INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1;
ISOLATION OF THE GENE AND CHARACTERIZATION OF THE PROTEIN.

INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1;
ISOLATIE VAN HET GEN EN KARAKTERISERING VAN HET EIWIT.

PROEFSCHRIFT

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Voor: Ivonne, Stan & Eva.

CONTENTS

List of abbriviations

AIB	aminoisobutyric acid
AF	amniotic fluid
ALS	acid labile subunit
ATP	adenosine triphosphate
cDNA	copy DNA
CI-M6PR	cation independent mannose 6-phosphate receptor
DNA	deoxyribonucleic acid
2D NMR	2 dimensional nuclear magnetic resonance
EGF	epidermal growth factor
GH	growth hormone
IBP	insulin-like growth factor binding protein
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
kb	kilobase
kD	kilo dalton
mRNA	messenger RNA
MSA	multiplication stimulating activity
NSILA	non suppressible insulin-like activity
PAGE	polyacrylamide gel electrophorese
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PHA	phytohemagglutinin
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SM	somatomedin

1. Introduction

Fetal and postnatal growth is the ultimate result of a delicate balance between processes of proliferation, differentiation and death of cells. Proliferation and differentiation are strictly controlled by growth factors and hormones. A large number of growth factors has been characterized thus far; among them are the Insulin-like Growth Factors I and II (IGF-I,-II). IGFs were identified originally as mediators of Growth Hormone (GH) action (Salmon and Daughaday, 1957). Both IGFs are small, single chain polypeptides, with structural and functional similarity to insulin. They display insulin-like mitogenic and differentiation stimulating activities (Froesch et al, 1963; Rinderknecht and Humbel, 1976; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel ,1978b). The biological response of the IGFs is mediated by specific receptors (Rechler et al, 1980; Rechler and Nissley, 1986).

Serum levels of IGF-I and to a lesser extent IGF-II are largely controlled by GH (Van Wyk, 1984; Berelowitz et al, 1981). In addition, the presence of IGFs in serum and tissues is also modulated by age, nutritional status and various hormones such as insulin, thyroid hormone and glucocorticoids (Clemmons et al, 1981; Clemmons and Shaw, 1983; Clemmons and Van Wyk, 1984; Binoux et al, 1986). The liver has been considered to be the primary site of IGF production and the IGFs were thought to act in an endocrine fashion (Daughaday et al, 1976; Schwander et al, 1983). Since the discovery that the synthesis of these factors occurs in many cell types, paracrine or autocrine mechanisms have been postulated as well (D'Ercole et al, 1984).

Unlike insulin, the IGFs circulate in plasma bound to specific proteins, the so-called IGF binding proteins (IGFBPs). Initially two major forms have been identified: in serum a GH dependent complex with a molecular weight of 150 kD in which the majority of the IGFs is present, and a second class of binding proteins with molecular weights varying from 30-40 kD accounting for most of the unsaturated binding sites (Hintz and Liu, 1977). Although the precise function of the binding proteins is still unclear it has been suggested that they play a modulatory role in IGF action. To gain more insight in the complex interaction of the IGFs, their receptors and their binding proteins, identification and characterization of the various IGFBPs is of particular interest.

The subject of the underlying thesis is the elucidation of the gene encoding the IGFBP originally identified in human amniotic fluid (AF). In addition, structural and functional

aspects of this IGFBP protein were studied. In order to place the results of the thesis in a wider perspective, a brief overview on the structural and functional aspects of the IGFs, their receptors and binding proteins is presented in the remainder of this chapter. For more detailed information the reader is referred to various reviews (Ooi and Herington, 1988; Sara and Hall, 1990; Clemmons, 1991a; Drop et al, 1991).

1.1. The Insulin-like growth factors (IGFs)

Three separate lines of evidence resulted in the discovery of polypeptide growth factors currently known as insulin-like growth factors IGF-I and -II. In 1957 Salmon and Daughaday demonstrated that the growth promoting activity of growth hormone (GH) was mediated by a factor in serum provisionally termed "sulphation factor". The observation was made that the *in vitro* sulphate incorporation into costal cartilage obtained from hypophysectomized (hypox) rats could be stimulated by serum of normal but not of hypox rats. GH treatment of hypox rats restored the serum growth promoting activity, while GH itself had no direct effect (Salmon and Daughaday, 1957). A second line of observations that led to the discovery of IGFs was the finding that serum contained factors that exerted insulin-like effects on muscle and adipose tissue which were not suppressible by insulin antibodies. This insulin-like activity was termed "non suppressible insulin-like activity (NSILA) (Froesch et al, 1963). A third line of evidence came from studies that indicated that factors secreted by cultured cells promoted cell proliferation. One of these factors termed "multiplication stimulating activity" (MSA) was identified in conditioned medium from rat liver cells (Dulak and Temin 1973). Later it became apparent that these three forms of bioactivities represented one group of factors that were at least partially GH dependent. They shared growth promoting activity on cartilage, mimicked insulin action and promoted cell division. Therefore, the term Somatomedin (SM) was introduced for the then uncharacterized factor that mediated these actions (Daughaday et al, 1972).

After purification and determination of the primary structure of these growth promoting and insulin-like factors it became evident that they consisted of two related peptides which were renamed insulin-like growth factor I and II (IGF-I and IGF-II) (Rinderknecht and Humbel, 1976; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel ,1978b). Since then no evidence has been found for the existence of IGFs other than IGF-I or -II. Only precursor and variant forms which differ in size and charge from both IGFs have been isolated or predicted from cDNA sequences. (Janssen et al, 1983; Janssen et al, 1985; Van den Brande, 1992).

Structure

The primary structure of both IGF-I and IGF-II (mol. weights approx. 7.5 kD.) consists of a single chain molecule of 70 and 67 amino acid residues respectively with three intrachain disulphide bonds. (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b; Raschdorf et al, 1988). In anology to proinsulin the amino acid sequences of IGF-I and -II can be grouped into an amino-terminal B-region and an A-region connected by a short C-region, as shown in Figure 1. These regions display a homology of respectively 62%, 43% and 41% to proinsulin. In addition, the IGFs contain a D-region which is absent in proinsulin. Due to the observed homology a tertiary structure similar to that of insulin has been proposed (Blundell and Humbel, 1980; Blundell et al, 1983).

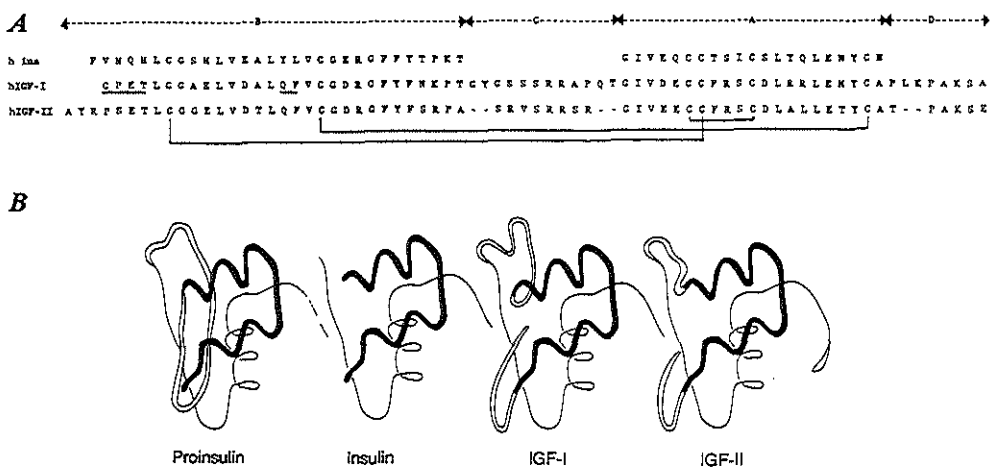

Fig.1 A: Primary structure of Insulin, IGF-I and IGF-II. Dashes are introduced for optimal alignment. Amino acid residues of IGF-I thought to be involved in interaction with the binding proteins are underlined. B: Proposed tertiary structure of Insulin, IGF-I and IGF-II.

The recent resolution of the tertiary structure of IGF-I by 2D NMR analysis confirmed that the conformation, with the exception of some minor differences, is similar to that of insulin (Cooke et al, 1991).

Computer modelling together with studies of IGF/insulin hybrid molecules and site-directed mutagenesis of IGF-I have led to the definition of various functionally active domains. The recognition site for the IGF binding proteins is enclosed in the N-terminal

B region of IGF-I. The first 4 amino acid residues of the amino-terminus plus residues 15 and 16 are of major importance for the interaction of IGF with the binding proteins. A possible involvement of residues 49-51 has also been suggested (Bayne et al, 1989; Cascieri et al, 1988a; Cascieri, 1989b).

The aromatic residues at positions 23-25 appear to be involved in the recognition of the ligand by the type 1 IGF receptor (Cascieri et al, 1988b). In addition, studies with hybrid Insulin-IGF molecules suggest that the A region of IGF is important for the mitogenic properties of IGF-I. (Sheikh et al, 1987). Binding to the type 2 IGF- receptor seems to be located in the A region with residues 49-51 as most important contributors (Cascieri et al, 1989a).

Apart from species differences in the primary structure, variant forms of both IGF-I and IGF-II have been isolated or predicted from cDNA sequences. A truncated IGF-I lacking the first three amino acid residues at the amino-terminus, probably as a result of alternative processing of the prepro-IGF-I, has been isolated from fetal and adult brain and has also been found in bovine colostrum and in porcine uterine tissues (Sara et al, 1988; Francis et al, 1988; Ogasawara et al, 1989). The reduced affinity of the truncated IGF-I for IGF binding proteins is thought to be responsible for the increased biological activity of this form being about 10 times higher than its full length counterpart. It is of interest that the N-terminal tripeptide appears to have intrinsic neurotransmitter activity (Sara et al, 1989). A variant precursor form of IGF-II has been described with a three amino acid insert that probably results from alternative splicing of the mRNA (Jansen et al, 1985). In addition, higher molecular weight forms (9 and 15 kD) of IGF-II have been identified in human brain and serum. Most likely they represent unprocessed precursor forms (Hasselbacher and Humbel, 1982; Gowan et al, 1987; Daughaday and Rotwein, 1989a).

Genes and expression

Both IGF-I and IGF-II are encoded by single genes. In man the IGF-I gene is localized on the long arm of chromosome 12(q21-q24). The IGF-II gene is situated on the short arm of chromosome 11(p15) only 1.4kb downstream of the insulin gene (Brissenden et al, 1984; Bell et al, 1985).

The human IGF-I gene extends over more than 73 kb and consists of six exons. It has at least four different promoter regions, and multiple potential polyadenylation sites of which only two are used (Sussenbach et al, 1991). Three major classes of mRNAs are transcribed from the gene with lengths of 7.6, 1.3 and 1.1 kb respectively. Two transcripts are generated due to alternative splicing and one by using a different polyadenylation site. Nevertheless, the three classes of transcripts all encode for functional IGF precursor peptides that only differ in their carboxyl-terminal sequences. A 195 amino acid preproIGF-IB precursor is encoded by exons 1-4, whereas exons 1-3 and 5 code for a 153 amino acid preproIGF-IA precursor (Rotwein, 1986). The mature IGF-I peptide is encoded by exons 2 and 3 (Figure 2).

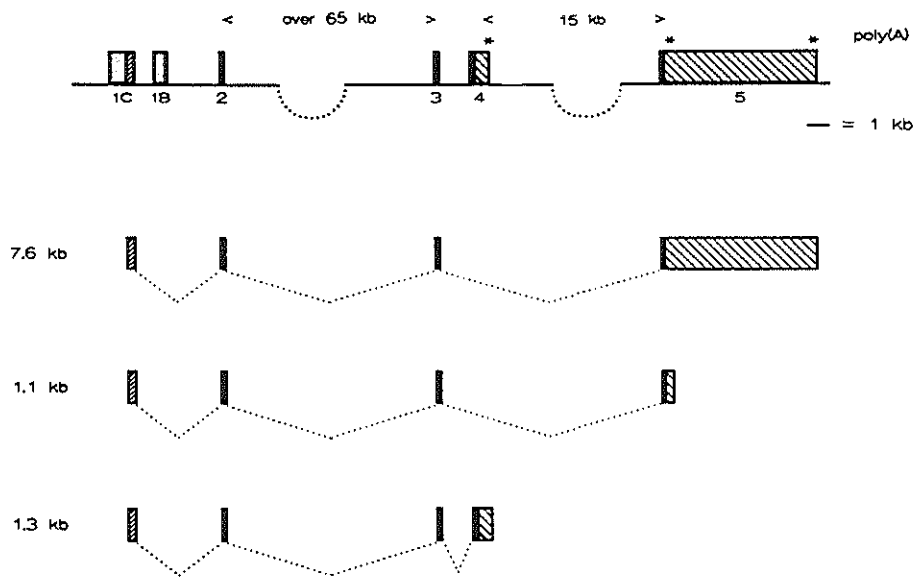

Figure 2. Structure of the human IGF-I gene and mRNA species. The mature IGF-I polypeptide is encoded by exons 2 and 3. The asteriks indicate different polyadenylation sites (from Sussenbach et al, 1991).

The human IGF-II gene spans about 30 kb of chromosomal DNA and consists of six non-coding and three coding exons (Bell et al, 1985; Janssen et al, 1985; Daughaday and Rotwein, 1989; Sussenbach et al, 1991). The IGF-II gene possesses four different promoter regions that are regulated in a tissue specific and developmental manner. Various IGF-II mRNA transcripts varying at their 5' ends are generated by transcription

initiation at different promoter regions (De Pagter-Holthuizen, 1988). In addition, multiple polyadenylation signals can be used. All transcripts include the coding region for the complete IGF-II precursor protein (Figure 3).

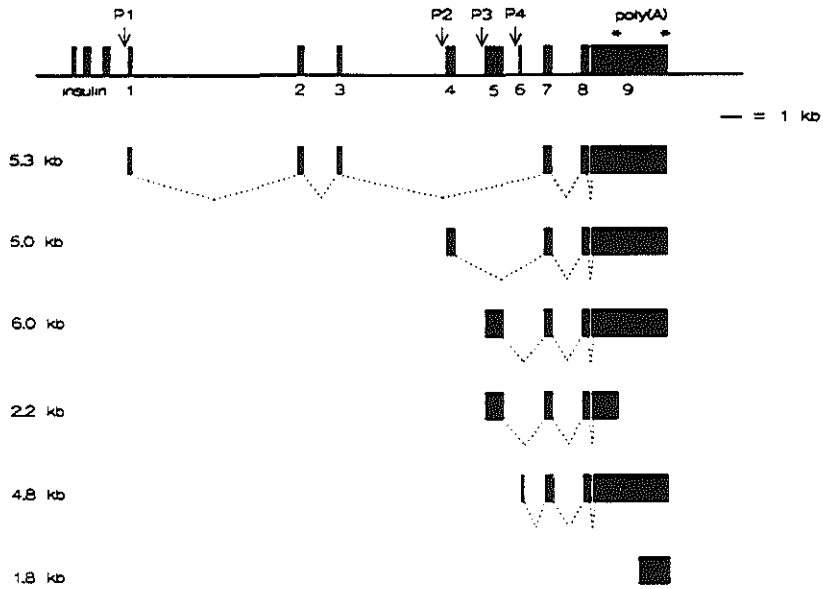

Figure 3. Structure of the human IGF-II gene and mRNA species. The promoters are indicated by the arrows (P1-4). The asteriks show two alternative polyadenylation sites. The mature IGF-II polypeptide is encoded by exons 7, 8 and part of 9 (from Sussenbach et al, 1991).

GH has been established as a primary regulator of IGF-I gene expression in adults (Hynes et al, 1987; Matthews et al, 1986), whereas IGF-II is less GH dependent. Several other factors like age, nutrition, insulin, thyroid hormones and glucocorticoids have also been demonstrated to be important regulators of IGF-I synthesis (Clemmons et al, 1981; Clemmons and Shaw, 1983; Clemmons and Van Wyk, 1984; Binoux et al, 1986). In addition IGF-I biosynthesis is enhanced in response to local tissue injury (Hanson et al, 1987, Jennische et al, 1987). These findings reflect that the proliferative effects of many hormones are mediated by stimulation of local IGF-I production.

The factors involved in activation of the tissue-specific and developmentally dependent promoters of the IGF-II gene are still unknown. Recent evidence demonstrated that the IGF-II gene is subject to parental imprinting. Transgenic mice carrying a hemizygous disruption of the IGF-II gene demonstrated a marked fetal growth retardation. This

mutation appeared to be transmitted only through the male germ line. Analysis of the transcripts from the wild-type and mutated alleles indicated that only the paternal allele is expressed in embryos, with the exception of the chorioid plexus and leptomeninges where both paternal and maternal alleles were active (DeChiara et al, 1991). In man the IGF-II gene is also found to be parentally imprinted as in the mouse (Ohlsson et al, 1993; Giannoukakis et al, 1993).

The analysis of the IGF genes has revealed that the biosynthesis and functioning of both IGFs can be controlled by multiple regulatory mechanisms at different levels: by imprinting, transcription initiation/termination. In addition, alternative splicing, translational control, and processing of the precursor proteins can take place. This variety of control mechanisms allows a flexible regulation of IGF expression under diverse conditions.

Physiological effects of IGF

Although the major source of IGFs is the liver, it has been firmly established that IGFs are synthesized and secreted in response to local stimuli in many cells throughout the body. This finding has challenged the original concept that IGFs only act via an endocrine mechanism as a mediator of growth hormone in the stimulation of longitudinal body growth (Daughaday et al, 1966). Many studies have demonstrated that IGF peptides are widely expressed and exert profound biological actions on target cells. Stimulation of metabolic activity, proliferation and differentiation has been demonstrated.

Although less potent than insulin, IGFs exert metabolic effects on insulin target tissues *in vitro*. Like insulin, IGFs stimulate glucose transport into muscle and adipose cells. In addition they promote lipolysis and glycogen and protein synthesis in adipose tissues (Froesch et al, 1985). The acute metabolic effects of IGFs appear to be mediated through both insulin and type 1 IGF receptors (Froesch et al, 1985;). Bolus injections of IGF-I in man as well as in rats resulted in a transient decrease of blood glucose and serum free fatty acid concentrations (Guler et al, 1987; Zapf et al, 1986). Continuous infusion of IGF-I decreases insulin secretion and results in lower GH and IGF-II concentrations (Guler et al, 1989). In addition, IGFs influence the rate of differentiation processes in fetal and neonatal cells, such as muscle, cartilage, bone, brain and adrenal gland (Han and Hill, 1991). In myoblast cell lines established from neonatal rat muscle,

IGF-I or -II enhanced the differentiation into contracting myotubes (Ewton and Florini, 1981). In fetal rat calvaria, IGF-I stimulated bone matrix formation in addition to bone cell replication (Schmid et al, 1984). Moreover, effects of IGF-I on differentiation of ovarian granulosa cells (Adashi et al, 1985; Veldhuis et al, 1985) and on androgen production in ovarian thecal-interstitial cells have been reported (Cara et al, 1988).

The growth promoting activities of the IGFs have received most attention. Both IGF-I and -II are mitogenic for isolated fetal rat or human mesenchymal tissues such as fibroblasts, myoblasts, chondrocytes and osteoblasts, for fetal hepatocytes, glial cells and adrenal cells (Han and Hill, 1991). However, IGF-I as a single growth factor is usually not enough to achieve a complete cell cycle; additional growth factors are needed for a full effect (Froesch et al, 1985). Platelet-derived growth factor (PDGF), and/or epidermal growth factor (EGF) bring the cells from G_0 to G_1, thereby rendering them competent for further progress (Pledger et al, 1977). Subsequently, IGF-I stimulates the cell to proceed through the rest of the cell cycle. (Wharton et al, 1981; Wharton et al, 1983; Chen et al, 1989).

The biological role of IGF-I in postnatal growth has been well established. IGF-I mediates the mitogenic action of GH in cartilage tissue (Van Wijk, 1984; Zapf, 1984). GH has been shown to stimulate long bone growth by inducing local production of IGF-I, which in turn stimulates proliferation of the bone growth plates (Isakson et al, 1987). Long term treatment with IGF-I at concentrations that do not cause hypoglycaemia results in an increase in body weight, tail length and weight of single organs in hypophysectomized rats (Schoenle et al, 1982), normal rats (Hizuka et al, 1986) and Snell dwarf mice (Van Buul-Offers et al, 1986). Similar effects were obtained in transgenic mice expressing human IGF-I (Quaife et al, 1989).

Though IGF-I is a more potent growth promoter than IGF-II, the latter has been inferred to be important in particular during fetal development. High concentrations of mRNA and, the IGF-II peptide are manifest *in utero* (Han et al, 1987a; Han et al ,1987b; Han et al ,1988). Moreover, studies with transgenic mice bearing a disruption of the IGF-II gene provided direct evidence for a role of IGF-II in fetal growth. Chimaeric animals demonstrated a growth deficient phenotype that became apparent during embryonic growth and persisted after birth. Apart from the body weights which were about 60% of

that of the wild type littermates, the growth deficient animals where apparently normal and fertile (DiChiara et al, 1990; DiChiari, 1991).

There is no doubt that IGFs have several biological effects that are tissue specific and depend on the developmental stage of the cells and it appears that the net effect is stimulation of fetal as well as postnatal growth.

1.2 The IGF receptors

Two classes of receptors have been identified which bind IGFs with high affinity. The type 1 receptor binds both IGFs with approximately similar affinity, and insulin with lower affinity. This receptor mediates metabolic and mitogenic responses to either ligand. In contrast, the type 2 receptor binds IGF-II with high affinity and IGF-I with low affinity, but has no affinity for insulin. The function of the latter receptor in the IGF system is presently unclear.

Structure and function

With the isolation of type 1 receptor cDNA it became apparent that the type 1 receptor not only shares structural and functional homology with the insulin receptor, but also that the sequence homology between the two receptors was very definite (Figure 4). The most prominent homology (84%) was found in the ß-subunit of the receptors enclosing tyrosine kinase activity (Ullrich et al, 1985; Ullrich et al, 1986). The mature forms of both receptors are synthesized from an αß polypeptide precursor. In the endoplasmatic reticulum and the Golgi apparatus the precursor undergoes glycosylation and proteolytic cleavage, and is assembled into an $\alpha_2ß_2$ heterotetrameric complex linked by disulphide bonds (Hedo et al, 1981; Jacobs et al, 1983a; Ronnet et al, 1984). The α-subunits of approx. 130kD constitute the extracellular part of the receptor molecule. They enclose a cysteine-rich domain that contributes to the high affinity binding of IGF-I and insulin (Waugh et al, 1989; Andersen et al, 1990; Gustafson et al, 1990). For IGF-I and IGF-II two binding sites with different affinities have been demonstrated. Site Ia with the highest affinity for IGF-I is blocked by an antibody raised against the 130 kD α-subunit, whereas site Ib, with preference for IGF II is not blocked by the same antibody (Casella et al, 1986; Lee et al, 1986). The ß-subunit of approx. 95 kD encloses a membrane spanning region, an ATP binding domain and tyrosine-specific autophosphorylation sites. The intracellular part exhibits intrinsic protein kinase activity (Jacobs et al, 1983b; Rechler and Nissly, 1985; Tornqvist et al, 1988). In addition to the insulin and IGF-I receptors several variant receptor subtypes have been observed with differences in binding and response to IGF-I, IGF-II and insulin. These subtypes are heterogeneous in their immunological and structural properties and may be the result of alternative mRNA spli cing or different posttranslational modifications. Part of these variant receptors with

unique properties were found to be assembled from insulin and IGF-I αß receptor subunits (Moxham et al, 1989; Soos et al, 1989; Soos et al, 1990). The function of these hybrid receptors is unclear.

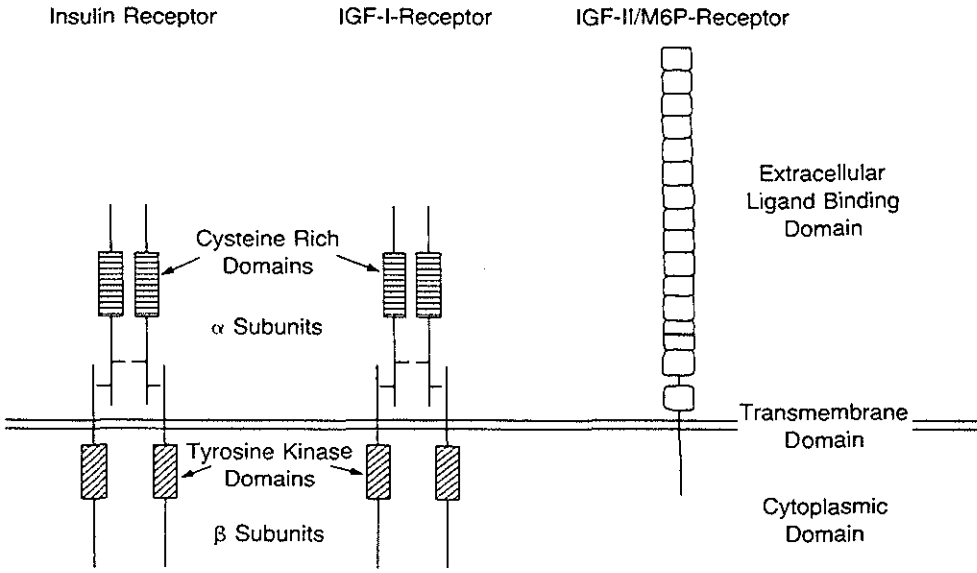

Figure 4. Schematic representation of the structure of Insulin, Type 1 (IGF-I) and Type 2 (IGF-II) receptors (from Bach et al, 1991).

