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Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue-specific expression of its mRNA

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The low-molecular-mass insulin-like growth factor-binding protein (IGF-BP) and placental protein 12 (PP12) are identical proteins that are present in human serum, amniotic fluid, secretory endometrium and decidua. IGF-BP/PP12 is believed to act as an autocrine or paracrine regulator of cell growth. A cDNA clone encompassing the entire protein coding region of this protein was isolated from a human decidual cDNA library. The authenticity of the cDNA was verified by in vitro transcription/translation experiments and by the identity of the 10 N-terminal amino acids deduced for the mature peptide with those obtained by direct protein sequencing. The amino acid sequence indicates that pre-IGF-BP/PP12 consists of 259 amino acid residues. The putative signal peptide is 25 residues long, and the mature protein thus contains 234 amino acids and has a molecular mass of 25293 Da. The sequence is very cysteine-rich at the N-terminus after which there are regions of clustered Pro, Glu, Ser and Thr residues (so-called PEST regions), which exist in proteins with short half-lives. The amino acid sequence also includes an Arg-Gly-Asp tripeptide that may function as a cell recognition signal. The IGF-BP/PP12 gene encodes a single 1.6 kb mRNA species that is expressed in decidua, secretory endometrium, liver and a human hepatoma cell line (HepG2). Southern blot analysis suggests that there is a single IGF-BP/PP12 gene in the human genome.

Insulin-like growth factor-binding protein; Placental protein 12; Amino acid sequence; mRNA expression

1. INTRODUCTION

Insulin-like growth factors 1 and 2 (IGF-I and IGF-II) interact with soluble extracellular binding proteins (IGF-BPs) the exact biological functions of which are poorly understood [1]. In man, the main circulating IGF-BP is a growth hormone-dependent macromolecule with a molecular mass of about 150 kDa [2]. Another class of IGF-BP, with molecular mass estimates from 25 to 45 kDa, is present in human serum and amniotic fluid [3,4]. It is also synthesized and secreted by a human hepatoma cell line, HepG2 [5]. A glycoprotein termed placental protein 12 (PP12) was originally isolated from soluble extracts of human placenta and fetal membranes but was subsequently shown to be synthesized in secretory endometrium [6,7], dedicua [8] and granulosa cells [9], but not in the placenta [8]. PP12 is present in serum of healthy men and women, its concentration increases during pregnancy [10] and is apparently regulated by insulin [11]. PP12 binds avidly IGF-I and IGF-II [12]. By all available criteria, including the molecular mass estimation by SDS-polyacrylamide gel electrophoresis, amino acid composition, N-terminal amino acid sequence and ligand-binding properties, PP12 and the smaller IGF-BP are identical [5,12–14]. Here, we use therefore the term IGF-BP/PP12 for this protein. We have isolated a cDNA clone for this protein, determined its com-

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plete amino acid sequence and studied the sites of IGF-BP/PP12 mRNA expression.

2. MATERIALS AND METHODS

2.1. *Preparation of a human decidual cDNA library*

Human decidual RNA was isolated by the LiCl-urea method [15] and enriched in poly(A)$^+$ RNA by two cycles of oligo(dT)-cellulose chromatography [16]. Synthesis of double-stranded cDNA was carried out according to Gubler and Hoffman [17]. The cDNAs were methylated and ligated to *Eco*RI linkers. After cleavage with *Eco*RI, the linker-containing cDNAs were ligated to *Eco*RI-cleaved, alkaline phosphatase-treated λgt11 arms (Statagene, La Jolla, CA). The DNA was packaged in vitro and amplified in *Escherichia coli* Y1088 cells.

2.2. *Screening of the cDNA library*

About 10^5 phage plaques were plated on *E. coli* Y1090 cells and probed [18] with two polyclonal antisera for IGF-BP/PP12 raised in rabbits, 245 II [19] and 461ZA (obtained from Dr Hans Bohn, Behringwerke AG, Marburg, FRG). Five recombinant phage clones which were strongly positive with both antisera were plaque purified and phage DNA isolated [20].

