Characterization of the binding defect in insulin-like growth factor binding protein-3 from pregnancy serum

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During pregnancy, insulin-like growth factor (IGF) binding protein-3 (IGFBP-3) undergoes proteolysis, rendering it undetectable by radioligand binding techniques. This study examines the physical and functional defect in pregnancy IGFBP-3. Ternary complex formation has been measured by the binding of the acid-labile subunit of the circulating IGFBP-3 complex, which also requires IGF-I or IGF-II binding. IGF-depleted pregnancy IGFBP-3, prepared by size-exclusion chromatography at low pH, could not form a ternary complex in the presence of [¹²⁵I]IGF-I or of an IGF-I analogue extensively altered in the A-domain, whereas analogues altered in the C- or D-domains complexed as well as native IGF-I. After purification by immunoaffinity chromatography, non-pregnancy and pregnancy

IGFBP-3 formed ternary complexes with IGF-I equally well, although the pregnancy-proteolysed protein appeared degraded to ~30 kDa. On analysis by affinity labelling, cross-linked ternary complexes containing non-pregnancy or pregnancy IGFBP-3 were predominantly 135–140 kDa, with an additional complex of 110–115 kDa in the pregnancy preparation. After reverse-phase h.p.l.c., affinity-isolated pregnancy IGFBP-3 was inactive, whereas the protein from non-pregnancy serum retained activity. Thus pregnancy-proteolysed IGFBP-3 is altered in its specificity for IGF analogues, and is more labile than non-pregnancy IGFBP-3, but shows little impairment in normal IGF binding or ternary complex formation.

INTRODUCTION

During human pregnancy, the total circulating level of insulin-like growth factors (IGFs) increases, the increase being predominantly due to an elevation in IGF-I (Wilson et al., 1982; Hall et al., 1984). When detected by radioimmunoassay, the concentrations of the two proteins with which IGFs form ternary complexes in serum, i.e. the IGF binding protein IGFBP-3 and the acid-labile subunit (ALS), also increase as gestation progresses (Baxter and Martin, 1986; Baxter, 1990; Suikkari and Baxter, 1992). The size distribution of IGFs, IGFBP-3 and ALS, determined by gel chromatography, does not differ between pregnancy and non-pregnancy serum samples, with the majority of IGFs and IGFBP-3 being found at approx. 140 kDa, the expected size of the ternary complex (Davies et al., 1991a; Gargosky et al., 1991; Suikkari and Baxter, 1992). Circulating IGFs are believed to be stabilized when in this complex, with an estimated half-life of 12–15 h compared with 10–12 min in the uncomplexed state (Guler et al., 1989).

It has been reported recently that IGFBP-3 in human pregnancy serum, while measurable by radioimmunoassay, cannot be detected by ligand blotting after SDS/PAGE, a technique which readily detects the protein in non-pregnancy serum (Giudice et al., 1990; Hossenlopp et al., 1990). A similar phenomenon is seen with rodent serum (Davenport et al., 1990; Fielder et al., 1990; Gargosky et al., 1990) while in porcine serum, IGFBP-3 remains detectable by ligand blotting throughout pregnancy (McCusker et al., 1989). The apparent disappearance of IGFBP-3 during pregnancy has been attributed to the presence of a cation-dependent serine protease (Giudice et al., 1990; Hossenlopp et al., 1990; Davies et al., 1991a) which has also been reported to be present in critically ill patients and in growth-hormone-insensitive subjects (Davies et al., 1991b; Cotterill et al., 1992).

Since IGFBP-3 from pregnancy serum has been shown to form the ternary complex quite readily in the presence of endogenous or exogenous IGFs, implying that IGFs were still able to bind (Suikkari and Baxter, 1991), the question arose as to whether the binding defect in pregnancy IGFBP-3 is exacerbated when the ligand is iodinated. The demonstration that iodo-IGF-I cannot form the ternary complex with pregnancy IGFBP-3, while reacting normally with non-pregnancy IGFBP-3 (Suikkari and Baxter, 1991), lends support to this hypothesis. In the present study we have further characterized the defect in pregnancy IGFBP-3, first by defining more closely the structural determinants in IGF-I which appear to exacerbate the binding defect, and secondly by comparing the physical and functional properties of purified IGFBP-3 preparations from non-pregnancy and pregnancy serum.

