Synthesis of the Blood Circulating C-terminal Fragment of Insulin-like Growth Factor (IGF)-binding Protein-4 in Its Native Conformation

CRystallization, Heparin AND IGf Binding, AND OsteoGeneic ACTIVITY**§§

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Insulin-like growth factor-binding proteins play a critical role in a wide variety of important physiological processes. It has been demonstrated that both an N-terminal and a C-terminal fragment of insulin-like growth factor-binding protein-4 exist and accumulate in the circulatory system, these fragments accounting for virtually the whole amino acid sequence of the protein. The circulating C-terminal fragment establishes three disulfide bridges, and the binding pattern of these has recently been defined. Here we show that the monodimensional 1H NMR spectrum of the C-terminal fragment is typical of a protein with a relatively close packed tertiary structure. This fragment can be produced in its native conformation in Escherichia coli, without the requirement of further refolding procedures, when synthesis is coupled to its secretion from the cell. The recombinant protein crystallizes with the unit cell parameters of a hexagonal system. Furthermore, it binds strongly to heparin, acquiring a well defined oligomeric structure that interacts with insulin-like growth factor-binding proteins, and promotes bone formation in cultures of murine calvariae.

The insulin-like growth factors (IGFs)1 are mitogens produced by many tissues and involved in numerous physiological processes. Their activity is strongly influenced by the IGF-binding proteins (IGFBPs), a family of proteins to which the IGFs bind with high affinity. IGFBPs may directly transport IGFs to different tissues and can either inhibit or enhance IGF activity, depending upon the conditions and form of IGFBP. To date, there are six members of the IGFBP family that share a high degree of sequence similarity in the first and last 100 residues of the N- and C-terminal domains, respectively. A highly variable region of ~30 amino acids separates these two conserved stretches. There are 12–14 conserved cysteines in the N-terminal domain and six in the C-terminal region, each forming internal disulfide bonds (1).

The IGFs are generally found throughout the body at concentrations far higher than those required for maximal stimulation of their quite ubiquitous cell receptors. However, despite their abundance, the effective concentrations of the IGFs are considerably lower in circulation due to their binding to the IGFBPs, with which they associate with a much higher affinity than to the cell receptors that mediate most of their cellular activity. The release of adequate amounts of IGFs, as required at the appropriate sites, is at least in part a consequence of the selective and highly regulated hydrolysis of the IGFBPs associated with them (2–4). This hydrolysis occurs at single or multiple sites in a quite precise fashion through specific and highly regulated protease(s). The sites at which each IGFBP is hydrolyzed are located toward the end of the variable domain, an area rich in basic amino acids. As a consequence, the molecular masses of the fragments produced by these specific proteases are characteristic of each IGFBP, irrespective of the tissue of origin or the species concerned. Indeed, in the case of IGFBP-3 and -4, cleavage sites have also been identified at the beginning of the variable domains (5–7). Furthermore, nerve growth factor seems to display potent protease activity toward IGFBP-4, and IGF-dependent proteolytic activity specific to IGFBP-4 has also been demonstrated in conditioned media from a wide variety of cells such as fibroblasts, osteoblasts, and smooth muscle and neuronal cells.

Recombinant DNA techniques constitute, in general, a more than adequate approach to obtain proteins for a wide variety of studies. However, the synthesis of eukaryotic proteins with a high content of disulfide bonds, such as IGFBP's, is normally extremely difficult. These proteins must be folded correctly, and often, microheterogeneous material is produced in this process. Whereas these preparations are often suitable for physiological studies, they are usually not apt to establish the structural basis of their biological properties at high resolution. Thus, of the two N-terminal fragments of IFGBP-5 that have
been synthesized by recombinant techniques (containing 45 and 104 residues, respectively), only the first was suitable for three-dimensional NMR structural determination, this containing only four Cys residues (8).

Recently, we isolated a C-terminal fragment of IGFBP-4 (CBP4) that extends from Lys136 to Glu237 from human hemofiltrates (9). Furthermore, we defined the pattern of disulfide bonds in this fragment (residues 153–183, 194–205, and 207–228). The stability of this fragment suggests that it is a well-structured domain of the whole protein, since poorly structured proteins are easily hydrolyzed by proteases (10–12). Here we show that high yields of CBP4 in its native conformation can be obtained from Escherichia coli, specifically when its synthesis is coupled to secretion of CBP4 into the periplasm, where protein refolding systems are coupled to thiol oxidation and disulfide isomerase (13–17). In addition, we show that this fragment binds to heparin, acquiring a well-defined oligomeric structure, and, in the presence of heparin, to IGF-I in solution. We also show that CBP4 crystalizes with unit cell parameters of a hexagonal system and that it can promote bone formation in cultures of murine calvariae.

**EXPERIMENTAL PROCEDURES**

*Gene Construction, Cloning, and Protein Purification—*The general procedures for DNA manipulation and cloning were carried out as described previously (18). For reverse-phase purification of recombinant CBP4 (rCBP4) from the periplasm (13–17), the extract was directly loaded onto a C4 analytical column (5-μm particle size, 300-A pore size; The Separation Group) equilibrated in 10 mM trifluoroacetic acid and eluted with a 30-min linear gradient of acetonitrile (0–70%). Fractions showing in SDS-PAGE polypeptides with an Mr value comparable with that of the hemofiltrate-purified CBP4 were subsequently diluted 4 times with 10 mM trifluoroacetic acid, reloaded on the same C18 resin, and eluted with a 40-min linear gradient (0–100%) of acetonitrile/isopropanol alcohol/water mixture (6:3:1). PAGE analysis under reducing conditions of the fractions containing rCBP4 was performed. Then the fractions were pooled together and subsequently loaded onto a 16-mm heparin-Sepharose column equilibrated with 10 mM sodium formiate (pH 4.0) and gradient of 1 M NaCl. The fractions containing rCBP4 were pooled together and subsequently loaded onto a 16-mm heparin-Sepharose column equilibrated with 10 mM sodium formiate (pH 4.0) and gradient of 1 M NaCl. The fractions containing rCBP4 were loaded on the same C18 resin, and eluted with a 40-min linear gradient (0–100%) of acetonitrile/isopropanol alcohol/water mixture (6:3:1).

