

# BEYOND CARRIER PROTEINS

## Comparative endocrinology of the insulin-like growth factor-binding protein

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### Abstract

Emerging early in chordate evolution, the IGF-regulatory axis diverged from an insulin-like predecessor into a vertebrate regulatory system specializing in cell growth activation and allied anabolic functions. Essential to the divergence of the IGF and insulin systems was an early presence of soluble IGF-binding proteins (IGFBPs), which bind IGF peptides at much higher affinity than that of the insulin receptor but at comparable affinities to that of the IGF receptor. IGFBPs have no homology with IGF receptors. Instead, IGFBPs are a derived group of proteins within a superfamily of cysteine-rich growth factors, whose members are found throughout the animal taxa. While blocking IGF actions through the insulin receptor is a fundamental role, IGFBPs evolved within the vertebrate line into centralized, 'integrators' of the endocrine growth-regulatory apparatus. IGFBPs have substantial influences on the distribution and bioavailability of IGF peptides in the cellular and physiological environments, but they have a variety of other properties. The six

principal mammalian IGFBPs exhibit an array of specialized properties that appear to be derived from a complex evolutionary history (including cell membrane association, interaction with proteins that post-translationally modify them, direct IGF-independent effects on cells, and others) and they are regulated by a diversity of 'outside' factors (e.g. other hormones, metabolic status, stress). Thus, IGFBPs are multifunctional integrators having diverse physiological 'agendas'. Much less is known about IGFBPs and their properties in the other vertebrate taxa. Increasingly, however, it is being recognized that they play equally important endocrine roles, in both conserved and non-conserved ways, when compared with those currently defined in mammals. This review highlights selected 'comparative aspects' in current IGFBP research, in an attempt to view this essential group of endocrine regulators from a wider, biological perspective.

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### A natural history introduction to the insulin-like growth factor (IGF) axis: emergence in the chordate line

#### IGF peptides

Insulin-like peptides and their specific cellular receptors represent two superfamilies of regulatory proteins whose common ancestry extends back to early metazoan evolution (e.g. see Duret *et al.* 1998, Reinecke & Collet 1998, Kelley *et al.* 2000). The involvement of these proteins in cellular anabolic processes is widespread throughout the animal world, where both ligand and receptor homologs are seen to regulate metabolite uptake, mitogenesis, synthetic and growth functions, and developmental processes (reviewed by Bern *et al.* 1991, de Pablo *et al.* 1993, Reinecke & Collet 1998, Upton *et al.* 1998, Kelley *et al.* 2000).

Earlier in the evolution of the chordates, an ancestral insulin-like gene is believed to have duplicated, giving rise to the predecessors of 'insulin' and the 'IGFs' of today's vertebrates. In the cephalochordate, *Amphioxus californiensis*, a single insulin-like peptide (ILP) exists as a lone gene product (Chan *et al.* 1990), whereas in the urochordate (tunicate), *Chelyosoma productum* (McRory & Sherwood 1997), and in the chordate agnathan, *Myxine glutinosa* (Chan *et al.* 1981, Nagamatsu *et al.* 1991), there are two separate insulin-like molecules, one more allied with vertebrate insulins and the other more allied with IGF. To date, no separate IGF-like molecule has been described among non-chordates.

The subsequent molecular divergence of the insulin and the IGF peptides is represented in extant vertebrates by differences in nucleotide and amino acid sequence,

mRNA slicing, and prohormone processing to the mature peptides. Comparison of the cloned vertebrate insulins with IGFs indicates an overall amino acid sequence identity of ~50% or less between the two peptides, even within species (e.g. hagfish IGF and insulin are only ~45% identical; Chan *et al.* 1981, Nagamatsu *et al.* 1991).

Later in vertebrate evolution, the IGF precursor duplicated, giving rise to IGF-I and IGF-II. Distinct IGF-I and IGF-II cDNAs have been cloned in the elasmobranch, *Squalus acanthias* (Duguay *et al.* 1995), as well as in a variety of bony fish species (reviewed by Duan 1997, 1998, Nicoll *et al.* 1998, Reinecke & Collet 1998, Kelley *et al.* 2000), indicating that the prototypical IGF gene duplicated in a gnathostome ancestor. The overall sequence homologies of teleost IGF-I and IGF-II to their mammalian counterparts is around 80% at the deduced amino acid level, indicating a strong conservation at the molecular level after the initial divergence of the two genes.

#### IGF receptors

To develop distinct anabolic actions of insulin versus IGF, duplication and molecular divergence of their specific cellular receptors was clearly also necessary, events that had to occur early in vertebrate evolution. In mammals, the genes for the homologous insulin and IGF ('type-I IGF') receptors both encode separate  $\alpha$ - (135 kDa) and  $\beta$ - (90 kDa) subunits, with the respective mature receptor protein complexes consisting of  $\alpha_2\beta_2$ -heterotetramers of around 350 kDa (reviewed by Cheatham & Khan 1995, LeRoith *et al.* 1995). The extracellular  $\alpha$ -subunits, possessing the ligand-binding domains, are covalently linked to each other and the  $\beta$ -subunits via disulfide bridges; the  $\beta$ -subunit proteins possess the transmembrane domain and mostly extend into the cytoplasm, where their tyrosine kinase domains initiate intracellular signaling cascades (outlined below). The insulin and IGF-I receptors of mammals can bind their heterologous ligand, but with less than 1% of the affinity of homologous ligand binding (Czech 1989), and they show varying degrees of conservation in their primary sequences dependent upon the region of receptor – e.g. as low as 40% identity in the transmembrane domain to as high as 85% in their tyrosine kinase domains. Although they have functional overlap physiologically, in general the insulin receptor transduces a greater number of metabolic functions in cells, whereas the type-I IGF receptor specializes in cell growth activation and allied anabolic functions.

Insulin and IGF receptor cDNAs have been cloned and sequenced in some non-mammalian vertebrates. Among fishes, only partial clones of the tyrosine kinase domains and proximal regions have thus far been reported (reviewed in more detail by Kelley *et al.* 2000). The clones – from coho salmon (Chan *et al.* 1997), rainbow trout (Green & Chen 1999a,b), turbot *Scophthalmus*

*maximus* (Elies *et al.* 1996), and seabream *Sparus aurata* (Perrot *et al.* 1999) – exhibit >80% sequence identity with the tyrosine kinase domains of their respective human genes while, in the juxtamembrane domains, additional sites have been identified that play important roles in signaling of the mammalian receptors (see Green & Chen 1999a,b). In addition, the fish receptor sequences are more conserved with their mammalian counterparts than they are with each other. For example, comparison of the trout insulin receptor (rtIR- $\alpha$ ) with the trout IGF receptor (rtIGFR-I $\alpha$ ) demonstrates 78% and 53% identity in the deduced amino acid sequences of the tyrosine kinase and juxtamembrane domains respectively. Thus, substantial molecular divergence between the insulin and IGF receptors is at least observable at the level of teleost fishes. DNA encoding receptors for insulin and IGF-I have also been cloned in an amphibian (*Xenopus laevis*; Scavo *et al.* 1991, Groigno *et al.* 1996, Zhu *et al.* 1998), the chicken (see de Pablo *et al.* 1993, Holzenberger *et al.* 1996, McMurtry *et al.* 1997), and Japanese quail (*Coturnix coturnix japonica*; Fu *et al.* 2001), but not in any reptilian vertebrate.

