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# The Insulin-Like Growth Factor-Binding Protein (IGFBP) Superfamily*

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## I. Introduction

THE insulin-like growth factor (IGF) system (Fig. 1) is well defined, with profound effects on the growth and differentiation of normal and malignant cells. The established components of the IGF system include IGFs (IGF-I and IGF-II), type I and type II IGF receptors, IGF-binding proteins (IGFBPs), and IGFBP proteases. IGF-I and IGF-II, which are structurally similar to insulin, are two highly homologous small hormone peptides of approximately 7 kDa molecular mass. First identified in 1957, they were originally named sulfation factors (1), nonsuppressible insulin-like activity (2), and multiplication-stimulating activity (3). They were renamed somatomedins (4) and subsequently IGFs (5). IGFs are ubiquitously expressed and are important mitogens that affect cell growth and metabolism. In addition to endocrine effects exerted by circulating IGFs (6, 7), locally produced IGFs exert paracrine, as well as autocrine, effects on cell proliferation (8–10). The IGFs interact with specific cell surface receptors, designated type I and type II IGF receptors, and can also interact with the insulin receptor. The mitogenic effects of IGF are mediated mainly through interactions with the type I IGF receptor, which, like the insulin receptor, is a receptor with tyrosine kinase activity. The type II IGF receptor is structurally distinct, binds primarily IGF-II, but also serves as a receptor for mannose-6-phosphate-containing ligands (11). The role(s) of the type II receptor in mediating IGF action is less well defined (12).

In biological fluids, IGFs are normally bound to IGFBPs. There are, at present, six well characterized mammalian IGFBPs, designated IGFBP-1 through -6. IGFBPs have higher affinities for IGFs ($k_d \sim 10^{-10}$ M) than do the type I IGF receptors ($k_d \sim 10^{-8}$–$10^{-9}$ M). Therefore, IGFBPs act not only as carriers of IGFs, thereby prolonging the half-life of the IGFs, but also function as modulators of IGF availability and activity (see review in Ref. 10). In the past several years, knowledge of the biological roles of IGFBPs has expanded, with a steady accumulation of data indicating that, in addition to modulating IGF bioactivity, IGFBPs are capable of important biological actions independent of their abilities to bind IGFs (13). Evidence implicates the direct association of IGFBPs with a variety of extracellular and cell surface molecules (14–16), with consequent effects upon important biological processes such as modulation of bone cell proliferation (17) and growth arrest of breast and prostate cancer cells (15, 18–20). There are numerous data, *in vitro* and *in vivo*, supporting the importance of IGFBPs for cell growth by both IGF-dependent and IGF-independent mechanisms.

Of particular interest is the recent discovery of several groups of cysteine-rich proteins with discrete, but striking, structural and functional similarities to the IGFBPs (21–24). This has led to the proposal of an IGFBP superfamily, comprised of the IGFBPs and these IGFBP-related proteins

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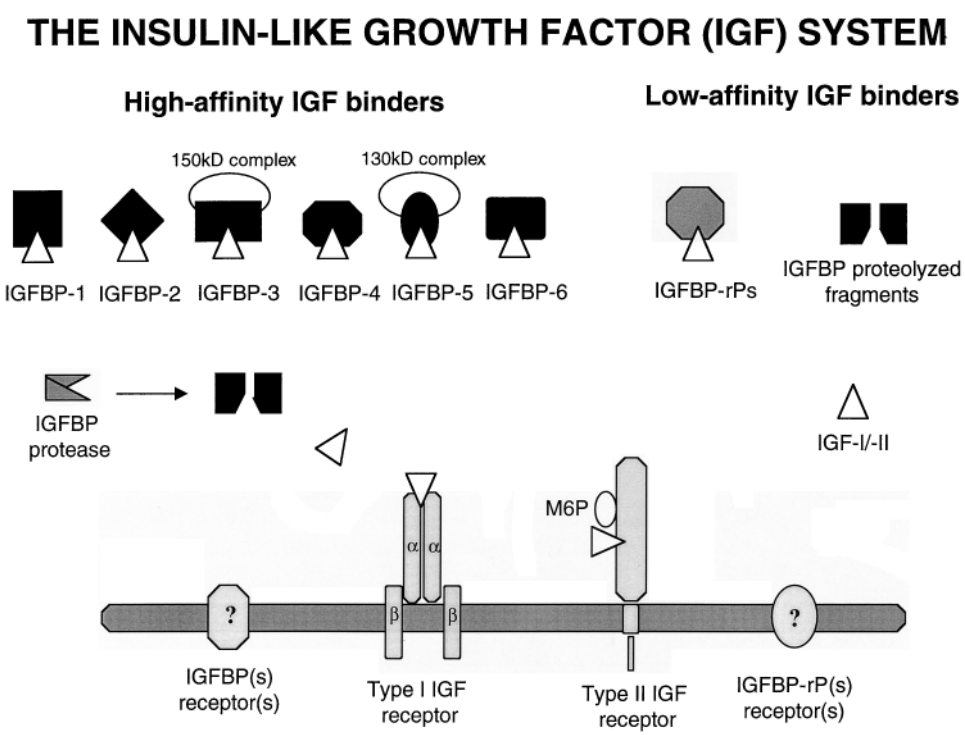


FIG. 1. The IGF system. The components of this system include the peptides IGF-I and -II, IGFBP-1 to -6, IGFBP-rPs, IGFBP proteases, type I and type II IGF receptors, and potential IGFBP(s) and IGFBP-rP(s) receptors. M6P, Mannose-6-phosphate.

(IGFBP-rPs) (23). Since several comprehensive reviews on IGFBPs are available (10, 25, 26), the present review will include only the most recent information on IGFBP structure and function and will focus on the IGFBP-rPs and their structural, functional, and evolutionary relationships with the conventional IGFBPs.

*A. Concept of an IGFBP superfamily*

First coined by Dayhoff in 1978, the term "superfamily" was used to discriminate between closely related and distantly related proteins (27). The relatedness of proteins was based solely on similarities between the primary protein structures, with amino acid similarities set arbitrarily at equal to or greater than 50% for closely related proteins (considered a family), and less than 50% for those more distantly related (considered a superfamily). With the wealth of information now available on protein structures from different organisms, the more acceptable classification of proteins into families and superfamilies is determined not only by amino acid similarities, but also by considering ancillary features such as tertiary structures (conformational similarities), functional similarities, and even tissue specificity (28). Furthermore, establishing relatedness among proteins requires that the evolutionary relationships be considered. A current definition of a superfamily, therefore, is a number of families who share some structural and functional characteristics that have been conserved through evolution. The list of superfamilies of genes is long, and includes the globins, collagens, actins, immunoglobulins, serine proteases, and, more recently, the transforming growth factor-$\beta$ and the nuclear receptor superfamilies.

The existence of proteins able to bind IGFs with high affinity had been suspected since the late 1960s [see review (29)]. The first IGFBP to be purified and its cDNA cloned was IGFBP-1 (30–35). Development of the Western ligand blot techniques, using $^{125}$I-IGF ligands to probe for proteins immobilized on nitrocellulose filters (36) facilitated elucidation of the IGFBPs. By 1991, six IGFBPs (IGFBP-1 to IGFBP-6) demonstrating high IGF binding affinity had been identified from a variety of biological fluids, mammalian and nonmammalian, and, in many cases, their respective cDNAs and genes had been cloned and characterized. The structural characteristics of the human IGFBPs are summarized in Table 1.

The ease with which IGFBPs are detected by ligand blotting techniques has, inadvertently, limited the IGFBPs identified to those that bind IGFs with high affinity. Peptides with lower affinities for IGFs, such as proteolysed IGFBP fragments and the "new" IGFBP-rPs, would not be readily detected using this technique. Thus, IGFBP-rPs were discovered and characterized in systems not involving the IGF axis, and only in the past few years were realized to be related to the IGFBPs through amino acid sequence similarities.

The discovery of cysteine-rich proteins sharing similarities with the IGFBPs led to the proposal of a new superfamily, an IGFBP superfamily (23), an hypothesis consistent with the current definition for a superfamily. The six established IGFBPs were classified as a family based on two key features. First, the IGFBPs are cysteine-rich proteins (16–20 cysteines in the pre-peptides) sharing high similarity in their primary amino acid sequences. Structurally, the cysteines are clustered at the conserved N-terminal third (12 cysteines in IGFBP-1 to -5; 10 in IGFBP-6) and at the conserved C-terminal third (6 cysteines) of the proteins (Fig. 2). The N and C domains are separated by a midregion of little similarity among the IGFBPs. The second key feature of the IGFBPs is their unique ability to bind IGFs with high affinity, presumably as a result of the N and C domains forming the correct

TABLE 1. Characteristics of the human IGFBP superfamily

| | Molecular mass (kDa)[a] | No. of amino acids[b] | No. of cysteines[c] | Glycosylation (N- or O-) | Chromosomal localization | mRNA size (kb) | Gene size (kb) | No. of exons | Exon encoding N terminus |
|---|---|---|---|---|---|---|---|---|---|
| IGFBP: high-affinity IGF binder | | | | | | | | | |
| IGFBP-1 | 25.3 | 234 | 18 | | 7p | 1.6 | 5.2 | 4 | 1 |
| IGFBP-2 | 31.4 | 289 | 18 | | 2q | 1.5 | 32.0 | 4 | 1 |
| IGFBP-3 | 28.7 | 264 | 18 | N | 7p | 2.4 | 8.9 | 5 | 1 |
| IGFBP-4 | 26.0 | 237 | 20 | N | 17q | 1.7 | 15.3 | 4 | 1 |
| IGFBP-5 | 28.6 | 252 | 18 | O | 2q | 1.7, 6.0 | 33.0 | 4 | 1 |
| IGFBP-6 | 22.8 | 216 | 16 | O | 12 | 1.1 | nd[d] | nd | nd |
| IGFBP-related protein:[e] low affinity IGF binder | | | | | | | | | |
| IGFBP-rP1 | 26.4 | 256 | 18 | N | 4q | 1.1 | >30 | 5 | 1 |
| IGFBP-rP2 | 35.5 | 323 | 38 | N | 6q | 2.4 | nd | 5 | 2 |
| IGFBP-rP3 | 36.0 | 329 | 38 | N | 8q | 2.4 | ~7 | 5 | 2 |
| IGFBP-rP4 | 39.5 | 358 | 38 | nd | 1p | 2.5, 4.0 | nd | nd | nd |
| IGFBP-rP5 | 49.0 | 458 | 16 | nd | nd | 2.3 | nd | nd | nd |
| IGFBP-rP6 | 18.1 | 165 | 18 | nd | nd | 2.2 | nd | nd | nd |
| IGFBP-rP7 | 24.4 | 228 | 28 | | 20q | nd | nd | nd | nd |
| IGFBP-rP8 | 38.0 | 345 | 38 | N | 8q | nd | nd | nd | nd |
| IGFBP-rP9 | 37.1 | 334 | 34 | N | 6q | nd | nd | nd | nd |

[a] Predicted molecular mass (kDa) of nonglycosylated, mature, protein.
[b] Number of amino acids of mature protein.
[c] Number of cysteines in mature protein.
[d] nd, Not determined.
[e] Nomenclature for the IGFBP-rPs: IGFBP-rP1, Mac25/TAF/PSF/IGFBP-7; IGFBP-rP2, CTGF; IGFBP-rP3, NovH; IGFBP-rP4, Cyr61; IGFBP-rP5, L56/HtrA; IGFBP-rP6, ESM-1; IGFBP-rP7, WISP-2/CTGF-L; IGFBP-rP8, WISP-1; IGFBP-rP9, WISP-3.

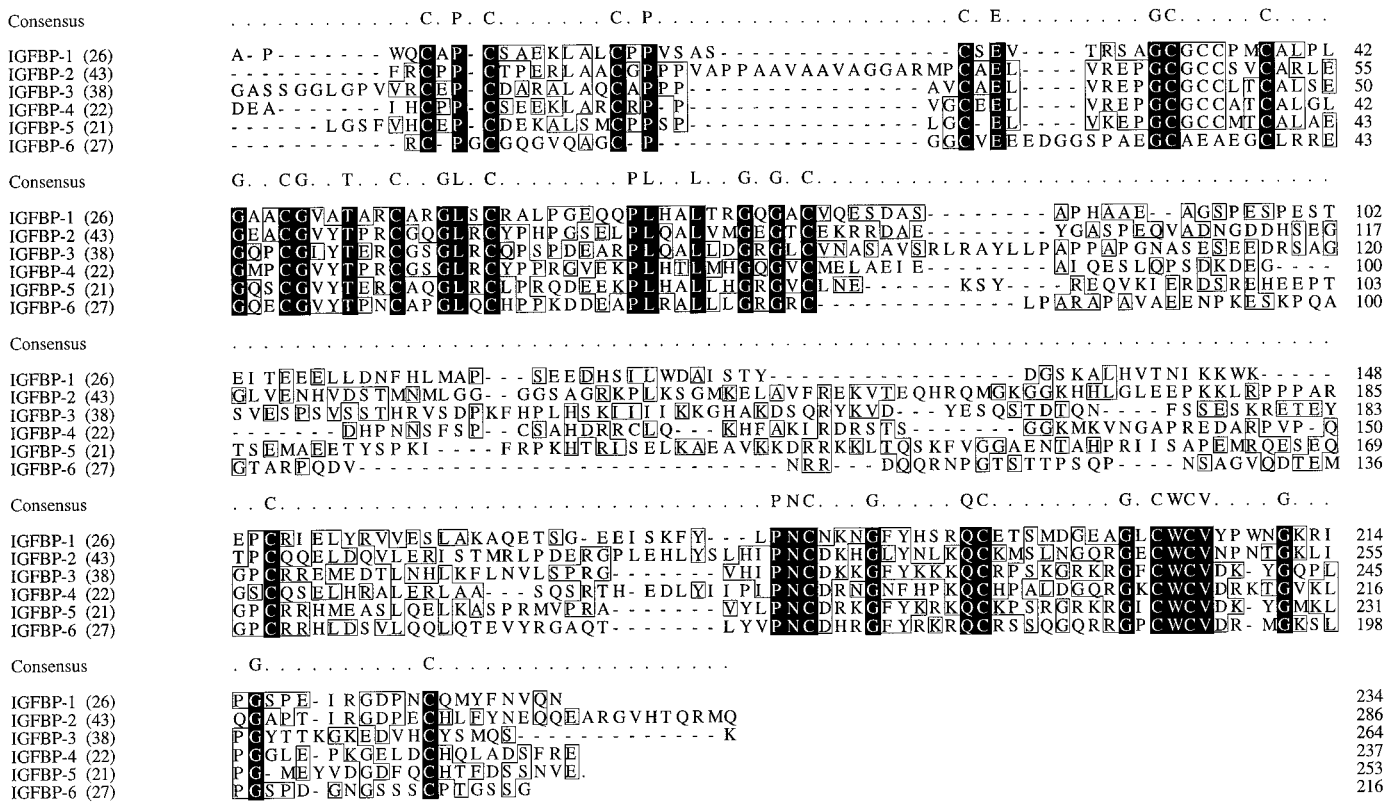


FIG. 2. Amino acid sequence alignment of human IGFBP-1 to -6. The signal peptides (number of amino acid residues are indicated in *brackets*) were not included in the analysis. The numbering system (*left* of the figure) is that of the mature peptide. Alignment was performed using the Clustral method in the DNA STAR program. Small gaps were introduced to optimize alignment. Consensus amino acid residues are as indicated (*shaded solid black*); *boxed* amino acid residues indicate matched residues.

tertiary configuration for high-affinity IGF binding. These two criteria, used to distinguish and classify conventional IGFBPs, were recently reevaluated in light of the identification of additional cysteine-rich proteins that share structural similarities with the IGFBPs: they carry the N-terminal domain of IGFBPs, but deviate from the common IGFBP struc-

ture in the midregion and C terminus. Functionally, at least four of these proteins are able to bind IGFs in *in vitro* assays, albeit at 100-fold or lower affinity than that observed with IGFBPs (22, 23, 37–39). These results, thus, substantiated that these proteins, while not falling into the classical definition of IGFBPs, are certainly related to the IGFBPs.

It was further proposed that this new IGFBP superfamily be subgrouped by their ability to bind IGFs, into those that bind IGFs with high-affinity (IGFBP-1 to -6), and those that bind IGFs with low-affinity (the IGFBP-rPs).

### *B. Superfamily nomenclature*

The various IGFBP-rPs were discovered by a number of different groups and designated accordingly (Table 2). Although both mammalian and nonmammalian IGFBP-rPs have been described, only the human IGFBP-rPs are presented in Table 2. At present, there are four proteins/families that are related to the IGFBPs. Mac25 was originally identified as a cDNA derived from leptomeninges (40); the *mac25* cDNA was subsequently expressed in a baculovirus system, and the synthesized protein was shown to bind IGFs and was renamed IGFBP-7 (22). Independently, the same protein has been purified from human diploid fibroblast cells and named prostacyclin-stimulating factor [PSF (41)] and from human bladder carcinoma cells [tumor adhesion factor (TAF) (42)]. The CCN family of proteins consists of a human growth factor-inducible, immediate-early gene [*cyr61* (43), connective tissue growth factor (CTGF) (44), and the human nephroblastoma overexpression gene (*novH*) (45)]. Recently, three new members of the CCN family have been identified in Wnt-1 (cysteine-rich glycosylated signaling proteins that are oncogenic) transformed cells: WISP-2 (46) and its rat counterpart, rCop-1 (47); WISP-1 (46) and the mouse orthologue, Elm-1 (48); and WISP-3 (46). Independently, WISP-2 was identified in primary human osteoblast cells and designated CTGF-L [CTGF-like (39)]. Two other proteins related to the IGFBPs are L56 (49), also named HtrA (50), a potential serine protease of IGFBPs, and endothelial cell-specific molecule, ESM-1 (51).

The physiological role of these IGFBP-rPs in the IGF system remains undefined at this time. Nevertheless, their structural relationship with IGFBP-1 to -6, although limited, is unequivocal, and the ability of some of these proteins to bind IGFs has been established (22, 23, 37–39). The nomenclature options that are currently being considered for the newly identified genes and proteins are summarized in Table 2 and include 1) retention of the multiple published names; 2) naming the proteins IGFBP-7 through -12 (and higher); and 3) naming the proteins IGFBP-rPs (IGFBP-rP)-1 to -6 (and higher). The latter nomenclature is recommended (52) since these IGFBP-rPs do not fall into the conventional definition of IGFBPs, and this nomenclature will be employed in this review.

The IGFBP-rPs will be discussed in depth in *Sections III–VI*. Their structural and functional relationships with the IGFBPs will be presented in *Sections VII* and *VIII*, respectively.

## II. IGFBP Family

Since the advent of the Western ligand blot technique (36), biological fluids from numerous systems have been studied for the presence of IGFBPs. In mammalian species (human, mouse, rat, bovine, and, more recently, water buffalo), IGFBP-1 through -6 have been well documented, their expression and regulation studied, their proteins purified, and their cDNAs and genes cloned and characterized (10, 25, 26, 53). In nonmammalian species, IGFBPs have been described as well, but are less well characterized. The cDNAs for IGFBP-2 and -5 from chicken have been cloned (54, 55), as is a partial *Xenopus* IGFBP-5 sequence (56). IGFBPs, as detected by Western ligand blot, are present in the serum of reptiles, such as crocodile (*Crocodilian johnsonii*), lizard (*Tiliqua rugosus*), snake (*Notechis scutatus*), tortoise (*Chelodina longicollis* and *Emydura macccquarii*), and tuatara (*Sphenodon punctatus*) (57). Whether these reptilian IGFBPs correlate to any mammalian IGFBPs is unknown, but since they are readily detectable by the Western ligand blot technique, these proteins probably have high affinities for IGFs, and, therefore, are likely to be structurally similar to mammalian IGFBPs (57). IGFBPs with molecular weights similar to, and similarly regulated as, the mammalian counterparts have been detected in bony fish [see review (58)]. Even the serum from the agnathan lamprey contains IGFBPs, as detected by ligand

TABLE 2. Proposed nomenclature for the (human) IGFBP-related proteins

| Published names | IGFBP name | IGFBP-related protein |
|---|---|---|
| Mac25 | | |
| Mac25 (40) | IGFBP-7 (provisional) (22) | IGFBP-rP1 |
| Tumor adhesion factor, TAF (42) | | |
| Prostacyclin stimulating factor, PSF (41) | | |
| CCN family | | |
| Connective tissue growth factor, CTGF (44) | IGFBP-8 (provisional) (23) | IGFBP-rP2 |
| Nephroblastomas overexpression gene, NovH (45) | IGFBP-9 (provisional) | IGFBP-rP3 (provisional) |
| Cyr61 (43) | IGFBP-10 (provisional) | IGFBP-rP4 (provisional) |
| WISP-2 (46); CTGF-L (39) | | IGFBP-rP7 (provisional) |
| WISP-1 (46) | | IGFBP-rP8 (provisional) |
| WISP-3 (46) | | IGFBP-rP9 (provisional) |
| L56 | | |
| L56 (49) | | IGFBP-rP5 (provisional) |
| HtrA (50) | | |
| ESM-1 | | |
| Endothelial cell-specific molecule, ESM-1 (51) | | IGFBP-rP6 (provisional) |

blot (57). Again, the structures of these ancient IGFBPs are unknown, but, like the reptilian IGFBPs, they presumably are structurally similar to the mammalian IGFBPs.

In this section, an analysis of IGFBP structure is summarized, based on information gathered from studies with mammalian IGFBPs. Correlation between IGFBP structure and function at the molecular level has only recently begun to be elucidated. Particular attention has been paid to the unique attributes of the N-terminal domain, alone and in combination with other IGFBP domains, since it is this portion of the molecule that is conserved exclusively in the IGFBP-rPs.