**PAGE analysis under reducing conditions of the major peak of the second round of chromatography appears in the Supplemental Data. This peak shows an electrophoretic mobility equivalent to that of the hemofiltrate-purified CBP4.**

For large scale protein expression, BL21 cells harboring the pN-III-ompA-2(CBP4) vector were grown at 37°C in a shaking incubator. Protein synthesis was induced with 1 mM isopropyl β-d-thiogalactopyranoside, and the cells were collected by centrifugation 60 h later. The supernatant from 2 liters of culture was reduced to 50 ml by ultrafiltration using a 3-kDa Ultrasette (PALL-FILTRON). This was then loaded onto a 16 × 200-mm heparin-Sepharose column equilibrated with 10 mM sodium formiate, 0.1% trifluoroacetic acid, and eluted with a 40-min linear gradient (0–100%) of a 0.1% aqueous solution of acetonitrile/isopropanol alcohol/water mixture (6:3:1). PAGE analysis under reducing conditions of the major peak of the second round of chromatography appears in the Supplemental Data. This peak shows an electrophoretic mobility equivalent to that of the hemofiltrate-purified CBP4.

**Biophysical and Physicochemical Protein Characterization—*N-terminal amino acid sequencing was carried out in an Applied Biosystems 494 microsequencer. SDS-polyacrylamide gel electrophoresis (under reducing conditions) and staining of the gels was carried out as described (19).** The mass spectra were acquired in a MALDI-TOF Biflex (Bruker) mass spectrometer in the linear mode, previously calibrated with cytochrome c (12361 Da) and myoglobin (16946 Da). Protein samples were prepared in volatile buffers, lyophilized, and dissolved in TA buffer (30% acetonitrile + 0.1% trifluoroacetic acid) saturated with synapinic acid. The samples were applied to the probe and dried in a stream of air at room temperature.

The free SH content of CBP4 was determined by mass spectrometry in a MALDI-TOF Biflex (Bruker). For this purpose, 40-μl samples (protein content 100 μg/ml) were incubated in the presence of 6 μM guanidinium HCl at room temperature for 180 min in 100 mM Tris, pH 8.4, either in the presence or in the absence of 20 mM dithiothreitol in the dark under argon. After 90 min, 2 pl of 4-vinylpyridine was added to the samples. Immediately following the incubation, the samples were desalted using ZipTipC18 (Millipore Corp.) and loaded into the mass spectrometer.

**NMR Spectroscopy—*For 1H NMR studies, the protein obtained from a large scale purification was concentrated to 0.2 mM in a 3-kDa Microsep filter (Filtron). The sample buffer was switched to 10 mM sodium phosphate, pH 7.2, 200 mM NaCl during the centrifugation by successive dilution and concentration in the filter with this solution. D2O was added prior to taking measurements at a ratio of 1:9 of the final sample volume. NMR experiments were performed on a Bruker AMX-600 spectrometer, at 25°C, using sodium 3-trimethylsilyl propionate as an internal reference. Water suppression was carried out by selective presaturation, placing the carrier on the solvent resonance. Conditions for the hemofiltrate-purified protein were the same, except that the concentration of protein was 0.1 mM.

**Analytical Ultracentrifugation—*Short column (70-μl) sedimentation analysis of experiments were carried out at 5°C with a 40-min linear gradient (0–100%) of a acetonitrile/isopropyl alcohol/water mixture (6:3:1). PAGE analysis under reducing conditions of the major peak of the second round of chromatography appears in the Supplemental Data. This peak shows an electrophoretic mobility equivalent to that of the hemofiltrate-purified CBP4.

**Biochemical and Physicochemical Protein Characterization—*N-terminal amino acid sequencing was carried out in an Applied Biosystems 494 microsequencer. SDS-polyacrylamide gel electrophoresis (under reducing conditions) and staining of the gels was carried out as described (19).**

**RESULTS**

**Synthesis of CBP4 in E. coli Cultures—A DNA fragment for the expression of CBP4 in E. coli was initially assembled using the procedure described by Didonato et al. (25). The DNA sequence was optimized to establish an even distribution of unique restriction enzyme target sequences along the gene, avoiding the formation of very stable secondary structures. It was also optimized to produce efficient termination of translation and, whenever possible, incorporate the amino acid codons most frequently used in E. coli. To facilitate the purification of the synthetic polypeptide by affinity chromatography, the DNA fragment was designed to be cloned into a pair of vectors that
add tags to the N terminus of the recombinant polypeptide (26, 27). Furthermore, the DNA fragment was also designed so that these affinity tags could be removed from the recombinant polypeptide by the human rhinovirus 3C protease. The overall structure of this DNA fragment is laid out in Fig. 1.

