Glycosaminoglycans inhibit formation of the 140 kDa insulin-like growth factor-binding protein complex

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The 140 kDa insulin-like growth factor (IGF)-binding protein complex in human serum consists of three subunits: an acid-labile, non-IGF-binding glycoprotein (α -subunit), an IGF-binding glycoprotein known as BP-53 or IGFBP-3 (β -subunit), and IGF-I or IGF-II (γ -subunit). This study investigates the regulation, by salt and glycosaminoglycans, of ternary (α - β - γ) complex formation, measured by incubating radioiodinated α -subunit with a mixture of IGF-I and IGFBP-3 and precipitating bound radioactivity with an anti-IGFBP-3 antiserum. Increasing NaCl concentrations progressively decreased ternary complex formation without any effect on binary (β - γ) complex formation. In 0.15 M NaCl, the association constant for the ternary complex was $0.318 \pm 0.092 \text{ nm}^{-1}$, 100-fold lower than that for the binary complex. Glycosaminoglycans also inhibited ternary complex formation without affecting the binary complex. Heparin [50 % inhibition at $0.27 \pm 0.08 \text{ units/ml}$ ($1.5 \pm 0.4 \text{ } \mu\text{g/ml}$)] was more potent than heparan sulphate (50 % inhibition at $15 \pm 7 \text{ } \mu\text{g/ml}$), with chondroitin sulphate even less potent. The inhibition by heparin was due principally to a decrease in binding affinity, from 0.604 ± 0.125 to $0.151 \pm 0.024 \text{ nm}^{-1}$ in the presence of 0.25 units of heparin/ml, with a slight decrease in the number of apparent binding sites from 1.05 ± 0.08 to $0.85 \pm 0.15 \text{ mol of } \alpha\text{-subunit bound/mol of } \beta\text{-subunit}$. Since the ternary IGF-binding protein complex cannot cross the capillary barrier, it is proposed that a decrease in the affinity of the complex, mediated by circulating or cell-associated glycosaminoglycans, may be important in the passage of IGFs and IGFBP-3 to the tissues.

INTRODUCTION

The insulin-like growth factors IGF-I and IGF-II circulate predominantly in a complex of approximate molecular mass 140 kDa [1–3]. Furlanetto [4] originally suggested that in this complex the IGF molecules were bound to an acid-stable IGF-binding protein in association with an acid-labile non-IGF-binding protein, a model also proposed by Hintz & Liu [5]. In a recent series of studies, we have purified both the acid-stable and acid-labile components of this complex, and demonstrated how they interact with each other and with the IGFs [6–9].

Initially, the acid-stable IGF-binding protein (IGFBP) was purified from human plasma and characterized [6]. This protein appears on SDS/PAGE as a glycoprotein doublet of apparent molecular mass 53 kDa (major band) and 47 kDa (minor band) (non-reduced), or 43 kDa and 40 kDa respectively after reduction. A similar doublet is seen for the protein purified from rat [10,11] or pig [12] serum or follicular fluid [13], or by ligand blotting of human serum after SDS/PAGE [14]. The acid-stable IGFBP, previously termed BP-53 [15] and now known as IGFBP-3, binds IGF-I or IGF-II at a single binding site with an association constant of $20\text{--}30 \text{ nm}^{-1}$ [6]. Iodinated IGF-I or IGF-II, when bound to IGFBP-3, is converted from a form of $\approx 60 \text{ kDa}$ to one of $\approx 150 \text{ kDa}$ in the presence of an acid-labile human serum factor [7]. This factor has been purified from serum and shown by SDS/PAGE to be a glycosylated doublet of 84–86 kDa [8]. The acid-labile glycoprotein has no intrinsic IGF-binding activity, but binds to IGFBP-3 with an association constant of $0.4\text{--}0.6 \text{ nm}^{-1}$ only when the binding protein is occupied by IGF-I or IGF-II [8].

