Biochem. J. (1991) 275, 441–446 (Printed in Great Britain)

Synthesis of the acid-labile subunit of the growth-hormone-dependent insulin-like-growth-factor-binding protein complex by rat hepatocytes in culture

Carolyn D. SCOTT* and Robert C. BAXTER
Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, N.S.W. 2050, Australia

Insulin-like growth factors (IGFs) circulate predominantly in a growth-hormone-dependent ternary complex of 125–150 kDa. This study investigates the production of the α-subunit of this complex, an acid-labile glycoprotein without intrinsic IGF-binding activity, which binds to the IGF-binding protein IGFBP-3 in the presence of IGFs. Medium conditioned by primary cultures of rat hepatocytes produced α-subunit with similar complex-forming activity to purified rat serum α-subunit. Bovine growth hormone stimulated hepatocyte production of both IGF-I and α-subunit. IGF-I tracer bound to pure rat IGFBP-3 was converted from approx. 60 kDa to 150 kDa by serum α-subunit, whole rat serum or rat hepatocyte culture medium; this converting activity was destroyed by transient acidification. In contrast, IGF-I bound to hepatocyte-medium IGF-binding proteins could not be converted into a high-molecular-mass form by purified rat serum α-subunit. Rat serum and hepatocyte-medium α-subunit appeared identical by electrophoretic analysis, since reaction of either with cross-linked IGF-I·IGFBP-3 tracer resulted in bands of molecular mass 130 kDa and 160 kDa, probably representing intact and partially deglycosylated complexes. However, IGF-binding proteins in rat serum and hepatocyte medium were different, in that affinity labelling of medium binding proteins, depleted of endogenous IGFs, showed no evidence of the 50–60 kDa cluster of bands characteristic of rat serum IGFBP-3. We conclude that rat hepatocytes in primary culture produce α-subunit similar to that in rat serum; however, α-subunit is unable to form ternary complexes with hepatocyte IGF-binding proteins, since cultured hepatocytes do not secrete IGFBP-3.

INTRODUCTION

Serum insulin-like growth factors (IGFs) are found in both the human and rat circulation predominantly as part of a growth-hormone (GH)-dependent complex of 125–150 kDa [1–4]. The complex in human serum is composed of three subunits: IGF-I or IGF-II (γ-subunit), an acid-stable IGF-binding protein (IGFBP-3 or β-subunit) of about 50 kDa [5–7], and an acid-labile subunit (α-subunit) of approx. 85 kDa which does not bind IGFs [8]. The existence of this acid-labile subunit was originally postulated by both Furlanetto [5] and Hintz & Liu [9], and later confirmed after its purification from human serum in our laboratory [10]. A binding protein homologous to human IGFBP-3 (hIGFBP-3) has been purified from rat serum [11,12], but the structure of the high-molecular-mass complex in rat serum has not yet been shown to be comparable with that in human serum.

Although a variety of rat tissues express the IGF-I gene [13,14], it appears that the liver is the major site of synthesis, and the predominant contributor to circulating IGF-I levels, in the adult rat [15–17]. The origin of circulating IGFBP-3 is less clear. In man, this protein is secreted by fibroblasts [18], vascular endothelial cells [19] and other cell types in culture, and its mRNA has been demonstrated in the liver [20]. In the rat, IGFBP-3 has been identified in cultures of osteoblasts [21] and other cell types [22]; however, no rat liver-derived cell line or primary hepatocyte culture has been shown to produce IGFBP-3. Thus the BRL-3A cell line produces rIGFBP-2 [22,23], and H_4EIIC_3 cells produce rIGFBP-1 [24]. The lack of IGFBP-3 production by liver-derived cells is unexpected, since rat liver, like human liver, is known to express the IGFBP-3 gene [25].

The site of origin of the α-subunit is unknown. Although a specific radioimmunoassay now exists for the human protein [26], no human cell type has yet been demonstrated to synthesize it. In the present study we demonstrate that primary cultures of rat hepatocytes produce α-subunit with activity similar to that of the purified rat serum protein. However, IGFBPs released by rat hepatocytes are unable to combine with rat α-subunit to form high-molecular-mass complexes.

