Effect of protein deprivation on insulin-like growth factor-binding proteins in rats

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The effect of protein deprivation on plasma concentration of insulin-like growth factor-binding proteins (IGFBP) was studied in rats. A significant decrease in the concentration of IGFBP of molecular weight (mass) approximately 40 kDa was observed in protein-deprived rats. There was no prominent effect of protein deprivation on the concentration of IGFBP with molecular weights of about 30 kDa or 22–24 kDa. The binding capacity to plasma IGFBP of exogenously-added ^{125}I-labelled insulin-like growth factor-1 (^{125}I-IGF-1) was also studied. IGFBP of molecular weight about 30 and 22–24 kDa (the native form of this protein is presumed to be 29 kDa) in protein-deprived rat plasma bound more ^{125}I-IGF-1 than those in protein-fed rat plasma. This suggested that these IGFBP in protein-deprived rat plasma are relatively unsaturated by endogenous IGF-1. The response of IGFBP to protein deprivation which was elucidated in the present investigations add further evidence to our previous assumption that IGFBP play an important role in protein nutrition.

Insulin-like growth factor-1: IGF-binding protein: Protein deprivation: Rat

Insulin-like growth factor-1 (IGF-1) is a protein, the concentration of which is largely affected by growth hormone and nutritional status of man and animals (for review, see Van Wyk, 1984; Froesch *et al.* 1985; LeRoith & Raizada, 1989). In a previous paper, we demonstrated that the plasma concentration of IGF-1 is regulated by the quantity and nutritional quality of dietary proteins (Takahashi *et al.* 1990).

This hormone has been shown to circulate in blood in forms conjugated with several kinds of proteins known as IGF-binding proteins (IGFBP) (Cohen & Nissley, 1975; Hintz & Liu, 1977). At least three groups or species of IGFBP have been demonstrated in human or rat plasma (Kaufmann *et al.* 1977, 1978; Binoux *et al.* 1982) (Fig. 4 explains the species of IGFBP). The first involves a complex of the molecular weight (mass) 150 kDa. This IGFBP can be chemically dissociated into two components which have been named as acid-labile subunit (ALS) and acid-stable subunit (ASS) (Furlanetto, 1980; Martin & Baxter, 1985; Wilkins & D'Ercole, 1985; Baxter, 1988). IGF is bound to ASS. ASS is a glycoprotein of molecular weight approximately 53 kDa (Martin & Baxter, 1986). However, sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) gives a smaller molecular weight component, i.e. about 40 kDa (Rechler *et al.* 1989*a*). This subunit has been recently named IGFBP-3 (Ballard *et al.* 1989). The second IGFBP has a molecular weight of approximately 30 kDa. This protein has been purified from amniotic fluid (Drop *et al.* 1984; Povoa *et al.* 1984; Busby *et al.* 1988*a*) and the recent nomenclature is IGFBP-1 (Ballard *et al.* 1989). The third is that with a molecular weight approximately 22–24 kDa (Hardouin *et al.* 1987). However, this protein may exist as a glycoprotein of molecular weight a little smaller (approximately 29 kDa) than IGFBP-1, and may be

converted to the 22–24 kDa molecule by the action of N-glycanase (Rechler *et al.* 1989*a*, *b*; Yang *et al.* 1989). These authors suggested that this 29 kDa protein is a N-terminal 29 kDa fragment of IGFBP-3.

In human plasma, IGF-1 is primarily found to be conjugated with IGFBP-3 and shows an apparent molecular weight of 150 kDa (Moses *et al.* 1979; Hardouin *et al.* 1987). The plasma concentration of this IGFBP has been shown to be affected largely by growth hormone (Moses *et al.* 1976; White *et al.* 1981; Grant *et al.* 1986). The physiological significance of other IGFBP is controversial (Knauer & Smith, 1980; Cornell *et al.* 1987; Elgin *et al.* 1987; DeMellow & Baxter, 1988; Ritvos *et al.* 1988; Busby *et al.* 1989).

In the previous paper (Takahashi *et al.* 1990), we demonstrated that IGF-1 is found in rat plasma in two forms, i.e. one associated with a protein of molecular weight 150 kDa, and another with that of 40 kDa or smaller. The total and relative amounts of IGF-1 bound to each protein were affected by dietary protein source. Furthermore, there was a large difference between the dietary groups in the capacity of plasma fractions to bind exogenously-added ^{125}I-labelled IGF-1 (^{125}I-IGF-1). The explanation of these was based on the extent of saturation of IGFBP with endogenous IGF-1.