### *A. Structure of IGFBPs*

The primary structures of mammalian IGFBPs appear to contain three distinct domains of roughly equivalent sizes: the conserved N-terminal domain, the highly variable midregion, and the conserved C-terminal domain. Alignment [Clustral method (59), DNA Star program] of the human IGFBPs indicates that, overall, the human IGFBPs share approximately 36% similarity (defined as "the direct comparisons of amino acid sequence without accounting for phylogenetic relationships", DNA Star), although as presented below, alignment of the conserved N and C domains shows significantly higher similarities. Between mammalian species, each IGFBP is highly conserved.

*1. N-terminal domain.* In the mature IGFBP peptides, the N terminus third of the proteins contains 80–93 amino acid residues after the signal peptides (Fig. 2) and shares approximately 58% similarity. Ten to 12 of the 16–20 cysteines found in the prepeptides are located within this domain. In IGFBP-1 to -5, these 12 cysteines are fully conserved, whereas in IGFBP-6, 10 of the 12 cysteines are invariant. Interestingly, rat IGFBP-6 is missing an additional 2 cysteines (the first 2 cysteines in the primary sequence) in the N-terminal domain (60). The high number of cysteines within such a small domain suggests that this domain is highly structured, with a maximum of 6 disulfide bonds formed (5 in the case of IGFBP-6). The even number of cysteines suggests that intradomain disulfide bond formation is more likely than interdomain disulfide linkages with cysteines in the C-terminal domain. Indeed, two recent studies have provided data supporting the hypothesis that the N-terminal and C-terminal domains are not linked by disulfide linkages (61, 62). Furthermore, analysis of tryptic digested fragments of human IGFBP-6 indicated that the native disulfide linkages in IGFBP-6 occur between cysteines that are close together in the primary sequence, forming sequential subdomains with at least 2 disulfide-linked subdomains in the N-terminal domain (61, 63). In the N-terminal domain, the first 6 cysteines form the first subdomain, and the next 4 cysteines form the second subdomain. Interestingly, in rat IGFBP-3 (64) and human IGFBP-5 (65), it was similarly demonstrated that the last 4 cysteines of the N-terminal domain formed overlapping disulfide linkages ($Cys^{56}$-$Cys^{69}$ and $C^{63}$-$C^{89}$ for rat IGFBP-3 (64), consistent with the second subdomain structure proposed by Neumann *et al.* (61). These results have been recently supported by nuclear magnetic resonance (NMR) spectroscopy of this subdomain from human IGFBP-5, which shows a rigid, globular structure stabilized by the two disulfide bridges (65).

Within the N-terminal domain, a local motif (GCGCCxxC) is well conserved among the IGFBPs. The exception is in IGFBP-6, which substitutes a GCAEAEGC sequence, thereby accounting for the two "missing" cysteines in IGFBP-6. The significance of this motif is as yet unknown, but, as indicated below, is also highly conserved in the IGFBP-rPs. One hypothesis is that the motif may be important in interactions with IGFs. However, IGFBP-6 has retained the high affinity for IGFs (in particular IGF-II), although the internal GCC amino acids are replaced with AEA. A search of the protein data bank (BLAST search, SwissProt) revealed several other proteins containing the GCGCCxxC motif, including a cysteine-rich protein found in the eggshell of the silk moth [chorion protein (66)]. Variants of this motif are also found in GP40, a small undefined protein found in Mycobacteriophage 15 (67), in the peplomer protein [a viral spike glycoprotein (68)], and in metallothionein-like protein 1 (69). The role(s) of the GCGCCxxC motif, or its close variants, are not known. Clearly, the GCGCCxxC motif appears to be highly conserved in, but not unique to, IGFBPs.

*2. Midregion.* For the human IGFBPs, the midprotein segment, ranging in size from 55 amino acid residues to 95 amino acids, separates the N-terminal domain from the C-terminal domain. The amino acid sequence for each midsegment appears to be unique to the protein, with shared similarity less than 15%. The belief is that this region acts structurally as a hinge between the N- and C-terminal domains.

Intriguingly, posttranslational modifications (glycosylation, phosphorylation) of the IGFBPs have been found so far in the midregion, but not in the N- or C-terminal domains. There has been no clear evidence to date that IGFBP-1 or -2 are glycosylated (an early paper suggests that IGFBP-1 was glycosylated (70), whereas IGFBP-3 and -4 are N-glycosylated, and IGFBP-5 and -6 are O-glycosylated. N-glycosylation occurs only on an asparagine that is part of the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline. Consistent with this prediction, three N-glycosylation sites in the mature protein, $Asn^{89}$, $Asn^{109}$ and $Asn^{172}$ (corresponding to prepeptide, $Asn^{116}$, $Asn^{136}$, and $Asn^{199}$) have been characterized in IGFBP-3 (71), and one, $Asn^{104}$ ($Asn^{125}$ of prepeptide), in IGFBP-4 (72). Although there is one potential N-glycosylation site in the C terminus of IGFBP-6, this site does not appear to be glycosylated (73). In contrast to the N-glycosylation sites, there are no consensus sequences for predicting O-glycosylation sites. Nevertheless, it has been demonstrated that IGFBP-5 (74) and IGFBP-6 (61, 73) are both O-glycosylated. Very recently, the O-glycosylation sites in human IGFBP-6 were determined to be within the midregion at 5 residues, $Thr^{126}$, $Ser^{144}$, $Thr^{145}$, $Thr^{146}$, and $Ser^{152}$ (61). The O-glycosylation states of other mammalian IGFBP-6 indicate that in the rat, mouse (75, 76), and pig (53) IGFBP-6 is glycosylated to a lesser extent than human IGFBP-6 (61); bovine IGFBP-6 may be similarly glycosylated as human IGFBP-6. The ability to bind IGFs with high affinity appears not be influenced by N- or O-glycosylation, although

there may be effects on other function(s) of IGFBPs, such as resistance to proteolysis (61).

Three of the six IGFBPs, IGFBP-1, -3, and -5, are posttranslationally modified by phosphorylation (77). Phosphorylation of proteins is an important and critical posttranslational modification mechanism that is used by cells to stringently regulate the activities of numerous intracellular proteins, including proteins involved in the signal transduction pathways, in the cell cycle, and in gene expression. The purpose of phosphorylating secreted proteins like IGFBPs is unclear, but there is evidence that, at least for human IGFBP-1, phosphorylation enhances the affinity of hIGFBP-1 for IGFs by 5-fold (78, 79). Phosphorylation in all three IGFBPs is predominantly at serine residues found in the midregion of the IGFBPs. Phosphorylation of hIGFBP-1, first described by Frost and Tseng (80) and by Jones *et al.* (78), is at three serine residues of the mature peptide: $Ser^{101}$ and $Ser^{119}$, both in the midregion of the protein, and $Ser^{169}$ (located at the amino-terminal end of the hIGFBP-1 C-terminal domain) (81). Frost and Tseng (80) demonstrated that, under *in vitro* conditions, casein kinase II and cAMP-dependent protein kinase are able to phosphorylate IGFBP-1. Recently, phosphorylated rat IGFBP-1 was described (82). Unlike hIGFBP-1, however, only two serine residues were phosphorylated ($Ser^{107}$ and $Ser^{132}$ in the nonconserved midregion), and more importantly, phosphorylation did not appear to affect IGF binding.

The phosphorylation status of human IGFBP-3 was analyzed by Hoeck and Mukku (83), who showed that $Ser^{111}$ and $Ser^{113}$ ($Ser^{138}$ and $Ser^{140}$ of prepeptide), which are within the consensus sequences for protein kinase CKII, were phosphorylated. Both serines are also in close proximity to one of the N-glycosylation sites, $Asn^{109}$ ($Asn^{136}$ of prepeptide). Further, they demonstrated that phosphorylation did not appear to affect IGF binding by IGFBP-3. Interestingly, phosphorylation of IGFBP-3 can be up-regulated by IGFs, through a mechanism involving IGF-I-type I IGF receptor interaction (84). The significance of phosphorylated IGFBP-3, and the significance of IGF regulation of phosphorylation, are unclear but may affect IGFBP-3 interactions with acid-labile subunit (ALS) or with the cell surface (84).

Evidence for the phosphorylation of IGFBP-5 at serine and threonine residues is limited to one report (85), although, like all the IGFBPs, there are several potential phosphorylation sites (84). The biological significance of IGFBP-5 phosphorylation is unknown.

*3. C-terminal region.* The C-termini of IGFBPs, like the N-terminal domain, are highly conserved and, among the human IGFBPs, share a similarity of approximately 34%. The remaining 6 cysteines of the total 16–20 cysteines are found in the C terminus and are strictly conserved (Fig. 2). Evidence from two independent studies indicated that the 6 cysteines are involved in intradomain disulfide bond formation (58, 59, 63). Neumann and Bach (63), in their studies of human IGFBP-6, and Forbes *et al.* (62), in their studies of bovine IGFBP-2, deduced that the disulfide bonding pattern of the C-terminal region was between adjacent cysteines.

The primary sequence of all members of the IGFBP family surrounding the last 5 cysteines is strikingly similar (~40%), implying that the tertiary structure of the C-terminal domain should be almost identical. Interestingly, the amino acid sequences encompassing these last 5 cysteines share 37% similarity with the thyroglobulin-type-I domain (86). The thyroglobulin-type I domain consists of about 65 amino acid residues, which are repeated 10 times in the N-terminal part of thyroglobulin (86). Its function(s) is unknown, but the domain is found in a number of proteins with varying physiological functions in different organisms (87). These include the major histocompatibility complex class II-associated p41 invariant chain fragment (88), nidogen (89), saxiphilin (90,91), a tumor-associated cell surface antigen known also as GA733 (92), a cysteine protease inhibitor from the egg of Chum salmon (93), equistatin, a new inhibitor of cysteine proteinases from sea anemones (94), and entactin-2, a new basement membrane protein (95). In IGFBPs, the role of this domain has yet to be determined, but is likely to affect binding to IGFs, and perhaps participate in the binding of IGFBPs to cell surfaces and/or to the extracellular matrix (ECM) proteins via heparin-binding sites. Consistent with this is the observation that IGFBP-1 and -2 contain RGD motifs, which are known to be involved in binding integrins (96). Similarly, heparin binding motifs (xBBBxxBx, where B is a basic residue, Arg, Lys, or His, and X is any residue) are found within the C-terminal domains of IGFBP-3, -5, and -6, and, for IGFBP-3 and -5, are involved in binding to cell surface and/or the ECM (16, 97–100).

### *B. Correlations between structure and function*

*1. IGF-IGFBP interactions.* The IGFBPs were so designated because of their abilities to bind IGFs with high affinity ($K_d$ $\sim 10^{-10}$ M). However, the precise molecular interactions between IGFBPs and IGFs are still unclear. It has also become apparent that IGFBPs can interact with proteins other than IGFs, including the ALS from serum, insulin, components of the cell surface, ECM proteins, and, potentially, intracellular components. These additional interactions may result in biological consequences not directly related to IGF action. Correlations between IGFBP structure and function have recently begun to emerge and will be summarized here.

It is worth noting here that the methods used to detect and study IGF-IGFBP interactions have become increasingly sensitive, permitting better assessment of both high and low IGF-affinity binding. Although the ligand blotting technique is the preferred and most commonly used method for detecting IGF-IGFBP interactions, more sensitive methods include affinity cross-linking assays, charcoal solution binding, solid-phase binding, and, recently, BIACORE analysis. Since the methods used vary among research groups, discrepancies in IGF-binding affinities among different studies are likely to arise, and it is often not obvious whether such differences reflect technical variations or underlying biology.

The high-affinity binding of IGFs by IGFBPs has long been hypothesized to involve interactions between the conserved N-terminal and C-terminal domains. Support for this hypothesis initially came from *in vivo* observations that in biological fluids, IGFBPs can be proteolysed resulting in diminished affinities for IGFs. Proteolysis was, thus, proposed as a mechanism for modulating IGF bioavailability (101–105). Proteolysis of IGFBPs was first observed in serum from

pregnant women, where it was demonstrated that IGFBP-3 was proteolytically cleaved to yield a predominant 29–30 kDa form that was still capable of binding IGFs, but with reduced affinity (101, 106). Since those observations, proteolysis of IGFBP-2 to -6 has been described in numerous studies of various biological fluids from different organisms, generating IGFBP fragments with decreased or no apparent affinity for IGFs.

*In vitro* generation of IGFBP fragments by limited proteolysis supports the *in vivo* data. For example, limited proteolysis of recombinant human IGFBP-3 (nonglycosylated and glycosylated) with the serine protease prostate-specific antigen (PSA) (102,107) or with plasmin (108, 109) generated a 22/25 kDa fragment with weak affinity for IGFs (residues 1–160) and a 16-kDa fragment (residues 1–95 that includes the N-terminal domain) with no detectable affinity for IGFs by affinity cross-linking assays. Vorwerk *et al.* similarly generated an approximately 15-kDa plasmin-digested IGFBP-3 fragment, corresponding to the N-terminal domain and part of the midregion (amino acid residues 1–97), capable of weakly binding to IGF-I, and detectable by both Western ligand blot and affinity cross-linking assays (110). Proteolytically modified IGFBP-4 generated a 16-kDa fragment that also could be affinity cross-linked specifically to IGF-I and IGF-II, although with a 20-fold lower affinity compared with intact IGFBP-4 (111). This 16-kDa IGFBP-4 fragment, like the 16-kDa N terminus IGFBP-3 fragment, corresponds to the N-terminal region and a small portion of the midregion (112). Similarly, a 23-kDa IGFBP-5 fragment from osteoblast-like cells that is carboxy truncated (113, 114) and a 10-kDa fragment (residues 1–94 of mature peptide) corresponding to the N-terminal fragment of endoproteinase Asp N-digested IGFBP-5 (65), demonstrated decreased binding affinity for IGF-I and IGF-II. Thus it appears that the N-terminal domain, with perhaps part of the midregion, can bind IGFs, but high-affinity IGF-binding also requires the added presence of the C-terminal domain.

The ability of IGFBP proteolytic fragments to bind IGFs, albeit with reduced affinity, has been further investigated using *in vitro* generated recombinant IGFBP peptides. Spencer and Chan (115) generated IGFBP-3 fragments that essentially corresponded to the N-terminal half (residues 1–147) and the C-terminal half (residues 151–263) of the IGFBP and showed that each of these fragments bound IGFs, but with less affinity than intact IGFBPs. Only a handful of studies have examined in depth the ability of the N-terminal domain to interact with IGFs, and results have been mixed. Baxter and Firth synthesized IGFBP-3 fragments that correlated to the N-terminal domain, the N-terminal domain plus the midregion, and a mutant IGFBP-3 in which the midregion was deleted (116). None of the fragments, however, was detectable by ligand blotting, although recent data indicate the N-terminal domain bound $^{125}$I-labeled IGF-II in solution binding assays (117). In contrast, recombinant N-terminal domain fragments generated by Yamanaka *et al.* (118) and by Vorwerk *et al.* (110), were detectable by ligand blotting, as well as by the more sensitive affinity cross-linking assay.

Further delineation of specific regions and subdomains of IGFBPs involved in IGF binding has come from limited chimera studies, as well as regional mutagenesis of the N and C domains. In the IGFBP N-terminal domain, the conserved GCGCCxxC motif was thought to be important for interactions with IGFs. The fact that the motif is incompletely conserved in IGFBP-6 (GCAEAxxC), however, would suggest that the role of this motif in the binding of IGFs may be subtle, or that there may be other explanations for its conservation in the IGFBPs and IGFBP-rPs.

In a recent study of human IGFBP-4 deletion mutants, Qin *et al.* (119) concluded that Leu$^{72}$-Ser$^{91}$ is important for IGF-II binding, as deletion of this region rendered the N-terminal peptide undetectable by ligand blot. Further, within this segment, a structural disruption generated by a His$^{74}$ (a basic amino acid conserved in IGFBP-4 from different species, but not conserved among the IGFBPs) to Pro$^{74}$ point mutation reduced the affinity of full-length IGFBP-4 for IGF-II by 50-fold. The N-terminal domain of rat IGFBP-3 demonstrated a reduction in IGF-II binding to less than 12% relative to full-length IGFBP-3 as determined by a sensitive solid-phase binding assay (64). A smaller fragment of the rat IGFBP-3 corresponding to the last 4 cysteines of the N-terminal domain [*i.e.*, the second N-terminal subdomain described by Neumann *et al.* (61) and Kalus *et al.* (65)] dramatically reduced IGF-II binding by four-logs. The same subdomain in recombinant human IGFBP-5 demonstrated 10- to 200-fold reduced affinity for IGFs by BIACORE analysis (65). The remaining N-terminal region, *i.e.*, the N-terminal subdomain encompassing the first 6 (IGFBP-6) or 8 cysteines (IGFBP-1–5), has not been tested for IGF affinity. Interestingly, Hobba *et al.* (120, 121) showed that in bovine IGFBP-2, Tyr$^{60}$, which is within the second subdomain and highly conserved among the IGFBPs and across species, substitution by Ala$^{60}$ or Phe$^{60}$ reduced, but did not abolish, affinity for IGF-I (4-fold and 8.4-fold, respectively) and for IGF-II (3.5-fold and 4-fold, respectively). These results were consistent by both charcoal binding assays and BIACORE analysis (120, 121). Mutations of adjacent residues, which are well conserved, did not reduce affinity. From these results, it can be deduced that Tyr$^{60}$ is probably one of the many contact points with IGFs. This is supported by NMR studies of IGFBP-5-IGF-II complexes, in which the analogous Tyr (Tyr$^{50}$) is proposed to interact with IGF-II, as are residues Val$^{49}$, Pro$^{62}$, and Lys$^{68}$-Leu$^{74}$ (65). Based on this handful of studies, it can be inferred that the two N-terminal subdomains proposed by Neumann *et al.* (61) are important for the integrity of the (partial) IGF binding pocket. Further studies are required to elucidate the precise points of contact with IGFs, which may vary from IGFBP to IGFBP.

The C-terminal domain of IGFBPs, without question, is essential for high affinity IGF binding, although more data are available regarding their non-IGF binding properties than their IGF binding characteristics (see below). Chimeras constructed between rat IGFBP-3 and IGFBP-2 indicated, not surprisingly, that the C-terminal domain from IGFBP-3 can be exchanged for the C-terminal domain of IGFBP-2 with no loss of IGF-II binding (64). However, replacement of the IGFBP-3 midregion with the IGFBP-2 midregion reduced the relative affinity of the resultant chimera for IGF-II by at least 37%, suggesting that the midregion of each IGFBP may maximize high-affinity IGF binding by the specific IGFBP.

Mutagenesis of the carboxy end of the IGFBP-1 cDNA

(122) showed that deletion of the C-terminal 20 amino acids resulted in loss of IGF binding by ligand blotting. In contrast to IGFBP-1, deletion of a similar region in human IGFBP-4 (C-terminal $Lys^{215}$-$Glu^{237}$) had no effect on relative IGF binding (bands in ligand blot assessed by radioactivity), but an additional deletion of 10 amino acids (removal included the highly conserved Cys-Trp-Cys-Val motif) reduced relative binding to less than 15% of wild-type IGFBP-4 (119). Similar sequential C-terminal deletion studies, with recombinant bovine IGFBP-2 and using charcoal-binding assays, suggested that loss of the region spanning the last four cysteines reduced IGF binding (62). In particular, results suggest that residues $Lys^{222}$–$Asn^{236}$ may be in close proximity to the N-terminal domain, to allow both domains to interact with IGF. Consistent with this proposal, recent site-specific mutagenesis of the strictly conserved amino acids, $Gly^{203}$ or $Gln^{209}$, within the corresponding region in rat IGFBP-5, reduced IGF-I binding affinity by 8- and 6-fold, respectively (123). In contrast, mutagenesis of adjacent basic amino acid residues in the equivalent region of human IGFBP-5 (amino acids 201–218) did not alter IGF-I binding affinity, although ability to interact with ECM was affected (see below and Ref. 124). The IGF binding properties of the C-terminal domain, itself, have yet to be tested thoroughly, although a recent study indicated that a natural C-terminal fragment of human IGFBP-2 retained partial IGF-binding activity (125). This observation is consistent with an earlier study where it was observed that a proteolyzed rat IGFBP-2 fragment containing half the midregion and the C-terminal domain showed similar reduced IGF binding compared with full-length rat IGFBP-2 (126). In contrast, a synthetic peptide corresponding to half the midregion and C-terminal domain of IGFBP-4 (as defined in this review; see Fig. 2), $His^{121}$-$Glu^{237}$, did not show detectable IGF binding (radioactivity in the bands from ligand blots were quantitated) (119); neither did an analogous C-terminal peptide, $Asp^{135}$-$Phe^{246}$, from IGFBP-5 demonstrate IGF binding (65). A comparable region in IGFBP-3, on the other hand, had demonstrable IGF binding capabilities by solution assays (115) and by ligand blot (G. R. Devi, D.-H. Yang, R. G. Rosenfeld, and Y. Oh, unpublished).

The midregion of the IGFBPs does not appear to bind IGFs; its contribution to the high-affinity binding of IGFs is likely to relate to its ability to promote a tertiary structure, which permits optimal relationships between the N-terminal and C-terminal domains.

*2. Effect of posttranslational modification on IGF binding.* Limited data are available on the effects of posttranslational modification of IGFBPs on IGF binding. Results so far indicate that neither glycosylation nor phosphorylation appear to have much influence on the IGF binding affinities of IGFBPs (83, 84). The exception is the phosphorylation of human IGFBP-1, where it has been shown that phosphorylation enhances IGF binding by at least 5-fold (78). Similar results were not observed with rat IGFBP-1 (82).

