Recognition of insulin-like-growth-factor-binding proteins in serum and amniotic fluid by an antiserum against a low-molecular-mass insulin-like-growth-factor-inhibitor/binding protein

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An antiserum (R8) raised against a purified specific low- M_r (16000–18000) insulin-like-growth-factor (IGF)-inhibitor/binding protein, which is immunologically related to the native growth hormone (GH)-dependent M_r -150000 IGF-binding protein in serum, has been used to probe a possible additional relationship to the predominant non-GH-dependent IGF-binding protein ($M_r \sim 30000$) of human amniotic fluid. Amniotic-fluid fractions and an IGF-inhibitory fraction of serum were analysed by covalent cross-linking, ligand-blotting and immunoblotting techniques. Western blotting of the serum fraction using the R8 antiserum gave five immunoreactive (ir) bands, of which at least three (M_r 38000, 34000 and 23000) possessed IGF-binding activity, as indicated by ligand (^{125}I -IGF-I) blotting. Western blotting of two differently prepared amniotic-fluid fractions, which both showed potent IGF-inhibitory bioactivity, gave several (M_r range 14500–73000) ir bands, of which two (M_r 28000 and 17000) were most prominent. Ligand-blotting analysis gave a single intensely labelled band at M_r 28000, consistent with the major presence of the M_r -28000–30000 amniotic-fluid IGF-binding protein. Covalent cross-linking of the amniotic-fluid fractions to ^{125}I -IGF-I gave three specifically cross-linked complexes (M_r 36000, 32000 and 23000), which, assuming a 1:1 binding stoichiometry with IGF (M_r 7500), represent binding proteins of M_r 28500, 24500 and 15500 respectively. The M_r -15500 binding protein, very similar to the M_r -17000 ir band, in all likelihood represents the IGF-inhibitor protein. Our results indicate that inhibitor-sized binding proteins and IGF-inhibitor bioactivity are present in human amniotic fluid, and that the IGF-inhibitor protein (M_r 16000) and amniotic-fluid IGF-binding protein (M_r 28000) are immunologically related. Since the IGF-inhibitor protein is also immunologically and structurally related to the native, GH-dependent, M_r -150000 binding protein in serum, our data suggest a heretofore-unrecognized immunological similarity between the M_r -150000 binding protein and the amniotic-fluid binding protein and its serum analogue, the $M_r \sim 30000$, non-GH-dependent, binding protein.

INTRODUCTION

The insulin-like growth factors (IGFs) circulate in serum completely bound to specific binding proteins of which there are two major classes (Ooi & Herington, 1988a): the high- M_r (150000) form, which is growth-hormone (GH)-dependent and appears to predominate in adult plasma, and the smaller (M_r 30000–34000) non-GH-dependent species, which is more prevalent in fetal plasma (D'Ercole *et al.*, 1985). The M_r -150000 binding protein can be dissociated *in vitro* with acid to give an M_r 50000–60000 IGF-binding component (Furlanetto, 1980; Hintz, 1984), now referred to as 'IGFBP-3' (see the Abbreviations footnote) and has been reported to be further dissociated by alkali into M_r -36000 and -46000 species (Morris & Schalch, 1982). The non-GH-dependent, M_r -30000–34000 binding protein, denoted 'IGFBP-1' in the present study is stable to acid and is regarded as distinct from the GH-dependent binding protein, but equivalent to the M_r -30000–34000 amniotic-fluid binding protein (Drop *et al.*, 1984; D'Ercole *et al.*, 1985), placental protein 12 (Koistinen *et al.*, 1986), and the binding protein secreted by human hepatoma cells (HEPG2) in culture (Povoa *et al.*, 1985). Recent cloning studies have allowed elucidation of the full amino acid sequences of IGFBP-3 (Wood *et al.*, 1988) and

IGFBP-1 (Brewer *et al.*, 1988; Lee *et al.*, 1988; Brinkman *et al.*, 1988). These sequences are distinct, although some evolutionary and structural relationship is in evidence, for example with absolute conservation of all 18 cysteine residues. Recently, Baxter *et al.* (1987) described the presence of at least two immunoreactive forms of IGF-binding protein in amniotic fluid: the classical IGFBP-1, representing the major component, and another form, related to IGFBP-3. The nature of the relationship, structural and/or functional, between these amniotic-fluid binding proteins remains unclear.