In the previous experiments, we employed a method of gel filtration for separation of IGFBP. That method could not differentiate all the molecular species of IGFBP. A recently-introduced method which separates IGFBP on sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) makes it possible to differentiate the small differences among IGFBP (Hossenlopp *et al.* 1986; Hardouin *et al.* 1987). In the present investigations, SDS–PAGE was used and the previous studies on the effect of protein status on plasma IGFBP in rats extended.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats were obtained from Japan SLC company (Shizuoka, Japan) and used throughout the experiments. The body-weight was approximately 100 g. The protein-free and casein (120 g/kg diet) diets were prepared as described previously (Takahashi *et al.* 1990). Rats were fed on these diets and water *ad lib.* for 1 week. At 11.30 hours on the 8th day they were anaesthetised with Nembutal, and blood samples were taken from the carotid artery.

Materials

IGF-1 was purchased from Boehringer Mannheim-Yamanouchi (Japan), and bovine serum albumin (BSA) from Sigma. Nylon membrane (GeneScreen Hybridization Transfer Membrane) was from NEN Research Products, Boston, MA, USA. Na^{125}I ($3{\cdot}7 \times 10^9$ Bq/ml) was obtained from Amersham (Japan Radioisotope Association). A molecular marker kit for SDS–PAGE was obtained from BioRad. Other chemicals were of the purest grade available commercially.

Determination of immunoreactive IGF-1 concentration and that after extraction with acid–ethanol (or total immunoreactive IGF-1 concentration)

The methods were the same as described previously (Takahashi *et al.* 1990).

Iodination of IGF-1 with ^{125}I

The method was similar to that reported previously (Takahashi *et al.* 1990). Briefly, 10 μl Na^{125}I (37 MBq) and 10 μl chloramine T solution (1 mg dissolved in 1 ml 0·2 M-phosphate buffer, pH 7·4) were added to 10 μl IGF-1 solution (recombinant human IGF-1, 1 μg/10 μl

water). The mixture was mixed well and kept at room temperature for 60 s. Then sodium metabisulphite solution (2·5 mg sodium metabisulphite dissolved in 1 ml 0·05 M-phosphate buffer, pH 7·5) was added in order to stop the reaction. From this mixture, the labelled IGF-1 was extracted three times with 100, 50 and 50 μl of transfer solution (1 litre of the solution contained 10 g potassium iodide, 80 g sucrose and 1 g Triton X-100 in distilled water). The extract was subjected to gel-filtration on a plastic column packed with Sephadex G-25M (bed volume 9 ml, 50 mm height; Pharmacia) previously equilibrated with phosphate-buffered saline solution (0·14 M-sodium chloride-0·01 M-sodium phosphate, pH 7·5) containing 10 g BSA/l. The labelled IGF-1 was eluted with the same phosphate-buffered saline solution. Two peaks with radioactivity were eluted. The first peak was labelled IGF-1. The preparation was stored at −20°. Just before use, this stored preparation was purified further by gel-filtration using an acryl column (15 mm diameter and 500 mm height) packed with Sephacryl S-200. The labelled IGF-1 was eluted with a solution containing 0·15 M-sodium chloride, 0·01 M-Tris–hydrochloric acid, 10 g BSA/l and 0·5 g sodium azide/l. Three radioactive peaks were obtained; i.e. the peak associated with BSA, free IGF-1 and inorganic $^{125}I^-$. The second peak was used as the ^{125}I-IGF-1 preparation.

