25-dihydroxyvitamin D_3.

I. Introductory Overview

A. IGFBP structure

THE IGF BINDING PROTEIN (IGFBP) gene family consists of six well characterized members that encode a family of homologous multifunctional proteins, IGFBP-1 to IGFBP-6. The genes share a common structural organization, in which four conserved exons are located within genes ranging from 5 kb (IGFBP-1) to more than 30 kb (IGFBP-2 and IGFBP-5) (1). The IGFBP genes, like those of the IGFs themselves, are believed to have emerged early in vertebrate evolution (2). Exon 1 of the IGFBP gene family is shared by several other genes encoding a variety of proteins, leading to proposals that they might be members of a larger gene superfamily (3, 4).

The precursor forms of all six IGFBPs have secretory signal peptides of between 20 and 39 amino acids, and the mature proteins are all found extracellularly. They share a highly conserved structure that is generally described as consisting of three domains of approximately equal size. In addition, important subdomains, or functional motifs, within each domain are now recognized as contributing to their diverse actions. Structural aspects of the IGFBPs have been extensively reviewed (3, 5–7). The mature proteins have between 216 and 289 amino acids, giving core molecular masses of between 22.8 and 31.3 kDa.

The conserved amino-terminal domain contains six disulfide bonds in all but IGFBP-6, which has five. The organization of these disulfides has been determined for several IGFBPs, revealing that, despite differences in the pairing of cysteines among IGFBP-1, -4, and -6 (8, 9), all form disulfide bonds within the domain. As recently reviewed (6), important IGF-binding residues are found in the amino-terminal

domain (Table 1), predicted by nuclear magnetic resonance studies on IGFBP-5 (10) and confirmed for IGFBP-3 and IGFBP-5 by mutagenesis studies (11–13). Although no other major functional motifs have been identified in the amino-terminal domain, the observation that amino-terminal proteolytic fragments of IGFBP-3 cause IGF-independent inhibition of mitogenesis (14, 15) implies the presence of another active subdomain in this region.

The conserved carboxyl-terminal domain is also cysteine rich, with three disulfide bonds in all IGFBPs, formed by the pairing of adjacent cysteines within the domain (8, 9, 16). IGF-binding residues are also present in this domain (Table 1), demonstrated by the binding activity of natural carboxyl-terminal fragments of IGFBP-2 (17, 18) and recombinant carboxyl-terminal IGFBP-3 fragments (19, 20), and mutagenesis of IGFBP-5 residues (21). The observation that residues involved in IGF binding occur in both amino- and carboxyl-terminal domains implies the existence of an IGF-binding pocket involving both domains. As shown in Fig. 1, other important subdomains have also been identified within the carboxyl-terminal region of various IGFBPs; for example, Arg-Gly-Asp (RGD) integrin-binding motifs are located at residues 221–223 of IGFBP-1 (22) and residues 265–267 of IGFBP-2 (23). Functionally important 18-residue basic motifs with heparin-binding activity have also been identified at residues 215–232 of IGFBP-3 and residues 201–218 of IGFBP-5 and are involved in interaction with the serum glycoprotein ALS (acid-labile subunit) (24–26) and other ligands such as plasminogen activator inhibitor-1 (27) and transferrin (28), cell and matrix binding (24, 29), and nuclear transport (30), as discussed in detail later.

The central domain of the IGFBPs shows essentially no structural conservation among any members of the family. It contains no disulfide bonds apart from an intradomain bond in IGFBP-4 (9). Three sites of N-linked glycosylation in IGFBP-3 (31) and one in IGFBP-4 (32) are found in this region. Other sites of posttranslational modification are also found in the central domain: potential phosphoacceptor sites on all IGFBPs, some of which are phosphorylated in IGFBP-1, -3, and -5 (33), and proteolytic cleavage sites in some of the proteins (15, 34, 35). Secondary IGFBP-5 binding sites for ALS (36) and heparin (37), and a potential cell-association domain of IGFBP-3 (38), are also found in this region (Fig. 1).

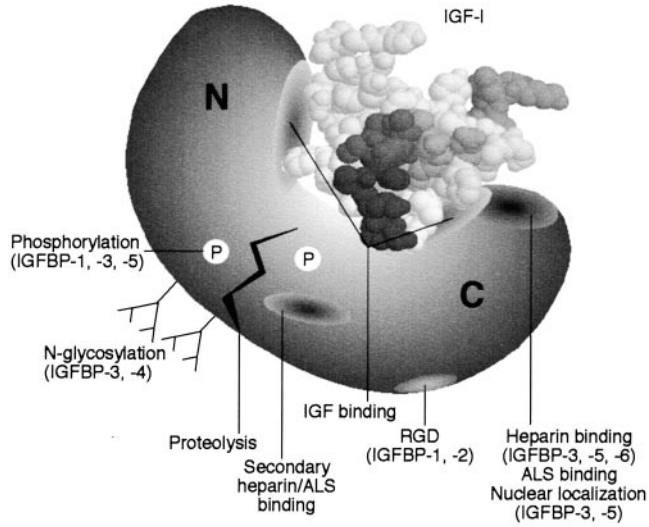

FIG. 1. Generalized diagram of IGFBP structure showing proposed interaction with IGF-I through both N and C domains. Functional domains and sites of posttranslational modification are indicated.

TABLE 1. Functional domains of IGFBPs

Domain	Function	IGFBP	Ref.
Amino terminal	IGF binding	IGFBP-1	375
		IGFBP-2	145, 376
		IGFBP-3	11, 19, 377
		IGFBP-5	10, 11
	Insulin binding	IGFBP-3	377
	Inhibition of insulin receptor autophosphorylation	IGFBP-3	377
	Inhibition of mitogenesis	IGFBP-3	14, 15
Central	Heparin binding[a]	IGFBP-2	242
		IGFBP-3	199
		IGFBP-5	213
	ALS binding[a]	IGFBP-5	36
	Cell binding	IGFBP-3	38
Carboxyl terminal	IGF binding	IGFBP-1	378
		IGFBP-2	17, 18, 145
		IGFBP-3	19
		IGFBP-5	21
	Nuclear localization signal[b]	IGFBP-3	30, 275
		IGFBP-5	
	Heparin-binding[b]	IGFBP-3	29
		IGFBP-5	
	ALS binding[b]	IGFBP-3	24
		IGFBP-5	25, 26
	Cell binding[b]	IGFBP-3	24, 29
		IGFBP-5	29
	Integrin binding	IGFBP-1	22

[a] Central domain ALS and heparin-binding sites may only be unmasked when carboxyl-terminal domain has been deleted.
[b] Carboxyl-terminal nuclear localization signal encompasses heparin-binding domain, which interacts with numerous other ligands.

B. Relationship between serum and tissue IGFBPs

The term "multifunctional" is very aptly applied to the IGFBPs. Originally described as passive circulating transport proteins for IGF-I and IGF-II, IGFBPs are now recognized as playing a variety of roles in the circulation, the extracellular environment, and inside the cell. The regulation and actions of circulating IGFBPs have been addressed in several reviews (39–43). In brief, the major IGF transport function can be attributed to IGFBP-3, the most abundant circulating IGFBP. It carries 75% or more of serum IGF-I and IGF-II in heterotrimeric complexes that also contain ALS, a leucine-rich glycoprotein of approximately 85 kDa (44). IGFBP-5, present at about 10% of the molar concentration of IGFBP-3, can form similar ternary complexes (45). Approximately 90% of IGFBP-3 and 55% of IGFBP-5 circulate in these complexes in healthy adults (46). All six IGFBPs are also found in the circulation in the free form or in binary complexes with IGFs. Free or binary-complexed IGFBPs are believed to exit the circulation rapidly, whereas ternary complexes appear to be essentially confined to the vascular compartment (47–49).

IGFBPs exert a complex array of functions at the cellular level. There is little information on the exact relationship between IGFBPs in the circulation and those in the cellular environment, but it appears that the IGFBPs may be differentially targeted to different tissues depending on both their primary structure and their posttranslational modifications. In some situations, endogenous IGFBPs from circulating ternary complexes may be found at low concentration in the tissues, as first implied by comparison of IGFBPs in serum and lymph (50). Using exogenous IGFBPs, Boes *et al.* (51) demonstrated in an isolated perfused heart model that IGFBP-4, after crossing the capillary endothelium, preferentially localizes to connective tissue rather than cardiac muscle, the exact distribution depending on the glycosylation state. In contrast, IGFBP-1, -2, and -3 are preferentially localized to cardiac muscle. IGFBP-3 injected iv appears initially in the liver (40% of injected dose) and kidney (4%), within 5 min of administration (52). Uptake by muscle was not examined in this study.

IGFBP-4 administered systemically to mice has also been shown to act on bone, stimulating bone alkaline phosphatase activity and serum osteocalcin by a mechanism that appears to involve IGFBP-4 proteolysis and increased IGF-I availability (53). The influence of circulating IGFBPs at the tissue level is further indicated by the observation that IGFBP-1 administered iv in rats inhibits IGF-I-stimulated 2-deoxyglucose uptake in cardiac and skeletal muscle (54). Similarly, exogenous IGFBP-3 administered to rats blocks the hypoglycemic effect of coadministered IGF-I, an effect that depends explicitly on its ability to form complexes with ALS in the circulation (55).

In addition to these effects of IGFBPs derived from the circulation, there are undoubtedly important local actions of IGFBPs, both autocrine and paracrine. As well as modulating activation of the type I IGF receptor (IGFRI) by IGFs (20, 56, 57), IGFBPs are documented to affect cell motility and adhesion (22, 58), apoptosis and survival, and cell cycle (59–61). They interact with diverse previously characterized signaling pathways (22, 62–64) and may have unique signaling pathways of their own (65). Their cellular effects are likely to be influenced by posttranslational modifications, *e.g.*, glycosylation affecting cell interaction (31, 66), phosphorylation affecting IGF binding affinity (67) and susceptibility to proteases (68), and proteolysis affecting both IGF-independent and IGF-dependent actions (15, 69). The purpose of this review is to document and provide a critical discussion of current knowledge on these and related topics.

II. Modulation of IGF Activity by IGFBPs and Their Proteases

Two known receptors present on most cell types specifically recognize the IGFs. IGFRI, a heterotetrameric tyrosine kinase that is homologous to the insulin receptor, has been shown definitively to mediate the effects of both IGF-I and IGF-II (70). The type II IGF receptor (IGFRII), which is structurally distinct from IGFRI and also binds glycoproteins containing mannose 6-phosphate moieties, has been reported to interact with G protein pathways (71) but its role in IGF-II signal transduction remains controversial. By virtue of its high specificity for IGF-II, it is thought to regulate the level of extracellular IGF-II by targeting it for degradation, and hence inhibit the autocrine/paracrine actions of IGF-II mediated through IGFRI (72). In its soluble form, the receptor may sequester IGF-II, thus inhibiting its cellular actions (73).

It is well established, from *in vitro* systems, that the IGFs acting through the IGFRI have acute anabolic effects on metabolism as well as longer term effects on cell replication and differentiation (40). Apart from their mitogenic activity, the IGFs also have potent inhibitory effects on apoptosis (74, 75). However, the bioactivity of IGFs is not only dependent on their interaction with IGFRI but is also influenced by the family of IGFBPs in the local cellular environment, which can potentially either inhibit or enhance IGF actions depending on the complement of IGFBPs present. Because most cells express more than one IGFBP, it is clear that the regulation of each IGFBP plays an important role in regulating the cellular effects of IGFs. Furthermore, a wide range of proteolytic enzymes can catalyze the limited hydrolysis of IGFBPs. Documented cleavage sites in IGFBP-3, -4, and -5 are shown in Table 2. Some of the resulting fragments have been reported to retain biological activity (76, 77). In certain cell types, IGF-I itself regulates the expression of specific IGFBPs (78) or their proteases (79, 80), thus adding further complexity.

A. IGFBP-4, -5, and -6

Vascular smooth muscle cells express both IGF-I and IGFRI, and IGF-I is a potent regulator of migration, proliferation, and apoptosis in these cells (81–85). Duan and Clemmons (86) showed that IGFBP-4 and IGFBP-5 expression was regulated differentially by IGF-I in porcine vascular smooth muscle cells. IGF-I decreased IGFBP-4 levels by activating an IGFBP-4-specific protease and increased IGFBP-5 levels by stimulating gene expression (86, 87). In addition, exogenous IGFBP-4 and IGFBP-5 have opposing effects on IGF-I-induced DNA synthesis in these cells; IGFBP-4 inhibits whereas IGFBP-5 potentiates IGF-I effects. Therefore, the

TABLE 2. Proteolytic sites of IGFBPs confirmed by sequencing

Proteolytic sites	Protease (Ref.)
IGFBP-3	
Arg^{97}-Ala^{98}, Lys^{160}-Val^{161}	Plasmin (379)
Arg^{95}-Leu^{96}, Lys^{160}-Val^{161}	Plasmin (380)
Arg^{97}-Ala^{98}, Arg^{206}-Gly^{207}	Thrombin (379)
Arg^{97}-Ala^{98}, Lys^{149}-Lys^{150}, Lys^{150}-Gly^{151}, Lys^{154}-Asp^{155}	Serum (379)
Arg^{97}-Ala^{98}, Arg^{132}-Val^{133}, Tyr^{159}-Lys^{160}, Phe^{173}-Ser^{174}, Arg^{179}-Glu^{180}	Seminal plasma PSA (381)
Arg^{97}-Ala^{98}, His^{131}-Arg^{132}, Tyr^{159}-Lys^{160}	Urinary PSA (382)
Arg^{97}-Ala^{98}	Cysteine protease from MCF-7 cells (15)
Tyr^{99}-Leu^{100}	MMP-1, MMP-2 (383)
Leu^{96}-Arg^{97}, Leu^{141}-His^{142} (minor sites)	
Tyr^{99}-Leu^{100}, Asn^{109}-Ala^{110}	MMP-3 (383)
Glu^{176}-Ser^{177}	
Lys^{144}-Ile^{145}	Cathepsin L (384)
IGFBP-4	
Lys^{120}-His^{121}	Calcium-dependent serine protease from smooth muscle cells (79, 88, 89)
Met^{135}-Lys^{136}	PAPP-A (34, 98)
IGFBP-5	
Arg^{138}-Arg^{139}	Serine protease from smooth muscle cells (35)
Ser^{143}-Lys^{144} (secondary cleavage site)	
Ser^{143}-Lys^{144}	PAPP-A2 (105)
Lys^{120}-His^{121}, Arg^{156}-Ile^{157}, Arg^{192}-Ala^{193}	Thrombin (385)

References are shown *in parentheses*. PSA, Prostate-specific antigen; MMP, matrix metalloproteinase.

balance between levels of IGFBP-4 and IGFBP-5 regulated by IGF-I has a direct impact on cellular proliferation.