MATERIALS AND METHODS

Reagents and columns

The Superose 12 HR 10/30 column, Protein A and Protein A-Sepharose were from Pharmacia, North Ryde, NSW, Australia. Affigel 10 (activated agarose) and the Hi-Pore RP 318 column were from Bio-Rad, Richmond, CA, U.S.A. Centricon 10 concentrators were obtained from Amicon, Beverly, MA, U.S.A., and the Protein Pak 125 column was from Waters, Milford, MA, U.S.A. Disuccinimidyl suberate was from Pierce, Rockford, IL, U.S.A. BSA (radioimmunoassay grade) was from Sigma, St. Louis, MO, U.S.A. Human ALS and ¹²⁵I-labelled ALS were prepared as previously described (Baxter et al., 1989; Baxter, 1990). The use of anti-IGFBP-3 serum R-7 has been described previously (Suikkari and Baxter, 1992); R-100 is a new, high-titre, rabbit antiserum raised with 100 µg immunization doses of pure human IGFBP-3.

IGF-I and analogues

Natural IGF-I was purified from Cohn fraction IV of human plasma as previously described (Baxter and De Mellow, 1986). Recombinant human IGF-I, and the following IGF-I analogues, prepared and isolated as described, were generously donated by Drs. M. A. Cascieri and M. L. Bayne, Merck Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. The analogues tested were [Ser²⁴][IGF-I], [Ala³¹][IGF-I] and [Leu⁶⁰][IGF-I], in each of which a tyrosine residue is substituted (Bayne et al., 1990); A-chain mutant IGF-I, in which A-domain residues 42–56 are replaced with the corresponding residues of insulin, and Thr⁴¹ is changed to Ile (Cascieri et al., 1989); and IGF-I-(1–62) and IGF-I-(1–27-Gly₄-38–70), the first having the C-terminal D-domain deleted, and the second having the C-domain substituted by a Gly₄ bridge (Bayne et al., 1989). Iodo-IGF-I (non-radioactive) was prepared as previously described (Suikkari and Baxter, 1991).

IGFBP-3 preparations

IGFBP-3 from Cohn fraction IV of human plasma was prepared by IGF affinity chromatography and reverse-phase h.p.l.c. as previously described (Martin and Baxter, 1986). IGFBP-3 fractions from pregnant and non-pregnant individuals were prepared by two methods: size-exclusion and immunoaffinity chromatography. Serum samples from pregnant women of 24–28 weeks' gestation were from specimens collected for other purposes (glucose tolerance testing); non-pregnancy samples were from female volunteers in the same age range.

In the size-exclusion chromatography method, 200 μ l of each serum was applied to a Superose 12 column eluting under previously described conditions (Baxter, 1988). The three 0.5 ml fractions corresponding to the 140 kDa peak were pooled with the equivalent fractions from a duplicate run, concentrated to approx. 200 μ l by ultrafiltration (Centricon 10), adjusted to the high-performance chromatography conditions of 0.2 M acetic acid, 0.1 M trimethylamine, pH 2.8, by the addition of 5 $\times$ concentrated buffer, and injected on to a Protein Pak 125 column eluting at 1.7 ml/min in the same buffer. The 2.2 ml fraction eluted between 3.9 and 5.2 min was collected in 25 μ l of 10% (w/v) BSA, and the sample was immediately neutralized by the addition of 0.2 ml of 2 M Tris base. The binding proteins in this fraction are depleted of their IGFs, which elute in the following 2.2 ml fraction (Scott et al., 1985).

To purify IGFBP-3 by immunoaffinity chromatography, a high-titre IGFBP-3 antiserum, R-100, was fractionated on a column of Protein A-Sepharose according to the manufacturer's instructions, to prepare an IgG fraction, which was then coupled to Affigel 10 (IgG from 6 ml of serum per 5 g moist weight of gel) according to the manufacturer's instructions. Non-pregnancy and pregnancy serum samples (12 ml; diluted to 36 ml with PBS) were pumped through separate 0.7 cm $\times$ 4 cm affinity columns at 2 ml/h, the columns were washed with 50 ml of PBS at 1 ml/min, and the bound proteins were eluted at 1 ml/min with 0.1 M acetic acid (pH 2.9) into siliconized glass tubes. Except when further purification was performed, fractions containing the peak IGFBP-3 immunoreactivity were pooled, adjusted to pH 6.5 with 1 M NaOH, and BSA was added to 2 g/l (final). For further purification by reverse-phase h.p.l.c., 2 ml of pooled eluted IGFBP-3 was injected on to a Hi-pore RP-318 column and eluted with an acetonitrile gradient as previously described for plasma IGFBP-3 purification (Martin and Baxter, 1986). Fractions containing IGFBP-3 immunoreactivity, eluting at approx. 16 min, were further purified by re-injecting on to the same column.