The construct was cloned into four common expression vectors: pCE17, which produces a fusion protein with the carboxyterminal domain of the major autolysin from *S. pneumoniae* (26); pHT2, a T7 promoter controlled system that adds a His tail to the N terminus of the cloned protein (27); pNIIII-3A (28); and pNIIII-ompA-2 (29). In the pHAT2 and pNIIII-3A vectors, the induction of protein expression only resulted in the formation of inclusion bodies, independent of the temperature used (between 28 and 37 °C). In the case of the pCE17 vector, part of the material produced was soluble when expression was carried out at 28 °C, but cleavage with the rhinovirus 3C protease only yielded the moiety corresponding to the pneumococcal hydrolase. Although this phenomenon was not further investigated, it strongly suggests that the CBP4 moiety failed to adopt an adequate higher order structure and thus was an easy target for nonspecific proteolysis frequently associated with even the most narrow spectrum proteases.

It is generally accepted that the -SH groups of Cys residues are mostly maintained in a reduced state in the cytoplasm of *E. coli* due to the existence of two efficient thiol reducing systems: the thioredoxin and the glutathione/glutaredoxin systems. In the latter case, disulfide bonds are reduced by the thioredoxin reductase enzyme (trxB mutants) can be successfully used to synthesize some eukaryotic proteins as the thioredoxin reductase enzyme (trxB mutants) can be successfully used to synthesize some eukaryotic proteins.

SDS-PAGE analysis under -SH reducing conditions of the periplasmal polypeptide content of cells transfected with the pIN-III-ompA-2 and pIN-III-ompA-2(CBP4) vectors, respectively, showed that a protein accumulated in this compartment in the later case with an M*, that corresponded to that of the CBP4 polypeptide purified from hemolofiltrates (Supplemental Data) (9). Maximal expression of this polypeptide was obtained 24–48 h after the induction of protein expression with isopropyl 1-thio-β-D-galactopyranoside. This polypeptide could be purified from the periplasm preparation by reverse-phase chromatography as detailed under “Experimental Procedures” (see Supplemental Data). N-terminal Edman degradation of this polypeptide yielded two equimolar sequences that corresponded to CBP4, one beginning at Asn<sup>138</sup> of IGFBP-4 and the other at the second Leu residue of the rhinovirus 3C protease recognition sequence (Fig. 1). This analysis also showed that removal of the ompA signal peptide had proceeded beyond its normal processing site (Val-Ala-Gln-Ala; not shown). This is in accordance with a relaxation of the specificity in removing the ompA signal peptide previously reported (15).

Nonreducing SDS-PAGE showed that the purified polypeptide is a monomer (not shown), and two peaks, with an M* of 12105 and 11176, were obtained by mass spectrometry of the final preparation purified by reverse-phase chromatography (not shown). Considering these data in conjunction with data from the Edman degradation, the C-terminal residue of the two rCBP4 forms detected in the periplasm by mass spectroscopy most probably correspond to the Glu of CBP4 (Fig. 1; theoretical M*, 12019 and 11119, respectively). In order to avoid disturbance from the N-terminal tag, a second gene construct was generated in which the triplet coding for Asn<sup>138</sup> was placed at the end of the ompA signal peptide. In this case, a double N-terminal sequence was again identified by Edman sequential degradation of the protein obtained, one beginning at Asn<sup>138</sup> and a second one at Ala<sup>140</sup> (not shown). Unless specifically indicated, the products of the first construct were used in the subsequent experiments reported here.

Analysis of the protein content in the supernatant and in the periplasmatic fraction of cultures induced for 48 h shows that, unlike other proteins, rCBP4 preferentially accumulated in the supernatant (Supplemental Data). Protein accumulation reached a maximum in both the periplasm and the supernatant fractions at 48 h after the induction (not shown). In the case of cultures grown in LB medium, we detected many biochemical products derived from the yeast extract that stick nonspecifically and fairly strongly to the chromatography columns, making large scale purification of rCBP4 feasible from the supernatant of this culture medium. However, despite the poor secretion of proteins fused to the OmpA secretion signal in minimal medium (32), approximately equivalent amounts of rCBP4 were recovered from cells cultured in minimal medium supplemented with an acid hydrolysate of casein (casamino acids) as were obtained from cells in LB medium (data not shown). In agreement with the results reported by Bénéf et al. (32), the secretion of rCBP4 into the medium was extremely low when *E. coli* was cultured in minimal medium alone (not shown).

**Preparative Purification of rCBP4**—Reverse-phase chromatography is not the best method to apply as the first step for large scale protein purification from culture media, even when minimal medium plus casamino acids are used. Many of the biochemical ingredients that irreversibly poison the columns accumulate in the media during fermentation. In addition, the harsh conditions of reverse-phase chromatography may introduce modifications in the protein, making it unsuitable for many biochemical and biophysical studies. The efficiency of other alternative chromatography methods for preparative purification of rCBP4 was therefore explored. The isoelectric point estimated for rCBP4 from its amino acid composition was
incorporation was detected in the samples not treated with CBP4 isolated from the hemofiltrate (9). Since no pyridyl-ethyl were involved in disulfide bond formation, as is the case of the irreversible oxidized state as would be expected if they were in the reversible oxidized state, these results indicate that the six -SH groups of rCBP4 appear to have a reasonably close packed tertiary structure. Furthermore, retention of a considerable number of nucleic acids other than rCBP4 was observed with anionic exchangers.