Analysis of IGFBP by Western ligand blotting

The method of Hossenlopp *et al.* (1986) and Hardouin *et al.* (1987) was employed. Briefly, 40 μl plasma was mixed with an equal volume of SDS solution (1 litre of the solution contained 100 g β-mercaptoethanol, 200 g glycerol, 50 g SDS, 20 mmol Tris and 2 mmol EDTA, pH 6·8) and incubated for 15 min at 60°. A portion (40 μl) of the mixture was used for SDS–PAGE. The gel was 135 × 135 mm and 2 mm thick with 125 g polyacrylamide/l. The polyacrylamide gel was prepared according to Laemmli (1970). The electrophoresis was performed at 180 V. After electrophoresis, proteins were transblotted to nylon membrane. After blotting, the membrane was incubated for 30 min at 4° in 30 g NP40/l saline solution (0·15 M-NaCl, 0·01 M-Tris–HCl, 0·5 g sodium azide/l). Then the membrane was again incubated in 10 g BSA/l in the saline solution for 6 h at 4°. By these incubation procedures, non-specific binding of labelled IGF-1 was minimised. The membrane was incubated further in 1 g Tween 20/l saline for 10 min at 4° in order to remove BSA. The membrane was then incubated with 1·8 ml ^{125}I-IGF-1 solution (2–3 × 10^6 counts/min (cpm)) for 10 h at 4° and then washed twice with the Tween 20 solution (each washing was for 15 min at 4°). The radioactivity which was bound to IGFBP was detected by autoradiography. After autoradiography, the portion with radioactivity on the filter was cut out and the radioactivity was determined with a gamma-counter (Aloka Auto Well Gamma System ARC-500).

Cross linking of ^{125}I-IGF-1 with IGFBP

In order to determine the amount of IGFBP not associated with endogenous IGF-1, the following procedure (Hardouin *et al.* 1987) was used.

Purified ^{125}I-IGF-1 (10 μl, approximately 50000 cpm, the amount was not exactly estimated but was more than that to saturate the vacant IGFBP) was mixed with 20 μl plasma and the mixture was incubated for 16 h at 4°. Then 10 μl disuccimydyl suberate (DSS) solution (10 mg DSS/ml dimethylsulphoxide–water mixture (80:20, v/v)) was added and the mixture was incubated for 15 min at 4°. By this incubation, ^{125}I-IGF-1 was covalently bound to IGFBP which had not been saturated with endogenous IGF-1. To this mixture, 40 μl SDS solution was added and incubated for 15 min at 60°. A 40 μl portion of the solution was used for SDS–PAGE. Electrophoresis conditions were as described

previously except the gel was 1 mm thick. After electrophoresis, the gel was dried by a gel-dryer and the radioactivity in the dried gel was detected by autoradiography. The results of autoradiography were analysed using a densitometer (Computing densitometer ACD-18; ATTO, Tokyo, Japan). This method may make it possible to determine the amount of IGFBP not saturated with endogenous (unlabelled) IGF-1. However, other possibilities cannot be excluded (see p. 113).

Statistical analysis

The results were analysed statistically by one way classifications (analysis of variance, Snedecor & Cochran, 1967).

RESULTS

Table 1 shows the body weight changes and plasma immunoreactive IGF-1 or total IGF-1 concentration in the rats fed on the casein or protein-free diet. The results confirm our previous observations (Takahashi *et al.* 1990).

Fig. 1 shows the results of autoradiography after Western ligand blotting. Four radioactive bands were observed, i.e. two bands at about 40 kDa, one at about 30 kDa and one at about 22 kDa. The two bands found in the area of 40 kDa were found in the A fraction of the earlier gel filtration (Fig. 2). These two bands probably are the same protein but glycosylated to different extents (Rechler *et al.* 1989*a*, *b*; Yang *et al.* 1989). The bands at about 30 kDa and about 22 kDa were in the C fraction obtained earlier. In Fig. 2, 30 kDa IGFBP showed two bands (the larger one will be referred to as 30 kDa IGFBP and smaller one as 29 kDa IGFBP). The amount of 22 kDa band was very small. Therefore, we concluded that the two bands at about 40 kDa (Fig. 1) were derived from the large binding protein (Takahashi *et al.* 1990) and bands at about 30 and 22 kDa were derived from the small binding protein. Tentatively, we refer to the two bands at 40 kDa as IGFBP-3, and the band at 30 kDa as IGFBP-1. From the assumption described on p. 113, 29 kDa IGFBP is tentatively presumed to be undegraded or native 29 kDa IGFBP and 22 kDa IGFBP as degraded 29 kDa IGFBP. Rechler *et al.* (1989*a*, *b*) and Yang *et al.* (1989) suggested that this protein is the N-terminal fragment of IGFBP-3.

Table 2 shows the results of the determination of radioactivity found in the fractions of Fig. 1. These results were obtained by counting the radioactivity after dissecting the bands. The total radioactivity of the bands at about 40 kDa decreased significantly after protein deprivation.