Complex formation assay

Ternary complex formation was measured by the binding of ¹²⁵I-labelled ALS to IGFBP-3 preparations in the presence of IGF-I, as described in previous studies (Baxter et al., 1989, 1992). Radioactivity bound to IGFBP-3 was precipitated with the antiserum R-7 (1 μ l per 300 μ l incubation). Binding studies for Scatchard plots were performed in the presence of 5 ng of IGFBP-3, and unlabelled ALS was added over the range 3–150 ng/300 μ l incubation. For IGF dose-response curves, performed on IGFBP-3 preparations depleted of endogenous IGFs, non-specific binding was defined as radioactivity precipitated in the absence of IGFs. When using IGFBP-3 preparations prepared by immunoaffinity chromatography, where endogenous IGFs would be expected to be present, non-specific binding was defined as radioactivity precipitated in the absence of IGFBP-3.

SDS/PAGE, immunoblotting and affinity labelling

For immunoblotting studies, approx. 50 ng of serum IGFBP-3 preparations eluted from immunoaffinity columns was subjected to SDS/PAGE on 12% gels as previously described (Martin and Baxter, 1990). Pure plasma IGFBP-3 (50 ng) was run as a control. Immunoblotting was performed using a high-titre IGFBP-3 antiserum, R-100, with detection using ¹²⁵I-labelled Protein A (Martin and Baxter, 1990). Similar results were obtained when antiserum R-7 was used. In affinity labelling studies, performed essentially as previously described (Baxter and Martin, 1989), 100 ng of each IGFBP-3 preparation was incubated for 2 h at 22 °C with 50 ng of IGF-I and ¹²⁵I-labelled ALS (approx. 0.1 μ Ci; 500 Ci/mmol), and bound proteins were covalently cross-linked with disuccinimidyl suberate (0.5 mM). Complexes were immunoprecipitated by adding anti-IGFBP-3 serum R-7 (20 μ l in a total of 300 μ l), incubating for 4 h at 22 °C, adding 40 μ l of Protein A-Sepharose beads, standing for 30 min with occasional mixing, and centrifuging to precipitate the beads, which were then washed three times by resuspending in PBS and re-centrifuging. Proteins were removed from the washed beads by boiling in electrophoresis sample buffer, and were separated on 9% acrylamide gels (Martin and Baxter, 1990). For autoradiography, immunoblots and gels containing affinity-labelled samples were exposed to Hyperfilm-MP (Amersham) for 1–3 days at –70 °C.

RESULTS

IGFBP-3 from pregnancy serum forms a ternary complex with iodo-IGF-I much less efficiently than with native IGF-I (Suikkari and Baxter, 1991). To determine which of the three tyrosine residues in IGF-I was responsible for this phenomenon, IGF-I analogues with specific amino acid substitutions were tested. In initial studies, [Ser²⁴][IGF-I], [Ala³¹][IGF-I] and [Leu⁶⁰][IGF-I] were each radioiodinated, and the binding of each radioligand to non-pregnancy and pregnancy IGFBP-3 was tested as described in our previous studies (Suikkari and Baxter, 1991). Whereas each of these analogues, when iodinated, bound to non-pregnancy IGFBP-3, none showed any binding activity towards pregnancy IGFBP-3 (results not shown). Similarly, analogues with two tyrosines substituted, i.e. [Ala³¹,Leu⁶⁰][IGF-I] and [Leu²⁴,Leu⁶⁰][IGF-I] (Bayne et al., 1990), did not bind to pregnancy IGFBP-3, while showing variable (but always measurable) binding to normal IGFBP-3 (results not shown).