MATERIALS AND METHODS

Materials

BSA (RIA grade), bovine insulin and Williams' E medium were purchased from Sigma (St. Louis, MO, U.S.A.). The Superose 12 HR 10/30 column was obtained from Pharmacia, Sydney, N.S.W., Australia, and the Protein Pak 125 column was from Waters, Milford, MA, U.S.A. Tissue-culture dishes (60 mm diameter) were bought from Costar (Cambridge, MA, U.S.A.). Centricon-10 and Centricon-30 microconcentrators were purchased from Amicon (Danvers, MA, U.S.A.). Protein standards for electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.). $Na^{125}I$ and Hyperfilm-MP were bought from Amersham International (Amersham, Bucks, U.K.). Natural human IGF-I and ^{125}I-labelled IGF-I were as described in previous studies [10]. A covalent complex of hIGFBP-3 and ^{125}I-IGF-I formed by cross-linking with disuccinimidyl suberate was prepared and purified as described previously [27]. Rat IGFBP-

Abbreviations used: IGF, insulin-like growth factor; GH, growth hormone; bGH, bovine GH; IGFBP, IGF-binding protein; α-subunit, acid-labile subunit of the GH-dependent IGFBP complex.

* To whom correspondence and reprint requests should be addressed.

3 (rIGFBP-3) was prepared from rat serum as described previously [11]. Rat serum α-subunit was prepared from a 100 ml pool of rat serum by ion-exchange chromatography and affinity chromatography on an agarose–IGF column saturated with human IGFBP-3, essentially as described for the human α-subunit preparation [10]. Bovine GH (bGH), lot S-32-34, was generously given by Dr. M. Sonnenberg (Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.).

Animals

Adult female Wistar–Furth rats (10–12 weeks) were maintained on a standard chow diet in a 12 h-light/12 h-dark cycle. Hypophysectomy was carried out under ketamine anaesthesia (10 mg/100 g body wt.) by the transaural route, and was confirmed by serum IGF-I levels less than 15% of normal, measured 1 week post-operatively.

Rat hepatocyte culture

Rat hepatocytes were prepared and cultured as described previously [17]. After 20 h in serum-free culture (2×10^6 cells/60 mm culture dish), cells were incubated with 2.5 ml of serum-free Williams' E medium supplemented with 0.3 μM-insulin, 2 g of BSA/l and various test substances. Incubations were carried out in duplicate, and conditioned medium was harvested 24 h later and stored at −20 °C until use.

IGF-I and IGFBP assays

Hepatocyte-medium IGF-I and IGFBP were separated by h.p.l.c. on a Protein Pak 125 column as described previously [17]. IGF-I was assayed by radioimmunoassay, and IGFBP was assayed by incubation with ^{125}I-IGF-I followed by separation of free from bound tracer by charcoal, as in our previous studies [17].

Routine α-subunit assay

Samples were assayed for α-subunit activity essentially as described previously [28]. To concentrate and decrease ionic strength, hepatocyte medium (0.5 ml) was ultrafiltered by Centricon-10 and then diluted to 0.25 ml with α-subunit assay buffer (50 mM-sodium phosphate, 0.15 M-NaCl, 0.2 g of NaN_3/l, pH 6.5, and 10 g of BSA/l). Rat sera to be assayed for α-subunit activity were diluted 100-fold with assay buffer. Samples (100 μl) were then incubated with cross-linked hIGFBP-3·IGF-I tracer (30000–50000 c.p.m.; 3 ng) in a final volume of 250 μl of the same buffer for 30 min at 22 °C. The incubation mixture (200 μl) was then injected on to a Superose 12 gel-permeation column (30 cm × 1 cm) eluted at 1 ml/min in assay buffer without BSA. Fractions (0.5 ml) were collected, and fractions 20–31 were counted for radioactivity in a gamma counter. The column was washed for a further 15 min before the next sample was applied. α-Subunit activity was calculated as a ratio ('150K/60K ratio') of the complexed tracer eluted at 150 kDa (fractions 22–24) to the unconverted tracer eluted at 60 kDa (fractions 25–27). Quantification was carried out by comparison with a standard curve prepared by using dilutions of a sample with high α-subunit activity. All incubations were repeated two or three times.

Reconstitution of 150 kDa complex

In order to test the ability of rat α-subunit, serum or hepatocyte medium to form a 150 kDa complex with IGF-I and rat IGFBP-3, incubations were carried out with 5 ng of rat IGFBP-3 and ^{125}I-IGF-I (20000–30000 c.p.m.; 60–90 pg) in 150 μl of assay buffer for 2 h at 22 °C, followed by addition of the sample to be tested for α-subunit activity in 100 μl of assay buffer and incubation for 30 min at 22 °C. Media samples were concentrated up to 10-fold by Centricon-10 as described for routine α-subunit assay. Incubation mixtures (200 μl) were applied to a Superose 12 column under conditions described above, and radioactivity in fractions 20–36 was determined by gamma counting. Unbound ^{125}I-IGF-I tracer reached a peak in fraction 34. All incubations were repeated two or three times.

SDS/PAGE

Samples incubated with cross-linked IGFBP-3·IGF-I tracer or IGF-I tracer were subjected to SDS/PAGE after cross-linking with 0.2 mM-disuccinimidyl suberate, without reduction, on 6–15%-acrylamide gradient gels. For autoradiography, gels were dried and exposed to film for 72 h at −70 °C.