Following our report on the isolation from serum of a specific inhibitor of IGF bioactivity *in vitro* (Herington & Kuffer, 1981), we subsequently showed that this inhibitor is a low- M_r (16000–18000) binding protein that may represent either a primary IGF-binding subunit of the native binding protein (M_r 150000) in serum, as suggested by the studies of Wilkins & D'Ercole (1985), or more likely a fragment, albeit biologically active, of the same binding protein. We now describe studies with a polyclonal antiserum raised against a highly purified preparation of this serum inhibitor (Ooi & Herington, 1988b) which demonstrates an equivalent-size binding protein (M_r 16000) in amniotic fluid and identifies an immunological similarity between amniotic-fluid binding protein(s) and the GH-dependent binding protein species in serum.

Abbreviations used: the nomenclature for insulin-like-growth-factor (IGF)-binding proteins agreed upon at an International Workshop on IGF-Binding Proteins (Vancouver, June 1989) has been used in this paper: IGFBP-1, the non-growth hormone (GH)-dependent, acid-stable IGF-binding protein of M_r 28000–30000, isolated from amniotic fluid [previous nomenclature included BP-28 (Baxter *et al.*, 1987)]; IGFBP-2, the binding protein equivalent to that secreted by the rat liver cell line BRL-3A (Binkert *et al.*, 1989); IGFBP-3, the GH-dependent, acid-derived subunit of the native, high- M_r (150000) binding protein [previous nomenclature included BP-53 (Martin & Baxter 1986)]; SP-, sulphopropyl; ir, immunoreactive.

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MATERIALS AND METHODS

Reagents

SP-Sephadex C-25, Sephadex G-50, Sephadex G-75, octyl-Sepharose for chromatography and M_r marker proteins for SDS/PAGE were purchased from Pharmacia, Uppsala, Sweden. Bio-Gel P30, dithiothreitol, nitrocellulose (0.45 μ m pore size), TransBlot apparatus and other reagents for SDS/PAGE and electroblotting were from Bio-Rad Laboratories, Sydney, Australia. Aurodye Forte for protein staining was from Janssen Life Sciences, Olen, Belgium. Disuccinimidyl suberate and Iodogen (1,3,4,6-tetrachloro-3 α -6 α -diphenylglycoluril) were from Pierce Chemical Co., Rockford, IL, U.S.A. Streptavidin-biotinylated peroxidase complex for Western blotting, Hyperfilm for autoradiography, and an IGF-I analogue ([Thr⁵⁹]IGF-I), prepared by recombinant DNA techniques by Amgen (Thousand Oaks, CA, U.S.A.), were purchased from Amersham Australia (Sydney, Australia). Biotinylated anti-rabbit antibody was from Vectalabs, Burlingame, CA, U.S.A. Inhibitor antibody (R8) was raised as previously reported (Ooi & Herington, 1988b) in rabbits using highly purified inhibitor/binding protein as antigen. General laboratory reagents were from Ajax Chemicals, Sydney, Australia.

Samples

Normal serum from 100 donors was obtained from the Red Cross Blood Bank, Melbourne, Australia, and these were pooled together to give a representative sample.

A pool of amniotic fluid was taken from 33 subjects during amniocentesis at 16 weeks gestation of normal pregnancies from the Royal Women's Hospital, Melbourne, Australia.

Assay of inhibitor bioactivity

Inhibitory bioactivity of the binding-protein fractions was assessed by their ability to inhibit IGF-stimulated [¹⁴C]glucose incorporation into [¹⁴C]lipids by isolated rat epididymal adipocytes as previously described (Herington & Kuffer, 1981).