2.3. *DNA sequencing*

The longest cDNA insert was cleaved with *Aval*, *Pst*I, *Acc*I, *Bam*HI and *Hin*cII restriction enzymes, which were found to have unique sites in the cDNA in preliminary restriction mapping analysis. The fragments were subcloned into the corresponding sites of pGEM-3 blue vector. Sequencing of double-stranded DNA was performed by the dideoxynucleotide chain-termination method using Sequenase™ enzyme (US Biochemical Corp., Cleveland, OH) and SP6 and T_7 promoter primers [21,22]. In addition, two internal 20-mer oligonucleotide primers were synthesized to permit unequivocal sequence determination for both strands of the IGF/BP/PP12 cDNA insert.

DNA sequences were aligned and joined using DBSTART, DBUTIL, and DBAUTO programs [23]. Comparison of two sequences was performed using the FASTN and FASTP programs [24]. A hydropathicity plot of the amino acid sequence deduced from the cDNA was constructed with the algorithm described by Kyte and Doolittle [25], using a window of 7 amino acids. Comparison of IGF-BP/PP12 cDNA and deduced amino acid sequences to known protein sequences was performed by searching the currently available data resources.

2.4. *In vitro transcription/translation studies*

The isolated cDNA was cloned in both orientations into the *Eco*RI site of pGEM-3 blue, the chimeric plasmids were linearized with *Sal*I, and capped sense and complementary RNA strands were synthesized using T_7 RNA polymerase in the presence of 5′-7meGpppG-3′ [26]. The RNAs were translated in vitro in a reticulocyte lysate (Bethesda Research Labs, Gaithersburg, MD) with [^{35}S]methionine as the labeled amino acid. Radioactive peptides were analyzed, before and after immunoprecipitation with anti-IGF-BP/PP12 antiserum, on 10% polyacrylamide gels containing 0.1% SDS [27], essentially as described [28].

2.5. *RNA blot hybridization analysis*

Samples of apparently normal human liver were obtained from a female patient undergoing hepatic resection because of hepatocellular carcinoma. Normal human renal and adrenal tissue were obtained from nephrectomies performed for renal cancer. Decidual samples were obtained from women undergoing legal abortions at the 10–12th week of pregnancy and from women undergoing caesarean section at the 40th week of pregnancy. Endometrial tissue was from curettage samples taken for medical reasons. Poly(A)-containing RNA samples (2 μg) were fractionated on 0.6% agarose gels containing 2.2 M formaldehyde [29] and transferred to nitrocellulose filters. Hybridization of the filter-bound RNA with the ^{32}P-labelled IGF-BP/PP12-cDNA and washing of the filters were carried out as described by Thomas [30]. Autoradiography of the dried filters was conducted by exposing them to Kodak XAR film for 1–3 days at −70°C.

2.6. *Southern blotting of genomic DNA*

Human leukocytic DNA was digested with the different restriction enzymes, fractionated by gel electrophoresis on 0.6% agarose and transferred to nitrocellulose filters. Hybridization, washing and autoradiography of the filters were conducted as described above for the RNA blots.

3. RESULTS

Screening of human decidual cDNA library with the two IGF-BP/PP12 antisera yielded five immunopositive clones. *Eco*RI digestion of the plaque-purified phage DNA preparations revealed inserts that ranged from 700 to 1500 base pairs in size and cross-hybridized with each other (not shown). The longest insert of a clone designated IGF-BP/PP12-5 was subcloned into plasmid vectors and used for subsequent studies.