Parker *et al.* (79) described a calcium-dependent serine protease secreted by smooth muscle cells, whose activity is induced by IGFs, that specifically cleaves IGFBP-4 into fragments with low affinity for IGF-I. As a consequence, the IGFBP-4 fragment is less inhibitory to IGF-I-stimulated thymidine uptake compared with intact IGFBP-4 (88). It was determined biochemically that the major cleavage site on IGFBP-4 was Lys^{120}-His^{121} (Table 2), resulting in a 16-kDa amino-terminal fragment (88). This was confirmed by a protease-resistant mutant form of IGFBP-4 (Lys^{120} and His^{121} substituted by Asn) that could inhibit DNA synthesis, cell migration, and muscle growth in response to IGFs, similar to intact IGFBP-4 (89, 90).

Similar IGF-dependent IGFBP-4 protease activity has been described in a variety of cells, including fibroblasts (91), osteoblasts (92–94), endometrial stromal cells (95), decidual cells (96), and granulosa cells (97). Recently, the IGFBP-4 protease was purified from human fibroblasts and identified as pregnancy-associated plasma protein-A (PAPP-A) (98), and the cleavage site on IGFBP-4 was determined to be Met^{135}-Lys^{136} (Ref. 34 and Table 2). Preexposure of human fibroblasts to IGF-II potentiated subsequent IGF-I-induced DNA synthesis, and this was inhibited by protease-resistant IGFBP-4 mutants but not wild-type IGFBP-4 (34). More recently, it was shown in human osteoblasts using non-IGF-binding mutants that direct interaction between IGFBP-4 and IGF-II was required for optimal proteolysis of IGFBP-4 by PAPP-A (99, 100). Taken together, these studies suggest that IGFBP-4 acts as a potent inhibitor of the anabolic effects of IGF-I or -II by regulating IGF bioavailability, with the corollary that factors that regulate protease activity may thus regulate IGF actions. For example, the high PAPP-A level in estrogen-dominant ovarian follicles has been proposed to account for IGFBP-4 proteolysis leading to IGF release and subsequent dominant follicle development (101, 102). IGFBP-4 proteolysis may also have a role in the repair of arterial injury, because PAPP-A has been shown to increase in injured vascular smooth muscle cell cultures and injured arteries *in vivo* and might act in these situations by releasing IGF-I from IGFBP-4 (103). Interestingly, both PAPP-A and a related metalloproteinase (PAPP-A2) were reported to have IGFBP-5 proteolytic activity, but in contrast to the requirement of IGF for the cleavage of IGFBP-4 by PAPP-A, cleavage of IGFBP-5 by either PAPP-A or PAPP-A2 was IGF independent (104, 105).

The ability of IGFBP-5 to potentiate the response to IGF-I in smooth muscle cells is dependent upon its binding to extracellular matrix (ECM). As described further in *Section V*, a highly charged region of IGFBP-5 that contains ten basic amino acids in residues 201–218 (Table 3) has been shown to mediate binding of IGFBP-5 to the ECM of porcine smooth muscle cells (106), and a synthetic peptide containing this sequence inhibited IGFBP-5 binding resulting in reduced cellular responses to IGF-I (107). IGFBP-5 interacts specifically with two ECM proteins, thrombospondin-1 and osteopontin, which not only potentiate the IGF-I effect but may modulate the cooperative interaction between the IGFRI and integrin receptor pathways (108). In human fibroblasts, ECM-bound IGFBP-5 has a 7-fold loss of IGF-I affinity, suggesting that the potentiation of the IGF-I effect may be due to the increased bioavailability of the IGF-I to IGFRI after sequestration and concentration by IGFBP-5 to the cell surface.

However, IGFBP-5 is also secreted into medium of cultured cells and may be proteolyzed into fragments with reduced affinity for IGF-I. The proteolysis site on IGFBP-5 was determined to be Arg^{138}-Arg^{139} (Table 2), and protease-resistant IGFBP-5 (Arg^{138} and Arg^{139} substituted with Asn)

TABLE 3. The conserved carboxyl-terminal basic domain and surrounding residues in human IGFBP-1 to -6

IGFBP-1	^{178}LPNCN KNGFYHSRQCETSMDGEA GLCWCVYPWNGKRIPGSPEI**RGD**PNC^{226}
IGFBP-2	^{222}IPNCD KHGLYNLKQCKMSLNGQR GECWCVNPNTGKLIQGAPTI**RGD**PEC^{270}
IGFBP-3	^{210}IPNCD **KK**GFY**KKK**QCRPS**KGRKR** GFCWCVDKYGQPLPGYTTKGKEDVHC^{258}
IGFBP-4	^{180}IPNCD RNGNFHPKQCHPALDGQR GKCWCVDRKTGVKLPGGLEPKGELDC^{228}
IGFBP-5	^{196}LPNCD **RK**GFY**KRK**QCKPS**RGRKR** GICWCVDKY GMKLPGMEYVDGDFQC^{243}
IGFBP-6	^{163}VPNCD HRGFYRKRQCRSSQGQRR GPCWCVDRM GKCLPGSPDGNGSSSC^{210}

Consensus heparin-binding motifs of the type B-B-B-X-X-B, where B is a basic residue (198), are *underlined.* Residues comprising the nuclear localization signal of IGFBP-3 and IGFBP-5 (30) and the potential integrin-binding motif of IGFBP-1 and IGFBP-2 are *bold.*

inhibited IGF-I-stimulated DNA and protein synthesis and migration of porcine smooth muscle cells (35). This is consistent with the finding that proteolyzed IGFBP-5 in the conditioned medium had no effect on the IGF-I stimulation of growth in cultured fibroblasts (109) and suggests that, in contrast to ECM-bound IGFBP-5, soluble IGFBP-5 acts as an inhibitor of IGF-I-stimulatory effects and that proteolysis of IGFBP-5 may serve as an important regulatory mechanism of this function. Although the IGFBP-5 protease in medium conditioned by smooth muscle cells has not been identified, similar proteolytic activity against IGFBP-5 in fibroblast-conditioned medium has been attributed to the complement components C1s and/or C1r (110). The biological significance of IGFBP-5 cleavage by these enzymes is as yet unknown.

Analogous to the situation in fibroblasts and smooth muscle cells, the IGF-dependent actions of IGFBP-5 in bone cells appear to be dependent on its location. IGFBP-5 is thought to act as a depot for IGF-II in bone via its high affinity for ECM proteins and hydroxyapatite, the mineral constituent of bone, and potentiates the proliferative actions of IGF-II on osteoblastic cells (111, 112). In addition, the stimulation of osteoclastic activity by IGFBP-5 can be blocked by IGF-I antibody (113). These results have led to the proposal that the IGFs, which are sequestered and concentrated in bone by IGFBP-5, may be released during osteoclastic resorption, thus leading to stimulation of osteoblastic activity during bone remodeling. In contrast, IGF-I- and IGF-II-stimulated DNA and glycogen synthesis in a human osteoblastic cell line was inhibited by soluble recombinant IGFBP-5 (114). Likewise, the relative insensitivity of U2 osteosarcoma cells to IGF-I compared with the non-IGFBP-binding analog, des(1–3)IGF-I, was attributed to the inhibitory effect of endogenously secreted intact IGFBP-5 (115). However, proteolyzed IGFBP-5 derived from the medium of U2 cells enhanced IGF-I-stimulated osteoblast mitogenesis (116). Intriguingly, the amount of intact IGFBP-5 was increased significantly in the medium of these cells treated with IGF-I without a concomitant increase in mRNA levels or reciprocal decrease in proteolytic fragments (115), suggesting that IGF-I not only has a protective effect on IGFBP-5 proteolysis, but may also affect the compartmentalization of IGFBP-5.

It is also well established that IGFs can stimulate both proliferation and differentiation of skeletal muscle cells and these actions are mediated through IGFRI. However, the actions of IGFs are modulated by the expression of IGFBP-4, -5, and -6. As described for other cell types, IGFBP-4 is mainly inhibitory (117, 118) whereas IGFBP-5 could be either inhibitory or stimulatory to IGF actions (119, 120). By employing IGF-II analogs, Bach *et al.* (121) demonstrated that the inhibition of IGF-II-induced proliferation and differentiation of L6A1 rat myoblasts by IGFBP-6 was correlated to its affinity for the analogs. It would appear that the IGFBPs, on balance, are generally inhibitory to IGF actions in myoblasts.

There are relatively few studies on the function of IGFBP-6 and generally, IGFBP-6 appears to inhibit the actions of IGF-II with some selectivity because it has 20- to 100-fold higher affinity for IGF-II than IGF-I (122, 123). Cell systems in which IGFBP-6 has been shown to inhibit IGF-II-induced effects such as proliferation, differentiation, cell adhesion, and colony formation include osteoblasts, keratinocytes, myoblasts, and colon cancer cells (121, 124–128).

B. IGFBP-1, -2, and -3

Several studies have described both the inhibition and potentiation of IGF actions by IGFBP-1 in a variety of cells (40). The positive or negative modulation by IGFBP-1 is thought to be related to its phosphorylation state because dephosphorylation of human IGFBP-1 reduces its affinity for IGF-I by 6-fold (129). Curiously, phosphorylation appears to have no effect on the affinity of rat IGFBP-1 (130), raising the question whether IGFBP-1 regulatory mechanisms described in humans are relevant to other species. Dephosphorylated IGFBP-1 has been shown to enhance IGF-I-induced DNA synthesis (131–133), whereas phosphorylated IGFBP-1 inhibits IGF-I effects (131, 133, 134). Although high-affinity phospho-IGFBP-1 is assumed to act by blocking IGF access to the IGFRI, no experimental evidence has yet explained how the low-affinity dephosphorylated form could enhance IGF action (rather than simply not inhibiting it). Polymerization of IGFBP-1 by tissue transglutaminase has also recently been shown to ablate its inhibition of IGF-I-stimulated protein synthesis (135), but the contribution of this effect to net IGFBP-1 cellular activity is not clear.

In the extensively studied paracrine interactions at the maternal-fetal interface during pregnancy, it is thought that IGFBP-1 secreted by the maternal decidua inhibits placental trophoblast invasiveness (further discussed in *Section IV*), but this inhibitory effect is repressed by the down-regulation of IGFBP-1 production by placental trophoblast-derived IGF-II (136, 137). An alternative mechanism was proposed by Gibson *et al.* (138), who identified both phosphorylated and nonphosphorylated isoforms of IGFBP-1 secreted by decidualized endometrium under basal conditions. In the presence of trophoblast-derived IGF-II, the nonphosphorylated form of IGFBP-1—noted above to have lower IGF affinity than phospho-IGFBP-1 (67)—was predominantly produced by decidual cells. Placental phosphatases could also generate this form by dephosphorylating phospho-IGFBP-1. In addi-

tion, a protease was found to act specifically on nonphospho-IGFBP-1, which would be expected to further decrease its IGF affinity. The net result of these posttranslational modifications would be IGFBP-1 forms with reduced affinity for IGF-I, thus increasing IGF-I bioavailability to enhance tissue growth (138).

IGF-I is actively involved in the process of dermal wound healing by stimulating reepithelialization of the wounds, and this action is potentiated by IGFBP-1 (139–143), although the contribution of its phosphorylation state to this action is not known. The enhancement of IGF-I actions appears to be related to the ability of IGFBP-1 to bind to $\alpha_5\beta_1$ integrin (discussed further in *Sections III* and *IV*) because a non-integrin-binding IGFBP-1 mutant had no effect (144). IGFBP-2, which has the integrin-binding motif RGD, like IGFBP-1, was ineffective in augmenting the IGF-I enhancement of wound repair (144).

In general, IGFBP-2 appears to inhibit IGF actions, in particular those of IGF-II, possibly related to its higher affinity for IGF-II (40), although this affinity difference is in fact only 2-fold (145). Overexpression of IGFBP-2 in human embryonic kidney fibroblasts results in inhibition of cell proliferation, which can be reversed by the addition of exogenous IGFs, thus suggesting that IGFBP-2 has an inhibitory effect on IGF action (146). This is supported by a previous study that showed growth stimulation of intestinal epithelial cells transfected with an antisense IGFBP-2 construct (147). In addition, Höflich *et al.* (148) recently reported that giant GH transgenic mice, which had 2- to 3-fold increased expression of serum IGF-I levels, had a significant reduction in growth parameters when crossed with IGFBP-2 transgenic mice, suggesting that IGFBP-2 is also inhibitory to IGF-I actions *in vivo*.

Addition of equimolar concentrations of IGFBP-2 completely inhibited IGF-II-stimulated DNA synthesis in non-small-cell lung carcinoma (NSCLC) cells but had no significant effect on IGF-I-stimulated DNA synthesis (149, 150). This is not easily explained because, as noted above, the relative affinities for IGF-I and IGF-II do not differ greatly. Interestingly, IGFs bind predominantly to IGF receptors in NSCLC cells, which have relatively low levels of membrane-associated IGFBP-2. In contrast, IGFs bind to high levels of membrane-associated IGFBP-2 in small-cell lung carcinoma (SCLC), which do not respond to IGFs even though IGFRI is present (149). This suggests that both soluble and membrane-associated IGFBP-2 may be competing with the IGF receptors for ligand and may therefore be regulating IGF responsiveness in lung carcinoma.

Proteolyzed IGFBP-2 has been detected in serum (151), milk (18), and cerebrospinal fluid (152) and has decreased affinity for IGFs compared with intact IGFBP-2. Serum withdrawal from porcine aortic smooth muscle cells induces the secretion of a calcium-dependent serine protease for IGFBP-2, the activity of which is relatively more enhanced by IGF-II than IGF-I (153, 154). Menouny *et al.* (155) reported that the interaction between the plasmin system and IGFBP-2 can modulate the bioavailability of IGF-II, which mediates autocrine proliferation in neuroblastoma cells. However, in the majority of cell studies in which IGFBP-2 is detected by immunoblot, it appears in culture medium at its intact size of approximately 34 kDa. Thus, IGFBP-2 proteolysis may not be a very widespread regulatory mechanism.

Potentiation and inhibition of IGF actions by IGFBP-3 have been demonstrated in many cell culture systems (6, 40). It is thought that cotreatment of cells with IGFBP-3 and IGF-I causes IGFBP-3 to inhibit IGF-I-mediated effects via high-affinity sequestration of the ligand (15, 156–158), presumably leading to prevention of IGF-I-induced IGFRI autophosphorylation and signaling (20). In contrast, preincubation of cells with IGFBP-3 before IGF-I treatment leads to the accumulation of cell-bound forms of IGFBP-3 with lowered affinity for IGF, which may enhance the presentation of IGF to IGFRI (156, 159, 160). However, this mechanism has never been proven explicitly, and Karas *et al.* (56) found that cell-bound IGFBP-3 could still attenuate IGF-I-mediated IGFRI signaling. It has also been reported, based on competitive ligand-binding studies, that IGFBP-3 can interact with IGFRI, causing inhibition of IGF-I binding to its receptor (161), although a direct physical interaction between IGFRI and IGFBP-3 has not been demonstrated, for example, by coprecipitation. It is therefore not clear that cell association of IGFBP-3 is the key factor in determining its IGF-stimulatory effects. In addition, it has been suggested that the enhanced IGF-I stimulation of DNA synthesis in MCF-7 cells transfected with IGFBP-3 might result from IGFBP-3 protecting the cells from IGF-I-mediated down-regulation of IGFRI (162) as initially proposed in a previous study in bovine fibroblasts (163). More recently, it has been suggested that the potentiation of IGF action by IGFBP-3 may be mediated through the phosphatidylinositol 3 (PI3)-kinase pathway (62) (*Section VI*).