RESULTS

Assay of α-subunit produced by rat hepatocytes

α-Subunit activity in rat hepatocyte medium was assayed as described previously for human α-subunit [28], by determining the conversion of 60 kDa covalently complexed hIGFBP-3·IGF-I tracer into the 150 kDa form. Fig. 1 shows a dose–response curve for complex conversion by increasing concentrations of medium conditioned for 24 h by cultured rat hepatocytes (2.5 ml of medium/2×10^6 cells in a 60 mm dish). To concentrate and minimize ionic strength, medium was ultrafiltered to 10% of the starting volume in a Centricon-30 micro-concentrator, then diluted to the original volume in α-subunit assay buffer without BSA and re-filtered to 10% of the initial volume. This concentrated medium caused conversion of 60 kDa cross-linked tracer into the 150 kDa complex, producing a dose–response curve parallel to that obtained with purified rat serum α-subunit, which could be used as a standard to quantify changes in α-subunit concentration in hepatocyte medium.

Pituitary dependence of hepatocyte production of α-subunit

The pituitary regulation of rat serum α-subunit *in vivo* was determined by examining the effect of hypophysectomy on levels

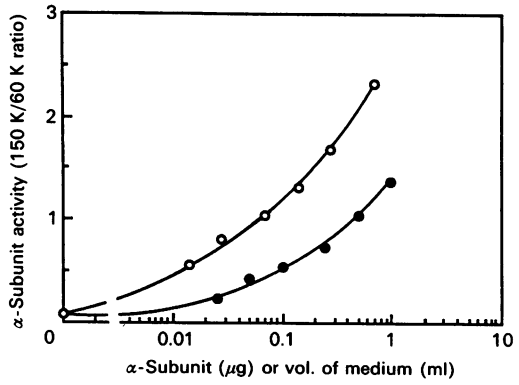

Fig. 1. Dose–response curve for increasing volumes of hepatocyte-conditioned medium in the routine α-subunit assay

The ability of hepatocyte medium to convert cross-linked 60 kDa hIGFBP-3·IGF-I tracer into the 150 kDa complex was determined by Superose 12 permeation chromatography as described in the Materials and methods section. The α-subunit activity is shown as a ratio (150K/60K ratio) of the complexed tracer eluted at 150 kDa (fractions 22–24) to the unconverted tracer eluted at 60 kDa (fractions 25–27) for increasing volumes of medium (●) and compared with pure rat α-subunit (○).

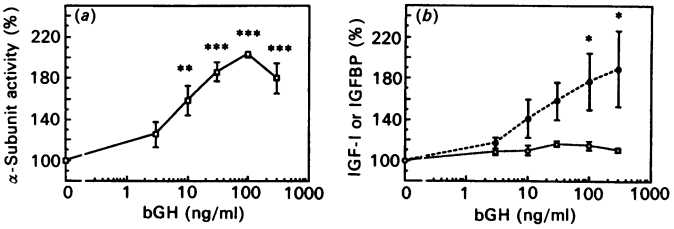

Fig. 2. Stimulation of hepatocyte α-subunit production by bGH

α-Subunit (*a*) and IGF-I (●) and IGFBP (○) (*b*) concentrations were determined in medium conditioned by cultured rat hepatocytes incubated for 24 h with various concentrations of bGH. Results are expressed as a percentage of the basal activity measured in cells incubated in the absence of bGH. The results of four experiments were pooled and shown as means ± S.E.M.: $*P < 0.05$, $**P < 0.005$, $***P < 0.001$ for bGH treatment compared with basal levels, as determined by Duncan's multiple-range test.

of free α-subunit in serum. Serum (1 μl) from rats 2–4 weeks post-hypophysectomy ($n = 4$) was assayed for free α-subunit activity by its ability to convert 60 kDa cross-linked IGFBP-3·IGF-I tracer into the 150 kDa complex, and compared with serum from intact animals ($n = 3$) after construction of a standard curve as described above. Serum concentrations of free α-subunit in hypophysectomized rats were decreased to 18.2 ± 5.4 % of that in control rats ($P < 0.001$ by Student's *t* test). As confirmation of the effectiveness of hypophysectomy, serum IGF-I levels were also determined, by radioimmunoassay, and were found to be decreased to 13.5 ± 2.4 % of those levels in control animals ($P < 0.001$).