The type 2 receptor is structurally distinct from the type 1 and insulin receptor. The mature receptor consists of a glycosylated single chain polypeptide of approx. 250 kD without kinase activity. The primary structure has been deduced from the cDNA and appeared to be identical to the human cation independent mannose 6-phosphate receptor (CI-M6PR) (Morgan et al, 1987; Oshima et al, 1988). The receptor is build from a repetition of 15 cysteine containing units and a transmembrane domain. Most of the IGF-II receptor (92%) is oriented extracellularly. This part of the molecule encloses two high affinity binding sites for mannose 6-phosphate (M6P)-containing proteins and a single binding site for IGF-II (Tong et al, 1988; Tong et al, 1989; Tong and Kornfeld, 1989). Both binding domains display reciprocal inhibition upon binding of the two classes of ligands (Nissley and Kies, 1991).

Confusingly, the CI-M6P/IGF-II receptor has no function related to any known function of IGF-II. The CI-M6P/IGF-II receptor is located on both the cell surface and Golgi apparatus where it is involved in sorting lysosomal enzymes from the Golgi apparatus and/or plasma membranes to lysosomes (Von Figura et al, 1986). The CI-M6P/IGF-II receptor features a rapid internalization and recycling (Oka et al, 1986). In this manner the receptor may serve to clear IGF-II from the circulation. IGF-II from the cell surface is internalized and degraded by lysosomal enzymes. This process can be induced by both GH as well as insulin (Oka et al, 1987; Lonnroth et al, 1987).
From all studies concerning IGF receptors the overall picture emerges that most if not all biological responses of IGF-I and -II are mediated by the type 1 receptor and only rarely by the type 2 receptor. Nevertheless, a number of reports indicate that IGF-II does mediate responses through its own receptor. At present the role of this receptor in the IGF system is puzzling.

Genes and expression

Little is known about the genes that code for the IGF receptors. Both receptors appear to be encoded by single genes that map at different chromosomes. In human the IGF-I receptor gene is located at chromosome 15q25-26 (Ullrich et al, 1986) and the IGF-II receptor gene at the long arm of chromosome 6 (Laureys et al, 1988). Both receptors appear to be regulated by different mechanisms. The IGF-I receptor is down-regulated by both IGFs and insulin (Rosenfeld et al, 1980; Rosenfeld, 1982). In addition, IGF-I receptor expression is influenced by nutritional (Lowe et al, 1989), hormonal (Werner et al, 1990), developmental and tissue-specific factors (Werner et al, 1989; Bondy et al, 1990; Bach et al, 1991).
The 5' flanking sequences of the IGF-I receptor gene enclose a transcription initiation site that lacks consensus TATA and CCAAT elements. Instead of these elements a specific "initiator" sequence is present that facilitates transcription initiation from a single site in the absence of TATA or CCAAT motifs (Werner et al, 1990). Multiple potential binding sites for a number of transcription factors have been identified upstream of the transcription start site. The physiological importance is presently unclear.

1.3. The IGF binding proteins (IGFBPs)

An important aspect of IGF functioning is represented by the IGF binding proteins (IGFBPs). Soon after the discovery of IGFs it has been noticed that the molecular size of the component displaying IGF activity in plasma did not correspond to the 7.5 kD of purified IGF. Gel filtration of serum and studies with radiolabeled IGF demonstrated specific association of IGF with two classes of serum proteins with molecular weights of approximately 150 kD and 30-40 kD. The majority of the IGFs in serum (>95%) was found to be associated to the high molecular weight binding proteins (150 kD), whereas the low molecular weight binding proteins (30-40 kD) accounted for most of the unsaturated binding sites in plasma (Hintz and Liu, 1977; Hintz and Liu, 1981). Upon acidification, the high molecular weight binding protein dissociated into subunits. For a long time it was not known whether the 30-40 kD binding protein forms were subunits of the high molecular weight binding protein or a separate class of binding proteins (Furlanetto, 1980).

A major breakthrough was the development of the ligand blot for binding proteins. With this method the IGFBPs are separated with SDS/PAGE and subsequently the proteins are transferred onto nitrocellulose. Following incubation with radiolabeled IGF the IGFBPs can be visualized by autoradiography (Hossenlopp et al, 1986a). The presence of multiple forms of binding proteins has been demonstrated in a variety of tissues and body fluids. The appearance and ratio of the different forms of IGFBPs varied depending on the tissue or body fluid analyzed (Chochinov et al, 1977; Schwander, 1983; Hossenlopp et al, 1986a; Bar et al, 1987; Binoux et al, 1988). These observations suggest specific roles for the various binding protein forms. At least five different forms were recognized with molecular weights ranging from 24-42 kD (Binoux et al, 1986). The 38 and 42kD binding proteins were found to be immunologically related and appeared to be differentially glycosylated forms of the same IGFBP (Baxter et al, 1986a).

Another important finding resolved the relation between these low molecular weight IGFBP forms and the 150 kD high molecular weight form (Martin and Baxter, 1986). Under natural conditions one of the binding proteins (now known as IGFBP-3) forms a ternary complex with an acid labile glycoprotein (ALS) in the presence of IGF. This ALS protein has a molecular weight of approx. 85 kD and is unrelated to the IGFBPs or the IGF receptors (Baxter, 1988; Baxter and Martin, 1989).

Evidently, the IGFBPs constitute a single class of related proteins of which only one is capable of forming a ternary complex. With the isolation of the various forms and the introduction of DNA cloning techniques a new impulse was given to the identification and characterization of IGFBPs

Structure of the Binding Proteins

At present six different binding proteins, termed IGFBP-1 to -6, have been isolated and their primary structure has been elucidated from the respective cDNAs (Chapter II; Brewer et al, 1988; Lee et al, 1988; Wood et al, 1988; Binkert et al, 1989; Zapf et al, 1990a; Kiefer et al, 1991; Shimasaki et al,1990; Shimasaki et al, 1991a; Shimasaki et al, 1991b). Comparison of the different IGFBP amino acid sequences clearly demonstrates that these proteins comprise a family of related proteins (Figure 5).

Figure 5. Comparison of the primary structure of IGFBPs1-6. Conserved amino acids are shaded and cysteine residues are indicated with an asterisk. The RGD sequence in IGFBP-1 and -2 is boxed. The serine phosphorylation sites in IGFBP-1 are circled. (Adapted from Rechler and Brown, 1992).

Notably the amino- and carboxyl-terminal regions of the IGFBPs display a large degree of homology. In IGFBP-1,-2,-3 and -5 there is a strict conservation of the position of the 18 cysteines in the N- and C-terminal parts of the proteins. In addition to these cysteines, IGFBP-4 also has two cysteine residues in the less conserved middle part of the protein. The recently characterized IGFBP-6 differs somewhat from the others in that it lacks 4 cysteine residues (Figure 5).

The positional conservation of the cysteines suggests that these residues contribute to an overall similar tertiary structure of the binding proteins. Indeed, site-directed mutagenesis

of several cysteine residues of IGFBP-1 demonstrated the importance of these residues for the integrity of the molecule and IGF binding capability. Substitution of Cys38 and Cys226 for tyrosine residues resulted in structural changes, shown as abnormal migration in SDS/PAGE, dimer formation and loss of IGF binding capability (Brinkman et al, 1991a; Brinkman et al, 1991b). Similarly, conversion of Cys16 and 35 into serine residues abolished IGF binding capability (Powell et al, 1989).

However, apparently not all cysteine residues are equally important for the structure and IGF binding properties (Zapf et al, 1990a; Shimasaki et al, 1991). Substitution of Cys34 of IGFBP-1 does not result in a notable change in apparent molecular weight or ligand binding potential (Brinkman et al, 1991a). This residue is also one of the 4 cysteine residues that are absent in IGFBP-6.

The region between the conserved extremities varies greatly between the different IGFBPs. The middle part typically carries a considerable number of charged and polar amino acids. It is conceivable that the variance in this region is responsible for the physical diversity displayed by the different members of the IGFBP family. For instance IGFBP-3 is the only binding protein known that is capable of forming a ternary complex together with IGF and the ALS protein. IGFBP-3, IGFBP-4 and IGFBP-6 exhibit potential N-linked glycosylation sites that are absent in IGFBP-1, IGFBP-2 and IGFBP-5 (Fig. 3). Treatment of purified IGFBP-3 and -4 with N-glycase not only demonstrated that these binding proteins are actually glycosylated *in vivo* but also that IGFBP-3 exists in multiple differentially glycosylated forms (38-42kD).

An Arg-Glu-Asp (RGD) sequence is present near the carboxyl-terminus in IGFBP-1 and IGFBP-2. Such an amino acid sequence motif is found to be involved in binding of various matrix proteins such as fibronectin, vitellogenin and von Willebrandts factor to a family of matrix receptors at the cell-surface (Ruoslathi and Pierschbacher, 1986; Ruoslathi and Pierschbacher, 1987). Although evidence has been presented that some binding proteins can adhere to cells (McCusker et al, 1990), it is not known whether the RGD motif in IGFBP-1 and IGFBP-2 is functionally involved.

Recently, phosphorylated forms of IGFBP-1,-2 and -3 have been demonstrated. At least 5 different phosphorylated isoforms of IGFBP-1 are secreted by the human hepatoma cell line HEPG2 and decidual cells. The ratio between the non-phosphorylated binding proteins and the phosphorylated forms appears to be dependent on the source from

which the IGFBPs were isolated (Jones et al, 1991, Frost et al, 1991). Phosphorylation of IGFBP-1 takes place exclusively on serine residues 101, 119 and 169, from which Ser101 appears to be the major site of phosphorylation. Serine phosphorylation results in a six fold increase in the affinity of IGFBP-1 for IGF-I. Moreover, it appears to modify the capacity of IGFBP-1 to potentiate or to inhibit IGF bioactivity (Jones et al, 1993).

Thus far, neither the IGF ligand binding site of the IGFBPs nor the site of interaction between the ALS protein and IGFBP-3 have been defined. One line of experimental data suggests that the cysteine rich amino-terminal region encloses the ligand binding domain. Partial deletion of the amino-terminus of IGFBP-1 resulted in loss of IGF binding capability (Chapter V), while truncated forms of IGFBP-1 having an intact amino-terminus have been found to be capable of binding IGF (Huthala et al, 1986). In accordance with this observation is the finding that the amino-terminal one-third of IGFBP-3 retains IGF binding capability *in vitro* (Sommer et al, 1991). However, this truncated protein did not inhibit IGF action *in vivo* as did the wild type IGFBP-3 (Schmid et al, 1991).

In contrast to these observations, other experiments favour the carboxyl-terminal region as a potential ligand binding site: a truncated form of rat IGFBP-2 that lacks the amino-terminal cysteine rich region was found to be capable of binding IGF, albeit with reduced capacity (Wang et al, 1988). The introduction of a small deletion or frame shift in the carboxyl-terminus of human IGFBP-1 resulted in loss of ligand binding capability (Brinkman et al, 1989; Chapter IV). However, site directed mutagenesis in well conserved areas in both the amino- and the carboxyl-terminal parts of IGFBP-1 failed to affect ligand binding. So far, only substitutions of residues that presumably affect the tertiary structure of the protein, like Cys38 and Cys226, or other substitutions that might interfere with disulphide bond formation such as mutation of Gly222, which also leads to dimer formation, resulted in loss of IGF binding (Brinkman et al, 1989; Chapters IV and V).

It appears that the IGFBPs are tightly folded molecules and it could very well be that both the N- and C-terminal regions are spatially very close and that both contribute to the ligand binding site. The less conserved middle part of the molecule, enclosing most of the potential glycosylation and phosphorylation sites may determine the individual

properties of IGFBPs such as: association with the cell surface, the ALS protein or preference for IGF-I or -II.

IGFBP genes

With the isolation of cDNAs for IGFBP-1-6 it became feasible to localize the genes in the human genome and to study the genomic organization. The IGFBP-1 and -3 genes have been regionally localized at chromosome 7p12-13. The genes are located in a tail to tail arrangement separated by 19.3 kb of DNA (Chapter III; Luthman et al, 1989; Cubbage et al, 1989; Cubbage et al, 1990). The IGFBP-2 gene is located at chromosome region 2q33-q34 (Shimasaki et al, 1991a; Ehrenborg et al, 1991) and, the IGFBP-4 and 5 genes are on chromosomes 17 and 5 respectively (Shimasaki et al 1991a). In Chapter III the genomic organization of the human IGFBP-1 gene is discussed. With the isolation of the IGFBP-2 and IGFBP-3 genes it appeared that IGFBP-1,-2 and -3 are all single copy genes spanning respectively 5.2, 32 and 8.9 kb. The intron/exon organization of the IGFBP-2 and -3 genes is very similar to that of the IGFBP-1 gene.

In all IGFBP genes, the protein coding region is divided over 4 exons and the intron/exon boundaries are more or less conserved among the genes. The exons 1,3 and 4 are approximately of the same size and code for the conserved amino- and carboxyl-terminal regions in the binding proteins. Exon 2 is similar in size in the IGFBP-1,-2 and 3 genes and codes for the less well conserved middle part of the proteins. In contrast to the IGFBP-1 and -2 genes, the IGFBP-3 gene has an additional fifth exon which encompasses the entire 3' untranslated region. The genomic organization of the IGFBP-4,-5, and -6 genes has not yet been resolved. Since the IGFBP-1,-2 and -3 genes have a similar genomic organization it may be anticipated that the genomic organization of the other binding protein genes will be similar to that of those already known.

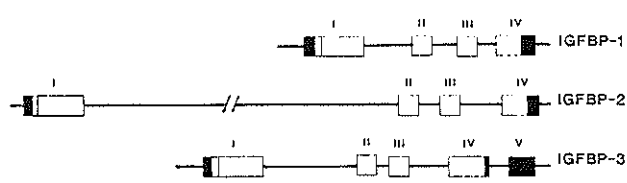

Figure 6. Genomic organization of the genes for IGFBP-1,-2 and -3. The exons are displayed as boxes in which the filled areas represent the 5' and 3' untranslated regions.

In all three IGFBP genes that have been studied, exon 1 and the promoter regions overlap with a CpG island. The promoters of both IGFBP-1 and -3 have an arrangement characteristic for many eukaryotic promoters (Chapter III; Suwanichkul et al, 1990; Cubbage et al, 1990). Both promoters encompass a single transcription start site downstream of a TATA box. Upstream of these elements a CCAAT box is present in the IGFBP-1 promoter, while for IGFBP-3 a GC element is found. Putative binding sites for transcription factors AP-2 and Sp-1 overlap with this GC element. Recently it has been shown that the liver specific transcription factor LF-B1 binds to a site overlapping with the CCAAT box of the IGFBP-1 promoter (Cubbage et al, 1990). This factor appeared to be crucial for basal IGFBP-1 promoter activity in HEPG2 cells. In addition, an insulin responsive element has been identified in the IGFBP-1 promoter region which is in accordance with many observations that suggest that insulin is a primary regulator of IGFBP-1 expression *in vivo* and *in vitro* (Powell et al, 1991). In contrast to the aforenamed promoters the IGFBP-2 promoter region does not contain a TATA box. However, chimaeric constructs with 438 bp of the 5' flanking region of the IGFBP-2 gene appeared to be sufficient to direct expression of a reporter gene in an orientation specific fashion. Upstream of the transcription start site several putative binding sites for the transcription factors AP-1, AP-2, Sp1 and liver factor B1 have been found (Rechler and Brown, 1991).

Thus, although the genomic organization of IGFBP-1, -2 and -3 is very similar, the promoter regions and upstream elements are quite different. It is obvious that the promoter activity of the different genes is controlled by various factors, that may appear to be responsible for the distinct tissue distribution, developmental and hormonal regulation of the IGFBPs.

Tissue specific and developmentally regulated expression of IGFBPs

From many studies it has become apparent that the IGFBPs are differentially and/or tissue specifically expressed (Han et al, 1998; Shimisaki et al, 1991a; Schuller et al, 1993). As discussed previously, the nature of the factors controlling the level of expression of the various IGFBPs is up till now poorly understood. Under normal conditions, the liver seems to be the major site of expression for most of the IGFBPs, but many other tissues also express IGFBPs depending on age or stage of development of the organ. In contrast

to the other IGFBPs, IGFBP-5 is more ubiquitously expressed with as major site of expression the kidneys (Shimasaki et al, 1991a). In addition to apparent regulation of IGFBP expression in a tissue specific manner, the expression level of the different IGFBPs is also regulated during development. IGFBP-3 is mainly expressed in adult tissues. IGFBP-1 and -2 however, are predominantly expressed during fetal development in respectively the endometrium and fetal brain. After birth the IGFBP-2 expression is rapidly turned off in most tissues except in the choroid plexus of the brain.

The expression of IGFBP-3 is found to be GH dependent (Moses et al, 1976; Copeland et al, 1980; Hintz et al, 1981; White et al, 1981). Serum levels of the large IGFBP/IGF/ALS complex rise with age especially during puberty (Baxter and Martin, 1986b). In addition, serum levels are decreased in hypopituitarism and increased in acromegaly. Low levels are also found in patients suffering from liver cirrhosis, in poorly controlled diabetes and in patients with Cushing's syndrome. Elevated serum levels are found in patients with chronic renal failure and during pregnancy (Zapf et al, 1980; Binoux et al, 1986; Martin and Baxter, 1986b; Hokken-Koelenga et al, 1991).

During pregnancy, IGFBP-1 levels rise, and peak between 20-25 weeks of gestation (Drop et al, 1982; Drop et al, 1984a; Hall, 1986). The protein is most abundant in midterm amniotic fluid (Chochinov et al, 1977; Bala et al, 1978; Póvoa et al, 1984; Drop et al 1979). Maternal serum levels parallel those in amniotic fluid although the levels are 100-1000 times lower (Rutanen et al, 1982; Koistinen et al, 1987). In situ hybridization with IGFBP-1 probes and immunohistochemistry with IGFBP-1 antibodies have demonstrated that in endometrium the stromal cells are responsible for the high level expression during pregnancy (Inabe et al, 1980; Rutanen et al, 1984a; Fasleabas et al, 1989; Julkunen et al, 1990; Brinkman, unpublished results). Serum levels of IGFBP-1 seem to be inversely related to the GH status, i.e. levels are elevated in patients with GH deficiency and decreased in patients with acromegaly (Drop et al, 1984b; Hall et al, 1988; Hardouin et al, 1989). In addition, elevated levels are found in patients with chronic renal failure (Drop et al, 1984b; Lee et al, 1989; Hokken-Koelenga et al, 1991), in diabetic and non-diabetic pregnant women (Hall et al, 1986) and in patients with Laron type dwarfism (Hardouin et al, 1989). Increased IGFBP-1 levels have been found in 94% of patients with primary liver cancer and in 50% of patients with cirrhosis (Rutanen et al 1984b). In addition, serum IGFBP-1 levels show a marked diurnal rhythm

with high early morning levels, unrelated to the GH status (Baxter and Cowell, 1987; Yeoh and Baxter, 1988). The observation that serum levels are elevated in type 1 diabetics and decreased in patients with insulinoma (Suikari et al, 1988) suggested that serum insulin concentration plays a role in the regulation of the IGFBP-1 levels. Similarly, *in vivo* experiments with diabetic rats suggested regulation of IGFBP-1 by insulin (Ooi et al, 1990). Indeed, a putative insulin responsive element has been found in the promoter region of the IGFBP-1 gene (Powell et al, 1991).

It is evident that dependent on the developmental, metabolic and hormonal state, the relative levels of the different IGFBPs can vary considerably. The consequences of these varying IGFBP profiles are at present not well understood.

Physiological role of the IGF binding proteins

In serum the main function of the IGFBPs seems to be sequestering of IGFs in order to arrest acute metabolic effects of free IGF and to maintain a stable storage depot of IGFs (Baxter, 1991; Zapf et al, 1991). Tracer experiments have demonstrated that most (secreted or injected) IGF is bound by IGFBP-3 in a ternary complex which protects the IGF peptides from degradation and rapid clearance from the circulation (Hintz et al, 1984; Zapf et al, 1986; Zapf et al, 1989). Approx. 80% of total serum IGF is associated with this complex; therefore, IGFBP-3 can be considered as the major circulating carrier of the IGFs. The remaining IGF (approx. 20%) is bound to IGFBP-1, -2, -4 and other proteins, of which IGFBP-2 is the most prevalent (Zapf et al, 1990b). Only 1-5% of total IGF-I and 1-2% of IGF-II are found in free form (Guler et al, 1989; Zapf et al, 1991). Association of IGFs to the binding proteins prolongs the serum half-life of IGFs considerably from 12 minutes in its free form to 30 minutes when associated to one of the IGFBPs. Moreover, when the IGFs are complexed in the 150 kD ternary complex the half-life increases up to 14-18 hrs.

Injection of des(1-3)IGF-I, a derivative of IGF-I with a decreased affinity for IGFBPs, resulted in an enhanced uptake of this derivative into tissues as compared to IGF-I or IGF-II, suggesting that the IGFBPs restrict transfer of IGFs to their target tissues (Cascieri et al, 1988a; Ballard et al, 1991). However, the situation appears to be even more complicated. Recently, it was shown that binary IGF/IGFBP complexes (50kD) can leave the circulation by crossing the capillary barrier. Infusion of labelled IGFBP-1 or -

3, or a bovine endothelial cell derived IGFBP in perfused rat hearts or in intact animals demonstrated a preferential localization of IGFBP-1 in the cardiac muscle while IGFBP-3 was predominantly found in connective tissue (Bar et al, 1990a). Insulin increased transendothelial transfer of IGFBP-1 and decreased that of IGFBP-3. This proves that re-entering and distribution in the tissues appears to be hormone dependent and selective for IGFBP-1,-2 and -3 complexes (Bar et al, 1990b).

In contrast to the binary IGF/IGFBP complexes, the high molecular weight ternary complex is not able to cross the capillary boundary. This explains why IGFs present in the low molecular weight pool have a much higher turnover rate then IGFs contained in the 150 kD ternary complex (Zapf et al, 1986; Guler et al, 1989). Thus the complex formation between IGFBP-3/IGF and ALS might be a key control mechanism in keeping a stable storage depot of inactivated IGFs in the circulation and to adjust influx and efflux of IGFs from the circulation to the tissues.

It has become increasingly clear that several factors influence the ternary IGFBP-3 complex. Evidence has been put forward that specific mechanisms, involving proteolytic enzymes and heparin, allow dissociation of the complex (Chatelain et al, 1983; Clemmons et al, 1983). In late pregnancy, specific protease activity seems to affect the stability of the ternary complex (Hossenlopp et al, 1990; Guidice et al, 1990), though IGFBP-3 is still present and also functionally intact (Suikkari and Baxter, 1991: Suikkari and Baxter, 1992). Proteolysis of IGFBP-3 has been reported also in serum of patients suffering from prostate cancer, GH deficiency or a GH receptor defect, and in patients in a catabolic state. These alterations may result in an increased bioavailability of the IGFs from the blood and in an altered distribution of IGF among the different IGFBPs.

Formation of the ternary complex is also affected by a high molecular weight form of IGF-II, which is present in serum of patients with tumour-related hypoglycemia (Daughaday and Kapadia, 1989b). When bound to IGFBP-3, this "big" IGF-II seems to prevent association with ALS (Baxter and Daughaday, 1991). Since the total IGF level is not markedly elevated in these patients and all IGF appears to be bound in complexes of approximately 60kD, it has been suggested that these binary complexes may still allow insulin-like to be activity responsible for the hypoglycemic state of these patients (Baxter, 1991).

Although the 150 kD IGFBP-3/IGF/ALS complex most likely is the major factor that determines the influx and efflux of IGF, it is conceivable that other IGFBPs contribute to the regulation of IGF availability as well. Overloading of the ternary complex by IGF-I infusions is partly compensated by elevation of IGFBP-2 levels in the circulation (Zapf et al, 1990b; Baxter, 1991). Such a compensation is also found in serum of patients with tumour related hypoglycemia.