Preparation of IGF inhibitor

Inhibitor was purified from Cohn Fraction IV-1 of human serum using ion-exchange chromatography on SP-Sephadex followed by sequential gel-permeation chromatography on Sephadex G-75 and Bio-Gel P30 as previously described (Herington & Kuffer, 1981). Unlabelled IGF required for determination of non-specific binding was prepared as a relatively impure, but bioactive, fraction by the same methodology (Herington & Kuffer, 1981). IGF inhibitor was eluted from Bio-Gel P30 gel-permeation chromatography at a K_{av} of 0.10–0.54, whereas IGF activity was eluted at K_{av} values between 0.54 and 0.88. An IGF-inhibitory fraction of serum (Serum Peak II), which was enriched in IGF-binding proteins, was prepared by hydrophobic-interaction chromatography using octyl-Sepharose as previously described (Ooi & Herington, 1986).

Isolation of amniotic-fluid binding proteins

Pooled amniotic-fluid samples (5 ml) were applied to a column (2.2 cm $\times$ 60 cm) packed with Bio-Gel P30 (bed volume 230 ml) and eluted at 0.1 ml/min with 0.1% trifluoroacetic acid. Collected fractions (2 ml) containing inhibitory bioactivity (fraction no. 40–50; K_{av} 0.09–0.18) were pooled and freeze-dried.

An alternative isolation procedure, based on the (NH₄)₂SO₄-precipitation protocol of Pova et al. (1984a) was also used. Protein material, precipitated between 35 and 50% (NH₄)₂SO₄ saturation, was prepared from 50 ml of pooled amniotic fluid. The precipitate fraction was dissolved in a minimal volume of distilled water, dialysed overnight against water and then

reconstituted to a final volume of 10 ml. The mean total amount of protein obtained in the precipitated fraction for three such extractions was 5.4 ± 0.2 mg.

Radioiodination of IGF-I

A pure IGF-I analogue ([Thr⁵⁹]IGF-I) (0.5 μ g) was iodinated by the Iodogen method (Salacinski et al., 1981) to a sp. radioactivity (assessed by percentage incorporation of ¹²⁵I) of ~ 350 μ Ci/ μ g. The iodinated mixture was then purified on a Sephadex G-50 column (1.0 cm $\times$ 20 cm) equilibrated in 50 mM-Tris/HCl, pH 7.5.

Electrophoresis, immunoblotting and ligand blotting

Samples were dissolved in Tris/HCl sample buffer [65 mM-Tris (pH 6.8)/3% (w/v) SDS/10% (v/v) glycerol] incubated at room temperature overnight, and then subjected to electrophoresis on 15%-polyacrylamide gels [30:0.8 (w/w) acrylamide bisacrylamide ratio as described by Laemmli (1970)]. Gels were then electroblotted on to nitrocellulose using the TransBlot apparatus as previously described (Ooi & Herington, 1988b). The nitrocellulose blots were cut into identical strips, and were either stained with Aurodye Forte for total proteins, Western-blotted with the R8 inhibitor antibody or ligand-blotted with ¹²⁵I-IGF-I. The procedures used have been described previously (Ooi & Herington, 1988b).

Covalent cross-linking of ¹²⁵I-IGF-I to binding proteins

The method used has been described previously (Ooi & Herington, 1986). Briefly, samples (75 μ g) in 0.1 M-sodium phosphate, pH 7.4, were incubated with 500 000 c.p.m. of ¹²⁵I-IGF-I (± 0.25 munit of insulin equivalents of excess unlabelled IGF for determination of specific binding) for 4 h at 20 °C. Disuccinimidyl suberate in dimethyl sulphoxide was added to a final concentration of 1 mM, and the cross-linking reaction allowed to proceed for 30 min at 20 °C. Excess Tris/HCl electrophoresis sample buffer (see above) was added to terminate the reaction, and the cross-linked samples were analysed by SDS/PAGE and autoradiography.

RESULTS

IGF inhibitor

Partially purified inhibitor, when subjected to SDS/PAGE, electroblotted on to nitrocellulose and then analysed with inhibitor antiserum R8 (Western blotting) gave four ir bands of M_r 34 500, 23 000, 16 000 and 12 000 (results not shown; see Ooi & Herington, 1988b). The most intense ir band occurred at M_r 16 000, identical with the M_r of the pure inhibitor (Ooi & Herington, 1988b). To detect IGF-binding activity, an adjacent strip of the nitrocellulose blot was probed with ¹²⁵I-IGF-I and autoradiographed (ligand blotting). IGF-binding activity was detected at M_r 29 000, 26 500, 16 000 and 12 000, indicating that at least two of the ir bands (M_r 16 000 and 12 000) were indeed binding proteins. The bindings were specific for IGF, since these bands were absent in the presence of 0.25 munit of unlabelled IGF (see Ooi & Herington, 1988b).