As with the other IGFBPs, specific proteases for IGFBP-3 in a variety of cell culture systems have been described (164), including serine proteases, cathepsins, and matrix metalloproteinases. Proteolysis results in IGFBP-3 fragments with decreased affinity for IGFs and is therefore assumed to enhance the availability of IGFs to the cell (77, 165, 166). However, several studies have described the inhibition of IGF actions by IGFBP-3 fragments with low (sometimes undetectable) affinity for IGFs (14, 15, 77, 167). It is unclear whether this inhibitory action of IGFBP-3 may, in some cases, be mediated via its sequestration of IGFs (despite its low affinity) or via an IGF-independent mechanism such as the proposed interaction with the IGFRI directly to prevent IGF-IGFRI interactions as described above.

Figure 2 summarizes proposed IGFBP actions that depend on binding of IGFs and modulation of IGFRI activation. Although studies describing the potentiation or inhibition of IGF activity by IGFBPs have been reported for at least two decades, there is currently no unifying mechanism that would explain these opposite actions. What is evident is that the complex interaction between IGFs and IGFBPs is further complicated by the fact that 1) the production of IGFBPs and IGFBP-specific proteases are often regulated by IGFs, and 2) IGFs can regulate the activity of these proteases.

III. IGF- and IGFRI-Independent Effects of IGFBPs

It is becoming increasingly clear that, apart from modulating IGF actions, IGFBPs may exert intrinsic bioactivity

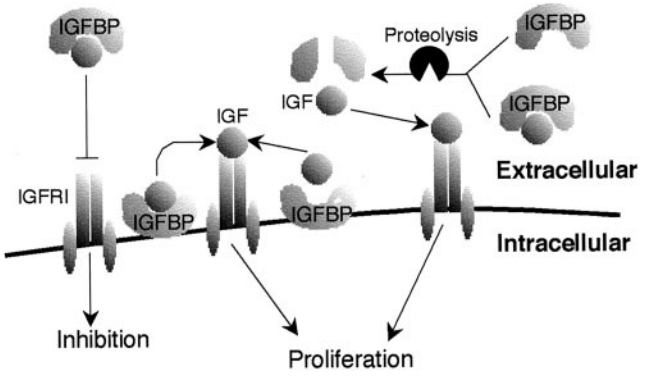

FIG. 2. Proposed pathways of IGF-dependent IGFBP action. The mitogenic activity of IGFs, mediated through IGFRI, is inhibited by their sequestration by soluble IGFBPs. Proteolysis of IGFBPs leads to the release of IGFs from the binary complexes and hence a potentiation of IGF activity. Cell-associated IGFBPs have been reported to either potentiate or inhibit the IGF effects. Refer to the text for further details of these interactions.

either in the absence of IGFs (IGF-independent effects) or in the presence of IGFs without triggering IGFRI signaling (IGFRI-independent effects). Once again, these effects may be mediated by either intact or proteolyzed fragments of IGFBPs and are likely to involve structural domains that are distinct from the IGF-binding determinants on IGFBPs. Recently, there has been considerable interest in the ability of IGFBPs, especially IGFBP-3, to induce or modulate apoptosis independently of inhibiting the survival functions of IGF-I (*Section VII*). This section will discuss what is currently known about other IGF-independent effects of IGFBPs.

One of the first reports of IGF-independent actions of IGFBPs was the effect of IGFBP-1 on cell motility and adhesion. There is good evidence that this effect is mediated by the RGD integrin-binding motif present in the carboxyl-terminal domain of IGFBP-1 (Table 3), interacting with $\alpha_5\beta_1$ integrin (22), as further discussed in *Section IV*. Although a homologous RGD motif is also present in IGFBP-2, the evidence supporting IGF-independent actions of IGFBP-2 mediated by integrin binding is still preliminary (*Section IV*).

IGFBP-2 has been shown to be mitogenic for uterine endometrial epithelial cells and osteosarcoma cells in the absence of IGFs (168, 169). Overexpression of IGFBP-2 in Y-1 adrenocortical tumor cells resulted in enhanced proliferation and increased cloning efficiency of IGFBP-2-secreting cells (170). In a subsequent preliminary report, several proliferation-associated genes including glutathione *S*-transferase were shown to be up-regulated in these IGFBP-2-transfected cells, leading to the suggestion that the induced glutathione *S*-transferase levels may confer resistance to oxidative stress (171). In these studies it was proposed that the proliferative effect of IGFBP-2 overexpression was IGF independent because IGFRI was down-regulated and an IGF-I analog with decreased IGFBP interaction had the same mitogenic potency as IGF-I (170). Although a mechanism of growth stimulation by IGFBP-2 is yet to be established, it may tentatively be concluded that, whereas inhibitory IGFBP-2 effects are likely to involve the sequestration of IGFs (*Section II*), some of the positive growth effects of IGFBP-2 appear to occur independently of IGF action. This may have implications for the treatment of IGFBP-2-expressing tumors, which include those of colon (172) and prostate (173).

IGF-independent effects of IGFBP-4 and IGFBP-6 are not well established and, as discussed in the previous section, it is generally accepted that IGFBP-4 and IGFBP-6 act primarily through inhibition of IGF actions. Indeed, the majority of reports on IGF-independent effects of IGFBPs have focused on IGFBP-3. The earliest of these is attributable to Harel and her colleagues (174), who determined that murine IGFBP-3 (initially referred to as "inhibitory diffusible factor 45"), in addition to acting through IGF binding, could inhibit DNA synthesis in chick embryo fibroblasts stimulated by serum, fibroblast growth factor, or TGFβ, but not insulin or platelet-derived growth factor (175, 176). Subsequently, mouse fibroblasts transfected with IGFBP-3 were shown to have a markedly reduced growth rate and were arrested at a lower cell density than vector-transfected cells. This growth-inhibitory effect, which was demonstrated in the presence of serum-containing media, was not reversible by insulin, leading to the proposal that the IGFBP-3 effect was IGF independent (177).

Although the possibility remained that IGF signaling somehow contributed to these early observations, IGFBP-3 was subsequently shown unambiguously to have IGFRI-independent growth-inhibitory effects in a mouse fibroblast cell line derived from IGFRI-knockout mice where transfection of IGFBP-3 resulted in cell growth inhibition (178). Whether IGFBP-3 might have acted in these cells by blocking IGF activity through a different receptor [*e.g.*, IGF-II signaling through the type A insulin receptor (179)] has never been explicitly excluded. This illustrates the need to distinguish between IGFRI independence (which was clearly demonstrated in this study) and IGF independence, which is difficult to prove rigorously in cell culture studies.

An interesting illustration of transition from IGF-independent to -dependent IGFBP-3 action is seen in the changing response to exogenous IGFBP-3 in differentiating chondrocytes (180). In this study in a chondrogenic cell line, IGFBP-3 inhibited thymidine incorporation stimulated by insulin, IGF-I, or low-IGFBP-affinity IGF-I analogs, when the cells were in the predifferentiated state. In contrast, when the cells had undergone terminal differentiation, only IGF-I action was inhibited by IGFBP-3, suggesting that it could only inhibit the actions of ligands that it could sequester with high affinity. Although there is as yet no mechanistic explanation for this transition, this cell system appears to offer a valuable model in which to examine the molecular basis of IGF-independent inhibitory signaling by IGFBP-3.

Several studies using human breast cancer cells have correlated the induction of IGFBP-3 mRNA and protein expression with growth-inhibitory effects of various antiproliferative agents including TGFβ , retinoic acid (181), antiestrogens (182), vitamin D analogs (183, 184), and TNFα (Ref. 185 and Fig. 3). IGFBP-3 expression is also up-regulated by the transcription factor p53 in colon carcinoma cells (186), and by the histone deacetylase inhibitors trichostatin A (187) and butyrate (188). Studies from several independent laboratories using breast or prostate cancer cell lines have shown that decreasing the expression of IGFBP-3 by antisense IGFBP-3 oligodeoxynucleotide treatment, or sequestering IGFBP-3

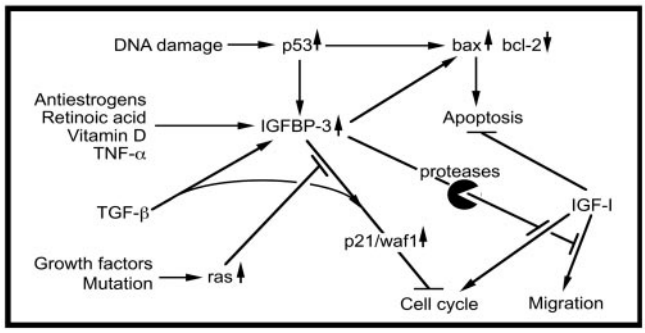

FIG. 3. Overview of antiproliferative pathways potentially involved in IGFBP-3 action. A variety of agents and cellular effectors, including p53 (186), up-regulate IGFBP-3. IGFBP-3 can induce apoptosis by a mitochondrial pathway involving bax, even in the absence of wild-type p53 (299). IGFBP-3 inhibition of DNA synthesis may involve p21/waf1 up-regulation (184). It can be blocked by activation of the ras-dependent MAPK pathway (281). IGF stimulation of cell cycle and migration, and inhibition of apoptosis, may be inhibited by IGFBP-3, this effect being potentially reversible by the limited proteolysis of IGFBP-3.

using antibodies, partially abrogates the antiproliferative effects of some of these factors. This phenomenon, demonstrated for TGFβ, retinoic acid, the antiestrogen ICI 182780, and 1,25-dihydroxyvitamin D_3, has been cited as evidence that the induction of IGFBP-3 contributes to the growth-inhibitory functions of these agents (181, 182, 184, 189). Interestingly, the induction of IGFBP-3 by TGFβ has also been associated with growth-stimulatory effects of TGFβ in airway smooth muscle cells and colon cancer cells (190, 191), and this TGFβ-induced cell growth can also be blocked by antisense IGFBP-3 oligodeoxynucleotides or neutralizing antibodies. These studies raise interesting questions, representing as they do a powerful accumulation of evidence that IGFBP-3 plays a mediating role in the actions of a diverse range of growth effectors. Balancing this is abundant literature describing alternative mechanisms of action of these effectors, so that it will be important to determine the extent to which IGFBP-3 induction is necessary to mediate their actions.

The IGF-independent growth-inhibitory and stimulatory effects described above are all associated with the intact form of IGFBP-3. Several studies have reported IGF-independent effects of proteolyzed forms of IGFBP-3 that have little or no demonstrable affinity for IGFs. For example, cleavage of nonglycosylated IGFBP-3 by plasmin results in a 22- to 25-kDa fragment with 50-fold loss in IGF-I affinity and a 16-kDa fragment that does not bind IGF-I, as detected by ligand blot (14). The 16-kDa amino-terminal fragment, IGFBP-3[1–95], was almost as effective as intact IGFBP-3 in inhibiting IGF-I-induced DNA synthesis in chick embryo fibroblasts. Surprisingly, whereas intact IGFBP-3 had no effect on insulin-induced stimulation, the 16-kDa fragment inhibited the action of insulin, suggesting a mechanism independent of IGF-I. These observations were further extended by the demonstration that the 16-kDa fragment, but not intact IGFBP-3, inhibited the mitogenic action of fibroblast growth factor on both wild-type and IGFRI-knockout mouse fibroblasts (192), making it unlikely that IGF sequestration had any involvement in the effect. Recently, this fragment has also been shown to induce apoptosis in MCF-7 cells (193).

Other, independent evidence supports the proposal that IGFBP-3 amino-terminal fragments have intrinsic activity. Using a semipurified mixture of plasmin-digested IGFBP-3, Booth *et al.* (194) demonstrated the bioactivity of approximately 20-kDa amino-terminal and about 8-kDa carboxyl-terminal fragments in stimulating glucose uptake by microvessel endothelial cells, in contrast to intact IGFBP-3, which was ineffective. It was not clear which fragment was responsible for the activity and whether the approximately 20-kDa fragment was the same as the 16-kDa fragment described above. A similar 16-kDa amino-terminal fragment, derived from proteolysis by an enzyme secreted by MCF-7 breast cancer cells (80), was identified by sequencing and mass spectrometry as IGFBP-3[1–97]. This fragment inhibits basal DNA synthesis in MCF-7 cells in the absence of IGF-I (15). The mechanism by which intact IGFBP-3, and amino-terminal IGFBP-3 fragments, might exert their effects independent of the modulation of IGF-IGFRI interactions remains to be determined. As described in *Section V*, possible cell-interacting sites have been identified in the central (38) and carboxyl-terminal domains (24) of IGFBP-3 but not in its amino-terminal domain.

Apart from binding its well described ligands, IGF-I and IGF-II, IGFBP-3 has a number of other interacting partners, as summarized in Table 4. In the serum, the binary complex of IGF-IGFBP-3 can bind ALS, to form high molecular mass heterotrimeric complexes (195). These complexes are thought to prevent the efflux of the IGFs from the vasculature and hence play a crucial role in regulating IGF bioavailability to target tissues. In pregnancy serum, IGFBP-3 appears to undergo limited proteolysis but still circulates in the full-sized ternary complex form (196). However, it is hypothesized that IGFs in complexes containing proteolyzed IGFBP-3 are more readily bioavailable, and it was suggested recently that ADAM 12, a disintegrin metalloprotease, may be involved in this process (197). Residues 228–232 of IGFBP-3 play an important role in ALS binding (24); these residues are part of the 18-residue basic domain implicated in cell binding by IGFBP-3 described above (Table 3). It may be speculated that competition between the endothelial cell surface and ALS for the same residues on IGFBP-3 is involved in the dissociation of the ternary complex and the release of IGFs.