IGFBPs are ubiquitously expressed and many cells from normal tissues or tumours release various forms of IGFBPs (Chapter II; DeLeon et al, 1989; Yee et al, 1989; Shimasaki et al, 1991a: Daughaday et al, 1991; Reeve et al, 1992a; Reeve et al, 1992b, Schuller et al, 1993). The relative proportions may vary considerably, depending on developmental, hormonal, metabolic and pathological conditions. No evidence has been found for the presence of the ternary complex in tissues. This observation prompts questions regarding the functional role of these IGFBPs and binary complexes. The consensus view is that the IGFBPs inhibit IGF bioactivity. Because the affinity constant (Ka 0.2-$1*10^9$ M^{-1}) of the various IGFBPs is of the same order of magnitude as that of the IGF-receptors, it has been suggested that a possible function of IGFBPs bioactivity is that the binding proteins can compete with IGF-receptors for IGF. Indeed, many *in vitro* studies support this view. IGFBPs have been shown to inhibit IGF action on: human adipose tissue, human and rat fibroblasts, rabbit chondrocytes, rat osteoblasts, chick embryo fibroblasts, and mononuclear blood cells (Drop et al, 1979; Zapf et al, 1979; Ritvos et al 1988; Liu et al, 1991; Kooyman et al, 1992). IGFBP-1 inhibits IGF-I binding to its receptors on human secretory endometrial membranes *in vitro* (Rutanen et al, 1988) or placental membranes (Orlowski et al, 1990). In addition, an IGFBP blocked FSH stimulated steroid production by ovarian granulosa cells (Ui et al, 1989). However, potentiation of IGF action by IGFBPs has been reported as well (Elgin et al, 1987; De Mellow and Baxter, 1988; Blum et al, 1989; Blat et al, 1989; Koistinen et al, 1990; Jones et al, 1991; Andress et al, 1991).

It can be envisaged that subtle changes in local levels of the binding proteins may govern an IGFBP specific action on IGF activity. Local levels of the IGFBPs may vary significantly as a result of their tightly regulated expression. As discussed before the expression of the different IGFBPs is cell- and tissue-specific and regulated by various

factors. In addition, the observed hormone dependent and selective transfer of some of the IGFBPs (Bar et al, 1990a; Bar et al, 1991) may also change local levels. A third factor affecting local IGFBP concentrations is the capability of some IGFBPs to adhere to the extracellular matrix or cell plasma membrane (McCusker et al, 1990; McCusker et al, 1991; Conover et al, 1990). These IGFBP-IGF complexes associated with the cell surface may form a large local pool of IGFs to be used for a prolonged stimulation of the receptor. Enhancement of IGF action by binding proteins is best documented for IGFBP-3. Co-incubation of IGF-I with purified bovine IGFBP-3 resulted in a dose-dependent inhibition of IGF-I-stimulated aminoisobutyric acid (AIB) uptake in fibroblasts. Pre-incubation with IGFBP-3, however, resulted in a dose-dependent enhancement of AIB uptake. It was shown that free IGFBP-3 inhibited, while membrane bound IGFBP-3 stimulated, IGF action (De Mellow et al, 1988). This also seems to be true for IGFBP-1 (Blum et al, 1989). Two fractions of IGFBP-1 have been purified from amniotic fluid: one enhancing and one inhibiting IGF-stimulated DNA synthesis in porcine aortic smooth muscle cells. The fraction that was stimulatory did adhere better to the cell surface than the fraction that had an inhibitory action (Busby et al, 1988).
Another feature that may play a role in putative differential functions of the IGFBPs is their specific affinity for both IGFs. Between the various binding proteins there exist small differences in affinity for IGF-I or -II. IGFBP-1,-3 and -4 bind IGF-I and -II with about equal affinities whereas IGFBP -5,-6 have a preference for IGF-II (Hossenlopp et al, 1986b; Roghani et al, 1989; Bautista et al, 1991). In addition, it was recently established that IGFBP-1,-2 and -3 can be phosphorylated *in vivo*, and it appears that there is a relation between the grade of phosphorylation and the affinity for IGF. (Jones et al, 1991) Moreover, serine phosphorylation seems to modify the capacity of IGFBP-1 to potentiate or to inhibit IGF bioactivity (Jones et al, 1993). It has been shown that the non-phosphorylated form of IGFBP-1 can potentiate the biological effects of IGF-I on porcine smooth muscle cells (Elgin et al, 1987) and in wound healing (Jyung et al, 1992), while the phosphorylated form inhibits the growth promoting action of IGF-I in chondrocytes (Burch et al, 1990). It is of particular interest that in proliferating endometrium, IGFBP-1 is secreted in a non-phosphorylated form whereas nonproliferating endometrium secretes phosphorylated IGFBP-1 (Frost and Cheng, 1991). Therefore,

serine phosphorylation of IGFBP-1 may potentially be an important mechanism that can modulate IGF-I bioactivity.

In addition, evidence has been presented suggesting that IGFBPs may have direct and IGF independent interactions with cells (Biscak et al ,1990; Booth et al, 1990; Liu et al, 1991).

It is clear that the IGFBPs play an important role in the IGF system, and it is likely that specificitiy in IGFBP action is the result of the relative amounts of the various IGFBPs at a given place. Due to the intrinsic properties of the various IGFBPs, IGFs can be routed from or to the tissues. At a local level, IGF gradients may be induced by the IGFBPs, and IGF concentrations may be influenced in such a way that the response is limited to only a few cells. Whether the IGFBPs indeed have additional functions independent of the IGF system remains to be established.

1.4 Scope of the thesis.

IGF-I and IGF-II are growth factors that play an important role in fuel metabolism, cell growth and differentiation. These growth factors exert their bioactivity via specific receptors, of which the biosynthesis is also regulated at various levels. In addition to the already complex regulated system of IGF/IGF receptor interaction, a third component namely the IGFBPs appears to play an important role. Although it is obvious that the IGFBPs can modulate IGF bioavailibility and bioactivity, the precise nature of the interaction and the functional role of the various IGFBPs is only poorly understood.
The work discussed in this thesis is focused on a characterization of IGFBP- 1. The isolation of cDNAs and the gene is described in Chapters II and III. In addition, the chromosomal localization of the IGFBP-1 gene was determined (Chapter II). The primary structure of the protein was deduced from the cDNA sequence. Mutagenesis of specific parts of the protein was used to identify regions that are important for IGF binding (Chapters IV and V). A simple procedure was developed to purify IGFBP-1 from amniotic fluid in sufficient amounts. This opened a way to study the properties of the IGFBP-1 protein *in vitro*. Initial experiments in several cell systems demonstrated a clear inhibiting effect of IGFBP-1 on the bioactivity of IGF (Chapter VII; Kooyman et al, 1992).

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Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IBP-1)

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IGF-I and IGF-II are growth-stimulating peptides with strong mitogenic properties. These polypeptide growth factors circulate in serum bound to specific binding proteins. We report the cloning and complete sequence of a cDNA encoding a low mol. wt IGF-binding protein from a human placenta cDNA library. We propose the designation IGF-binding protein 1 (IBP-1) for the gene and corresponding protein. Expression of the cDNA encoding IBP-1 in COS cells resulted in the synthesis of a 30-kd protein which binds IGF-I and is immunologically indistinguishable from the IGF-binding protein isolated from amniotic fluid or human serum. Northern blotting analysis demonstrated that expression of the IBP-1 gene is highly tissue specific and limited to placental membranes and fetal liver suggesting a rigid control. The IBP-1 gene is a single copy gene, located on chromosome 7. The results obtained suggest that most, if not all, lower mol. wt IGF-binding proteins originate from this gene.
Key words: IGF-binding protein/human placenta/human fetal liver/HEPG2 cell line/cDNA

Introduction

Insulin-like growth factors (IGF) I and II are small polypeptide hormones structurally related to insulin (Rinderknecht and Humbel, 1978a,b). The IGFs evoke a number of biological activities including cellular induction of DNA synthesis and cell multiplication (Rechler *et al.*, 1974). IGF-I is thought to mediate the growth-promoting effects of growth hormone (Van Wyk *et al.*, 1974), while IGF-II is primarily expressed in first trimester human fetal tissue (Scott *et al.*, 1985).

The biological effects of the IGFs are exerted through association to specific receptors. The IGF-I receptor binds IGF-I and IGF-II with high affinity and insulin with lower affinity, the IGF-II receptor binds IGF-II with high affinity, IGF-I with lower affinity but has no significant affinity for insulin (Massague and Czech, 1982).

Unlike insulin both IGFs circulate in plasma tightly bound to specific binding proteins (BP). Two major forms of IGF–BPs with apparent mol. wts of 150 and 30–35 kd have been identified in human plasma (Smith, 1984). In addition, a wide variety of IGF–BPs have been isolated from various biological sources, e.g. tissue extracts (brain, pituitary and cerebrospinal fluid) and cell culture media (human hepatoma cell line HEPG2). The mol. wts range from 24 to 150 kd (Wilkins and D'Ercole, 1985; Binoux *et al.*, 1986). Some of these proteins are related to the 150-kd BP (Baxter *et al.*, 1986a), some are thought to be derived from the low mol. wt IGF–BP by proteolytic processing (Huhtala *et al.*, 1986). The NH_2-terminal amino acids of the 30–35-kd BPs derived from amniotic fluid, human serum and the HEPG2 cell line are completely homologous, suggesting that these proteins are in fact identical (Drop *et al.*, 1984a; Póvoa *et al.*, 1985). Recently, another 35-kd IGF–BP has been isolated from soluble extracts of term human placental/fetal membranes (Koistinen *et al.*, 1986). The protein has been designated placental protein 12 (PP12) and the preferential site of synthesis of PP12 is in the secretory/decidualized endometrium (Rutanen *et al.*, 1986). Of the 15 NH_2-terminal amino acids of PP12 that have been determined 13 are identical to the sequence of the low mol. wt BPs found in amniotic fluid, serum and HEPG2 cells (Koistinen *et al.*, 1986). Because of the confusing terminology, the abbreviation IGF–BP28 has been suggested for the lower mol. wt binding proteins and for similar proteins sharing the same NH_2-terminal amino acid sequence. The designation is based on electrophoretic mobility of these proteins in non-reduced SDS–PAGE (Baxter *et al.*, 1987).

In various bioassays partly purified BP fractions were shown to inhibit the insulin-like and growth-promoting activities of IGF (Drop *et al.*, 1979; Zapf *et al.*, 1979). *In vivo* the BPs increase the biological half-life of IGFs (Hintz, 1984). Human amniotic fluid is an abundant source of 30–35-kd IGF–BP and very high levels are found in fetal serum suggesting a possible role during embryonal and fetal development (Drop *et al.*, 1979, 1984b). However, the precise biological function of these proteins is still unclear.

As a first step towards a better insight into the function of IGF–BP we have screened a cDNA library with an antibody to the low mol. wt IGF–BP and obtained cDNA clones encoding this protein from two different cDNA libraries. We propose the designation IBP-1 for the gene and

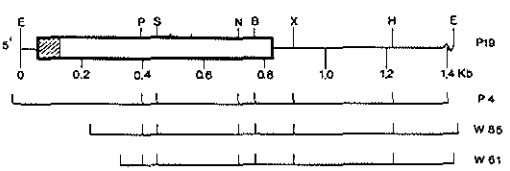

Fig. 1. Restriction map for human IBP-1. The four cDNA clones are aligned. p4 and p19 are representatives from the placenta cDNA library, w61 and w85 originate from the liver cDNA library. The composition restriction map is shown at the top of the figure. The open box represents translated regions. The putative leader sequence is shaded. Solid lines represent untranslated regions. The zigzag line represents the poly(A) tail. E = *Eco*RI, P = *Pst*I, B = *Bam*HI, H = *Hin*dII, S = *Sst*I, X = *Xba*I, N = *Nco*I.

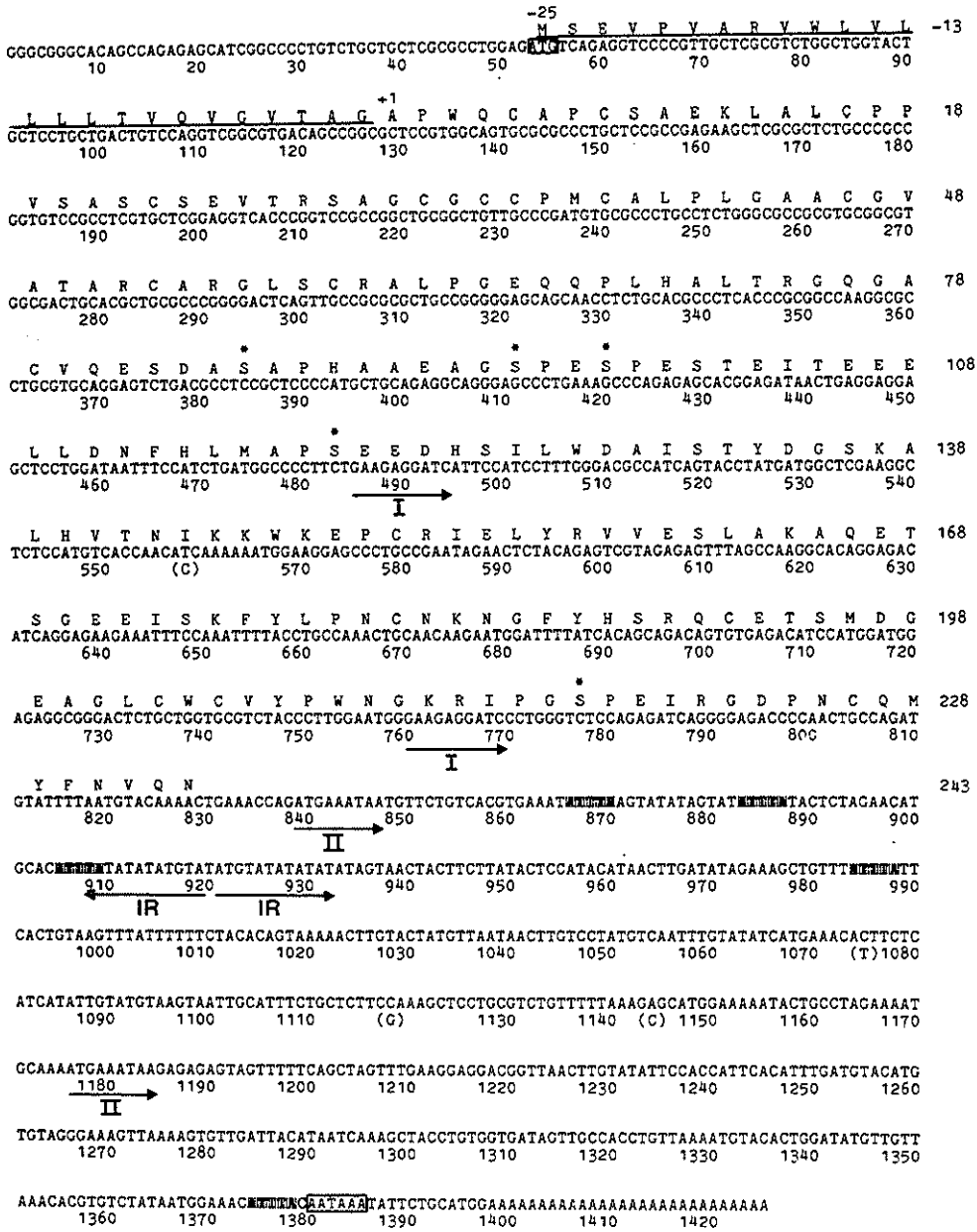

Fig. 2. Nucleotide and deduced amino acid sequence of human IBP-1. The putative initiating codon and the polyadenylating signal are boxed. A thin line represents the leader sequence. Also indicated are possible O-linked glycosylation sites (*), two repeated sequences (I and II) and an inverted repeat (IR). Differences between the placental cDNA sequence and the liver cDNA sequence are shown in parentheses.

corresponding protein. Northern blot analysis revealed that expression of the IBP-1 gene is highly regulated with specific expression in placental membranes and also in fetal and adult liver. In addition, we show that the IBP-1 gene is a single copy gene and is located on chromosome 7.

Results

Isolation of cDNA clones encoding IBP-1

We have previously reported the isolation of a polyclonal antibody raised against IGF–BP isolated from amniotic fluid

(Drop *et al.*, 1984a). The antibody was used to screen a human placental cDNA library in λ gt11. In this library 33 plaques which strongly cross-reacted with the polyclonal antibody were identified. Ten of them were rescreened and the inserts were isolated. The inserts, varying from 0.9 to 1.5 kb, were isolated and subcloned in the vector PTZ19. Restriction enzyme analysis of the subclones and Southern blot experiments showed that all clones but one originated from the same mRNA. In addition two other clones were isolated from a human-liver-derived cDNA library. Restriction enzyme analysis indicated that the clones isolated from the placenta library and the clones isolated from the liver library were colinear, supporting their candidacy as IGF−BP clones. Figure 1 shows the physical map and size of four representative cDNA clones.

Nucleotide sequence of IBP-1

The complete nucleotide sequence of the cDNA insert of two clones (p19 and w85) has been determined (Figure 2). The 1425-nt sequence of p19 contains one major open reading frame of 777 nucleotides. The potential initiation codon at position 53 is flanked by sequences matching Kozak's criteria for an initiation codon (Kozak, 1986). At the 3′ end the putative open reading frame is flanked by a translation termination codon (TGA) and a 596-nt-long 3′ untranslated sequence. At the 3′ end a canonical polyadenylation signal (AATAAA) is found 13 nt upstream of the poly(A) tail. The coding region contains a 10-nt-long repeat (Figure 2, I) and in the 3′ untranslated region a 9-nt-long repeat (Figure 2, II) and a 12-nt-long inverted repeat can be found (Figure 2, IR).

The nucleotide sequence is characterized by an unusually high content of CpG dinucleotides in the 5′ coding region. Between nucleotides 1 and 361 the content of C plus G is 74% and the level of the dinucleotide CpG is 94% of that expected on a random basis. Thus this region does not show marked suppression of the CpG dinucleotides as commonly seen in eukaryotic DNA (Gardiner-Garden and Frommer, 1987).

The 3′ untranslated region contains AT-rich sequences which are frequently found in transiently expressed genes of certain lymphokines, cytokines and proto-oncogenes. A common motif to these genes is the pentanucleotide sequence ATTTA, which has been proposed to destabilize the mRNAs of these genes (Shaw and Kamen, 1986).

Primary structure of the IBP-1 precursor

The open reading frame in cDNA clone p19 has a coding capacity for a protein of 259 residues, with a calculated M_r of 28 172 daltons. The proposed initiating methionine is the first amino acid of a series of highly hydrophobic amino acids, suggesting the presence of a signal peptide. A recently developed method for predicting signal sequence cleavage sites (Von Heijne, 1987) revealed a favourable signal peptide cleavage site after the glycine shown in Figure 2 as residue −1. The thus predicted NH_2 terminus of the mature protein is identical to the chemically determined NH_2-terminal sequence of the IGF-binding protein isolated from amniotic fluid (Pòvoa *et al.*, 1984a,b; Baxter *et al.*, 1986a) and the HEPG2 cell line (Pòvoa *et al.*, 1985). In view of these findings we propose that the ATG codon at position −25 in Figure 2 is indeed the ATG where translation initiation takes place.

Fig. 3. Comparison of the deduced NH_2-terminal amino acid sequence of human IBP-1 with the NH_2-terminal rat IGF−BP. The conserved amino acid regions between the human and rat proteins are boxed.

The nucleotide sequences of the liver cDNA clones differ in four nucleotides from the placenta clones. Of these three are located in the 3′ untranslated region. The coding region bears a transition resulting in the incorporation of a Thr instead of an Ile (Figure 2). These differences may be caused by allelic variation of the IBP-1 gene.

The mature protein is unusually rich in tryptophans. We have identified six Trp residues in the amino acid sequence accounting for 2.6% of the total amino acid sequences. Other characteristic features are a cysteine-rich region with nine cysteine residues concentrated between residues 1 and 46. The primary structure of the protein encloses three regions (80−102, 105−127 and 141−178) with prominent α-helix properties, separated by short unordered segments. A short stretch of amino acids with the features of a phosphoryl binding site is located in the COOH-terminal part of the protein. Although the low mol. wt IGF−BP was found to be glycosylated (Bohn *et al.*, 1980; Koistinen *et al.*, 1987), the deduced amino acid sequence did not enclose N-linked glycosylation sites (N−T and N−S). However, at least five potential O-linked glycosylation sites (Takahashi *et al.*, 1984) were found in the NH_2-terminal two thirds of the molecule.

The IBP-1 protein did not show any significant homology to 4612 entries of the National Biomedical Research Foundation, protein data base release no. 11.0 and no. 26.0, suggesting that IBP-1 is a unique protein. Comparison of the amino acid sequence of IGF-I, IGF-II, insulin, IGF-I receptor and the IGF-II receptor with that of IBP-1 revealed no considerable homology.

Recently the NH_2-terminal amino acid sequence of the low mol. wt IGF−BP synthesized in rat BRL 3A cells has been presented (Mottola *et al.*, 1986). With the complete amino acid sequence available we demonstrate here that the NH_2-terminal part of human IBP-1 is strongly homologous with that of the rat IGF−BP (Figure 3), suggesting that the gene is conserved during evolution. In addition, no homology was found with the reported NH_2-terminal amino acid sequence of the rat high mol. wt IGF−BP (Baxter *et al.*, 1986b), indicating that these proteins are distinct.

Expression in COS-1 cells, immunoprecipitation and IGF-I binding

We constructed the expression plasmid pSV19 by inserting cDNA p19 (Figure 1) into expression vector pSV328 (Van Heuvel *et al.*, 1986). In pSV19 the IBP-1 coding sequence is under direction of the SV40 early promoter. Monkey COS-1 cells were transfected with this plasmid and pSV328 as a negative control. Samples of the medium of transfected COS-1 cells were used for non-reduced SDS−PAGE and subsequently transferred to nitrocellulose filters. The filters were incubated with either [^{125}I]IGF-I or antibody against low mol. wt IGF−BP. The results are shown in Figure 4. In lane B (pSV19) a band of 30 kd cross-reacting with the antibody is visible, which is absent in lane A (pSV328). This band migrates at the same position as the IGF−BP present in amniotic fluid (lane C). When incubated with [^{125}I]IGF-I

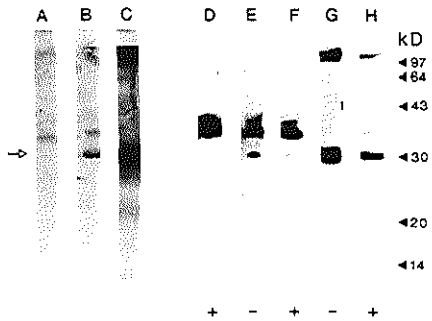

Fig. 4. Binding protein analysis in culture media from COS-1 cells transfected with pSV328 (lanes A and D), pSV19 (lanes B, E and F) and amniotic fluid as a positive control (lanes C, G and H). The IGF−BPs were separated by SDS−PAGE (12.5% acrylamide) and transferred onto nitrocellulose by Western blotting. The filters were incubated with >200 000 c.p.m. [^{125}I]IGF-I with (+) or without (−) 150 ng cold IGF-I, washed and autoradiographed (lanes D−H). After autoradiography the same filters were incubated with antibody against low mol. wt IGF−BP (lanes A−C). The immunoreactive bands were made visible by immunostaining as described for the screening of the cDNA libraries. The IBP-1 protein is indicated by an arrow.

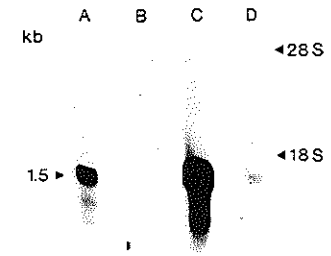

Fig. 5. Northern blot analysis of total mRNA isolated from HEPG2 cell line (lane A), human placenta (lane B), placental membranes (lane C) and human fetal liver (lane D). The samples containing 50 μg of RNA were electrophoresed on 0.8% agarose containing formaldehyde, transferred to nitrocellulose and hybridized with the 5′ *Eco*RI−*Pst*I fragment of IBP-1 cDNA as probe.

the same 30-kd band binds [^{125}I]IGF-I (lane E). This binding is specific because it can be displaced by cold IGF-I (lane F). Lanes C, G and H are control samples of IGF−BPs present in amniotic fluid showing a 30-kd band which reacts with the antibody (lane C) and with [^{125}I]IGF-I (lanes G and H without and with excess cold IGF respectively). Incubation with [^{125}I]IGF-II gave similar results (data not shown). Thus the IBP-1 cDNA encodes a IGF−BP which has the same M_r, immunoreactivity and IGF-binding properties as the IGF−BP present in amniotic fluid. In addition slower migrating bands of ~34 kd can be found in medium from pSV19 and control transfected cells (lanes A, B and D−F). These bands cross-react with the antibody (lanes A and B) and also bind [^{125}I]IGF-I which cannot be displaced by cold IGF (lanes D−F). They presumably represent non-specific cross-reacting proteins from COS-1 cells. The high mol. wt band in amniotic fluid which specifically binds IGF-I probably is the high mol. wt IGF−BP (lanes F and G).