*3. Other structure-function associations of IGFBPs.* The regions of IGFBPs that are involved in functions unrelated to IGF binding appear to be predominantly in the mid- and C-terminal domains. To date, the only function clearly associated with the N-terminal domain is IGF binding, and more recently, insulin binding (110, 118). This does not rule out other potential functions for the N-terminal domain, either alone or in concert with other IGFBP domains. Lalou *et al.* (108), for example, have reported that IGFBP-3 (residues 1–95) inhibits cell replication. For the midregions, aside from simply acting as a "hinge" between the N-terminal and C-terminal domains, the fact that these regions are posttranslationally modified suggests that specific functions, as yet undefined, may be associated with this region. For IGFBP-3, the midregion appears to be involved in specific membrane association (127). Interestingly, the proteolytic sites for a number of IGFBP proteases are found in the midregion. It is possible that individual characteristics of each IGFBP reside in these nonconserved regions.

In the C-terminal domain, more information is available about functions other than IGF binding. The C-terminal domain of IGFBP-3, irrespective of its ability to bind IGF-II, has been shown to be essential for interactions with the acid-labile subunit (64, 116), most likely through the IGFBP-3 basic region, $Lys^{228}$-$Arg^{232}$ (116). A recent report suggested that in addition to ALS, IGFBP-3 can interact with other high mol wt proteins found in human serum (128); whether these interactions are through the C-terminal region or midregion is unknown. Interestingly, IGFBP-5 also forms a ternary complex with ALS and IGFs (129). Since the C-terminal domain between IGFBP-3 and -5 is highly similar (54%), particularly in the sequences spanning the basic region (see Fig. 2), IGFBP-5 presumably also interacts wth ALS through this domain.

The other notable motifs in the C-terminal domains are the RGD sequence found in analogous positions in IGFBP-1 (amino acid residues 221–223) and IGFBP-2 and the highly basic heparin-binding sequences found in the thyroglobulin type I domain in IGFBP-3, -5, and -6. The RGD motif in IGFBP-1 was shown by Jones *et al.* (14) to interact with integrins, which are a large family of heterodimeric cell adhesion receptors involved in both cell-cell and cell-ECM interactions (130). It has been hypothesized that interactions of IGFBPs with the ECM, via the integrins, may allow the IGFBPs to provide a reservoir of IGFs (26). In IGFBP-3, the heparin-binding motif can associate with glycosaminoglycan-containing molecules, like proteoglycans found on cell surfaces and in ECM (116, 131). While the consequences of IGFBP-3 interacting with glycosaminoglycan are unclear, these interactions may enhance localization of IGFBP-3 to the cell suface and, perhaps, the ECM (131). The same motif in IGFBP-5 and IGFBP-6 may have similar functions and, in fact, there is strong evidence that the highly basic region surrounding the heparin-binding motif ($Arg^{201}$-$Arg^{218}$) mediates binding of IGFBP-5 to osteoblast cells (16), to ECM (124, 132, 133), and to mesangial cell surface (134). The highly basic regions from all three IGFBPs (IGFBP-3, -5, and -6) are capable of specifically inhibiting IGFBP-4 degradation, and the inhibition of IGFBP-4 degradation is abrogated by IGFs (98). The mechanisms by which this inhibition is mediated are not understood, but since IGFBP-3, -5, and -6 are not themselves substrates for the IGFBP-4 protease, one hypothesis is that the highly basic region in these IGFBPs may act as a protease inhibitor (98). The physiological ramifications

of inhibiting IGFBP-4 degradation are unclear, although IGFBP-4 is known to be a potent inhibitor of IGF actions and proteolysis of IGFBP-4 could, therefore, potentiate IGF actions.

One of the most intriguing observations made within recent years has been evidence for the targeting of IGFBP-3 and IGFBP-5, but not IGFBP-1 or -2, to the nucleus. Although there are no definitive consensus amino acid sequences for nuclear localization signals (NLS) (135), many proteins do contain sequences rich in basic amino acids similar to the NLS (PKKKRKV) of SV40 large T antigen. Potential NLS sequences in IGFBP-3 and in IGFBP-5 were first noted in 1994 by Radulescu (136). It was not until 1997, however, that the evidence supporting nuclear IGFBP-3 was published (137, 138). Not only was endogenous IGFBP-3 clearly found in the nucleus of lung cancer cells (138), but labeled recombinant IGFBP-3 added exogenously to wounded opossum kidney cells was transported into the nucleus, whereas in resting cells, IGFBP-3 was internalized and accumulated in the endosomal compartment (137). Intriguingly, IGFs bound to IGFBP-3 can also localize to the nucleus. In human keratinocytes, nuclear IGFBP-3 was detected in cells undergoing division (139). Recent *in vitro* studies have demonstrated that both recombinant IGFBP-3 and IGFBP-5, but not IGFBP-1 or -2, can translocate from the extracelluar compartment to the nucleus in rapidly dividing human breast cancer cells (140). Site-specific mutagenesis confirmed that the putative NLS in IGFBP-3 is the predicted basic sequence in the C-terminal domain ($Lys^{215}$-$Arg^{232}$) (139). The biological significance of translocating IGFBPs into the nucleus is unclear at present but is consistent with potential IGF-independent actions of some IGFBPs (see below).

### C. Biological functions of IGFBPs

The detailed biological functions of IGFBPs have been well reviewed in recent years (10, 13, 24, 25) and will not be reiterated in this review. The aim in this section is to put into perspective the correlations made between the structure of IGFBPs and their functions in the context of IGF-dependent *vs.* IGF-independent actions of IGFBPs.

*1. IGF-dependent actions of IGFBPs.* The term "IGF-dependent" functions of IGFBPs has been used to define functions of IGFBPs, both positive and negative, that are directly linked with IGF bioactivities (10, 13, 25, 26). Since IGFBPs are well established secreted proteins, this inevitably meant that the focus has been on the extracellular sequestration of IGFs by IGFBPs, and the effects this sequestration has on the consequent loss of interactions between IGFs and the type I IGF receptor. There is a plethora of *in vivo* and *in vitro* studies describing and supporting this mechanism of IGFBP action. Most recently, the approach taken has been to directly test this hypothesis by generating recombinant mutated forms of IGFBPs with reduced affinities for IGFs, and subsequently testing whether these mutants have effects on IGF bioactivities. This was most clearly demonstrated in the case of IGFBP-4, an IGFBP known to inhibit the mitogenic effect of IGFs on bone cell growth. Mutations in human IGFBP-4 that greatly reduced its affinity for IGF-II resulted in an inability of the mutant IGFBP-4 to inhibit IGF-II-induced human osteoblast proliferation (119).

An extension of the IGF-dependent actions of IGFBPs is investigations into the mechanisms of IGF release from IGFBPs. Reducing affinity for IGFs is an obvious mechanism for the release of IGFs and is achieved by proteolysis of IGFBPs, alteration in phosphorylation status of IGFBP-1 (78), and perhaps also by IGFBP conformational changes, such as via binding of the IGFBPs to ECM and/or to the cell surface. Molecular evidence for the importance of IGFBP proteolysis to IGF-dependent actions was provided recently by site-specific mutagenesis of the proteolytic site, resulting in enhanced IGFBP growth-inhibitory effects (141, 142). In contrast, there is yet to be evidence for release of IGFs by conformational change of IGFBP-IGF interactions, although interactions between IGFBPs and ECM and/or cell surface (see above) would support this postulate. An alternative hypothesis, suggested in a recent study, implicate a physical occlusion effect based on the observation that, in IGFBP-5, the regions involved in IGF and ECM interactions overlap (123).

In contrast to the extracellular effects of IGFBP on IGF-type-I IGF receptor complexes, intracellular effects of IGFBPs, particularly any effects on type-I IGF receptor signaling pathway, have yet to be addressed. In light of very recent data indicating the internalization and nuclear localization of IGFBP-3 and of IGFBP-5, it may be necessary to redefine "IGF-dependent" actions.

*2. IGF-independent actions of IGFBPs.* Given the classical definition of IGF-dependent actions of IGFBPs, IGF-independent actions of IGFBPs are defined as biological effects exerted by IGFBPs that involved neither binding of IGFs nor activation or inhibition of the type I IGF receptor. There has been a steady accumulation of data supporting the existence of IGF-independent actions for IGFBP-3 and IGFBP-5 (13) and limited data for IGFBP-1 (14). The recent demonstration of IGFBP-3 and -5 translocation to, and localization in, the nucleus support the concept that these two IGFBPs have functions unrelated to direct IGF actions. Presumably, these IGF-independent functions are through the C-terminal domains and perhaps also the midregions of the IGFBPs (see *Section II.B.3.* above).

As initially demonstrated by Oh *et al*. (18) in breast cancer cells, the epithelial growth-inhibitory actions of IGFBP-3 are mediated through specific binding of IGFBP-3 to cell surface molecules that are not type I IGF receptors. The purification and cDNA cloning of a specific IGFBP-3 receptor, however, remains elusive, but, using the yeast two-hybrid system, cDNAs encoding IGFBP-3 interacting proteins have been obtained (Y. Oh, unpublished). Interestingly, a recent report suggests that the type V TGF$\beta$ receptor could be the putative IGFBP-3 receptor (143, 144) and that IGFBP-4 and IGFBP-5 may also interact with this receptor (144). Presence of this receptor, however, has not been convincingly demonstrated in breast cancer cells (Y. Oh, unpublished).

A similar sequence of events has led to the conclusion that IGFBP-5 also has biological actions that are IGF independent (16). Supporting this hypothesis, a novel, putative IGFBP-5 membrane receptor, a 420-kDa membrane protein, was very

recently purified from osteoblast cells (145). Although not fully characterized and the cDNA not cloned, it would appear, at least *in vitro*, that the binding of IGFBP-5, through the basic region in its C-terminal domain, to the receptor stimulated phosphorylation of the receptor.

### *D. Genomics of IGFBPs*

*1. Chromosomal locations of IGFBPs.* The genomic locations of all human IGFBPs are known and are summarized in Table 1. Interestingly, the genes for IGFBP-1 and IGFBP-3 not only reside on the same chromosome, at the locus 7p14-p12, but are only 20 kb apart, with transcription orientated in a tail-to-tail configuration (146). IGFBP-2 and IGFBP-5 constitute another gene pair, located 20–40 kb apart on chromosome 2q. Based on amino acid similarity analysis, IGFBP-1 is more closely related to IGFBP-2 than to IGFBP-3, which, in turn, is more closely related to IGFBP-5. IGFBP-4, found on chromosome 17q12–21.1, is more closely related to IGFBP-1 and -2, whereas IGFBP-6, located on chromosome 12q13, appears to be the most divergent of the IGFBPs. The similarity in configuration of the human IGFBP genes, especially the gene pairs, is striking, and, together with analysis of the protein sequences, has led to the hypothesis that a tandem gene duplication and inversion occurred early in the evolution of the IGFBPs [one suggestion is that IGFBP-6 is the proto-IGFBP, (58)], and subsequent gene duplications primarily involved partial chromosome duplication (see review in Ref. 58).

An intriguing observation that was made in the analysis of chromosomal locations of the human IGFBP genes is that the genes appear to co-map with genes encoding homeoboxes (HOX) and epidermal growth factor receptors [see review by Reinecke and Collet (58)]. Homeobox is a conserved element of 180 bp that is found in all homeotic (and also nonhomeotic) genes. The importance of homeotic genes is that they are the master control genes that regulate development of higher organisms. Thus, by association, the inference is that IGFBPs are important and fundamental proteins in development. The evolutionary implication is that there may be an association between the evolution of the vertebrate homeobox genes, the epidermal growth factor receptors, and the IGFBPs. Since this area is summarized in a very recent, comprehensive review on IGF phylogeny by Reinecke and Collet (58), readers are referred to that review for more details.

*2. IGFBP gene structures.* The gene structures of human IGFBPs are highly similar, although the sizes of the genes vary from 5.7 kb for IGFBP-1 to 33 kb for IGFBP-5 (Table 1), due to variations in the sizes of the introns. All of the IGFBPs are encoded by four exons, with the exception of IGFBP-3, which carries an extra exon, exon 5, that is not translated. The corresponding exons among the IGFBPs are equivalent in size, with exon 1 less than 600 bp, exons 2 and 3 both small exons of less than 230 bp, and exon 4 more variable in size. There is a stiking correlation between these exons and the three protein domains of IGFBPs. The N-terminal domain, as defined in Fig. 2, is encoded within exon 1 in all of the IGFBPs, as is the 5′-untranslated region and a few amino acids of the midregion. Exon 2 encodes for the nonconserved midregion. Both exon 3, which ends precisely at the invariant Gln (Q) residue in the thyroglobulin domain, and exon 4 encode for the conserved C-terminal domain. The fact that the N-terminal domain is contained within one exon strongly supports the concept of an IGFBP superfamily, as will be discussed in *Section IX*.

## III. Mac25

### *A. IGFBP-rP1 (MAC25/TAF/PSF)*

The (human) Mac25 was the first protein proven to be functionally related to the IGFBPs (22, 37). It was, therefore, provisionally named IGFBP-7 (22), and, subsequently, redesignated IGFBP-rP1 (Table 2). The gene for human IGFBP-rP1 has been localized to chromosome 4q12–13 (147). A mouse homolog, sharing 87.5% nucleotide identity and 94.4% similarity with human IGFBP-rP1, has been described (148). Three groups independently identified the human IGFBP-rP1 protein, and each has continued to use its own designations. Without a doubt, however, they are the same protein. The structural relationship to the IGFBPs was initially noted by Murphy *et al.* (40), who were the first to identify the putative protein, deduced from the cDNA clone, *mac25*. Akaogi *et al.* (42) and Yamauchi *et al.* (41) independently purified and characterized the protein, which they designated TAF and PSF, respectively. Oh *et al.* (22) synthesized the Mac25 protein in a baculovirus system, demonstrated its ability to bind IGFs, and provisionally named the protein IGFBP-7, later to be redesignated IGFBP-rP1. Structurally, the region of similarity of IGFBP-rP1 (Mac25/TAF/PSF) to IGFBPs is confined to the N-terminal domain (see *Section VII*). Functionally, the protein appears to have multiple roles, including the ability to bind IGFs and insulin (see *Section VIII*), but the physiological significance of this protein is still largely unknown. The structural and binding characteristics of IGFBP-rP1 will be discussed in more detail in *Sections VII–VIII*. Below, a historical perspective of IGFBP-rP1 will be presented.

*1. Mac25.* Murphy *et al.* (40) employed subtractive hybridization to search for genes whose expression were altered in meningioma cell lines, compared with normal leptomeningeal cells. The cDNA clone they designated *mac25* was found to be preferentially expressed in normal leptomeningeal cells, compared with meningiomas. *mac25* Expression in breast carcinomas has also been examined and it was noted that expression may be related to the estrogen receptor status of the cancer cells: that is, the presence of estrogen receptor (ER) mRNA appeared to be negatively correlated to expression of *mac25* mRNA. A more extensive examination of *mac25* expression between ER+ *vs.* ER− breast cancer cell lines indicated that some ER− cancer cells also did not express *mac25* mRNA (147). *mac25* cDNA was identified by differential display, as one of the genes overexpressed in senescent normal human mammary epithelial cells (HMEC) (147), and as one of the genes that was down-regulated in breast carcinomas (149). Furthermore, there appeared to be a significant (5/10 tumor tissues examined) loss of heterozygosity in

the *mac25* gene in breast tumors (149). Consistent with these observations was a recent *in situ* hybridization study of IGFBP-rP1 (mac25/TAF/PSF) expression in normal prostate tissue *vs.* prostate tumors, where a marked decrease in IGFBP-rP1 expression was associated with increasing malignancy (150). Interestingly, a malignant prostatic cell line stably transfected with IGFBP-rP1 cDNA was shown to be poorly tumorigenic in both *in vitro* and *in vivo* assays, when compared with cells stably transfected with empty vector, suggesting a potential tumor-suppressive function for IGFBP-rP1 (151).

Expression of IGFBP-rP1 is regulated by growth factors. In midpassage HMEC (147), breast cancer cells Hs578T (Y. Oh, unpublished), and in immortalized prostate epithelial cells (P69) (150), IGFBP-rP1 expression is up-regulated by retinoids. TGF$\beta$ also up-regulates IGFBP-rP1 expression, both at the mRNA and protein levels, in Hs578T and P69 cells (150). Whether IGFBP-rP1 mediates the epithelial growth-inhibitory effects of TGF$\beta$ and retinoic acids has yet to be determined.

One recent study indicated that *mac25* mRNA expression is higher in dividing mouse myoblasts than in nondividing, undifferentiated myotubes (152), suggesting that IGFBP-rP1 may play a role in differentiation of muscles. IGFBP-rP1 may also play a role in differentiation of rat osteoblast cells, as PTH and the glucocorticoid, cortisol, both increase IGFBP-rP1 mRNA (153, 154).

*2. TAF.* TAF, a 30-kDa protein isolated from the conditioned media of a human bladder carcinoma cell line, was so named because it was tumor derived and promoted cell adhesion activity. Initial studies of the purified protein showed that it promoted the attachment and spreading of rat liver cells and human endothelial cells, but did not stimulate endothelial cell growth (42). Subsequent structural analysis of the purified protein and its cDNA indicated identity with PSF (Ref. 41 and see below) and close similarity with Mac25 (37, 42). The discrepancies in primary amino acid sequence between PSF and Mac25 will be discussed below. A monoclonal antibody generated against a C-terminal peptide of purified TAF was used to determine the distribution of TAF in various human cancer tissues (155). Results, based solely on immunohistochemical staining of tissues using this monoclonal antibody, indicated that TAF appears to specifically accumulate in new blood vessels in various human cancer tissues, but not in those of normal tissues, and also in capillary tube-like structures of cultured vascular endothelial cells. These observations, in conjunction with an affinity of TAF for type IV collagen, that was inhibitable by heparin, suggested that TAF may be involved in the formation of new capillary vessels by vascular endothelial cells. This led to the suggestion of renaming the protein "angiomodulin" (155). TAF, at high concentrations (1 $\mu$g/ml), also appears to be capable of stimulating and enhancing IGF and insulin-mediated fibroblast cell growth (37). The seemingly diverse functions of IGFBP-rP1 (TAF/Mac25) can be reconciled by the fact that expression and function of this protein are most likely cell type specific, but further studies are clearly necessary.

*3. PSF.* Yamauchi *et al.* (41) was the third group to purify "IGFBP-rP1." Their interest was in an activity found in plasma that stimulated prostacyclin production in endothelial cells but that was reduced in patients with diabetes mellitus (156, 157). Prostacyclin is a vasodilator and inhibitor of platelet adhesion and aggregation, whose synthesis is stimulated by many factors, including proteases such as thrombin (158). The prostacyclin-stimulating activity in serum was relatively heat stable, acid labile, and nondialyzable (156, 157). A similar activity was detected in the conditioned media of human diploid fibroblast cells (41). Purified PSF was approximately 31 kDa on SDS-PAGE and was able to stimulate prostacyclin production in endothelial cells at a concentration as low as 10 ng/ml (41). PSF was subsequently identified to be the same protein as Mac25 and TAF (159). An antibody generated to a synthetic PSF C-terminal peptide indicated that PSF is expressed in arterial endothelial cells and in smooth muscle cells of human tissues (160, 161).

Although PSF, Mac25, and TAF are the same protein, there are a few discrepancies in the published cDNA nucleotide sequences between PSF and Mac25. Four nucleotides differ in the signal peptide region, resulting in three amino acid substitutions; one nucleotide differs in the N-terminal domain of the molecules resulting in an Arg for Mac25, and Lys for PSF, both basic amino acids. Finally, the major difference is an extra nucleotide found near the C terminus of Mac25 that results in a stop codon within 5 amino acids of the insertion. In PSF, the lack of this one extra nucleotide generated a completely different sequence and extended the sequence by 10 amino acids. Of the two sequences, the PSF sequence is most likely the correct sequence, as it concurs with amino acid sequencing data (41), and with the cloned genomic IGFBP-rP1 gene (V. Hwa and R. G. Rosenfeld, unpublished and Ref. 159). In addition, TAF has an identical sequence with PSF (37). It is quite likely, therefore, that the discrepancy between the Mac25 and PSF cDNAs was the result of errors in the sequencing of *mac25*.

In summary, IGFBP-rP1 appears to be involved in diverse biological functions, from regulation of epithelial cell growth, to stimulation of fibroblast cell growth, to stimulation of prostacyclin production in endothelial cells. Further, it can associate with type IV collagen (37) and can bind IGFs and insulin (22, 37, 118). Its expression is regulated, not only by specific growth factors such as IGFs, PTH, cortisol, TGF$\beta$, and retinoic acid, but by unknown factors involved in the progression of tumorigenesis, in senescing epithelial cells, in diabetes, and in vascular development. IGFBP-rP1 has thus been hypothesized to have a significant biological role in senescence, tumor suppression, and vascular disease; these multiple effects will need to be substantiated.