The 18-residue basic domain—also present in IGFBP-5—contains a heparin-binding motif of the type B-B-B-X-X-B where B is a basic residue and X is undefined (198) as shown in Table 3. Binding of heparin and certain other glycosaminoglycans, as well as cell surface proteoglycans, to this heparin-binding motif of IGFBP-3 has been demonstrated (29, 199, 200). Recently, it was found that both IGFBP-3 and IGF-I-IGFBP-3 complexes bind fibrinogen, fibrin (201), and plasminogen (202) with high affinity via the heparin-binding domain (HBD) (Table 3). It was also shown that activation of plasminogen to plasmin, a specific protease for IGFBP-3, is not inhibited by IGFBP-3 binding (202). In addition, fibronectin binding to IGFBP-3 and IGF-I-IGFBP-3 complexes has also been recently demonstrated (203). Because all of these proteins are involved in the process of wound healing, these studies suggest a role for IGFBP-3 in concentrating IGF-I at

TABLE 4. Proteins known to interact with IGFBPs

IGFBP	Cell-surface and extracellular binding partners	Intracellular binding partners
IGFBP-1	IGF-I and IGF-II	
	$\alpha_5\beta_1$ integrin (22)	
IGFBP-2	IGF-I and IGF-II	
	$\alpha_5\beta_1$ integrin (233)	
	Glycosaminoglycans (242, 243)	
IGFBP-3	IGF-I and IGF-II	Importin β (30)
	ALS (195)	RXR (64)
	Latent TGFβ binding protein-1 (386)	E7 oncoprotein (311)
	Type 1α collagen (387)	
	IGFRI (161)	
	TGFβ type V receptor (257)	
	Transferrin (28)	
	Lactoferrin (207)	
	Glycosaminoglycans (29, 199, 200)	
	Fibrin and fibrinogen (201)	
	Plasminogen (202)	
	Fibronectin (203)	
IGFBP-4	IGF-I and IGF-II	
IGFBP-5	IGF-I and IGF-II	
	Importin β (30)	
	ALS (25)	FHL2 (312)
	Plasminogen activator inhibitor-1 (27)	
	Glycosaminoglycans (211, 212)	
	Osteopontin (108)	
	Thrombospondin (108)	
	Vitronectin (388)	
	Hydroxyapatite (214)	
	IGFBP-5 receptor (65)	
IGFBP-6	IGF-I and IGF-II	

References are shown *in parentheses.*

wound sites, and, conceivably, after proteolysis of IGFBP-3 by plasmin, IGF-I is released to exert its mitogenic effects.

IGFBP-3 has also been shown to bind to human serum transferrin, the effect depending on the degree of iron saturation (28). In an independent study, transferrin itself was shown to bind IGFs, although with an affinity 200-fold lower than the IGF-IGFBP-3 affinity (204). Interestingly, the affinity of IGF-II for IGFBP-3 was increased 5-fold in the presence of transferrin. Transferrin, a key component of iron transport and metabolism, is involved in various aspects of cell-cell interactions and cell viability (205) and reported to regulate programmed cell death (206). IGFBP-3 induced cell proliferation in bladder smooth muscle cells, and apoptosis in prostate cancer cells was blocked by transferrin cotreatment (28). However, it is not clear whether these effects were due to the modulation of IGFBP-3 or transferrin (or both) interacting with their respective receptors, or whether IGF binding by either protein was involved. The related iron-binding protein, lactoferrin, has also been described as binding IGFBP-3, competing with IGF-binding, and affecting IGFBP-3 nuclear entry in mammary cells (207). Because IGFs may modulate the cellular interactions between IGFBP-3 and transferrin or lactoferrin, or may even be central to their effects, these studies do not necessarily represent IGF-independent actions of IGFBP-3. They do, however, raise the possibility of cellular entry of IGFBP-3 through transferrin receptors.

The first description of IGF-independent effects of IGFBP-5 was reported in 1992 when a 23-kDa IGFBP-5 fragment was shown to stimulate normal osteoblast mitogenesis in the absence of IGF-I (116). Using the recombinant amino-terminal fragment IGFBP-5[1–169], Andress *et al.* (208) confirmed the initial study and suggested that the IGFBP-5 fragment-stimulated mitogenesis may be mediated by low-affinity binding of the fragment to the osteoblast surface. This is further supported by reports that IGFBP-5 binds to cell surfaces via its basic carboxyl-terminal region (residues 201–218; Refs. 29 and 209), shown in Table 3. In contrast, IGFBP-5[1–169] did not display intrinsic bioactivity in mesangial cells but inhibited IGF-I-stimulated migration (210). It is currently unexplained how IGFBP-5[1–169], shown to have decreased affinity for IGF-I (208), could be a more potent inhibitor of IGF-I than intact IGFBP-5.

Analogous to IGFBP-3, the HBD region of IGFBP-5 has been implicated as the binding region for several different molecules including ALS (25, 26), heparin, and various glycosaminoglycans (211, 212), IGF-I (21, 213), plasminogen activator-1 (27), osteopontin (108), thrombospondin (108), hydroxyapatite (214), and importin β (30) as well as to the ECM (Refs. 29 and 209 and Table 4). At present, it is not entirely clear if the same specific residues within the 201–218 region of IGFBP-5 (Table 3) are required for interacting with this diverse group of molecules. This issue is addressed in more detail in a recent review of IGFBP mutagenesis studies (7).

As described above, IGFBP-5 binds to ECM (215) and hydroxyapatite (214), indicating that it may accumulate in bone and sequester IGFs to bone. IGFs are important regulators of bone formation because IGF-I knockout mice show severe impairment of bone growth (216). Administration of recombinant human IGFBP-5 to mice increased bone formation parameters and decreased bone resorption parameters. The increase in bone formation was not mediated by in-

creases in circulating levels of IGF-I, providing indirect evidence of an IGF-independent effect (217). This finding was further extended by a similar study in IGF-I knockout mice. Treatment of osteoblast cells, derived from IGF-I knockout mice, with recombinant human IGFBP-5 significantly increased proliferation and alkaline phosphatase activity, a marker of osteoblast differentiation. When injected *in vivo* into IGF-I knockout mice, recombinant human IGFBP-5 increased local levels of alkaline phosphatase activity and osteocalcin, markers of bone formation, whereas an equimolar administration of IGF-I did not have a significant effect (112). A second study using ovariectomized mice also showed that administration of recombinant human IGFBP-5 stimulated osteoblast activity and bone accretion in the femur and spine (218). Although the mechanism involved in this IGF-independent effect of IGFBP-5 is yet to be established, IGFBP-5 has been shown to stimulate the binding of GH to GH receptors, resulting in the potentiation of GH-stimulated mitogenesis in rat osteoblasts (219), an intriguing result if confirmed.

The concept of IGF-independent effects by IGFBPs on cellular growth has gained wide acceptance in recent years, yet the mechanisms underlying these activities are still poorly understood. Although many questions remain unanswered regarding specific receptors and intracellular signaling (*Sections V* and *VI*), the complexity of having several IGFBPs expressed in the same cell system, each possibly existing in different isoforms due to posttranslational modifications, and potentially having opposing effects that may be IGF dependent or independent, adds to the challenge faced by researchers. Recent studies using overexpression systems have shown such complexity of IGFBP regulation and the necessity for caution in interpreting the data. Transfection of IGFBP-2 into an epidermoid carcinoma cell line, which normally does not secrete IGFBP-2, resulted in increased tumor growth. However, concomitant with the expression of IGFBP-2, there was a decrease in IGFBP-1 expression and an increase in IGFBP-3 proteolysis (220). When IGFBP-4 was transfected into prostate carcinoma cells, the delayed onset of tumorigenesis was accompanied by a decrease in IGFBP-2 expression (221). However, when an antisense IGFBP-4 construct was transfected into the same cell line, tumor growth was also decreased but in this instance, this was accompanied by an increase in IGFBP-3 and IGFBP-6 expression (222). Thus, it may well be that changes in cell activity attributed to a single IGFBP are, in reality, the result of alterations in several proteins.

IV. Adhesion and Migration

The regulation of cell adhesion to, and release from, the ECM is recognized as an active process involving complex signaling events that can influence cytoskeletal rearrangement, cell motility, and tumor invasiveness (223, 224). IGFs are well known to increase cell migration (225, 226), whereas IGF-increased cell adhesion to matrix proteins has also been described, effects that can be blocked by IGFBPs (35, 126, 227). The possibility that IGFBPs might have an effect on cell adhesion and motility independent of their IGF-binding function was first suggested by the observation that IGFBP-1 and IGFBP-2 contain an RGD integrin-binding motif in their carboxyl-terminal domain (Ref. 5 and Table 3). Integrins function as cell adhesion receptors, transducing extracellular signals both through phosphorylation cascades and through direct connection with cytoskeletal elements (228).

Jones *et al.* (22) first reported the increased migration of Chinese hamster ovary (CHO) cells transfected to express human IGFBP-1. Cells expressing a mutated form of IGFBP-1 containing WGD in place of the ^{221}RGD motif failed to show increased migration, and the stimulatory effect of wild-type IGFBP-I could be blocked by the addition of a synthetic peptide containing the RGD sequence. The interacting cell surface protein, isolated by affinity chromatography on immobilized IGFBP-1, was identified as $\alpha_5\beta_1$ integrin, the fibronectin receptor (22). Despite its RGD motif, IGFBP-2 was unable to stimulate smooth muscle cell migration under conditions where IGFBP-1 was stimulatory (229). However, both IGFBP-1 and IGFBP-2 were found to inhibit IGF-stimulated migration of smooth muscle cells, in contrast to the stimulatory effect of IGFBP-1 seen in the absence of IGFs.

IGF-independent actions of IGFBP-1 mediated by integrins have been demonstrated in several systems, including the stimulation of healing in a dermal wound model (144) and the stimulation of cell detachment and apoptosis in breast cancer cells (58). Although focal adhesion kinase (FAK) has been implicated in the IGFBP-1-induced changes in cellular adhesion and migration, the mechanism is unclear, with both dephosphorylation (58) and increased phosphorylation (230) of FAK reported as a consequence of IGFBP-1 action. The most extensively studied system for IGFBP-1 signaling through $\alpha_5\beta_1$ integrin is that of human trophoblast cell migration. Gleeson *et al.* (230) reported that IGFBP-1 stimulated the migration of extravillous trophoblast cells, the effect again being dependent on RGD interaction with $\alpha_5\beta_1$ integrin. *In vivo*, the IGFBP-1 is assumed to come from the decidua, an abundant source of this protein (231). In contrast, decidua-derived IGFBP-1 has been shown by others to prevent cytotrophoblast attachment to fibronectin and was inhibitory to cytotrophoblast invasiveness (137). As noted earlier, trophoblast-derived IGF-II, by inhibiting decidual IGFBP-1 production, has been proposed to overcome the inhibitory effect, thus allowing the trophoblast to invade (136).

There is considerably less evidence that the RGD motif in IGFBP-2 can initiate IGF-independent signaling. Indeed, only preliminary data in published abstracts currently support this hypothesis. Mutation to Arg-Gly-Glu (RGE) decreased IGFBP-2 cell association in ovine choroid plexus cells (232); in a more recent report, IGFBP-2 was shown to cell associate through its RGD to $\alpha_5\beta_1$ integrin (233), similarly to IGFBP-1. In contrast, IGFBP-2 with the RGE mutation was identical with the wild-type protein in cell association and growth inhibition when expressed in a transgenic mouse model (234). In two adhesive tumor cell lines (Ewing sarcoma A673 and Hs578T breast cancer) IGFBP-2 was reported to induce FAK dephosphorylation and affect cell adhesion and migration, suggesting that its cell interaction is functional (233). Overall, the lack of published data on an IGFBP-2-integrin interaction, together with cell migration studies

where IGFBP-2 does not mimic IGFBP-1 (229), makes this an area where further investigation is needed.

The effect of IGFBP-5 on the migration of glomerular mesangial cells has been studied by Abrass *et al.* (210). Although IGFBP-5 was inhibitory to IGF-I-stimulated migration, it was stimulatory when added alone. At high concentration, the 18-residue carboxyl-terminal fragment IGFBP-5[201–218] also showed potent stimulatory activity. This peptide represents the basic motif (Table 3) known to be involved in ALS binding, cell and matrix interaction, and nuclear translocation of IGFBP-3 and IGFBP-5. IGF-I-induced cell migration involves $\alpha_V\beta_3$ integrin and is blocked by the disintegrin kistrin (235); however, migration induced by IGFBP-5[201–218] was not inhibited by kistrin (210), indicating a different mechanism. IGFBP-5 also induced marked morphological changes in mesangial cells, with multiple filopodia developing (236). The small GTPase Cdc42, known to be involved in filopodia formation, was shown to be activated by the IGFBP-5 peptide, and early addition of staurosporine inhibited the IGFBP-5 effect, consistent with signaling through a serine-threonine protein kinase. The possible role of a putative IGFBP-5 receptor in this function is discussed in *Section V*.

V. Cell-Binding Sites and Putative Receptors

Beyond regulation of cell adhesion and migration, IGFBPs have major effects in regulating cell cycle and apoptosis, as discussed in *Section VII*. Identification of the signaling pathways that mediate these effects on cell proliferation, and the receptors that initiate signaling, has been among the major goals in IGFBP research in recent years. In the previous section, $\alpha_5\beta_1$ integrin, the fibronectin receptor, was discussed as a cell surface protein complex of known structure that binds IGFBP-1 through an identified domain, initiating a definable response of cellular events. Despite intensive investigation and the reporting of numerous putative receptor proteins, there are, to date, no other examples in the literature of a fully characterized cell surface protein that would satisfy the usual criteria for a signaling receptor for any IGFBP, namely reversible and saturable ligand binding, and the initiation of a definable intracellular signaling pathway.

Although the characterization of cell surface IGFBP receptors has proved elusive, cell binding of IGFBPs has been reported in many systems, resulting in the partial description of several interacting proteins. In some of the early literature, before the identification of the six IGFBPs and the establishment of specific analytical reagents, it was unclear which IGFBP was being studied. Clemmons *et al.* (239) used affinity labeling to demonstrate the association of a 35- to 40-kDa IGFBP with fibroblast monolayers. Although no cell binding site was identified, the interaction was proposed to modify cell responsiveness to IGFs (237). By using [$Q^3A^4Y^{15}L^{16}$]IGF-I, which has greatly reduced affinity for IGFBPs but near-normal affinity for the IGFRI, it was estimated that surface-bound IGFBPs could contribute up to 80% of the total IGF-I binding sites in human glioblastoma cells and fetal fibroblasts (238). Data from the laboratories of Clemmons (239) and Conover (240) further showed, using IGF-I analogs, that fibroblasts exposed to IGF-I released an unidentified IGFBP of approximately 40 kDa from the cell surface, independently of IGFRI interaction. Martin *et al.* (241) extended these observations using specific immunological detection of IGFBP-3 to show IGF-I-dependent release of IGFBP-3 from the fibroblast cell surface, with reciprocal appearance in the culture medium. Receptor-inactive IGF-I analogs were fully active in this process. It thus appears that, whereas IGFs can bind to cell surface IGFBP-3 and other IGFBPs, the IGF-IGFBP interaction may, paradoxically, also act to release IGFBP-3 from the cell.