Tissue specific expression of IBP-1 mRNA

Total RNA prepared from human fetal liver, the HEPG2 cell line, human placental tissue and placental membranes from term pregnancy were examined for IBP-1 expression by Northern blot analysis. Using a ^{32}P-labelled 5′ *Eco*RI−*Pst*I fragment derived from cDNA clone p19 as probe, a single transcript of ~1.5 kb was demonstrated in HEPG2 and in fetal-liver-derived RNA (Figure 5, lanes A and D). Since the cDNA clone p19 was isolated from a placental cDNA library it was surprising to find that RNA isolated from placental tissue did not show any expression (Figure 5, lane B). However, analysis of RNA isolated from placental membranes showed a strong hybridizing band of ~1.5 kb (Figure 5, lane C).

In addition, Northern blots of RNAs isolated from a wide range of human fetal tissues (14−16 weeks gestation) and adult liver and kidney were hybridized with IBP-1 and human actin probes. None of the tissues, with the exception of fetal and adult liver, showed hybridization to the cDNA probe, whereas all RNA samples tested were positive for actin expression (Table I). Apparently expression of the IBP-1 gene is highly tissue specific and of all tested tissues limited to liver and placental membranes. Expression of the IBP-1 gene in fetal liver was found to be 5−10 times higher than in adult liver (results not shown).

Table I. Northern blot analysis of total RNA from different tissues hybridized to human placental IBP-1 cDNA and to actin cDNA

RNA[a]	IBP-1	Actin
Liver	++	+
Kidney	−	+
Lung	−	+
Heart	−	+
Brain	−	+
Adrenal	−	+
Tongue	−	+
Muscle	−	+
Jejunum	−	+
Thymus	−	+
Spleen	−	+
Pancreas	−	+
Liver[b]	+	+
Kidney[b]	−	+

Northern blots were kindly provided by Dr M.Jansen.
[a]Total RNA was isolated from fetal tissues at 14−16 weeks gestation.
[b]The marked RNAs originated from adult tissues.

The IBP-1 clones hybridize to a single mRNA of ~1500 nt. This suggests that clone p4 which contains an insert of 1470 bp may represent a full-length cDNA clone.

Genomic DNA analysis

To determine the size and the number of IBP-1 genes present in the human genome, Southern blot analysis was performed using a 5′-(*Eco*RI−*Pst*I) and a 3′-(*Pst*I−*Eco*RI) fragment of clone p19. Figure 6 demonstrates that both probes hybridize with single restriction fragments. In most cases the probes recognized different restriction fragments. However, in *Eco*RI-digested DNA the 5′ probe hybridized with two fragments from which the larger also hybridized to the 3′ probe. In *Pst*I-digested DNA an additional 2.7-kb fragment

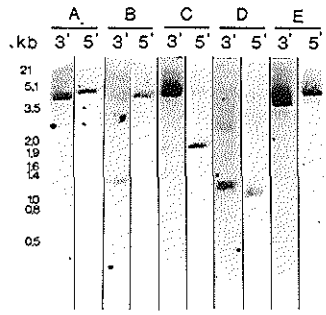

Fig. 6. Southern blots of genomic DNA hybridized to 3′ and 5′ fragments of IBP-1 cDNA. About 10 μg genomic DNA was digested with *Hin*dIII (**lane A**), *Bgl*II (**lane B**), *Sst*I (**lane C**), *Pst*I (**lane D**) and *Eco*RI (**lane E**). After digestion the samples were electrophoresed on 0.8% agarose and subsequently transferred to nitrocellulose and hybridized using either the 3′ *Pst*I–*Eco*RI fragment or the 5′ *Eco*RI–*Pst*I fragment of IBP-1 cDNA as probe.

Table II. Segregation of human IBP-1 gene in rodent–human hybrids

Human chromosome	Chromosome/IBP-1				% discordancy
	+/+	+/−	−/+	−/−	
1	9	6	6	8	41
2	4	2	11	12	45
3	8	2	7	12	31
4	9	4	6	10	34
5	10	2	5	12	24
6	12	4	3	10	24
7	14	1[a]	1[b]	13	7
8	10	7	5	7	41
9	9	6	6	8	41
10	7	6	8	8	48
11	7	5	8	9	45
12	10	6	5	8	38
13	9	6	6	8	41
14	11	9	4	5	45
15	8	5	7	9	41
16	9	5	6	9	38
17	14	9	1	5	34
18	8	1	7	13	28
19	11	7	4	7	38
20	11	7	4	7	38
21	12	7	3	7	34
22	12	7	3	7	34
X	8	9	7	5	52

[a]Exception may be due to small deletion of chromosome 7 not detected by cytogenetic analysis in this clone.
[b]Weak hybridization signal.

was found, which weakly hybridized to the 3′ probe and probably encodes only a small part of the cDNA. Genomic DNA digested with *Bgl*II reveals fragments of 1.2 and 4.5 kb, which hybridize to the 5′ and 3′ probes respectively. These data suggest that the IBP-1 gene is a single copy gene spread over at most 5.7 kb in genomic DNA.

Chromosomal localization

Southern blot analysis of genomic DNA isolated from 29 human–rodent somatic cell hybrids was used for the chromosomal localization of the IBP-1 gene. Filters containing *Pst*I- and *Sst*I-digested DNA from hybrids and their parental cell lines were hybridized with ^{32}P-labelled IBP-1 cDNA clone w85 (Figure 1). Specific 1.1- and 1.2-kb *Pst*I and 5- and 2.1-kb *Sst*I hybridizing fragments were clearly distinguishable in size from rodent cross-hybridizing IBP-1 fragments (results not shown). The presence or absence of human-specific *Pst*I and *Sst*I fragments was correlated with the presence or absence of human chromosomes in the hybrids. The results of this concordance analysis are summarized in Table II. The human IBP-1 fragments appeared to segregate with chromosome 7 with high (93%) concordance. Only two exceptions were observed. One discordant clone did contain chromosome 7, but did not hybridize to the IBP-1 probe. This may be due to a small deletion of chromosome 7 not detected by cytogenetic analysis in this clone, a phenomenon which is known to occur in hybrid cells. The other discordant clone hybridized only weakly with the probe. For all other human chromosomes high discordance scores were obtained ranging from 24 to 52%, confirming the assignment of the IBP-1 gene to human chromosome 7.

Discussion

IGF–BPs with apparent mol. wts of 24–150 kd have been isolated from different biological sources. The designation IGF–BP28 has been proposed for IGF–BPs with an apparent mol. wt of 28 kd under non-reducing conditions and for BPs with the same NH_2-terminal sequence (Baxter *et al.*, 1987). In this paper we describe the isolation of several clones from λ gt11 cDNA libraries using an antibody raised against the low M_r IGF–BP from amniotic fluid. All cDNA clones were found to derive from at most two alleles of the same single copy gene. These results strongly suggest that most, if not all, lower mol. wt IGF–BPs originate from this gene. In view of this finding and the confusing terminology for IGF–BPs, we propose the designation IBP-1 (IGF-binding protein 1) for the gene and corresponding protein.

The IBP-1 cDNA sequence predicts a mol. wt of 25 kd for the 234-amino-acid-long mature protein. The M_r found under non-reducing conditions is 30 kd. It has been reported that the protein is glycosylated. If true, this cannot be due to N-glycosylation because putative N-glycosylation sites are absent from the primary sequence of the protein. Although no definitive consensus sequence for O-glycosylation is known, recently some structural similarities in O-linked glycosylation sites have been observed (Takahashi *et al.*, 1984). Several such sites are found in the NH_2-terminal part of IBP-1. Thus, it is possible that O-linked glycosylation is partly responsible for the observed difference in M_r. Besides this discrepancy there is a difference between the M_r under non-reducing (30 kd) and reducing conditions (34 kd). This is probably due to the high cysteine content of the protein, enabling the formation of several S–S bridges, with as a consequence a faster migration in the gel.

Isolation of the BP from placental membranes, PP12, also yields lower mol. wt peptides which are thought to arise through proteolytic processing (Huhtala *et al.*, 1986). The presence of two putative proteolytic processing sites in the primary sequence of IBP-1 supports this finding. Some of the PP12-derived fragments were still able to bind IGF. They were shown to have the same NH_2-terminal sequence as PP12, suggesting that the IGF-binding site is located in the NH_2-terminal part of the protein. From our data it appears

that this part of the protein is very rich in cysteines. Cysteine-rich regions are frequently found in the binding portion of hormone receptors and are thought to define ligand specificity (Ullrich *et al.*, 1984, 1985; Yamamoto *et al.*, 1984). Similarly, the cysteine-rich region at the NH_2-terminal part of IBP-1 might be the portion of the molecule which binds IGF.

There is some evidence that IBP-1 may be involved in IGF–IGF receptor interaction. Binding studies with IGF-I on cultured fibroblasts demonstrated that these cells synthesize and secrete a 35-kd IGF–BP, which is associated with the cell surface. This BP is immunologically indistinguishable from IBP-1 (Clemmons *et al.*, 1987). Addition of increasing concentrations of IGF-I resulted in augmented binding of IGF-I to this cell-surface-associated BP and to the IGF-I receptor (Elgin *et al.*, 1987). In the primary sequence of IBP-1 as presented here no hydrophobic region which could serve as a membrane anchor is evident. Recently, the human GH-binding protein was found to be a truncated form of its receptor (Leung *et al.*, 1987). Similarly, evidence has been presented that a rat high mol. wt IGF–BP might be a truncated form of the rat IGF-II receptor (Morgan *et al.*, 1987). The lack of significant homology between IBP-1 and either type of IGF receptor proves that IBP-1 is not derived from one of the growth factor receptors. However, it cannot be excluded that IBP-1 might be associated with one or both types of IGF receptors and plays a role in IGF binding. IBP-1, the IGF-I and the IGF-II receptor all bind IGF-I as well as IGF-II, albeit with different affinities. An intriguing consequence of the difference in primary structure between IBP-1 and both receptors could be that the relatively small IGF peptides contain distinct binding sites for each of these three proteins.

The primary structure of the IBP-1 cDNA shows that the sequence ATTTA occurs several times in the 3′-non-coding region. This sequence is present in a similar position in several hormone mRNAs and has been shown to decrease mRNA half-life (Shaw and Kamen, 1986).

Northern blot analyses of RNA from different fetal and adult organs show that expression of the IBP-1 gene is highly tissue specific. Very large amounts of mRNA are found in placental membranes, with at most a very low expression in the placenta itself. Of the other organs tested only fetal and, to a lesser extent, adult liver were positive for IBP-1 mRNA. This type of expression correlates with the high amounts of IGF–BP that were found in amniotic fluid and in fetal serum. Thus, synthesis of IBP-1 is primarily taking place during fetal life, suggesting a specific role of this protein during fetal development. This is in accordance with the observation that synthesis of IGF–BP PP12, which presumably is identical to IBP-1, depends on progesterone stimulation (Rutanen *et al.*, 1985). As is generally known, progesterone levels increase during pregnancy, reaching a maximum shortly before birth.

The availability of complete cDNA encoding human IGF-binding protein IBP-1 will enable the investigation of the function of IBP-1 as a mediator in the complex action of insulin-like growth factors (IGF-I and IGF-II), the intriguing regulation of the gene and its putative role in fetal development.

Materials and methods

Isolation of cDNA clones and sequencing

A polyclonal antibody to the low mol. wt IGF–BP isolated from human amniotic fluid was raised and purified as described previously (Drop *et al.*, 1984a). The antibody preparation was depleted for *Escherichia coli* and λgt11 proteins and by incubation with nitrocellulose filters that had been lifted from confluent lysis plates of *E.coli* Y1090/λgt11, induced with 10 mM isopropyl β-*d*-thiogalactopyranoside (IPTG). The antibody was further absorbed against human serum albumin immobilized on nitrocellulose filters. The thus purified antibody was used to screen a human placenta cDNA library in λgt11 (kindly provided by Dr J.Groffen, Children's Hospital, Los Angeles) and a λgt11 library of RNA isolated from human liver (Chandra *et al.*, 1983) according to the procedure described by Young and Davis (1983).

Approximately 4×10^5 clones of the placental library and $\sim 0.5 \times 10^5$ clones of the liver library were screened with the purified antibody. The plates were overlaid with nitrocellulose filters (Millipore HATF) saturated with 10 mM IPTG, and they were incubated for 2 h at 37°C. The filters were removed, rinsed with Tris-buffered saline (TBS; 10 mM Tris–HCl, pH 7.5/150 mM NaCl) at room temperature and incubated with 3% BSA in TBS for 30 min at room temperature. The filters were incubated overnight with a 1:125 dilution of antibody in TBS, 3% BSA, 0.02% sodium azide for 16 h at 4°C. Subsequently the filters were washed and incubated for 60 min at room temperature with horseradish peroxidase conjugated goat anti-rabbit IgG (Tago) diluted 1:200 in 3% BSA in TBS. The filters were washed and stained with amidophenyl and naphthol AS-MX phosphate in 0.2 M Tris– HCl pH 9.2, 10 mM $MgCl_2$ at room temperature.

Isolation and analysis of DNA from positive phages was performed using standard methods (Maniatis *et al.*, 1982). Restriction fragments were subcloned in the vectors PTZ18 or PTZ19 (Pharmacia) and sequenced according to the chain termination method (Sanger *et al.*, 1977). In regions that lacked convenient restriction sites, appropriate clones were generated by *Bal*3I nuclease digestion.

DNA analysis

Genomic DNA was extracted from cultured HEPG2 cells (Blin and Stafford, 1976) and was digested with various restriction endonucleases (BRL, NEN, Boehringer) according to the suppliers' directions, electrophoresed in 0.7% agarose, and transferred to nitrocellulose filters according to the method of Southern (1975).

Chromosomal mapping of the IBP-1 gene was performed using Southern blots containing DNA from a panel of human–rodent somatic cell hybrids segregating human chromosomes. DNA was extracted from 25 human–hamster and four human–mouse hybrids, digested with *Pst*I or *Sst*I and subsequently separated on 0.7% agarose gels and blotted onto nitrocellulose filters. The resulting blots were hybridized to the IBP-1 cDNA clone w85. Chromosome analysis of hybrid cells was carried out using reverse (R) banding with acridine orange after heat denaturation. The same batches of cells were used for karyotyping and DNA extraction.

Northern analysis

RNA was extracted from fetal liver, HEPG2 cells, placental membranes and placenta tissue from term pregnancy by the thioguanidyl method as described (Ullrich *et al.*, 1977). The RNA was denatured with dimethylsulfoxide and glyoxal, electrophoresed in 1% agarose and transferred to nitrocellulose filters (Thomas, 1980). The filters were hybridized with fragments derived from IBP-1 cDNA clones. In addition, filters containing poly(A)$^+$ RNAs of various fetal tissues of 12–16 weeks gestation (kindly provided by Dr M.Jansen, Wilhelmina Children's Hospital, Utrecht) were hybridized with the same fragments.

Transfection of COS-1 cells and IGF–BP analysis

The cDNA clone p19 encoding the IBP-1 gene was inserted into the *Eco*RI site of pSV328, which expresses cloned inserts using the simian virus 40 (SV40) early promoter (Van Heuvel *et al.*, 1986). COS-1 cells (Gluzman, 1981) were transfected with the expression vector by incubation with 100 μg/ml DEAE–dextran for 2 h (McCutchan and Pagano, 1968) followed by treatment with 100 μM chloroquine in Dulbecco's MEM (DMEM) for 4 h. After this treatment the cells were fed with DMEM plus 5% fetal calf serum for 24 h. Subsequently, the cells were washed extensively with DMEM and incubated for 72 h with DMEM without serum.

The culture medium was concentrated ~20-fold using centricon microcon-

centrators (Amicon) and desalted on Sephadex G25 columns (Pharmacia). Samples of culture medium were used for SDS–PAGE (12.5% acrylamide) and transferred onto nitrocellulose filters (Millipore HATF) by Western blotting. Production of IBP-1 protein in culture media was determined by binding with [^{125}I]IGF-I (Hossenlop *et al.*, 1986) and by cross-reaction with the antibody against the low mol. wt IGF–BP. The nitrocellulose filters were incubated with >200 000 c.p.m. [^{125}I]IGF-I (kindly provided by Dr M.Jansen, Wilhelmina Children's Hospital, Utrecht) for 16 h at 4°C with or without 150 ng cold IGF, washed and autoradiographed. After autoradiography the same filters were incubated with antibody against low mol. wt IGF–BP. The immunoreactive bands were made visible by immunostaining as described for the screening of the cDNA libraries.

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ORGANIZATION OF THE GENE ENCODING THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN IBP-1

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Human genomic clones encompassing the tissue specific expressed gene IBP-1. an insulin-like growth factor binding protein were isolated and characterized. The gene is organized in four exons and spans 5.9 kb. S1 nuclease analysis determined a single transcription start site. The first exon and 5' flanking region are highly GC rich and located in a CpG island. The CpG island enclose the CAAT box. the TATA box. the transcription start site and a potential SP1 transcription factor binding site. The presumptive promoter region is characteristic for genes expressed in a tissue specific fashion. All signals required for cleavage/ polyadenylation are located within exon IV. predicting a mRNA of 1.5 kb which is consistent with the size seen on RNA blots.

Insulin-like growth factor (IGF) I and II are growth stimulating peptides which circulate in serum complexed to specific binding proteins (BPs) (1). BPs with molecular weights ranging from 150 to 24 kd have been isolated from several sources (2). At least two different BPs can be distinguished. the 150 kd high molecular weight IGF-BP and the 32 kd low molecular weight IGF-BP (IBP-1). They are immunologically and structurally distinct (3,4).

At present the function of these binding proteins is still unclear, although growing evidence suggests that they regulate the interaction of the IGFs with their cellular receptor (5).

Recently we and others (3,6,7) reported the cloning of the low molecular weight IGF-BP cDNA. Although the nucleotide sequence reported by Brewer et. al. (6) differs in some details from the cDNA sequence reported by the other groups (3,7). We isolated the IBP-1 cDNA from a placenta and a liver cDNA library. The corresponding protein appeared to be identical to the IGF-BPs isolated from human amniotic fluid. the Hepg2 cell line and the placental IGF-BP. PP12 (3). The amino terminus of IBP-1 is homologous to the amino terminal sequence of the low molecular weight IGF-BP isolated from rat BRL3A cells (8,9).

The IBP-1 gene is a single copy gene spread over at the most 5.9 kb on chromosome 7 and is expressed in a strictly tissue specific manner (3). So far detectable levels of

IBP-1 mRNA have been demonstrated only in Hepg2 cells, in human liver and decidualized endometrium, Wilm's tumour (7) but not in other human tissues (3) or in human fibroblasts (authors unpublished results).

In this paper we report the isolation of genomic clones containing the IBP-1 gene and the structural organization of the entire trancriptional unit of the IBP-1 gene.

Methods and Materials

Screening of a genomic library

A genomic library constructed from MBOI partially digested high molecular weight human DNA in lambda EMBL3 (kindly provided by dr G.C. Grossveld, Dept. Cellbiology and Genetics, Erasmus University, Rotterdam) was used to elucidate the genomic organization of the IBP-1 gene. A 5'EcoRI-PstI fragment and a 3'HindII-EcoRI fragment of the IBP-1 cDNA (3) were ^{32}P-labeled by random priming (10) and used to screen approx. $2*10^6$ clones of the library by standard hybridization of replica filters (11). Positive plaques were purified by standard methods (12).

Restriction enzyme and Southern blot analysis

Isolation and analysis of DNA from phages was performed using standard methods (12). DNA of isolated clones was digested with various restriction enzymes (BRL, NEN, Boehringer) according to the suppliers' directions. The fragments were electrophoresed in 0.7% agarose and transferred to nitrocellulose filters conform the method of Southern (13). Restriction fragments of the cDNA were ^{32}P-labeled and hybridized to the filters using standard methods (12).

Subcloning and DNA-sequencing

Relevant restriction fragments were subcloned in the vector PTZ19 (Pharmacia). Either single stranded or double stranded DNA was sequenced by the dideoxy chain termination method (14,15) using the reverse M13 primer or specifically designed oligo nucleotide primers, synthesized on a Biolabs DNA Synthesizer.

S1 nuclease analysis

From the genomic subclone pG1.15 a 560 bp RSA1 fragment, contain-ing part of exon I, was isolated and ^{32}P-end-labeled by using polynucleotide kinase. Digestion with HindIII, removed a small fragment at the 5' end leaving a 540 bp fragment in which only the noncoding strand is end labeled. Total RNA (10µg) previously isolated from placental membranes (3) and the end labeled probe were hybridized at 55 °C. Subsequently S1 nuclease digestion was carried out (12). The resulting fragments were analyzed on a 6% denaturating acrylamide gel.

Results

Isolation and characterization of the human IBP-1 gene

We have recently reported the isolation and characterization of cDNA clones from the human IBP-1 gene (3). ^{32}P-labeled fragments derived from the cDNA clone p19 were used to screen the human genomic library in lambda EMBL3. The probes included an EcoRI-PstI fragment representing the extreme 5' end (5'probe), a PstI- HindII fragment and a HindII- EcoRI fragment, encompassing the most 3' end of the 3' untranslated region of the IBP-1 cDNA, (3' probe).

Approx. $2*10^6$ clones were screened with the 5' and 3' probe. Rescreening of potential positive clones revealed five independent clones, designated λG1 to λG5, which hybridized with the 3' probe. Two clones, λG1 and λG3 hybridized also with the 5'

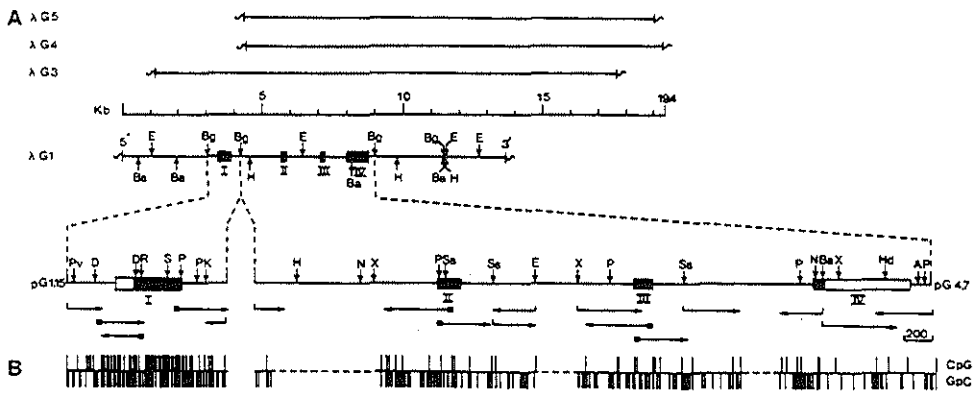

Figure 1.
A Physical map and sequence strategy of the human IBP-1 gene. At the top, the position of the inserts of 4 overlapping genomic clones. λG1, λG3, λG4 and λG5 are shown. The restriction map of the representative DNA region is shown below a scale in kilobases. The position of exon I-IV is indicated. Relevant subcloned regions containing the exons are illustrated in an expanded scale with additional restriction sites. The exons are displayed as solid boxes in which the open areas represent the 5' and 3' nontranslated regions. At the bottom the dideoxy sequence strategy is shown in which the arrows represent the direction and extend of each reaction. The small boxes indicate where synthetic oligo nucleotides were used. Ba, BamH1; Bg, Bgl2; D, Dra1; E, EcoR1; H, HindIII; Hd, HindII; K, Kpn1; N, Nco1; P, Pst1; Pv, Pvu1, R, Rsa1; S, Sma1; Ss, Sst1; X, Xba1.
B Analysis of the distribution of CpG dinucleotides in the IBP-1 gene. The position of each CpG and GpC dinucleotide relative to position in the sequence is indicated by a vertical line.

probe, indicating that these clones potentially enclosed the complete IBP-1 gene. These five clones were purified, grown in culture and their DNA was subjected to restriction enzyme analysis. The results are summarized in figure 1A. The inserts of these five clones, varying from 13-16 kb, are overlapping, spanning a total region of approx. 19 kb. The region which hybridized to the three IBP-1 probes is located on two BglII fragments of respectively 1.15 and 4.8 kb (Fig.1A). Both BglII fragments were subcloned in the vector PTZ19, resulting in the plasmids pG1.15 and pG4.6 respectively. These clones were used for high resolution mapping and sequencing (Fig 1A).