Surprisingly, although IGFBP-4 is not considered as a heparin binding IGFBP (33, 34), we observed that hemofiltrate-purified CBP4 bound quite strongly to heparin-Sepharose columns, even at its isoelectric point, when it is not retained by other strong cationic exchangers of the sulfonate-derived solid phase type (not shown). This specific affinity of CBP4 toward heparin was therefore used to purify CBP4 from the isopropyl 1-thio-β-D-galactopyranoside-induced liquid cultures of E. coli harboring pIN-III-ompA-2(CBP4) vector. A peak highly enriched in CBP4 was separated by chromatography of culture supernatants with heparin-Sepharose at neutral pH (Fig. 2, A (top plot, fraction C) and B (lane C)). From this, a fairly homogeneous preparation of rCBP4 could be extracted by a subsequent chromatography with SP Sepharose at pH 4 (Fig. 2, A (middle plot, fraction E) and B (lane E)). The remaining CBP4 included in the tail of peak E (Fig. 2A) could then be recovered by subsequent chromatography with heparin-Sepharose at neutral pH (Fig. 2, A (bottom plot) and B (lane I)). The overall yield of the procedure, by pooling together fractions E and I (Fig. 2A), was ~8 mg of rCBP4/liter of culture. When culture volumes above 1 liter were used for purification, an ultrafiltration step was carried out before performing the first chromatographic purification (see “Experimental Procedures”).

In order to assess the native folding of rCBP4, mass spectrometry was initially used to determine the free SH content of the polypeptide produced above. This analysis showed that the addition of 4-vinylpyridine altered the mass spectrum of the sample when it had been incubated in the presence of dithiothreitol (Fig. 3). Furthermore, the increment of 637 mass units/charge in M0 observed corresponded quite well to the incorporation into the polypeptide of six pyridyl-ethyl groups. Thus, these results indicate that the six -SH groups of CBP4 appear in the reversible oxidized state as would be expected if they were involved in disulfide bond formation, as is the case of the CBP4 isolated from the hemofiltrate (9). Since no pyridyl-ethyl incorporation was detected in the samples not treated with dithiothreitol, no free SH groups appear to exist in the rCBP4 polypeptide prepared by this procedure (Fig. 3).

1H NMR Spectra of Recombinant and Hemofiltrate-purified CBP4—The absence of a clearly identifiable heparin-binding consensus sequence in CBP4 suggests that the region of the polypeptide responsible for its affinity is the consequence of the protein adopting its native conformation. In this way, residues that may be separated in the primary structure may be capable of mediating heparin binding once correct folding has taken place. Thus, the similar affinity for heparin of the hemofiltrate-purified CBP4 and rCBP4 strongly suggests that both polypeptides adopt a similar, well defined, three-dimensional structure. The involvement of the six Cys residues of rCBP4 in establishing intramolecular disulfide bonds also indicates that the recombinant protein folds in the same way as the hemofiltrate-purified protein (9). To gain some insight into the structural features of the recombinant and the hemofiltrate-purified CBP4 and to investigate whether both polypeptides show equivalent and specific three-dimensional structures, 1H NMR spectroscopy was used.

The partial monodimensional 1H NMR spectra of the recombinant and hemofiltrate-purified CBP4 obtained is shown in Fig. 4. The presence in these spectra of some very high field signals (δ < 0.8 ppm), and the large signal dispersion in the NH region (~9.5–6.0 ppm) clearly indicate that both polypeptides have a reasonably close packed tertiary structure. Furthermore, the strong similarity between the two spectra also implies that the purified recombinant and the hemofiltrate protein adopt a similar three-dimensional structure. Preliminary two-dimensional NOESY spectra of the recombinant and hemofiltrate-purified CBP4 were also examined. The clustering of the cross-peaks in the fingerprint region of the CH dimension (~4.6–3.5 ppm) suggests the predominance of α-helical components in the secondary structure of this protein (data not shown). Finally, it is worth noting that the width of the NMR signals agrees well with the particle size (a monomer of CBP4) sedimented in analytical ultracentrifugation experiments,
where the protein concentration was on the same order as that used for the $^1$H NMR experiments (see below).

**Crystallization of rCBP4**—Crystallization requires homogeneous solutions of protein with a well defined and reasonably compact three-dimensional structure (35). To confirm the compact structure suggested by the $^1$H NMR studies, attempts were made to crystallize the rCBP4 protein. Reproducible crystals (maximum dimensions $-0.2 \times 0.2 \times 0.02$ mm) were obtained in sitting drop CryoChem screening plates at pH 8.0 in a solution of 30% polyethylene glycol 6 kDa (0.1 M Tris-HCl; Fig. 5A). A set of diffraction images of one of such crystal collected under synchrotron radiation (Hamburg-DESY BW7A beamline) showed that these crystals diffracted up to 2.3 Å (Fig. 5B). The data obtained from these showed that there was twinning along the long axis of the crystal. According to the MOSFLM indexing method (36), the crystals belong to the hexagonal system, the approximate unit cell parameters being as follows: $a$ and $b$, 44 Å; $\alpha$, $\beta$, and $\gamma$, 90, 90, and 120° ($c$ could not be accurately estimated because of the twinning).

**Binding of rCBP4 to Heparin**—The capacity to perform heparin-Sepharose chromatography suggests that CBP4 specifically binds to this glycosaminoglycan, particularly considering that it is retained at pH values close to its theoretical isoelectric point and that it does not bind appreciably to other common cation exchange chromatographic media. Therefore, the interaction of rCBP4 with heparin was further characterized by analytical ultracentrifugation. In the absence of heparin, rCBP4 (42 $\mu$m) sedimented as a single species with a $bM_w$ of $\sim3400$ (Table I). This value is compatible with that theoretically calculated for the rCBP4 monomer from its amino acid composition and its partial specific volume ($bM_w = 3431$). At concentrations higher than 200 $\mu$m, the sedimentation profiles clearly indicated that rCBP4 began to form oligomers (data not shown). Thus, in order to avoid interference due to this effect, the interaction of rCBP4 with heparin was characterized using concentrations of around 40 $\mu$m.