Cell-free translation of the capped RNAs transcribed from both strands of the cDNA showed that each strand supported synthesis of a labeled peptide with molecular masses of about 33 and 20 kDa, respectively (fig.1A). Only the 33 kDa peptide was immunoprecipitable with anti-IGF-BP/PP12 antiserum; the antibody precipitated 100% of this peptide as judged by radioactivity measurements. The same antiserum immunoprecipitated from the labeled translation products of decidual poly(A) RNA a peptide whose mobility on polyacrylamide gel electrophoresis was identical with the 33 kDa peptide encoded by the synthetic RNA (fig.1B). These data provided strong evidence that the cDNA corresponds to IGF-BP/PP12 and contains the entire protein coding sequence.

DNA sequencing indicated that the IGF-

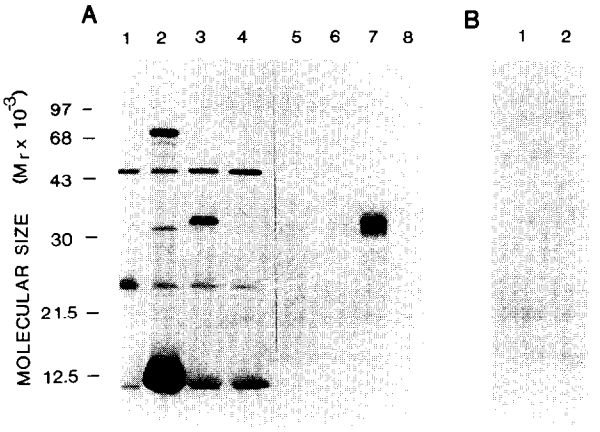

Fig.1. Analysis of cell-free translation products encoded by RNAs transcribed from IGF-BP/PP12 cDNA and by poly(A)-containing RNA from human decidua. Capped RNAs corresponding to both strands of the cDNA were synthesized with T_7 RNA polymerase as described in section 2. The peptides labelled with [^{35}S]methionine were analyzed, before and after immunoprecipitation with IGF-BP/PP12 antiserum, on 10% polyacrylamide gels containing 0.1% SDS. (A) Lanes: 1–5, no exogenous RNA added; 2,6, reticulocyte RNA (1 μg); 3,7, IGF-BP/PP12 RNA (sense strand, 100 ng); 4,8, IGF-BP/PP12 RNA (complementary strand, 100 ng). Lanes 1–4 correspond to 1/5th of the translated peptides before immunoprecipitation and lanes 5–8 to 3/5th of the peptides after immunoprecipitation. (B) Immunoprecipitated products from cell-free translation of decidual poly(A) RNA. Lanes: 1, decidual RNA (1 μg); 2, reticulocyte RNA (1 μg). Exposure times during fluorography were 2 h and 3 days for A and B, respectively.

BP/PP12 cDNA is 1443 nucleotides long and contains an open reading frame of 777 bases encoding a 259-amino-acid-long peptide (fig.2) on the strand that supported cell-free translation of the 33 kDa peptide immunoprecipitable with IGF-BP/PP12 antiserum (fig.1A). The predicted molecular mass of the peptide deduced from the cDNA sequence is 27926 Da. The amino acid contains a 25-residue-long putative signal peptide rich in hydrophobic amino acids (fig.2). The 10 N-terminal amino acids of the mature protein (APWQCAPCSA) are identical with those published for the N-termini of human amniotic fluid IGF-BP [13] and PP12 [14], thus confirming the authenticity of the cDNA clone. The mature peptide has a calculated molecular mass of 25293 Da. The IGF-BP/PP12 peptide is very rich in Cys, Pro, Glu and Asp residues, which may explain its aberrant mobility, even in non-glycosylated form, on SDS-containing polyacrylamide gels; the cell-free translation product has an apparent molecular mass of 33 kDa as opposed to 27.9 kDa deduced from the cDNA. The peptide sequence does not contain recognition sequences for *N*-linked glycosylation (Asn-X-Ser or Asn-X-Thr) but has regions rich in Ser and Thr (e.g. residues 101–102, 131–132, 168–169, 194–195) which could serve as acceptors for *O*-linked carbohydrates.