The effect of GH *in vitro* on production of α-subunit in cultured hepatocytes was determined by incubating cells with a range of bGH concentrations (3–300 ng/ml) for 24 h. The combined results of four such experiments are shown in Fig. 2. As shown in Fig. 2(*a*), α-subunit production was increased by bGH in a dose-related manner, half-maximally at approx. 10 ng of bGH/ml, with a maximum 2-fold stimulation at 100 ng of bGH/ml ($P < 0.001$ by Duncan's multiple range test). As reported previously, IGF-I production was stimulated to a similar extent by bGH ($P < 0.05$ at 100 ng of bGH/ml), although the slight stimulation of IGFBP activity was not statistically significant (Fig. 2*b*).

To determine whether new protein synthesis is required for secretion of α-subunit by cultured hepatocytes into the medium, cells were incubated for 24 h with or without bGH (100 ng/ml) in the presence or absence of cycloheximide (15 μg/ml). α-Subunit production by cultured hepatocytes in the absence of bGH was partially inhibited by cycloheximide, to 60 ± 2 % ($n = 4$) of basal levels ($P < 0.001$ by Student's *t* test). bGH stimulated α-subunit secretion to 190 ± 18 % ($n = 4$) of basal levels ($P < 0.001$), and this was totally prevented by addition of cycloheximide, which decreased secretion to 68 ± 8 % ($n = 4$) of the basal value.

Reconstitution of the rat 150 kDa IGFBP complex

The ability of rat serum and hepatocyte medium to convert rIGFBP-3 and IGF-I to the 150 kDa IGFBP complex was tested by incubating ^{125}I-IGF-I (20000 c.p.m.) with rIGFBP-3 for 2 h at 22 °C, followed by addition of rat serum, rat hepatocyte-conditioned medium or purified rat α-subunit for 30 min at 22 °C. Gel-permeation chromatography on a Superose 12 column showed a single peak of radioactivity at fraction 34 for un-incubated IGF-I tracer (Fig. 3*a*). After incubation with 5 ng of rIGFBP-3, 79 % of this radioactivity was found in a single peak at fraction 26, corresponding to 60 kDa, as seen previously for covalently bound hIGFBP-3·IGF-I tracer (Fig. 3*b*). Addition of 70 ng of purified rat α-subunit caused about half this rIGFBP-3·IGF-I complex to be converted into a larger species giving a

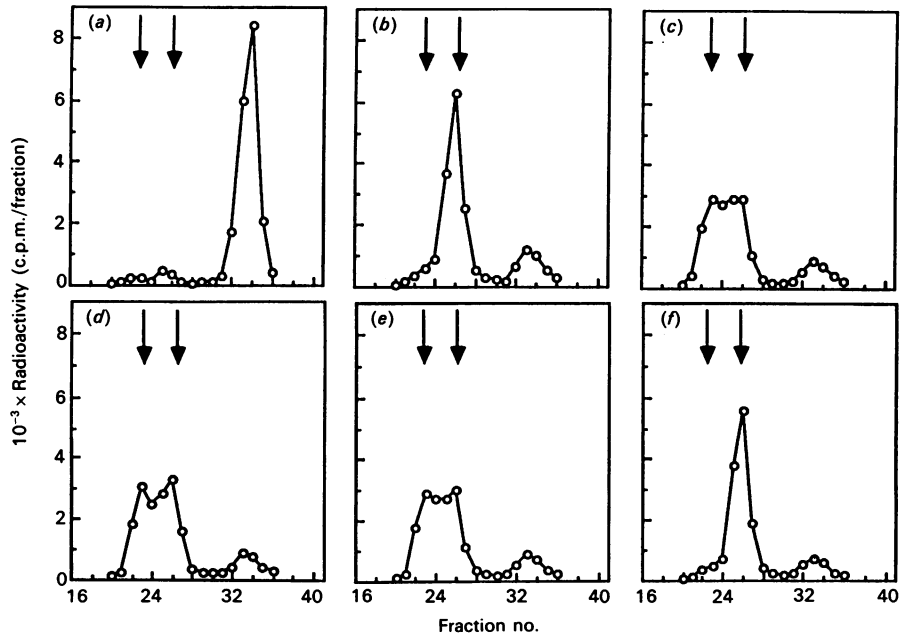

Fig. 3. Ability of rat hepatocyte α-subunit to form 150 kDa complex with rIGFBP-3 and [^{125}I]IGF-I

Radioactivity profiles of IGF-I tracer after Superose 12 gel-permeation chromatography were determined after 2 h incubation without (*a*) or with 5 ng of rIGFBP-3 (*b*) or with 5 ng of rIGFBP-3 followed by 30 min incubation with 70 ng of purified rat α-subunit (*c*), 1 μl of rat serum (*d*), 500 μl equivalent of rat hepatocyte-conditioned medium (*e*) or 500 μl of acidified/re-neutralized hepatocyte medium (*f*). Arrows indicate the peak positions of 60 kDa (fraction 26) and 150 kDa (fraction 23) markers.