The addition of $-3000$-Da heparin ($bM_w = 1470$) to the rCBP4 solution at a molecular ratio of 1:1 drastically modified the sedimentation behavior of the recombinant protein. The gradient was much steeper than that of rCBP4 alone and corresponded to a $bM_w$ of $\sim11,000$ (Table I). This value is compatible with the formation of a complex between three molecules of rCBP4 and one of heparin. Accordingly, a change in the molar ratio of rCBP4:heparin to 3:1 did not modify the $bM_w$ of the protein in the mixture, in support of this proposed stoichiometry (Table I). Moreover, the buoyant molar mass of the complex was not affected by a 10-fold molar excess of heparin (Table I), additionally suggesting that rCBP4 adopts a well defined quaternary structure when it binds to heparin. Furthermore, these data point to the existence of cooperative effects in the formation of the complex.

When $-3000$-Da heparin was substituted by $-6000$-Da heparin ($bM_w = 2940$), the analysis of the sedimentation equilibrium gradients of the rCBP4:heparin mixtures at molar ratios of 1:1 yielded a $bM_w$ value of $\sim20,000$ (Table I). This value suggests the formation of complexes involving five polypeptide units per heparin molecule instead of the six expected. Taking into account the possibility of an accumulation of errors in the calculations, the experiment was repeated at rCBP4:heparin ratios of 6:1. The $bM_w$ of $\sim20,000$ observed in these experiments clearly precludes the coexistence in the solution of unbound rCBP4 and 5:1 complexes of protein-heparin and instead

**FIG. 3.** Mass spectrometry and free SH content determination of rCBP4. The green plot is the spectrum of the untreated rCBP4 preparation. The red plot is the spectrum of rCBP4 treated with 4-vinylpyridine after a 90-min incubation in the presence of 6 M guanidinium HCl. The blue plot is the spectrum of rCBP4 treated with 4-vinylpyridine after a 90-min incubation in the presence of 6 M guanidinium HCl and 20 mM dithiothreitol. The rCBP4 preparation yielding a double sequence beginning at Asn$^{138}$ and Ala$^{149}$ (theoretical $M_w$, 11,125 and 10,954, respectively) was used in the experiment shown. Equivalent results were obtained with the higher $M_w$ forms of rCBP4.

**FIG. 4.** Selected regions of the one-dimensional $^1$H NMR spectra of rCBP4 (top) and hemoflate-purified CBP4 (bottom).

### TABLE I

| Solution content (molar ratio between the different components) | $bM_w \pm 2$ S.D.$^a$ |
|---------------------------------------------------------------|---------------------|
| rCBP4$^b$                                                   | 3430 ± 306          |
| rCBP4, heparin$^c$ (1:1)                                      | 11,082 ± 434        |
| rCBP4, heparin$^c$ (3:1)                                      | 10,998 ± 725        |
| rCBP4, heparin$^c$ (1:10)                                     | 10,989 ± 409        |
| rCBP4, heparin$^c$ (6:1)                                      | 19,557 ± 195        |
| rCBP4, heparin$^c$ (6:1)                                      | 20,422 ± 200        |
| rCBP4, MIHS (1:10)                                           | 4219 ± 488          |
| rCBP4, heparin$^c$, MIHS (1:1:71)                             | 4233 ± 420          |
| rCBP4, heparin$^c$, MIHS (1:1:10)                             | 5439 ± 343          |
| rCBP4, IGF-I$^d$ (1:1)                                       | 2621 ± 224          |
| rCBP4, IGF-I, heparin$^c$ (3:3:1)                             | 8305 ± 313          |
| IGF-I                                                        | 2143 ± 057          |
| IGF-I, heparin$^c$ (1:1)                                     | 2184 ± 098          |
| IGF-I, MIHS (1:10)                                           | 2424 ± 299          |
| rCBP4, IGF-I, MIHS (1:1:10)                                   | 5558 ± 389          |

$^a$ Sedimentation profiles are included in the Supplemental Data.

$^b$ 42 $\mu$m in all cases.

$^c$ Heparin $M_w$ = 3000.

$^d$ Heparin $M_w$ = 6000.
suggests that all of the polypeptide is bound to heparin at a ratio of 6:1. Heparin did not show any tendency to self-associate in solution, and equivalent results were obtained using CBP4 isolated from the hemo filtrate (data not shown). Because of its complexity, a more detailed characterization of the energetics of the association between rCBP4 and heparin is currently under way, taking advantage of a combination of different biophysical approaches.

MIHS has been reported to substitute functionally for heparin in certain circumstances, and it is a compound with a simpler and better defined chemical structure than heparin (37). Indeed, MIHS has enabled certain high resolution structural consequences of heparin binding to be defined for some proteins (38). When rCBP4 was incubated in the presence of a 10-fold molar excess of MIHS, the protein sedimented as a single molecular species with a \( bM_w \) compatible with one molecule of rCBP4 being complexed with two molecules of MIHS (monomer \( bM_w = 360 \); Table I). The number of ligands per rCBP4 molecule does not apparently increase when the protein is incubated in the presence of a 30-fold molar excess of MIHS (data not shown). Decreasing the molecular ratio of MIHS to rCBP4 to 1:1 did not cause any aggregation of the sedimenting particles through the binding of several polypeptide units to a single MIHS molecule (data not shown). Disruption of the rCBP4-heparin complex by MIHS provided additional evidence that this compound does interact with rCBP4. Thus, when 3 mM MIHS was added to a mixture of rCBP4-heparin (42 \( \mu \)M; molar ratio 1:1), the sedimenting particle showed a \( bM_w \) of \(~\sim~\)2600 (Table I), a value clearly different to that obtained when MIHS is omitted (\( bM_w = 11,082 \); Table I). However, the \( bM_w \) observed when the concentration of MIHS in the mixture was decreased could have derived from the coexistence in the solution of rCBP4-heparin and rCBP4-MIHS complexes (Table I).