After the first 80 N-terminal residues of the mature peptide the amino acid sequence of IGF-BP/PP12 contains several regions very rich in Pro, Glu, Ser and Thr residues (fig.2). Clusters of these amino acids are known to be present in proteins with short intracellular half-lives [31], and have been termed PEST regions. Of particular significance is the sequence encompassing residues 89–114 (HAAEAGSPESPESTEITEEELLDNFH) which is a typical PEST sequence in that it contains clusters of the above-mentioned four residues and is flanked with a positively charged amino acid (His) on each side. An Arg-Gly-Asp sequence, which has been shown to serve as a cell attachment site of several proteins [32], is present close to the carboxy-terminal end of the protein (residues 221–223).

The hydropathicity profile of the pre-IGF-BP/PP12 is illustrated in fig.3. The signal peptide region is very hydrophobic as expected. The other relatively hydrophobic region of the protein is also at its N-terminus and covers residues 30–50, which contains a cluster of 5 cysteines. It is tempting to speculate that this part of the peptide is involved in ligand binding. The rest of the protein sequence comprises several very hydrophilic regions that are mainly due to clustered Glu residues.

The 1443-nucleotide-long IGF-BP/PP12 cDNA sequence has 5′- and 3′-noncoding regions of 102 and 564 bases in length, respectively (fig.2). The sequence flanking the initiation codon, GGAG(ATG)T, agrees in part with the consensus sequence of Kozak [33]. A putative polyadenylation signal (AATAAA) starts 13 nucleotides from the 3′-end of the cDNA, i.e. the cloned sequence is missing a few nucleotides between the polyadenylation signal and the poly(A) tail. Whether the GC-rich 5′-noncoding region of the cDNA reaches the cap site of IGF-BP/PP12-mRNA is not known, and primer exten-