Serum Peak II

A serum fraction (serum Peak II), known to contain binding proteins and having inhibitory bioactivity, was prepared by hydrophobic-interaction chromatography on octyl-Sepharose as described elsewhere (Ooi & Herington, 1986). Western blotting of this fraction using the R8 antiserum gave five distinct ir bands of M_r 83 000, 47 000, 38 000, 35 000 and 23 000, plus a less distinct band of M_r 28 000 (Fig. 1). Ir bands of M_r > 94 000 were also observed. Ligand-blotting analysis with ¹²⁵I-IGF-I gave specific

Fig. 1. Western and ligand blotting of serum peak II

Serum peak II, an IGF-inhibitory fraction of serum prepared by hydrophobic-interaction chromatography, was separated by SDS/PAGE (non-reducing conditions), electroblotted on to nitrocellulose and analysed for IGF-binding activity (ligand blotting), immunoreactivity with inhibitor antiserum (Western blotting) or stained for total proteins (Aurodyne staining). Control antiserum for Western blotting was pre-immune serum from rabbit R8. The M_r values for marker proteins are shown on the right; the M_r values for detected bands are shown on the left.

Fig. 2. Gel-permeation chromatography of amniotic fluid on Bio-Gel P30

Pooled human amniotic fluid (5 ml) was chromatographed on a Bio-Gel P30 column (2.2 cm $\times$ 60 cm) using 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min. Fractions (2 ml) were collected, and the absorbance of the eluate was monitored at 280 nm. Aliquots (200 μ l) of the fractions were freeze-dried in the presence of BSA and assayed for both IGF activity and inhibitor bioactivity. IGF activity is indicated by the cross-hatched bar; inhibitory bioactivity is denoted by the vertically hatched bar. In subsequent runs, inhibitory fractions (nos. 40–50) were pooled and freeze-dried. The column was calibrated with Blue Dextran (void volume), ovalbumin (43 kDa), 125 I-hGH (22 kDa), 125 I-IGF (7.5 kDa), 125 I-insulin (5.6 kDa) and Na^{125}I ('salt volume'). The chromatogram is representative of 11 such separations.

Fig. 3. Western and ligand blotting of amniotic-fluid fractions 40–50

Fractions 40–50, corresponding to an M_r range of 34000–45000, were pooled and subjected to SDS/PAGE, electroblotted on to nitrocellulose and analysed by Western- and ligand-blotting procedures. The data are presented as described for Fig. 1.

binding-protein bands at M_r 42000, 38000, 34000, 28000 and 23000, indicating that at least four of the ir bands (M_r 38000, 34000, 28000 and 23000) possessed and/or retained IGF-binding activity. The detection of these IGF-binding species is entirely consistent with the data of Hossenlopp *et al.* (1986) and Hardouin *et al.* (1987), but contrasts slightly with our own data (see above) obtained with a purified IGF inhibitor fraction, where the bands at M_r 34000 and 23000 were not detected by ligand blotting. This may be due to a combination of (i) the more involved, low-pH, procedure used for the inhibitor purification (Herington & Kuffer, 1981) compared with the two-step procedure used here for serum fractionation and (ii) a relative sensitivity of the IGF-binding potency of these two particular binding species to such a purification scheme.

Amniotic fluid

Pooled amniotic fluid was fractionated by two different procedures for analysis by Western- and ligand-blotting techniques: gel permeation chromatography and $(\text{NH}_4)_2\text{SO}_4$ fractionation.