The transcribed region of the human IBP-1 gene consists of four exons (I-IV) of 480, 170, 129 and 698 bp respectively, interrupted by three introns of approximately 1.8, 1.4 and 1.2 kb. The precise locations of the introns were delineated by sequencing through the exon-intron junctions (Table I). All splice junctions conform to the GT/AT rule for exon-intron junctions (16). Exon I contains the first 348 bp of the coding sequence. Exon 2 and exon 3 both map within the coding region. Exon IV contains the last 128 coding nucleotides and the complete 3' nontranslated region.

Table 1

Position and sequence of intron-exon junctions in the human IBP-1 gene

Intron	Position[a]	Splice junction sequence and intron size			
		Exon[b]	Intron[c]		Exon[b]
A	499/500	90 91 GCT GCA G ala ala g	gtaccacagt.....~1.6 Kb.....tcctttccag		92 93 94 AG GCA GGG lu ala gly
B	2266/2267	147 148 TGG AAG trp lys	gtgaggcccca.....~1.24 KB.....attctcttag		149 150 GAG CCC glu pro
C	3525/3526	190 191 AGA CAG arg gln	gtaggtggcc.....~1.24 Kb.....gtctttgcag		192 193 TGT GAG cys glu

[a]Base pair number starting with base pair no. -348 in the genomic sequence (Fig. 2).

[b]Exon nucleotide sequence in upper case and amino acid translation (with residue number) are shown.

[c]Intron nucleotide sequence in lower case and approximate size in kilobases (Kb) are shown.

Characterization of exon I and the 5' flanking region

The transcription start site for exon I was mapped by S1 nuclease analysis using the noncoding strand of a HindIII-RsaI fragment of genomic clone pG1.15. This fragment contains approximately 540 nucleotides upstream of the translation start site in exon I. Total RNA of placental membranes was hybridized at several temperatures with the end-labeled fragment and subsequently digested by S1 nuclease. Analysis of the resulting fragments revealed a single band of 193 bp (Fig 3). This maps the transcription start, indicated by +1 in figure 2. The sequence of the determined transcription start corresponds to a cap site consensus py---pyApypy (17). Thus the RNA contains a 5' nontranslated region of 155 nucleotides and extends 97 nucleotides further upstream than the previously isolated cDNA. These results correspond with data from Northern blot analysis of several polyA+ RNA preparations which show a single transcript of 1.5 kb (Fig 4) and ref.3.

The nucleotide sequence of the 5' flanking region of the IBP-1 gene was determined up to position -350. A TATA box sequence 5'TTTATAAA-3' was found 34 bp upstream from the transcription start. A sequence 5'GCCCAATC-3' homologous to the CAAT element, commonly found 60-100 bp upstream of transcription start sites, is located at position -83.

Examination of the 5' exon and the 5' flanking sequences reveals a region with a high content of CpG dinucleotides (figure 1B). The nucleotide sequence between position-100 and 610 has a content of C plus G of > 70 % and CpG dinucleotides occur with a frequency that would be expected on a random base. Thus this region does not show the marked suppression of CpG dinucleotides as commonly seen in eukariotic DNA (18). Such regions, often referred to as CpG islands, are commonly found in all housekeeping genes and in many genes which are expressed in a tissue specific manner. The CpG island spans the promoter region, the transcription start site, the 5' untranslated region and also part of the 5' coding region of the IBP-1 gene (Fig 2).

Genomic DNA preparations isolated from several tissues digested with either HpaII or MspI were hybridized with either clone pG1.15, which comprises the CpG island, or with clone pG4.6. The results sugeested that the 5' part of the IBP-1 gene enclosing the CpG island is hypomethylated (data not shown). Although the methylation status

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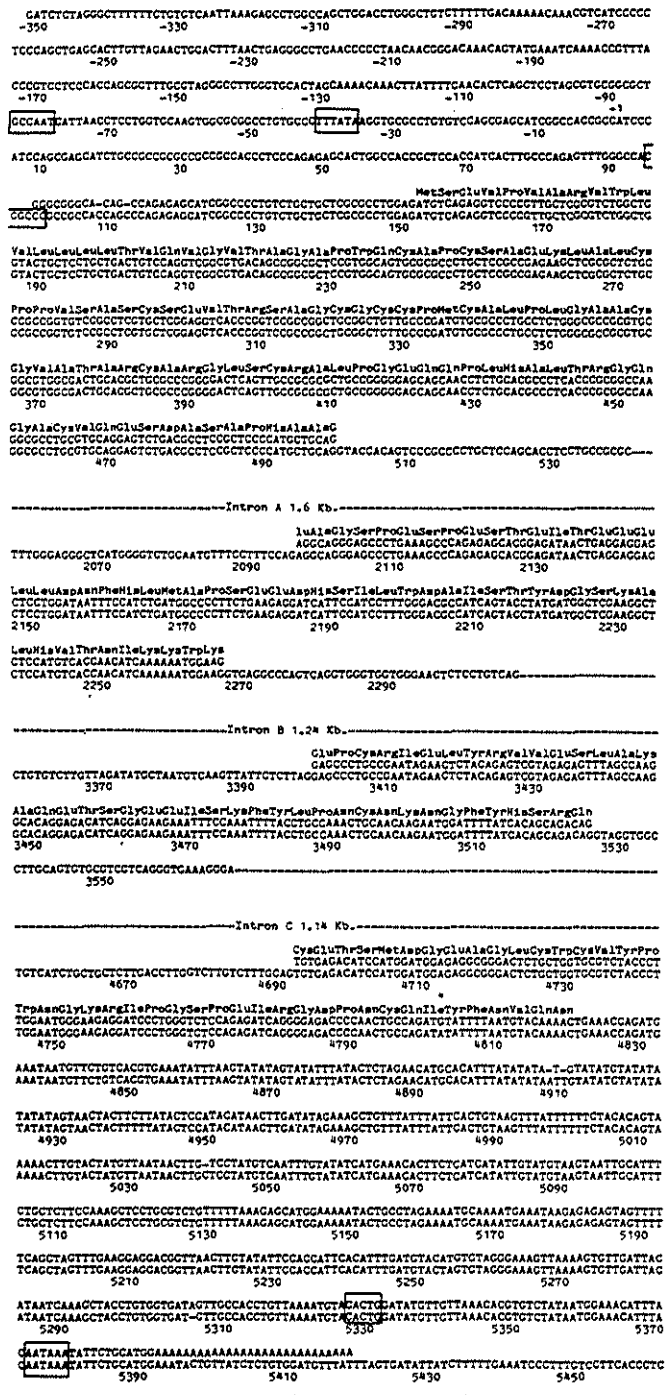
Figure 2.
The nucleotide sequence of the IBP-1 gene was determined from the subclones pG1.15 and pG4.6. The sequence of the cDNA as previously determined (3) is aligned with the exon sequences. Dashes are introduced for optimal alignment. The cap site (+1) is indicated, the TATA box, the CAAT box, a potential Sp1 binding site and the polyadenylation signals are boxed. The deducted amino acid sequence of the IBP-1 protein is given along with the exons. A (*) represents a mismatch in the coding sequence of the IBP-1 gene, resulting in a different amino acid in the previously determined amino acid sequence (3). This difference was also reported by Lee et. al. (7).



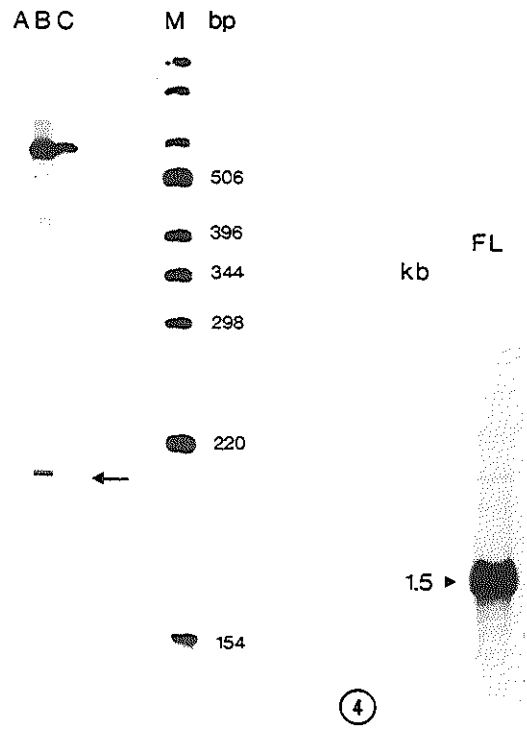


Figure 3.
Nuclease S1 analysis of the 5' end of the IBP-1 gene. For the nuclease S1 experiment, a ^{32}P-end-labeled RsaI to HindIII genomic fragment of the noncoding strand, spanning the region from approximately 540 bases upstream of the translation start to the first RsaI site in exon 1. This labelled probe was hybridized to human placental membrane RNA (10 μg) at 55 °C overnight, digested with nuclease S1 as described, and run on a polyacrylamide-urea gel. Lane A, no RNA. Lane B, RNA 55 °C. Lane C no RNA, no S1. Molecular mass markers are indicated to the right. In lane B a band of 193 nucleotides protected from nuclease S1 digestion is indicated by an arrow. The exact size of the band was determined by running this reaction next to a sequence ladder.

Figure 4.
Northern blot analysis of poly A+ RNA (10 μg), isolated from human fetal liver, of 16 weeks gestation, hybridized with IBP-1 cDNA. A single band of 1.5 kb, indicated by an arrow, was detected.

of CpG islands is known for only a few genes, they all have been identified to be hypomethylated.

A search for important DNA binding domains did not reveal sequences for known transcription regulatory proteins (19). Only one G/C rich motif (sequence CCGCCC) is found in the 5' non-translated region of exon 1. This motif is the reverse complement of a G/C box to which transcription factor Sp1 binds. Sp1 facilitates the transcription of several viral and cellular promoters (20,21).

Characterization of exon 4

The sequence of the 4th exon is shown in figure 4 . The sequence reveals a 704 bp region identical to the 3' part of the IBP-1 cDNA previously determined (3,6,7). Four mismatches were found in this region. Three of these are located in the 3' non-translated region. These regions are known to be the most diverging sequences in human genes. The one in the coding region was also present in a cDNA clone (5). The 3' region of the IBP-1 gene revealed all signals required for appropriate cleavage/polyadenylation: a CA$\frac{C}{T}$G motif, the canoni-cal poly A site (AAUAAA) and a region with a high incidence of the trinucleotide TGT in conjunction with oligo-T stretches, a so called GT-cluster (22) (Fig.2). In the IBP-1 cDNA a stretch of A's is found 13 bp downstream the polyadenylation signal indicating that this site is actually used for cleavage/polyadenylation (3,6,7).

Discussion

The characterization of the human IBP-1 genomic clones revealed a gene composed of 4 exons spanning 5.9 kb. The sequence determined for the portion of the genomic clones corresponding to the coding region is identical to that obtained for cDNAs isolated from human placenta (3) and from the HepG2 cell line (7). The nucleotide sequence of the 5'flanking region of the IBP-1 gene was determined up to -355 nucleotides of the transcription start. The 5' flanking region encloses a typical promoter, including a TATA box and a CAAT box at respectively -34 bp and -84 of the transcription start. Results of S1 nuclease analysis using placental membrane derived mRNA indicated a single transcription start site. Northern blot analysis of human liver derived mRNA showed only a single transcript for the IBP-1 gene. So far, in all tissues and cell lines tested, no evidence has been found for more than one transcript of the IBP-1 gene is (3,6,7). However, a variety of low molecular weight IGF-BPs have been described (2) some of these are immunologically distinct, others have identical amino terminal sequences (23). Our results of nuclease S1 and Northern blot analysis demonstrate that it is unlikely that the IBP-1 gene has more than one promoter or that the IBP-1 mRNA can be processed in an alternative manner.

Therefore, the range of low molecular weight IGF-BPs present in human serum cannot be the result of alternative splicing or the occurrence of variant forms from the IBP-1 gene but are most probably derived from different genes and/or post-translational processing.

The 5' end of the IBP-1 gene comprises, besides the TATA and CAAT boxes, a characteristic region with a high content of G plus C nucleotides combining all features of a CpG island (18). CpG islands associated with the 5' ends of genes are common for 'housekeeping' genes and genes whose expression is regulated in a tissue specific manner. In this respect it may be relevant that the more ubiquitously

expressed genes have CpG islands which lack a TATA box, but none of the tissue specific regulated genes with 5' CpG islands miss a TATA box (18). These results are in accordance with our earlier findings that the IBP-1 gene is expressed in a highly tissue specific way (3).

Several elements which are involved in regulation of transcription and which bind specific transcription factors are known. No such element was found in the promoter region of the IBP-1 gene. Only a potential SP1 binding site is found 95 bp downstream from the transcription start. Although SP1 binding sites have been demonstrated downstream of the transcription start, all functional copies are found upstream of the TATA box. Further analysis of the 5' region by deletion mapping and footprinting will be of importance to define regions involved in the tissue specific regulation of the IBP-1 gene.

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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

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In an attempt to define domains in insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) that are involved in IGF binding, we subjected the carboxyl end of the coding region of IGFBP-1 cDNA to mutagenesis. Mutant cDNAs were isolated, characterized by sequencing, and cloned in an expression vector under control of the simian virus-40 (SV40) early promoter. The constructs were transfected into COS-1 cells, and the mutant proteins, secreted into the culture medium, were analyzed for IGF binding by ligand blotting.

The results obtained show that deletion of the C-terminal 20 amino acids or introduction of frameshifts in this region resulted in loss of IGF binding and for some mutants in the formation of dimeric IGFBP-1 molecules. These dimers are probably formed when cysteine-226 (Cys-226) is missing, and its putative partner is able to form intermolecular disulfide bonds. Site-directed mutagenesis demonstrated that most of the introduced point mutations in the C-terminal region did not affect IGF binding. Only mutation of Cys-226 to tyrosine completely abolished IGF binding, as did the introduction of a negatively charged amino acid in the vicinity of this residue. Again, dimers were observed, supporting that Cys-226 is essential for the conformation of IGFBP-1. In addition, our data suggest that an IGF-binding domain may be located in the vicinity of the intramolecular disulfide bond formed by Cys-226 and its putative partner. (Molecular Endocrinology 5: 987–994, 1991)

INTRODUCTION

Insulin-like growth factors (IGF-I and IGF-II) are small polypeptide hormones with potent metabolic and mitogenic activities. They exert their action via specific receptors. Unlike insulin, to which they are structurally related, they do not exist as free hormones in body fluids, but are complexed to specific proteins, termed IGF-binding proteins (IGFBPs). At least five distinct classes of IGFBPs with mol wt ranging from 24–45 kDa can be recognized in serum, extracelluar fluid, and culture medium from various cell types in tissue culture. At present, the primary structure of four different types of IGFBPs has been fully characterized. IGFBP-1, originally purified from amniotic fluid (1, 2) and placenta (3, 4), was the first IGFBP whose complete primary structure was deducted from cDNA clones (5–9). A second IGFBP, IGFBP-2, was cloned from human and rat cDNA libraries (10–12). IGFBP-3 is the IGF-binding subunit of the 150-kDa ternary complex found in plasma. From various species cDNAs were isolated, and the primary structure was deduced (13–15). IGFBP-4 cDNAs were recently cloned from rat and human libraries (16).

Although IGFBPs and receptors both bind IGFs with comparable affinity, there is no considerable homology between the two groups of proteins. Therefore, it seems likely that the ligand-binding domains of IGFBPs and receptors are different. Indeed, Cascieri *et al.* (17, 18) demonstrated that IGF-I has different interaction sites for receptors and IGFBPs.

Comparing the primary structures of the different IGFBPs, it appeared that they are distinct but clearly related. The strongest homology is found in the N- and C-terminal region. These regions typically enclose 18 Cys residues in identical positions. Analogous to hormone and growth factor receptors, in which ligand-binding domains frequently constitute Cys-rich regions, the N- and C-terminal parts of the IGFBPs are the most likely candidates for such an IGF-binding domain. Studies thus far have been conflicting, since fragments from either end capable of binding IGF have been described (19, 20). However, N-terminal sequencing of a 34-kDa IGFBP isolated from human cerebrospinal fluid and a

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homologous one isolated from culture medium from a simian virus-40 (SV40)-transformed fibroblast cell line suggested that this type of IGFBP lacks most of Cys-rich N-terminal region (21, 22). This finding favors the C-terminus as the potential ligand-binding domain. The middle section of the protein is only poorly conserved among the IGFBPs. Only IGFBP-4 accommodates two additional Cys residues in this region (16).

It is of interest to note that IGFBP-1 and -2 contain a RGD sequence in the C-terminal part of the molecule. Such a sequence is considered to be of importance for the adherence of proteins to the cell surface by interaction with integrin-like receptors (23). Although a specific IGF-binding domain has not been identified, it is tempting to speculate that conserved stretches contribute to an IGF-binding site. In a first attempt to define domains involved in IGF binding, we introduced mutations in the C-terminal part of the coding region of the IGFBP-1 cDNA and analyzed the mutant proteins for IGF binding. The results obtained suggest that Cys-226 is essential for the structural integrity of the protein. Substitution of this residue leads to dimer formation and loss of IGF binding.

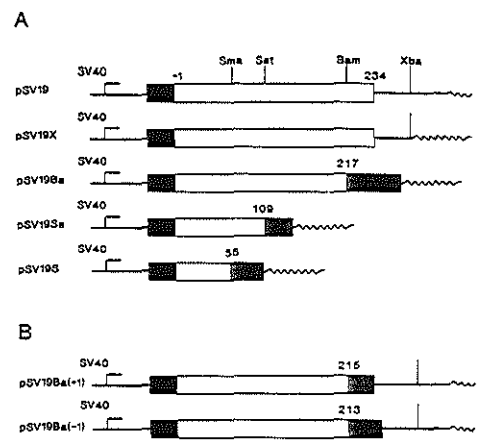

Fig. 1. Diagram of Expression Plasmids Carrying IGFBP-1 cDNA Fragments with Increasing C-Terminal Deletions (A) or Enclosing a +1 or −1 Frameshift (B)

On *top* is a construct encompassing the complete IGFBP-1 cDNA under control of the SV40 promotor. The *wavy line* represents the rabbit β-globin polyadenylation region. The coding region of IGFBP-1 is *boxed*, with the signal peptide in *black*. Stretches of nonspecific amino acids residues are *hatched*.

RESULTS

Deletion and Frameshift Mutants

In a first attempt to locate regions in IGFBP-1 participating in IGF binding we truncated the IGFBP-1 cDNA insert of expression plasmid pSV19 (6) using the restriction enzymes *Xba*I, *Bam*HI, *Sst*I, and *Sma*I. This resulted in plasmids carrying IGFBP-1 cDNA fragments with increasing C-terminal deletions of, respectively, 17 (pSV19Ba), 125 (PSV19Ss), or 179 (pSV19S) amino acids. An outline of the constructs is shown in Fig. 1A. In addition, two variants of the *Bam*HI deletion were constructed by either blunting [pSV19Ba(−1)] or filling in [pSVBa19(+1)] of the *Bam*H1 site in the IGFBP-1 cDNA, shown in Fig. 1B. These manipulations introduced a frameshift in the coding region of IGFBP-1, resulting in a stretch of 17 nonspecific amino acids in clone pSV19Ba(+1) or 24 residues in clone pSV19Ba(−1) (Fig. 5).

To demonstrate the functional activity of the constructs, we transfected COS-1 cells with expression plasmids containing the various fragments of the IGFBP-1 coding sequence under control of the SV40 promoter. Samples of the culture medium were separated by nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto nitrocellulose filters. The filters were incubated with either [^{125}I]IGF-I (Fig. 2A) or polyclonal antibody against IGFBP-1 (Fig. 2B). The same samples were separated under reduced conditions, and the nitrocellulose filter was incubated with polyclonal IGFBP-1 antibody (Fig. 2C).

Figure 2A shows the results of ligand binding. Culture medium from mock-transfected cells or cells transfected with the expression vector pSV328a itself contained three proteins that bind [^{125}I]IGF-I. These proteins with apparent mol wt of 47, 45, and 40 kDa, presumably represent binding proteins secreted by COS-1 cells (COS-1 and pSV328a). An additional [^{125}I] IGF-I-binding band of 28 kDa was detected in culture medium from COS cells transfected with pSV19 or pSV19X, two constructs containing the full-length IGFBP-1-coding sequence. That this extra IGFBP represents IGFBP-1 is suggested by the finding that it migrates at the same position as IGFBP-1 from amniotic fluid. None of the constructs containing C-terminal deletions in the IGFBP-1-coding region showed ligand binding different from the background COS-1-binding proteins (pSV19Ba, pSV19Ss, and pSV19S).

In Fig. 2B the results are shown when the same filter is incubated with antibody against IGFBP-1. This reveals a 28-kDa band in the culture medium from pSV19- and pSV19X-transfected COS cells and amniotic fluid. This confirms the results obtained with [^{125}I]IGF-I incubation. In contrast to the ligand blot several additional bands reacting with IGFBP-1 antibody were detected in culture medium from COS-1 cells transfected with the various mutant plasmids. Bands with mol wt of 64, 58, 32, and 24 kDa were observed with pSV19Ba. The band at approximately 32 kDa migrates at the expected position of the IGFBP-1 read-through product of pSV19Ba. The minor 24-kDa band presumably represents a breakdown product of this fusion protein. A truncated IGFBP-1 protein of about the expected size of 15 kDa is found with pSV19Ss. No immunoreactive

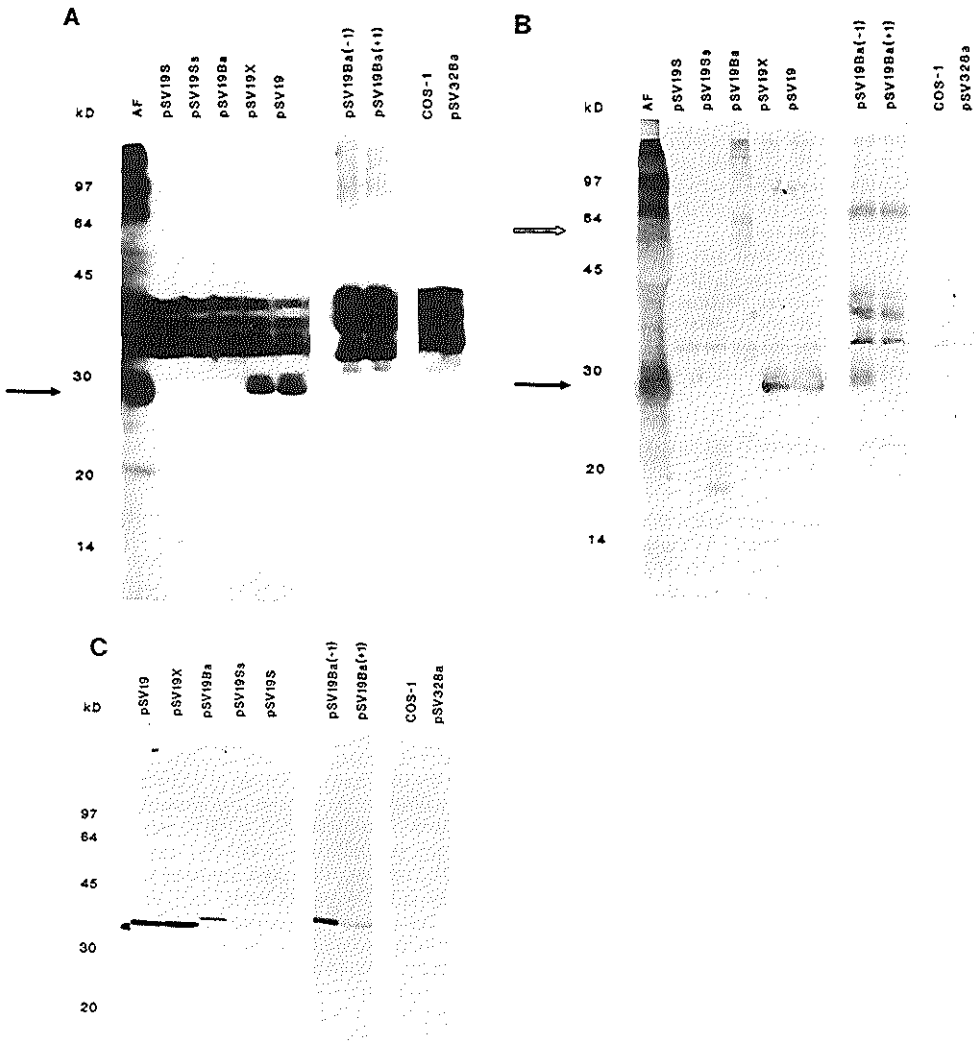

Fig. 2. Analysis of Mutant IGFBP-1 Proteins in Culture Medium of COS-1 Cells Transfected with the Expression Plasmids from Fig. 1

Culture medium of mock-transfected COS-1 cells or cells transfected with pSV328a was used as a negative control; amniotic fluid (AF) was used as a positive control. The binding proteins were separated by SDS-PAGE (12.5% acrylamide) and transferred onto nitrocellulose by Western blotting. The filters were incubated with more than 200,000 cpm [^{125}I]IGF-I, washed, and autoradiographed (A). After autoradiography, the same filters were incubated with polyclonal anti-IGFBP-1 and made visible by immunostaining (B). C shows immunostaining of the mutant IGFBP-1 proteins separated by SDS-PAGE after reduction with β-mercaptoethanol. The position of IGFBP-1 is indicated with *black arrows*, and the position of IGFBP-1 dimers with an *open arrow*.

band can be seen when COS-1 cells were transfected with pSV19S, although RNA isolated from pSV19S-transfected COS-1 cells showed positive hybridization with an IGFBP-1 cDNA probe (results not shown), demonstrating that the cells were successfully transfected. Most likely the antigenic determinants of the chimeric protein are lost by using an IGFBP-1 sequence coding only as far as residue 55. From the two frameshift mutants, only culture medium from pSV19Ba(−1)-transfected cells showed an immunoreactive band of 28 kDa, which failed to bind [^{125}I]IGF-I [Fig. 2A, pSV19Ba(−1)]. In addition, an extra immunoreactive

band of approximately 50–64 kDa was present. The same phenomenon was observed with pSV19Ba and pSV19Ba(+1), in which the high mol wt band is more prominent. Figure 2C shows that under reducing conditions these high mol wt forms resolve into bands of 32 kDa with pSV19Ba(+1) and pSV19Ba(−1) or into a band of 35 kDa with pSV19Ba. As the higher mol wt forms are approximately twice the size of IGFBP-1, the most likely explanation for this finding is that the mutant proteins form dimers.