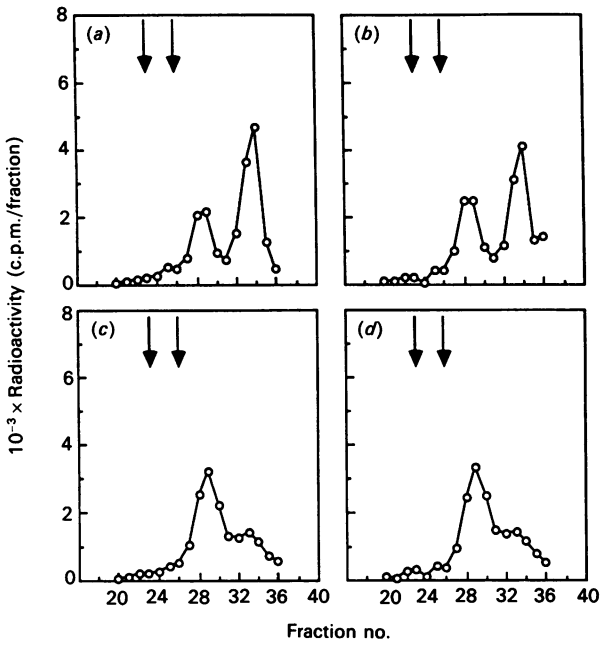

Fig. 4. Inability of rat-hepatocyte-produced IGFBP to form 150 kDa complex

Radioactivity profiles after Superose 12 fractionation were determined for IGF-I tracer incubated for 2 h with 500 μl equivalent of rat hepatocyte-conditioned medium (*a*, *b*) or hepatocyte-medium IGFBP (equivalent to 100 μl of medium) after h.p.l.c. fractionation (*c*, *d*), without (*a*, *c*) or with a 30 min incubation with 280 ng of purified rat α-subunit (*b*, *d*). Arrows indicate the peak elution positions of 60 kDa (fraction 26) and 150 kDa (fraction 23) markers.

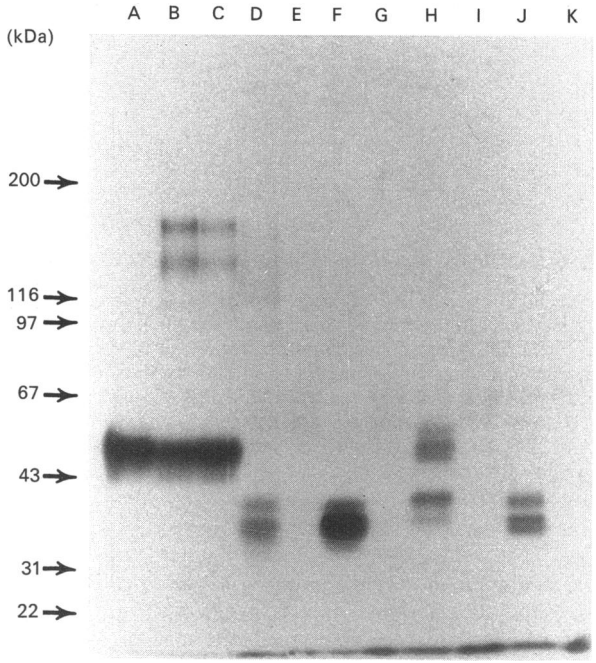

Fig. 5. SDS/PAGE of rat serum and hepatocyte medium incubated with IGF-I and IGFBP-3·IGF-I cross-linked tracers

Electrophoresis on 6–15 %-polyacrylamide gradient gel under non-reducing conditions was carried out for samples incubated with 25000 c.p.m. of IGFBP-3·IGF-I cross-linked tracer (lanes A–C) or 50000 c.p.m. of IGF-I tracer (lanes D–K) after cross-linking with disuccinimidyl suberate (0.2 mM). IGFBP-3·IGF-I cross-linked tracer was incubated alone (lane A), with 1 μl of rat serum (lane B) or with 30 μl equivalent of rat hepatocyte medium (lane C). IGF-I tracer was incubated with 1 μl of rat serum (lanes D, E), 60 μl equivalent of rat hepatocyte medium (lanes F, G), 1 μl equivalent of h.p.l.c.-fractionated rat serum (lanes H, I) or 60 μl equivalent of h.p.l.c.-fractionated hepatocyte medium (lanes J, K) in the absence (lanes D, F, H, J) or presence of 100 ng excess of unlabelled IGF-I (lanes E, G, I, K). Arrows indicate the position of molecular-mass markers shown in kDa.