Fig. 3 shows the results of cross-linking experiments. Three bands, one at 150 kDa and two bands at about 35 and 33 kDa, were found in protein-deprived rats and two additional bands, one at about 25 kDa and another at about 20 kDa in casein-fed animals. The dense bands at the bottom of the figure show the unbound ^{125}I-IGF-1 which is comparable to peak D reported in the previous paper (Takahashi *et al.* 1990, Fig. 2). This confirms that an excess amount of labelled IGF-1 for saturation of vacant IGFBP was used in the experiments. The molecular weight of IGF-1 cross-linked to IGFBP must be apparently larger than the binding protein not associated with IGF-1 by the molecular weight of IGF-1, 7 kDa. Besides, the acid-labile subunit, the molecular weight of which has been reported to be about 90–110 kDa (Baxter *et al.* 1989), and acid-stable subunit, *i.e.* 40 kDa binding protein (Furlanetto, 1980; Martin & Baxter, 1985, 1986; Baxter, 1988), are assumed to be non-covalently bound to each other in native condition. This complex is dissociated into subunits during SDS–PAGE. However, if plasma is treated with DSS, covalent bonds between ASS, ALS and IGF-1 are presumed to be formed and this complex

Table 1. *Effect of protein deprivation on plasma immunoreactive (IR) insulin-like growth factor-1 (IGF-1) concentration in rats*

(Values are means for five rats)

Diet	Initial body-wt (g)	Final body-wt (g)	IR-IGF-1 (A) (U/ml)	Total IGF-1 (B) (U/ml)	A:B
Protein-free	106	97	1·12	6·78	0·16
Casein (120 g/kg diet)	107	147	6·49	25·1	0·26
Residual mean square	7·2	20·6	0·10	30·8	0·003
F	0·35	306	716	27·4	9·75
P	> 0·05	< 0·01	< 0·01	< 0·01	< 0·05

For details of dietary regimen, see p. 106.

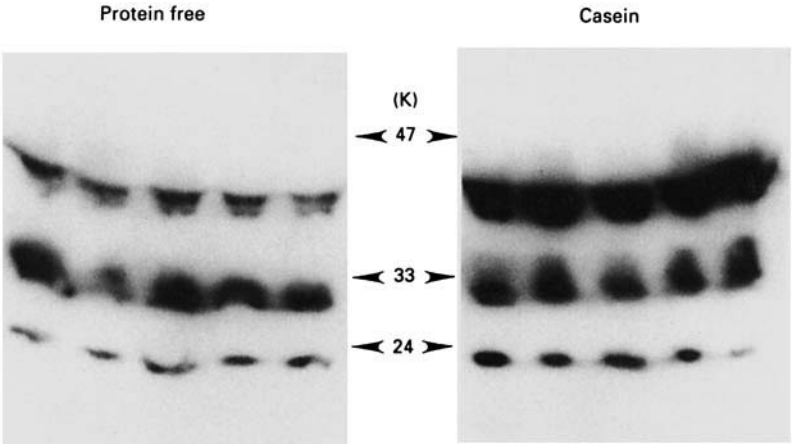
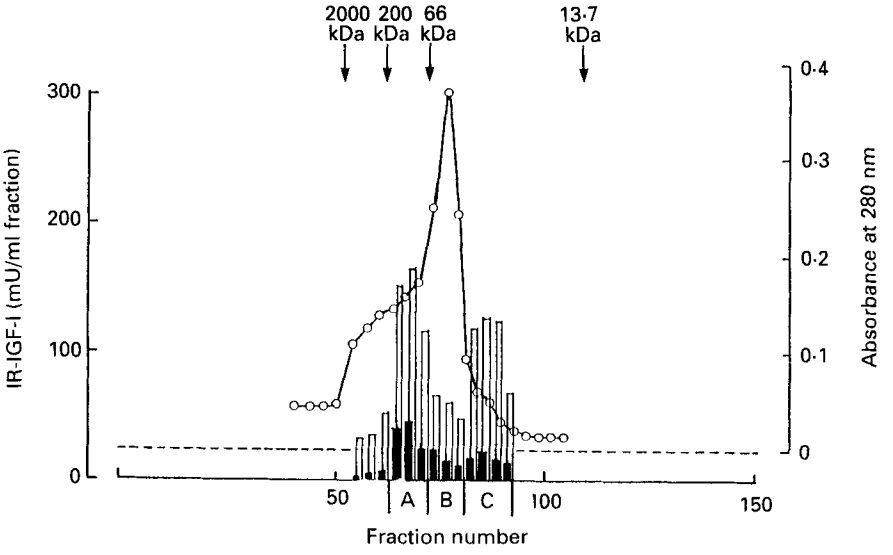