Gel-permeation chromatography. Fig. 2 shows a typical elution profile of amniotic fluid when subjected to gel-permeation chromatography under acid conditions on BioGel P30. Major absorbance peaks (280 nm) were associated with both the void- and total-elution volumes, indicating molecular species of $M_r > 45000$ and < 4000 respectively. IGF-inhibitory bioactivity, as measured by the ability to inhibit IGF-stimulated lipogenesis in isolated rat adipocytes, was detected in fractions 42–48 (K_{av} 0.09–0.18), corresponding to an M_r range of 34000–45000. When the bioactive fractions were pooled, subjected to SDS/PAGE and Western-blotting analysis with inhibitor antiserum (R8), three immunoreactive bands of M_r 34000, 28000 and 17000 were observed (Fig. 3). The most intense ir band was at M_r 28000, consistent with the M_r of the predominant IGF-binding protein in amniotic fluid (Baxter *et al.*, 1987).

Fig. 4. Autoradiograph of ^{125}I -IGF-I covalently cross-linked to fractions 40–50 of amniotic fluid

Fractions 40–50 were pooled and incubated with ^{125}I -IGF-I in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of unlabelled IGF (0.25 munit). After cross-linking with disuccinimidyl suberate the covalent complexes were analysed by SDS/PAGE and autoradiography under non-reducing (lanes 1 and 2) or reducing (100 mM-dithiothreitol; lanes 3 and 4) conditions. The gel was exposed to autoradiographic film for (a) 25 h and (b) 17 days. The M_r values for specifically labelled bands are indicated on the left; the M_r values for marker proteins are shown on the right.

Ligand-blotting analysis also gave an intensely labelled band at M_r 28 000, confirming this ir band as the predominant binding-protein species present (Fig. 3). The M_r 17 000 ir band is identical in molecular size with the inhibitor (M_r 17 000) isolated from serum. No binding activity was observed by ligand blotting at the inhibitor M_r region (16 000–17 000). However, by using a more sensitive method, covalent cross-linking of ^{125}I -IGF-I using disuccinimidyl suberate and subsequent analysis by SDS/PAGE and autoradiography (Figs. 4a and 4b), this fraction gave three cross-linked complexes of M_r 36 000, 32 000 and 23 000 (un-reduced). Under reducing conditions, these cross-linked complexes were of M_r 39 000, 37 000 and 23 000 respectively. The M_r

Fig. 5. Western and ligand blotting of amniotic-fluid fraction precipitated by $(\text{NH}_4)_2\text{SO}_4$

An IGF-inhibitory fraction of amniotic fluid was precipitated by 35–50% $(\text{NH}_4)_2\text{SO}_4$ saturation. The fraction was subjected to SDS/PAGE under non-reducing conditions, electroblotted and analysed by Western- and ligand-blotting procedures. The data are presented as described for Fig. 1.

39 000 and 37 000 species are the dominant binding complexes observed, the M_r 23 000 complex only being detected after a much longer (17-day) autoradiographic exposure. Assuming a 1:1 binding stoichiometry with IGF (M_r 7500), these three complexes would represent binding proteins of M_r 28 500, 24 500 and 15 500 (unreduced) respectively. The M_r 15 500 binding protein is very similar to the M_r 17 000 ir band and in all likelihood represents the IGF inhibitor protein.

$(\text{NH}_4)_2\text{SO}_4$ fractionation. A binding protein-enriched fraction of amniotic fluid, precipitated by 35–50% saturation of $(\text{NH}_4)_2\text{SO}_4$, was prepared as described by Povia *et al.* (1984a). It showed potent IGF-inhibitory bioactivity (results not shown). Western-blotting analysis using inhibitor antiserum (R8) gave six ir bands of M_r 73 000, 41 500, 37 000, 28 000, 17 000–18 000 and 14 500 (Fig. 5). Ligand-blotting analysis using ^{125}I -IGF-I gave an intensely labelled band at M_r 28 000, again indicating that this binding protein is the predominant form in amniotic fluid. No binding activity was detected for the other ir bands, again suggesting that differences in abundance and/or binding potency can markedly affect ligand-blotting sensitivity. Previous studies from this laboratory (Ooi & Herington, 1988b) have demonstrated that the M_r 17 000 binding species can be detected by ligand blotting when highly concentrated, partially purified, fractions from serum are used. M_r 41 500 and 37 000 binding-protein species have also been detected by others, using equivalent ligand-blotting techniques, in serum (Hardouin *et al.*, 1987) and in liver-explant conditioned medium (Hossenlopp *et al.*, 1986). The M_r 73 000, 17 000 and 14 500 ir bands correspond closely in M_r to the serum-binding protein isolated by Grant *et al.* (1987) (M_r 73 000) and to the IGF inhibitor (M_r 16 000–18 000) respectively. Covalent cross-linking of this fraction to ^{125}I -IGF-I gave four specifically labelled complexes of M_r 36 000, 34 000, 32 000 and 24 000 (Figs. 6a and 6b). The M_r 32 000–36 000 complexes represent binding proteins of M_r 24 500–28 500, whereas the M_r 24 000 complex, observed only after extended