A solitary insulin-like receptor gene exists in the amphioxus (Pashmforoush *et al.* 1996). As was discussed earlier, ILP is also alone as the only insulin-type molecule in amphioxus. In cyclostomes, however, insulin- versus IGF-specific binding sites are seen (Drakenberg *et al.* 1993), suggesting that duplication and divergence of the insulin and IGF receptor genes occurred early in chordate evolution. IGF-specific binding sites clearly distinguishable from insulin-specific binding sites have also been demonstrated in a variety of non-mammalian vertebrates, including chondrichthyan and bony fishes (reviewed by Leibush *et al.* 1996, Nicoll *et al.* 1998, Navarro *et al.* 1999, Kelley *et al.* 2000). Using affinity cross-linking techniques, it has been demonstrated that IGF binds to a 110–130 kDa  $\alpha$ -subunit in all vertebrates (e.g. see Fig. 5) and that the entire receptor protein complex has a comparable molecular weight (~350 kDa) to that of the mammalian type-I IGF receptor (see above reviews).

Studies on non-mammalian vertebrate IGF receptor signaling pathways are very limited; however, it has been shown that IGF binding to fish IGF receptors activates autophosphorylation, receptor tyrosine kinase, MAP kinase, and phosphatidylinositol 3 (PI3) kinase pathways (e.g. Gutierrez *et al.* 1995, Parrizas *et al.* 1995a,b, Leibush *et al.* 1996, Pozios *et al.* 2001). While the IGF and insulin receptors activate many common signal transduction pathways in mammalian cells, as well as in non-mammalian cells where information has been available, it is also clear that the two receptor systems allow for ligand-specific cellular responses. The degree to which IGF and insulin induce distinct signaling versus signaling cross-talk, and the importance of these aspects to physiological processes, is not well worked out in any vertebrate model to date.

A second IGF receptor, the type-II IGF receptor, is not homologous to the insulin and IGF type-I receptors.

Instead, it is the cation-independent mannose-6-phosphate receptor (MPR-300) which binds IGF-II with high affinity (see Braulke *et al.* 1994). When bound, the MPR-300/type-II IGF receptor translocates bound IGF-II to lysosomes for degradation, but it has no known growth-signaling functions. Instead, the growth-promoting actions of both IGF-II and IGF-I are mediated solely through binding to the type-I receptor. Several studies have reported the existence of the MPR-300 in fish, amphibians, reptiles, and birds (Canfield & Kornfeld 1989, Clairmont & Czech 1989, Janicot *et al.* 1991, Yang *et al.* 1991, McFarland *et al.* 1992, Drakenberg *et al.* 1993, Goddard *et al.* 1993, Zhou *et al.* 1995, Nadimpalli *et al.* 1997, Yerramalla *et al.* 2000), but there has been no evidence that the non-mammalian receptor binds IGF-II (in the amphibian and avian receptors in which it has been directly tested). This has prompted a widely held hypothesis that the IGF-II-binding property was isolated to the mammalian MPR-300. However, a recent study of embryonic brown trout, *Salmo trutta*, reported the presence of an MPR-300/type-II IGF receptor similar in structure and specificity for IGF-II to that seen in mammals (Méndez *et al.* 2001). The biological functions of the MPR-300/type-II IGF receptor is a major unanswered issue in the IGF/IGF-binding protein (IGFBP)/IGF receptor field.

### IGFBPs

Alongside the development of molecular and functional differences in the ligands and receptors themselves, as just described, there was another fundamental event in the evolutionary divergence of insulin and IGF: introduction of the IGFBP. IGF peptides are bound in biological fluids with IGFBPs, a family of six homologous proteins that bind IGF peptides with affinities generally greater than that of the type-I IGF receptor itself ( $K_d$  of  $\sim 10^{-10}$  M versus  $\sim 10^{-9}$  M respectively). With such high affinities for IGFs, but no binding affinity for insulin, IGFBPs block the significant potential of IGFs to bind the insulin receptor and exert insulin-like side-effects. This important physiological role for IGFBPs is hypothetically their most fundamental one relative to the evolutionary divergence of IGF- from insulin-mediated pathways (Kelley *et al.* 1996).

Adding to this more simple, but crucial, physiological purpose, IGFBPs developed into centralized 'integrators' in the endocrine growth-regulatory apparatus. Through differential tissue expression of IGFBPs, various post-translational modifications affecting IGFBP functions (e.g. limited proteolysis, glycosylation, phosphorylation, multimerization), and specific cell surface- and extracellular matrix (ECM)-binding activity, the IGFBPs substantially affect how and where IGF peptides may act at cellular targets. In addition to these influences on IGF bioactivity, several of the IGFBPs are now known to exert direct, IGF-independent actions in a variety of cell models. Many

excellent reviews are available that discuss the diversified roles of IGFBPs in mammalian models, including Duan *et al.* (2002), Lee & Cohen (2002) and Mohan & Baylink (2002) published in this special issue. In this review, the regulatory roles of IGFBPs in certain 'comparative' models will be highlighted. Finally, it should be added that the complexity does not end with the IGFBP principals themselves, as they are part of a diverse superfamily of cysteine-rich growth factors (discussed in the next section).

### IGFBPs through the vertebrate line: evolution of diverse functions

In addition to the six mammalian IGFBPs, certain non-mammalian IGFBPs have recently been cloned and sequenced. All characterized IGFBPs share an  $\sim 50\%$  homologous protein sequence overall, with up to an 80% homology between corresponding IGFBPs of different species (e.g. between mammalian species; Lamson *et al.* 1991, Shimasaki & Ling 1991). Among the non-mammalian cDNA sequences reported (some of which are partial), chicken IGFBP-5 (Allander *et al.* 1995, 1997) and chicken IGFBP-2 (Schoen *et al.* 1995) exhibit 83% and 66% amino acid sequence identity with their mammalian counterparts respectively, while IGFBP-1 of zebrafish (*Danio rerio*; Maures *et al.* 2002), rainbow trout (*Oncorhynchus mykiss*; Bauchat *et al.* 2001) and goby (*Gillichthys mirabilis*; Gracey *et al.* 2001), IGFBP-2 of zebrafish (Duan *et al.* 1999) and seabream (Funkenstein *et al.* 2002), and IGFBP-5 of *Xenopus* (James *et al.* 1993) all show around 40–50% overall sequence identity with their mammalian counterparts. The genes for mammalian IGFBPs 1–6 all have a similar organization, comprised of four exons (except IGFBP-3 which has a fifth 3' untranslated exon), of which exons 1 and 4 contain highly conserved sequences encoding the N- and C-terminal domains. The gene for chicken IGFBP-2, the only complete gene sequence for a non-mammal yet characterized, also has four similarly positioned exons (Schoen *et al.* 1995). The N- and the C-terminal domains of mammalian IGFBPs possess 12 and 6 highly conserved cysteines respectively (except IGFBP-6 which lacks two cysteines from its exon 1), that are important in maintaining the high-affinity IGF-binding site. Among the non-mammalian IGFBPs thus far characterized, these 18 cysteines are similarly conserved, an expected observation given the ability of non-mammalian IGFBPs to bind mammalian IGF peptides with high affinity (discussed further below). The C-termini of IGFBPs also include specialized domains that function in protein–protein and/or protein–cell membrane interactions of the IGFBPs. In contrast to the N- and C-terminal domains, the mid-regions of all known IGFBP sequences show considerable variation.