Fig. 1. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) of plasma insulin-like growth factor (IGF)-binding proteins detected by Western ligand blotting (Hossenlopp *et al.* 1986; Hardouin *et al.* 1987). Rat plasma was subjected to SDS–PAGE. After electrophoresis, proteins were transferred to nylon membrane. The proteins which bound ^{125}I-labelled-IGF-1 were detected by autoradiography. Rats were fed on protein-free or casein (120 g/kg diet) diets (for details of dietary regimen, see p. 106).

is probably found at 150 kDa. The band at about 35 kDa is presumed to be 30 kDa IGFBP (IGFBP-1) (Fig. 1) with bound radioactive IGF-1. The difference from the expected molecular weight (if 30 kDa IGFBP binds IGF-1, the molecular weight must be 37 kDa) is probably due to the three-dimensional conformation of the complex (Baxter *et al.* 1987; Rosenfeld *et al.* 1989). The band at about 33 kDa may be 22 kDa IGFBP in Fig. 1. However, 22 kDa IGFBP is presumably produced during incubation with SDS (for discussion, see p. 113) and the native form of the proteins may be 29 kDa IGFBP in Fig. 2. If plasma is treated with DSS before treatment with SDS, conversion of 29 kDa IGFBP to 22 kDa is presumed to be inhibited. Therefore, undegraded 29 kDa IGFBP is found at the position of 33 kDa. We assume that the small difference in expected *v.* observed molecular weight (a 29 kDa IGFBP plus IGF-1 should give 36 kDa complex) in this case is also due to the conformation of the complex. The proteins which appear as the bands at about 25 and 20 kDa after conjugation with ^{125}I-IGF-1 were not detected by Western ligand blotting. These proteins may be sensitive to SDS–PAGE and lose the ability to bind



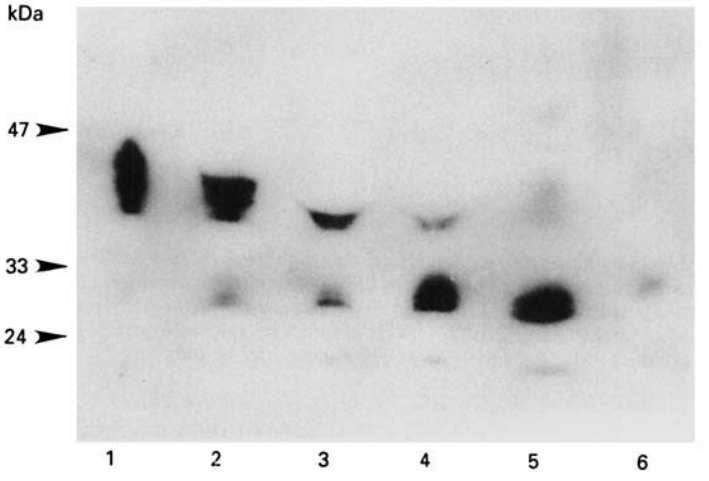


Fig. 2. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) of the fractions of Sephacryl S-200 gel filtration of rat plasma proteins and detection of insulin-like growth factor-binding proteins by Western ligand blotting (Hossenlopp *et al.* 1986; Hardouin *et al.* 1987). Rat plasma was subjected to Sephacryl S-200 gel filtration. The pooled fractions were used as the samples for SDS–PAGE. After SDS–PAGE, insulin-like growth factor-binding proteins were detected by Western ligand blotting (see Fig. 1). (1), Fractions 56–61; (2), fractions 62–67; (3), fractions 68–73; (4), fractions 74–79; (5), fractions 80–85; (6), fractions 86–91. For gel filtration, see Takahashi *et al.* (1990). IR-IGF-I, immunoreactive IGF-I.

IGF-1 after SDS–PAGE. The origin of these proteins will also be discussed later. Figure 4 explains the relationship of the bands among Figs. 1, 2 and 3.