Pure acid-labile protein, IGFBP-3 and IGF-I or IGF-II can be reconstituted into a ternary complex which, after covalent cross-linking, appears to have a molecular mass of 140 kDa as measured by SDS/PAGE and autoradiography [9]. The same radioactive complex is formed whether the radioactive label is on the acid-

labile protein, the binding protein or the IGF; it can also be detected in human serum when the endogenous 140 kDa complex is affinity-labelled by exchange with IGF-II tracer [9]. These studies have thus confirmed the original hypothesis of Furlanetto [4] and Hintz & Liu [5]. To simplify the nomenclature of the three components that participate in complex formation, we have termed them the α - (acid-labile), β - (binding) and γ - (growth factor) subunits of the complex [9].

This paper describes studies in which the regulation of ternary complex formation has been investigated. The complex is formed in these experiments by incubating radiolabelled α -subunit with unlabelled β - and γ -subunits, and detected after precipitation with an antiserum specific for the β -subunit (i.e. IGFBP-3). This study demonstrates that formation of the complex decreases with increasing salt concentration, and that glycosaminoglycans, notably heparin, are potent inhibitors of complex formation.

EXPERIMENTAL

Human IGF-I [16], IGFBP-3 [6] and α -subunit [8] were purified as previously described. Heparin (porcine intestinal mucosa) was purchased from Fisons (Sydney, N.S.W., Australia) or Sigma (St. Louis, MO, U.S.A.); the latter preparation had an activity of 178 units/mg. Protamine sulphate, heparan sulphate (bovine kidney) and chondroitin sulphate (bovine trachea) were from Sigma. Poly(ethylene glycol) 6000 was obtained from Merck (Darmstadt, Germany), and goat anti-(rabbit immunoglobulin) was from Bioclone Australia (Sydney, N.S.W., Australia). Radioiodinated α -subunit (500 Ci/mmol) was prepared using chloramine-T and purified by ion-exchange chromatography before use; approx. 90 % of radioactivity was precipitable by excess anti- α -subunit antiserum [17]. IGF-I (1500 Ci/mmol) was radioiodinated and purified by hydrophobic interaction chromato-

graphy as previously described; approx. 80% of radioactivity was precipitable by excess anti-IGF-I antiserum [18].

Formation of the ternary (α - β - γ) complex was determined by measuring the binding of radiolabelled α -subunit to a mixture of unlabelled IGFBP-3 (β -subunit) and IGF-I (γ -subunit), essentially as described previously [8]. Duplicate incubations in 0.3 ml of 50 mM-sodium phosphate buffer and BSA (2.5 g/l), pH 6.5, contained 125 I-labelled α -subunit (approx. 10000 c.p.m.; 1 ng), 10 ng of pure IGFBP-3, 10 ng of IGF-I and other additions as indicated. After 2 h at 22 °C, 25 μ l of 25-fold-diluted antiserum R7 (against IGFBP-3) was added, and the incubation was continued for 4 h at 22 °C. The radioactive complex was precipitated by adding 25 μ l of 5-fold-diluted goat anti-(rabbit immunoglobulin), incubating for a further 30 min, adding 1 ml of cold poly(ethylene glycol) (60 g/l in 0.15 M-NaCl) and centrifuging after 15 min. Non-specific binding was measured by omitting IGFBP-3 and IGF-I from the incubation.

Formation of the binary (β - γ) complex was measured as the binding of radiolabelled IGF-I (γ -subunit) to 10 ng of unlabelled IGFBP-3 (β -subunit). This was performed as for ternary complex formation, except that the tracer was 125 I-labelled IGF-I (approx. 10000 c.p.m.; 30 pg), and unlabelled IGF-I was omitted. Non-specific binding was measured by omitting IGFBP-3 from the incubation.

In the competitive binding experiments, unlabelled α -subunit was added over the range 10–400 ng/tube. Binding parameters were estimated by Scatchard analysis after subtraction of non-specific binding. For the calculation of the number of binding sites for α -subunit on IGFBP-3, the molecular masses of the proteins were assumed to be 85 kDa and 43 kDa respectively.