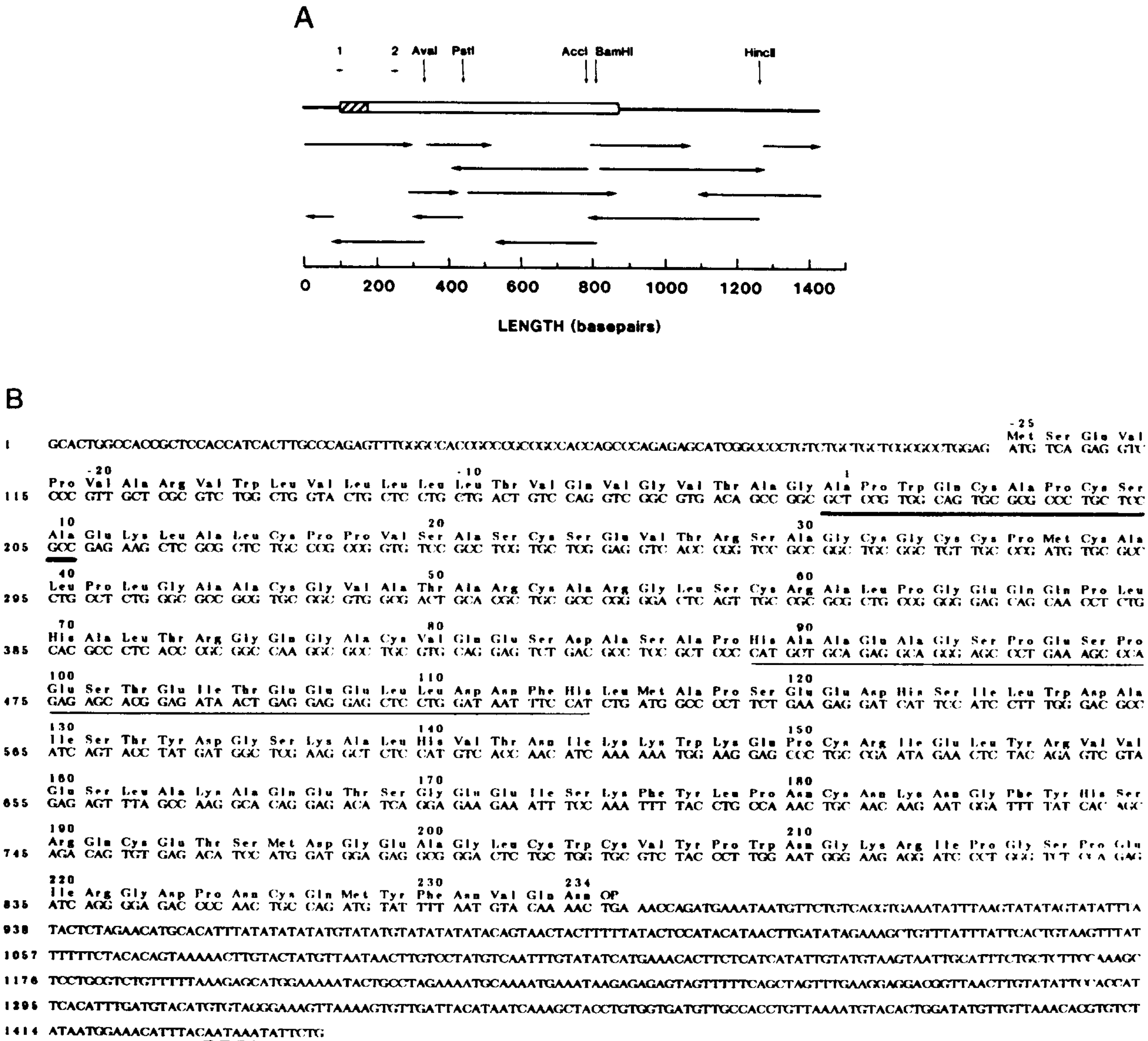

Fig.2. Sequencing strategy and nucleotide and deduced amino acid sequences of IGF-BP/PP12 cDNA. (A) Arrows show the direction and extent of each sequence determination. The locations of the two internal primers (1,2) are depicted by the short arrows. Restriction sites shown in the map are those used for subcloning. (——) 5′- and 3′-nontranslated regions; (▭) the protein-coding sequence with the cross-hatched area corresponding to the signal peptide. (B) Nucleotide sequence and deduced amino acid sequence. The N-terminal amino acid residues of the mature peptide identical with those determined by direct protein sequencing are depicted by a thick line under the sequence. The residues corresponding to the PEST region are identified by a thin line. The poly(A) addition/termination signal (AATAAA) is underlined by a dashed line.

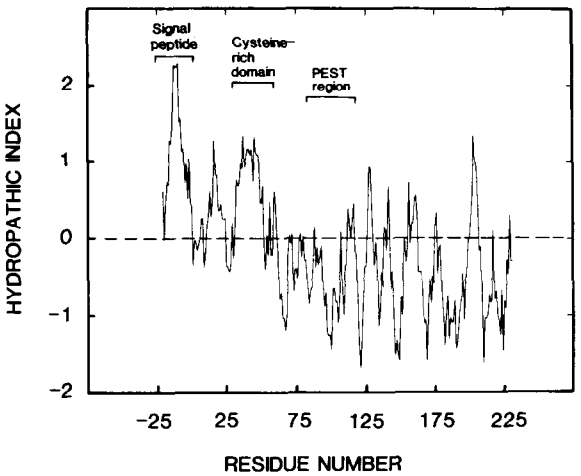

Fig.3. Hydropathicity plot of IGF-BP/PP12 amino acid sequence. The algorithm described by Kyte and Doolittle [25] and a window of 7 amino acids were used to construct the plot. The numbers in the abscissa refer to those of the amino acids, with the amino-terminal residue of the mature peptide being no.1.