All vertebrate IGFbps characterized to date are between 216 and 289 amino acids in length. Among the mammalian IGFbps, final molecular weights will be dependent upon several possible post-translational modifications, which thus far have only been found to occur in the variable mid-region. Such modifications may include phosphorylation (IGFBP-1, -3, and -5; Elgin *et al.* 1987, Frost & Tseng 1991, Hoeck & Mukku 1994, Coverley & Baxter 1997, Clemmons *et al.* 1998), N-glycosylation (IGFBP-3 and -4; Wood *et al.* 1988, Ceda *et al.* 1991), O-glycosylation (IGFBP-5 and -6; Bach *et al.* 1992), and limited proteolysis (IGFBP-2, -3, -4, and -5; Collett-Solberg & Cohen 1996, Conover 1996, Kelley *et al.* 1996, Clemmons *et al.* 1998, Baxter 2001). While glycosylation accounts for variation in molecular size (e.g. serum IGFBP-3 often exists as a 45–50 kDa doublet, but deglycosylated IGFBP-3 is ~30 kDa), it has been difficult to define the regulatory impacts of glycosylation (however, see Sommer *et al.* 1992, Neumann *et al.* 1998). Phosphorylation, on the other hand, may alter IGF-binding affinity, as has been demonstrated for IGFBP-1 in which phosphorylation increases its IGF-binding affinity and, therefore, its inhibitory influence on IGF actions (see above citations and Lee *et al.* 1997). In all reports on IGFBP proteolysis, the limited degradation results in reduced IGF-binding affinity of the IGFBP, with other possible effects (e.g. Andress & Birnbaum 1992, Mohan *et al.* 2002). Post-translational modifications of non-mammalian IGFbps have not yet been described; however, IGFBP proteins of various molecular weights have been observed (e.g. Park *et al.* 2000), suggestive of a similar scenario.

With increasing availability of molecular cloning data for non-mammalian vertebrates, the molecular evolution of the IGFBP family, much of which presumably occurred through the vertebrate line, should become clearer in the near future. Using amino acid sequence homologies among mammalian IGFbps, the IGFBP family can be broken into two allied groups: (I) IGFbps -3, -5, and -6: IGFBP-3 and IGFBP-5 are most similar to each other, with IGFBP-6 more divergent, and (II) IGFbps -2, -4 and -1: IGFBP-2 and IGFBP-4 share closest homology, with IGFBP-1 more divergent. Interestingly, the genes for IGFBP-3 (group I) and IGFBP-1 (group II) are found together in a tail-to-tail orientation on the same chromosome (no. 7 in human, no. 11 in mouse), while the genes for IGFBP-5 (group I) and IGFBP-2 (group II) are also found together in a tail-to-tail orientation on a second chromosome (no. 2 in human, no. 1 in mouse) (see Ehrenborg *et al.* 1992, Allander *et al.* 1994, Kou *et al.* 1994). This physical linkage of IGFBP genes suggests simultaneous duplication and translocation of DNA containing the IGFBP-3 and -1 genes, resulting in corresponding IGFBP-5 and -2 genes respectively. Curiously, the IGFBP-3 and -1 genes are also co-localized with the homeobox (HOX)-A gene and epidermal growth factor receptor (EGFR) gene, whereas the IGFBP-5 and -2

genes are co-localized with the HOX-D gene and ERBB2 gene (an oncogene homolog of the EGFR gene), indicating some linkage between evolution of the HOX, IGFBP, and additional gene families (see Acampora *et al.* 1989, Scott 1992, Garcia-Fernandez & Holland 1994). As IGFBP-6 is the most divergent of the IGFbps, its co-localization with HOX-C and ERBB3 genes has led to the idea that this 'cassette' of genes was the predecessor of the IGFBP gene family, although there are alternative possibilities (see Reinecke & Collet 1998, Kelley *et al.* 2000).

The relatively high sequence similarity among the IGFbps suggests that their individual genes were derived from successive gene duplications in a relatively short time-frame in evolution and, additionally, this presumably occurred entirely within the vertebrate line, given that no IGFbps have been identified in non-vertebrate taxa. Hypotheses about the origins of the IGFBP family have proliferated in recent years. Recognizing structural and functional similarities between several other cysteine-rich growth factors and the IGFbps, Kim *et al.* (1997) proposed that 'IGFBP-related proteins' (IGFBP-rPs) were part of a greater IGFBP superfamily. A variety of IGFBP-rPs are now recognized, including members of the large connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) growth factor family (reviewed by Brigstock 1999, Hwa *et al.* 1999). More recently, Vilmos *et al.* (2001) proposed that the IGFbps, together with the twisted gastrulation (TSG; see Chang *et al.* 2001, Ross *et al.* 2001, Scott *et al.* 2001) and CCN families of growth factors, constitute a larger 'TIC superfamily', named after the subfamilies of TSG, IGFBP, and CCN. All of the members of this superfamily share a common conserved, cysteine-rich N-terminus and a similar overall topology, as was described above for IGFbps: a highly conserved 5' cysteine-rich domain(s), a poorly-conserved cysteine-free intermediate region, and a 3' cysteine-rich domain. As the TSG family includes proteins present within both invertebrates and vertebrates, while the CCN and IGFBP families are vertebrate only, it has been suggested that the TSG family may pre-date the other two branches of the TIC superfamily (Vilmos *et al.* 2001). This is in agreement with the notion that IGFbps emerged and evolved within the vertebrate line.

While the N-terminal domains of all members of the TIC superfamily are highly conserved, the C-termini can be very different among and even within the sub-families. Typically, the C-terminus will be conserved within groups within families, although for the IGFBP family it exhibits relatively high sequence identity but with certain specific, functionally relevant differences – e.g. IGFbps -1 and -2 possess the cell surface integrin interaction motif, RGD (see Jones *et al.* 1993). It has been suggested that the functionally distinct domains – such as the RGD motif or heparan-binding domain – have arisen in the C-termini of TIC members through a process of 'domain shuffling',

resulting in the evolution of a greater diversity of properties and regulatory functions in the proteins (Hwa *et al.* 1999). The N-terminal domain, on the other hand, has been proposed to have disseminated to all TIC superfamily members through a process of 'exon shuffling', as the highly conserved domain is encoded by a single exon in a majority of TIC members (see Vilmos *et al.* 2001). It therefore seems probable that the IGFBP sub-family and the diversity of its known physiological roles emerged through a process of 'accumulating' functionally useful parts from other genes.

A recently isolated 84 amino acid long protein from the abalone (*Haliotis laevis*), named perlustrin, has been found to exhibit 40% sequence identity with the N-terminal domain of mammalian IGFbps, which includes all 12 of the conserved cysteines (Weiss *et al.* 2001); thus, perlustrin is the first putative molluscan member of the TIC superfamily. Interestingly, perlustrin was found to bind human IGFs with a  $K_d \sim 10^{-7}$  M, an affinity similar to that of IGFBP-rPs, certain proteolyzed IGFbps, as well as truncated IGFbps containing only the N-terminus (reviewed by Hwa *et al.* 1999); it bound human insulin with a slightly reduced relative affinity (two- to sevenfold lower than to IGFs). The biological function of such a protein, which is largely present in the matrix of the nacreous layer of shells (=calcium carbonate in the form of aragonite, combined with organic matrix), is not known; however, nacre has been shown to contain water-soluble proteinaceous factors that have bone differentiative and growth effects in mammalian cells and tissues (e.g. Lamghari *et al.* 1999, Almeida *et al.* 2000). Whether it works through binding a growth factor (remember from above that no IGF molecule has yet been found among non-chordates) or has direct actions itself (as does IGFBP-5 in bone; Mohan *et al.* 2002) is of course not yet defined, but an exciting possibility. It might then be speculated that an ancestral protein like perlustrin could have been a predecessor to the IGFBP family (in addition to other TIC families), which then developed specialized C-termini to derive the IGFbps of extant vertebrates.