RESULTS

We previously reported that the affinity constant for α -subunit binding to the β - γ complex (IGFBP-3 plus IGF-I or -II) was almost two orders of magnitude lower than for the formation of the β - γ complex [8]. Because these studies were done at relatively low ionic strength, complex formation was re-tested in the presence of increasing concentrations of NaCl. Fig. 1 shows that, whereas β - γ complex formation was unaffected by salt concentrations up to 400 mM, the formation of the α - β - γ complex was decreased by increasing concentrations of NaCl in a dose-dependent manner. For three such experiments, the concentration

of NaCl required for 50% inhibition was 140 ± 36 mM (mean $\pm$ S.D.).

The nature of this inhibition was investigated by performing competitive binding studies of ternary complex formation in the presence of increasing salt concentrations. As illustrated in Fig. 2(a), α -subunit binding to the β - γ complex was inhibited by NaCl at all α -subunit concentrations. When expressed as Scatchard plots (Fig. 2b), the inhibition was seen to be due predominantly to a decrease in binding affinity with increasing salt concentration. In five similar experiments the mean association constant for α -subunit binding to the β - γ complex in the absence of salt was 0.604 ± 0.125 nM $^{-1}$, and the mean binding site concentration was 1.05 ± 0.08 mol/mol (Table 1). These estimates are essentially identical to those derived from three separate experiments which we reported previously [8]. The addition of NaCl lowered the binding affinity, with little effect on the number of α -subunit binding sites per mol of β -subunit, such that, in the presence of 0.15 M-NaCl, the mean association constant was approx. 50% of that measured in the absence of salt (Table 1).

Fig. 1. Effect of increasing NaCl concentrations on the formation of the binary and ternary IGFBP complexes

■, Binding of iodinated IGF-I to IGFBP-3 (10 ng); □, binding of iodinated α -subunit to a mixture of IGFBP-3 and IGF-I (10 ng of each). Both complexes were precipitated with the anti-IGFBP-3 antiserum R7. Both data sets have been corrected for non-specific binding (4.3% for IGF-I tracer; 3.7% for α -subunit tracer).

Fig. 2. Effect of NaCl on ternary complex formation

(a) Effect of NaCl on competitive binding curves for α -subunit binding in the ternary complex. Mean non-specific binding was 3.8%. (b) Scatchard plots of the binding data shown in (a). Lines were fitted by the least squares method after correcting data for non-specific binding. The binding site concentration is expressed as mol of α -subunit bound/mol of IGFBP-3, assuming molecular masses of 85 kDa and 43 kDa respectively. A value of 1 mol/mol corresponds to approx. 20 ng of α -subunit bound to 10 ng of IGFBP-3. ■, No addition; ●, 75 mM-NaCl; ▲, 150 mM-NaCl.

Table 1. Effect of NaCl and heparin on binding parameters for ternary complex formation

Values are expressed as means $\pm$ S.D., for *n* determinations. The binding site concentration is expressed as mol of α -subunit bound/mol of β -subunit, assuming molecular masses of 85 kDa and 43 kDa respectively. All experiments were performed in the presence of 10 ng (0.23 pmol) of β -subunit. For heparin, 1 unit/ml = 5.6 μ g/ml. Comparisons (Student's *t* test) with corresponding values for 'No addition': **P* < 0.05; ***P* < 0.02; ****P* < 0.01; *****P* = 0.001.

Addition	Association constant (nM ⁻¹)	Binding site concentration (mol/mol)	<i>n</i>
None	0.604 $\pm$ 0.125	1.05 $\pm$ 0.08	5
0.075 M-NaCl	0.432 $\pm$ 0.077	0.94 $\pm$ 0.06	2
0.15 M-NaCl	0.318 $\pm$ 0.092**	0.90 $\pm$ 0.08*	3
Heparin (0.1 unit/ml)	0.569 $\pm$ 0.169	0.73 $\pm$ 0.08***	3
Heparin (0.175 unit/ml)	0.246 $\pm$ 0.013**	0.77 $\pm$ 0.08***	2
Heparin (0.25 unit/ml)	0.151 $\pm$ 0.024****	0.85 $\pm$ 0.15*	3

Fig. 4. Inhibition of ternary complex formation by increasing concentrations of glycosaminoglycans

For comparison with other glycosaminoglycans, heparin is expressed in μ g/ml (1 μ g = 0.178 units). ■, Heparin; ●, heparan sulphate; ▲, chondroitin sulphate.