A. Glycosaminoglycan-binding domains on IGFBPs

In addition to the integrin system discussed earlier for IGFBP-1 and IGFBP-2, other mechanisms of IGFBP-2 interaction with cells have been reported. In the rat olfactory bulb, IGFBP-2 has been shown to interact with cell surface proteoglycan binding sites. *In vitro*, IGFBP-2 bound to chondroitin-4 and -6-sulfate, keratan sulfate, and the proteoglycan aggrecan (242). Arai *et al.* (243) also demonstrated IGFBP-2 interaction with glycosaminoglycans, but only if IGF-I or IGF-II was present. IGFs were similarly required for IGFBP-2 interaction with cell matrix in fibroblast cultures. Residues ^{180}KKLR in the central domain of human IGFBP-2 represent a consensus short HBD of the form B-B-X-B, where B is a basic residue (198), although the role of these residues in IGFBP-2 cell binding has not been demonstrated. The consequence of IGFBP-2 binding for cell function is unknown, but it may serve to concentrate IGFs near type I IGF receptors as it can increase IGF-stimulated proliferation in some cell types (170, 220), although paradoxically it is growth inhibitory in other cells (146), and when overexpressed *in vivo* (244).

IGFBP-4 has no consensus HBD, whereas a putative long HBD of the form B-B-B-X-X-B (198) is found in human IGFBP-6 residues ^{173}RKRQCR (Table 3). IGFBP-4 is not known to associate with cell surface-binding sites, but a single report describes nonglycosylated IGFBP-6 binding to heparan sulfate, chondroitin sulfate, and other glycosaminoglycans (66), as further discussed below. In contrast, there are numerous reports of the cell association of IGFBP-3 and IGFBP-5, and evidence for growth-regulatory signaling by these proteins. The observation that heparin, like IGF-I, was effective in releasing IGFBP-3 from the fibroblast cell surface into the culture medium led to the suggestion that its binding sites might involve proteoglycans (241). A variety of sulfated glycosaminoglycans in addition to heparin are partially effective in competing with radioiodinated IGFBP-3 or IGFBP-5 for binding to endothelial cell monolayers, as is an 18-residue basic peptide corresponding to IGFBP-3 [215–232] (29). This sequence, like the corresponding motif in IGFBP-5 (and IGFBP-6), contains a consensus long HBD at residues ^{220}KKKQCR (Table 3). However, removal of sulfated proteoglycans by growing various cell types in 5 mM sodium chlorate, or treating them with a mixture of heparinases, was unable to prevent IGFBP-3 or IGFBP-5 binding (29, 245), leading to the conclusion that the inhibitory effect of heparin probably resulted from a direct interaction between heparin and the binding protein. The interaction of heparin with

these basic residues presumably also accounts for its ability to block IGFBP-3 binding to ALS (246).

Site-directed mutagenesis of IGFBP-3 residues ^{228}KGRKR to the corresponding IGFBP-1 residues MDGEA (Table 3) substantially abolished binding to Chinese hamster ovary cells (24, 247) and other cell types (our unpublished data). This region is adjacent to, but not overlapping, the carboxyl-terminal consensus HBD, and the mutant protein retained considerable affinity for a heparin-agarose column (24), despite the loss of cell binding. A contribution to the residual heparin binding may come from a secondary short consensus HBD at residues ^{149}KKGH, which has been shown to be functional (199), and provides further evidence against glycosaminoglycans as IGFBP-3 cell surface-binding sites.

Although IGFBP-3[1–184], representing the amino-terminal and central domains, shows no binding to CHO cells, in contrast to the full-length protein (24), a central domain cell-binding site on IGFBP-3 has been proposed on the basis of competition studies between nonglycosylated IGFBP-3 and the central domain peptides IGFBP-3[88–148] and IGFBP-3[88–183] for binding to Hs578T human breast cancer cells (38). This result, contrary to the observation of Firth *et al.* (24), is possibly explained by cell-specific differences in IGFBP-3 binding sites. It remains unclear whether a central domain cell-binding site for IGFBP-3 would involve the HBD residues ^{149}KKGH, because a peptide containing these residues had extremely low activity in competing for bound IGFBP-3 (38). A central domain heparin binding site, involving consensus short HBD residues in this region, has also been described for IGFBP-5 (37). This appears to be masked by the carboxyl-terminal domain in the intact protein, but is active in a carboxyl-terminally truncated form of IGFBP-5. Whether it has a role in cell association of IGFBP-5 is unknown.

B. The role of posttranslational modifications in cell surface association

Of the four IGFBPs with well documented cell and matrix association, only IGFBP-3 has consensus sites for N-glycosylation (248), whereas IGFBP-5 is reported to be O-glycosylated (249), and the low level of carbohydrate on IGFBP-1 is presumably also O-linked (Ref. 250 and Table 5). In contrast to IGFBP-1 and IGFBP-5, the primary structures of IGFBP-2 and IGFBP-3 predict no O-glycosylation sites (Ref. 31 and Table 5). In IGFBP-3, carbohydrate increases the core protein size of 29 kDa to forms estimated to be 40–43 kDa. Of the three potential glycosylation sites at Asn^{89}, Asn^{109}, and Asn^{172}, the first two are always used, carrying an estimated 4 kDa and 4.5 kDa of carbohydrate, respectively, whereas the third site alternatively contains either undetectable or about 5 kDa of carbohydrate, accounting for the characteristic doublet form of the protein (31). Comparison of *Escherichia coli*-derived and CHO cell-derived IGFBP-3 indicates that glycosylation has no significant effect on the binding of IGF-I (251) or ALS (31).

Although nonglycosylated *E. coli* IGFBP-3 appeared similar to the glycosylated protein in its effect on IGF-I-stimulated aminoisobutyrate uptake by fibroblasts, no direct comparison of their ability to cell associate was reported (159). However, IGFBP-3 forms in which various N-glycosylation sites have been altered by mutagenesis reveal that decreasing glycosylation tends to increase cell surface association, so that nonglycosylated IGFBP-3 shows approximately 3-fold higher binding to both CHO cells and T47D breast cancer cells compared with the fully glycosylated protein (Refs. 31 and 252 and Table 6). This suggests that the carbohydrate present in natural IGFBP-3 might mask potential cell-association sites and raises the question whether cell binding studied using *E. coli* IGFBP-3 reflects the binding of the native protein.

Interestingly, cell association by IGFBP-6 also appears to be inhibited by carbohydrate (Table 6). Binding to glycosaminoglycans is greatly inhibited by O-glycosylation, and the nonglycosylated protein, which is not known to occur in nature, has been shown to bind to PC12 cell membranes, whereas the natural, O-glycosylated form shows no binding (66). This suggests that, as seen to a smaller degree in IGFBP-3, cell binding sites of IGFBP-6 are permanently masked by carbohydrate.

IGFBP-1, IGFBP-3, and IGFBP-5 are all secreted as phosphoproteins (Ref. 33 and Table 5). IGFBP-1 phosphorylation increases IGF-I affinity 6-fold for the human protein (67) but, as noted in *Section II*, has no effect on the rat protein (130). Phosphorylation differences in IGFBP-1 appear to account for two distinct species isolated from human amniotic fluid, the more weakly anionic of which was found to enhance IGF-I-stimulated DNA synthesis (131), whereas the more strongly anionic was inhibitory. Only the less anionic form, presumably in a lower phosphorylation state, bound to smooth muscle cells (131), suggesting that phosphorylation may be inhibitory to cell surface interaction of IGFBP-1. Although, as described earlier, IGFBP-1 can interact with cells through $\alpha_5\beta_1$ integrin, it is not clear how this interaction is modulated by phosphorylation.

There is also evidence that phosphorylation inhibits IGFBP-3 cell binding (Table 6). Human skin fibroblasts secrete IGFBP-3 into the culture medium as a phosphoprotein, but release of surface-bound IGFBP-3 from fibroblasts using an IGFRI-inactive IGF-I analog was found to increase total IGFBP-3 but not phospho-IGFBP-3 in the culture medium, implying that surface-bound IGFBP-3 was nonphosphorylated (253). More recently, phosphorylation of IGFBP-3 *in vitro* by protein kinase CK2 has been shown by direct binding studies to be inhibitory to cell surface association (68).

The functional implications of the effects of phosphorylation and glycosylation on cell binding, especially in the case of IGFBP-3, are uncertain. Clearly, if cell signaling by IGFBP-3 can be initiated by cell surface binding, this process may be modulated by phosphorylation, possibly in a dynamic way. Modification of signaling by changes in glycosylation is less likely to be biologically relevant, at least as a form of acute regulation. A key limitation in interpreting these studies is the unknown relationship between general cell surface binding of IGFBPs, *e.g.*, the heparin-displaceable binding of IGFBP-3 by fibroblasts (241), and binding to true functional receptors. For example, if phosphorylated IGFBP-3 shows decreased cell surface binding (Table 6), does this imply decreased signaling through a cell surface receptor? In the case of IGFBP-3 and IGFBP-5 it would be pre-

TABLE 5. Sites of potential glycosylation and phosphorylation on IGFBPs

	Predicted glycosylation sites[a]		Predicted phosphorylation sites[a]			
IGFBP	N-linked[b]	O-linked	PKA[c]	PKC[c]	CK2[c]	MAPK[c]
IGFBP-1		T^{27} S^{95}		**T^{50}AR** **S^{58}CR**	**S^{22}CSE** T^{105}EEE **S^{119}EED** S^{131}TYD T^{168}SGE S^{169}GEE **T^{194}SMD**	G^{94}SPE E^{97}SPE G^{216}SPE
IGFBP-2				T^{66}PR **T^{204}MR** T^{254}GK **T^{263}IR** T^{285}QR		C^{9}TPE Y^{65}TPR A^{105}SPE **R^{188}TPC**
IGFBP-3	**N^{89}ASA** **N^{109}ASE** **N^{172}FSS**		**K^{178}RET**	**T^{58}ER** **T^{130}HR** **S^{156}QR** **S^{177}KR** **S^{204}PR** T^{249}TK	S^{70}PDE **S^{111}ESE** **S^{113}EED** **S^{177}KRE**	P^{69}SPD E^{123}SPS **L^{203}SPR**
IGFBP-4	**N^{204}NSF**			T^{50}PR **S^{95}DK** S^{234}FR	**S^{95}DKD** **S^{111}AHD** **T^{171}HED** **S^{234}FRE**	**Y^{49}TPR** F^{107}SPC
IGFBP-5		**T^{103}** **T^{104}** **T^{111}**	**K^{138}KLT**	**T^{51}ER** S^{85}YR **S^{113}PK** **S^{186}PR**	**S^{85}YRE** **T^{103}TSE** S^{159}APE **S^{179}LQE** **S^{249}NVE**	**P^{20}SPL** **Y^{112}SPK** **A^{185}SPR**
IGFBP-6		**T^{119}** S^{120} T^{121} T^{122} S^{124} S^{208} T^{212}		T^{102}AR	**S^{28}PAE**	**G^{27}SPA** Y^{50}TPN T^{121}TPS **G^{200}SPD**

[a] The potential N-linked glycosylation and phosphorylation sites in human IGFBP sequences were predicted by ProfileScan using the PROSITE database at http://www.expasy.org/prosite/ (389), and the potential O-linked glycosylation sites were predicted by NetOGlyc 2.0 at http://genome.cbs.dtu.dk/services/NetOGlyc/ (390). Sites in *bold* indicate conservation across species with known sequences.

[b] The consensus pattern for N-linked glycosylation site is N-{P}-[ST]-{P} where N is the carbohydrate acceptor site, ST means S or T, and {P} indicates any residue except P.

[c] The consensus patterns for phosphorylation sites are as follows: cAMP- and cGMP-dependent protein kinase (PKA): [RK](2)-x-[ST]; protein kinase C (PKC): [ST]-x-[RK]; casein kinase II (CK2): [ST]-x(2)-[DE]; MAPK: x-[ST]-P-x, where ST means S or T (the phospho-acceptor site), RK means R or K, and x is any residue. Glycosylation has been reported for IGFBP-1 (250), IGFBP-3 (391), IGFBP-4 (69), IGFBP-5 (115), and IGFBP-6 (392), whereas phosphorylation has been reported for IGFBP-1 (67) and IGFBP-3 (393).

TABLE 6. Posttranslational modification of IGFBPs: effects on cell interaction

Modification	IGFBP	Effect	Ref.
Glycosylation			
N-Glycosylation[a]	IGFBP-3	Inhibitory	31, 252
O-Glycosylation	IGFBP-1	Not reported	
O-Glycosylation	IGFBP-5	Not reported	
O-Glycosylation	IGFBP-6	Inhibitory	66
Phosphorylation			
	IGFBP-1	Inhibitory	131
	IGFBP-3	Inhibitory	68, 253
	IGFBP-5	Not reported	
Limited proteolysis	IGFBP-1	Not reported	
	IGFBP-3	C-terminal truncation abolishes binding	24
	IGFBP-5	C-terminally truncated form retains binding	208
	IGFBP-5	Central HBD unmasked by C-terminal truncation	37

[a] IGFBP-4 can also exist in an N-glycosylated form (394) but is not reported to cell associate.

mature to conclude that the extensively studied binding involving carboxyl-terminal basic residues, which appears to account for the majority of cell-binding sites (29), represents receptor binding. As discussed elsewhere, IGFBP-3 mutated in these residues, and truncated IGFBP-5 lacking these residues, both elicit biological effects. Therefore it may be surmised that functional, low-abundance receptors do not necessarily make a major contribution to overall cell binding of IGFBPs.

C. Other putative IGFBP receptors

A number of other cell surface proteins have been designated in the literature as IGFBP receptors, with varying degrees of experimental support. Iodinated nonglycosylated IGFBP-3 exhibited cation-stimulated binding to Hs578T breast cancer cells, displaceable by unlabeled ligand with an EC_{50} of approximately 10 nM (254). Subsequent kinetic studies yielded affinity constants of 8 nM for IGFBP-3 binding to both Hs578T and MCF-7 breast cancer cells (38). Iodo-IGFBP-3 binding to other cell types has revealed a higher apparent affinity, with a K_d estimated as 0.22 nM in Ishikawa endometrial cancer cells (56) and approximately 0.5 nM in platelets (255). In contrast to the observation in Hs578T cells, the binding to Ishikawa cells was not stimulated by divalent cations, and neither sulfated proteoglycans nor glycosylphosphatidylinositol linkage appeared to be involved.

Affinity labeling was used to identify Hs578T cell surface-interacting proteins of 20, 26, and 50 kDa, immunoprecipitable by anti-IGFBP-3 antibody (256). In mink lung cells, where IGFBP-3 binding to the 400-kDa type V TGFβ receptor was observed (*Section VI*), affinity labeling also revealed cross-linked bands of 64–70 kDa, consistent with IGFBP-3 binding to proteins of 20–30 kDa (257). Whether these bands represent IGFBP-3 dimerizing with its own proteolytic products, as suggested, or interaction with other cell proteins in this size range, is unclear. Ligand blotting with iodo-IGFBP-3 has also been used to identify IGFBP-3-interacting proteins. In solubilized membranes isolated from PC-3 prostate cancer cells, interacting proteins with sizes estimated as 18, 68, and 150 kDa were observed (59). The nature of these cell-associated interacting proteins is unknown, and it remains to be demonstrated that any of them is involved in IGFBP-3 signal transduction. A preliminary report of a better-characterized protein, designated 4–33, suggests a role in mediating or modulating IGFBP-3 effects. This protein, identified from a yeast two-hybrid screen, is detectable both on the cell surface and intracellularly in Hs578T breast cancer cells (258). Overexpression of 4–33 increases IGFBP-3 cell binding, and in the presence of IGFBP-3 it decreases DNA synthesis and induces apoptosis. Although these studies demonstrate the involvement of 4–33 in IGFBP-3 cellular functions, its precise structure and mechanism of action are yet to be described.