Fig. 6. Autoradiograph of ^{125}I -IGF-I covalently cross-linked to an amniotic-fluid fraction precipitated by $(\text{NH}_4)_2\text{SO}_4$

An amniotic-fluid fraction precipitated by 35–50 % $(\text{NH}_4)_2\text{SO}_4$ saturation was cross-linked to ^{125}I -IGF-I and analysed by SDS/PAGE and autoradiography. The gel was exposed to autoradiographic film for (a) 25 h and (b) 17 days. The data are presented as described for Fig. 4.

autoradiography, corresponds to the inhibitor, assuming a 1:1 binding stoichiometry. Cross-linked bands of M_r 19000 and 15500 did not appear to be specific IGF complexes.

DISCUSSION

Two major IGF-binding protein classes exist in human serum: one GH-dependent (IGFBP-3), the other non-GH-dependent (IGFBP-1) (Ooi & Herington, 1988a; Baxter & Martin, 1989). A variety of subunits/fragments/dissociated forms of these binding proteins have been identified in serum and other fluids, e.g.

amniotic fluid, by covalent cross-linking, Western-blotting and ligand-blotting techniques.

The relationship between the IGF inhibitor (M_r 17000) isolated in this laboratory (Herington & Kufer, 1981) and the higher- M_r binding proteins has not been fully elucidated. Initial experiments indicated that it was immunologically related to the native M_r -150000 GH-dependent binding protein of serum, since an antibody against the GH-dependent, M_r -53000, binding protein (IGFBP-3) derived from the M_r -150000 native form, showed strong cross-reactivity with the inhibitor (Baxter & Martin, 1986). In the present study we have used an inhibitor antiserum (R8) to determine more clearly the possible relationship between the inhibitor and both the GH-dependent and non-GH-dependent binding proteins.

Western-blotting analysis, using inhibitor antiserum, of an IGF-inhibitory fraction of serum (prepared by hydrophobic-interaction chromatography on octyl-Sepharose) showed the presence of several distinct ir bands, of which four (M_r 38000, 34000, 28000 and 23000) possessed IGF-binding activity on ligand blotting. Work by Binoux's group (Hossenlopp *et al.*, 1986; Hardouin *et al.*, 1987) has shown previously, using equivalent ligand-blotting techniques, that serum contained at least five binding-protein subunits (M_r 41500, 38500, 34000, 30000 and 24000), of which the M_r -41500 and -38500 forms were GH-dependent, whereas the M_r -34000, -30000 and -24000 species were not. Our results are very similar and indicate, therefore, that the inhibitor antiserum recognized both classes of binding-protein species (GH-dependent and -independent), suggesting possible immunological similarity between them. This possibility was further investigated by using similar Western- and ligand-blotting analysis on two different chromatographic preparations of human amniotic fluid.