A true understanding of how the IGFBP family emerged will depend upon the acquisition of additional sequence data, most importantly from 'earlier' vertebrate or chordate representatives, since the cloned IGFBP sequences currently available for bony fish (references cited above) indicate a relatively high degree of conservation with that of the mammalian IGFbps. Nonetheless, sequence data among 'later' vertebrates are likely to help define more subtle differences in IGFBP properties that may have evolved more recently along the vertebrate line. Functional studies are also crucial, in order to determine whether molecular differences are actually translated into physiologically relevant property differences in the proteins. Thus, there is a large opening for interested researchers working at all levels of investigation (molecu-

lar, cellular, physiological, even ecological) to contribute crucial pieces to the story of how this important family of proteins has developed through the vertebrates. Given the myriad of physiological adaptations represented among vertebrate species living today, this story is likely to have many fascinating sub-plots.

## Comparative endocrinology of the IGFbps

Current understanding of the physiological roles of IGFbps is based largely on studies using mammal-based cellular or physiological models. However, the functional significance of IGFbps in growth and other physiological processes in non-mammalian species is gaining widespread recognition and experimental support. In the rest of this review, the comparative physiology of IGFbps will be considered. However, given the large number of 'comparative' IGFBP studies published as of 2002, it is not our aim here to provide a comprehensive review of the entire field of research. Rather, by highlighting a subset of the lines of current research that we find of interest, for several reasons to be discussed, it is hoped that a greater biological perspective – that comparative biologists enjoy – can be shared. Comprehensive reviews on the IGF/IGFBP/IGF receptor system of non-mammals have been published recently (Nicoll *et al.* 1998, Reinecke & Collet 1998, Kelley *et al.* 2000, Moriyama *et al.* 2000).

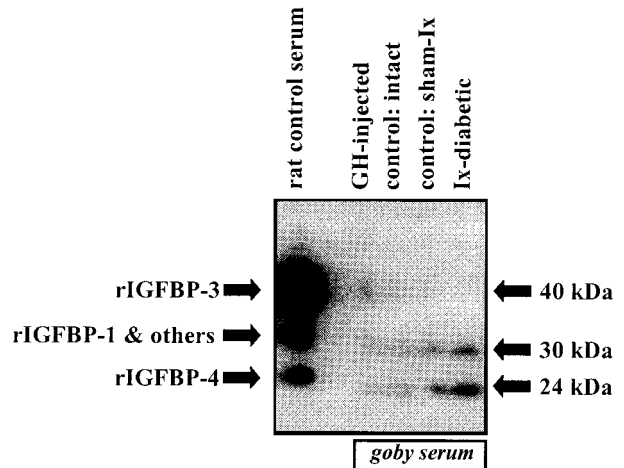
It has been evident since 1992 that IGFbps are present throughout the vertebrates. IGFbps in non-mammalian vertebrates were initially identified based on their ability to bind  $^{125}$ I-labeled human IGF specifically (Kelley *et al.* 1992, Upton *et al.* 1992). Among the various vertebrates tested, at least three distinct serum IGFbps were identified. Indicating that IGFbps were likely to be present very early in vertebrate evolution, Upton *et al.* (1992) demonstrated that three major IGFbps of 50, 32, and 28 kDa exist in the serum of the lamprey, *Geotria australis* (cyclostomes diverged from the main vertebrate line ~550 million years ago). Kelley *et al.* (1992) showed that serum levels of the higher molecular weight IGFBP (~40 kDa) of different teleost fish species could be increased by treatment with GH and were positively correlated with somatic growth rate, raising this IGFBP as a candidate for the fish counterpart to mammalian IGFBP-3; alternatively, they demonstrated that levels of two 24–30 kDa fish IGFbps were reduced by insulin treatment and inversely correlated with somatic growth rate, suggesting a possible relationship to mammalian IGFBP-1 (and possibly IGFBP-2). Although their actual identities are yet to be confirmed, the recent cDNA cloning of non-mammalian versions of IGFbps -1, -2, and -5 (discussed earlier) provide some confidence that the serum proteins will be confirmed as counterparts to mammalian IGFbps. The study by Upton *et al.* (1992) also detected IGFbps in the serum of a variety of different

reptilian species as well as mammalian species, with the overall result that, in general, there was a consistent presence of a higher molecular weight (40–50 kDa) IGFBP and two IGFBPs in the 24–30 kDa size range, although other putative IGFBPs sizes were noted. Since 1992, a large number of studies in a spectrum of non-mammalian vertebrates (see recent reviews by Duan 1997, Nicoll *et al.* 1998, Reinecke & Collet 1998, Kelley *et al.* 2000, Noguchi 2000) have corroborated the presence of similar, as well as some variant (e.g. Park *et al.* 2000) serum IGFBPs. Therefore, the evidence to date indicates that IGFBP proteins circulate in the serum of all vertebrates tested.

#### Circulating IGFBP-3–IGF-I ‘reservoir’: are mammals the exception?

The IGFBP in mammals that carries most of the circulating IGF (>90% of total IGF) is IGFBP-3. A ~140 kDa ‘ternary’ complex of IGFBP-3 (45 kDa), IGF (7 kDa), and a bound ‘acid-labile subunit’ (ALS; 90 kDa) dominates the serum IGF-binding capacity of mammals. Pituitary growth hormone (GH) stimulates the production of all three subunits (IGF-I, IGFBP-3, ALS) and their levels in the circulation are positively correlated with somatic growth rate. The large collective molecular size of the IGFBP-3 ternary complex functions to block IGF movement across the capillary barrier, and the complex serves as a substantial and functionally important reservoir for IGF peptides (reviewed by Baxter 2001); IGF peptides do not exhibit intracellular storage (e.g. in hepatocytes) from which they can be released, as do other hormones. It has been estimated that the ternary IGFBP-3 complex in the human circulation can increase the half-life of IGF-I up to 12 h, from less than 10 min for uncomplexed IGF-I (Guler *et al.* 1989); insulin, which typically has no serum-binding proteins (however, see Yamanaka *et al.* 1997), also has a half-life of less than 10 min. The other IGFBPs do not appear to play any appreciable role in stabilizing IGFs in the blood circulation. Rather, the more important functional aspects of the other IGFBPs – as well as IGFBP-3 without bound ALS – appear to be their ability to cross the capillary barrier. In this manner, they may then move to the tissue/cellular microenvironment to exert their biological effects, which can include regulating IGF bioactivity, tissue (cellular) distribution of IGF, as well as IGF-independent actions (as outlined earlier). Such ‘local’ roles of IGFBPs are beginning to be defined in a few non-mammalian models, a subject taken up later.

Despite numerous attempts in our laboratory as well as in several others, it has not been possible to demonstrate that a large molecular weight IGFBP complex, comparable to the ternary IGFBP-3 complex of mammals, exists in the serum of non-mammalian vertebrates. Using size-exclusion chromatography under neutral conditions, which allows detection of the 140 kDa ternary complex in



**Figure 1** IGFBPs in blood serum from the rat and goby, *Gillichthys mirabilis*, as measured by human  $^{125}\text{I}$ -IGF-I binding in a Western ligand blot (Kelley *et al.* 1999) procedure. Rat serum contains 45 kDa rat (r) IGFBP-3, a 30 kDa band that may represent several rIGFBPs (-1, -5, and possibly others), and 28 kDa rIGFBP-4. IGFBPs in fish serum have similar sizes, including 40, 30, and 24 kDa, although they are present in substantially lower concentrations. When gobies are injected with 0.1  $\mu\text{g}$  bovine GH/g body weight, the 40 kDa IGFBP shows increased levels, while the 30 and 24 kDa IGFBPs are difficult to detect. However, when fish are made diabetic by pancreatic isletectomy (Ix-diabetic), they exhibit a loss of the 40 kDa IGFBP simultaneously with increased levels of the 30 and 24 kDa IGFBPs, as compared with intact and sham-Ix controls. See text for further discussion.

rat serum, no IGF-binding activity larger than 50 kDa can be detected in serum from chicken and lamprey (Upton *et al.* 1992), in serum of the teleost fishes, barramundi (*Lates calcarifer*; Degger *et al.* 2000) and coho salmon (*Oncorhynchus kisutch*; Shimizu *et al.* 2000), in the frog, *Rana pipiens*, or in the teleost, *Gillichthys mirabilis* (G Ngan & K M Kelley, unpublished data). Although a possible large molecular weight IGFBP species was reported in an earlier study on tilapia (Drakenberg *et al.* 1989), our group has not been able to detect any in this species.