peak at fraction 23, corresponding to the 150 kDa complex, giving a 150K/60K ratio of 1.10 (Fig. 3*c*). Incubation of rat α-subunit (70 ng) alone with ^{125}I-IGF-I caused no change in the column profile seen in Fig. 3(*a*) (result not shown). Addition of 1 μl of rat serum (Fig. 3*d*) or the equivalent of 500 μl of rat hepatocyte-conditioned medium (Fig. 3*e*) caused a similar conversion of 60 kDa rIGFBP-3·IGF-I complex into the 150 kDa species (150K/60K ratio of 1.07 for rat serum and 0.96 for hepatocyte medium). However, as shown in Fig. 3(*f*), hepatocyte-conditioned medium, adjusted to pH 4 for 30 min and then neutralized before incubation with rIGFBP-3 and IGF-I tracer, was not able to convert the 60 kDa complex into the 150 kDa form, confirming that the converting activity is indeed acid-labile.

The ability of IGFBPs produced by cultured rat hepatocytes to form the 150 kDa complex with rat α-subunit was also tested by incubating IGF-I tracer and rat α-subunit with whole rat hepatocyte-conditioned medium and the neutralized IGF-binding fraction from h.p.l.c.-separated medium (as described for assay of hepatocyte IGFBP production in the Materials and methods section). The column profile of IGF-I tracer alone was as shown in Fig. 3(*a*). Incubation of the equivalent of 500 μl of whole hepatocyte medium (50 μl of 10-fold concentrate) with ^{125}I-IGF-I caused 30 % of the IGF-I tracer to appear as a larger-molecular-mass species, with a peak of radioactivity at fraction 29 (Fig. 4*a*), suggesting that the binding species is somewhat smaller (approx. 35–40 kDa) than the authentic rIGFBP-3·IGF-I complex (approx. 60 kDa), which gives a peak at fraction 26, as seen in Fig. 3(*b*). Addition of 280 ng of rat α-subunit to this incubation of IGF-I tracer with hepatocyte medium caused no change in fractionation of the tracer, with no high-molecular-mass complex formation evident (Fig. 4*b*). Similar results were seen for medium IGFBPs depleted of endogenous IGFs by acidification and h.p.l.c. Incubation of IGF tracer with IGFBPs from the equivalent of 100 μl of hepatocyte medium after this treatment resulted in substantial conversion of the tracer into a higher-molecular-mass form, again giving a peak at fraction 29 (Fig. 4*c*), and no change in this profile was observed in incubations containing 280 ng of rat α-subunit (Fig. 4*d*).

The nature of the binding complexes formed in the incubations performed above were examined by non-reducing SDS/PAGE on 6–15 % gradient polyacrylamide gels (Fig. 5). Incubation of cross-linked hIGFBP-3·IGF-I tracer (25000 c.p.m.) with 1 μl of rat serum (lane B) or 30 μl rat hepatocyte conditioned medium (lane C) results in conversion of the low-molecular-mass tracer (lane A) into two larger radioactive species, of approx. 130 kDa and 160 kDa. Incubation of ^{125}I-IGF-I (50000 c.p.m.) with either 1 μl of whole rat serum (lanes D, E) or 60 μl of whole hepatocyte medium (lanes F, G) resulted in the appearance of a band of radioactivity at 40 kDa and a second band at 32–35 kDa, which may in fact be a doublet. IGF-I binding activity was abolished in both cases by addition of excess quantities of unlabelled IGF-I (200 ng), as shown by lanes E and G. After removal of endogenous IGFs by h.p.l.c. separation, further bands of radioactivity at 50–60 kDa could be seen in rat serum (lane H), which

were absent in the presence of excess IGF-I (lane I). No extra bands were observed in hepatocyte medium after h.p.l.c. separation of endogenous IGFs (lane J). Detailed interpretation of these results is given below in the Discussion.

DISCUSSION

This study demonstrates that rat hepatocytes are a site of production of the acid-labile subunit of the high-molecular-mass IGFBP complex. The existence of such a complex in rat serum, similar to the human serum IGFBP complex [1,3], has been recognized for many years [2]. However, confirmation of the structure of the human complex was not possible until human α-subunit was purified [10], enabling complex-reconstitution studies to be performed [8]. These studies demonstrated the ternary nature of the complex, consisting of IGFBP-3 occupied by IGF-I or IGF-II, in association with the 85 kDa glycoprotein termed the α-subunit. Since no human cell line producing α-subunit has yet been identified, and no probes for α-subunit mRNA have been developed, the sites of origin of human α-subunit remain to be established. However, the present study demonstrates that, at least in the rat, the liver is a site of synthesis of α-subunit. When examined by SDS/PAGE, rat serum or hepatocyte α-subunit appeared to form two distinct ternary complexes, of approx. 130 kDa and 160 kDa, with human IGFBP-3 and IGF-I. Two complexes, of approx. 140 kDa and 120 kDa, were also seen in reconstitution experiments using human α-subunit [8], although in that study the larger complex was distinctly the more prominent of the two, whereas the complexes containing rat α-subunit appear approximately equal. It is not yet clear how these two complexes differ, although we previously suggested that the smaller complex might be a partially deglycosylated form of the larger [8].