## IV. CCN Family

A group of highly related, cysteine-rich proteins was recently identified and shown to contain a N-terminal domain that has significant structural similarity with the N terminus of IGFBPs (21). Coined the CCN family by Bork (21), they include human CTGF, a growth factor-inducible immediate-early gene; *cyr61* and its chicken ortholog *cef10*; a potential oncogene, *nov* (for nephroblastomas overexpressing gene); and, more recently, three genes (*WISP-1*, *WISP-2*, and

*WISP-3*) that are up-regulated in Wnt-1-transformed cells and are aberrantly expressed in human colon tumors (46); [see also recent review by Brigstock (162) and Lau and Lam (163)]. The first members of this family to be described were *cef10*, an immediate-early gene, from chicken, detected after induction by the viral oncogene $pp60^{v\text{-}src}$ (164), and a related protein from the mouse, *cyr61* (165). Structurally, the proteins share an overall similarity of 46% and range in sequence length from 349 to 381 amino acids (prepeptides). There are 38 completely conserved cysteines in the mature proteins. Each of the proteins is comprised of four domains: the N-terminal domain of the IGFBP family, the Von Willebrand factor type C repeat (VWC), the thrombospondin type I repeat, and the C-terminal (CT) domain. A central "fifth" domain is variable among the CCN proteins. Detailed structure and function analysis of the N-terminal domain, particularly in relationship to the IGFBP family, will be discussed in *Sections VII* and *VIII*. The specific functions of the latter three domains are still unclear, but are believed to be involved in dimerization, oligomerization, and interactions with the ECM through heparin binding regions. The biological roles of the CCN family are under investigation and appear to be important in the regulation of cellular proliferation. Below, a summary of each CCN family member will be described.

### *A. IGFBP-rP2 (CTGF)*

Connective tissue growth factor was the first human protein of the CCN family to be described (44) and the second protein tested and shown to be related to the IGFBPs (23). CTGF (IGFBP-rP2) was purified from media conditioned by human umbilical vein endothelial cells (HUVEC) as the major secreted protein that was immunoreactive with antibody against platelet-derived growth factor (PDGF) (44). Interestingly, it was subsequently shown that the two proteins did not share any regions of amino acid sequence similarity (44). The purified protein is a monomer of 36–38 kDa that demonstrated mitogenic activity and chemotactic activities for fibroblastic cells. Furthermore, the protein could bind to the cell surface of endothelial cells and was competitively displaced by purified PDGF (44). Subsequent studies have demonstrated that a 10-kDa proteolytic fragment of CTGF, corresponding to the CT domain, can associate with the cell surface of fibroblasts and is capable of inducing cell proliferation (166–168). These activities are inhibitable by heparin and clearly do not involve the PDGF receptor (168). The specific cell surface receptor for CTGF is not known, although a recent study has suggested that, at least for human chondrocytes, CTGF, which participates in endochondral ossification (169), interacts with an uncharacterized 280-kDa membrane protein (170).

The chromosomal location for the CTGF gene has been determined to be 6q23.1 and proximal to the oncogene, c-*myb* (45). The cDNA for CTGF encodes a 38-kDa protein with two potential glycosylation sites and hybridizes to a single 2.4- kb mRNA species in Northern blots (44). Expression of CTGF is regulated in a manner consistent with an immediate-early gene. In fibroblast cells, it is selectively up-regulated by TGF$\beta$, a potent stimulator of fibroblast cell proliferation and a critical factor in cell regeneration and wound repair, within an hour of exposure to TGF$\beta$ (171). Unlike other immediate-early genes, however, short-term exposure to TGF$\beta$ induces prolonged CTGF mRNA expression, for up to 36–48 h (171, 172). A novel TGF$\beta$ response element found in both the human and murine *CTGF* promoters, but absent in other genes regulated by TGF$\beta$, suggests that regulation of *CTGF* gene expression may function by a mechanism distinct from other TGF$\beta$-regulated genes (172). Importantly, some of the biological effects of TGF$\beta$ on fibroblast and endothelial cells appear to be mediated by the up-regulated CTGF protein (173–177).

Since the discovery and initial characterization of CTGF, there has been considerable research into the regulation, biology, and clinical implications of this protein, which will be briefly summarized here. The readers are referred to recent reviews for more comprehensive coverage (162, 174, 178–180). Because of clinical implications in fibrosis and mucosal repair, IGFBP-rP2 (CTGF) research has focused on its role(s) in fibroblast and endothelial systems. However, IGFBP-rP2 may also be important for epithelial growth, as recent data suggest that TGF$\beta$, which is inhibitory for epithelial cell proliferation, up-regulates IGFBP-rP2 expression (mRNA and protein) in mammary cells (181). Interestingly, *in situ* studies of mammary tumors have suggested that IGFBP-rP2 mRNA expression is exclusively in the fibrous stroma (182). The implications are unclear at present.

### *B. IGFBP-rP3 (NovH)*

The gene *nov* was first discovered in myeloblastosis-associated virus type I-induced avian nephroblastomas (183). Expression of *nov* was elevated in these nephroblastomas, compared with normal adult avian kidney cells, suggesting that *nov* may be a protooncogene. Supporting this concept, human *nov*H (184) expression was shown to be elevated in Wilms tumors of the stromal type, which histologically are similar to avian nephroblastomas (45, 184). Of particular interest is that the *nov*H gene maps to chromosome 8q24.1, proximal to c-*myc* (45), a region often involved in chromosomal abnormalities associated with human tumors, including Wilms tumor. The expression of *nov*H appears to be inversely correlated with the expression of the tumor suppressor gene, WT1 (185,186), whose inactivation is postulated to participate in the etiology of Wilms tumors. Indeed, recent studies indicate that WT1 does transcriptionally down-regulate *nov*H expression (187).

Aside from its oncogenic potential, *nov*H and *nov* are involved in other biological processes. For example, the effects of *nov* on chicken embryo fibroblast (CEF) cells are quite different: overexpression of *nov* inhibits fibroblast cell growth, although, interestingly, overexpression of an N-terminally truncated form of *nov* (which deleted the N-terminal domain) induced cellular transformation of the fibroblast cells (183). Consistent with the growth-inhibitory effects observed in CEF cells, it was demonstrated that *nov* was expressed only in quiescent CEF cells, and that transformation of CEF by $p60^{v\text{-}src}$ oncogene down-regulated expression of the *nov* gene (188). In humans, *nov*H is associated with the developing kidney, where observations suggest that NovH protein is stably accumulated in embryonic kidney in glo-

merular podocytes undergoing differentiation, and, after birth, the persistence of high levels of NovH protein may be required for maintenance of podocyte structure and/or for specfic podocytic functions (186).

The structures of mammalian and nonmammalian Nov proteins are similar to that of Cef-10 (164), Cyr61 (165), Fisp-12 (189), and CTGF (44). The human NovH protein, deduced from the cloned cDNA, indicated that the cDNA encodes a putative 39-kDa secreted polypeptide (184, 186). Immunoblots of biological fluids and media conditioned by various cell lines indicate that NovH is at least 44 kDa and is N glycosylated (38, 186). Interestingly, intracellular isoforms of NovH were detected and appeared to be less stable than the extracellular form (186). Like all members of the CCN family, Nov/NovH consists of four domains (21), of which the first domain (after the signal peptide) is an IGFBP N-terminal domain, leading to the redesignation of NovH as IGFBP-rP3. Recently, Burren *et al.* (38) demonstrated that IGFBP-rP3 could bind IGF with low affinity, similar to that detected for IGFBP-rP1 and -rP2. More structural and functional information will be given in *Sections VII* and *VIII* below.

### C. IGFBP-rP4 (Cyr61)

The *cyr61* gene was originally identified in mouse 3T3 fibroblasts as an immediate-early gene that was rapidly activated by serum, PDGF, fibroblast growth factor, and 12-*O*-tetradecanoylphorbol-13-acetate (165). Unlike other immediate-early genes, but similar to CTGF, induced *cyr61* mRNA persists for a considerable time after induction. The human *cyr61* gene, cloned recently, and mapped to chromosome 1p22-p31 (43), is similar to mouse *cyr61* in both structure [sharing 85% amino acid similarity (190)] and function. Both *cyr61* mRNAs are not detected in quiescent fibroblasts, but are abundant in logarithmically growing cells and serum-stimulated cells (165, 190). Human *cyr61* mRNA was also recently shown to be up-regulated by factors important for osteoblast function and differentiation, such as 1$\alpha$,25-dihydroxyvitamin $D_3$, EGF, tumor necrosis factor $\alpha$ (TNF$\alpha$), and interleukin-1 (191).

Cyr61 protein, like the rest of the CCN family, is a secreted protein. However, unlike the other members, it is not readily detected in conditioned media of cell lines examined, apparently because it associates with the ECM and cell surfaces, most likely through its heparin binding regions (192, 193); (see recent review in Ref. 163). In fact, it was recently demonstrated that Cyr61 protein adheres to HUVEC cells through integrin $\alpha_v\beta_3$ (194). This adherence of Cyr61 to HUVEC cells may be a mechanism by which Cyr61 promotes the attachment and spreading of endothelial cells (193). Support for this hypothesis comes from recent studies in which purified Cyr61 was shown to promote angiogenesis through an $\alpha_v\beta_3$-dependent pathway (195). Cyr61 also promotes the adhesion of fibroblasts and epithelial cells (193, 196), induces chemotaxis of fibroblasts (193), enhances growth factor-stimulated DNA synthesis in both fibroblast and endothelial cells (176, 193, 196), and plays a role in chondrogenesis (197).

There is recent evidence that Cyr61 may also play a role in tumorigenesis. Stably *cyr61*-transfected gastric adenocarcinoma cells demonstrated increased tumor growth when tested in a nude mouse model, suggesting that Cyr61 promoted tumor growth (195). However, Cyr61 protein expression was down-regulated in prostate carcinomas (198). Although the role of Cyr61 in cancer is unclear, it is of interest to note that the human *cyr61* gene is mapped to chromosome 1p22-p31 (43), as abnormalities of chromosome 1p have been shown to correlate with breast cancer (199), neuroblastoma (200), and pheochromocytoma (201).

The human *cyr61* cDNA encodes a 381-amino acid protein rich in cysteine and proline residues (43, 190). There are two distinct mRNA species, a major one at 2.5 kb and a minor one at about 4.0 kb, which are believed to be either alternatively spliced transcripts or transcripts with different polyadenylation signals (43). Cyr61 is structurally consistent with other members of the CCN family, but has yet to be tested for its ability to bind IGFs. It is predicted, however, that it will prove capable of binding IGFs with low affinity, similar to that observed with CTGF and NovH.

### D. New members

*1. IGFBP-rP7 (rCOP-1/WISP-2/CTGF-L).* The gene *rCop-1* was very recently identified by differential display from rat embryo fibroblasts (REFs) as one of three genes whose expression was lost specifically upon cell transformation (47). By sequence comparison, it appears that rCop-1 belongs to the CCN family of proteins, but, unlike the other CCN proteins, it only has the first three conserved protein domains and lacks the last domain (the CT domain). The cDNA encodes a unique 250-amino acid protein with a signal peptide and is detectable as a single 1.7 kb transcript, thereby ruling out the possibility that rCop-1 mRNA is a result of alternative splicing of other CCN transcripts. However, until the gene for rCop-1 is fully characterized, this possibility cannot be completely ruled out. Although rCop-1 has a signal peptide, it is not detectable in conditioned media of fibroblast cells, nor is it associated with the ECM, like Cyr61, perhaps due to loss of the CT domain. Rather, it seems to be predominantly cell surface associated. Not only is the structure of rCop-1 distinct from the other CCN proteins, but the pattern of expression of rCop-1 mRNA indicates that its regulation may be through different mechanisms than for the rest of the CCN family. It is not an immediate-early gene, like the *CTGF* and *cyr61* genes; it is not serum inducible, and, in fact, expression is inversely related to that of *cyr61* in normal fibroblast cells. Overexpression of rCop-1 in transformed cells reduced tumorigenicity and increased cell death. In primary cultures of rat and mouse fibroblasts, the rCop-1 gene was detected only when cells became senescent during passage in culture.

A human ortholog of the rCop-1 gene, *WISP-2* (46) and CTGF-L (39), has been subsequently identified and redesignated IGFBP-rP7 in this review. The gene maps to human chromosome 20q12–20q13 (46) and appears to be linked to tumorigenesis (46) as well as to the modulation of bone turnover (39). WISP-2/IGFBP-rP7 mRNA expression was reduced in human colon tumors and is one of three *WISP* genes that are regulated by Wnt-1, a glycosylated signaling protein critical in developmental processes and linked to tumorigenesis (202, 203).

Kumar *et al.* (39) identified a CTGF-like cDNA by analysis of an expressed sequenced tag cDNA library derived from primary human osteoblasts. Expression of CTGF-L/IGFBP-rP7 appeared to be predominantly in osteoblasts at sites of high bone turnover. Functionally, recombinant CTGF-L/IGFBP-rP7 had a 10-fold higher affinity for IGF-II than for IGF-I by both Western ligand blotting and cross-linking assays. Further, in osteoblast cells, it inhibited osteocalcin (a marker of mineralizing osteoblasts) production and promoted cell adhesion and integrin binding. The implication is a role of CTGF-L/IGFBP-rP7 in bone turnover, through mechanisms yet to be determined.

*2. IGFBP-rP8 (ELM1/WISP-1).* The *Elm1* gene ("expressed in low-metastatic type 1 cells") was cloned by differential display as a novel mouse gene that was preferentially expressed in low, but not high, metastatic type 1 murine melanoma cells (48). Functionally, overexpression by stable transfection indicates that it can suppress the *in vivo* growth and metastatic potential of murine melanoma cells. Elm1 cDNA encodes a predicted secreted protein of 367 amino acids, which conserves the four CCN protein domains. An alignment analysis with other members of the CCN family, including mouse Nov and mouse Fisp12, indicates it is not an ortholog of other CCN family members. Recently, the human ortholog, designated WISP-1, was identified (46). Unlike WISP-2 (CTGF-L/IGFBP-rP7), WISP-1 mRNA is overexpressed in human colon tumors. Interestingly, the *WISP-1* gene is mapped to the human chromosome 8q24.1–8q24.3, in the same region as the *novH* (IGFBP-rP3) gene (chromosome 8q24.1).

*3. IGFBP-rP9 (WISP-3).* The third new member of the CCN family is WISP-3 (46). The WISP-3 gene is mapped to human chromosome 6q22–6q23, in close proximity to the *CTGF* (IGFBP-rP2) gene [chromosome 6q23.1 (45)]. The deduced amino acid sequence from the WISP-3 cDNA indicates that it contains the four typical protein domains of the CCN family. Like WISP-1, WISP-3 expression is up-regulated in human colon tumors (46).

In summary, it seems likely that the CCN family of proteins will continue to expand as new members are identified. The implication for the IGFBP superfamily is that the number of IGFBP-rPs will also continue to expand.

## V. L56

### A. IGFBP-rP5 (L56/HtrA)

L56 is a cDNA cloned from a subtractive library constructed between human fibroblast cells and the respective SV40-transformed fibroblast cells (49). The cDNA hybridizes to a 2.3-kb mRNA and encodes a predicted secreted protein of 51 kDa. Structurally, the mature protein is composed of three domains: an N-terminal domain with significant similarity to the IGFBPs, a KI domain similar to that found in IGFBP-rP1, and a large domain (330 amino acids) devoid of cysteines and sharing a 58% similarity with a bacterial serine protease of the HtrA class, which allows bacteria to survive at elevated temperatures (204). It was postulated, therefore, that L56 may function as a protease (49). Interestingly, recombinant human L56 protein, generated in a baculovirus expression system, demonstrated that the protein is an active serine protease that specifically cleaves IGFBP-5 and is capable of binding heparin (205).

L56 was independently identified by differential display analysis of transcripts expressed in osteoarthritic cartilage (50). HtrA (L56) expression (mRNA and protein) was up-regulated in osteoarthritis and demonstrated endoproteolytic activity (50). Mutagenesis of the putative active site ($Ser^{328}$ to Ala) eliminated the enzymatic activity (50).

## VI. ESM-1

### A. IGFBP-rP6 (endothelial-specific molecule-1)

There is limited information concerning ESM-1, an endothelial cell-specific molecule whose cDNA was cloned from a HUVEC cDNA library (51). Expression of this molecule appears to be restricted to human lung tissue, although extensive characterization has not yet been performed. In HUVECs, mRNA expression is up-regulated by cytokines TNF$\alpha$, interleukin-1$\beta$, and interferon d. The biological actions of the protein are unknown. Structurally, the cDNA encodes a small secreted protein of about 20 kDa, consisting of two potential protein domains: a larger N-terminal domain that is similar to the IGFBP N-terminal domain and a C terminus that does not appear to share significant similarities with any known proteins. Of the proteins that contain the IGFBP N-terminal domain, ESM-1 shows the least, but still significant, similarity to the IGFBPs (see below). The ability to interact with IGFs is unknown.

## VII. Structural Relationships Within the IGFBP Superfamily

The primary sequences of IGFBPs, with the clustering of invariant cysteines, have indicated three distinct regions within these proteins: the conserved N-terminal third of the protein, the conserved C-terminal third, and a nonconserved midregion. It has been hypothesized that the N- and C-terminal domains are capable of acting independently of each other, based on the fact that the cysteines within each of the conserved regions are even numbered, and that proteolytic fragments of IGFBPs contain either the N- or C-terminal regions. Indeed, recent disulfide linkage mapping has demonstrated that disulfide linkages are typically formed within each conserved domain, rather than between domains (61, 62). The modularity of these domains, however, has only been recently appreciated. In the C-terminal domain, a subdomain with resemblance to the thyroglobulin type I domain had been previously noted. It was not until the IGFBP N-terminal domain was identified in other cysteine-rich proteins (21) that it became clear that 1) there exist proteins structurally related to the IGFBP family; and 2) the N- and C-domains of the IGFBPs are best thought of as discrete modules of the IGFBPs, which may be considered mosaic proteins.

*A. Protein domains (modules)*

Conceptually, a domain may be defined as a region of a protein that can fold into a tertiary structure independent of neighboring sequences. A domain found in two otherwise nonhomologous proteins is considered to be evolutionarily mobile, and, therefore, termed a module (206). Under these definitions, the N-terminal domain of IGFBPs may properly be considered a module, as it is found in the IGFBP-rPs, as well as in the IGFBPs. The primary sequences of human IGFBPs and IGFBP-rPs (schematically presented in Fig. 3) indicate that the only common region among the present members of the IGFBP superfamily is the N-terminal domain. An optimized alignment (Clustral method, DNA Star program) of these N-terminal domains, and the (averaged) similarity matrix based on this alignment, are shown in Fig. 4 and Table 3. The alignment also suggests that the N-terminal domain extends up to the last conserved cysteine (xGxCx sequence, where x is any amino acid residue). The N-terminal domain (excluding the signal pepide) is, thus, fairly consistent in size, ranging from 70 amino acids (IGFBP-rP2) to 93 amino acids (for IGFBP-3). The exception is IGFBP-rP6 [ESM-1 (51)], which has the largest N-terminal domain of the IGFBP superfamily (130 amino acid residues, not counting the putative signal peptide), and carries all of its 18 cysteines within its N-terminal domain.

The conservation of the 12 cysteines in the N-terminal domains is striking, with 10 of 12 cysteines conserved in IGFBP-6, 11 of 12 cysteines conserved in the CCN family (the fourth conserved cysteine is replaced by a valine), and the remaining IGFBPs and IGFBP-rPs conserving all 12 cysteines. It is noted that although IGFBP-rP2, -rP4, and -rP7-rP9 carry 12 cysteines in their N-terminal domains, only 11 of the 12 can be aligned with those in the IGFBPs. Within the N-terminal domains, the (G)CGCCxxC motif (Fig. 4) is well conserved; as noted above, the motif in IGFBP-6 shows the least homology.

Based on the alignment shown in Fig. 4, the similarity matrix (Table 3) indicates that the N-terminal domains of the IGFBP-rPs have significant similarities to that in the IGFBPs (40–57%). Among the IGFBP-rPs, IGFBP-rP6 has the least similarity (37–41% similarity) to members of the IGFBP superfamily. As expected, the N-terminal domains among the IGFBP family show a high 68% relative similarity, as does the N-terminal domains among the CCN family (66%). The N-terminal domains of IGFBP-rP1 and IGFBP-rP5 also share a higher similarity (61%) compared with their similarities with either the CCN family or IGFBPs.

The similarity between the IGFBP family and the IGFBP-rPs significantly decreases to less than 15% beyond the N-terminal domain. Unlike the IGFBPs, the IGFBP-rPs do not contain the thyroglobulin-type I domain (see above) at the C terminus. Analysis of the IGFBP-rP1 amino acid sequence (24) using the Gapped BLAST database search program reveals that immediately adjacent to the N-terminal domain is a stretch of 30–45 amino acid residues that has 30% similarity to the Kazal family of serine proteinase inhibitors, including the (human) pancreatic secretory trypsin inhibitor (207). This domain, known as a KI domain, is also found in follistatin, leading to the hypothesis that Mac25 was a follistatin-like protein (148). Interestingly, the KI domain can be found in IGFBP-rP5, in the analogous position (Fig. 5). The remainder of the IGFBP-rP1 sequence shares (41%) similarity with the Ig-like domains found in heparin sulfate proteoglycan (208, 209) and in the fibroblast growth factor receptor (210). The functions of these regions with respect to the biological activities of IGFBP-rP1 are not known. The C terminus of IGFBP-rP5 is highly similar (58%) to the family of HtrA/Do serine proteases found in bacteria (49).

As indicated above, the C-terminal half of the CCN family (IGFBP-rP2–4) contain three domains: the VWC repeat, the thrombospondin type I repeat, and the CT domain (21). Hence, the common feature among the IGFBPs and the IGFBP-rPs is limited to the N terminus, with the conserved cysteines and the (G)CGCCxxC motif; the remaining protein domains have clearly diverged.