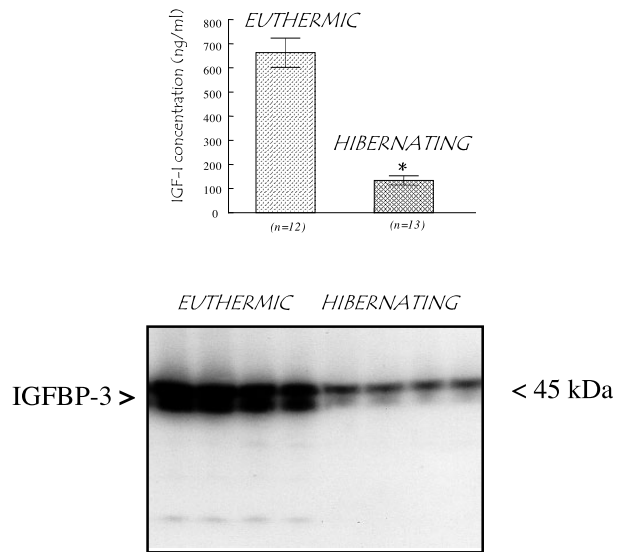
In addition to this key difference between mammals and the other vertebrates, it also appears that IGFBP-3 typically circulates at much higher levels in mammalian serum than that seen for the comparable IGFBP (40 kDa) of non-mammals. Figure 1 illustrates  $^{125}\text{I}$ -IGF-I binding to 4  $\mu\text{l}$  rat serum as compared with the same volume of serum from the goby, *G. mirabilis*. Within the same Western ligand blot assay, the levels of rat IGFBP-3 – as well as all IGFBPs in rat serum – are substantially higher than levels of IGFBPs in the fish serum. Indeed, sera from all fish species that we have tested (goby, jack mackerel, *Trachurus symmetricus*, tilapia, rainbow trout, coho salmon, *O. kisutch*), as well as serum from an amphibian (*Rana pipiens*) and a reptile (marine iguana, *Amblyrhynchus cristatus*), have all shown similarly low relative levels of IGFBPs when compared against those in mammalian serum (Kelley *et al.*

1992, 2000, 2001, A Hawayek, JA Reyes, MM Galima, G Ngan & KM Kelley, unpublished data). The above findings are consistent with several additional published studies on non-mammals (e.g. Cortizo *et al.* 1993, Niu & Le Bail 1993, Siharath *et al.* 1996, Shimizu *et al.* 2000), although direct comparisons with mammalian serum have not been made in most studies. Consistent with this difference in IGFBP levels are the concentrations of IGF-I found in mammalian serum versus fish serum: in mammals, IGF-I typically circulates at levels between 500 and 800 ng/ml (e.g. Schmidt & Kelley 2001, discussed later), whereas in fish, serum IGF-I levels are typically ~10–40 ng/ml, similar to insulin concentrations in both mammals and fish (see Baños *et al.* 1999, Shimizu *et al.* 2000, Larsen *et al.* 2001). Similar findings have been reported in the toad, *Bufo arenarum* (Cortizo *et al.* 1993). Therefore, it appears that mammals may be the exception with respect to exhibiting a substantial IGF-binding capacity, as well as up to 100 times more IGF-I, in their blood circulation.

Thus, at least under basal ('normal') physiological conditions, IGFbps and IGFs are far less prominent features of the serum of non-mammals as compared with that of mammals. Indeed, in many instances, at least in fishes, serum IGFBP may be difficult to detect by standard binding assays. On the other hand, if the animals are provoked physiologically (e.g. hormone injection or stress induction), levels of one or more particular IGFBP will be substantially increased and easy to measure. Figure 1 illustrates this in the goby: low but detectable levels of the 40 kDa IGFBP in serum from control animals, but a several-fold increase in gobies injected with a moderately growth-stimulatory dose of GH (0.1 µg/g). Alternatively, the lower molecular weight goby IGFbps (30 and 24 kDa) are up-regulated under catabolic circumstances in which body growth is inhibited (shown in Fig. 1 is serum from uncontrolled type-I diabetics). In coho salmon, the same dose of GH similarly increases serum levels of the 40 kDa IGFBP, which occurs in parallel with increasing IGF-I concentrations, from <10 to 30 ng/ml (Shimizu *et al.* 2000). Based on such findings, we propose the following physiological hypothesis: in fishes, and probably in a majority of other non-vertebrates, enhancement of IGF-mediated anabolism may be somewhat like 'filling an empty glass', in that the serum requires addition of the growth factors (IGF, IGFBP) from a low basal level. In contrast, mammals retain a 'filled glass', in that they maintain very high levels of IGF/IGFBP that serve as a continuing reservoir of growth factor (Baxter 2001).

#### Hibernation in the golden-mantled ground squirrel

The golden-mantled ground squirrel, *Spermophilus lateralis*, is a winter hibernator inhabiting the mountainous areas of the western United States, and typically will be found at elevations between 1800 and 3000 meters (Jameson & Peeters 1988). At these high elevations, small mammals



**Figure 2** Serum IGF-I and IGFBP levels in golden-mantled ground squirrels, *Spermophilus lateralis*, during hibernation as compared with those in euthermic control animals. IGF-I concentrations were measured by radioimmunoassay and IGFBP levels were measured by Western ligand and immunoblot analyses as described by Schmidt & Kelley (2001). Serum IGF-I concentrations were reduced by 75% in hibernating squirrels, with a similar reduction in IGFBP-3 levels, as compared with levels in the euthermic controls.

like *S. lateralis* are subjected to severe winter conditions. Given the animals' high metabolic rate, combined with freezing ambient temperatures and the absence of significant food availability, an adaptive physiological response is required if they are to survive winter. *S. lateralis* undergo a profound winter hibernation, in which their core body temperatures are lowered to a few degrees above ambient (as low as 2–5 °C) and their metabolic rates are reduced by more than 90%. In addition to these dramatic metabolic changes, other adaptations include ceasing energetically expensive physiological processes, such as reproduction (see Barnes 1986, Kenagy & Barnes 1988).