Table 3 shows the results of densitometry of Fig. 3. The results are expressed as the relative density of the bands and are not absolute values. The bands of about 35 and 33 kDa (IGFBP-1 and 29 kDa IGFBP conjugated with ^{125}I-IGF-1) increased significantly in protein-deprived rats. This is presumed to be the increase in unsaturated IGFBP, because

Table 2. *The effect of protein deprivation on the plasma insulin-like growth factor (IGF)-binding proteins which were detected by the activity to bind ^{125}I-labelled IGF-1 after SDS-polyacrylamide gel electrophoresis (counts/min × 10^{-2})*

(Values are means for five rats)

Protein species... Diet	40 kDa	30 kDa	22 kDa	Total
Protein-free	37	60	11	108
Casein (120 g/kg diet)	130	55	15	200
Residual mean square	222	71	19	391
F	96·5	1·09	1·75	52·7
P	< 0·01	> 0·05	> 0·05	< 0·01

For details of dietary regimen, see p. 106.

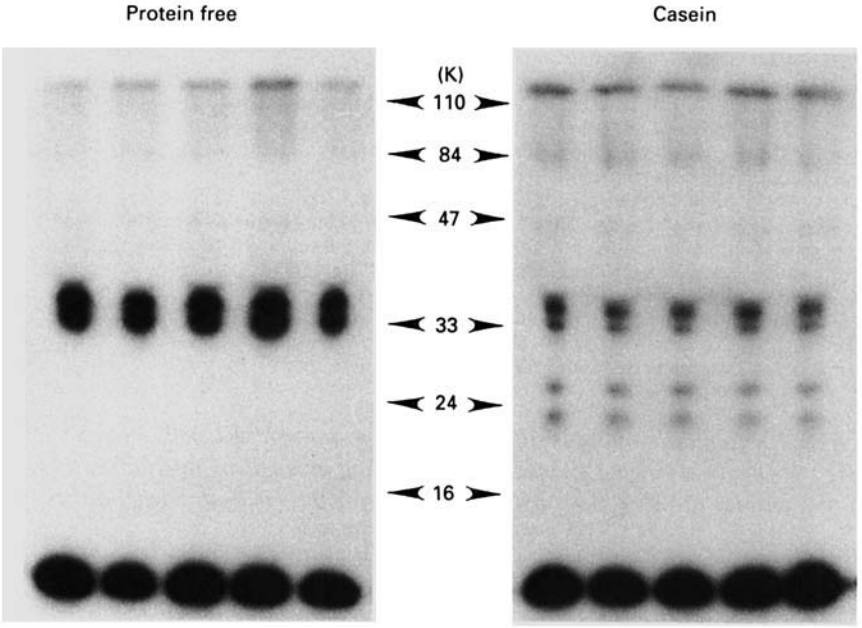


Fig. 3. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) of the insulin-like growth factor-binding proteins covalently bound with labelled IGF-1. ^{125}I-labelled IGF-1 was covalently bound to IGF-binding proteins in rat plasma which had the capacity to bind exogenously added IGF-1. Then the plasma proteins were subjected to SDS–PAGE. The labelled proteins were detected by autoradiography. Rats were fed on protein-free or casein diets (for details of dietary regimen, see p. 106).

the total amount of these IGFBP did not change significantly (Table 2). These observations are consistent with the former observation that the small BP in protein-deprived rats binds a larger amount of labelled IGF-1 than that in protein-fed rats (Takahashi *et al.* 1990). On the contrary, the bands at 150 kDa, 25 kDa or 20 kDa increased in casein-fed rats (Fig. 3). The increase in radioactivity at 150 kDa is probably due to the increase in unsaturated 150 kDa IGFBP, because the total amount of its subunit, i.e., 40 kDa IGFBP in Fig. 1, increased significantly. The reason for the increase in the 25 and 20 kDa bands, *i.e.* whether this is due to the increase in either unsaturated BP or in the total amount of the BP, cannot