The striking feature of the IGFBP-rPs is the modular architecture of the protein domains, first noted by Bork (21) for the CCN family. Each of the domains noted above

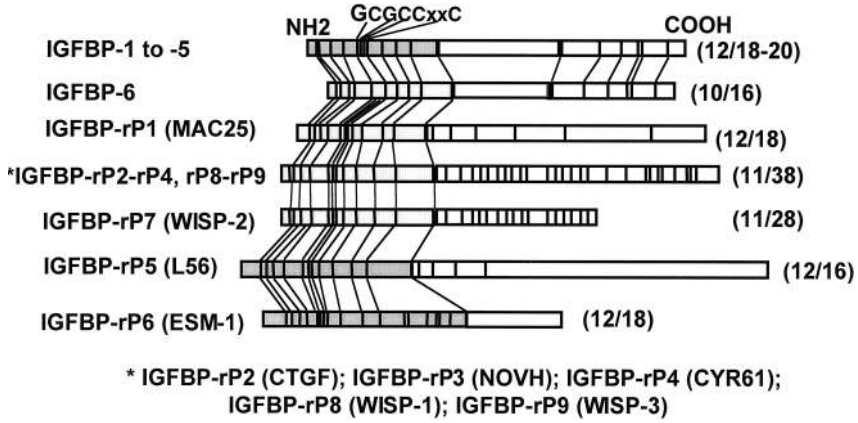


FIG. 3. Schematic primary structures of IGFBP-1 to -6 and IGFBP-rPs. Number of conserved cysteines in the N-terminal domain, out of the total number of cysteines in each protein, is indicated on the *right* of each structure. Locations of the cysteines are indicated as *vertical lines*. The N-terminal domains are *shaded*. Nonconserved segments between the IGFBPs and IGFBP-rPs are represented by *white bars*. The consensus motif GCGCCxxC in the N-terminal domains is as indicated.

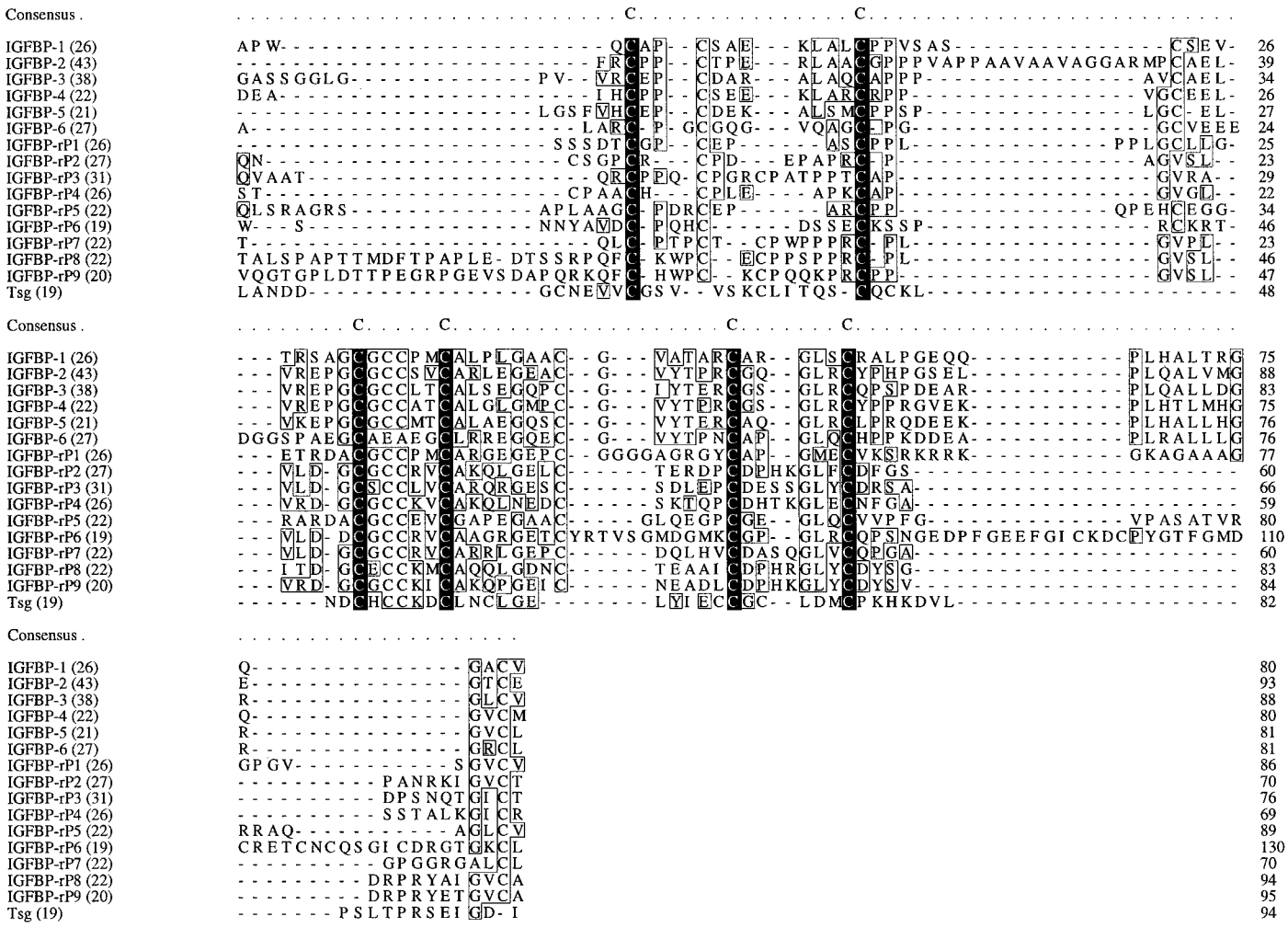

FIG. 4. Amino acid sequence alignment of the N-terminal domains from human IGFBP-1 to -6, human IGFBP-rPs, and from the Tsg (*Drosophila*). Signal peptides (number of amino acid residues are indicated in *brackets*) were not included in the analysis, and the first amino acid residue shown is that for the mature peptide. Alignment was performed using the Clustral method (DNA STAR program), as for Fig. 2. Small gaps were introduced to optimize alignment. Consensus amino acid residues are *shaded solid black*; *boxed* amino acid residues indicate that the residue is found in the majority of peptides. IGFBP-rP1, Mac25/PSF/TAF/IGFBP-7; IGFBP-rP2, CTGF; IGFBP-rP3, NovH; IGFBP-rP4, Cyr61; IGFBP-rP5, L56/HtrA; IGFBP-rP6, ESM-1; IGFBP-rP7, WISP-2/CTGF-L; IGFBP-rP8, WISP-1; IGFBP-rP9, WISP-3; Tsg, twisted gastrulation protein.

is modular, in that they are found in combination with other domains, generating mosaic proteins (28, 206, 211). It is noteworthy, as suggested above, that the IGFBP N-terminal domain is a bona fida module, and that the IGFBPs, like the IGFBP-rPs, therefore conform to a mosaic protein structure. A schematic presentation of the mosaic structures of members of the IGFBP superfamily is shown in Fig. 5, where the N-terminal domain is the conserved module.

### *B. Gene structure and correlation with protein domains*

The gene locations for all members of the human IGFBP superfamily were discussed in *Sections II* and *III*, and are summarized in Table 1. IGFBP genes consist of four exons, with the exception of IGFBP-3, which has an additional untranslated fifth exon. The IGFBP-rP1, IGFBP-rP3 (*novH*), and IGFBP-rP2 genes each contain five exons (174, 184, 212); presumably, IGFBP-rP4 gene also has five exons, as the mouse IGFBP-rP4 gene has five exon-coding sequences (213).

Analysis of known gene structures for the IGFBPs and IGFBP-rPs (IGFBP-rP1, -rP2, and -rP3) supports the concept of conservation of the N-terminal domain (Fig. 6). The N-terminal domain in the IGFBPs is encoded by exon 1. In IGFBP-rP1, the N-terminal domain, like in the IGFBPs, is encoded within one exon, exon 1 (Fig. 6). Exon 1 of IGFBP-rP1, additionally, encodes the 5′-untranslated region, signal peptide, and the complete KI domain. The remaining exons, 2–5, encode the Ig-like domain. Strikingly, for IGFBP-rP2 and -rP3, each exon encodes one protein domain. Thus the N-terminal domain ($T^{1}$QR–TGICT$^{76}$) is encoded by exon 2, VWC by exon 3, thrombospondin type I repeat by exon 4, and CT and 5′-untranslated region by exon 5.

Although in most genes exons typically do not encode discrete domains, and DNA coding regions for many movable protein modules are interrupted by introns (206), it is

TABLE 3. Similarities between the N-terminal domains from human IGFBPs, human IGFBP-rPs, and Tsg (fruit fly)

| | Relative similarity (%)[a] | | | | | |
|---|---|---|---|---|---|---|
| | IGFBPs | IGFBP-rP1 | IGFBP-rP2-rP4-rP7-rP9 | IGFBP-rP5 | IGFBP-rP6 | Tsg (fruit fly) |
| IGFBPs | 68[b] | 57 | 48 | 52 | 40 | 44 |
| IGFBP-rP1 | | 100 | 47 | 61 | 41 | 41 |
| IGFBP-rP2-rP4-rP7-rP9 | | | 66[c] | 46 | 37 | 50 |
| IGFBP-rP5 | | | | 100 | 39 | 40 |
| IGFBP-rP6 | | | | | 100 | 34 |

[a] Similarities were generated from the alignment (Clustral method, DNA STAR program) of the N-terminal domains (see Fig. 3A) of human IGFBPs and IGFBP-rPs, and the fruit fly Tsg. Note that the similarities, rounded up to the nearest percentage, will fluctuate, depending on the number of sequences aligned. Hence the percentage similarity presented is relative to the other proteins in this alignment.
[b] Mean value for IGFBPs (IGFBP-1 to -6).
[c] Mean value for IGFBP-rP2 to -rP4, and IGFBP-rP7 to -rP9 (CCN family).

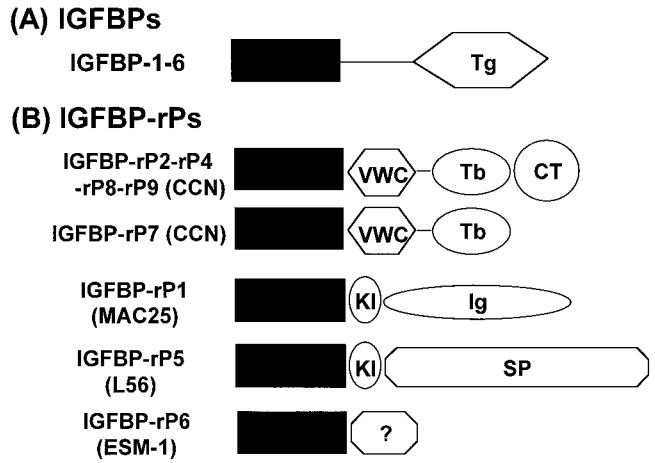


FIG. 5. Modular architecture of protein domains in the IGFBP superfamily. The IGFBP superfamily is subgrouped into (A) IGFBPs (IGFBP-1 to -6); and (B) IGFBP-rPs (as indicated). The N-terminal module is shown as a *black box*. The other modules are as indicated and are abbreviated as follows: Tg, thyroglobulin-type I domain; VWC, Von Willebrand factor type C repeat; Tb, thrombospondin type I repeat; CT, C-terminal domain; KI, Kazal-type serine proteinase inhibitor; Ig, immunoglobulin-like domain; SP, serine protease. The "?" indicates no obvious similarity to known domains or protein motifs.

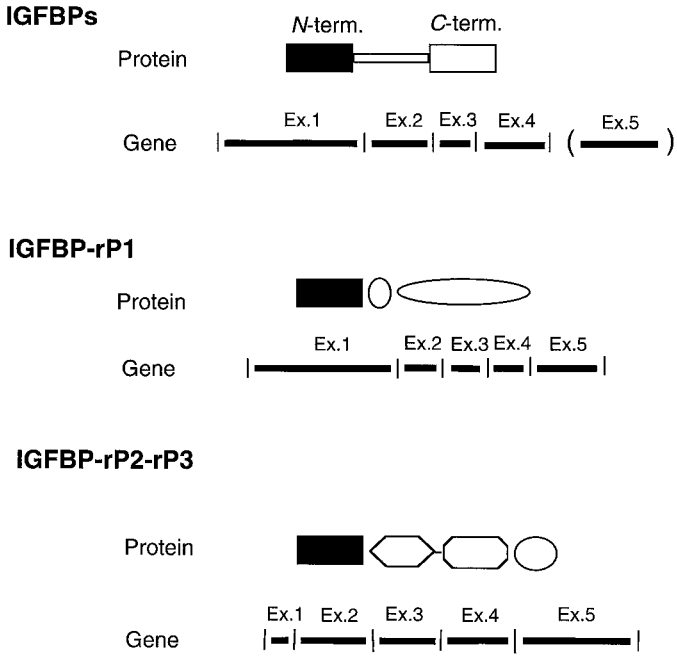


FIG. 6. Schematic presentation correlating (human) IGFBP and IGFBP-rP gene structures to protein domain structures. The protein domain structures are as shown in Fig. 5. Exons (Ex) are as indicated. Exon 5 (untranslated) for the IGFBPs is in *brackets* as it is found only in the IGFBP-3 gene. The exon structures of the human IGFBP genes were summarized from Ehrenborg *et al*. (146), Allander *et al*. (219), and Zazzi *et al*. (220); for human IGFBP-rP1, from Mizushima *et al*. (212) and V. Hwa and R. G. Rosenfeld, unpublished; for human IGFBP-rP2 and -rP3, from Martinerie *et al*. (184) and Grotendorst (174).

all the more remarkable, but certainly not unique, that the N-terminal domain is encoded by one exon. The implications of this observation will be discussed when considering the evolutionary aspects of the N-terminal domain (see *Section IX*).

## VIII. Functional Relationships Within the IGFBP Superfamily

### *A. IGF binding*

The ability of IGFBPs to bind IGFs with high affinity is well established, although the precise amino acid residues involved are still largely unknown. There is ample evidence indicating that both the N terminus and the C terminus are necessary for high-affinity binding of IGFs. Thus, as summarized in *Section II*, loss of the C-terminal domain significantly reduces, but does not abolish, the ability to interact with IGFs. Therefore, since the structural relationship between the IGFBPs and IGFBP-rPs is confined to the N-terminal domain, it was hypothesized that the IGFBP-rPs may be capable of low-affinity IGF binding through their conserved N-terminal domain. Consistent with this hypothesis, IGFBP-rP1 (Mac25/TAF/PSF/IGFBP-7), IGFBP-rP2 (CTGF/IGFBP-8), and IGFBP-rP3 (NovH) were shown by Oh *et al*. (22), Kim *et al*. (23), and Burren *et al*. (38), respectively, to have at least 100-fold lower affinity for IGFs, as assessed by competitive affinity cross-linking assays. Akaogi *et al*. (37) similarly demonstrated that IGFBP-rP1 (TAF) interacts with IGF with low affinity ($k_a$ was $\sim 3 \times 10^{-8}$ M), as does IGFBP-rP7 [CTGF-L (39)]. The binding of IGFs by the IGFBP-rPs, although of lower affinity, was, nevertheless, specific, as PRL, GH 23 (118), and the C-peptide of proinsulin (38) have been shown to be unable to bind to the IGFBP-rPs. Interestingly, the N terminus of IGFBP-3 is capable of binding IGF, but with significantly reduced affinity (110, 118). The decrease in af-

finity for IGFs by both the IGFBP-rPs and the recombinant IGFBP-3 N terminus was attributed to the lack of the appropriate C terminus, although it is certainly possible that the N-terminal domain of the IGFBP-rPs is not "optimally" configured.

The fact that IGFBP-rPs appear to have at least 100-fold lower affinity for IGFs, compared with the IGFBPs, suggests that there are two distinct classes of IGF binders in the IGFBP superfamily: the low-affinity IGF binders (IGFBP-rPs and IGFBP proteolytic fragments) and the high-affinity IGF binders (IGFBP-1–6). The role(s) of the high-affinity IGF binders in modulating IGF activity is well established. In contrast, the role(s) of the low-affinity IGF binders in modulating IGF activity is less clear. It is likely, however, that regulation of IGF activity is not the major function of the IGFBP-rPs, and that the IGFBP-rPs may, in fact, be more involved in functions independent of their direct effects on IGFs. In this regard, it is worth pointing out that IGF-independent actions have also been demonstrated for some of the conventional IGFBPs (see *Section II*).

### *B. Insulin binding*

An unexpected action of IGFBP-rP1, and most recently, IGFBP-rP3 is their ability to bind insulin at an affinity at least equal to their ability to bind IGFs (37, 38, 118). To date, no insulin-binding proteins have been described, and none of the IGFBPs was believed to bind insulin with any degree of significance. In retrospect, it was only the lack of an appropriate detection technique that led to this incorrect conclusion. Yamanaka *et al*. (118) have recently demonstrated that, in a Western ligand blot using reduced, denaturing conditions, IGFBP-1 to -6 are capable of binding $^{125}$I-labeled insulin, although with considerably lower affinity than IGFBP-rP1 has for insulin.

Based on the ability of IGFBPs and IGFBP-rP1 to bind IGFs and insulin, a model for the secondary/tertiary structure of the IGFBPs that would accomodate IGF binding, as well as insulin binding, active sites was proposed (Ref. 118 and Fig. 7). The model suggests that the folding of IGFBPs is such that the N and C termini together form a high-affinity IGF-binding site, which simultaneously partially hides an insulin-binding site deep within the protein. Disruption of this tertiary structure through proteolysis of the IGFBPs, or by reducing the protein through the use of agents such as dithiothreitol, not only disrupts the high-affinity IGF-binding site, but also exposes the partially hidden insulin-binding site. The result is an increase in affinity for insulin, with a concomitant decrease in affinity for IGFs. Consistent with this hypothesis, *in vitro* generation of the N terminus of IGFBP-3 (110, 118), as well as *in vivo* proteolysed N-terminal fragments of IGFBP-3 from human urine, demonstrate reduced affinity for IGFs and enhanced insulin binding (110, 214). For IGFBP-rP1 and IGFBP-rP3, the presence of a different C terminus from that of the IGFBPs results in essentially the same effect and may explain both the decrease in affinity of IGFBP-rP1 for IGFs and its ability to bind insulin (118). Additionally, it has been shown that the synthetic N terminus [amino acid residues 1–97 (110)], as well as IGFBP-rP1 (118), can effectively inhibit the autophosphorylation of the $\beta$-subunit of the insulin receptor in insulin receptor-overexpressing NIH3T3 cells, indicating a biological effect of the N terminus in the inhibition of insulin action. It is thus suggested that the N terminus of IGFBP-3 (and of the IGFBP-rPs) may have important biological roles in the modulation of both IGF and insulin actions.

### *C. IGF/insulin-independent actions*

Although the biological functions of IGFBPs are predominantly IGF dependent, there is strong evidence for the IGF-independent actions of IGFBPs, as presented in *Section II* above. The IGFBP regions involved in these IGF-independent interactions (such as interactions with the cell surface, ECM, potential intracellular proteins) appear to be either in the midregion or the C-terminal domains.

Given their relatively low affinity for IGFs, the IGFBP-rPs, as suggested above, are likely to be involved primarily in functions independent of their effects on IGF and insulin binding. Their initial discoveries were in systems unrelated to IGF or insulin actions. For example, IGFBP-rP1 was purified as a PSF (41) and as a TAF (42); IGFBP-rP2 was shown

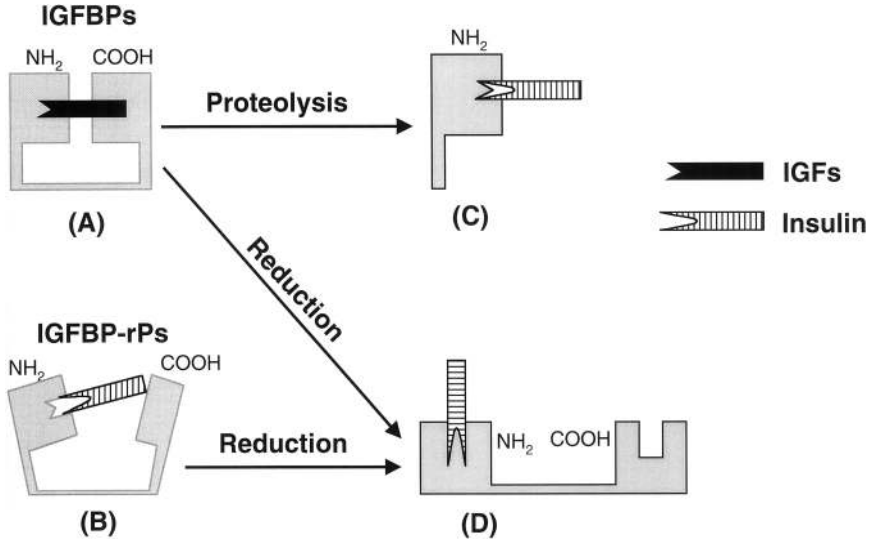


FIG. 7. Model of IGF and insulin binding by IGFBPs and IGFBP-rPs [modified from Yamanaka *et al*. (118)]. A, The conventional tertiary structure of IGFBPs binds IGFs with high affinity, and with no detectable insulin binding. B, IGFBP-rPs bind IGFs with low affinity and bind insulin with equal or higher affinity. C, Upon proteolysis of IGFBPs, IGF binding is reduced with concomitant increase in insulin binding as insulin binding sites are revealed. D, Reduction of IGFBPs and IGFBP-rPs, like proteolysis of IGFBPs, demonstrate reduction in IGF binding and, simultaneously, the peptides demonstrate increased binding to insulin.

to be a mitogen and to be chemotatic for fibroblast cells (44); IGFBP-rP3 was proposed to be a potential oncogene (183). Subsequent studies have suggested that the IGFBP-rPs have multiple functions (see *Section III*), with no evidence for the direct involvement of IGFs or insulin, although Akaogi *et al.* (37) reported that IGFBP-rP1 enhanced the mitogenic actions of IGF and insulin. The domains responsible for these functions are not known, although for IGFBP-rP2, it has been demonstrated that the CT domain mediates IGFBP-rP2 binding to fibroblast cells, resulting in cell proliferation (166). Interestingly, a truncated form of chicken IGFBP-rP3 (Nov) lacking the N-terminal domain was capable of inducing cellular transformation of CEF cells (183). It is thus predicted that the IGF/insulin-independent actions of the IGFBP-rPs most probably are mediated through the CT domains. Whether the N-terminal domain may participate in these actions has yet to be elucidated and is an area that requires further investigation.