As noted in *Section III*, Andress *et al.* (208) have reported the cell binding and stimulation of mitogenesis by carboxyl-terminally truncated IGFBP-5[1–169]. Both intact and truncated IGFBP-5 were shown to bind to osteoblasts with K_d values around 10 nmol/liter, but only intact IGFBP-5 was heparin displaceable (259). Affinity-labeling studies led to the identification of a membrane protein estimated as 420 kDa, which was purified by IGFBP-5 affinity chromatography (259). Subsequently, purification from solubilized mouse osteoblast membranes was achieved on an affinity column of the octadecapeptide IGFBP-5 [201–218], again yielding a 420-kDa protein (Ref. 65 and Fig. 4). This might not be expected to be the same protein that bound IGFBP-5 [1–169], which does not include the affinity peptide sequence, in the previous study (259), although competition studies on the purified protein were not reported.

The 420-kDa protein has apparent serine/threonine kinase activity, becoming phosphorylated when exposed to intact IGFBP-5, IGFBP-5[201–218] on which it was affinity purified, and also IGFBP-5[1–169] (65). The activity of these nonoverlapping sequences suggests that both a carboxyl-terminal domain, and a more amino-terminal site, must be involved in the interaction that stimulates this activity. Autophosphorylation was assumed to account for the kinase activity observed, although the possibility of a small associated protein kinase was not specifically excluded. Signaling downstream of this protein has not yet been elucidated, but the observation that early addition of staurosporine inhibits biological actions initiated by IGFBP-5[201–218] in mesangial cells suggests that serine/threonine kinase activity is an ini-

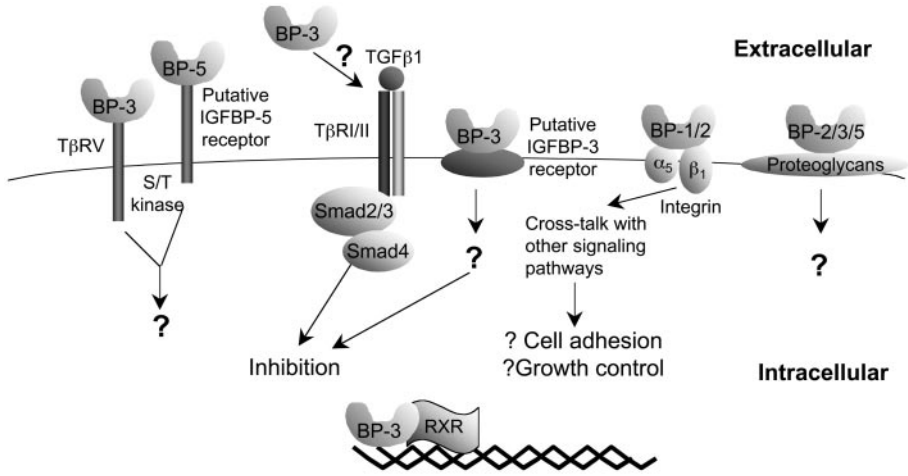

FIG. 4. Proposed pathways of IGF-independent IGFBP action—a composite figure combining data from studies in a variety of cell types. TβRV and a 420-kDa protein have been proposed as serine/threonine kinase receptors for IGFBP-3 and IGFBP-5, respectively. Other putative IGFBP-3 receptors with undemonstrated signaling capacity have been proposed. IGFBP-3 stimulates TGFβ signaling via the Smad pathway, and this activity requires TβRII and is enhanced in the presence of TGFβ. IGFBP-1 (and possibly IGFBP-2) binds to $\alpha_5\beta_1$ integrin, which could lead to cross-talk with other growth control-signaling pathways. IGFBP-2, IGFBP-3, and IGFBP-5 bind to proteoglycans but no signaling has been demonstrated. IGFBP-3 and IGFBP-5 are translocated to the nucleus by importin β and IGFBP-2 has also been identified there; IGFBP-3 has an identified nuclear partner, RXRα. Refer to the text for further details of these interactions.

tiating event in the action of IGFBP-5 (236). A very recent report also implicates serine phosphorylation of Ras in IGFBP-5 signaling (260).

VI. Interactions of IGFBP-3 with Known Signaling Pathways

A. IGFBP-3 and TGFβ signaling

A protein of similar high molecular mass to the putative IGFBP-5 receptor has been designated a receptor for IGFBP-3 in mink lung epithelial cells (Ref. 257 and Fig. 4). The type V receptor for TGFβ (TβRV) is an incompletely characterized serine/threonine kinase of approximately 400 kDa, first described a decade ago (261). Although its relative importance in TGFβ signaling, compared with the type I and type II TGFβ receptor (TβRI and TβRII) system (262), is unclear, it is reported to mediate growth inhibition by TGFβ in cells lacking TβRI and TβRII (263). Recombinant nonglycosylated IGFBP-3 was shown to bind to this protein and to inhibit TGFβ binding, and IGFBP-3-induced growth inhibition in TβR-deficient mink lung cells could be blocked by a TGFβ1 peptide antagonist (257). The interaction of IGFBP-3 with a 400-kDa protein in mink lung cells has been confirmed independently using a partially glycosylated IGFBP-3 mutant, IGFBP-3[N109D, N172D] (264). Judged by its comigration with a similar protein that binds TGFβ, the 400-kDa protein could be identical with the TβRV. In this study, Leu^{60}-IGF-I, an analog with decreased IGFRI binding activity, inhibited both the interaction of IGFBP-3 with the 400-kDa protein, and the inhibition of DNA synthesis seen with IGFBP-3 alone, leading to the suggestion that the 400-kDa protein might be involved in IGFBP-3 inhibitory signaling (264).

In addition to IGFBP-3, IGFBP-4 and -5 bind to the TβRV (265), raising the possibility of a similarity between this protein and the IGFBP-5-binding 420-kDa serine/threonine kinase described by Andress (65). However, IGFBP-4 and -5 are weak receptor ligands, and weak inhibitors of DNA synthesis in mink lung cells, compared with IGFBP-3 (265). It is therefore possible that TβRV is a receptor in mink lung cells with relative specificity for IGFBP-3, but its role in IGFBP-3 signal transduction, its presence in other cell types, and its complete structural characterization remain to be determined.

The possibility that IGFBP-3 signals through a TGFβ-related pathway is of particular interest because IGFBP-3 is believed to mediate some of the growth effects of TGFβ in various cell types. TGFβ was initially shown to stimulate IGFBP-3 production by skin fibroblasts and was suggested to modulate IGF action by this mechanism (266). More recently, both growth-inhibitory (59, 189) and growth-stimulatory (190, 191) actions of TGFβ have been shown to involve the induction of IGFBP-3, the effects being blocked when IGFBP-3 induction is abrogated. These and similar studies, described more fully in *Section III*, have implicated IGFBP-3 in TGFβ action but did not address the concept that IGFBP-3 might interact with a TGFβ signaling pathway, as implied by the studies with TβRV.

Recently, the TβRII has been shown to play an important role in IGFBP-3 action in T47D breast cancer cells. Many T47D cell lines lack TβRII and are TGFβ resistant. Restoration of the receptor by cDNA transfection restored sensitivity to TGFβ and rendered the cells sensitive to synergistic growth inhibition by IGFBP-3 and TGFβ (63). IGFBP-3 has subsequently been shown to stimulate the phosphorylation of the TβRI and of the signaling intermediates Smad2 and Smad3 (63, 267) in both T47D and MCF-7 breast cancer cells. This distinguishes IGFBP-3 action involving TβRII from effects mediated by TβRV, which do not involve Smad phosphorylation (265). IGFBP-3 has reduced activity in stimulating this pathway after immunoneutralization of endogenous TGFβ1, suggesting that IGFBP-3 can act independently, but its effects are enhanced by endogenous TGFβ (267).

Interestingly, IGFBP-3 mutated in its nuclear localization domain (^{228}KGRKR→MDGEA), which has greatly reduced cell surface binding (24) and fails to translocate to the nucleus (268), is able to induce TβRI and Smad phosphorylation and to increase plasminogen activator inhibitor-1 transcriptional activity, a Smad-dependent process (267). These observations distinguish IGFBP-3 actions through the Smad pathway from IGFBP-3 antiproliferative effects mediated through nuclear interactions, described below. It is therefore now possible to define an intracellular signaling cascade initiated by IGFBP-3 (Fig. 4) and dependent upon the presence of a defined cell surface receptor system. However, it is not yet known whether IGFBP-3 is a direct ligand for TβRII or initiates signaling in an indirect manner; therefore, this study does not yet define an IGFBP-3 receptor. Moreover, it remains to be shown whether this is a ubiquitous pathway for IGFBP-3 action or is confined to the breast cancer cell lines in which it was first demonstrated.

B. IGFBP-3, retinoids, and nuclear signaling

All-*trans*-retinoic acid (RA) is a potent inducer of IGFBP-3 in breast and other cancer cells (269–272). This effect, which is believed to contribute to the growth-inhibitory effect of RA, requires the presence of RA receptor (RAR)-β (273) and can be blocked by retinoid X receptor (RXR)-specific retinoids (274). Although signaling through RAR stimulates IGFBP-3 production, experiments using a RA response element reporter system suggest that IGFBP-3 actually inhibits RA signaling (64). If these *in vitro* data can be extrapolated to RA action *in vivo*, this observation could have important implications for the regulation of sensitivity to retinoids as therapeutic agents.

In contrast to possible effects on RA signaling, IGFBP-3 has been shown in one study to enhance signaling through RXR, as determined using an retinoid X response element reporter, and has been observed by a variety of techniques to interact directly with RXRα. IGFBP-3 and RXRα have been colocalized by confocal microscopy in the cytoplasm and nucleus of LAPC-4 prostate cancer cells, with nuclear localization more evident after exposure to an RXR-specific ligand. The IGFBP-3 residues involved in the RXR interaction appear to be located within the 18-residue basic domain sequence 215–232 (64). As previously noted, IGFBP-3 and IGFBP-5 share this carboxyl-terminal domain, which resembles a consensus bipartite nuclear localization sequence (Table 3 and Ref. 275).

A variety of studies have identified IGFBP-3 in cell nuclei or directly demonstrated its nuclear transport (172, 268, 276–

278); competition studies suggest that IGFBP-5 uses the same transport system as IGFBP-3 (268). Nuclear transport of IGFBP-3 appears to be favored in dividing cells and can serve to cotransport IGF-I to the nucleus (277, 278). The plasma membrane may present a major barrier to the transport of extracellular IGFBP-3 to the nucleus, because extracellular IGFBP-3 translocates to a relatively small percentage of cell nuclei, but in cells with a permeabilized plasma membrane, IGFBP-3 can be found in almost all nuclei, demonstrating that transport from the cytoplasm to the nucleus occurs readily (268). Its retention within the nucleus when the nuclear membrane is permeabilized suggests that it interacts with insoluble elements there (30, 268). The nuclear transport protein importin-β has been shown to mediate translocation of both IGFBP-3 and IGFBP-5 (30). This interaction appears predominantly to require residues 228–232 of IGFBP-3, and 214–218 of IGFBP-5, although other basic residues within the 18-residue basic domain are also involved (Table 7).

IGFBP-3 interacts with an RXR-retinoid X response element complex, as determined by EMSA, and in F9 embryonal carcinoma cells appears to require this interaction to induce apoptosis, as the viability of RXRα-deficient F9 cells is unaffected by IGFBP-3 whereas a control cell line expressing RXRα has reduced viability when exposed to IGFBP-3. Furthermore, an RXR-specific ligand has been reported to enhance IGFBP-3-induced apoptosis (64). However, a recent study (279) shows that the mutant form of IGFBP-3 that fails to translocate to the nucleus (IGFBP-3[^{228}MDGEA]) can still induce apoptosis in breast cancer cells. These data imply that, in these cells, either RXR-IGFBP-3 interaction is not required for apoptosis, or interaction between these proteins in the cytoplasm may be sufficient. RXR can dimerize with numerous nuclear receptors including RAR, vitamin D receptor, thyroid receptor, peroxisome proliferator-activated receptors, and others, as well as itself (280); thus, it may be speculated that IGFBP-3, by binding RXR, could also influence signaling by these other receptors. Because this could have wide implications for understanding nuclear actions of IGFBPs, it will be important to confirm this study and demonstrate other cell systems in which the IGFBP-3-RXR interaction occurs.

TABLE 7. Effect of mutations within the nuclear localization signals of IGFBP-3 and IGFBP-5 on their nuclear accumulation

Nuclear localization sequence	Nuclear accumulation
IGFBP-3	
K K G F Y K K K Q C R P S **K G R K R**	+ +
– N – – – – – – – – – – – – – – – –	+ +
– – – – – H S R – – – – – – – – – –	+
– – – – – – – – – – – – – M D G E A	–
– N – – – H S R – – – – – – – – – –	+
– N – – – – – – – – – – – M D G E A	–
A A – – – – – – – – – – – – – – – –	+
IGFBP-5	
R K G F Y K R K Q C K P S **R G R K R**	+ +
– N – – – – – – – – – – – – – – – –	+ +
– – – – – H S R – – – – – – – – – –	+
– – – – – – – – – – – – – M D G E A	–
A A – – – – – – – – – – – – – – – –	+

The bipartite nuclear localization signal sequences of IGFBP-3 and IGFBP-5 are shown with the basic residues indicated in *bold*. Changes in amino acid residues are indicated for the different IGFBP-3 and IGFBP-5 mutants. The effect of each mutation on nuclear accumulation was reported by Schedlich *et al.* (30).

C. Other pathways

The mechanism underlying the phenomenon of enhancement of IGF-I activity by IGFBP-3, discussed in *Section II*, is not fully understood. A recent study by Conover *et al.* (62) demonstrated that LY294002, an inhibitor of PI3-kinase activity, could block the ability of IGFBP-3, when preincubated with bovine fibroblasts, to stimulate IGF-I-stimulated aminoisobutyrate uptake, implying that this pathway was required to mediate the IGFBP-3 effect. Although IGFBP-3 alone did not directly stimulate PI3-kinase, as assessed by immunoblot for the active phosphoform of its downstream effector protein kinase B (PKB)/Akt, it enhanced the effect of IGF-I on this phosphorylation (62). IGFBP-5 is also reported to activate signaling through PI3-kinase. LNCaP prostate cancer cells stably transfected to overexpress IGFBP-5 were found to have an increased proliferation rate, concomitant with increased PKB/Akt phosphorylation (61). PI3-kinase blockade by LY294002 induced apoptosis independent of IGFBP-5 expression, but IGF-I rescued the cells from death only if IGFBP-5 was overexpressed. This effect parallels the effect of IGFBP-3 described above, to the extent that both IGFBPs appear capable of sensitizing cells to IGF-I action. In the study of Conover *et al.* (62), a decrease in PKB/Akt phosphorylated at a Thr-Pro site in cells preincubated with IGFBP-3 alone suggested that dephosphorylation of this site might be involved in the heightened IGF-I sensitivity; consistent with this observation, okadaic acid, a serine/threonine phosphatase inhibitor, blocked the potentiating effect of IGFBP-3 on IGF-I. These data provide evidence that IGFBP-3 might influence growth factor signaling by activating a specific protein phosphatase system; whether IGFBP-5 acts through a similar mechanism is unknown.