The data presented here demonstrate that mutant IGFBP-1 are produced by COS-1 cells and secreted into the culture medium despite the introduction of frameshifts in the coding region. However, changing the extreme C-terminal part of the IGFBP-1 molecule abolished IGF binding regardless of the kind of change introduced.

Site-Directed Mutagenesis in the C-Terminal Region of IGFBP-1

To investigate the C-terminal region of IGFBP-1 in more detail, point mutations were introduced between the *Bam*HI and *Xba*I sites in the IGFBP-1 cDNA. This was performed by random deamination of cytosines of partly single stranded DNA, followed by transfection of the modified DNA into *E. coli* (24). DNA from single colonies was isolated and sequenced to locate the mutations. We routinely found that about 30–50% of the isolated clones had single or double mutations. This is in agreement with the results of Pine and Huang (24). No mutations were found in the sequences surrounding the region that was subjected to the mutagenic agent. In Fig. 3 the amino acid sequence of the 24 C-terminal residues of the isolated mutants (I to XII) is compared to that of the wild-type (wt) protein.

The IGFBPs encoded by the mutant genes were analyzed analogous to the frameshift mutants. Figure 4A shows the results obtained with [^{125}I]IGF-I binding. As was also shown in Fig. 2, medium from mock-transfected COS-1 cells only contained COS-1-specific

	211	226
pSV19	- - - G K R I P G S P E I R G D P N C Q M Y F N V Q N	
I	- I - - - - - -	
II	- - - - - - - - - - - - - - R - - - - - I - - - I - -	
III	- Y - - - - - - -	
IV	- I - -	
V	- - - - - - - - E - - - - - - - - - Y - - - - - - -	
VI	- - - - - - - - - - - - - - - N - - - - - - - - - -	
VII	- - - - - - - - - - - - - K E - - - - - - - - - - -	
VIII	- - - - - - - - - - - - - - R - - - - - - - - I - -	
IX	- - - - - - - - - - - - - K - - - - - - - - - - - -	
X	- - - - - - - - - - - - - - - - S - - - - - - - - -	
XII	- - - - - - - - - - - - - - I - - - - - - - - - - -	

Fig. 3. The Amino Acid Sequence of the C-Terminal Region of IGFBP-1 Mutants

Changes in amino acid residues are indicated for the different IGFBP-1 point mutants (I-XII). On *top* are shown the 24 terminal residues of the wt sequence.

binding proteins. Ligand binding to IGFBP-1 was present in culture medium from pSV19 transfected COS-1 cells. Most of the IGFBP-1 point mutants are positive for [^{125}I]IGF-I binding (Fig. 4A and Table 1). Mutants III(Y226) and VII(K221-E222) failed to bind [^{125}I]IGF-I. However, the ligand-binding capacity of mutant XII(I222) seems to be slightly reduced when it is compared with the that of COS-1-binding proteins from the same lane. Figure 4B shows the results when the same blot is incubated with anti-IGFBP-1. It is obvious that several mutant proteins, III(Y226), VII(K221-E222), and XII(I222), predominantly migrate with an apparent mol wt that corresponds to twice the size of native IGFBP-1, suggesting the formation of IGFBP-1 dimers. Separation under reduced conditions, as shown in Fig. 4C, leads to immunoreactive bands of the expected 28-kDa size.

In Table 1 the results regarding IGF-binding and dimer-forming capacities of the various mutant proteins are summarized. From these, the overall picture emerges that dimer formation with concomitant loss of IGF binding is enhanced when Cys-226 is absent or when Gly at position 222 is replaced by Glu or Ile. In parallel experiments similar data were obtained with [^{125}I]IGF-II (results not shown).

DISCUSSION

In the present study we introduced mutations in the C-terminal conserved region to analyze whether this part of the molecule contributes to ligand binding. Our data obtained on the deletion and frameshift mutants demonstrate that changing of the C-terminal 20 or more amino acid residues of IGFBP-1 completely abolished IGF-I binding. This was observed regardless of the change in the reading frame, suggesting that this part of the protein is essential for IGF binding and may encompass the binding site. However, a more structural role for the C-terminal part of the IGFBP is favored by the finding that mutant pSV19Ba, pSV19Ba(+1), and pSV19Ba(−1) have an increased tendency to form IGFBP-1 dimers. These dimers are not capable of IGF binding. In constructs pSV19Ba, pSV19Ba(+1), and pSV19Ba(−1) the Cys at position 226 is absent (Fig. 5). As IGFBP-1 is an extracelluar protein, we assume that this Cys residue is normally involved in an intramolecular disulfide bond. When absent, the free sulfhydryl group of its putative partner may be able to form intermolecular disulfide bonds, resulting in IGFBP-1 dimers. With mutant pSV19Ba(−1), a fraction of monomeric IGFBP-1 is formed. Due to the frameshift this mutant has a Cys residue at position 227, which is more or less at the same position as in the wt IGFBP, although of course in a different context. (Fig. 5). This Cys may be able to form an intramolecular disulfide bond.

Further support for our hypothesis that Cys-226 is important for the tertiary structure came from the data

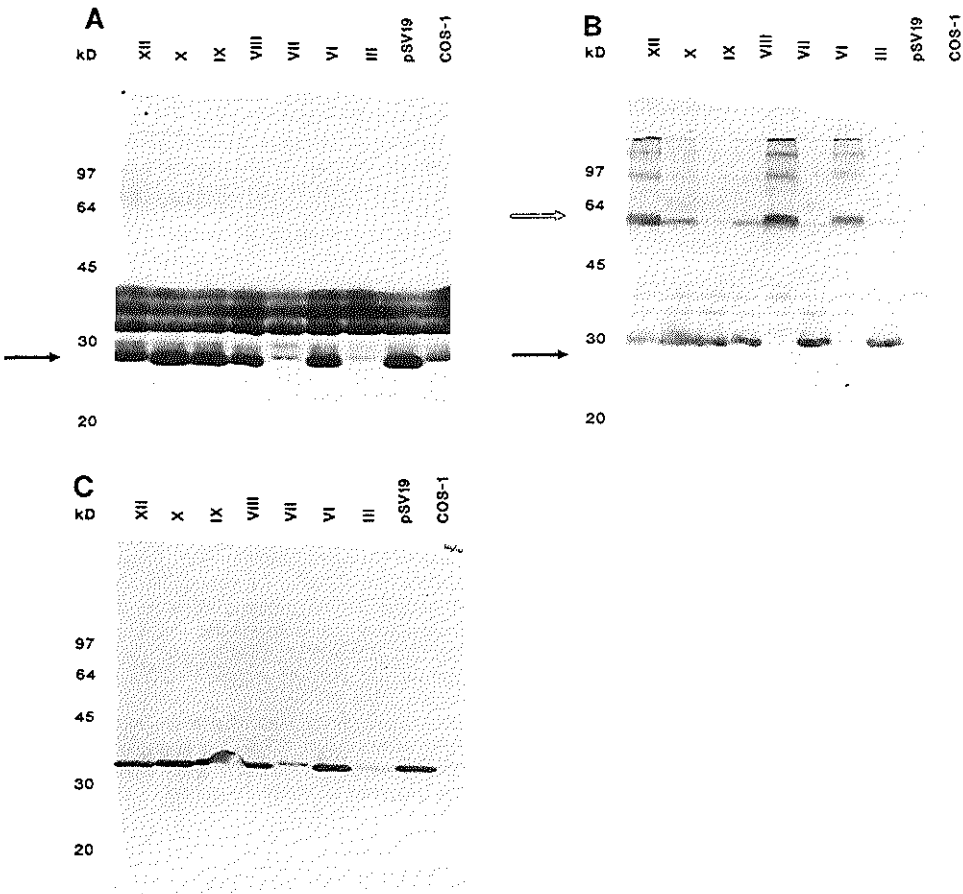

Fig. 4. Analysis of Mutant IGFBP-1 Proteins in Culture Medium of COS-1 Cells Transfected with the Expression Plasmids from Fig. 3

Culture medium of mock-transfected COS-1 cells was used as a negative control. Culture medium of cells transfected with pSV19, which encompasses the complete IGFBP-1 cDNA, was used as a positive control. The culture media were processed in the same way as the media shown in Fig. 2. A and B represent the same filter, on which the binding proteins are separated under nonreduced conditions and incubated with, respectively, more than 200,000 cpm [^{125}I]IGF-I or polyclonal anti-IGFBP-1. C shows the IGFBP-1 point mutants separated by SDS-PAGE after reduction with β-mercaptoethanol and subsequently immunostained. *Arrows* indicate the position of IGFBP-1 (dimers), as described in Fig. 2.

obtained with the point mutants. Most of the introduced mutations did not cause major alterations in the IGF-binding properties of the IGFBP-1 protein. Exchanging Cys-226 for a Tyr in mutant III(T226) resulted in dimer formation and loss of IGF-binding capacity. Mutant VII(K221-E222) does not bind IGF, while mutant IX(K221) binds normally. Therefore, the loss of IGF-binding capability of mutant VII,presumably results from Glu-222. Dimer formation was promoted by a Glu and to a lesser degree an Ile at position 222. In mutant XII(I222) dimer formation is enhanced, although to a lesser extent. These results suggest that a negatively charged or a hydrophobic residue, rather than a positively charged residue, in position 222 prohibits the formation of an intramolecular disulfide bond of Cys-226 with its normal partner. A positively charged residue such as Arg at the same position in mutant VIII(R222-I232) had only a minor effect.

The formation of IGFBP-1 dimers with concomitant loss of ligand-binding capacity may be caused by inaccessibility of the IGF-binding site in the dimers. Another explanation may be that the conformation of the binding site is dramatically changed when Cys-226 is prevented from forming an intramolecular disulfide bond. In this

Table 1. IGF-Binding and Dimer-Forming Capacities of the Various IGFBP-1 Mutant Proteins

Type	Mutation	IGF Binding	Dimer Formation
pSV19	wt	+	±
pSV19X	wt	+	±
pSV19Ba	Del 217-end	−	++
pSV19Ss	Del 109-end	−	−
pSV19S	Del 55-end	−	−
pSV19Ba (+1)	215-end	−	+++
pSV19Ba (−1)	213-end	−	++
I*	Ile-228	+	±
II*	Arg-222, Ile-228, Ile-232	+	+
III	Cys-226	−	+++
IV*	Ile-232	+	±
V*	Glu-216, Cys-226	−	+++
VI	Asn-223	+	±
VII	Lys-221, Glu-222	−	+++
VIII	Arg-222, Ile-232	+	+
IX	Lys-221	+	±
X	Ser-224	+	+
XII	Ile-222	±	++

* Results not shown.

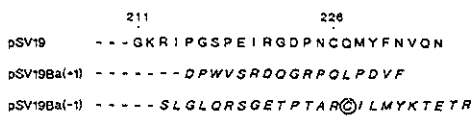

Fig. 5. The Amino Acid Sequence of the C-Terminal Region of the wt IGFBP-1 Compared to the Same Region of the Two Frameshift Mutants, pSV19Ba(+1) and pSV19Ba(−1)

The Cys residue in construct pSV19Ba(−1) potentially capable of forming intramolecular disulfide bonds is *circled*.

view it is of interest to note that although most mutations in the C-terminal region did not affect IGF binding, the monomeric form of mutant pSV19Ba(−1) fails to bind IGF (Fig. 2). This mutant, which produces both monomers and dimers, has a major change in the C-terminal region due to the frameshift. However, with the Cys residue present at approximately the same position as in the wt protein, part of the mutant molecules may be able to form an intramolecular disulfide bond, resulting in monomeric molecules. The finding that these monomers do not bind IGF suggests that in addition to the intramolecular disulfide bond, which appears to be essential, neighboring residues of Cys-226 may contribute to the ligand-binding domain of IGFBP-1. This implies that Cys-226, its partner, and the surrounding residues may be in the vicinity of the ligand-binding domain of IGFBP-1. Of course, this does not prove that this region encloses the binding site. It could be that both the N- and C-terminal regions, when brought into close proximity by disulfide bonds, contribute to a binding site. It is also possible that the pSV19Ba-1 monomers contain aberrant intramolecular disulfide bonds, or that the additional nonspecific C-terminal amino acids interfere with normal ligand binding.

Most mutations in the C-terminal RGD sequence did not affect IGF binding. However, it is still possible that these mutations have biological implications *in vivo*. In a diversity of biological systems IGFBPs have been found to inhibit as well as potentiate IGF action (29–35). Evidence has been reported that adherence to or release from the cell surface of IGFBPs plays an important role in modulating IGF binding to the receptor in human fibroblasts (34). This is supported by data which show that soluble bovine IGFBP-3 inhibits IGF-I action in fibroblasts by preventing IGF-I from binding to the receptor, whereas this IGFBP, when associated with the cell surface, potentiates IGF-I action (35). In addition, it has been demonstrated that IGFBPs can reenter tissues from the circulation and distribute themselves in a selective manner (36). This may suggest that cell adherence and release are general concepts for IGFBP functioning.

A dynamic balance between soluble and cell surface-attached IGFBPs could provide a flexible system for intercellular distribution of IGF and its action. Cell adherence is a prerequisite for such a mechanism. Both IGFBP-3 and IGFBP-4 are glycosylated proteins, and it is thought that the glycosylated nature of the IGFBP-3 protein accounts for its specific membrane interaction (34, 35). In contrast, IGFBP-1 and IGFBP-2 are not glycosylated, but both enclose a RGD sequence which may facilitate binding to the cell surface through integrin-like receptors. Therefore, those mutations in the RGD sequence of IGFBP-1 that do not affect IGF binding may be very useful to study the significance of this sequence for IGFBP-1 function.

MATERIALS AND METHODS

Construction of IGFBP-1 Deletion and Frameshift Mutants

The cDNA clone PTZp19 (6) encoding the IGFBP-1 gene was digested with various restriction enzymes. Complementary DNA fragments with decreasing lengths of IGFBP-1-coding region were isolated and inserted into the polylinker of expression vector pSV328a using standard methods (25). The pSV328a expresses cloned inserts using the SV40 early promotor and the rabbit β-globin polyadenylation signals (28). Note that because no stop codons were introduced, the reading frame of the various constructs continues into the β-globin polyadenylation region of the vector. As a consequence, the proteins encoded by clones pSV19Ba, pSV19Ss and pSV19S were elongated with stretches of nonspecific amino acids (*bold letters*), respectively: GKRIP**GRARIHSSGAGCLSEGGGWCGQCPGSQIPLRSFSLCQKLWGHHEAP** (pSV19Ba); **EEELLFTPQVQAAYQRVVAGVANALAHKYH** (pSV19Ss), and **ARCARASSNSLLRCLPIRRWWLVWPMPWLTNTTEIFFPLPRIMGTS** (pSV19S).

In addition 4-basepair (bp) deletions or insertions were introduced in the IGFBP-1 cDNA by blunting or filling in the *Bam*HI site. T7 polymerase deletes GATC, resulting in a −1 shift of the reading frame; Klenov polymerase duplicates GATC, resulting in a shift to the +1 reading frame. The amino acid sequences resulting from these frameshifts are presented in Fig. 5.

Random Mutagenesis by Chemical Modification

Random mutagenesis was performed essentially according to the method described by Pine and Huang (24). Single stranded DNA of PTZp19 was isolated and annealed to double stranded PTZp19 lacking the *BamHI-XbaI* fragment encoding the C-terminal part of IGFBP-1. The resulting gapped duplexes were subjected to the mutagenic agent sodium bisulfite, causing C to U transitions in the single stranded DNA. Finally, mutated gapped molecules were recovered and directly transfected. Instead of introducing the treated DNA into an *E. coli* strain carrying the ung mutation, which prevents the uracil DNA from degradation, we used *E. coli* strain POP101. With this strain we found that the transfection efficiency of modified, partially single stranded DNA was only 100 times lower than that of double stranded unmodified DNA. Single colonies were isolated and characterized by sequencing (26).

Site-Directed Mutagenesis using a Polymerase Chain Reaction (PCR)

Two-point mutations were introduced in the C-terminal part of IGFBP-1 (mutant X and XII) using a PCR, as described by Higuchi *et al.* (27). Mutant primers differing one and two bases (underlined), respectively, from the wt sequence and a reverse primer were synthesized on an Applied Biosystem Oligonucleotide Synthesizer (Foster City, CA). Both primers started 2 bp up-stream of the *BamHI* site in the wt IGFBP-1 cDNA. In primer 1 a Ser residue is substituted for Pro-224 [mutant X(S224)]: 5′-GAGGATCCCTGGGTCTCCAGAGATCAGGGGAGACTCCA-ACTGCC-3′. In primer 2 an Ile is substituted for Gly-222 [mutant XII(I222)]: 5′-GAGGATCCCTGGGTCTCCAGAGAT-CAGGATAGACTCCAACTGCC-3′. The reverse primer encloses the *XbaI* site and starts 76 bp 3′ down-stream of the IGFBP-1 reading frame: 5′-TGTGCATGTTCTAGAGTATA-3′. After PCR the modified DNA sequence was isolated, digested with *BamHI* and *XbaI* and inserted instead of the wt sequence. The introduced mutations were confirmed by sequencing.

Expression in COS-1 Cells, Immunostaining, and IGF Binding

Mutated cDNAs were inserted into the polylinker of the expression vector pSV328a (28) by standard DNA procedures. This vector expresses cloned inserts using the SV40 early promotor and the rabbit β-globin polyadenylation region. Subsequently, wt and mutant plasmids were transfected into monkey COS-1 cells, as described previously (6). Trichloroacetic acid was added to medium collected from the transfected COS-1 cells (7%, vol/vol). The proteins were allowed to precipitate for 16 h at 4 C. After centrifugation for 10 min at 1000 × *g*, the pellet was dissolved in sample buffer [0.06 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 6 M ureum, and 0.02% bromophenol blue], heated for 5 min at 100 C, and applied to a 12.5% SDS-polyacrylamide gel. For reducing conditions, 5% β-mercaptoethanol was added to the sample buffer. After separation the proteins were transferred onto nitrocellulose filters (Millipore HATF, Millipore Corp., Bedford, MA) by Western blotting. The filters were successively incubated with more than 200,000 cpm [^{125}I]IGF-I or [^{125}I]IGF-II (kindly provided by Dr. S. van Buul-Offers, Wilhelmina Children's Hospital, Utrecht, The Netherlands) and polyclonal anti-IGFBP-1, as previously described (6). Filters from reduced gels were incubated only with anti-IGFBP-1.

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Site-directed mutagenesis of the N-terminal region of IGF binding protein 1; analysis of IGF binding capability

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To define domains involved in IGF binding 60 N-terminal amino acid residues of IGFBP-1 were deleted. This deletion resulted in loss of IGF binding suggesting that the N-terminus may enclose an IGF binding domain. However, most point mutations introduced in this region did not affect IGF binding. In contrast to Cys-34, only substitution of Cys-38 for a tyrosine residue abolished IGF binding. With the determination that all 18 cysteine residues are involved in disulphide bond formation our data suggest that, although not all cysteines contribute to the same extent, the ligand binding site may be spatially organized.

IGF binding protein; N-terminus deletion mutant; Site-directed mutagenesis; Point mutation; IGF binding site; Disulphide bond

1. INTRODUCTION

Insulin-like growth factor (IGF) binding proteins (IGFBPs) form a family of structurally related proteins. All IGFBPs are capable of binding IGF-I and -II, which are small peptide hormones closely related to insulin. So far six IGFBPs have been isolated and characterized and even more IGFBPs, albeit less well characterized, have been identified [1–10]. IGFBPs are generally accepted as modulators of IGF action although their precise function remains unclear. Both inhibition and potentiation of IGF action on cells has been reported [11–14].

The amino- and carboxyl-termini of the IGFBPs are extremely well conserved. This probably also holds for the tertiary structure of the proteins because of the positional conservation of 18 cysteine and most of the glycine residues. The middle part is less well conserved and may serve as a spacer between the conserved termini.

Because of the high content of cysteine residues frequently found in ligand binding domains of hormone receptors, and the conservation, the N- and the C-terminal regions are the most likely candidates for an IGF binding domain. Evidence for both possibilities has been presented since fragments from either end capable of binding IGF have been described [15,16]. In addition, we recently presented evidence that an IGF binding domain may be located in the vicinity of a putative intramolecular disulphide bond formed by cysteine residue 226 and its partner cysteine [17].

In this study we present evidence that probably all cysteine residues in IGFBP-1 are involved in the formation of disulphide bonds. We demonstrate that in contrast to Cys-34, Cys-38 is essential for appropriate folding of IGFBP-1. In addition, our data show that most of the mutations introduced in one of the most strongly conserved regions in the N-terminus of IGFBP-1 do not affect IGF binding.

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2. MATERIALS AND METHODS

2.1. *Construction of a deletion mutant*

A deletion spanning amino acid residues −1 to 60 was introduced in IGFBP-1 expression clone pSV19. pSV19 expresses IGFBP-1 using the simian virus 40 (SV40) early promoter and the β-globin polyadenylation region [1]. Using standard DNA procedures a 5' *Hin*dIII-*Bss*HII cDNA fragment in pSV19 enclosing residues −25 to 60 was substituted for a 5' *Hin*dIII-*Hpa*II fragment enclosing amino acid residues −25 to −1. The deletion was confirmed by sequence analysis.

2.2. *Random mutagenesis by chemical modification*

Random mutagenesis in the N-terminal region of IGFBP-1 cDNA was performed as described previously [17]. Briefly, a 5' *Eco*RI-*Sma*I cDNA fragment encoding the N-terminal region of IGFBP-1 was subcloned in the vector PTZ19 (Pharmacia). Single stranded DNA was isolated from this construct and annealed to double stranded DNA of the same construct lacking a *Bst*EII-*Sma*I fragment in the N-terminal region of IGFBP-1. The resulting gapped duplexes were subjected to the mutagenic agent sodium bisulphite, causing C to U transitions in the single stranded DNA. Finally, mutated gapped molecules were recovered and directly transfected into *E. coli* strain POP101. DNA from single colonies was isolated and characterized by sequencing. We found that approximately 20–30% of the isolated clones had single or double mutations. From those clones that contained interesting mutations *Eco*RI-*Sma*I fragments were isolated and used to replace the corresponding *Eco*RI-*Sma*I region in expression plasmid pSV19.

2.3. *Expression in COS-1 cells, immunostaining and IGF binding*

Plasmids carrying the wild type IGFBP-1 sequence or mutations in the N-terminal region were transfected into monkey COS-1 cells. Samples of the medium of the transfected cells were separated with SDS/PAGE, transferred onto nitrocellulose filters and analyzed for IGF binding and IGFBP-1 immunoreactivity as described previously [17].

2.4. *Determination of disulphide bonds in the IGFBP-1 protein*

The number of disulphide bonds was determined by comparing the number of free sulfhydryl groups in the unfolded protein before and after reduction essentially as described by Ellman [18]. IGFBP1 was isolated from pooled mid-term amniotic fluid as described before [12]. Silver staining of the preparations showed a purity of >98%. 30–50 μg of this material was dissolved in 5 M guanidinium chloride (GdCl). To reduce the protein fresh dithiothreitol (DTT) was added to a final concentration of 100 mM. Reduced or untreated protein was precipitated with cold 5% w/v TCA and collected by centrifugation. Residual reducing agent was removed by washing five times with 5% TCA. The precipitate was dissolved in 5 M GdCl. After removing traces of turbidity by centrifugation, the protein concentration was determined by absorbance at 280 nm. The sulfhydryl content was determined by reacting the dissolved protein with an equal volume of 0.1 M Tris-HCl, pH 9.1, 5 M GdCl and 0.2 mM 5'-dithionitrobenzoic acid (DTNB). The absorbance of the mixture and a reference sample was measured at 412 nm. From the increase in absorbance caused by DTNB, the sulfhydryl content was calculated, using a molar extinction coefficient of 13 600.