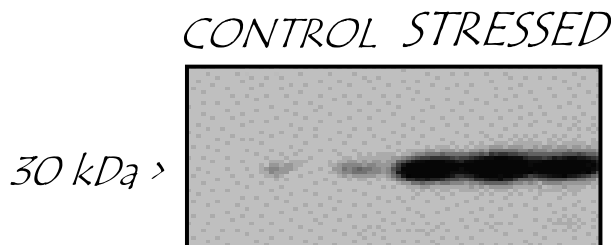
We hypothesized that there may also be significant alterations in the endocrine axis that regulates growth, with an expectation that the IGFbps would exhibit changes reflective of reduced anabolism. As represented in Fig. 2, hibernating *S. lateralis* exhibit dramatic depression in serum IGF-I levels (by 75%), with parallel reductions in serum IGFBP-3. In accordance with these changes, when serum from hibernating animals was tested for its anabolic effect on cartilage *in vitro*, it had no ability to alter the <sup>35</sup>S-proteoglycan synthetic rate, whereas serum from euthermic control squirrels significantly stimulated synthetic activity (Schmidt & Kelley 2001). Since hibernation represents, among other changes, a prolonged period of starvation, it was surprising that there was no detectable 30 kDa IGFBP-1, since fasting strongly up-regulates IGFBP-1 in mammals (see Lee *et al.* 1997). As discussed

further in the next section, increases in circulating IGFBP-1 serve to reduce growth under catabolic conditions, which would be a conceivable mechanism to suppress growth during hibernation. Instead, it appears that hibernation does not look like a typical catabolic effect, at least with respect to IGFBP-1 expression. In support of the absence of this 'growth inhibitor' in serum, it is of note that the serum of hibernating squirrels did not suppress cartilage biosynthetic activity, and rather simply had no stimulatory effects consistent with reduced IGFBP-3 and IGF-I. It is now of interest to determine how glucocorticoids are altered as part of hibernation adaptation in *S. lateralis*, since glucocorticoids are important factors up-regulating IGFBP-1 under catabolic conditions (a subject taken up in the next section).

*IGFBPs under catabolic circumstances: do they serve as markers of growth status in fishes?*

In all animals in which it has been tested, the ~40 kDa IGFBP is typically the most prominent IGFBP present in the circulation under anabolic conditions (e.g. after injection of GH), whereas the lower molecular weight (30 and possibly 24 kDa) IGFBPs are usually at a more basal level, at the limit of detection (e.g. see Fig. 1 for goby serum, as well as Kelley *et al.* 1992, 2001, Moriyama *et al.* 1994, Siharath *et al.* 1996, Shimizu *et al.* 1999, Park *et al.* 2000). In the studies in which it has been tested (e.g. Kelley *et al.* 1992, 1993, Siharath *et al.* 1995), elevated levels of the ~40 kDa IGFBP are correlated with significantly enhanced anabolism (e.g. cartilage <sup>35</sup>S-proteoglycan synthetic rate). Insulin represents another anabolic regulator of the ~40 kDa IGFBP, as insulin-dependent diabetes mellitus (IDDM) in the goby, induced by a surgical pancreatic isletectomy (Kelley 1993, Haigwood *et al.* 2000), reduces serum levels of the protein in parallel with decreased anabolism, while insulin replacement therapy corrects these deficits (Kelley *et al.* 2000). Similar observations have been made on the effects of fasting and refeeding (see Duan 1998, Reinecke & Collet 1998, Kelley *et al.* 2000, 2001). In mammals, insulin is an important permissive factor for somatic growth, since its presence is necessary for maintenance of hepatic IGFBP-3 and IGF-I expression, and thereby growth. Available information indicates that a comparable relationship exists with respect to the ~40 kDa IGFBP.

Under catabolic circumstances, on the other hand, an opposite picture emerges: serum levels of the ~40 kDa IGFBP are consistently below detection, while the 30 and 24 kDa IGFBPs are substantially elevated in association with a significant inhibition of growth and anabolic function. Kelley *et al.* (2001) compared three catabolic states in fish: fasting, untreated IDDM, and stress, and in all conditions there were consistently elevated serum cortisol concentrations, increased levels of one or both (depending on species) of the 30 and 24 kDa IGFBPs, and



**Figure 3** Levels of the 30 kDa IGFBP in serum of jack mackerel, *Trachurus symmetricus*, as measured by binding of <sup>125</sup>I-labeled recombinant barramundi (*Lates calcarifer*) IGF-I in a Western ligand blot procedure. Mackerel subjected to handling (touching tails every 10 min for 1 h) showed increased serum levels of the IGFBP as compared with non-handled controls.

a clear suppression of somatic growth and/or anabolic activity (see Kelley *et al.* 2000, 2001). This relationship between low molecular weight IGFBP(s) and cortisol, and the inverse correlation with anabolic function, serum insulin, and serum GH, strongly suggest a scenario comparable with the physiology of mammalian IGFBP-1. Expression of mammalian IGFBP-1 is regulated by the same factors in a similar manner, and it is a key player in growth inhibition: increases in high-affinity IGFBP-1 will bind and block IGF peptides from reaching their target receptors, serving to shut down energy-expensive anabolic processes under catabolic circumstances (see Underwood 1996, Lee *et al.* 1997). It would therefore seem that a comparable system exists in non-mammals, although the identification of which of the two IGFBPs is actually IGFBP-1 still awaits the development of specific antisera to the IGFBPs of non-mammalian species. If one of them is arguably IGFBP-1, then the other may be IGFBP-2, in as much as mammalian IGFBP-2 is also known to be increased in the circulation of catabolic mammals (see Reinecke & Collet 1998, Duan *et al.* 1999, Kelley *et al.* 2000).

The connection between stress and an altered growth–endocrine axis, via alterations in IGFBPs, has raised an interesting possibility: the 'applied' use of IGFBP measurement to assess the physiological status of fishes and, for that matter, other vertebrate species. In Fig. 3, serum from jack mackerel subjected to handling stress (contact by touching their tails) for 60 min exhibited dramatic elevations in levels of their serum 30 kDa IGFBP, as compared with animals under similar housing conditions but without handling (Galima & Kelley 2001). Correlated with this change were significant increases in circulating cortisol concentrations, indicating the induction of a stress response in the animals. In mammals, the IGFBP-1 gene possesses glucocorticoid-regulated elements, and the steroid can potentially increase IGFBP-1 levels (see Lee *et al.* 1997). Therefore, it is evident that human handling induces significant physiological stress responses in these fish (see also Schreck 1993, Pankhurst & van der Kraak 1997),



including alteration of an IGFBP(s) with potential to exert negative impacts on growth and other anabolic processes. This has led to the suggestion that blood plasma measurements of this IGFBP, which require only a few microliters of serum, may serve as an informative biomarker of catabolic growth inhibition in fishes (Kelley *et al.* 2001). Furthermore, it would appear to have excellent potential as a tool for understanding the negative impacts of stress responses in fishes, particularly those related to human activities. Our group, along with two collaborating laboratories at Oregon State University (Dr Carl Schreck) and here in Long Beach (Dr Chris Lowe), are now working to understand the secondary physiological impacts of stress as related to the IGF axis generally, and IGFBPs in particular. 'Catch-and-release' of wild-caught fish is a common governmental management tool designed to protect both marine and freshwater fisheries. Is it truly effective?

#### *Local IGFBPs and the cellular IGF axis in non-mammalian vertebrates*

It is now widely recognized that, as in mammals, IGFBPs are produced by a variety of tissue and cell types in other vertebrates. Among all mammalian cells studied to date, typically two or more of the six different IGFBPs genes will be expressed. In the cells of other vertebrates studied to date, this does not appear to be similar. Instead, typically one, and only sometimes two, IGFBPs are produced by tissues or cells of non-mammalian vertebrates. This should not be overlooked, as it points to non-mammalian cells as exceptionally good experimental cell models in which one IGFBP can be studied without interference from other expressed IGFBPs, a situation typical in mammalian cell studies.