Since it appeared that either iodination or substitution of IGF-I tyrosine residues was sufficient to impair binding to pregnancy IGFBP-3, analogues were tested for their ability to allow ALS

Figure 1 Binding of ALS tracer to IGF-depleted IGFBP-3 from non-pregnancy (a) or pregnancy (b) serum in the presence of increasing concentrations of IGF-I (●, ○), [Leu⁶⁰]IGF-I (■, □), or iodo-IGF-I (▲, △)

Incubations contained approx. 3 ng of IGFBP-3, prepared by size-exclusion chromatography at pH 2.8.

Figure 2 Binding of ALS tracer to IGF-depleted IGFBP-3 from non-pregnancy (●) or pregnancy (○) serum in the presence of increasing concentrations of [Ala³¹]IGF-I (a), IGF-I(1-27-Gly₄-38-70) (b), IGF-I(1-62) (c) or A-chain mutant IGF-I (d)

binding to IGFBP-3, which requires occupancy by IGF-I (Baxter et al., 1989). Substitution of Tyr⁶⁰ was examined first, as this residue has previously been shown to be important in the IGF-IGFBP interaction (Moss et al., 1991). Figure 1 compares the ability of IGF-depleted IGFBP fractions, isolated from non-

Figure 3 Binding of ALS to IGFBP-3 from pregnancy and non-pregnancy sera

(a) Binding of ALS tracer to increasing concentrations of IGFBP-3 isolated from non-pregnancy (●, ■) and pregnancy (○, □) sera by immunoaffinity chromatography. These IGFBP-3 preparations contain endogenous IGFs; in addition, excess IGF-I (5 ng) was added to each incubation. (b) Scatchard plots of ALS binding to IGFBP-3 preparations from non-pregnancy (●) and pregnancy (○) serum as described in (a). Incubations contained 5 ng of IGFBP-3; excess IGF-I (5 ng) was added to each incubation.

pregnancy and pregnancy serum by size-exclusion chromatography, to bind ALS tracer in the presence of native IGF-I, iodo-IGF-I or [Leu⁶⁰]IGF-I. In the presence of IGFBPs from non-pregnancy serum, iodo-IGF-I was slightly less active than native IGF-I in allowing ternary complex formation, while [Leu⁶⁰]IGF-I appeared more active than IGF-I. However, when IGFBPs from pregnancy serum were tested, iodo-IGF-I and [Leu⁶⁰]IGF-I showed little activity relative to native IGF-I in complex formation.

A similar study utilizing other IGF-I variants is shown in Figure 2. IGF-I in which Tyr³¹ is substituted by Ala was little different from native IGF-I in complex formation with pregnancy and non-pregnancy IGFBP-3, whereas [Ser²⁴]IGF-I, in which Tyr²⁴ is substituted, showed such reduced complex formation with both pregnancy and non-pregnancy IGFBP-3 (results not shown) that a conclusion about the altered binding of this analogue to pregnancy IGFBP-3 could not be reached. Also shown in Figure 2 are results for IGF-I(1-62), which lacks the

Table 1 Binding parameters for ALS binding to IGFBP-3 isolated from non-pregnancy and pregnancy serum

Data are derived from Scatchard plots similar to those shown in Figure 3(b). Incubations contained 5 ng of IGFBP-3, isolated (together with endogenous IGFs) from pregnancy or non-pregnancy serum by immunoaffinity chromatography, to which excess (5 ng) IGF-I was added. To calculate molar binding ratios, the molecular masses of ALS and IGFBP-3 were assumed to be 85 kDa and 43 kDa respectively. Values are means $\pm$ S.D. for three determinations. Differences between non-pregnancy and pregnancy values are not significant (paired two-tail *t*-test).

Source of IGFBP-3	Association constant (nM^{-1})	Binding sites (mol of ALS/mol of IGFBP-3)
Non-pregnancy serum	1.52 ± 0.62	0.96 ± 0.36
Pregnancy serum	1.19 ± 0.29	0.91 ± 0.26

Figure 4 Absorbance profiles at 280 nm of IGFBP-3 preparations from non-pregnancy (a) and pregnancy (b) serum, fractionated by h.p.l.c.

After immunoaffinity chromatography, IGFBP-3 samples were subjected to reverse-phase h.p.l.c. on an RP-318 (C_{18}) column eluted at 1.5 ml/min with a 30-min linear acetonitrile gradient (15–60%) in 0.1% trifluoroacetic acid. Only the peaks emerging at 16 min showed IGFBP-3 immunoreactivity.