Western blotting, using inhibitor antiserum, of both the amniotic-fluid fraction prepared by gel-permeation chromatography and $(\text{NH}_4)_2\text{SO}_4$ precipitation, gave an intense ir band at M_r 28000, a value identical with that for the predominant binding protein (IGFBP-1) in amniotic fluid (Baxter *et al.*, 1987). Ligand-blotting analysis confirmed that this intense ir band is an IGF-binding protein. Since the M_r -28000 binding protein (IGFBP-1) of amniotic fluid is not GH-dependent (Baxter *et al.*, 1987), our observations indicated that the inhibitor is immunologically related to the non-GH-dependent class of binding protein. This contrasts with the observation of Martin & Baxter (1985), whose antibody against the GH-dependent IGFBP-3 did not cross-react with the non-GH-dependent IGFBP-1 of either serum or amniotic fluid. Conversely, an antibody against IGFBP-1 did not recognize IGFBP-3 of serum (Baxter *et al.*, 1986). The ability of the inhibitor antiserum to recognize both binding proteins indicates that, structurally, these two binding proteins contain regions of internal sequence similar to the inhibitor sequences defining its antigenic epitopes. Studies by others support this: Povia *et al.* (1984b) found that, on acidification, the large serum binding protein generated a smaller binding protein that cross-reacted in a radioimmunoassay for amniotic-fluid binding protein. The most definitive evidence for structural similarity comes from the recently reported full amino acid sequences derived from the cDNAs for these binding proteins (Wood *et al.*, 1988; Brewer *et al.*, 1988). These data reveal limited, but significant, areas of amino acid sequence similarity in both the N- and C-terminal domains, including conservation of all cysteine residues.

In the present study, inhibitor-sized ir protein (M_r 17000) was demonstrated in amniotic-fluid samples after Western-blotting analysis. Although no IGF-binding activity was observed at this M_r on ligand-blotting analysis, the presence of such low- M_r binding protein was demonstrated after covalent cross-linking of

^{125}I -IGF-I to amniotic-fluid samples. Interestingly, the M_r -17000 binding protein can also be found (see Fig. 3) in the amniotic-fluid fraction taken from the M_r -34000–45000 region of the Bio-Gel P30 gel-permeation-chromatography step. The M_r -17000 protein could thus be a degradative fragment of higher- M_r binding proteins, possibly derived during the denaturing SDS/PAGE. Inhibitor-sized degradative fragments (M_r 17000–20000) still capable of binding IGF have been reported by others to be derived from the amniotic-fluid binding protein (Huhtala *et al.*, 1986).

Amniotic-fluid binding protein can inhibit IGF actions *in vitro* (Drop *et al.*, 1979), and in the present study we have confirmed that it similarly inhibits IGF-stimulated lipogenesis. In contrast, however, an amniotic-fluid IGF-binding protein of M_r ~30000 has been reported to enhance the mitogenic actions of IGF on cultured fibroblasts (Elgin *et al.*, 1987). The relationship between this binding protein and the IGF-inhibitory binding proteins described previously, however, is unclear, since an amniotic-fluid binding protein that inhibited IGF-stimulated mitogenesis was also isolated by the same authors (Elgin *et al.*, 1987). The inhibitory activity of IGF-binding proteins has normally been attributed to the prevention of IGF binding to its cell-surface receptor. However, we have been unable to demonstrate inhibitory bioactivity in the rat adipocyte lipogenesis assay of higher- M_r (M_r > 17000) serum binding proteins, and intact IGF-II-binding protein complexes have been shown to be biologically active in this bioassay (Cornell & Herington, 1983; Cornell *et al.*, 1985, 1987). Furthermore, the demonstration of the synergistic effects of the amniotic-fluid binding protein described by Elgin *et al.* (1987) in enhancing the biological actions of IGF-I indicated that there may be a direct interaction between the binding protein and cell-surface receptors, by themselves binding to cell surfaces and delivering IGF to adjacent IGF receptors (Clemmons *et al.*, 1986; De Vroede *et al.*, 1986). This view has been strengthened by the recognition, within the amniotic-fluid binding-protein sequence, of an Arg-Gly-Asp tripeptide known to be involved in cell-attachment mechanisms (Brewer *et al.*, 1988).

The differential ability of the various IGF-binding protein species to either inhibit, enhance or not affect IGF actions *in vitro* points to an important aspect in the regulation of IGF physiology, namely that of the physiological role of the binding proteins *in vivo*. Irrespective of whether the inhibitor represents a true binding-protein subunit, or simply an active degradative fragment, important activities associated with IGF physiology (i.e. IGF binding and IGF inhibitory activities) can be ascribed to it. Our observation of immunological identity between the GH-dependent binding proteins in serum and the non-GH-dependent binding protein in amniotic fluid, as indicated by the inhibitor antibody, suggests that common structural elements exist within these two classes of binding proteins. It remains to be shown whether such common elements are related to, and/or important for, the biological activity of the various IGF-binding proteins.

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