An interaction between MAPK signaling and IGFBP-3 activity has also been established, with the demonstration that the inhibitory effect of IGFBP-3 on DNA synthesis in breast epithelial cells is blocked in cells expressing oncogenic *ras*, and restored by the MAPK/ERK pathway inhibitor PD98059 (Ref. 281 and Fig. 3). The sensitivity to IGFBP-3 of Hs578T breast cancer cells, which express oncogenic *ras*, can also be enhanced by treatment with PD98059. Changes in IGFBP-3 sensitivity were unrelated to the extent of IGFBP-3 cell association (281), suggesting that the regulation of cellular sensitivity to IGFBP-3 does not involve a mechanism as straightforward as modulating the interaction between IGFBP-3 and an abundant cell surface receptor.

Very recently, another signaling intermediate, STAT1 (signal transducer and activator of transcription-1) has been implicated in IGFBP-3 action during the process of chondrocyte differentiation (282). Predifferentiated chondrocytes were previously shown to provide a convincing example of IGF-independent IGFBP-3 action (180), as discussed in *Section III*. IGFBP-3 strongly up-regulated STAT1 mRNA in this system, and phospho-STAT1 protein was shown to increase and translocate to the nucleus. Furthermore, in the presence of a STAT1 antisense oligonucleotide, IGFBP-3 action was

ablated, strongly implicating STAT1 induction and phosphorylation in the antiproliferative action of IGFBP-3 in these cells (282). This is yet another example of an IGFBP-3-activated signaling mechanism observed in a single cell line. It should now be relatively easy to determine whether STAT1 induction by IGFBP-3 occurs in other systems.

Is it possible to integrate these reports of IGFBP-3 interaction with diverse signaling pathways into a single unifying mechanism of action? At present there is insufficient information available to do so, because each of the above-described studies has only been performed in a limited number of laboratories (often only one) and cell types. One well established fact is the translocation of IGFBP-3 to the cell nucleus, although the cell signals that initiate this are unknown, and the function of IGFBP-3, once inside the nucleus, is still unclear. As noted above, by mutating the nuclear localization signal of IGFBP-3, it is now possible to distinguish between cellular effects that require nuclear translocation and those that do not. A similar mutagenesis approach involving other key functional residues may eventually further delineate distinct pathways of IGFBP-3 action, *e.g.*, antiproliferative effects seen with non-IGF-binding forms of IGFBP-3 (282) add further weight to the concept of truly IGF-independent actions.

The relationship between MAPK activity and IGFBP-3 signaling, if confirmed in other laboratories, may help to integrate some of the pathways described earlier. Phosphorylation of effector proteins by MAPK can interrupt pathways involving both Smads (283) and RXRα (284), both reported to mediate some IGFBP-3 effects (63, 64). This suggests that cell sensitivity to growth-inhibitory effects of IGFBP-3 may be regulated by a variety of intersecting pathways. Understanding the mechanisms underlying IGFBP-3 sensitivity and resistance may be important in developing therapeutic approaches to cancer cell proliferation because, despite its antiproliferative and proapoptotic activity *in vitro* (*Section VII*), a high level of tumor IGFBP-3 has been associated with poor prognosis in breast malignancy (285). This apparent discrepancy highlights the fact that few of the regulatory mechanisms demonstrated in cell culture models have yet been confirmed *in vivo*.

VII. Cell Cycle, Apoptosis, and Survival

IGFs stimulate cell proliferation as well as inhibiting apoptosis, thus acting as cell survival factors. The antiapoptotic functions of IGF-I are mediated by IGFRI, independent of the receptor's ability to transduce mitogenic signals (70). Similar to their modulation of IGF-I's cellular proliferative effects, IGFBPs can also modulate the antiapoptotic effects of IGF-I by regulating the IGF-I-IGFRI interaction. Perturbations at each level of the IGF axis have been implicated in cancer development and progression in several cell types (75). For example, treatment of MCF-7 cells with either exogenous IGFBP-3 or an antiestrogen that stimulates the endogenous production of IGFBP-3 resulted in increased apoptosis. This effect could be inhibited by a non-IGFBP-binding IGF-I analog but not by IGF-I, suggesting, but not proving, that IGFBP-3 induced apoptosis by sequestering IGF-I from IGFRI (286). Similarly, serum withdrawal from human teratocarcinoma cells induced apoptosis that could be reversed by simultaneous treatment with IGF-I or IGF-II. However, the survival effect by IGF-II was abrogated by cotreatment with either IGFBP-2 or IGFRI blocking antibody, again suggesting that interference of the IGF-IGFRI interaction can abolish the antiapoptotic effect (287).

Modulation of IGFBPs by proteolysis also plays a role in regulating their antiproliferative effects. For example, the proliferation of DU145 prostate adenocarcinoma cells in serum-free medium is thought to occur through an autocrine IGF loop because these cells express IGF-I, IGF-II, IGFRI, and IGFBP-2, -3, and -4, although the IGFBP-3 is proteolyzed. However, addition of anti-IGFRI antibody or intact IGFBP-3 can inhibit this serum-free proliferation (288). Concurrent with the inhibition of DNA synthesis, exogenous IGFBP-3 promotes a decline in the number of cells accumulating in the G_2/M phase and a reduction in the levels of p34(cdc2), a protein required for G_2 transition to mitosis (289). The cells were shown to produce matrix metalloproteinase 9, a protease for IGFBP-3, and it was proposed that the equilibrium between proteolysis of IGFBP-3 by matrix metalloproteinase 9, resulting in low-affinity IGF binding fragments, and sequestration of IGF by intact IGFBP-3 may determine the outcome of cellular proliferation or inhibition (288).

Studies of this kind illustrate clearly that IGFBPs can affect cell cycle progression and apoptosis by preventing IGFRI activation. IGFBPs can also exert IGF-independent effects on cell cycle arrest and apoptosis (290, 291). As noted in *Section III*, previous studies have shown 1,25-dihydroxyvitamin D_3 (vitD) to be a potent growth inhibitor of some cancer cells and that it up-regulates IGFBP-3 (183, 292). Whereas in PC-3 prostate cancer cells, IGFBP-3-mediated growth inhibition by vitD appears to involve IGF sequestration (292), in Hs578T breast cancer cells the effects of vitD analogs are thought to be IGF independent because the cells are IGF unresponsive (183). The mechanism of inhibition by vitD may involve the accumulation of cells in the G_1 phase of the cell cycle. In support of this, it was recently reported that the inhibition of growth in LNCaP prostate cancer cells in an IGF-free system by vitD is mediated by the up-regulation of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 via the induction of IGFBP-3 (184) (Fig. 3). This effect can be abrogated by using IGFBP-3 immunoneutralizing antibodies. The inhibition of growth of T47D cells by overexpression of IGFBP-3 was also associated with an accumulation of cells in G_1 phase (60). However, because IGFs stimulate cell cycle progression at the G_1-S transition (293), the possibility of IGF involvement in these IGFBP-3 actions still needs to be considered.

Rajah *et al.* (59) provided the first unambiguous demonstration of the IGFRI-independent proapototic effects of IGFBP-3 in an IGFRI-negative mouse fibroblast cell line, by treating the cells with exogenous IGFBP-3 and by transfecting IGFBP-3 into the cells. In both instances, the basal level of apoptosis was significantly increased, a key observation that will be important to confirm in other laboratories. Using Hs578T breast cancer cells, which do not respond to IGF-I, as a model to determine IGF-independent effects of exogenous IGFBPs on apoptosis, Perks *et al.* (294) found that none of the IGFBPs alone had a significant inhibitory effect on cell

growth. However, when apoptosis was induced by a ceramide analog, C2, in these cells, IGFBP-3 accentuated the apoptotic effect whereas IGFBP-4 and IGFBP-5 reduced C2-induced apoptosis, and IGFBP-1, IGFBP-2, and IGFBP-6 had no significant effect. In addition, although IGFBP-3 had no effect on cell death induced by an RGD peptide through integrin detachment, IGFBP-5 inhibited integrin detachment-induced apoptosis (294, 295). These findings indicate that the IGFBPs have distinctive functional effects on different apoptotic signaling pathways even though they share structural homology.

A further study has suggested that the enhancement of C2-induced apoptosis in Hs578T by IGFBP-3 may involve cell association and subsequent proteolysis of the binding protein, and that this effect can be abrogated by cotreatment with IGF-I (296). IGFBP-3 has also been shown to enhance the apoptotic effect of paclitaxel, a cytotoxic drug that acts via TNFα and subsequent ceramide generation (297).

There is also evidence that IGFBP-3 can induce apoptosis by itself, as well as potentiate the apoptotic effects of other DNA-damaging stimuli such as ionizing and UV irradiation and chemotherapeutic drugs (298–301). Transfection of IGFBP-3 into breast cancer cells expressing either wild-type (MCF-7) or mutant (T47D) p53 resulted in an induction of apoptosis (299), suggesting that the IGFBP-3 effect can occur independently of functional p53. Furthermore, transfection of IGFBP-3 into T47D cells restored the sensitivity of these radiation-resistant cells to ionizing radiation. This IGFBP-3 action in MCF-7 and T47D cells is one of few cases where it has been shown to be apoptotic by itself: in most other studies, it enhances the apoptotic effect of other agents. Because all cell culture studies involve a degree of cell apoptosis, presumably due to limitations of the culture conditions, it is not clear what contributory role other factors may play in cells that appear to respond to IGFBP-3 alone. This may, in fact, be a semantic argument because all tissue and tumor growth is a balance between proliferative and apoptotic activities, and studies of xenograft tumors expressing IGFBP-3 show inhibition of tumor growth (302, 303).

The inhibition of protein kinase Cα, the expression of which is correlated to tumor progression in glioblastoma multiforme, by either antisense oligonucleotide (304) or a protein kinase Cα-specific inhibitor (305), was reported to be associated with the induction of p53 and IGFBP-3 concomitant with apoptotic cell death. A tumor suppressor, p53 regulates the transcription of many cellular genes that are involved in mediating its effects on cell cycle arrest and apoptosis (306). IGFBP-3 was identified in 1995 as one of the p53-inducible genes and a mediator of p53-dependent apoptosis in EB1 colon carcinoma cells in response to cellular stress (186). Consistent with this, Williams *et al.* (298) reported that IGFBP-3 potentiation of apoptosis after ionizing radiation in colonic adenoma cells was dependent on functional p53. In an esophageal carcinoma cell line, apoptosis after UV irradiation was accompanied by an increase in p53 levels, which was enhanced and prolonged by exogenous IGFBP-3 treatment (300). Although the level of endogenous IGFBP-3 was not examined, this study raises the possibility that IGFBP-3 may also act upstream or independently of p53 in an autocrine fashion as well as downstream of p53 as the mediator of its actions (Fig. 3).

The intracellular mechanisms that mediate the IGF- and IGFRI-independent antiproliferative and proapoptotic functions of IGFBP-3 are still being elucidated. The induction of apoptosis in breast cancer cells overexpressing IGFBP-3 was associated with an increase in the proapoptotic Bax and Bad and a decrease in the antiapoptotic Bcl-2 and Bcl-x_L (Ref. 299 and Fig. 3). This led to the suggestion that IGFBP-3 may exert its apoptotic effect by modulating the Bax-to-Bcl-2 ratio. Bcl-2 activity may be further decreased by its serine phosphorylation in response to IGFBP-3 (307). The ratio of proapoptotic and antiapoptotic members of the Bcl-2 family of cytoplasmic proteins is an important determinant of survival or death in cells (308). These proteins reside or assemble on the surface of the mitochondria during apoptosis. Indeed it has been shown that apoptosis induced by a mitochondrial respiratory chain inhibitor, antimycin A, was accentuated by IGFBP-3. Together with the observation that both antimycin A and IGFBP-3 can also enhance ceramide-induced apoptosis (295, 309), it would appear that IGFBP-3 accentuates apoptosis induced via pathways involving the mitochondria.

Caspases, a family of cysteine aspartic acid-specific proteases, occupy a central position as intracellular effectors of apoptotic signals. When activated by various apoptotic stimuli, caspases then activate numerous cellular substrates by restricted proteolysis, and may involve the release of mitochondrial proteins, resulting in cell death (310). Apoptosis induced by IGFBP-3 in PC-3 cells could be inhibited by a caspase inhibitor, thus implicating the involvement of this key pathway (59). However, it remains to be shown which caspases are involved and whether there is a direct or indirect interaction with IGFBP-3.

The E7 oncoprotein, encoded by the human papillomavirus type 16, has recently been identified as an IGFBP-3 interacting partner in a yeast two-hybrid screen (311). Coexpression of E7 with IGFBP-3 in PC-3 cells reduced the number of apoptotic cells compared with PC-3 cells transfected with IGFBP-3 alone, thus suggesting that E7 can inhibit IGFBP-3-induced apoptosis. Furthermore, it was shown that E7 and IGFBP-3 colocalize intracellularly, and degradation of intracellular IGFBP-3 was enhanced in E7 coexpressing cells, the effect being reversible by a proteasome inhibitor. E7 mutants with reduced oncogenic potential were not as effective in inhibiting IGFBP-3-induced apoptosis. This study suggests that the degradation of intracellular IGFBP-3, and hence the abrogation of the proapoptotic function of IGFBP-3, could potentially contribute toward the transforming capacity of E7. Functional inactivation of IGFBP-3 by an oncogene was also demonstrated in breast epithelial cells that were transformed by constitutively activated Ha-ras oncogene. However, in contrast to the E7 study, the cellular growth resistance to IGFBP-3 was not due to degradation of the protein but was associated with an increase in both secreted and cell-associated IGFBP-3 (281).

It remains unclear whether the proapoptotic function of IGFBP-3 is effected by secreted extracellular IGFBP-3, which is then internalized, or by intracellular IGFBP-3. The ability of exogenous IGFBP-3 to induce apoptosis in several cell systems (59, 294), and of IGFBP-3 neutralizing antibodies to

inhibit IGFBP-3-induced apoptosis in PC-3 cells (59), provides some evidence that the apoptotic signal may initiate at the cell surface. This raises questions about the identity of the signaling receptor for IGFBP-3, as discussed in *Section V*. Questions also remain about how IGFBP-3 exerts its apoptotic function. However, as discussed in *Section VI*, IGFBP-3 can translocate to the nucleus by binding to the importin β nuclear transport factor (30) and was recently shown to interact with the nuclear receptor, RXRα (64). Butt *et al.* (299) reported that IGFBP-3 modulated the mRNA levels of bcl-2 in IGFBP-3-transfected MCF-7 cells, raising the possibility that the regulation of gene expression by nuclear IGFBP-3 may effect its apoptotic function.