## IX. Evolutionary Relationships Within the IGFBP Superfamily

### A. N-terminal domains

The question arises as to the nature of any evolutionary relationship between the IGFBPs and their related proteins. The conventional IGFBPs appear to be conserved during evolution, as IGFBP activity has been detected in nonmammalian sera, including lamprey serum (57). The structures of these primitive IGFBPs are largely unknown, but, presumably, contain conserved *N*-terminal and, possibly, *C*-terminal regions. Of the IGFBP-rPs, CTGF-like cDNAs have been isolated from the frog (215); *nov* (183) and *cyr61* [*cef-10*, (164)] cDNAs were originally isolated from chicken. Analysis of the N terminus of the predicted protein structures based on these cDNAs indicates that an high degree of similarity exists between the IGFBPs and these human and nonmammalian IGFBP-rPs. Most interestingly, an N-terminal domain-like sequence also is found in the twisted gastrulation protein (Tsg) from the fruit fly *Drosophila* (216). Tsg is a secreted protein of approximately 25 kDa that is involved in embryonic development of *Drosophila*. Both the genomic and cDNA *tsg* subclones are colinear, indicating that the *tsg* gene contains no introns (216). Considered to be a CTGF/IGFBP-rP2-like protein (174, 216), Tsg actually shares only 18% overall similarity to the human and frog IGFBP-rP2/CTGF. The region of most similarity is confined to the N-terminal domain. An alignment of the N-terminal domains from Tsg and the human IGFBP superfamily (Fig. 4), with the corresponding similarity matrix (Table 3), indicates that the Tsg N-terminal domain shares 44% and 34%–50% similarity to the N termini of IGFBPs and IGFBP-rPs, respectively.

Since arthropods (like *Drosophila*) evolved well before vertebrates, it is clear that the N-terminal domain is ancient. As the amino acid residues of the N terminus share a significant similarity, a phylogenetic tree of the N-terminal domains can be generated (Cladogram, DNA Star program), that indicates the number of amino acid substitution events leading to divergence (Fig. 8). IGFBP-rP6 diverged from the rest of the proteins early on the evolutionary scale, whereas the point of divergence for the CCN proteins (IGFBP-rP2-rP4), Tsg, IGFBP-rP5, and the IGFBPs probably occurred later, and at relatively close points in evolutionary time, based on an estimated 52 substitution events for each branch in total.

### B. C-terminal domains

The ancient nature of the N-terminal domain indicates that there were constraints in the evolution of this domain. In contrast, the lack of similarity in the C termini among members of the IGFBP superfamily suggests that the evolution of the C termini is complex and may be independent from that of the N-terminal domain. Unlike the N-terminal domains, there is no similarity among the C-terminal domains from the different proteins/families of the IGFBP superfamily. The IGFBPs share a common C-terminal domain, although their midregions are distinct. In the C-terminal domains, a subdomain shares 37% similarity to the thyroglobulin type I domain. The thyroglobulin type I domain spans the amino acid sequence encompassing the highly conserved Cys-Trp-Cys-Val residues. This domain is found in a number of proteins with quite diverse physiological functions (see *Section II*). Within some of these proteins (such as ascidian nidogen and equistatin), there are more than one copy of this domain.

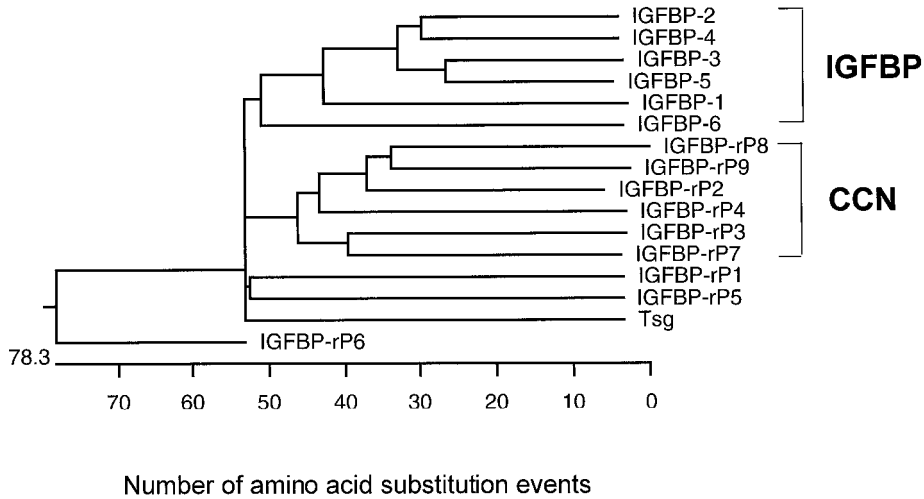


FIG. 8. Phylogenetic tree (Cladogram) of the N-terminal domains of the IGFBP superfamily. The N-terminal domains, human and Tsg protein from *Drosophila*, were aligned using the Clustral method (see Fig. 4), and a Cladogram was generated, based on the alignment (DNA STAR program). Small gaps were introduced to optimize alignment. The x-axis indicates the number of amino acid substitution events.

No specific function has been associated with this domain. Whether this domain, like the IGFBP N-terminal domain, can bind IGF has yet to be evaluated. Thus, for the present, proteins carrying the thyroglobulin type I domain have not been included as part of the IGFBP superfamily. The fact that this domain is found in a protein (equistatin, an inhibitor of cysteine proteinase) from the sea anemone *Actinia equina* (94) indicates that this domain is ancient and may even be older than the IGFBP N-terminal domain, since the sea anemone evolved well before the arthropods.

The IGFBP-rPs clearly have very different C-terminal domains from the IGFBPs and from each other (see *Section II* above and Fig. 5). In IGFBP-rP1, the Ig-like domain at the C terminus is one of the most common of rudimentary protein modules, found not only in antibodies and T cell receptors, but also in hundreds of other animal proteins involved in cell-cell attachment and communications, and even in several bacterial proteins (206). Proteins containing the Ig domain are considered part of the Ig superfamily (206). In the CCN family (IGFBP-rP2 to IGFBP-rP4), the three other protein domains are conserved in the frog IGFBP-rP2 (CTGF), and each domain can also be found in many other unrelated proteins (21). Interestingly, the thrombospondin domain is part of the TRAP protein from *Plasmodium falciparium*, a malarial parasite (21). Finally, the serine protease domain in IGFBP-rP5 shares a surprisingly high similarity with the bacterial HtrA protease. Clearly, each C-terminal protein domain in the IGFBP-rPs not only is modular, but is phylogenetically ancient.

### C. Evolutionary models

The structural modularity at both the genomic and protein levels suggests that the evolution of the IGFBPs and IGFBP-rPs can be examined in two ways: 1) the evolution of the conserved N-terminal domain; and 2) the evolution of the IGFBP superfamily. The evolutionary relationship among the N-terminal domains has been discussed above. More complex is the question of how the N-terminal domain was retained in the IGFBP superfamily as the remainder of each protein apparently diversified.

The evolutionary sequence of events that could account for the array of IGFBPs and IGFBP-rPs are shown in Fig. 9, and include (A) the IGFBPs and related proteins share a common ancestral gene, which over time, selectively retained the sequences encoding the N-terminal domain, while the remaining sequences evolved to encode for the various midsegments and C termini of the IGFBPs and IGFBP-rPs; (B) the N-terminal domain was originally part of the gene encoding the IGFBP family and, through gene fusion, was subsequently acquired by the genes encoding the IGFBP-rP; (C) a gene encoding an IGFBP-rP originally carried the N-terminal domain DNA sequence, which was later acquired by the genes encoding for the IGFBP family and other IGFBP-rPs; or (D) the DNA sequence for the N-terminal domain constitutes a module which, in the course of evolution, has been disseminated to genes by various DNA recombinational events.

The striking similarity of the N-terminal domains and the fact that they are encoded by a single exon in all IGFBPs and

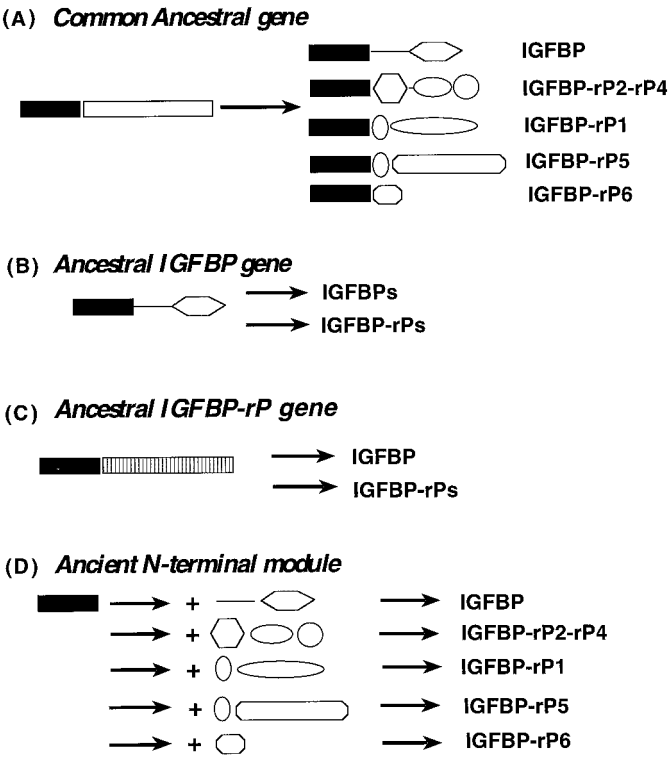


FIG. 9. Mechanisms of evolution of the IGFBP superfamily. The possible scenarios and explanation of each model is in the text. The protein modules are the same as in Fig. 5.

IGFBP-rPs strongly suggest that alternative D above, with a modular N-terminal domain, was a critical part of the evolutionary process. This would be consistent with the general "rule" (206) that genes with well delineated domains set off by introns have been fashioned more recently (within the last half billion years or so), and that exon shuffling was probably involved in the dissemination of, in this case, the N-terminal domain. Furthermore, the other domains are also modular, although, unlike the N-terminal domain, not all are encoded within one exon; exon shuffling, therefore, is unlikely to be involved in their formation. Domain shuffling (28, 206), however, is thus strongly suggested as a mechanism for the evolution of the IGFBP superfamily.

## X. Summary

Over the last decade, the concept of an IGFBP family has been well accepted, based on structural similarities and on functional abilities to bind IGFs with high affinities. The existence of other potential IGFBPs was left open. The discovery of proteins with N-terminal domains bearing striking structural similarities to the N terminus of the IGFBPs, and with reduced, but demonstrable, affinity for IGFs, raised the question of whether these proteins were "new" IGFBPs (22, 23, 217). The N-terminal domain had been uniquely associated with the IGFBPs and has long been considered to be critical for IGF binding. No other function has been confirmed for this domain to date. Thus, the presence of this important IGFBP domain in the N terminus of other proteins must be considered significant. Although these other pro-

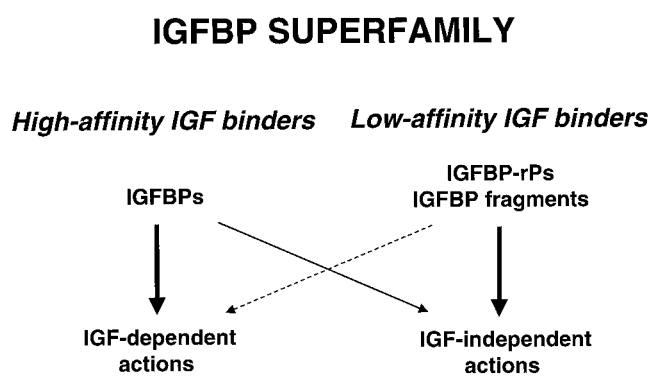


FIG. 10. The IGFBP superfamily is divided into two subclasses, high-affinity IGF binders (IGFBPs), and low-affinity IGF binders (IGFBP-rPs and proteolysed IGFBPs). The IGFBPs are known to mediate biological functions mainly through IGF-dependent mechanisms and also through IGF-independent mechanisms. IGFBP-rPs and IGFBP fragments, on the other hand, mediate biological effects mainly by IGF-independent mechanisms and may also act through IGF-dependent mechanisms.

teins appear capable of binding IGF, their relatively low affinity and the fact that their major biological actions are likely to not directly involve the IGF peptides suggest that they probably should not be classified within the IGFBP family as provisionally proposed (22, 23). The conservation of this single domain, so critical to high-affinity binding of IGF by the six IGFBPs, in all of the IGFBP-rPs, as well, speaks to its biological importance. Historically, and perhaps, functionally, this has led to the designation of an "IGFBP superfamily".

The classification and nomenclature for the IGFBP superfamily, are, of course, arbitrary; what is ultimately relevant is the underlying biology, much of which still remains to be deciphered. The nomenclature for the IGFBP related proteins was derived from a consensus of researchers working in the IGFBP field (52). Obviously, a more general consensus on nomenclature, involving all groups working on each IGFBP-rP, has yet to be reached. Further understanding of the biological functions of each protein should help resolve the nomenclature dilemma. For the present, redesignating these proteins IGFBP-rPs simplifies the multiple names already associated with each IGFBP related protein, and reinforces the concept of a relationship with the IGFBPs.

Beyond the N-terminal domain, there is a lack of structural similarity between the IGFBP-rPs and IGFBPs. The C-terminal domains do share similarities to other internal domains found in numerous other proteins. For example, the similarity of the IGFBP C terminus to the thyroglobulin type-I domain shows that the IGFBPs are also structurally related to numerous other proteins carrying the same domain (87). Interestingly, the functions of the different C-terminal domains in members of the IGFBP superfamily include interactions with the cell surface or ECM, suggesting that, even if they share little sequence similarities, the C-terminal domains may be functionally related.

The evolutionary conservation of the N-terminal domain and functional studies support the notion that IGFBPs and IGFBP-rPs together form an IGFBP superfamily. A superfamily delineates between closely related (classified as a family) and distantly related proteins. The IGFBP superfamily is therefore composed of distantly related families. The modular nature of the constituents of the IGFBP superfamily, particularly their preservation of an highly conserved N-terminal domain, seems best explained by the process of exon shuffling of an ancestral gene encoding this domain. Over the course of evolution, some members evolved into high-affinity IGF binders and others into low-affinity IGF binders, thereby conferring on the IGFBP superfamily the ability to influence cell growth by both IGF-dependent and IGF-independent means (Fig. 10).

A final word, from Stephen Jay Gould (218):

"But classifications are not passive ordering devices in a world objectively divided into obvious categories. Taxonomies are human decisions imposed upon nature—theories about the causes of nature's order. The chronicle of historical changes in classification provides our finest insight into conceptual revolutions in human thought. Objective nature does exist, but we can converse with her only through the structure of our taxonomic systems.

"We may grant this general point, but still hold that certain fundamental categories present so little ambiguity that basic divisions must be invariant across time and culture. Not so—not for these, or for any subjects. Categories are human impositions upon nature (though nature's factuality offers hints and suggestions in return)."

## References


1. **Salmon W, Daughaday W** 1957 A hormonally controlled serum factor which stimulates sulfation incorporation by cartilage *in vitro*. J Lab Clin Med 49:825–836
2. **Froesch ER, Muller WA, Burgi H, Waldvogel M, Labhart A** 1966 Nonsuppressible insulin-like activity of human serum. II. Biological properties of plasma extracts with nonsuppressible insulin-like activity. Biochim Biophys Acta 121:360–374
3. **Dulak NC, Temin HM** 1973 A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-simulating activity fro embryo fibroblasts. J Cell Physiol 81:153–160
4. **Daughaday WH, Hall K, Raben MS, Salmon Jr WD, van den Brande JL, van Wyk JJ** 1972 Somatomedin: proposed designation for sulphation factor. Nature 235:107
5. **Daughaday W, Rotwein P** 1989 Insulin-like growth factors I and II peptide messenger ribonucleic acid and gene structures, serum and tissue concentrations. Endocr Rev 10:68–91
6. **Rosenfeld R, Wilson D, Lee P, Hintz R** 1986 Insulin-like growth factors I and II in the evaluation of growth retardation. J Pediatr 109:428
7. **Rosenfeld R** 1987 Somatomedin action and tissue growth-factor receptors. In: Robbins R, Melmed S (eds) Acromegaly. Plenum Press, New York, pp 45–53
8. **Lowe L** 1991 Biological actions of the insulin-like growth factors. In: LeRoith D (ed) Insulin-like Growth Factors: Molecular and Cellular Aspects. CRC Press, Boca Raton, FL, pp 49–85
9. **Rotwein P** 1991 Structure, evolution, expression and regulation of insulin-like growth factors I and II. Growth Factors 5:3–18
10. **Jones JI, Clemmons DR** 1995 Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3–34
11. **Nissley P, Lopaczynski W** 1991 Insulin-like growth factor receptors. Growth Factors 5:29–43
12. **Kornfeld S** 1992 Structure and function of the mannose 6-phosphate/insulin-like growth factor II receptors. Annu Rev Biochem 61:307–330
13. **Oh Y, Yamanaka Y, Wilson E, Kim H-S, Vorwerk P, Hwa V, Spagnoli A, Wanek D, Rosenfeld RG** 1998 IGF-independent actions of IGFBPs. In: Takano K, Hizuka N, Takahashi S-I (eds)

Molecular Mechanisms to Regulate the Activities of Insulin-Like Growth Factors. Elsevier, Amsterdam, pp 123–133
14. **Jones JI, Gockerman A, Busby Jr WH, Wright G, Clemmons DR** 1993 Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the $\alpha 5\beta 1$ integrin receptor by means of its Arg-Gly-Asp sequence. Proc Natl Acad Sci USA 90:10553–10557
15. **Oh Y, Muller HL, Pham H, Rosenfeld RG** 1993 Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. J Biol Chem 268:26045–26048
16. **Andress D** 1995 Heparin modulates the binding of insulin-like growth factor (IGF) binding protein-5 to a membrane protein in osteoblastic cells. J Biol Chem 270:28289–28296
17. **Mohan S, Nakao Y, Honda Y, Landale E, Leser U, Dony C, Lang K, Baylink DJ** 1995 Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. J Biol Chem 270:20424–20431
18. **Oh Y, Muller HL, Lamson G, Rosenfeld RG** 1993 Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. J Biol Chem 268:14964–14971
19. **Oh Y, Gucev Z, Ng L, Muller HL, Rosenfeld RG** 1996 Antiproliferative actions of insulin-like growth factor binding protein (IGFBP)-3 in human breast cancer cells. Prog Growth Factor Res 6:503–512
20. **Rajah R, Valentinis B, Cohen P** 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-$\beta 1$ on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem 272:12181–12188
21. **Bork P** 1993 The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett 327:125–130
22. **Oh Y, Nagalla SR, Yamanaka Y, Kim H-S, Wilson E, Rosenfeld RG** 1996 Synthesis and characterization of insulin-like growth factor binding protein (IGFBP-7). J Biol Chem 271:30322–30325
23. **Kim H-S, Nagalla SR, Oh Y, Wilson E, Roberts Jr CT, Rosenfeld RG** 1997 Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. Proc Natl Acad Sci USA 94:12981–12986
24. **Hwa V, Oh Y, Rosenfeld RG** 1999 Insulin-like growth factor binding proteins: a proposed superfamily. Acta Paediatr Suppl 428: 37–45
25. **Kelley K, Oh Y, Gargosky S, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson D, Rosenfeld R** 1996 Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. Int J Biochem Cell Biol 28:619–637
26. **Rajaram S, Baylink DJ, Mohan S** 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr Rev 18:801–831
27. **Dayhoff MO** 1978 Atlas of Protein Sequence and Structure. National Biomedical Research Foundation, Washington, DC, p 5
28. **Li W-H** 1997 Molecular Evolution. Sinauer Associates, Inc., Sunderland, MA
29. **Rosenfeld RG, Lamson G, Pham H, Oh Y, Conover C, Leon DDD, Donovan SM, Ocrant I, Guidice L** 1990 Insulin-like growth factor-binding proteins. Recent Prog Horm Res 46:99–163
30. **Luthman H, Söderling-Barros J, Persson B, Engberg C, Stern I, Lake M, Franzén S-Å, Israelsson M, Rådén B, Lindgren B, Hjelmqvist L, Enerbäck S, Carlsson P, Bjursell G, Póvoa G, Hall K, Jörnvall H** 1989 Human insulin-like growth-factor-binding protein: low-molecular-mass form: protein sequence and cDNA cloning. Eur J Biochem 180:259–265
31. **Lee Y-L, Hintz RL, James PM, Lee PDK, Shively JE, Powell DR** 1988 Insulin-like growth factor (IGF) binding protein complementary deoxyribonucleic acid from human HEP G2 hepatoma cells: predicted protein sequence suggests an IGF binding domain different from those of the IGF-I and IGF-II receptors. Mol Endocrinol 2:404–411
32. **Brinkman A, Groffen CA, Kortleve DJ, Geurts van Kessel A, Drop SL** 1988 Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IBP-1). EMBO J 7:2417–2423
33. **Grundmann U, Nerlich C, Bohn H, Rein T** 1988 Cloning of cDNA encoding human placental protein 12 (PP12): Binding protein for IGF-1 and somatomedin. Nucleic Acids Res 16:8711
34. **Brewer MT, Stetler GL, Squires CH, Thompson RC, Busby WH, Clemmons DR** 1988 Cloning, characterization, and expression of a human insulin-like growth factor binding protein. Biochem Biophys Res Commun 152:1289–1297
35. **Julkunen M, Koistinen R, Aalto-Setälä K, Seppälä M, Jänne OA, Kontula K** 1988 Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue-specific expression of its mRNA. FEBS Lett 236:295–302
36. **Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M** 1986 Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding studies. Anal Biochem 154:138–143
37. **Akaogi K, Sato J, Okabe Y, Sakamoto Y, Yasumitsu H, Miyazaki K** 1996 Synergistic growth stimulation of mouse fibroblasts by tumor-derived adhesion factor with insulin-like growth factors and insulin. Cell Growth Differ 7:1671–1677
38. **Burren CP, Wilson EM, Hwa V, Oh Y, Rosenfeld RG** 1999 Binding properties and distribution of insulin-like growth factor binding protein-related protein 3 (IGFBP-rP3/NovH), an additional member of the IGFBP superfamily. J Clin Endocrinol Metab 84:1096–1103
39. **Kumar S, Hand AT, Connor JR, Dodds RA, Ryan PJ, Trill JJ, Fisher SM, Nuttall ME, Lipshutz DB, Zou C, Hwang SM, Votta BJ, James IE, Rieman DJ, Gowen M, Lee JC** 1999 Identification and cloning of a connective tissue growth factor-like cDNA from human osteoblasts encoding a novel regulator of osteoblast functions. J Biol Chem 274:17123–17131
40. **Murphy M, Pykett MJ, Harnish P, Zang KD, George DL** 1993 Identification and characterization of genes differentially expressed in meningiomas. Cell Growth Differ 4:715–722
41. **Yamauchi T, Umeda F, Masakado M, Isaji M, Mizushima S, Nawata H** 1994 Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. Biochem J 303:591–598
42. **Akaogi K, Okabe Y, Funahashi K, Yoshitake Y, Nishikawa K, Yasunitsu H, Umeda M, Miyazaki K** 1994 Cell adhesion activity of a 30-kDa major secreted protein from human bladder carcinoma cells. Biochem Biophys Res Commun 198:1046–1053
43. **Jay P, Berge-Lefranc J, Marsollier C, Mejean C, Taviaux S, Berta P** 1997 The human growth factor-inducible immediate early gene, CYR61, maps to chromosome 1p. Oncogene 14:1753–1757
44. **Bradham DM, Igarashi A, Potter RL, Grotendorst GR** 1991 Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J Cell Biol 114:1285–1294
45. **Martinerie C, Viegas-Pequignot E, Guenard I, Dutrillaux B, Nguyen V, Bernheim A, Perbal B** 1992 Physical mapping of human loci homologous to the chicken nov proto-oncogene. Oncogene 7:2529–2534
46. **Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, Melhem MF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D, Levine AJ** 1998 WISP genes are members of the connective tissue growth factor family that are up-regulated in Wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc Natl Acad Sci USA 95:14717–14722
47. **Zhang R, Averboukh L, Zhu W, Zhang H, Jo H, Dempsey PJ, Coffey RJ, Pardee AB, Liang P** 1998 Identification of rCop-1, a new member of the CCN protein family, as a negative regulator for cell transformation. Mol Cell Biol 18:6131–6141
48. **Hashimoto Y, Shindo-Okada N, Tani M, Nagamachi Y, Takeuchi K, Shiroishi T, Toma H Yokota J** 1998 Expression of the *Elm1* gene, a novel gene of the CCN (connective tissue growth factor, Cyr61/Cef10, and neuroblastoma overexpressed gene) family, suppresses *in vivo* tumor growth and metastatis of K-1735 murine melanoma cells. J Exp Med 187:289–296
49. **Zumbrunn J, Trueb B** 1996 Primary structure of a putative serine protease specific for IGF-binding proteins. FEBS Lett 398:187–192
50. **Hu S-I, Carozza M, Klein M, Nantermet P, Luk D, Crowl RM** 1998 Human HtrA, an evolutionarily conserved serine protease identi-