Fig. 3. Effect of increasing heparin concentration on the formation of the binary and ternary IGFBP protein complexes

Details are similar to those for the experiment shown in Fig. 1. Non-specific binding was 8.3 % for IGF-I tracer and 4.6 % for α -subunit tracer. ■, Binding of IGF-I to IGFBP-3; □, binding of α -subunit to a mixture of IGFBP-3 and IGF-I.

It was reported several years ago that heparin appeared to enhance the liberation of IGF-I from its 140 kDa serum complex [19]. It was therefore of interest to determine what effect heparin might have on the kinetics of complex formation. As shown in Fig. 3, increasing concentrations of heparin progressively inhibited α -subunit binding to the β - γ complex, while having little or no effect on the formation of the β - γ complex itself. As determined in five experiments, the heparin concentration causing 50 % inhibition was 0.27 ± 0.08 unit/ml, or 1.5 ± 0.4 μ g/ml. Other glycosaminoglycans were similarly tested in three experiments. Heparan sulphate showed a flatter inhibition curve, with 50 % inhibition at 15 ± 7 μ g/ml, and chondroitin sulphate inhibited relatively poorly, not reaching 50 % inhibition at 50 μ g/ml (Fig. 4).

Scatchard analysis was again used to determine the effect of heparin on binding parameters for ternary complex formation. As shown in Fig. 5, heparin at 0.1 unit/ml decreased binding with no effect on the affinity of the reaction, but caused a moderate decrease in the apparent number of binding sites on the β -subunit. In contrast, higher concentrations of heparin

Fig. 5. Effect of heparin on ternary complex formation

(a) Effect of heparin on competitive binding curves for α -subunit binding in the ternary complex. Mean non-specific binding was 4.4 %. (b) Scatchard plots of the binding data shown in (a), as in Fig. 2. ■, No addition; ●, 0.1 unit of heparin/ml; ▲, 0.175 unit of heparin/ml; ◆, 0.25 unit of heparin/ml.

progressively lowered the binding affinity, so that at 0.25 unit of heparin/ml, the association constant was decreased by over 70 % to $0.151 \pm 0.024 \text{ nm}^{-1}$. An identical pattern of inhibition was seen in three experiments, for which the derived binding parameters are summarized in Table 1. Since protamine, a polycation, may reverse the effects of polyanions such as glycosaminoglycans, the effect of protamine sulphate on ternary complex formation was also examined. Protamine sulphate was itself a weak inhibitor of α -subunit binding to the β - γ complex, with less than 50 % inhibition seen at a concentration of 50 $\mu\text{g}/\text{tube}$ or 167 $\mu\text{g}/\text{ml}$ (determined in two experiments). However, protamine sulphate at either 16.7 or 333 $\mu\text{g}/\text{ml}$ was able to reverse by approx. 50 % the inhibition of complex formation caused by 5 units of heparin/ml (28 $\mu\text{g}/\text{ml}$) (results not shown), indicating that the polyanionic charge on heparin was an important component of its inhibitory effect.

DISCUSSION

After remaining controversial for several years [4,20], the structure of the high-molecular-mass serum form of IGF-I and IGF-II was recently confirmed as a ternary complex containing α - (acid-labile, non-IGF-binding), β - (binding), and γ - (growth factor) subunits [8,9]. The only binding protein yet found to act as a β -subunit in this complex is the growth-hormone-dependent glycoprotein now known as IGFBP-3. The β -subunit is in fact a binding protein for both the α - and γ -subunits, each of which interacts at a single binding site [6,8]. These binding sites are apparently not entirely independent. Thus, whereas the presence of α -subunit has no effect on the affinity of IGF binding to β -subunit (association constant 30 nm^{-1}), prior occupancy of the IGF-binding site is an absolute requirement for α -subunit binding [8].