Like IGFBP-3, IGFBP-5 also translocates to the nucleus by binding to the importin β nuclear transport factor (30). Although the full spectrum of intracellular binding partners for IGFBP-5 (like IGFBP-3) is far from understood, the recent identification by two-hybrid screening, and confirmation by coprecipitation, of FHL2 as an IGFBP-5-interacting protein raises interesting questions (312). FHL2 interacts with a variety of cellular proteins including the androgen receptor, suggesting the possibility of a direct role for IGFBP-5 in transcriptional regulation, but the true significance of this interaction will require further extensive experimentation.

The role of IGFBP-5 in apoptosis and cell survival has been studied in models of mammary gland and prostate involution. GH and prolactin enhance mammary epithelial cell survival by increasing IGF-I production and decreasing IGFBP-5 production, respectively (313). Involution of the mammary gland involves apoptosis of the epithelial cells as well as extensive remodeling and degradation of the ECM. It has been proposed that IGF-dependent and -independent functions of IGFBP-5 may be involved in these processes. During mammary gland involution, there is a decrease in prolactin levels concomitant with a 50-fold increase in IGFBP-5 concentration in milk, which is thought to inhibit IGF-I-mediated cell survival, thus releasing the cells from suppression of apoptosis (314). Furthermore, IGFBP-5 binds to PAI-1, effectively increasing the activation of plasminogen to plasmin, which then initiates degradation and remodeling of the ECM (315), processes involved in mammary gland involution.

The IGF axis plays an important role in the maintenance of the normal prostate as well as in the regression of both normal prostate and prostate tumors after androgen withdrawal or castration. Increased expression of IGFBP-5 in castration-induced and androgen withdrawal- or ablation-induced apoptosis in the prostate gland or in the involuting prostate is associated with reduced IGF-I activity (316–319), suggesting that IGFBP-5 was inhibiting the antiapoptotic function of IGF-I. Miyake *et al.* (320) recently demonstrated that although there was no difference in prostate-specific antigen levels, tumor incidence, or growth rates between control and IGFBP-5 overexpressing LNCaP cells grown in intact mice, prostate-specific antigen levels and tumor growth rates were increased in mice bearing IGFBP-5 overexpressing LnCaP cells after castration. This indicates that after castration, IGFBP-5 can accelerate the rate of progression of prostate cancer cells to androgen independence. This was supported by the observation that there was a delay in the recurrence of androgen-independent Shionogi tumors in mice treated with IGFBP-5 antisense oligonucleotide compared with mice treated with control oligonucleotide. In cell culture studies *in vitro*, the treatment of Shionogi tumor cells with IGFBP-5 antisense oligonucleotide resulted in inhibition of cell growth that was reversed in the presence of IGF-I, suggesting that the IGFBP-5 effect was IGF dependent.

It was previously shown that IGF-II promotes differentiation of myoblasts to myotubes and acts as a survival factor (321, 322) whereas IGFBP-5 blocked IGF-stimulated myogenesis (120). Treatment of C2 myoblasts with TNFα downregulated the secretion of IGF-II and IGFBP-5 and was associated with the blockade of myoblast differentiation and induction of apoptosis (323). However, TNFα was unable to induce apoptosis in C2 cells transfected with IGFBP-5, thus indicating that IGFBP-5 may have an antiapoptotic role in the survival of these cells during differentiation.

The regulation of ovarian follicle selection and atresia is another biological process that is thought to depend on the balance between IGF-I and IGFBPs. Studies in a variety of species indicate that follicle atresia involves an apoptotic process that may be blocked by IGF-I, whereas follicle development and selection requires IGF-I (324–326). IGFBP-2, -4, and -5 have all been implicated in the regulation of ovarian IGF-I availability, and there is some evidence that IGFBP-4 plays a key role. As noted in *Section II*, IGFBP-4 degradation is IGF-stimulated, and in the human ovarian follicle has been shown to be due to PAPP-A activity (101). Other IGFBPs might modulate this process by regulating IGF-I availability (327). IGFBP-4 gene expression appears confined to apoptotic and atretic follicles (325), whereas dominant follicles acquire an IGFBP-4 protease, which would enhance IGF-I action (326). In the rat, IGFBP-5 proteolysis may play a parallel role to that of IGFBP-4 proteolysis in other species (328, 329). Although the details appear to differ between species, these and similar studies emphasize the central role of IGFBPs in regulating ovarian development.

IGFBP-1 has been shown to stimulate cell detachment and apoptosis in breast cancer cells through dephosphorylation of FAK (Ref. 58; see *Section IV*). The expression of IGFBP-1 in the endometrium during the secretory phase of the menstrual cycle (330) and the regulation of cytotrophoblast invasion by IGFBP-1 (136, 137) suggests that IGFBP-1 may play a role in apoptosis and remodeling. However, this awaits further investigation.

Exposure of lung epithelial cells to hyperoxia leads to inhibition of cell division and subsequently induction of apoptosis via the Fas pathway which is associated with an increased expression of IGFBP-2 and IGFBP-3 (331). Although previous studies have shown that IGFBP-2 is upregulated in growth-arrested cells (332–334), it remains to be shown that IGFBP-2 plays a role in the induction of apoptosis. Interestingly, the increased expression of IGFBP-2 was predominantly found intracellularly and in the nucleus whereas IGFBP-3 was found in the extracellular compartment (331). There are few reports of nuclear localization of IGFBP-2, and it will be important to confirm this observation in other cell systems.

IGFBP-4, known to be IGF inhibitory, when overexpressed

in M12 prostate epithelial cells inhibits IGF-I-induced proliferation. Apoptosis induced by 6-hydroxyurea is increased in the IGFBP-4 transfectants, but IGFBP-2 levels are decreased concomitantly. When injected into nude mice there is a delay in the onset of tumor formation by IGFBP-4 transfectants compared with controls (221). Surprisingly, when the same cell line was transfected with antisense IGFBP-4, the effects were decreased cellular proliferation, decreased colony formation in soft agar, decreased tumor formation, and increased apoptosis induced by etoposide. This was accompanied by an increase in IGFBP-3 and IGFBP-6 expression (222). Therefore, the role of IGFBP-4 in apoptosis remains controversial and will require further study in isolation of the other IGFBPs.

Infection of NSCLC cell lines with an adenovirus expressing human IGFBP-6 reduced cell numbers by inducing apoptosis, the effects of which were not reversible by IGF-I or IGF-II (335). Moreover, treatment of the cells with exogenous IGFBP-6 did not result in inhibition of cell growth, suggesting that the IGFBP-6 effect is mediated by an intracellular form of IGFBP-6 through an IGF-independent mechanism. In contrast, IGFBP-6 stimulated cell growth and decreased apoptosis in the Saos-2/B-10 cell line (336).

Because of the central position occupied by the IGF-IGFBP system in cell growth regulation, it is inevitable that the full extent of the specific functions of IGFBPs in the determination of cell survival or death remains to be revealed. It is, however, clear that these proteins interact with proliferative and apoptotic processes at multiple levels and in different tissues.

VIII. Implications for Animal Physiology

The previous sections have highlighted many of the important studies, mostly conducted at the level of cell biology, that shed light on the complex array of cellular actions of the IGFBPs. Transposing knowledge of these cellular actions to the areas of animal and human physiology will present many challenges. Gene deletion experiments in mice have the potential to provide unique information pointing to cellular actions of IGFBPs, but to date IGFBP-2 is the only IGFBP for which a mouse knockout model has been published. In this model there was no overall growth phenotype, although spleen size was reduced and liver size increased (337). Notably, altered levels of other IGFBPs pointed to the possibility that the deletion of one IGFBP could cause compensatory changes in others, making the interpretation of such experiments, in terms of normal physiology, extremely complex.

A report of other IGFBP gene deletions has also appeared in abstract form. Although still preliminary, these data suggest that IGFBP-4 is the only IGFBP that, when deleted, alters growth. Surprisingly, the phenotype was a reduction in growth (338), even though IGFBP-4 is generally inhibitory to cell proliferation. No cellular mechanism for the growth inhibition is currently available, although this animal model appears to offer a novel opportunity to study previously unrecognized effects of IGFBP-4. Another preliminary observation was the delayed mammary gland involution in IGFBP-5 knockout mice, consistent with the known role of IGFBP-5 in mammary epithelial cell apoptosis (*Section VII*). These are intriguing observations, but unpublished at the time of writing, and clearly just the tip of the iceberg in the use of gene deletions to study IGFBP cellular function.

Gene overexpression *in vivo* may also provide information on cellular IGFBP actions, although ectopic expression has the potential to yield misleading results. Transgenic mouse models including overexpression of IGFBP-1 (339–342), -2 (148, 244), -3 (343–345), and -4 (346) have been reported. IGFBP-1 overexpression was found to have no effect (339), or in another study to cause up to 20% reduction (340), in somatic growth, whereas IGFBP-2 and its RGD-to-RGE mutant led to growth reduction (234, 244); and IGFBP-3 caused no overall growth phenotype in one study (343) and modest growth reduction in another (345). The variation in phenotype for IGFBP overexpression models in different laboratories, and the contrast between lack of a growth effect in IGFBP-2 knockout mice and the growth reduction in IGFBP-2-overexpressing mice, highlights the difficulty in drawing simple conclusions from such studies.

In general, transgenic studies published to date have not provided major new insights into cellular actions of the IGFBPs. An interesting exception is the decreased apoptosis in the involuting mammary gland described in a tissue-specific IGFBP-3-overexpressing model (344). This observation certainly raises questions about the proapoptotic effects of IGFBP-3 consistently noted in cultured breast cells (*Section VII*). Other studies have supported earlier hypotheses concerning IGFBP actions; for example, the observation that several IGFBP-1-overexpressing models show fasting hyperglycemia (342, 347) is consistent with the hypothesis that IGFBP-1 has a counterregulatory role in glucose homeostasis (348), although it is unclear whether this is primarily an endocrine or a cellular action of IGFBP-1.

In other cases, tissue-specific changes resulting from IGFBP overexpression may be interpreted as indicating unexpected roles for IGFBPs, such as the consistently seen negative effect of IGFBP-1 overexpression on brain growth and development (349–351). However, there may be insufficient information available to distinguish true physiological IGFBP actions from the more general consequences of high-level IGF sequestration. For example, the hypoplastic action of smooth muscle-specific IGFBP-4 overexpression (346) has interesting implications for muscle IGF action but does not necessarily delineate the role of IGFBP-4 in physiological muscle growth regulation. Further refinement in the temporal and spatial regulation of IGFBP expression, and in transgene responsiveness to physiological influences, as attempted in a recent IGFBP-1 transgenic model (342), may allow more definitive conclusions on IGFBP action to be drawn in the future.

IX. Implications for Human Disease

The use of serum measurements of IGFBPs is well established as an aid to the diagnosis or monitoring of growth disorders (352, 353) and is developing in relation to some other diseases (354, 355). In the context of predicting cancer risk, there has been considerable recent interest in reports

that a high serum IGFBP-3 level reduces the relative risk of developing breast cancer predicted by high IGF-I levels in premenopausal women (356) and also tends to attenuate prostate and lung cancer risk in patients with high IGF-I (357, 358). Low serum IGFBP-3 may also be associated with increased risk of colorectal cancer (359), although another prospective study of colorectal cancer found that high IGFBP-3 did not modify the risk related to high IGF-I, and patients in the highest quintile for IGFBP-3 actually had an increased risk (360). Other studies of breast (361), lung (362), and prostate (363) cancer risk also challenge the predictive value of serum IGFBP-3 levels. Correlative studies of IGFBP-3 levels in women with breast cancer show variable results, with decreased (364, 365), increased (366), and unchanged (367) IGFBP-3 levels reported.

There has also been some interest in the measurement of other serum IGFBPs in relation to cancer status or development. Increasing IGFBP-1 levels have been associated with a decrease in colorectal cancer risk (360), and in correlative studies, breast cancer patients have been shown in one study to have lower serum levels of IGFBP-1 and IGFBP-6 than women with benign breast disease (365). Elevated serum IGFBP-2 levels are also associated with malignancies of the prostate (368–370) and ovary (371).

It is not at all clear how changes in serum IGFBP levels reflect changes at the level of specific tissues, and, in contrast to the growing use of serum IGFBP measurement in cancer and other disease states, there are currently no accepted diagnostic or therapeutic applications that exploit knowledge of changes in IGFBPs at the cellular level. This is largely due to the relative paucity of data on the measurement and interpretation of IGFBP protein levels in tumor samples.

In general, high levels of IGFBP-3 in breast tumor specimens are associated with unfavorable prognosis (285, 372), despite the antiproliferative and proapoptotic activity of this protein in many *in vitro* studies. This suggests that some malignancies can escape from the growth-inhibitory effects of IGFBP-3, or even become stimulated by it, as demonstrated *in vitro* (60). If this transition, which is not yet understood mechanistically, occurs preferentially *in vivo*, due to the presence of factors not studied in cell culture systems, it is possible that some mechanisms delineated *in vitro* may have limited relevance to tumor growth *in vivo*. Therefore the association of tissue IGFBP-3 levels with relevant signaling intermediates and cell growth markers in patient tumor samples will be an important area for study.

Similarly, the high tissue IGFBP-2 levels associated with some cancerous tissues, such as ovarian (373) and colorectal (374), are consistent with its actions being growth stimulatory rather than inhibitory in these tumor types, but whether tumor IGFBP-2 measurement will provide any prognostic information is not known.

X. Concluding Comment

How might the limited clinical information on IGFBPs in tumors and in other diseased tissues be reconciled with the vast accumulation of *in vitro* studies on cellular actions of IGFBPs, and exploited to clinical advantage? It needs to be stressed that many of the potential "breakthrough" discoveries on IGFBP signaling pathways, and other cellular phenomena involving IGFBPs, still await confirmation in other laboratories and other cell systems. Even when confirmed *in vitro*, the links between cell culture studies and *in vivo* IGFBP actions are likely to be complex. A primary aim should be to increase our understanding of the relationship between inhibitory and stimulatory actions of IGFBPs on cell proliferation and migration, because these are known in some cases to differ between cell culture and *in vivo* situations. Delineation of the signaling pathways involved in these dichotomous effects may allow the development of targeted therapeutics to activate growth inhibition (*e.g.*, in cancer) or stimulation (*e.g.*, in tissue engineering). IGFBPs themselves might be exploited as active agents in tissue-directed gene therapy approaches, or IGFBP mimetics may be developed when their receptor systems are more fully understood. Because the IGF-IGFBP system is already recognized as central to processes of cell growth, differentiation, and migration, and the understanding of the cellular pathways mediating these effects is progressing, the prospect of clinical applications based on the IGFBPs seems increasingly likely.

Acknowledgments

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