Hepatocyte production of α-subunit was found to be dependent on the pituitary status of the donor animal. Although hormonal replacement studies *in vivo* were not performed, the pituitary hormone involved in α-subunit regulation appears to be GH, since GH is a potent stimulator of α-subunit production by cultured hepatocytes. This is consistent with the observation that immunoreactive α-subunit in human serum is markedly GH-dependent [26]. The GH-dependence of hepatocyte α-subunit synthesis is similar to that of IGF-I synthesis, both requiring approx. 10 ng of bGH/ml for half-maximal stimulation. In contrast, IGFBP production by hepatocytes was not GH-dependent in the present study, although we previously reported a small degree of stimulation by bGH [29]. Rat hepatocyte α-subunit was able to convert a binary complex between ^{125}I-labelled IGF-I and rat IGFBP-3 from approx. 60 kDa to approx. 150 kDa, as determined by gel-permeation chromatography. The same activity was seen with an α-subunit preparation purified from rat serum. Similarly to α-subunit from human serum, the preparation from rat hepatocyte medium was irreversibly inactivated on acidification to pH 4.

Surprisingly, when the IGFBP produced by rat hepatocyte cultures was compared with serum IGFBP after binding ^{125}I-labelled IGF-I, it appeared distinctly different in several ways. First, it appeared somewhat smaller on Superose 12 chromatography: whereas rat serum IGFBP-3 bound to IGF-I was eluted in 13 ml, corresponding to a molecular mass of approx. 60 kDa, rat hepatocyte IGFBP-3 bound to IGF-I reached a peak at an elution volume of 14.5 ml, corresponding to 35–40 kDa. Second, unlike rat serum IGFBP-3, the IGFBP in rat hepatocyte medium was unable to react with purified rat serum α-subunit to form a high-molecular-mass complex. The distinction between rat serum and hepatocyte IGFBPs was seen more clearly when examined by SDS/PAGE. Both hepatocyte medium and whole serum gave two bands in the 35–40 kDa region when affinity-labelled with ^{125}I-IGF-I; these might represent IGFBP-1 and IGFBP-2 [22]. However, after acid-stripping to remove endogenous IGFs, serum also showed the 50–60 kDa labelled bands characteristic of rIGFBP-3 [11,12], whereas these bands were absent in hepatocyte medium. These observations are consistent with our earlier findings, based on examination by isoelectric focusing, that rat serum contained some IGFBPs identifiable in hepatocyte culture medium, but also some additional, more acidic, IGFBPs apparently not produced by hepatocytes [17].

These experiments indicate that rat hepatocytes do not produce IGFBP-3, or if so, not in its normally glycosylated form or able to combine with α-subunit. Although a similar conclusion was reached by Schmid *et al.* [21], this is surprising, since both human [20] and rat [25] liver contain IGFBP-3 mRNA. Several explanations for this observation are possible. Firstly, rat hepatocytes may in fact synthesize and secrete IGFBP-3, but this may be acted on by proteases in the medium, causing smaller IGF-binding fragments to appear which are unable to form high-molecular-mass complexes with α-subunit and IGF. However, we have found that purified rat serum IGFBP-3 added to cultured hepatocytes for 24 h is not degraded to smaller IGF-binding species, as detected by affinity labelling and SDS/PAGE, but appears intact and retains its ability to form high-molecular-mass complex with purified rat α-subunit (results not shown). This suggests that endogenously secreted IGFBP-3 should also remain intact in hepatocyte medium. Secondly, rat hepatocytes, while retaining the ability to secrete IGF-I and α-subunit in culture, might lose the ability to secrete IGFBP-3 during their adaptation to conditions *in vitro*. Thirdly, cultured hepatocytes might secrete a smaller form of IGFBP-3, presumably a form with defective or absent glycosylation. If this is not an artifact of cell culture, it implies that the fully glycosylated forms seen in serum are derived from an extrahepatic source. Furthermore, since hepatocyte IGFBPs do not form high-molecular-mass complexes with rat α-subunit, this would imply that IGFBP-3 glycosylation is required for ternary-complex formation. These questions are at present unresolved.

In conclusion, we have demonstrated GH-dependent production of α-subunit by rat hepatocytes. The protein in culture medium appears functionally similar to that isolated from rat serum. However, it is unable to form ternary complexes with rat hepatocyte IGFBPs, apparently because hepatocytes in culture do not produce structurally or functionally normal IGFBP-3.