3. RESULTS

3.1. *Deletion mutant*

To examine whether the N-terminal region of IGFBP-1 participates in IGF-binding we introduced a deletion in the cDNA insert of expression plasmid pSV19. We exchanged a 5' *Hin*dIII-*Bss*H1 cDNA fragment enclosing residues −25 to 60 for a 5' *Hin*dIII-*Hpa*II fragment enclosing amino acid residues −25 to −1. This procedure created a deletion spanning amino acid residues −1 to 60. An outline of the constructs is shown in Fig. 1. Note that this deletion resulted in the loss of the site from which the leader sequence is being processed in the wild type (wt) IGFBP-1 precursor protein. However, the newly created sequence may satisfy the criteria for a leader sequence processing site with Leu-62 as potential +1 residue [19].

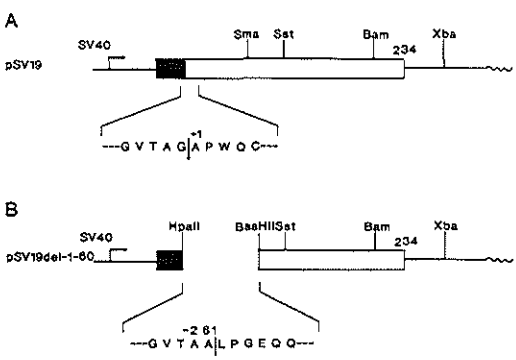

Fig. 1. Diagram of expression plasmids carrying the complete IGFBP-1 cDNA fragment under control of the SV40 promotor (A) and the construct with a N-terminal deletion spanning residues −1 to 60 (B). The wavy line represents the rabbit β-globin polyadenylation region. The coding region of IGFBP-1 is boxed with the signal peptide in black. An arrow indicates the putative leader peptide processing site.

To demonstrate the functional activity of the construct, we transfected COS-1 cells with the deletion mutant in which the coding sequence is under control of the SV40 promoter. Samples of the culture medium were separated by nonreduced and reduced SDS/PAGE and subsequently transferred onto nitrocellulose filters. The filters were incubated with either [^{125}I]IGF-I (Fig. 2A) or polyclonal antibody against IGFBP-1 (Fig. 2B and C).

Fig. 2A shows the results of ligand binding. Culture medium from mock-transfected COS-1 cells contained proteins that bind [^{125}I]IGF-I (COS). These proteins with apparent molecular weights of 47, 45, 40 and 24 kDa are presumably binding proteins secreted by the COS-1 cells themselves. An additional band of 28 kDa was observed with the construct enclosing the wild type (wt) IGFBP-1 sequence, pSV19. However, with deletion mutant pSV19del-1–60 no ligand binding different from the background bands was observed. Incubation of the same filters with IGFBP-1 antibody (Fig.2B) demonstrated the expected 28 kDa band in the culture medium from pSV19 transfected COS-1 cells and a 24 kDa band with pSV19del-1–60. Under reduced conditions (Fig. 2C) the latter band appears as a triplet with two major and one minor band with apparent molecular weights of 28, 27 and 26 kDa, respectively. These results demonstrate that deletion mutant pSV19del-1–60 is synthesized and secreted by the COS-1 cells. In addition, the finding that this truncated IGFBP-1 protein no longer binds IGF suggests that the deleted part of the protein may enclose an IGF binding site.

3.2. *Site directed mutagenesis in the N-terminal region of IGFBP-1*

Mutations were introduced in the N-terminal part of IGFBP-1 between amino acid residues 26 and 56, using the same procedure as described previously [17]. Fig. 3 shows the N-terminal amino acid sequence of the selected mutants NI-VIII when compared to the wt IGFBP-1 sequence. The IGFBPs encoded by the mutant genes were analysed analogous to the deletion mutant. The results in Fig. 4A demonstrate that COS-1 cells transfected with the mutant clones NI-VIII produce a protein capable of binding [^{125}I]IGF-I. Only mutant NI(Y38) failed to bind IGF. The latter mutant is produced by the COS-1 cells and is recognized by the antibody as is apparent from Fig. 4B. However, the mutant protein migrates in two bands with apparent molecular weights of approximately 30 and 50 kDa. Note that the other mutants also show some heterogeneity in apparent molecular weight (Fig. 4A and B). However, when separated under reduced conditions as shown in Fig. 4C it is obvious that all mutant proteins (NI-VIII) migrate with

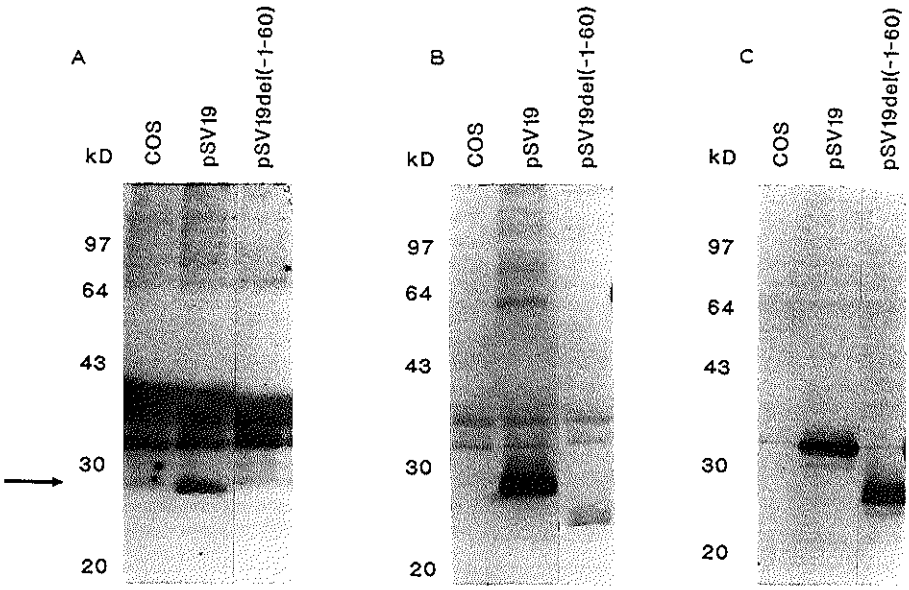

Fig. 2. Analysis of mutant IGFBP-1 proteins in culture medium of COS-1 cells transfected with the expression plasmids from Fig. 1. Culture medium of COS-1 cells transfected with pSV328a was used as a negative control, cells transfected with pSV19 as a positive control. The binding proteins were separated by SDS/PAGE (12.5% acrylamide) and transferred onto nitrocellulose by Western blotting. The filters were incubated with >200 000 cpm [^{125}I]IGF-I, washed and autoradiographed (A). After autoradiography the same filters were incubated with polyclonal anti-IGFBP-1 and made visible by immunostaining (B). C shows immunostaining of the mutant IGFBP-1 proteins separated by SDS/PAGE after reduction with β-mercaptoethanol. The position of IGFBP-1 is indicated with a black arrow.

the same molecular weight as the wt protein (pSV19), demonstrating that the primary structure of the mutant proteins is stil intact. These apparent heterogeneity in molecular weight is most likely due to changes in the conformation of the molecule introduced by the mutations. In parallel experiments similar data were obtained when [^{125}I]IGF-II was used (results not shown).

3.3. *Determination of disulphide bonds*

Reduced and non-reduced samples of three different batches of purified IGFBP-1, with a purity of >98% were treated with DTNB. From the increase in absorbance measured at 412 nm, the sulfhydryl content was calculated. We reproducibly found 4.7±0.9 (n=4) mole -SH groups per mole reduced IGFBP-1, corresponding to 26% ±5 of the 18 mole of -SH groups as predicted from the primary structure. Increasing the denaturating agent GdCl to a final concentration of 6 M with or without 5–10 min additional heating at 100°C did not improve the results. To verify whether reduction of the IGFBP-1 protein was complete, the protein samples were precipitated in the same test tube after reaction with DTNB and measurement of the absorbance. The precipitate was separated on a nonreduced SDS/PAGE and analysed with anti-IGFBP-1 according to the standard procedure. The results (not shown) indicated that the IGFBP-1 samples indeed were completely reduced. Apparently the absorbance is quenched by the IGFBP-1 protein. In a parallel experiment to determine the free -SH groups in human serum albumin (HSA) we found an efficiency of 64% for this reaction suggesting that quenching of the absorbance is inherent for this method and depends on the nature of the protein. In non-reduced IGFBP-1 preparations after correction for an efficiency of 26% we found 1.1±0.9 (n=4) mole free -SH groups per mole IGFBP-1. These results correspond to 0 or at most 2 free -SH groups suggesting that most if not all cysteines are involved in disulphide bonds.

4. DISCUSSION

In the present study we analyzed the effect of mutations in the N-terminal part of IGFBP-1 on IGF binding. In addition, we determined the number of disulphide bonds. Deletion of 60 N-terminal amino acid residues of IGFBP-1 resulted in loss of IGF binding suggesting a possible IGF binding site in this region. This finding is consistent with the report that N-terminal fragments are capable of binding IGF [15]. Therefore we focussed our attention on the most conserved stretch of amino acids in this region, spanning residues 26–50.

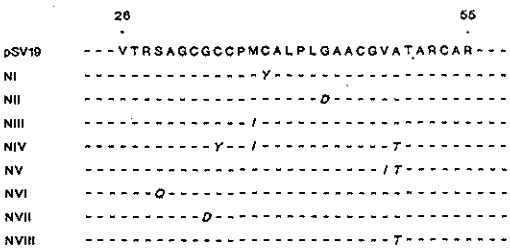

Fig. 3. The amino acid sequence of the N-terminal region of IGFBP-1 mutants. Changes in amino acid residues are indicated for the different IGFBP-1 point mutants (NI-VIII). On top the amino acid residues of the wild type sequence are shown.

However, our results show that most of the point mutations introduced in this part of the N-terminus did not affect IGF binding but merely resulted in minor changes in the conformation of the IGFBP-1 molecule. This effect, shown as a shift in apparent molecular weight, is most clearly seen with mutant NV(I48,T49) (Fig. 4A and B). Only substitution of Cys-38 for an tyrosine residue in mutant NI(Y38) resulted in loss of IGF binding and was accompanied by a dramatic change in conformation as suggested by the shift in migration on the gel (Fig. 4A and B). Such a structural change may influence the IGF binding site in a way that ligand binding is impossible. It has been reported by others [20] that substitution of Cys-16 and Cys-35 for serine residues also abolished IGF binding. It seems obvious that these three cysteine residues in the N-terminal region are essential for the integrity of the IGFBP-1 molecule due to their participation in disulphide bond formation.

Therefore, we also determined the number of free -SH groups in IGFBP-1. Our data suggest that at least 16 but most likely all cysteine residues form disulphide bonds, implying that IGFBP-1 indeed has a tightly fold-

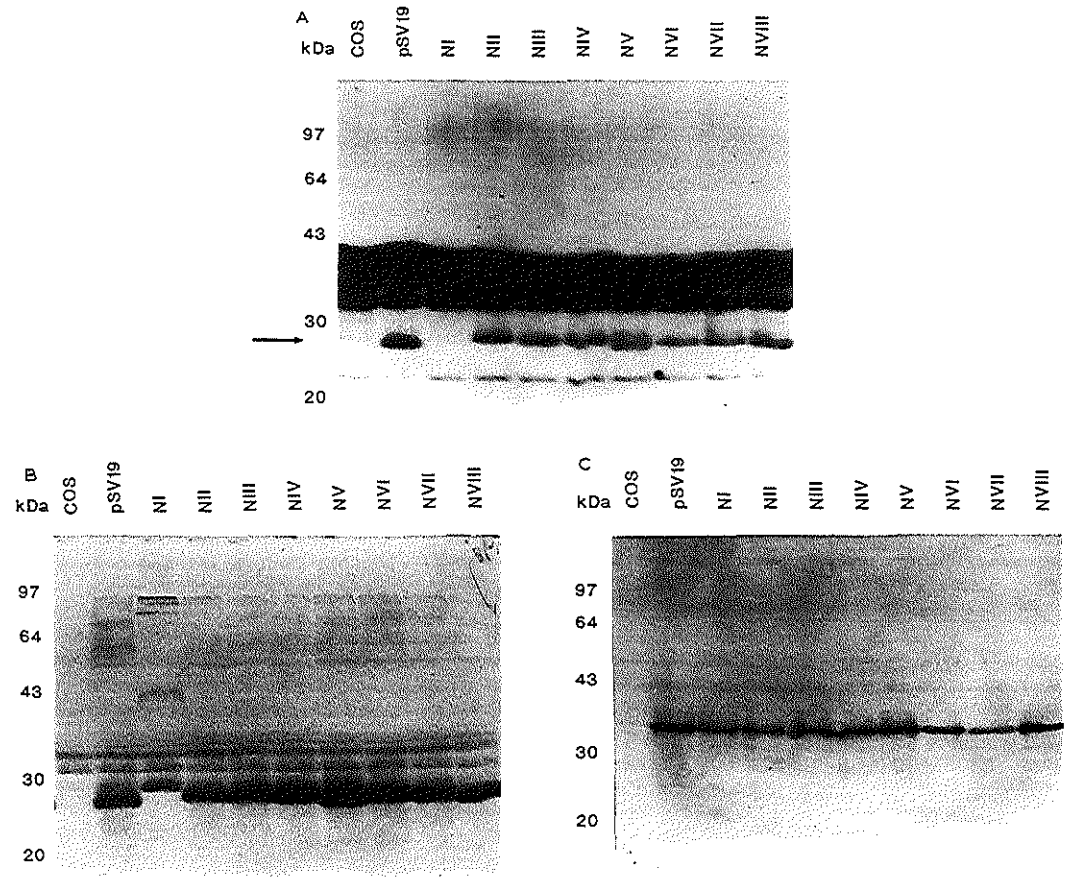

Fig. 4. Analysis of mutant IGFBP-1 proteins in culture medium of COS-1 cells transfected with the expression plasmids from Fig. 3. For further details see legend to Fig. 2.

ed conformation. Considering the conservation of the cysteine residues this is most likely true for all IGFBPs.

Although our data suggest that all cysteine residues are involved in disulphide bond formation apparently not all cysteine residues contribute to the structure to the same extent. The results obtained with the mutants NIII(I37), NVIII(T49) and NIV(Y34,I37,T49) indicate that substitution of Cys-34 for a tyrosine residue has no effect on IGF binding nor on the apparent molecular weight (Fig. 4A and B). Thus Cys-34 in contrast to its neighbouring cysteine residues (Cys-35 and Cys-38) is not essential for maintaining IGFBP-1 structure. Loss of ligand binding observed in some of the mutant IGFBP-1 proteins is most probably the result of major structural changes introduced by deleting significant parts of N-terminal region or by mutations presumably preventing cysteine residues to form disulphide bonds. The same seems to be true for the C-terminal region of IGFBP-1 in which we observed that Cys-226 is also essential for IGF binding [17]. Therefore, it may be possible that neither the N- nor the C-terminal ends separately comprise the IGF binding site but rather that both ends linked by disulphide bonds contribute to a spatially organized IGF binding site.

Future research to assign the disulphide bonds will be of great importance to understand the structure of IGFBP-1 and its interaction with IGF.

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IGFBP-1, AN INSULIN LIKE GROWTH FACTOR BINDING PROTEIN, IS A CELL GROWTH INHIBITOR

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SUMMARY : A novel cell growth inhibitor, IDF45 (inhibitory diffusible factor), was recently purified to apparent homogeneity. It is a bifunctional molecule : able to bind Insulin like growth factor (IGF) and to 100 % inhibit DNA synthesis stimulated by serum in fibroblasts. It was of interest to verify whether other members of the IGF-binding protein (IGFBP) family show the same bifunctional growth inhibitory properties.

In this paper we show that purified IGFBP-1 derived from amniotic fluid is a cell growth inhibitor. In chick embryo fibroblasts, it inhibited DNA synthesis stimulated by serum. However the stimulation was maximally 60 % inhibited and half of the inhibition was observed with 100ng/ml IGFBP-1. So the specific activity of IGFBP-1 is lower than that of IDF45. IGFBP-1 also reversibly prevented the CEF growth.

In the same cells IGFBP-1 inhibited DNA synthesis stimulated by IGF-I. We demonstrated that the same protein IGFBP-1 is able to inhibit DNA synthesis stimulated by serum and by IGF-I . The possibility that IGFBP-1 is a bifunctional molecule is discussed.

From medium conditioned by 3T3 cells, we recently (1) purified to apparent homogeneity a novel cell growth inhibitor of 45 kDa which we termed IDF45 (inhibitory diffusible factor). This molecule is an IGF-binding protein able to 100% inhibit DNA synthesis stimulated by serum in chick embryo fibroblasts (CEF) (2).

Our previous results (2) showed that, in CEF, the stimulation of DNA synthesis by IGF-I and stimulation by serum were additive. This suggests that mode of action of IGF-I and serum are different, and that stimulation by serum is not due to cell stimulation by the IGF-I present in the serum. Moreover, it was possible to dissociate the two inhibitory effects of IDF45 upon IGF-I and serum stimulation. Taken together, our results suggest that IDF45 is a bifunctional molecule.

Different IGF-binding proteins have recently been purified and cloned (3,4,5,6,7,8,9). It was of interest to verify whether other members of the IGFBP family show the same bifunctional growth-inhibiting properties. In the present paper, we demonstrate that purified IGFBP-1 derived

from amniotic fluid is also a cell-growth inhibitor, suggesting that this inhibition may be a general property of IGFBPs.

MATERIALS AND METHODS

Cells

Primary cultures of chick embryo fibroblasts were prepared from 10-day-old brown Leghorn chick embryos and were cultivated at 37°C in modified Eagle's medium supplemented with antibiotics and 5 % calf serum as described (10).

IGFBP-1 purification

IGFBP-1 was purified from pooled mid-term amniotic fluid in three steps. The first involved fractionated ammonium sulfate precipitation of the amniotic fluid, essentially as described by Povoa et al. (11). In the second step, IGFBP-1 containing the 45 % fraction was dissolved in 50 mM Tris buffer pH 7.5 and loaded onto a C18 packed column (Waters/Millipore). Subsequently, the column was eluted stepwise with 50 mM Tris buffer pH 7.5, containing, respectively : zero, 40 %, 50 % and 65 % methanol ; finally, the column was stripped with 100 % methanol. The final step consisted of concentrating the IGFBP-1 protein from the 65 % peak fraction by adding 7 % (end volume) trichloroacetic acid. After centrifugation (3800 g for 15 min), the pellet was lyophilized and stored at -20°C until use.

Aliquots of purified protein were reconstituted in 20 mM Tris buffer pH 7.5 and electrophorezed on 10 % polyacrylamide gel in the presence of SDS. Purity was determined by silver staining of the gel. With this procedure, we routinely find 95 % purity (fig. 1). Scatchard analysis demonstrated that affinity for IGF-I and II was unchanged (results not shown) after the final step of purification.

Assay of IGFBP-1 activity

Secondary cultures of CEF were seeded at 7 x 10^4 cells/well in 96-well culture plates. Medium was discarded 5 h later and cells were washed and maintained in serum free medium for 42 h before addition of calf serum and/or IGFBP-1. Lyophilized IGFBP-1 was solubilized in 0.1 M acetic acid + albumin (1 mg/ml) (stock solution). Aliquots were diluted with culture

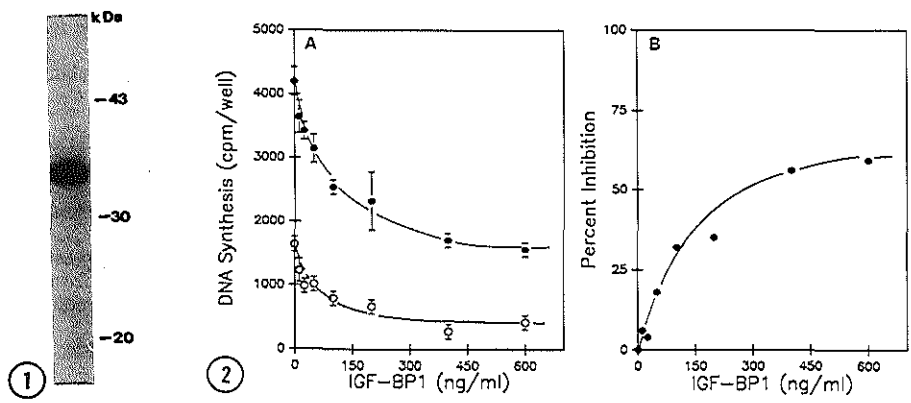


Fig. 1 . SDS-PAGE of IGFBP-1

About 1.5 µg of protein were separated by electrophoresis on 10 % polyacrylamide gel under reducing conditions and revealed by silver staining.

Fig. 2 . Inhibition by IGFBP-1 of serum induced stimulation of DNA synthesis.

Different concentrations of IGFBP-1 were added to quiescent CEF with (—●—) or without (—○—) serum (1 %). Cells were labelled 6 h later for 18 h with [^{14}C]-thymidine.
A : DNA synthesis (cpm thymidine incorporated/well). Data are means of 2 determinations.
B: Stimulation by serum of DNA synthesis and percent inhibition by IGFBP-1 of this stimulation were calculated.

medium before being added to the cells at the concentrations indicated. DNA synthesis was determined as described (12) by labeling the cells with [^{14}C]-thymidine (0.05 µCi/well) between 6 and 24 h after addition of IGFBP-1 with or without newborn calf serum or IGF-I (Amersham corporation).

RESULTS

Inhibition by IGFBP-1 of serum-induced stimulation of DNA synthesis

Quiescent CEF were stimulated by 1 % serum in the presence of different concentrations of IGFBP-1, and DNA synthesis was determined between 6 and 24 h after addition of serum and IGFBP-1. Figure 2A shows typical results. Addition of IGFBP-1 inhibited DNA synthesis in CEF which were non-stimulated or stimulated by serum. In the presence of large concentrations of IGFBP-1, DNA synthesis in stimulated cells decreased to the level observed in quiescent cells. In figure 2B, the inhibition by IGFBP-1 of the stimulation of DNA synthesis by serum was calculated. This stimulation was maximally 60 % inhibited in the presence of large concentrations of IGFBP-1. Half of this inhibition is observed with 100 ng/ml IGFBP-1.

Inhibition of cell growth

In the experiment shown in figure 3, 1 % serum was added to quiescent CEF in the presence or absence of IGFBP-1 . IGFBP-1 prevented cell growth. However, this inhibition was reversible : when the medium was changed 24 h after addition of IGFBP-1 plus serum and replaced by fresh medium with 1 % serum, cells immediately resumed their growth. The number

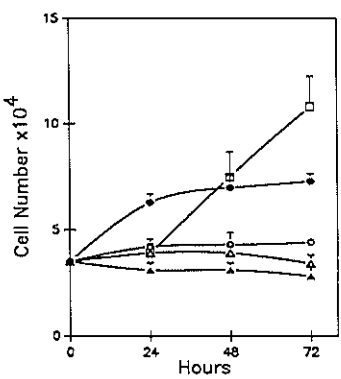


Fig. 3 . Inhibition of cell growth.
6 x 10^4 cells/well were seeded ; 5 h later, the medium was changed and replaced by fresh serum-free medium. Cultures were stimulated 42 h later (time 0) by addition of 1 % serum in the absence (— ● —) or presence (— ○ —) of IGFBP-1 (500 ng/ml). In some wells, cells were left in serum-free medium (— △ —). In some wells, IGFBP-1 was added in the absence of serum (— ▲ —). One part of the cultures incubated with serum and IGFBP-1 was washed 24 h later and maintained in fresh medium + 1 % serum (— □ —). Cells number was determined on the pool of two culture wells. Data are means of two determinations .