The first demonstration of 'peripheral' tissue/cellular IGFBP production was in a study of striped bass (*Morone saxatilis*) tissues, in which kidney, spleen, brain, gonads, pituitary, gill filaments, cartilage, bone, heart, muscle, and gut were all found to produce a single 30 kDa IGFBP *in vitro*, while liver produced both the 30 and 24 kDa IGFBPs (Siharath *et al.* 1995). Similarly, a reptilian heart cell in culture produces a single 30 kDa IGFBP (discussed below), while cultured liver explants of the goby (Roth 1999) and primary hepatocytes of the coho salmon (Moriyama *et al.* 1997) produce the two IGFBPs of similar size to that described for bass liver. Fukuzawa *et al.* (1995) reported that release of the 30 kDa IGFBP from bass liver slices *in vitro* was stimulated by mammalian GH, epidermal growth factor, and tri-iodothyronine, but inhibited by mammalian IGF-I, glucagon, and thyroxine. Release of the 24 kDa IGFBP, on the other hand, was reduced after treatment with mammalian insulin, IGF-I, or prolactin, while estradiol increased its release. While complicated, the results certainly suggest that, as for mammals, 'local' tissue IGFBP expression appears to be regulated by a variety of physiologically relevant factors. It is also worth

noting that the inhibitory effects of insulin on the release of the 30 and 24 kDa IGFBPs *in vitro* are consistent with the *in vivo* studies discussed earlier, indicating that these IGFBPs are inversely related to insulin (and to anabolism as well). Curiously, the 40 kDa IGFBP has not yet been observed in conditioned medium of cultured cells or tissues of non-mammalian vertebrates.

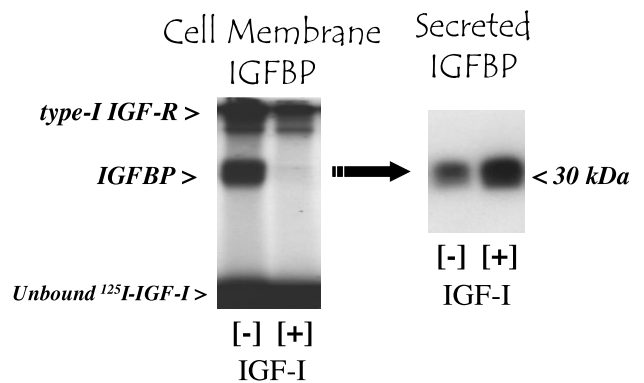
While there is a growing literature on the physiological regulation of IGFBPs and their relationships to growth and other physiological processes *in vivo*, there are only a very limited number of studies on the properties of IGFBPs at the cellular level using non-mammalian models. This is a very important, unanswered issue, as the dynamics of IGFBPs in the microenvironment of mammalian cells, particularly relating to their membrane association and/or binding, are now understood to be crucial events in IGFBP regulation of cellular activities (see reviews by Duan *et al.* 2002, Lee & Cohen 2002 and Mohan & Baylink 2002 in this special issue). As one mammalian example of this, studies in our laboratory on a murine glomerular mesangial cell line have shown that IGFBP-2 avidly binds to cell membranes and that this localization plays an important role in IGF-I-mediated events in the cells (Desai 1999, Berg *et al.* 2002). Utilizing, at least in part, its integrin-binding RGD domain to bind the membrane, IGFBP-2 localization on the membrane results in an approximately tenfold decrease in its binding affinity for IGF-I and enhances IGF-I-stimulated production of ECM components. Interestingly, when the mesangial cells are treated with diabetic factors (e.g. high glucose, angiotensin II), secreted and membrane-associated IGFBP-2 levels increase and the cells show activated expression of ECM components (Arope 1999, Song *et al.* 2000, Berg *et al.* 2002) similar to those observed in glomerular mesangium of patients and rat models with diabetic nephropathy (see Flyvbjerg 1997). Thus, the dynamics of IGFBPs in the cellular microenvironment of mammals, especially relating to their membrane-association properties, can be crucial in the regulation of normal (or abnormal) cell growth and function.

Our laboratory has therefore been interested in how IGFBPs of lower vertebrate species function at the cellular level, with an overall aim of understanding how the cell-regulatory roles of these proteins have evolved through the vertebrate line. Are IGFBPs from 'lower' vertebrate taxa capable of cell membrane association? If so, does this affect IGF binding/activation of cellular IGF receptors? Do they have any direct, IGF-independent effects on cells? At what point in the evolution of the IGFBPs through the vertebrate line did certain specialized properties arise and become functionally relevant? Answers to these questions require both a molecular characterization of the proteins themselves and, more important to the function-minded endocrinologist, investigation of how certain properties translate into regulatory roles known for mammalian IGFBPs and, perhaps more

interesting, into roles particular to the vertebrate group being studied and its biology.

Cell membrane association of IGFBPs has only been identified in two non-mammalian vertebrate systems thus far, in chicken hepatoma (LMH) cells by Duclos *et al.* (1994, 1998) and in reptilian heart cells by our group (discussed in next paragraph). Affinity cross-linking of bound  $^{125}\text{I}$ -human IGF-I to chicken LMH cell cultures identified a 28–30 kDa IGFBP that was both bound to cell membranes and present as a soluble protein in the medium (Duclos *et al.* 1994). When the effect of IGF-I on amino acid uptake was compared with that of the analog, Long-[Arg3]-IGF-I, which shows low affinity for IGFBP binding, it was found that the native IGF-I was less potent, presumably as a result of competitive inhibition of receptor binding by IGFBP binding (Duclos *et al.* 1998). These findings suggested that the presence of this avian IGFBP, which was capable of binding to the cell membranes, had an inhibitory effect on IGF-I action. The degree to which the membrane-bound state of the IGFBP plays a role in this effect, however, has not yet been characterized.

Whether IGFBPs from ectothermic vertebrates are capable of cell membrane association has been tested thus far in only two lines of study. Duan *et al.* (1999) found that recombinant zebrafish IGFBP-2 did not localize to zebrafish embryonic (ZF-4) cell membranes, and it was proposed that this lack of ability to associate to membrane may be accounted for by the lack (versus mammalian IGFBP-2) of a heparin-binding motif that may allow ECM association. The presence of an RGD sequence in the zebrafish IGFBP-2 was apparently not utilized either, at least in the ZF-4 cell tested. The presence of soluble IGFBP-2 inhibited IGF-I's proliferative actions, a result expected from a competition for ligand between receptor and IGFBP. Similarly, purified IGFBP-1 of rainbow trout also inhibits IGF-I action in ZF-4 cells (Bauchat *et al.* 2001), while its ability to associate with membranes is not known. In heart cells derived from the lizard, *Iguana iguana*, however, distinct cell membrane-association properties of an IGFBP has been demonstrated (Schmidt *et al.* 2002). A single 30 kDa IGFBP is produced by the cells and is present both in soluble form in the medium and localized to the cell membranes (Fig. 4). This clean 'single-IGFBP' cell system has allowed investigation of the protein's cell-regulatory properties in advance of its molecular identification and characterization (the latter is underway). The iguana IGFBP in its soluble form has been shown to bind  $^{125}\text{I}$ -IGF-I in a specific and high-affinity manner. Interestingly, when affinity cross-linking experiments of cell monolayers were attempted to assess whether cell membrane-associated IGFBP may have a changed (reduced?) affinity, the IGFBP was found to fall off the cell membranes in a rapid fashion. Indeed, when IGF-I was added during culture, it caused a complete removal of the IGFBP from its cell surface site(s) and its release into the medium (compare left and right panels in Fig. 4).