fied as a differentially expressed gene product in osteoarthritic cartilage. J Biol Chem 273:34406–34412
51. **Lassalle P, Molet S, Janin A, Heyden JV, Tavernier J, Fiers W, Devos R, Tonnel A-B** 1996 ESM-1 is a novel human endothelial cell-specific molecule expressed in lung and regulated by cytokines. J Biol Chem 271:20458–20464
52. **Baxter RC, Binoux M, Clemmons DR, Conover C, Drop SLS, Holly JMP, Mohan S, Oh Y, Rosenfeld RG** 1998 Recommendations for nomenclature for the insulin-like growth factor binding protein (IGFBP) superfamily. J Clin Endocrinol Metab 83:3213
53. **Shimasaki S, Ling N** 1991 Identification and molecular characterization of insulin-like growth factor binding Proteins (IGFBP-1, -2, -3, -4, -5 and -6). Prog Growth Factor Res 3:243–266
54. **Schoen TJ, Mazuruk K, Waldbillig RJ, Potts J, Beebe DC, Chader GJ, Rodriguez IR** 1995 Cloning and characterization of a chick embryo cDNA and gene fro IGF-binding protein-2. J Mol Endocrinol 15:49–59
55. **Allander SV, Ehrenborg E, Luthman H, Powell DR** 1995 Conservation of IGFBP structure during evolution: cloning of chicken insulin-like growth factor binding protein-5. Prog Growth Factor Res 6:159–165
56. **James PL, Jones SB, Busby WH, Clemmons DR, Rotwein P** 1993 A highly-conserved insulin-like growth factor-binding protein (IGFBP-5) is expressed during myoblast differentiation. J Biol Chem 266:22305–22312
57. **Upton Z, Chan SJ, Steiner DF, Wallace JC, Ballard FJ** 1993 Evolution of insulin-like growth factor binding proteins. Growth Regul 3:29–32
58. **Reinecke M, Collet C** 1998 The phylogeny of the insulin-like growth factors. Int Rev Cytol 183:1–94
59. **Thompson JD, Higgins DG, Gibson TJ** 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
60. **Shimasaki S, Gao L, Shimonaka M, Ling N** 1991 Isolation and molecular cloning of insulin-like growth factor-binding protein-6. Mol Endocrinol 5:938–948
61. **Neumann GM, Marinaro JA, Bach LA** 1998 Identification of O-glycosylation sites and partial characterization of carbohydrate structure and disulfide linkages of human insulin-like growth factor binding protein 6. Biochem J 37:6572–6585
62. **Forbes BE, Turner D, Hodge SJ, McNeil KA, Forsberg G, Wallace JC** 1998 Localization of an insulin-like growth factor (IGF) binding site of bovine IGF binding protein-2 using disulfide mapping and deletion mutation analysis of the C-terminal domain. J Biol Chem 273:4647–4652
63. **Neumann G, Bach L** 1999 The N-terminal disulfide linkages of human insulin-like growth factor-binding protein-6 (hIGFBP-6) and hIGFBP-1 are different as determined by mass spectrometry. J Biol Chem 274:14587–14594
64. **Hashimoto R, Ono M, Fujiwara H, Higashihashi N, Yoshida M, Enjoh-Kimura T, Sakano K-i** 1997 Binding sites and binding properties of binary and ternary complexes of insulin-like growth factor-II (IGF-II), IGF-binding protein-3, and acid-labile subunit. J Biol Chem 272:27936–27942
65. **Kalus W, Zweckstetter M, Renner C, Sanchez Y, Georgescu J, Grol M, Demuth D, Schumacher R, Dony C, Land K, Holak TA** 1998 Structure of the IGF-binding domain of the insulin-like growth factor-binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions. EMBO J 17:6558–6572
66. **Rodakis G, Moschonas N, Regier J, Kafatos F** 1983 The B multigene family of chorion proteins in saturniid silkmoths. J Mol Evol 19:322–332
67. **Hatfull GF, Sarkis GJ** 1993 DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. Mol Microbiol 7:395–405
68. **Jordi BJ, Kremers DA, Kusters HG, van der Zeijst BA** 1989 Nucleotide sequence of the gene coding for the peplomer protein (= spike protein) of infectious bronchitis virus, strain D274. Nucleic Acids Res 17:6726
69. **Evans IM, Gatehouse LN, Gatehouse JA, Robinson NJ, Croy RR** 1990 A gene from pea (*Pisum sativum* L.) with homology to metallothion genes. FEBS Lett 262:29–32
70. **Bohn H, Johannsen R, Kraus W** 1980 New placental protein (PP15) with immunosuppressive properties. Arch Gynecol 230:167–172
71. **Zapf J, Born W, Chang JY, James P, Froesch ER, Fischer JA** 1988 Isolation and NH2-terminal amino acid sequences of rat serum carrier proteins for insulin-like growth factors. Biochem Biophys Res Commun 156:1187–1194
72. **Ceda GP, Fielder PJ, Henzel WJ, Louie A, Donovan SM, Hoffman AR, Rosenfeld RG** 1991 Differential effects of insulin-like growth factor (IGF)-I and IGF-II on the expression of IGF binding proteins (IGFBPs) in a rat neuroblastoma cell line: isolation and characterization of two forms of IGFBP-4. Endocrinology 128:2815–2824
73. **Bach LA, Thotakura NR, Rechler MM** 1992 Human insulin-like growth factor binding protein-6 is O-glycosylated. Biochem Biophys Res Commun 186:301–307
74. **Conover CA, Kiefer MC** 1993 Regulation and biological effect of endogenous insulin-like growth factor binding protein-5 in human osteoblastic cells. J Clin Endocrinol Metab 76:1153–1159
75. **Bach LA, Tseng LY, Swartz JE, Rechler MM** 1993 Rat PC12 pheochromocytoma cells synthesize insulin-like growth factor-binding protein-6. Endocrinology 133:990–995
76. **Claussen M, Buergisser D, Schuller AG, Matzner U, Braulke T** 1995 Regulation of insulin-like growth factor (IGF)-binding protein-6 and mannose 6-phosphate/IGF-II receptor expression in IGF-IL-overexpressing NIH 3T3 cells. Mol Endocrinol 9:902–912
77. **Coverley JA, Baxter RC** 1997 Phosphorylation of insulin-like growth factor binding proteins. Mol Cell Endocrinol 128:1–5
78. **Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR** 1991 Phosphorylation of insulin-like growth factor (IGF)-binding protein-1 in cell culture and *in vivo*: effects on affinity for IGF-I. Proc Natl Acad Sci USA 88:7481–7485
79. **Westwood M, Gibson JM, White A** 1997 Purification and characterization of the insulin-like growth factor-binding protein-1 phosphoform found in normal plasma. Endocrinology 138:1130–1136
80. **Frost RA, Tseng L** 1991 Insulin-like growth factor binding protein-1 is phosphorylated by cultured human endometrial cells and multiple protein kinases *in vitro*. J Biol Chem 266:18082–18088
81. **Jones JI, Busby WH, Wright G, Smith CE, Kimack NM, Clemmons DR** 1993 Identification of the sites of phosphorylation in sinulin-like growth factor binding protein-1: regulation of its affinity by phosphorylation of serine 101. J Biol Chem 268:1125–1131
82. **Peterkofsky B, Gosiewska A, Wilson S, Kim YR** 1998 Phosphorylation of rat insulin-like growth factor binding protein-1 does not affect its biological properties. Arch Biochem Biophys 357:101–110
83. **Hoeck WG, Mukku VR** 1994 Identification of the major sites of phosphorylation in IGF binding protein-3. J Cell Biochem 56:262–273
84. **Coverley JA, Baxter RC** 1995 Regulation of insulin-like growth factor (IGF) binding protein-3 phosphorylation by IGF-I. Endocrinology 136:5778–5781
85. **Jones JI, Gockerman A, Clemmons DR**, Insulin-like growth factor binding protein-5 (IGFBP-5) binds to extracellular matrix and is phosphorylated. Program of the 74th Annual Meeting of The Endocrine Society, San Antonio, TX, 1992 (Abstract) p 372
86. **Malthiery Y, Lissitzky S** 1987 Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary DNA. Eur J Biochem 165:491–498
87. **Lenarcic B, Bevec T** 1998 Thyropins — new structurally related proteinase inhibitors. Biol Chem 379:105–111
88. **Bevec T, Stoka V, Pungercic G, Dolenc I, Turk V** 1996 Major histocompatibility compex class II-associated p41 invariant chain fragment is a strong inhibitor of lysosomal cathepsin L. J Exp Med 183:1331–1338
89. **Mann K, Deutzmann R, Aumailley M, Timpl R, Raimondi L, Yamada Y, Pan T, Conway D, Chu M** 1989 Amino acid sequence of mouse nidogen, a multidomain basement membrane protein with binding activity for laminin, collagen IV and cells. EMBO J 8:65–72
90. **Morabito MA, Moczydlowski E** 1994 Molecular cloning of bullfrog saxipholin: a unique relative of the transferrin family that binds saxitoxin. Proc Natl Acad Sci USA 91:2478–2482
91. **Morabito MA, Moczydlowski E** 1995 Molecular cloning of bull-

frog saxiphilin: a unique relative of the transferrin family that binds saxitoxin. (Correction). Proc Natl Acad Sci USA 92:6651
92. **Szala S, Froehlich M, Scollon M, Kasai Y, Steplewski S, Koprowski H, Linnenbach A** 1990 Molecular cloning of cDNA for the carcinoma-associated antigen GA733–2. Proc Natl Acad Sci USA 87:3542–3546
93. **Yamashita M, Konagaya S** 1996 A novel cysteine protease inhibitor of the egg of Chum salmon, containing a cysteine-rich thyroglobulin-like motif. J Biol Chem 271:1282–1284
94. **Lenarcic B, Ritonja A, Strukelj B, Turk B, Turk V** 1997 Equistatin, a new inhibitor of cysteine proteinases from *Actinia equina*, is structurally related to thyroglobulin type-I domain. J Biol Chem 272: 13899–13903
95. **Kimura N, Toyoshima T, Kojima T, Shimane M** 1998 Entactin-2: a new member of basement membrane protein with high homology to entactin/nidogen. Exp Cell Res 241:36–45
96. **Jones JL, Doerr ME, Clemmons DR** 1995 Cell migration: interactions among integrins, IGFs and IGFBPs. Prog Growth Factor Res 6:319–327
97. **Booth B, Boes M, Dake B, Linhardt R, Caldwell E, Weiler J, Bar R** 1996 Structure-function relationships in the heparin-binding c-terminal region of insulin-like growth factor binding protein-3. Growth Regul 6:206–213
98. **Fowlkes JL, Thrailkill KM, George-Nascimento C, Rosenberg CK, Serra DM** 1997 Heparin-binding, highly basic regions within the thyroglobulin type-I repeat of insulin-like growth factor (IGF)-binding protleins (IGFBPs) -3, -5, and -6 inhibit IGFBP-4 degradation. Endocrinology 138:2280–2285
99. **Smith E, Lu L, Chernausek S, Klein D** 1994 Insulin-like growh factor-binding protein-3 (IGFBP-3) concentration in rat Sertoli cell-conditioned medium is regulated by a pathway involving association of IGFBP-3 with cell surface proteoglycans. Endocrinology 135:359–364
100. **Jones JI, Gockerman A, Busby Jr WH, Camacho-Hubner C, Clemmons DR** 1993 Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. J Cell Biol 121:679–687
101. **Giudice L, Farrell E, Pham H, Lamson G, Rosenfeld R** 1990 Insulin-like growth factor binding proteins in maternal serum throughout gestation and in the puerperium: effects of a pregnancy-associated serum protease activity. J Clin Endocrinol Metab 71:806–816
102. **Cohen P, Graves H, Peehl D, Kamerei M, Giudice L, Rosenfeld R** 1992 Prostate specific antigen is an IGF binding protein-3 (IGFBP-3) protease found in seminal plasma. J Clin Endocrinol Metab 75:1046–1053
103. **Cohen P, Peehl D, Grave H, Rosenfeld R** 1994 Biological effects of prostate specific antigen (PSA) as an IGF binding protein-3 (IGFBP-3) protease. J Endocrinol 142:407–415
104. **Clemmons DR, Busby WH, Arai T, Nam TJ, Clarke JB, Jones JI, Ankrapp DK** 1995 Role of insulin-like growth factor binding proteins in the control of IGF actions. Prog Growth Factor Res 6:357–366
105. **Collett-Solberg PF, Cohen P** 1996 The role of the insulin-like growth factor binding proteins and the IGFBP proteases in modulating IGF action. Endocrinol Metab Clin North Am 25:591–614
106. **Hossenlopp P, Segovia B, Lassare C, Roghani M, Bredon M, Binoux M** 1990 Evidence of enzymatic degradation of the insulin-like growth factor binding proteins in the 150K complex during pregnancy. J Clin Endocrinol Metab 71:797–805
107. **Fielder P, Rosenfeld R, Graves H, Grandboist K, Maack C, Sawamura S, Ogawa Y, Sommer A, Cohen P** 1994 Biochemical analysis of prostate specific antigen-proteolyzed insulin-like growth factor binding protein-3. Growth Regul 1:164–172
108. **Lalou C, Lassarre C, Binoux M** 1996 A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. Endocrinology 137:3206–3212
109. **Lalou C, Sawamura S, Segovia B, Ogawa Y, Binoux M** 1997 Proteolytic fragments of insulin-like growth factor binding protein-3: N-terminal sequences and relationships between structure and biological activity. C R Acad Sci III 320:621–628
110. **Vorwerk P, Yamanaka Y, Spagnoli A, Oh Y, Rosenfeld RG** 1998 Insulin and IGF binding by IGFBP-3 fragments derived from proteolysis, baculovirus expression and normal human urine. J Clin Endocrinol Metab 83:1392–1395
111. **Cheung P-T, Wu J, Banach W, Chernausek SD** 1994 Glucocorticoid regulation of an insulin-like growth factor-binding protein-4 protease produced by a rat neuronal cell line. Endocrinology 135: 1328–1335
112. **Chernausek SD, Smith CE, Duffin KL, Busby WH, Wright G, Clemmons DR** 1995 Proteolytic cleavage of insulin-like growth factor binding protein 4 (IGFBP-4): localization of cleavage sit to non-homologous region of native IGFBP-4. J Biol Chem 270:11377–11382
113. **Andress DL, Birnbaum RS** 1992 Human osteoblast-derived insulin-like growth factor (IGF) binding protein 5 stimulates osteoblast mitogenesis and potentiates IGF action. J Biol Chem 267:22467–22472
114. **Andress DL, Loop SM, Zapf J, Kiefer MC** 1993 Carboxy-truncated insulin-like growth factor binding protein-5 stimulates mitogenesis in osteoblast-like cells. Biochem Biophys Res Commun 195:25–30
115. **Spencer EM, Chan K** 1995 A 3-dimensional model for the insulin-like growth factor binding proteins (IGFBPs); supporting evidence using the structural determinants of the IGF binding site on IGFBP-3. Prog Growth Factor Res 6:209–214
116. **Baxter RC, Firth SM** 1995 Modulations of human IGF binding protein-3 activity by structural modification. Prog Growth Factor Res 6:215–222
117. **Firth SM, Ganeshprasad U, Baxter RC** 1998 Structural determinants of ligand and cell surface binding of insulin-like growth factor-binding protein-3. J Biol Chem 273:2631–2638
118. **Yamanaka Y, Wilson EM, Rosenfeld RG, Oh Y** 1997 Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. J Biol Chem 272:30729–30734
119. **Qin X, Strong DD, Baylink DJ, Mohan S** 1998 Structure-function analysis of the human insulin-like growth factor binding protein-4. J Biol Chem 273:23509–23516
120. **Hobba GD, Forbes BE, Parkinson EJ, Francis GL, Wallace JC** 1996 The insulin-like growth factor (IGF) binding site of bovine insulin-like growth factor binding protein-2 (bIGFBP-2) probed by iodination. J Biol Chem 271:30529–30536
121. **Hobba GD, Lothgren A, Holmberg E, Forbes BE, Francis GJ, Wallace JC** 1998 Alanine screening mutagenesis established tyrosine 60 of bovine insulin-like growth factor binding protein-2 as a determinant of insulin-like growth factor binding. J Biol Chem 273:19691–19698
122. **Brinkman A, Kortleve DJ, Zwarthoff EC, Drop SL** 1991 Mutations in the C-terminal part of insulin-like growth factor (IGF)-binding protein-1 result in dimer formation and loss of IGF binding capacity. Mol Endocrinol 5:987–994
123. **Bramani S, Song H, Beattie J, Tonner E, Flint DJ, Allan GJ** 1999 Amino acids within the extracellular matrix (ECM) binding region (201–218) of rat insulin-like growth factor binding protein (IGFBP-5) are important determinants in binding IGF-I. J Mol Endocrinol 23:117–123
124. **Parker A, Rees C, Clarke J, Busby Jr WH, Clemmons DR** 1998 Binding of insulin-like growth factor (IGF)-binding protein 5 to smooth-muscle cell extracellular matrix is a major determinant of the cellular response to IGF-I. Mol Biol Cell 9:2383–2392
125. **Ho PJ, Baxter RC** 1997 Characterization of truncated insulin-like growth factor-binding protein-2 in human milk. Endocrinology 138:3811–3818
126. **Wang JF, Hampton B, Mehlman T, Burgess WH, Rechler MM** 1988 Isolation of a biologically active fragment from the carboxy terminus of the fetal rat binding protein for insulin-like growth factors. Biochem Biophys Res Commun 157:718–726
127. **Yamanaka Y, Fowlkes JL, Rosenfeld RG, Oh Y** 1999 Characterization of insulin-like growth factor binding protein-3 (IGFBP-3) binding to human breast cancer cells. Endocrinology 140:1319–1328
128. **Collett-Solberg PF, Nunn SE, Gibson TB, Cohen P** 1998 Identification of novel high molecular weight insulin-like growth factor-binding protein-3 association proteins in human serum. J Clin Endocrinol Metab 83:2843–2848
129. **Twigg SM, Kiefer MC, Zapf J, Baxter RC** 1998 Insulin-like growth factor-binding protein-5 complexes with the acid-labile subunit. Role of the carboxyl-terminal domain. J Biol Chem 273:28791–28798
130. **Hynes RO** 1992 Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25