Figure 5 Binding of ALS tracer to IGFBP-3 from non-pregnancy (●, ■, ▲) or pregnancy (○, □, △) serum in the presence of increasing concentrations of IGF-I (●, ○), [Leu⁶⁷]IGF-I (■, □), or [Ala³¹]IGF-I (▲, △)

Incubations contained 10 ng of IGFBP-3, prepared by h.p.l.c. after immunoaffinity chromatography as described in the legend to Figure 4.

C-terminal D-domain, and IGF-I(1–27-Gly₄-38–70), in which the C-domain is substituted by a Gly₄ bridge. Neither analogue differentiated between pregnancy and non-pregnancy IGFBP-3 to a greater extent than did native IGF-I. Finally, an IGF-I analogue in which residues 42–56 are replaced by the corresponding residues of insulin, and residue 41 is changed from threonine to isoleucine (A-chain mutant), resembled iodo-IGF-I and [Leu⁶⁷]IGF-I in that it formed a ternary complex with normal but not with pregnancy IGFBP-3.

High-performance size-exclusion chromatography at pH 2.8, although designed to minimize the time of exposure to low pH, might cause damage to proteolysed IGFBP-3 that could affect its binding activity. In an attempt to obtain pure IGFBP-3 from non-pregnancy and pregnancy sera by an alternative method, immunoaffinity chromatography was employed, using a column of high-titre IGFBP-3 antiserum coupled to agarose beads. IGFBP-3 bound to, and eluted from, this column would be expected to be occupied by endogenous IGFs.

Figure 3(a) shows the ability of increasing concentrations of two such IGFBP-3 preparations from non-pregnancy serum, and two from pregnancy serum, to form the ternary complex. No difference was seen between the ability of the pregnancy and non-pregnancy preparations to form the complex, consistent with the results reported initially with whole serum after transient acidification (Suikkari and Baxter, 1992). Scatchard plots of ALS binding to pregnancy and non-pregnancy IGFBP-3 isolated by immunoaffinity chromatography confirmed that the binding of ALS was unaltered in pregnancy (Figure 3b). Table 1 shows the mean association constants and binding site concentrations for non-pregnancy and pregnancy IGFBP-3, determined in three such experiments. Since the binding site concentration is thought to represent the concentration of binary IGF-IGFBP complex (Baxter et al., 1992), this indicates that IGF-I binding to pregnancy IGFBP-3 isolated in this manner is not impaired.

Since IGFBP-3 preparations at this stage of purity were indistinguishable by their binding characteristics, reverse-phase h.p.l.c. was used to further purify them. As shown in Figure 4, the IGFBP-3 emerged from the column in a single peak at 16 min for both pregnancy and non-pregnancy preparations, similar to the elution time initially reported for IGFBP-3 on this column (Martin and Baxter, 1986). Other minor peaks were not immunoreactive with IGFBP-3 antiserum. However, while the highly purified IGFBP-3 from non-pregnancy serum retained its binding characteristics, the preparation from pregnancy serum was entirely inactive (Figure 5), indicating that this preparation, which appeared to be fully active prior to h.p.l.c., was more labile than the corresponding protein from non-pregnancy serum.

Pregnancy IGFBP-3 has been reported to be smaller than the non-pregnancy protein, when analysed by SDS/PAGE; therefore we used this technique to compare the sizes of the proteins purified by immunoaffinity chromatography. Figure 6 shows that, when analysed by immunoblotting, non-pregnancy IGFBP-3 purified in this way contained the characteristic doublet, as seen in a standard preparation isolated by IGF affinity chromatography and h.p.l.c. In contrast, the preparation from pregnancy serum appeared as a single band of approx. 30 kDa, which was also visible as a minor component in the standard and non-pregnancy samples.