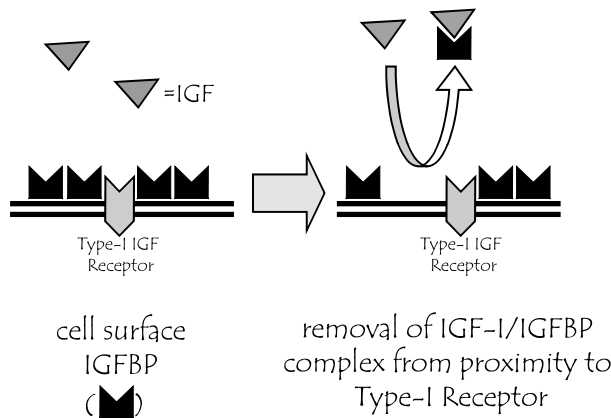
**Figure 4** Membrane-associated and soluble 30 kDa IGFBP in cultured heart cells derived from the lizard, *Iguana iguana*, as measured by affinity cross-linking (Kelley *et al.* 1999). The iguana IGFBP is localized to the cell surface at levels rivaling that of the type-I IGF receptor (upper, 135 kDa band represents  $\alpha$ -subunit of the receptor) under control conditions ([-] represents serum-free medium without added IGF-I). When IGF-I was added to cultures at 100 ng/ml ([+]), the presence of the IGFBP was completely lost from the membrane surface concomitant with increased IGFBP levels in the medium.

Furthermore, this removal effect is specific to IGF-I and IGF-II (e.g. insulin, DES(1–3)-IGF, and GH do not have this effect) and it has been determined to occur within minutes of the addition of IGF-I (Schmidt *et al.* 2002). These results led to the hypothesis that the iguana IGFBP serves an IGF-inhibitory role in the heart cells, since binding of IGF-I peptides is followed immediately by removal of the IGF/IGFBP complex from the membrane – and from proximity to the cellular IGF receptor (illustrated in Fig. 5). Continuing studies are now showing that the heart cells are significantly more responsive to IGF-I's proliferative actions under conditions in which the IGFBP has been experimentally removed from the cell surfaces, supporting the above hypothesis.

Although only a few studies have been carried out to date, it can now be stated that cell membrane IGFBP association, with physiologically relevant effects, are present among non-mammals at least to the level of reptiles. Studies of cells from additional ectothermic vertebrate representatives are certainly needed, as is a continued effort to characterize the non-mammalian IGFBPs at the molecular level. With additional information, there should emerge an interesting story of molecular and functional adaptations in IGFBPs through the evolution of the vertebrates.

#### *Other directions of research in the comparative endocrinology of IGFBPs*

In a review such as this, it is impossible to cover the entire field of work, let alone possible work, on the comparative endocrinology of IGFBPs. Although deficient in the



**Figure 5** Hypothesized inhibitory role of the membrane-bound iguana IGFBP shown in Fig. 4. As growth-stimulatory IGF peptide approaches the cell membrane, high levels of membrane-associated IGFBP mean that there is a strong likelihood that the peptide will bind IGFBP instead of the cellular IGF receptor. Immediately upon binding of IGF to IGFBP, the IGF/IGFBP complex leaves the membrane. Presence of IGFBP on the cell surface is therefore likely to represent a growth-inhibitory mechanism, whereby IGF arriving at the cellular site is specifically removed from proximity to the growth receptor. Recent studies on cellular proliferation responses support this hypothesis (see text).

breadth and interest of other research areas on IGFbps, we point out in this last section the additional arenas, not covered above, in which the IGFBP either is receiving, or deserves to be receiving, the attention of comparative and all biologists.

In mammals, there is an increasingly substantial body of work on the reproductive roles of IGFbps (reviewed by Irwin *et al.* 1999, Fowler *et al.* 2000, Erickson & Shimasaki 2001, Guidice 2001). Whereas the effects of IGF peptides in reproductive processes have been identified in a host of non-mammalian vertebrates (see Guillette *et al.* 1996, McMurtry *et al.* 1997, Kelley *et al.* 2000, Weber & Sullivan 2000, Fu *et al.* 2001), any roles of IGFbps are not currently known. As IGFbps in mammals have been implicated in several key reproductive processes, such as determining follicular fate (Adashi 1998), the comparative field will be certain to add insight into the evolution of important IGFbps functions in reproduction. Similarly sparse are comparative endocrinological studies on the developmental biology of IGFbps (see Schoen *et al.* 1995, Ernst *et al.* 1996, Radecki *et al.* 1997, Perrot *et al.* 1999, Allan *et al.* 2001, Fu *et al.* 2001, Pera *et al.* 2001). With respect to developmental biology issues, one can only imagine the kind of story that will emerge from studies on metamorphosis in amphibians, in which a vertebrate quickly develops from the fish-like, water-breathing larva (tadpole) into a tetrapod with lungs (frog)! Similarly, the 'metamorphic' changes in Pacific salmon undergoing the parr-to-smolt transformation in preparation for life in ocean salt water are likely to be influenced by IGFbps, as

several studies now show important IGF-I roles, particularly relative to osmoregulatory adaptation (e.g. see McCormick *et al.* 1991, Madsen *et al.* 1995, McCormick 1996, Beckman *et al.* 1998, Seidelin *et al.* 1999). It should also be noted that it has not yet been established whether proteolyzed IGFBP forms exist among non-mammalian vertebrates. At least some of the IGFbps currently detected by binding assays are sure to represent proteolytic IGFBP fragments, as is observable in serum of rats and humans assayed by Western ligand blot analysis (Conover 1996, Kelley *et al.* 1996, Clemmons *et al.* 1998, Maile & Holly 1999, Baxter 2000, 2001). Although not reviewed herein, IGFBP proteolysis represents a major regulatory mechanism in IGF-mediated processes both at the cellular and physiological levels. Finally (at least as included here) was the recent discovery in chickens that vitronectin is an IGF-II-selective, 70 kDa serum-binding protein by Upton *et al.* (1999), who proposed that the complex may serve to bring IGF-II to cell ECM. Recently, Nam *et al.* (2002) reported that vitronectin also binds IGFBP-5 with high affinity and that localization of IGFBP-5 within the ECM by vitronectin appears to modify cell responsiveness to IGF-I via modulation of IGFBP-5. The degree to which this type of regulatory apparatus exists widely among vertebrates will likely emerge in the next few years.

### A comparative commentary

In the IGF/IGFBP/IGF receptor system, a range of physiological signals are input and integrated, with a substantial role of the IGFbps in this integration, and the outcome is that an appropriate degree of anabolic activity will proceed in a particular way in a particular cell type, tissue, and/or organ system. The evolution of this complex system is an entirely vertebrate one, but the paths by which it developed are not understood.

One of the principal reasons comparative endocrinology, and comparative biology in general, exists is because its scientists derive a certain satisfaction from gaining the larger biological perspective on the systems they study. Life systems, and physiological systems, are the result of millions of years of trial and error and evolutionary change. To a comparative biologist, it is knowledge in a vacuum when there is only understanding in a single species, most notably, *Homo sapiens*! Rather, the more relevant questions would be the following. How did things become what they are today? How and why do systems exhibit different adaptations in different animal groups? How and why might they have evolved? What is fundamental versus derived? Taxon by taxon, study by study, the collective biological picture comes into focus.

As is true for most of the endocrinology sub-fields, the research field encompassing the biology of IGFbps is top heavy with mammal-based information, and most of the

available tools with which to analyze the system are mammal specific. Nonetheless, an increasing number of groups are entertaining comparative aspects of the system, with cDNA clones, recombinant proteins, and specific antibodies being progressively generated for non-mammalian vertebrates. The new comparative information is providing an important biological perspective on the entire growth-regulatory endocrine system, a perspective that has not before been available.

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