Interaction of IGF-I with rCBP4 Complexed with Heparin and with MIHS—When CBP4 was first described, it was shown to bind very weakly to IGFs by surface plasmon resonance spectroscopy (9). This interaction of CBP4 with IGF-I was reassessed in solution by analytical ultracentrifugation. The sedimentation equilibrium of a mixture of 42 \( \mu \)M rCBP4 and IGF-I indicated the sedimentation of a single species with a \( bM_w \) of \(~\sim~\)2600 (Table I). This result indicates that IGF-I (monomer \( bM_w = 2143 \)) and rCBP4 (\( bM_w = 3430 \); Table I) do not associate in solution. A similar behavior was observed when different concentrations of rCBP4 and IGF-I were mixed, ranging between 40 and 100 \( \mu \)M. Higher concentrations were not tested because ultracentrifugation analysis of rCBP4 alone showed that this polypeptide forms oligomers at concentrations above 200 \( \mu \)M. The failure to associate in solution of elements that interact relatively weakly in solid phase assays has been repeatedly reported (39–41).

Interestingly, the sedimentation equilibrium of a mixture of rCBP4 and 3000-Da heparin (molar ratio = 3:1) was clearly altered when the experiments were carried out in the presence of IGF-I (rCBP4/IGF-I molar ratio 1:1). This ternary mixture sedimented as a single sedimenting species with a \( bM_w \) of \(~\sim~\)8300 instead of \(~\sim~\)11,000 when IGF-I was omitted (Table I). The value of \(~\sim~\)8300 cannot be interpreted as the result of the average of the buoyant masses of the rCBP4-heparin complex and IGF-I (\(~\sim~\)11,000 and \(~\sim~\)2100, respectively) (Table I). Hence, this suggests that some sort of interaction occurs between the rCBP4-heparin complex and IGF-I that elicits the formation of new states of association. No appreciable interaction was observed between IGF-I and heparin in the absence of rCBP4 (Table I).

We also examined whether MIHS can promote the formation of complexes between rCBP4 with IGF-I. No appreciable interaction was detected between IGF-I and MIHS when 42 \( \mu \)M IGF-I was incubated in the presence of an \(~\sim~\)10-fold higher concentration of MIHS (Table I). Nevertheless, the incubation of an equimolar solution of rCBP4 and IGF-I (42 \( \mu \)M; \( bM_w = 2621 \)) in the presence of 500 \( \mu \)M MIHS yielded a complex whose \( bM_w \) was \(~\sim~\)5600. This finding strongly suggests that rCBP4 binds to IGF-I in the presence of MIHS, and the \( bM_w \) observed is compatible with a complex rCBP4-IGF-I-MIHS with \(~\sim~\)1:1:1 stoichiometry (\( bM_w = 3430 + 2143 + 360 \)). As in the case of the rCBP4-heparin association described previously, a more detailed analysis of the energetics of this association is currently being performed and will be the subject of a separate study.

Stimulation of Bone Formation in Vitro by rCBP4—It has recently been shown that systemic administration of IGFBP-4 promotes bone formation by enhancing the bioavailability of IGF through a mechanism dependent on the IGFBP-4 protease (42). Thus, it is clear that this hydrolysis will also increase the local concentration of the two IGFBP-4 fragments. The affinity of rCBP4 for sulfated glycosaminoglycans and its ability to bind IGF-I when associated with such heparin-like compounds suggest that rCBP4 could concentrate IGFs in the cell matrix, proximal to their cell membrane IGF receptors. Indeed, similar mechanisms have been described for other growth factors, considerably enhancing their signaling efficiency (43, 44). Thus,
we tested whether CBP4 might influence bone growth in a recently developed in vitro assay of calvarial bone formation (24, 45), since the bone osteoblasts synthesize and secrete both IGF-I and IGF-II into the culture media, in the assay conditions (46).

The addition of rCBP4 to calvariae clearly promoted the formation of new bone, as seen in histological sections from these bone cultures (Fig. 6). This effect was maximal at a concentration of ~400 pg/well and decreased at higher concentrations. Histological examination of calvariae treated with 400 pg/well of rCBP4 revealed a large population of mesenchymal cells in the osteogenic layer of the bone periosteum and endosteum. As a result, this layer adopted an irregular appearance characteristic of the intense stimulation of osteogenic activity in osteocytes not accompanied by an equivalent stimulation of osteoclast remodeling activity (Fig. 6C). Micrographs of sections of calvariae treated with 400 pg/well of rCBP4, using two complementary staining techniques, and at higher magnification, further illustrates those morphological features (Fig. 7). Abundance in spiculae, cuboidal (active) osteoblasts and osteocytes, the latter often enclosed in lacunae, as a consequence of new bone deposition in the osteogenic layers are readily observable in these micrographs. New bone formation is especially evident in the hematoxylin and eosin-stained micrograph (Fig. 7A). The new bone was woven and well mineralized, as judged by Masson’s trichrome staining (Fig. 7B). Some of these features, typical of active osteogenesis, were still evident in cultures treated with 1000 and 2500 pg/well, but were not observed in the vehicle-treated bone in which the cells maintained a flattened histological shape, typical of an inactive osteogenic layer (Fig. 6). The quantitative analysis of total area of new bone and the number of osteoblast cells in the calvarial cultures, in the presence and absence of rCBP4 at doses between 100 and 2500 pg/well (four bones per experiment; three independent experiments), revealed a significant induction of bone formation by 400 pg rCBP4 per well. At this concentration, rCBP4 induced a 2.7-fold increase in the cell density and a 3-fold increase in the new bone area of 2.22 mm²/mm² of bone when exposed to rCBP4; a new bone area of 16.47 ± 5.75 mm²/mm² of bone in the presence of rCBP4; p < 0.001 in both measurements).}

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**Fig. 6.** Effects of different concentrations of rCBP4 on cultures of murine calvarial bones after 72 h in culture. Calvarial bones were treated with vehicle (A) or 100 (B), 400 (C), 1000 (D), or 2500 (E) pg/well of rCBP4, respectively. The coronal sections were stained with Masson’s trichrome. b, bone material; e, endosteum; p, periosteum. Bar, 313 μm.