This work was supported by the National Health and Medical Research Council, Australia.

REFERENCES

1. Zapf, J., Waldvogel, M. & Froesch, E. R. (1975) Arch. Biochem. Biophys. **168**, 638–645
2. Moses, A. C., Nissley, S. P., Cohen, K. L. & Rechler, M. M. (1976) Nature (London) **263**, 137–140
3. Hintz, R. L. & Liu, F. (1977) J. Clin. Endocrinol. Metab. **45**, 988–995
4. Hall, K., Brandt, J., Enberg, G. & Fryklund, L. (1979) J. Clin. Endocrinol. Metab. **48**, 271–278
5. Furlanetto, R. W. (1980) J. Clin. Endocrinol. Metab. **51**, 12–19
6. Morris, D. H. & Schalch, D. S. (1982) Endocrinology (Baltimore) **111**, 801–805
7. Martin, J. L. & Baxter, R. C. (1986) J. Biol. Chem. **261**, 8754–8760

8. Baxter, R. C. & Martin, J. L. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6898–6902
9. Hintz, R. L. & Liu, F. (1980) in Growth Hormone and Other Biologically Active Peptides (Pecile, A. & Müller, E. E., eds.), pp. 133–143, Excerpta Medica, Amsterdam
10. Baxter, R. C., Martin, J. L. & Beniac, V. A. (1989) J. Biol. Chem. **264**, 11843–11848
11. Baxter, R. C. & Martin, J. L. (1987) Biochem. Biophys. Res. Commun. **147**, 408–415
12. Zapf, J., Born, W., Chang, J.-Y., James, P., Froesch, E. R. & Fischer, J. A. (1988) Biochem. Biophys. Res. Commun. **156**, 1187–1194
13. Murphy, L. J., Bell, G. I. & Friesen, H. G. (1987) Endocrinology (Baltimore) **120**, 1279–1282
14. Roberts, C. T., Lasky, S. R., Lowe, W. L., Seaman, W. T. & LeRoith, D. (1987) Mol. Endocrinol. **1**, 243–248
15. D'Ercole, A. J., Stiles, A. D. & Underwood, L. E. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 935–939
16. Schwander, J. C., Hauri, C., Zapf, J. & Froesch, E. R. (1983) Endocrinology (Baltimore) **113**, 297–305
17. Scott, C. D., Martin, J. L. & Baxter, R. C. (1985) Endocrinology (Baltimore) **116**, 1094–1101
18. Martin, J. L. & Baxter, R. C. (1988) Endocrinology (Baltimore) **123**, 1907–1915
19. Bar, R. S., Harrison, L. C., Baxter, R. C., Boes, M., Dake, B. L., Booth, B. & Cox, A. (1987) Biochem. Biophys. Res. Commun. **148**, 734–739
20. Wood, W. I., Cachianes, G., Henzel, W. J., Winslow, G. A., Spenser, S. A., Hellmiss, R., Martin, J. L. & Baxter, R. C. (1988) Mol. Endocrinol. **2**, 1176–1185
21. Schmid, C., Zapf, J., Meier, P., Boeni, M., Ernst, M. & Froesch, E. R. (1989) in Insulin-like Growth Factor Binding Proteins (Drop, S. L. S. & Hintz, R. L., eds.), pp. 267–272, Excerpta Medica, Amsterdam.
22. Yang, Y. W.-H., Brown, A. L., Orlowski, C. C., Graham, D. E., Tseng, L. Y.-H., Romanus, J. A. & Rechler, M. M. (1990) Mol. Endocrinol. **4**, 29–38
23. Margot, J. B., Binkert, C., Mary, J.-L., Landwehr, J., Heinrich, G. & Schwander, J. (1989) Mol. Endocrinol. **3**, 1053–1060
24. Unterman, T. G., Oehler, D. T., Gotway, M. B. & Morris, P. W. (1990) Endocrinology (Baltimore) **127**, 789–797
25. Albiston, A. L. & Herington, A. C. (1990) Biochem. Biophys. Res. Commun. **166**, 892–897
26. Baxter, R. C. (1990) J. Clin. Endocrinol. Metab. **70**, 1347–1353
27. Baxter, R. C. & Martin, J. L. (1986) J. Clin. Invest. **78**, 1504–1512
28. Baxter, R. C. (1988) J. Clin. Endocrinol. Metab. **67**, 265–272
29. Scott, C. D., Martin, J. L. & Baxter, R. C. (1985) Endocrinology (Baltimore) **116**, 1102–1107

Received 18 September 1990/6 December 1990; accepted 18 December 1990