Since this preparation appears to form the ternary complex normally, the question arises as to whether the complex that it forms is smaller than that seen in the presence of non-pregnancy IGFBP-3. Complexes were formed using labelled ALS, then cross-linked with disuccinimidyl suberate and analysed by SDS/PAGE. As shown in Figure 6, both IGFBP-3 preparations predominantly formed a complex of 135–140 kDa, similar to

Figure 6 SDS/PAGE of IGFBP-3

(a) SDS/PAGE on 12% polyacrylamide of IGFBP-3 preparations isolated from pooled human plasma by IGF affinity chromatography (C) or from individual non-pregnancy (NP) or pregnancy (P) serum by immunoaffinity chromatography. IGFBP-3 (50 ng/lane) was detected by immunoblotting with IGFBP-3 antiserum, followed by incubation with 125 I-labelled Protein A and autoradiography. (b) SDS/PAGE on 9% polyacrylamide of affinity-labelled complexes formed from IGFBP-3 preparations (100 ng) as designated in the left lane, IGF-I (50 ng), and 125 I-labelled ALS. Before electrophoresis, complexes were cross-linked with disuccinimidyl suberate and immunoprecipitated with IGFBP-3 antiserum; some free ALS tracer is also visible as an 85 kDa doublet. Arrows indicate molecular masses (kDa) of marker proteins; additional numbers on the right indicate calculated molecular masses (kDa) of the bands indicated.

that previously reported (Baxter and Martin, 1989). A second complex of 120 kDa, also described previously, formed more strongly from the non-pregnancy IGFBP-3 than from the pregnancy preparation, whereas a band of ~95 kDa, previously attributed to a cross-linked IGF-ALS complex, was stronger in the pregnancy sample. Also in pregnancy serum was a diffuse band of 110–115 kDa, possibly a doublet, of which only the 110 kDa component was present in the non-pregnancy sample.

DISCUSSION

The mechanism by which the circulating high-molecular-mass IGF complex releases its IGFs has been a subject of investigation for many years. The possibility of a mechanism involving proteolysis, first proposed a decade ago (Chatelain et al., 1983), has gained increased attention recently with the observation that a protease which impairs the ligand-binding activity of IGFBP-3 is present in the circulation of pregnant humans and some other species (Davenport et al., 1990; Fielder et al., 1990; Giudice et al., 1990; Hossenlopp et al., 1990). The products of this proteolytic action appear, when analysed by SDS/PAGE, to be a fragment of approx. 30 kDa which retains IGFBP-3 immunoreactivity (Hossenlopp et al., 1990) and smaller inactive fragments detectable only when iodinated IGFBP-3 is used as a substrate for the protease (Giudice et al., 1990).

The apparent decrease in IGF binding to IGFBP-3 in pregnancy has been postulated to result in either increased (Giudice et al., 1990) or decreased (Gargosky et al., 1990) IGF-I bioavailability. However, the biological significance of this phenomenon has been questioned on the grounds that IGFs and IGFBP-3 circulate in the normal ternary complex, and at normal to elevated concentrations, in pregnancy, implying that neither

IGF nor ALS binding is impaired (Davies et al., 1991a; Gargosky et al., 1991; Suikkari and Baxter, 1992). Furthermore, the fact that pregnancy IGFBP-3 forms a ternary complex with iodinated IGF-I much less effectively than with native IGF-I suggests that the use of radioiodinated ligands might artifactually exacerbate the IGF-binding defect in the proteolysed protein (Suikkari and Baxter, 1991).

In the present study, proteolysed IGFBP-3 has been characterized in two ways. First, it has been demonstrated that the proteolysed protein is unable to bind IGF-I in which the A-domain in the vicinity of residue 60 has been altered. Either iodination or substitution of Tyr⁶⁰ renders the IGF-I unable to complex with proteolysed IGFBP-3. This is surprising, since the A-domain of IGF-I is thought to have little involvement in the binding of IGF-I to intact IGFBP-3 (Cascieri et al., 1989; Bayne et al., 1990; Baxter et al., 1992), even though Tyr⁶⁰ has been implicated in binding to IGFBP-2 (Moss et al., 1991). Substitution of Tyr⁶⁰ with Leu is thought to change the tertiary structure of IGF-I slightly, possibly by disrupting the Van der Waals interaction between Tyr⁶⁰ and Ile⁴³ (Bayne et al., 1990). Proteolysis of IGFBP-3 may convert the protein to a conformation which, like the other IGFBPs (Clemmons et al., 1992), is sensitive to A-chain alterations. There was no evidence that Tyr³¹, or the C- or D-domains of IGF-I, are involved in the loss of binding to proteolysed IGFBP-3. The possible involvement of Tyr²⁴, which has some involvement in IGF-I binding to intact IGFBP-3 (Baxter et al., 1992), could not be evaluated in this study.