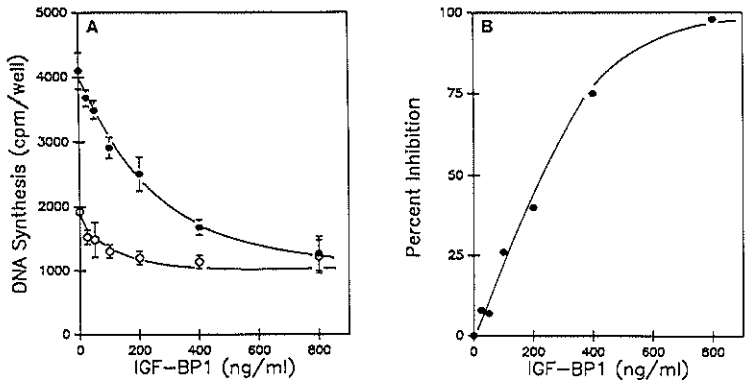


Fig. 4 . Inhibition by IGFBP-1 of DNA synthesis stimulated by IGF-I.
Different concentrations of IGFBP-1 were added to quiescent CEF in the presence (—●—) or absence (—o—) of IGF-I (20 ng/ml).
A : DNA synthesis determined between 6 and 24 h after addition of IGF-I and IGFBP-1. Data are means of two determinations .
B : Stimulation by IGF-I of DNA synthesis and percent inhibition of this stimulation were calculated.

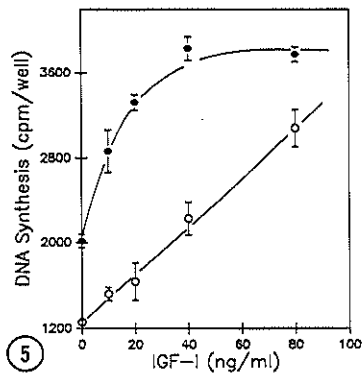
of cells incubated in serum free medium did not change between time 0 and 72 h incubation. When cells were incubated in serum-free medium but in the presence of IGFBP-1, the number of cells decreased. These results suggest that in CEF (secondary culture) in serum free-medium, some cells disappear and are replaced by new cells multiplying in the presence of a stimulating autocrine factor. Addition of IGFBP-1 prevents this multiplication.

Inhibition by IGFBP-1 of DNA synthesis stimulated by IGF-I

Quiescent CEF were stimulated or not by 20 ng/ml IGF-I in the absence or presence of different concentrations of IGFBP-1, and DNA synthesis was determined (fig. 4A). Figure 4B shows percent inhibition by IGFBP-1 of stimulation of DNA synthesis induced by IGF-I in the cells. Inhibition was proportional to the concentration of IGFBP-1 in the medium. Stimulation by IGF-I was 100 % inhibited by IGFBP-1.

Inhibition by IGFBP-1 of IGF-I stimulation depended on the concentration of IGF-I in the medium

IGFBP-1 (200 ng/ml) was added to cells incubated in the absence or presence of different concentrations of IGF-I, and DNA synthesis was determined. DNA synthesis was inhibited. However, this inhibition depended on the concentration of IGF-I and decreased significantly in the presence of large concentrations of IGF-I (fig.5).In the presence of 80 ng/ml of IGF-I, most IGFBP-1 molecules, which probably bound to IGF-I, were unable to inhibit stimulation induced



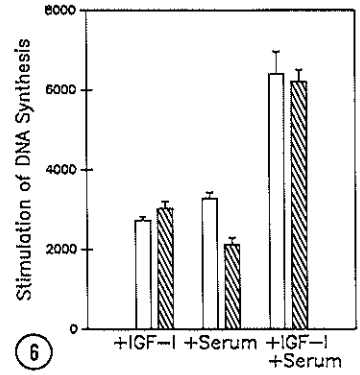


Fig. 5 . Inhibition by IGFBP-1 of IGF-I stimulation as a function of IGF-I concentration .
Different concentrations of IGF-I were added to quiescent culture of CEF in the absence (—●—) or presence (—○—) of IGFBP-1 (200 ng/ml). DNA synthesis was determined as described before.

Fig. 6 . Inhibitory activity of IGFBP-1 upon serum in the presence of high concentration of IGF-I.
IGF-I (120 ng/ml) or serum (1 %) or IGF-I (120 ng/ml) + serum (1 %) was added to quiescent cultures of CEF and the stimulation of DNA synthesis by IGF-I alone, serum alone, or by the mixture, was determined in the absence or presence of IGFBP-1 (200 ng/ml). Data are means of 3 determinations (± SD).

by free IGF-I molecules. Therefore, it was of interest to verify whether in these conditions IGFBP-1 was able to inhibit serum stimulation.

Inhibitory activity of IGFBP-1 upon serum in the presence of high concentrations of IGF-I.

Cells were stimulated either by serum (1 %) alone, by IGF-I (120 ng/ml) alone, or by a mixture of IGF-I + serum in the absence or presence of IGFBP-1 (200 ng/ml) (fig. 6). As observed previously (2), stimulation of CEF by serum and by IGF-I were additive.

Stimulation by IGF-I was not inhibited by IGFBP-1. Stimulation by serum was 35 % inhibited, but this inhibition was lost when IGF-I + serum were added simultaneously. This suggests that IGFBP-1 is involved in both inhibition of IGF-I and serum stimulation.

DISCUSSION

Our results show that IGFBP-1 is an inhibitor of CEF growth. It inhibited DNA synthesis in CEF maintained in serum-free medium and stimulation of DNA synthesis induced by addition of serum. IGFBP-1 decreased DNA synthesis stimulated by serum to the level of non stimulated CEF. However the inhibition of DNA synthesis stimulated by serum was maximally 60 % inhibited, whereas IDF45 was able to 100 % inhibit DNA synthesis stimulated by serum in the same cells and under the same conditions (1). Furthermore the specific inhibitory activity of IGFBP-1 is lower than that observed with IDF45.

IGFBP-1 was able to prevent CEF growth, but in a reversible manner; therefore,IGFBP-1 may be considered as a physiological cell growth inhibitor.

Two IGFBP-1 proteins were purified from amniotic fluid by Elgin et al (13) one which inhibits and an other one which stimulates DNA synthesis in the presence of IGF-I. Our preparation of IGFBP-1 inhibited IGF-I stimulation of DNA synthesis. Inhibition was proportional to the concentration of the binding protein and depended on the concentration of IGF-I. These results agree with the assumption that inhibition of stimulation of DNA synthesis induced by IGF-I is the result of the ability of IGFBP-1 to bind IGF-I.

We demonstrated that inhibition by IGFBP-1 of serum stimulation and of IGF-I stimulation are due to the same protein: when IGFBP-1 was in the presence of a large concentration of IGF-I, it lost its activity toward serum. If the two activities took place on two different molecules, the presence of IGF-I would not modify inhibition of serum stimulation.

Inhibition by IGFBP-1 of serum stimulation does not seem to result from its inhibitory effect upon IGF-I stimulation : in the present work, we verified that stimulation by serum and IGF-I are additive, suggesting that IGF-I and the growth factors of serum have different modes of action. Furthermore, in culture medium with 1 % calf serum, about 0.8 ng/ml of IGF-I and 2.8 ng/ml IGF-II (14) are present. From results described in figure 6, it is unlikely that 60 % of stimulation of DNA synthesis induced by 1 % serum is due to 4 ng/ml of IGF-I + IGF-II.

Our results suggest that IGFBP-1, like IDF45 (2), is bifunctional : it binds IGF-I and it also inhibits serum stimulation. Such bifunctionality for IGF-BP has recently been proposed : in rat granulosa cells, both IBP-2 and IBP-3 inhibited IGF-I-induced estradiol and progesterone production. They also inhibited FSH stimulation of thymidine incorporation in granulosa cells, while IGF-I had no effect on this parameter (15).

Taken together these results therefore indicate that the IGF- binding protein family may be considered as a new class of cell growth inhibitors.

ACKNOWLEDGMENTS

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Summary and concluding remarks

Since the discovery of the IGFs it has become evident that these growth factors play an important role in the growth, differentiation and metabolism of cells. In contrast to most growth factors the IGFs are almost completely bound to a group of proteins presently known as IGFBPs.

In Chapter II the isolation and characterization is described of cDNA for an IGFBP, that was originally identified in and purified from human amniotic fluid. The cDNA sequence predicts a molecular weight of 25 kD for the 324 amino acid mature protein. As this protein was the first IGF binding protein fully characterized,it was termed IGFBP-1. Expression of the cDNA encoding IGFBP-1 in COS cells resulted in the synthesis of a protein which binds IGF-I and which is immunologically indistinguishable from the IGF binding protein isolated from human amniotic fluid or human serum. Northern blotting analysis demonstrated that expression of the IGFBP-1 gene is highly tissue specific and limited to placental membranes and fetal liver.

The genomic organization of the gene encoding IGFBP-1 is presented in Chapter III. The gene is organized in 4 exons and spans 5.9 kb. S1 nuclease analysis determined a single transcription start site. The first exon and the 5' flanking region are highly CG rich and form a CpG island. The CpG island encloses the CAAT box, the TATA box, the transcription start site and a potential Sp-1 transcription factor binding site. All signals required for polyadenylation are located within exon IV, predicting a mRNA of 1.5 kb, which is consistent with the size seen on RNA blots.

Soon after the elucidation of the cDNA sequence of IGFBP-1, five additional IGFBPs have been discovered and characterized. Comparison of the different IGFBP amino acid sequences clearly demonstrated that these binding proteins comprise a family of related proteins. Notably, the amino- and carboxyl-terminal regions display a large degree of homology. In IGFBP-1, -2, -3 and -5 there is a strict conservation of the positions of 18 cysteine residues in the amino- and carboxyl-terminal parts of the proteins. In addition to these cysteines, IGFBP-4 has two other cysteine residues in the less conserved middle part of the protein. IGFBP-6 lacks 4 cysteine residues. The genomic organization of the IGFBPs is also very similar. However, the promoter regions and upstream elements are quite different.

The conservation of the cysteines suggest, that these residues contribute to an overall similar tertiary structure of the binding proteins. In IGFBP-1, all 18 cysteine residues are involved in disulphide bond formation. As described in Chapters IV and V, site-directed mutagenesis of several cysteine residues of IGFBP-1 demonstrated the importance of these residues for the integrity of the molecule and IGF binding capability. For instance, substitution of Cys38 and Cys226 for tyrosine residues resulted in structural changes shown as abnormal migration in a SDS/PAGE electrophoresis system, in dimer formation and in loss of IGF binding capability.

In addition, deletion of the carboxyl-terminal 20 amino acids or introduction of frame-shifts in this region resulted in loss of IGF binding and for some mutants in the formation of dimeric IGFBP-1 molecules. Similarly, deletion of the amino-terminal 60 amino acids resulted in loss of IGF binding. However, most point mutations either in the carboxyl- or amino-terminal regions did not affect IGF binding. These results suggest that IGFBP-1 is a tightly folded molecule. It is quite likely that both the N- and C-terminal regions are spatially very close and that they both contribute to the ligand binding site. This may hold true for the other IGFBPs as well. The less conserved middle part of these proteins enclosing most of the potential glycosylation and phosphorylation sites may determine the individual properties of the IGFPBs such as association to the cell surface or preferential binding to IGF-I or -II.

The IGFBPs bind IGF-I and -II with an affinity equal to that of the IGF receptors. Because of this property, these proteins have the potency to modulate the interaction of the IGFs with their receptors. For many cell types it has been demonstrated that IGFBPs were able to inhibit IGF bioactivity. The results presented in Chapter VI demonstrate that purified human IGFBP-1 protein inhibits IGF-induced DNA synthesis of chicken embryo fibroblasts.

Similarly, purified IGFBP-1 has been shown to inhibit the effects of IGF-I and -II on PHA-induced proliferation of peripheral blood mononuclear cells. However, potentiation of IGF action by various IGFBPs has been reported as well. It can be envisioned that subtle changes in molecular structure may govern IGFBP specific action on IGF activity, being inhibitory or under certain conditions stimulatory.

Many cells from normal tissues or tumours express and one or more IGFBP mRNAs and release various forms of IGFBPs. The relative proportions may vary considerably, depending on developmental, hormonal, metabolic and pathological conditions.
For instance, in a collaborative study, the secretion of IGFBP-s and the expression of the genes encoding IGFBP-1, -2 and -3 was studied in a panel of cell lines derived from breast carcinomas, Wilms tumors, neuroblastoma, retinoblastoma, colon carcinomas, liver adenocarcinoma, Burkitt lymphoma and non small-cell lung carcinoma. It was found that all cell lines with the exception of the Burkitt lymphoma cell lines, secreted IGFBPs as detected by affinity labeling. IGFBP-1 gene expression could be detected only by reverse transcriptase PCR (Reeve et al, 1992).

Notwithstanding the rapidly growing data, the picture of the role of the IGFBPs in the IGF system is far from complete, but it emerges that these proteins are likely to have an important impact on the regulation of the bioavailability of IGFs. Like IGFs, the scope of action of the IGFBPs seems to be endocrine as well as paracrine. In the circulation they prolong the half life of IGFs and at the same time they protect the body from metabolic effects of free IGF. In addition, association and dissociation of IGFBP-3/IGF with the ALS protein, combined with the potency from binary IGFBP/IGF complexes to cross the capillary barrier provides a sophisticated control of the bioavailability and routing of IGFs from and to cells. At a local level a dynamic balance between soluble and cell-surface attached IGFBPs gives a flexible system for intercellular distribution of IGFs.

The impact that IGFBPs have on the IGF system is obviously due to their ability to bind IGF. This property shared by all IGFBPs is best studied. In addition, IGFBPs may have direct and IGF independent interactions with the cell. Our results as discussed in Chapters IV and V demonstrate that both the well conserved amino- and carboxyl-terminal ends of the IGFBPs are involved in IGF binding. However, not only the similarity in the primary structure of the IGFBPs and their capability to bind IGFs is of interest, but also the differences between the IGFBPs are worthwhile to study althought as yet rather unexplored. The various IGFBPs differ in many ways, structurally and physically. Most variation is found in the central part of the IGFBPs. The relation between

structure and physical differences is presently unknown. For instance IGFBP-3 is the only binding protein known capable of forming a ternary complex with IGF and an unique protein termed acid labile subunit (ALS). This complex forms a stable storage depot of inactivated IGFs in the circulation. The molecular site of interaction of IGFBP-3 with the ALS protein is unidentified. Some but not all IGFBPs are glycosylated, a property that has been suggested to be important for adherence of IGFBP to the surface of cells. Cellular attachment may also be mediated by the RGD motif found in IGFBP-1 and -2. The central part of IGFBP-1 is rich in proline, glutamine, serine, and threonine residues and therefore potentially prone to protease activity that may affect the half-life of the protein. The serine residues in this region are also the sites where phosphorylation takes place. Variation of the state of phosphorylation is found to be a mechanism that determines the affinities for IGF-I and -II.

Much more molecular and biochemical analysis will be required to elucidate the functional relevance of the structural variability among the IGFBPs. At present one can only speculate whether these structural variations regulate the mode of IGF action or determine still unknown IGF independent functions.
Further studies using mutant IGFBPs such as presented in this study may lead to a better understanding of the very complex interactions between the IGFs, their receptors and the different IGFBPs.

Samenvatting

Sinds de ontdekking van de insuline-achtige groeifactoren (IGFs) wordt het steeds duidelijker dat zij een belangrijke rol spelen bij de groei, de differentiatie en het metabolisme van cellen. In tegenstelling tot de meeste andere groeifactoren komen IGFs niet vrij voor, maar zijn zij vrijwel volledig gebonden aan eiwitten, de zogeheten IGF bindende eiwitten (IGFBPs).

Hoofdstuk II beschrijft het isoleren en karakteriseren van een cDNA dat codeert voor een IGFBP, dat identiek bleek aan een uit humaan vruchtwater gezuiverd IGFBP. Deze cDNA sequentie voorspelt een eiwit van 324 aminozuren, met een moleculair gewicht van 25kD. Omdat dit het eerste volledig gekloneerde IGF bindende eiwit was, is het IGFBP-1 genoemd. Expressie van het voor IGFBP-1 coderende cDNA in COS cellen resulteerde in de synthese van een eiwit met dezelfde immunologische en IGF bindende eigenschappen als die van het uit vruchtwater geïsoleerde IGFBP. Northern blot RNA analyse laat zien dat het IGFBP-1-gen weefselspecifiek tot expressie komt; het is slechts aantoonbaar in foetale lever en in decidua weefsel.

De genomische organisatie van het IGFBP-1 gen wordt in Hoofdstuk III beschreven. Het gen ligt verspreid over ca. 5.9 kb en bestaat uit 4 exonen. Met behulp van S1 nuclease analyse is de transcriptie startplaats van het gen gelokaliseerd. Het eerste exon en het 5' flankerend gebied is rijk aan GC nucleotiden en vormt een zogeheten CpG-eiland. Naast exon I omsluit dit CpG-eiland de promotor elementen: CAAT en TATA, de transcriptie start van het IGFBP-1 gen en een potentiële bindingsplaats voor transcriptie factor Sp-1. In exon IV liggen alle signalen die nodig zijn voor polyadenylering van de mRNA. Het geheel voorspelt een mRNA voor het IGFBP-1-gen dat overeenkomt met de 1.5 kb die op RNA blots gevonden wordt.

Spoedig na de opheldering van de cDNA sequentie van IGFBP-1 zijn nog vijf andere IGFBPs ontdekt en gekarakteriseerd. Vergelijking van de aminozuurvolgorde van de verschillende IGFBPs laat duidelijk zien dat deze eiwitten nauw verwant zijn. Vooral de uiteinden van de verschillende eiwitten vertonen veel overeenkomst. Er is een strikte conservering van de positie van 18 cysteïne residuen in het amino- en carboxyl-uiteinde, alhoewel er ook verschillen zijn. IGFBP-4 bevat twee extra cysteïnes in het minst geconserveerde centrale deel van het eiwit, IGFBP-6, daarentegen mist 6 cysteïne residuen ten opzichte van IGFBP-1. Ook de genomische organisatie van de verschillende

IGF bindende eiwitten vertoont veel overeenkomst; de verschillen bevinden zich vnl. in de 5' flankerende gebieden en promotors.

De stringente conservering van de cysteïne residuen doet vermoeden dat ze van belang zijn voor de tertiaire structuur. Het is aannemelijk dat die voor de verschillende IGFBPs vergelijkbaar is. Met behulp van gerichte mutagenese is getracht het belang van enkele van deze cysteïne residuen voor de integriteit en voor de IGF bindende eigenschappen van IGFBP-1 eiwit aan te tonen (Hoofdstukken IV en V). Angetoond werd dat alle 18 cysteïne residuen in IGFBP-1 disulfide bruggen vormen. Vervanging van Cys38 en Cys226 door tyrosine residuen leidde tot veranderingen in de structuur van het eiwit. Dit uitte zich in abnormale migratie bij SDS/PAGE elektroforese, vorming van dimeren en verlies van IGF bindingscapaciteit. Deletie van 20 aminozuren van het carboxyl-uiteinde of introductie van een verschuiving in het leesraam in dit gebied, resulteerde in het verlies van IGF bindend vermogen en gaf, in sommige gevallen, ook dimeer vorming. Evenzo had een 60 aminozuren deletie van het amino-uiteinde verlies van ligand bindend vermogen tot gevolg. De meeste puntmutaties, die aangebracht werden in beide uiteinden, hadden daarentegen geen effect op de IGF bindende eigenschappen van IGFBP-1. Uit de resultaten kan worden geconcludeerd dat het IGFBP-1 een compact gevouwen molecuul is waarin het amino- en het carboxyl-uiteinde in elkaars nabijheid liggen, waardoor beide kunnen bijdragen aan de ligand bindingsplaats. Gezien de strikte conservering van de voor de structuur belangrijke cysteïne residuen geldt deze conclusie waarschijnlijk ook voor de andere IGF bindende eiwitten. Het minst geconserveerde, centrale deel van de IGFBPs, dat voor de individuele eiwitten glycosylerings- en fosforylering-plaatsen bevat, zou de specifieke eigenschappen van de verschillende IGFBPs kunnen bepalen, zoals: associatie aan het cel oppervlak of preferentiële binding van IGF-I of -II.

Doordat de affiniteit van de IGF bindende eiwitten voor IGF-I en -II van dezelfde grootte-orde is als die van de IGF-receptoren voor het ligand, kunnen ze de interactie van IGF met de receptor moduleren. Bij veel celtypen is aangetoond dat de IGFBPs de IGF bioactiviteit remmen. De resultaten gepresenteerd in Hoofdstuk VI laten zien dat gezuiverd IGFBP-1 de door IGF geïnduceerde DNA synthese in embryonale kippe fibroblasten remt. Ook in PHA-geaktiveerde mononucleaire cellen uit perifeer bloed remt IGFBP-1 het effect van IGF-I of -II op de proliferatie. Echter, stimulatie van IGF

bioactiviteit door IGFBPs wordt ook gevonden. Het is denkbaar dat kleine veranderingen in de moleculaire structuur van de IGFBPs resulteert in een remmend of een stimulerend effect op de IGF bioactiviteit.

IGFBPs blijken vrij algemeen tot expressie te komen; vele cellen uit normaal weefsel of uit tumor-weefsels scheiden diverse IGFBPs uit, alhoewel de relatieve verhoudingen tussen de IGFBPs aanzienlijk kunnen verschillen. De ratio tussen de IGFBPs blijkt afhankelijk van het stadium van ontwikkeling, de hormonale-, metabole-, en pathologische conditie van de cellen/weefsels. In een serie cellijnen verkregen uit borst carcinoma's, Wilms tumoren, neuroblastoma, retinoblastoma, colon carcinoma's, lever adenocarcinoma, Burkitt lymphoma en niet kleincellig long carcinoma, is de secretie en expressie van IGFBP-1,-2 en -3 onderzocht. Alle cellijnen, uitgezonderd de Burkitt lymphoma cellijnen, scheidden diverse IGFBPs uit. IGFBP-1 expressie kon slechts met reverse transcriptase PCR aangetoond worden.

Ondanks de snel toenemende kennis over de IGFBPs is de rol die deze bindende eiwitten in het IGF systeem spelen nog verre van duidelijk. Het lijkt alsof deze eiwitten een belangrijke rol spelen in de regulatie van de biologische beschikbaarheid van IGF. Net als de IGFs zijn de IGFBPs zowel endocrien als paracrien werkzaam. In de circulatie verlengen zij de halfwaarde tijd van de IGFs en tegelijkertijd beschermen zij het lichaam tegen de metabole effecten van vrij IGF. De mogelijkheid van het IGFBP-3/IGF complex om al dan niet een ternair complex te vormen met het ALS eiwit geeft, tesamen met het vermogen van binaire IGFBP/IGF complexen om de capillaire barrière te passeren, een verfijnde controle op de biologische beschikbaarheid en het transport van IGF van en naar de weefsels. Plaatselijk zorgt een dynamisch evenwicht tussen vrij en celgebonden IGFBPs voor een flexibele distributie van IGFs tussen de cellen.
De invloed die IGFBPs in het IGF systeem uitoefenen hebben onweerlegbaar te maken met hun vermogen om IGF-I of -II te binden. Deze voor alle IGFBPs gemeenschappelijke eigenschap is tot nu toe het meest bestudeerd. Tevens zouden de IGFBPs nog directe, IGF onafhankelijke, interacties met de cel kunnen hebben.
De resultaten beschreven in Hoofdstukken IV en V van dit proefschrift geven aan dat de beide sterk geconserveerde uiteinden van het IGFBP-1 eiwit, betrokken zijn bij de

binding van IGF. Echter, niet alleen de overeenkomsten in de primaire structuur van de IGFBPs zijn belangwekkend, maar ook de verschillen. De verschillen tussen de diverse IGFBPs worden met name in het centrale deel van het molecuul aangetroffen. Ook de fysische eigenschappen verschillen. De relatie tussen structuur en fysische eigenschappen is tot nu toe vrijwel onbekend. IGFBP-3, bijvoorbeeld, is het enige IGFBP dat in staat is een ternair complex te vormen met een ander eiwit, het zgn. ALS, waardoor een stabiel depot van geïnaktiveerde IGFs in de circulatie gevormd wordt. De plaats van interactie tussen IGFBP-3 en het ALS eiwit is onbekend. Sommige, maar niet alle, IGFBPs zijn geglycosyleerd, een eigenschap die in verband gebracht is met de associatie van de IGFBPs met het celoppervlak. Het RGD motief in het carboxyluiteinde van IGFBP-1 en -2 zou hierbij een rol kunnen spelen. Het centrale deel van IGFBP-1 is rijk aan proline, glutamine, serine en threonine residuen en daardoor potentieel gevoelig voor protease activiteit; hierdoor kan de halfwaarde tijd van het eiwit beïnvloed kan worden. Sommige serine residuen in dit gebied worden gefosforyleerd, met als gevolg een verandering in de affiniteit van het bindend eiwit voor IGF.

Meer moleculair biologisch en biochemisch onderzoek is nodig om de functionele betekenis van de diversiteit die tussen de IGFBPs bestaat op te helderen. Tot op heden kan men slechts speculeren of deze verschillen de werking van de IGFs reguleren, of dat ze nog onbekende, IGF onafhankelijke eigenschappen bepalen. Verdere studies die gebruik maken van mutant IGFBPs kunnen een beter inzicht geven in de complexe interacties tussen IGFs, hun receptoren en de verschillende IGFBPs.

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