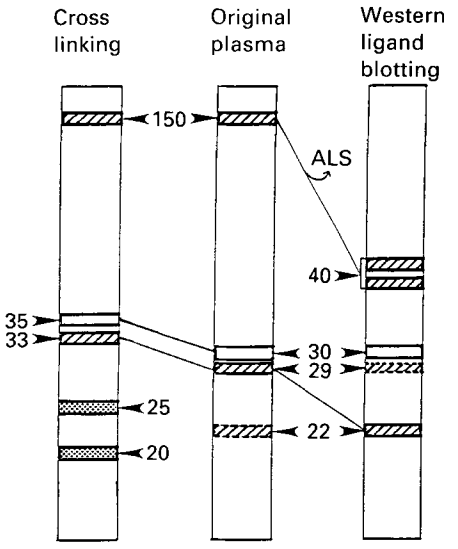


Fig. 4. Schematic representation of the relationship among the bands of IGF-binding proteins (IGFBP) shown in Figs. 1–3. Cross linking: IGFBP detected by autoradiography after sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) of the plasma mixed with ^{125}I-IGF-1. After mixing with ^{125}I-IGF-1, the plasma IGFBP were covalently linked with the labelled IGF-1 by means of disuccimidyl suberate. Therefore, the molecular weights of IGFBP are apparently larger than that of the original IGFBP by the molecular weight of IGF-1 (approximately 7,000). For details of the method, see Materials and Methods (pp. 107–108). Original plasma: supposed IGFBP in rat plasma deduced from evidence presented by the present authors and many others. Besides the molecular species presented in this figure, at least two species, which form 25 and 20 kDa bands after cross-linking, must be present in the plasma. Western ligand blotting: IGFBP detected by blotting with the ligand (^{125}I-IGF-1) after SDS–PAGE of the plasma. For details of the procedure, see Materials and Methods (p. 107). ALS: acid-labile subunit of 150 kDa binding protein. Bands existing in small amounts, if any (ZZZZ); IGFBP-3 (ZZZ); IGFBP-1 (═══); unidentified IGFBP (▒▒▒). Numbers are molecular weights (kDa). The band at 29 kDa is the N-terminal 29 kDa protein of 40 kDa IGFBP-3 (Rechler *et al.* 1989*a*, *b*).

Table 3. *The densitometric analysis of the autoradiogram of ^{125}I-labelled insulin-like growth factor (IGF)-1 which was bound to plasma IGF-binding proteins of rats fed on a protein-free or casein diet (values are expressed as the relative optical density by densitometry)*

(Values are means for five rats)

Protein species... Diet	150 kDa	35 and 33 kDa	25 kDa	20 kDa	Total
Protein-free	142	1865	ND	ND	2007
Casein (120 g/kg diet)	286	642	127	130	1185
Residual mean square or mean square (for 25 and 20 kDa)	1765	79973	170	553	93386
F	29·4	46·8			18·1
P	< 0·01	< 0·01			< 0·01

ND, not detected.

For details of dietary regimen, see p. 106.

The values are expressed as the relative density of the spots of the autoradiogram. Therefore, the absolute values are meaningless.

be explained at present because these proteins were not observed in Western blotting experiments.

DISCUSSION

Firstly, let us consider the molecular species of IGFBP in rat plasma. At present, we agree with the discussion of Rechler *et al.* (1989*a*, *b*) and Yang *et al.* (1989) and explain our results as follows. By SDS–PAGE, four IGFBP bands are detected. The two at about 40 kDa are IGFBP-3; these two bands are the same protein with different sugar contents. The band at 30 kDa is IGFBP-1. The band at 22 kDa was produced from 29 kDa IGFBP during incubation with SDS at 60°, probably by the endogenous glycosidase (or, less likely, by endogenous protease). Thus, if plasma is gel-filtrated before SDS–PAGE (Fig. 2), only a little 22 kDa protein is produced. The endogenous enzyme(s) must be contained in fractions other than those with 29 kDa protein (probably fraction A). Furthermore, if the original plasma is treated with DSS before SDS–PAGE, 22 kDa protein is not produced because DSS denatures the endogenous enzyme(s) or 29 kDa IGFBP cross-linked to IGF-1 is resistant to the endogenous enzyme(s). The 29 kDa IGFBP may be the N-terminal fragment of IGFBP-3 as suggested by Rechler *et al.* (1989*a*, *b*) and Yang *et al.* (1989). We presume that 29 kDa IGFBP exists in native plasma not bound with ALS (if 29 kDa IGFBP is found in plasma non-covalently bound with ALS, the complex will be recovered in the fraction A of Fig. 2), because this IGFBP was found in the experiments shown in Figs. 2 and 3. These hypotheses are summarized in Fig. 4.