**Fig. 7.** High magnification optical micrographs of coronal sections of calvarial bones treated with 400 pg/well after 72 h in culture. The sections were stained with either hematoxylin and eosin (A) or Masson’s trichrome (B). co, cuboidal osteoblast; lc, lacunae; nb, new bone material; o, osteocyte; sp, spicula. Bar, 6 5 μm.
above in calvariae treated with 1000 and 2500 pg/well of recombinant rCBP4, the differences were not statistically significant when compared with the control samples (p ≥ 0.05). We cannot readily explain this bell shape pattern of the proliferative response observed in the calvarial bone cultures at increasing rCBP4 concentrations. However, this type of observation is not uncommon among in vitro proliferation tests carried out with other growth factors (47).

**DISCUSSION**

The C- and N-terminal fragments of IGFBP-4 isolated from human hemofilters encompass practically the entire primary structure of the native protein, and probably represent two subdomains of its three-dimensional structure (9). We report here that the C-terminal fragment shows physical and chemical features typical of a polypeptide with a reasonably well-defined three-dimensional structure. We also show that this C-terminal domain can be synthesized in its native conformation in E. coli, provided that the nascent polypeptide is exposed to the bacteria's periplasmic protein-refolding machinery. Despite their physiological relevance, IGFBPs are still poorly characterized at the high resolution structural level. The synthesis of rCBP4 in a native conformation in conditions that are compatible with high resolution structural studies, reported here, constitutes a step forward in the structural characterization of IGFBPs.

Recombinant IGFBP-4 has previously been synthesized in E. coli (48), in an expression system in which one of the two thiol-reducing systems of the bacteria, normally suppressed to help eukaryotic proteins to fold correctly inside the cell, was simultaneously overexpressed (thioredoxin) (30, 31). Although the disulfide bonding pattern could not be fully determined, the recombinant protein displayed affinity toward IGF-I similar to the protein secreted by rat cells. As we report under "Results," rCBP4 was unable to refold properly within the cytoplasm of the bacteria, even given that the -SH reducing conditions were less efficient than those used for the full protein (30, 31, 48). A three-dimensional structure of the last 80 C-terminal residues of IGFBP-6 was recently reported (49), based on NMR spectroscopy of a refolded polypeptide that includes a 27-residue vector-encoded leader sequence unrelated to IGFBP-6. These additional residues did not seem to adopt a defined secondary structure but were necessary to maintain the polypeptide in solution (49, 50). They probably emulate structural elements corresponding to the first 30 residues of rCBP4, which seems to adopt a relatively compact structure. The resistance of the rCBP4 polypeptide to the unspecific hydrolytic activity of the proteases that remove the ompA signal peptide and to those that unavoidably accumulate in the media in the 60 h that the E. coli are cultured to produce rCBP4 is evidence of this compact structure (10–12, 15). Three-dimensional characterization of this polypeptide should help elucidate the structure of the complete C-terminal domain of the IGFBPs generated by their specific proteases, that in some cases acquire novel biological properties (6, 51). Nevertheless, the rCBP4 is probably going to be more accessible to proteolysis than that of the whole family, because of its peculiar affinity for heparin and IGF-I reported here. Actually, the structure reported for the C-terminal fragment of IGFBP-6 suggests that IGF-II and heparin partially share a binding site, which could explain the inhibition of IGF-II binding to IGFBP-6 in the presence of glycosaminoglycans (49). A surprisingly large number of residues of the C-terminal IGFBP-6 fragment (at least 30 of 80) seem involved in IGF-II binding, according to NMR data (reversible disappearance of 15N-2H HSQC peaks and significant increases of the spin-spin R2 upon the addition of the ligand) (49). The appearance of affinity of rCBP4 toward IGF-I in the presence of heparin, we show, is not easily explained on the basis of such data, a task additionally complicated by the peculiarities of the rCBP4 binding to heparin, which are not straightforwardly enlightened, either, by the reported IGFBP-6 structure (49).

The stoichiometry of the interaction between rCBP4 and heparin detected here is an unexpected feature of this polypeptide. IGFBP-4 has never been included in the group of IGFBPs with affinity for heparin; nor, as far as we know, has it been detected in the extracellular matrix. Accumulation in the extracellular matrix is a typical feature of polypeptides that associate with heparin due to their affinity for the sulfated glycosaminoglycans of this extracellular structure (34, 52). Therefore, the affinity for heparin of CBP4 shown here suggests that proteolysis of IGFBP-4 by its specific proteases unmask a heparin binding site within the C-terminal domain. Interaction of IGFBPs with heparan sulfate proteoglycans of the cell matrix was first demonstrated for IGFBP-5 (34), where it was shown to depend on the motifs 133–140 and 205–212 in its primary structure (33). This motif at 205–212 was subsequently found in IGFBP-3 (residues 209–216) and IGFBP-6 (residues 172–178). These stretches are characterized by a high density of positive charges (34). Nevertheless, this consensus sequence does not seem to be essential for all IGFBPs to associate with sulfated glycosaminoglycans. Whereas IGFBP-2 can also bind to heparin, it does not contain any canonical IGFBP heparin binding motifs in its primary structure, and these sequences are also absent in IGFBP-4.