131. **Fowlkes J, Serra D** 1996 Characterization of glycosaminoglycan-binding domains present in insulin-like growth factor-binding protein-3. J Biol Chem 271:14676–14679
132. **Parker A, Busby WH, Clemmons DR** 1996 Identification of the extracellular matrix binding site for insulin like growth factor binding protein-5. J Biol Chem 271:13523–13529
133. **Rees C, Clemmons DR** 1998 Inhibition of IGFBP-5 binding to extracellular matric and IGF-I-stimulated DNA synthesis by a peptide fragment of IGFBP-5. J Cell Biochem 71:375–381
134. **Abrass CK, Berfield AK, Andress DL** 1997 Heparin binding domain of insulin-like growth factor binding protein-5 stimulates mesangial cell migration. Am J Physiol 273:F899–F906
135. **Gorlich D, Mattaj IW** 1996 Nucleocytoplasmic transport. Science 271:1513–1518
136. **Radulescu RT** 1994 Nuclear localization signal in insulin-like growth factor-binding protein type 3. Trends Biochem Sci 19:278
137. **Li W, Fawcett J, Widmer HR, Fielder PJ, Rabkin R, Keller G-A** 1997 Nuclear transport of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in opossum kidney cells. Endocrinology 138:1763–1766
138. **Jaques G, Noll K, Wegmann B, Witten S, Kogan E, Radulescu RT, Havemann K** 1997 Nuclear localization of insulin-like growth factor binding protein-3 in a lung cancer cell line. Endocrinology 138:1767–1770
139. **Wraight CJ, Liepe IJ, White PJ, Hibbs AR, Werther GA** 1998 Intranuclear localization of insulin-like growth factor binding protein-3 (IGFBP-3) during cell division in human keratinocytes. J Invest Dermatol 111:239–242
140. **Schedlich LJ, Young TF, Firth SM, Baxter RC** 1998 Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-3 share a common nuclear transport pathway in T47D human breast carcinoma cells. J Biol Chem 273:18347–18352
141. **Imai Y, Busby Jr WH, Smith CE, Clarke JB, Garmong AJ, Horwitz GD, Rees C, Clemmons DR** 1997 Protease-resistant form of insulin-like growth factor-binding protein 5 is an inhibitor of insulin-like growth factor-I actions on porcine smooth muscle cells in culture. J Clin Invest 100:2596–2605
142. **Rees C, Clemmons DR, Horvitz GD, Clarke JB, Busby WH** 1998 A protease-resistant form of insulin-like growth factor (IGF) binding protein 4 inhibits IGF-I actions. Endocrinology 139:4182–4188
143. **Leal SM, Liu Q, Huang SS, Huang JS** 1997 The Type V transforming growth factor b receptor is the putative insulin-like growth factor-binding protein 3 receptor. J Biol Chem 272:20572–20576
144. **Leal SM, Huang SS, Huang JS** 1999 Interactions of high affinity insulin-like growth factor-binding proteins with the type V transforming growth factor-$\beta$ receptor in mink lung epithelial cells. J Biol Chem 274:6711–6717
145. **Andress DL** 1998 Insulin-like growth factor-binding protein-5 (IGFBP-5) stimulates phosphorylation of the IGFBP-5 receptor. Am J Physiol 274:E744–E750
146. **Ehrenborg E, Larsson C, Stern I, Janson M, Powell DR, Luthman H** 1992 Contiguous localization of the genes encoding human insulin-like growth factor binding proteins 1 (IGFBP1) and 3 (IGFBP3) on chromosome 7. Genomics 12:497–502
147. **Swisshelm K, Ryan K, Tsuchiya K, Sager R** 1995 Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. Proc Natl Acad Sci USA 92:4472–4476
148. **Kato MV, Sato H, Tsukada T, Ikawa Y, Aizawa S, Nagayoshi M** 1996 A follistatin-like gene, mac25, may act as a growth suppressor of osteosarcoma cells. Oncogene 12:1361–1364
149. **Burger AM, Zhang X, Li H, Ostrowski JL, Beatty B, Venanzoni M, Papas T, Seth A** 1998 Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. Oncogene 16: 2459–2467
150. **Hwa V, Tomasini-Sprenger C, Bermejo AL, Rosenfeld RG, Plymate SR** 1998 Characterization of insulin-like growth factor binding protein-related protein 1 in prostate cells. J Clin Endocrinol Metab 83:4355–4362
151. **Sprenger CC, Damon SE, Hwa V, Rosenfeld RG, Plymate SR** 1999 Insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) is a potential tumor suppressor protein for prostate cancer. Cancer Res 59:2370–2375
152. **Damon SE, Haugk KL, Swisshelm K, Quinn LS** 1997 Developmental regulation of Mac25/insulin-like growth factor-binding protein-7 expression in skeletal myogenesis. Exp Cell Res 237:192–195
153. **Pereira RC, Canalis E** 1999 Parathyroid hormone increases mac25/insulin-like growth factor-binding protein-related protein-1 expression in cultured osteoblasts. Endocrinology 140:1998–2003
154. **Pereira RC, Blanquaert F, Canalis E** 1999 Cortisol enhances the expression of mac25/insulin-like growth factor-binding protein-related protein-1 in cultured osteoblasts. Endocrinology 140:228–232
155. **Akaogi K, Okabe Y, Sato J, Nagashima Y, Yasumitsu H, Sugahara K, Miyazaki K** 1996 Specific accumulation of tumor-derived adhesion factor in tumor bascular endothelial cells. Proc Natl Acad Sci USA 93:8384–8389
156. **Inoguchi T, Umeda F, Watanabe J, Ibayashi H** 1986 Reducted serum-stimulatory activity on prostacyclin production by cultured aortic endothelial cells in diabetes mellitus. Haemostasis 16:447–452
157. **Inoguchi T, Umeda F, Ono H, Kunisaki M, Watanabe J, Nawata H** 1989 Abnormality in porstacyclin-stimulatory activity in sera from diabetes. Metabolism 38:837–842
158. **de Groot PG, Brinkman HJ, Gonsalves MD, Van Mourik JA** 1985 The role of thrombin in the regulation of the endothelial prostaglandin production. Biochim Biophys Acta 846:342–349
159. **Rosenfeld RG** 1998 Editorial: The blind men and the elephant - a parable for the study of insulin-like growth factor binding proteins. Endocrinology 139:5–7
160. **Masakado M, Umeda F, Takei A, Hashimo T, Sueishi K, Nawata H** 1995 Immunohistochemical localization of a novel peptide, prostacyclin-stimulating factor (PSF), in human tissues. Thromb Haemost 74:1407–1410
161. **Umeda F, Ono Y, Masakado M, Sekiguchi N, Yamauchi T, Hashimoto T, Nawata H** 1996 Prostacyclin-stimulating factor, novel protein, and diabetic angiopathy. Diabetes 45[Suppl 3]:S111–S113
162. **Brigstock DR** 1999 The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev 20:189–206
163. **Lau LF, Lam SC-T** 1999 The CCN family of angiogenic regulators: the integrin connection. Exp Cell Res 248:44–57
164. **Simmons D, Levy D, Yannoni Y, Erikson R** 1989 Identification of a phorbol ester-repressible v-src-inducible gene. Proc Natl Acad Sci USA 86:1178–1182
165. **O'Brien TP, Yang GP, Sanders L, Lau LF** 1990 Expression of cyr61, a growth factor-inducible immediate-early gene. Mol Cell Biol 10: 3569–3577
166. **Brigstock DR, Steffen CL, Kim GY, Vegunta RK, Diehl JR, Harding PA** 1997 Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. J Biol Chem 272:20275–20282
167. **Ball DK, Surveyor GA, Diehl JR, Steffen CL, Uzumcu M, Mirando MA, Brigstock DR** 1998 Characterization of 16- to 20-kilodalton (kDa) connective tissue growth factors (CTGFs) and demonstration of proteolytic activity for 38-kDa CTGF in pig uterine luminal flushings. Biol Reprod 59:828–835
168. **Steffen CL, Ball-Mirth DK, Harding PA, Bhattacharyya N, Pillai S, Brigstock DR** 1998 Characterization of cell-associated and soluble forms of connective tissue growth factor (CTGF) produced by fibroblast cells *in vitro*. Growth Factors 15:199–213
169. **Nakanishi T, Kimura Y, Tamura T, Ichikawa H, Yamaai Y-I, Sugimoto T Takigawa M** 1997 Cloning of a mRNA preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF) mRNA. Biochem Biophys Res Commun 234: 206–210
170. **Nishida T, Nakanishi T, Shimo T, Asano M, Hattori T, Tamatani T, Tezuka K, Takigawa M** 1998 Demonstration of receptors specific for connective tissue growth factor on a human chondrocytic cell line (HCS-2/8). Biochem Biophy Res Commun 247:905–909
171. **Igarashi A, Okoshi H, Bradham DM, Grotendorst GR** 1993 Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. Mol Biol Cell 4:637–645
172. **Grotendorst GR, Okochi H, Hayashi N** 1996 A novel transforming

growth factor $\beta$ response element controls the expression of the connective tissue growth factor gene. Cell Growth Differ 7:469–480
173. **Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR** 1996 Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. J Invest Dermatol 107:404–411
174. **Grotendorst GR** 1997 Connective tissue growth factor: a mediator of TGF-$\beta$ action on fibroblasts. Cytokine Growth Factor Rev 8:171–179
175. **Kothapalli D, Frazier KS, Welply A, Segarini PR, Grotendorst GR** 1997 Transforming growth factor $\beta$ induces anchorage-independent growth of NRK fibroblasts via a connective tissue growth factor-dependent signaling pathway. Cell Growth Differ 8:61–68
176. **Kothapalli D, Hayashi N, Grotendorst GR** 1998 Inhibition of TGF-$\beta$-stimulated CTGF gene expression and anchorage-independent growth by cAMP identifies a CTGF-dependent restriction point in the cell cycle. FASEB J 12:1151–1161
177. **Shimo T, Nakanishi T, Kimura Y, Nishida T, Ishizeki K, Matsumura T, Takigawa M** 1998 Inhibition of endogenous expression of connective tissue growth factor by its antisense oligonucleotide and antisense RNA suppresses proliferation and migration of vascular endothelial cells. J Biochem 124:130–140
178. **Oemar BS and Luscher TF** 1997 Connective tissue growth factor, friend or foe? Arterioscler Thromb Vasc Biol 17:1483–1489
179. **Haustein UF, Anderegg U** 1998 Pathophysiology of scleroderma: an update. J Eur Acad Dermatol Vernereol 11:1–8
180. **Dammeier J, Brauchle M, Falk W, Grotendorst GR, Werner S** 1998 Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? Int J Biochem Cell Biol 30:909–922
181. **Yang D-H, Kim H-S, Wilson EM, Rosenfeld RG, Oh Y** 1998 Identification of glycosylated 38-kDa connective tissue growth factor (IGFBP-related protein 2) and proteolytic fragments in human biological fluids, and up-regulation of IGFBP-rP2 by TGF-beta in Hs578T human breast cancer cells. J Clin Endocrinol Metab 83: 2593–2596
182. **Frazier KS, Grotendorst GR** 1997 Expression of connective tissue growth factor mRNA in the fibrous stroma of mammary tumors. Int J Biochem Cell Biol 29:153–161
183. **Joliot V, Martinerie C, Dambrine G, Plassiart G, Brisac M** 1992 Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type I-induced nephroblastomas. Mol Cell Biol 12:10–21
184. **Martinerie C, Huff V, Joubert I, Badzioch M, Saunders G, Strong L, Perbal B** 1994 Structural analysis of the human nov proto-oncogene and expression in Wilms tumors. Oncogene 9:2729–2732
185. **Martin JL, Baxter RC** 1994 Regulation and actions of the insulin-like growth factor binding proteins. In: Kohler PO (ed) Current Opinion in Endocrinology and Diabetes. Current Science, Philadelphia, pp 16–21
186. **Chevalier G, Yeger H, Martinerie C, Laurent M, Alami J, Schofield PN, Perbal B** 1998 novH: Differential expression in developing kidney and Wilms tumors. Am J Pathol 152:1563–1575
187. **Martinerie C, Chevalier G, Rauscher III FJ, Perbal B** 1996 Regulation of nov by WT1: a potential role for nov in nephrogenesis. Oncogene 12:1479–1492
188. **Scholz G, Martinerie C, Perbal B, Hanafusa H** 1996 Transcriptional down regulation of the nov proto-oncogenein fibroblasts transformed by p60v-src. Mol Cell Biol 16:481–486
189. **Ryseck RP, Macdonald-Bravo H, Mattei MG, Bravo R** 1991 Structure, mapping, and expression of fisp-12, a growth factor-inducible gene encoding a secreted cysteine-rich protein. Cell Growth Differ 2:225–233
190. **Kolesnilova TV, Lau LF** 1998 Human Cyr61-mediated enhancement of bFGF-induced DNA synthesis in human umbilical vein endothelial cells. Oncogene 16:747–754
191. **Schutze N, Lechner A, Groll C, Siggelkow H, Hufner M, Kohrle J, Jakob F** 1998 The human analog of murine cystein rich protein 16 is a 1$\alpha$,25-dihydroxyvitamin D3 responsive immediate early gene in human fetal osteoblasts: regulation by cytokines, growth factors, and serum. Endocrinology 139:1761–1770
192. **Yang GP, Lau LF** 1991 Cyr61, product of a growth factor-inducible immediate early gene, is associated with the extracellular matrix and the cell surface. Cell Growth Differ 2:351–357
193. **Kireeva ML, Mo F-E, Yang GP, Lau LF** 1996 Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. Mol Cell Biol 16:1326–1334
194. **Kireeva ML, Lam SC-T, Lau LF** 1998 Adhesion of human umbilical vein endothelial cells to the immediate-early gene product Cyr61 is mediated through integrin $\alpha_v\beta_3$. J Biol Chem 273:3090–3096
195. **Babic AM, Kireeva ML, Kolesnikova TV, Lau LF** 1998 CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. Proc Natl Acad Sci USA 95:6355–6360
196. **Kireeva ML, Latinkic BV, Kolesnikova TV, Chen CC, Yang GP, Abler AS, Lau LF** 1997 Cyr61 and Fisp12 are both ECM-associated signaling molecules:activities, metabolism, and localization during development. Exp Cell Res 233:63–77
197. **Wong M, Kireeva ML, Kolesnikova TV, Lau LF** 1997 Cyr61, product of a growth factor-inducible immediate-early gene, regulates chondrogenesis in mouse limb bud mesenchymal cells. Dev Biol 192:492–508
198. **Pilarsky CP, Schmidt U, Eissrich C, Stade J, Froschermaier SE, Haase M, Faller G, Kirchner TW, Wirth MP** 1998 Expression of the extracellular matrix signaling molecule Cyr61 is downregulated in prostate cancer. Prostate 36:85–91
199. **Hainsworth PJ, Raphael KL, Stillwell RG, Bennett RC, Garson OM** 1992 Rearrangement of chromosone 1p in breast cancer correlates with poor prognostic features. Br J Cancer 66:131–135
200. **Gehring M, Berhold F, Edler L, Schwab M, Amler LC** 1995 The 1p deletion is not a reliable marker for the prognosis of patients with neuroblastoma. Cancer Res 55:5366–5369
201. **Shin E, Fujita S, Takami K, Kurahashi H, Kurita Y, Kobayashi T, Mori T, Nishisho I, Takai S** 1993 Deletion mapping of chromosome 1p and 22q in pheochromocytoma. Jpn J Cancer 84:402–408
202. **Cadigan KM, Nusse R** 1997 Wnt signaling: a common theme in animal development. Genes Dev 11:3286–3305
203. **Dale TC** 1998 Signal transduction by the Wnt family of ligands. Biochem J 329:209–223
204. **Lipinska B, Fayet O, Baird L, Georgopoulos C** 1989 Identification, characterization, and mapping of the *Escherichia coli* htrA gene, whose product is essential for bacterial growth only at elevated temperatures. J Bacteriol 171:1574–1584
205. **Hou J, Conovor CA, Smeekens SP**, Selective cleavage of insulin-like growth factor binding protein-5 by a novel human stress response pathway serine protease: identification, expression and functional characterization. Program of the 80th Annual Meeting of The Endocrine Society, New Orleans, LA, 1998 (Abstract)
206. **Doolittle RF** 1995 The multiplicity of domains in proteins. Annu Rev Biochem 64:287–314
207. **Bartelt D, Shapanka R, Greene L** 1977 The primary structure of the human pancreatic secretory trypsin inhibitor. Amino acid sequence of the reduced S-aminoethylated protein. Arch Biochem Biophys 179:189–199
208. **Murdoch AD, Dodge GR, Cohen I, Tuan RS, Iozzo RV** 1992 Primary structure of the human heparan sulfate proteoglycan from basement membrane (HSPG2/Perlecan). J Biol Chem 267:8544–8557
209. **Kallunki P, Tryggvason K** 1992 Human basement membrane heparan sulfate proteoglycan core protein: a 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. J Cell Biol 116:559–571
210. **Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burgess WH, Jaye M, Schlessinger J** 1990 Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J 9:2685–2692
211. **Bork P** 1992 Mobile modules and motifs. Curr Opin Struct Biol 2:413–421
212. **Mizushima S, Sato H, Negishi T, Koushima H, Okamoto A, Nii A, Hashimoto T, Umeda F, Nawata H, Kanamori T** 1996 Isolation and characterization of the human chromosomal gene for prostacyclin-stimulating factor. J Biochem 120:929–933
213. **Latinkic BV, O'Brien TP, Lau LF** 1991 Promoter function and

structure of the growth factor-inducible immediate early gene cyr61. Nucleic Acids Res 19:3261–3267
214. **Spagnoli A, Rosenfeld RG** 1996 The mechanisms by which growth hormone brings about growth. Endocrinol Metab Clin North Am 25:615–631
215. **Ying Z King ML** 1996 Isolation and characterization of xnov, a *Xenopus laevis* ortholog of the chicken nov gene. Gene 171:243–248
216. **Mason E, Konrad K, Webb C, Marsh J** 1994 Dorsal midline fate in *Drosophila* embryos requires twisted gastrulation, a gene encoding a secreted protein related to human connective tissue growth factor. Genes Dev 8:1489–1501
217. **Collet C, Candy J** 1998 How many insulin-like growth factor binding proteins? Mol Cell Endocrinol 139:1–6
218. **Gould SJ** 1996 Full House. Harmony Books, New York
219. **Allander SV, Larsson C, Ehrenborg E, Suwanichkul A, Weber G, Morris SL, Bajalica S, Kiefer MC, Luthman H, Powell DR** 1994 Characterization of the chromosomal gene and promoter for human insulin-like growth factor binding protein-5. J Biol Chem 269: 10891–10898
220. **Zazzi H, Nikoshkov A, Hall K, Luthman H** 1998 Structure and transcription regulation of the human insulin-like growth factor binding protein 4 gene (IGFBP4). Genomics 49:401–410

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## Gordon Research Conference on Prolactin
## Harbortown, Ventura, California
## January 30–February 4, 2000
## Chair: Li-yuan Yu-Lee, yulee@bcm.tmc.edu
## Vice-Chair: Paul Kelly, kelly@necker.fr

The 2000 Gordon Research Conference on Prolactin (PRL) will provide an outstanding forum for the critical discussion of the functional, biochemical and molecular genetics aspects of PRL. The topics will include: pituitary development, synthesis and secretion of PRL, GH and PRL receptor signaling, cross talk between signaling molecules, and PRL action on the mammary gland, the utero-placental unit, immune diseases, angiogenesis and apoptosis.

The speakers include: George Stark (plenary), Steve Anderson, Nira Ben-Jonathan, Christy Carter-Su, Carmen Clapp, Charles Clevenger, Betty Diamond, Joelle Finidori, Prisilla Furth, Birgit Gellersen, Arthur Gutierrez-Hartmann, Lothar Hennighausen, Douglas Hilton, Shuji Hinuma, Kathryn Horwitz, Nancy Hynes, John Kopchick, Warren Leonard, Daniel Linzer, Joseph Martial, Shlomo Melmed, Jeff Rosen, Mike Soares, Ameae Walker, Christine Watson, Ron Wilder, David Wynick.

Enrollment is limited to 130. For more information, please visit the **GRC Web site** at:
**http://www.grc.url.edu/programs/2000/prolac.htm**

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## Satellite Conference on Prolactin and Autoimmunity
## Harbortown, Ventura, California
## February 4–5, 2000
## Sponsor: American Autoimmune Related Disease Association (AARDA)
## Organizers: Li-yuan Yu-Lee, yulee@bcm.tmc.edu
## Sara Walker, sewk@tranquility.net

The Prolactin and Autoimmunity Satellite Conference will immediately follow the PRL GRC, and will focus on the critical appraisal of PRL action on autoimmune diseases, PRL signaling, and cytokine-immune interactions. The speakers include: Noel Rose, Brigitte Bouchard, Arthur Buckley, George Chrousos, Charles Clevenger, Betty Diamond, Elisabeth Hooghe-Peters, Robert McMurray, Lina Matera, Sue Richards, Hallgeir Rul, Esther Sternberg, Ameae Walker, Sara Walker, Li-yuan Yu-Lee.

For more information, please contact the organizers above. Enrollment is open to all.