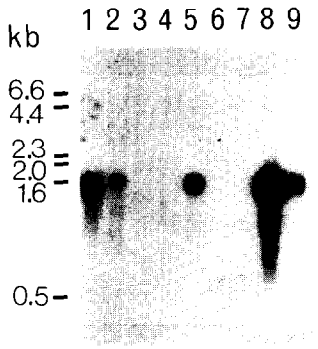

Fig.4. RNA blot hybridization analysis of poly(A)-containing RNA from human tissues. RNA was isolated from the following sources: HepG2 cells (lane 1), liver (2), kidney (3), adrenal (4), secretory endometrium (5), proliferative endometrium (6), placenta (7), late-pregnancy decidua (8) and early-pregnancy decidua (9). RNA samples (2 μg/lane) were fractionated by electrophoresis on 0.6% agarose containing 2.2 M formaldehyde, transferred to nitrocellulose filter and hybridized with the ^{32}P-labelled IGF-BP/PP12 cDNA probe. Molecular size markers are shown on the left.

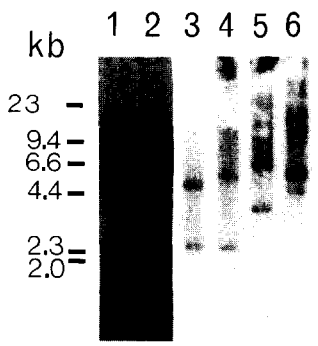

Fig.5. Southern blot analysis of human DNA. Samples of DNA (10 μg/lane) were digested with *Bgl*II (lane 1), *Eco*RV (2), *Pvu*II (3), *Sac*I (4), *Bam*HI (5) and *Eco*RI (6), fractionated by electrophoresis on 0.6% agarose, transferred to nitrocellulose filter and hybridized with the ^{32}P-labelled IGF-BP/PP12 cDNA probe. Molecular size markers are shown on the left (kb = thousands of base pairs).

sion and/or direct RNA sequencing studies remain to be carried out. There are four copies of the sequence motif ATTTA in the 3′-nontranslated region of IGF-BP/PP12 mRNA (fig.2), suggesting that, in addition to the encoded protein, the mRNA also has a short half-life [34].

As mentioned above, the RNA corresponding to the opposite strand of the cDNA also encoded a peptide during cell-free translation that, however, was not immunoprecipitated with IGF-BP/PP12 antiserum (fig.1A). This unexpected finding may be explained by the presence of two overlapping open reading frames on the opposite strand, corresponding to nucleotides 660 ⟶ 109 and 542 ⟶ 1. The first could encode the 20 kDa peptide (fig.1A), as the open reading frame has an initiation Met codon 65 nucleotides from the beginning of its sequence (nucleotides 597–595) and potentially codes for a peptide with 163 amino acid residues.

RNA from a number of human tissues, including liver, kidney, adrenal, endometrium, placenta and decidua, as well as RNA extracted from a human hepatoma cell line (HepG2), were analyzed for the presence of IGF-BP/PP12 mRNA sequences by RNA blot hybridization (fig.4). When present, a single mRNA species with a molecular size of 1.6 kb was found. This mRNA species was most abundant in decidua at term and a considerable amount was also present in decidua of early pregnancy and secretory endometrium; no hybridizable mRNA was found in proliferative endometrium, placenta at term, kidney or adrenal.

HepG2 cells expressed IGF-BP/PP12 mRNA at concentrations clearly higher than those in normal human liver cells.

Southern blot analysis of human leukocytic DNA using the restriction enzymes *Bgl*II, *Eco*RV, *Pvu*II, *Sac*I, *Bam*HI and *Eco*RI revealed the presence of one or two hybridizable DNA fragments with molecular sizes of 2.5–10 kb (fig.5). These data are compatible with the assumption that there is a single IGF-BP/PP12 gene in the human genome.