Binding of IGFBP-2 to sulfated glycosaminoglycans differs from that of the rest of IGFBPs in that it requires the binding of IGF, a polypeptide with no special affinity for heparin (53). By complexing with IGF, a heparin-binding domain in IGFBP-2 becomes accessible in a similar way that proteolysis reveals a heparin-binding domain in IGFBP-4. Since neither of these polypeptides (IGFBP-2 and IGFBP-4) includes sequences with a high density of positive charges in their primary structure, a different determinant of affinity must be present. It is possible that as in other proteins, like acidic fibroblast growth factor, the heparin binding domain of CBP4 and IGFBP-2 results from the three-dimensional folding of the protein (37, 54). To determine whether the appearance of this heparin binding domain in CBP4 is a consequence of protein folding or merely the unmasking of a hidden face will require considerable progress in resolving the structure of IGFBP-4 at high resolution. It is also noteworthy that whereas IGFBP-2 acquires affinity toward heparin upon IGF binding, IGFBP-4 acquires affinity for IGF upon binding heparin.

According to our data, the binding of CBP4 to heparin does not appear to involve indiscriminate oligomerization between compounds of opposite charges. Rather, it involves a precise interaction in which 3-kDa stretches of heparin nucleate the incorporation of a precise numbers of CBP4 units. Moreover, an excess of heparin does not lead to the dissociation of the complex. Such features suggest that a degree of cooperativity probably exists in forming the complex. Ultracentrifugation experiments show that, in solution, CBP4 by itself has a certain propensity to form polydisperse oligomers. However, oligomerization induced by heparin does not appear to represent the mere displacement of the oligomerization equilibrium of CBP4 in solution, since the bMw of the assembled oligomers is well defined and depends on the size of the heparin monomer that induces oligomerization. Although the N-terminal fragment of IGFBP-4 conserves some affinity for IGF, it seems likely that the C terminus will also be involved in IGF binding, since the affinity of the isolated N terminus is considerably lower than that of the whole protein (9). To decipher what the relationship
is between the contribution of the C terminus to the binding of IGFBP-4 and the affinity of IGFBP-4 in the presence of heparin reported here will probably require structural studies to be carried out at atomic resolution. IGFBPs have previously been envisaged as passive and indirect endocrine elements. Nevertheless, in recent years, our understanding of IGFBPs has progressed, and there is even increasing evidence that each IGFBP might have its own biological activities, beyond its ability to regulate IGF availability. Thus, growth-promoting activities have been attributed to several members of the IGFBP family, they can inhibit mitogenesis driven by other growth factors, and some may even be found in the nucleus (51). Furthermore, some unexpected physiological effects of the IGFBP fragments were reported recently that could account for some of the IGF-independent effects of intact IGFBPs (6, 51). Indeed, the influence of rCBP4 on cultured calvariae reported here could also be added to this list. It is evident that several mechanisms may account for the growth-promoting effects of rCBP4 described in this study. For example, rCBP4 could act to concentrate IGF in the extracellular matrix within the neighborhood of their cell receptors. This was a rational basis of our search for a growth-promoting effect of rCBP4 in calvarial cultures. Obviously, the experiments reported here do not exclude more direct growth-promoting activities of rCBP4. In any event, the proteolysis of IGFBP-4 may have a dual effect on bone formation, both increasing the availability of IGF and unmasking an osteogenic activity of CBP4. Finally, because of its affinity for sulfated glycosaminoglycans, CBP4 may constitute a component of the extracellular matrix signaling system responsible for integrating the cell within the framework of the physiology of the whole organism (55).

In conclusion, the information presented here may open new avenues to understand the physiology of IGF more thoroughly. Furthermore, these data may shed light on the molecular basis underlying this system and aid future high resolution structural studies.

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REFERENCES

1. Oei, G. T., and Boisclair, Y. R. (1999) in Contemporary Endocrinology: The IGF System (Rosenfeld, R., and Roberts, C., eds) pp. 111–139, Humana Press, Inc., Totowa, NJ
2. Lassarre, C., and Binoux, M. (1994) Endocrinology 134, 1254–1262
3. Blat, C., Villard, J., and Binoux, M. (1994) J. Clin. Invest. 93
4. Cohen, P., Pfehl, D. M., Lamson, G., and Rosenfeld, R. G. (1991) J. Clin. Endocrinol. Metab. 73, 401–407
5. Maile, L. A., Crown, A. L., and Holly, J. M. P. (1999) in Contemporary Endocrinology: The IGF System (Rosenfeld, R., and Roberts, C., eds) pp. 633–649, Humana Press, Inc., Totowa, NJ
6. Bineux, M., Lalou, C., and Mosheni-zadch, S. (1999) in Contemporary Endocrinology: The IGF System (Rosenfeld, R., and Roberts, C., eds) pp. 281–313, Humana Press, Inc., Totowa, NJ
7. Oei, G. T., and Boisclair, Y. R. (1999) in Contemporary Endocrinology: The IGF System (Rosenfeld, R., and Roberts, C., eds) pp. 281–313, Humana Press, Inc., Totowa, NJ
8. Oei, G. T., and Boisclair, Y. R. (1999) in Contemporary Endocrinology: The IGF System (Rosenfeld, R., and Roberts, C., eds) pp. 281–313, Humana Press, Inc., Totowa, NJ
9. Oei, G. T., and Boisclair, Y. R. (1999) in Contemporary Endocrinology: The IGF System (Rosenfeld, R., and Roberts, C., eds) pp. 281–313, Humana Press, Inc., Totowa, NJ