The present investigations extend the earlier observations and elucidate the mechanisms involved (Takahashi *et al.* 1990). The results show that the total amount of IGFBP and the amount of IGF-1-saturated binding proteins are affected greatly by the status of protein nutrition. As shown in the present paper, the amount of large binding protein or 150 kDa IGFBP containing the 40 kDa subunit, IGFBP-3, decreases greatly with protein deprivation. Although the total amount of small binding protein, which is composed of at least two components, did not change significantly, the saturability of the binding protein was affected greatly by protein deprivation. Even if 29 kDa IGFBP is a N-terminal fragment of IGFBP-3, it is probably not an artifact produced during plasma handling, because the response of this protein to nutritional status of the animals was clearly different from that of IGFBP-3 (Tables 1 and 2).

From these results and the previous observations, we conclude the effect of protein deprivation on IGF-1 and IGFBP is as follows. When rats are deprived of dietary protein, the amount of large binding protein decreases concomitant with a decrease in IGF-1 associated with this protein. At the same time, the amount of IGF-1 bound to small binding proteins decreases because the total amount of IGF-1 in plasma decreases. The total amount of small binding protein is not affected significantly but the saturability of small binding protein decreases significantly.

There is no clear explanation at present on the physiological role of different kinds or groups of binding proteins (Cornell *et al.* 1987). The 150 kDa IGFBP, which is composed of two subunits (Furlanetto, 1980; Martin & Baxter, 1985; Baxter, 1988), has been assumed to be IGF-1 reservoir protein in humans (Busby *et al.* 1988*a*; Blum *et al.* 1989) because almost all IGF-1 is found associated with this protein in plasma (Moses *et al.* 1979). The amount of this protein is presumably regulated by the amount of acid-stable subunit (40 kDa) or IGFBP-3 because relatively larger amounts of acid-labile subunit are assumed to be produced (Furlanetto, 1980; Martin & Baxter, 1985; Baxter, 1988). However, there is no clear information concerning the tissue which produces this protein or its subunits. Fibroblasts, osteoblasts or vascular epithelial or endothelial cells have been suggested as sources (Binoux *et al.* 1981; Rutanen *et al.* 1985, 1986; Koistinen *et al.* 1986; Bar *et al.*

1989; Schmid, *et al.* 1989). Very recently, IGFBP-3 mRNA was demonstrated in rat liver by Northern blot analysis (Albiston & Herington, 1990). Before final conclusions can be drawn, detection of mRNA (or other technical approaches) of the subunits in various tissues will be required (Brewer *et al.* 1988; Lee *et al.* 1988; Brown *et al.* 1989).

The role of small binding proteins is not known either. Although the total amount of these proteins was not affected significantly by protein deprivation, the amount has been shown to increase during fasting (Rechler *et al.* 1989*b*) and change diurnally (Baxter & Cowell, 1987; Busby *et al.* 1988*b*). It reaches maximum concentration before a meal. This diurnal rhythm will not affect the present results, because we took all the blood samples at around 11.30 hours in the morning. There is a discrepancy concerning the effect of these small binding proteins on the biological activity of IGF-1. Some experiments suggest that the binding proteins suppress the activity of IGF-1 (Knauer & Smith, 1980; Ritvos *et al.* 1988) and others that they enhance the activity (Elgin *et al.* 1987; Busby *et al.* 1989). It is possible that the binding proteins enhance the IGF-1 activity in some tissues and depress it in others (DeMellow & Baxter, 1988).

The origin of binding proteins in 22 kDa and 15 kDa fractions (Fig. 3) is not known yet. Baxter and his coworkers showed that the subunit of 150 kDa binding protein, i.e. IGF-BP-3, is easily dissociated to small fractions which still have a capacity to bind IGF-1 (Martin & Baxter, 1985; Baxter, 1988). The two small binding proteins may be the product of IGFBP-3. However, further studies are required to prove this. It is also possible that one or more of these binding proteins is the IGF-1 inhibitor reported by Herington and others (Herington & Kuffer, 1981; Ooi & Herington, 1986, 1988; Wang *et al.* 1988).

The present results clearly showed that the amount of IGFBP, and possibly their saturability, is largely affected by the status of protein nutrition. IGF-1 has been suggested to play important roles in protein anabolism. If IGFBP affect the activity of IGF-1 as suggested by many investigators, elucidation of the changes in IGFBP under various nutritional conditions will be very important in the assessment of the status of protein nutrition of man, particularly in protein-deprived children or patients losing body protein because of various diseases.

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