4. DISCUSSION

In this communication, we report cloning of a cDNA corresponding to the low-molecular mass IGF-binding protein, also known as placental protein 12. The authenticity of the cDNA was verified by cell-free transcription/translation experiments and by the identity of the 10 N-terminal amino acid residues deduced from the cDNA and obtained by direct protein sequencing. A single copy of the IGF-BP/PP12 gene appears to be present in the human genome, and its 1.6 kb mRNA is expressed in uterine cells. However, the mRNA was detectable only in decidua and secretory endometrium but not in proliferative endometrial tissue, suggesting that the expression of this gene is under the control of progesterone in human uterus. This is in agreement with previous studies in which immunoreactive protein concentrations in the uterus were investigated [7,35]. Furthermore, human liver and a hepatoma-derived cell line (HepG2) express IGF-BP/PP12 mRNA, suggesting that at least part of serum PP12 originates from the liver. Several other human tissues, including the placenta, do not express this gene.

Comparison of the IGF-BP/PP12 sequence to other reported protein sequences in most current data resources did not reveal any striking homologies. However, a stretch extending from residue 59 to 79 of IGF-BP/PP12 exhibited a 43% identity with a portion (amino acids 133–153) of human transforming-growth factor α precursor [36], and another stretch from residue 151 to 165 showed a 47% similarity with a region (amino acids 4–18) of human plasma retinol-binding protein [37]. It remains to be investigated whether these limited similarities have any functional implications.

The primary amino acid sequence of IGF-BP/PP12 revealed some interesting features that may be of functional and regulatory importance. First, the peptide is very cysteine-rich, with 11 of the 18 Cys residues being located among the 60 N-terminal amino acids. A hydrophobic domain covering residues 30–50 is particularly abundant in cysteines; the suggestion that this domain is involved in the ligand binding of the protein is in agreement with the previous demonstration that the N-terminal half of the PP12 is responsible for its IGF-I-binding activity [38]. Although the primary structure of IGF-BP/PP12 does not show any apparent similarity with those of the receptors for IGF-I [39] or IGF-II [40], the putative ligand-binding sites of these two other IGF-binding proteins also contain clusters of Cys residues.

Second, the protein contains a number of regions rich in Pro, Glu, Ser and Thr residues; one of these is a particularly typical PEST region (residues 89–114). The presence of this sequence along with the negatively charged nature of the protein suggest that its intracellular half-life is very short [31]. Many other proteins with PEST regions and rapid turnover rates have been shown to possess important intracellular regulatory functions. These include proteins such as c-*myc*, c-*fos*, v-*myb*, heat-shock protein 70 and ornithine decarboxylase and hydroxymethylglutaryl-CoA reductase [31]. Although the intracellular half-life of IGF-BP/PP12 is currently unknown, its fast rate of degradation may be inferred from the findings that the circulating IGF-BP/PP12 levels exhibit a clearcut diurnal variation [41,42]. Finally, in addition to being responsible for the rapid turnover rate of the protein, the hydrophilic C-terminal half could also be the region through which this protein adheres to the cell surface [43], since it includes the Arg-Gly-Asp (RGD) tripeptide known to represent the cell attachment site in proteins such as fibronectin, vitronectin and fibrinogen [32].

The function of the low-molecular-mass IGF-BP has remained poorly understood. While some studies have suggested that IGF-BPs may increase the amount of IGF-I that binds to the IGF receptors [43] and enhance the biologic response to IGF-I [44], other investigations have indicated that these binding proteins may in fact inhibit the interaction of IGF-I with its receptor and thus attenuate its biological function [45,46]. It is possible

that IGF-BPs represent paracrine regulatory proteins whose ultimate physiological effects, whether stimulatory or inhibitory, depend on their concentration, the type of the target cells and the presence of other available growth regulators. The present cloning of a full-length cDNA for IGF-BP/PP12 provides an opportunity to examine the sites and regulation of expression of the IGF-BP/PP12 gene under different experimental conditions, delineate the functional domains of the protein using site-directed mutagenesis, and overexpress the native or mutated proteins in cells that are regulated by insulin-like growth factors.

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