ALS binding (i.e. ternary complex formation) has been used as an indirect measure of IGF binding, since there are no direct methods presently available for studying binding of non-iodinated IGF-I. This approach is validated by the observations that (i) ALS does not bind to IGFBP-3 unless IGFs are present (Baxter and Martin, 1989; Baxter et al., 1989); (ii) when the binding affinity of the IGF-I-IGFBP-3 interaction is decreased, ternary complex formation is decreased (Baxter et al., 1992); and (iii) the presence of ALS has no effect on the binding of IGF-I or IGF-II to IGFBP-3 (Baxter et al., 1989).

Because of the indirect approach used, the possibility exists that pregnancy IGFBP-3 does not actually differ from intact IGFBP-3 in its binding of IGFs altered in the A-domain, but that the resulting binary complexes have reduced affinity for ALS. Other IGF-I variants with this property have been identified in studies with intact IGFBP-3 (Baxter et al., 1992). While it is not known exactly how IGF binding facilitates ALS binding, there may be some interaction between the IGF and ALS in the ternary complex, as suggested by affinity labelling studies (Baxter and Martin, 1989). However, this is considered an unlikely explanation for the reduced IGF analogue activity towards pregnancy IGFBP-3 for two reasons. First, the region of IGF-I which seems to be most important in permitting ALS binding to intact IGFBP-3 is the D-domain (Baxter et al., 1992), whereas in the present studies the D-domain-deleted analogue, although lower in activity than native IGF-I, did not distinguish between intact and proteolysed IGFBP-3 in ternary complex formation. Conversely, [Leu⁶⁰]IGF-I, which does not allow ALS to bind to proteolysed IGFBP-3, appears more active than IGF-I in facilitating ALS binding to intact IGFBP-3, indicating that alteration of residue 60 does not have a deleterious effect on the interaction with ALS. Thus the reduced activity of proteolysed IGFBP-3 in ternary complex formation with IGF-I iodinated or substituted in the A-domain appears to be at the level of IGF binding rather than ALS binding.

Proteolysed IGFBP-3 has also been characterized electrophoretically in this study. This has demonstrated that

immunoaffinity-purified pregnancy IGFBP-3, indistinguishable in binding activity from non-pregnancy IGFBP-3, is exclusively in a 30 kDa form after SDS/PAGE. This confirms the original observation of Hossenlopp et al. (1990), but demonstrates clearly that the decreased size of IGFBP-3 by this analysis does not necessarily imply reduced binding activity. The demonstration that an affinity-cross-linked band of 135–140 kDa is equally present in complexes formed from proteolysed or intact IGFBP-3 also suggests that the decrease in size of IGFBP-3 to 30 kDa may occur during electrophoresis. Another band of 110–115 kDa, evident in complexes formed from proteolysed IGFBP-3, may represent a cross-linked ternary complex containing the 30 kDa form of IGFBP-3, but it is considerably less abundant than the 135–140 kDa complex. The observation of a predominant 135–140 kDa complex from both proteolysed and intact IGFBP-3 contrasts with a recent study (Liu et al., 1992) in which IGFBP-3 from acid-chromatographed pregnancy serum yielded a 130 kDa cross-linked complex (equivalent to our 115 kDa band), with a band at 150 kDa (equivalent to our 135–140 kDa band) seen only in non-pregnancy serum. The lack of the larger complex in the pregnancy sample may be due to the different IGFBP-3 isolation procedure used in the study of Liu et al. (1992).

The question therefore arises: how much of the physical degradation of proteolysed IGFBP-3 occurs during the analytical process, and how much is present in the protein in the circulation? Our observation that reverse-phase h.p.l.c. of fully active immunoaffinity-purified pregnancy IGFBP-3 results in a total loss of activity suggests that continuing exposure to acids and other harsh conditions progressively decreases the binding activity of the proteolysed protein, while having little effect on the intact protein. The demonstration of profound degradation of IGFBP-3 exposed to proteases *in vitro* and subsequently analysed by SDS/PAGE (Giudice et al., 1990) may therefore not be the ideal parallel for the proteolysis which IGFBP-3 undergoes *in vivo* in pregnancy and other conditions. Nevertheless, it is well established that such limited proteolysis does occur, and the possibility clearly remains that this action may, by some as yet unknown mechanism, influence the bioavailability of the IGFs.

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