We have previously determined that the association constant for ternary (α - β - γ) complex formation was considerably lower than that for the formation of the binary (β - γ) complex [6,8]. However, in the course of early binding studies using gel chromatography [7], it was observed that increasing ionic strength inhibited complex formation, suggesting that the initial measurements of α -subunit binding, carried out at low ionic strength, may have overestimated the affinity. This has been investigated in the present study using a quantitative method of assessing complex formation, which demonstrated that the inhibitory effect of salt is due to a decrease in the affinity of α -subunit binding to the β - γ complex, and that the inhibition is half-maximal at a physiological NaCl concentration. In contrast, binary complex formation appears to be entirely unaffected by increasing ionic strength. Therefore, at physiological ionic strength, the association constant for ternary complex formation (0.3 nm^{-1}) is estimated to be 100-fold lower than that for formation of the binary complex.

Why, then, are most of the IGFBP-3 and IGFs in the circulation found in the 140 kDa complex, rather than in the binary 50–60 kDa form? One factor driving ternary complex formation is the relatively high concentration of α -subunit compared with that of IGFBP-3, which is itself roughly equimolar with that of the total IGF-I plus IGF-II [21]. When measured using a recently developed radioimmunoassay [17], the mean adult serum α -subunit concentration of approx. 24 mg/l or 0.28 μM is twice that of IGFBP-3, which is approx. 6 mg/l or 0.14 μM [22], assuming molecular masses of 85 kDa and 43 kDa respectively. This can be demonstrated directly if the ternary complex is removed from human serum by immunoaffinity chromatography on a column of anti-IGFBP-3 antibody: one-third to one-half of the α -subunit remains in the treated serum,

indicating that it was present in the free (i.e. not complexed) form [17].

An important consequence of the fact that the 140 kDa complex contains most of the IGFBP-3 and IGFs in serum is that the growth factors are apparently not able to cross the capillary barrier in this form [23], but presumably could pass from the circulation when in the binary complex. Therefore, if the circulating IGFs, the concentration of which can be accounted for by the hepatic production rate [24], have an endocrine function, and the 140 kDa complex acts as a storage and delivery system, the α -subunit must be able to dissociate readily from the ternary complex. This might happen simply as a result of the relatively low affinity constant for α -subunit binding; however, it is possible that passage of the IGFs from the circulation to the tissues might depend on specific dissociation mechanisms.

In investigating such dissociation mechanisms, I tested the effect of glycosaminoglycans. Clemmons *et al.* [19] reported several years ago that heparin could liberate IGF-I from its serum complex. Interestingly, in that study there appeared to be a direct effect of heparin on serum binding of IGF-I tracer, whereas in the present experiments with pure IGFBP-3, heparin had no effect on IGF-I binding. However, heparin was a potent inhibitor of the interaction between the α -subunit and the β - γ complex, and other glycosaminoglycans showed a similar, though weaker, effect. The inhibition apparently depended on the negative charge of the glycosaminoglycan, since protamine, a polycation, was able to partially reverse the effect of heparin.

Although circulating glycosaminoglycans might have a role in the dissociation of the 140 kDa complex, a more specific mechanism could involve cell-surface-bound polysaccharides. Glycosaminoglycans can be associated with the cell surface in two ways [25]. They may form part of proteoglycans which are integral membrane components, e.g. in fibroblasts, glial cells and endothelial cells [26], or they may be peripherally associated with cell membranes, binding to sites from which they can be displaced by added heparin [27]. Furthermore, cell-associated glycosaminoglycans can themselves act as 'receptors' for other circulating molecules [25]. Thus a plausible mechanism for the passage of circulating IGFs into the tissues could involve the interaction of the ternary binding protein complex with proteoglycans on the surface of capillary endothelial cells, resulting in the dissociation of the α -subunit from the complex. The remaining β - γ dimer might then be taken up by the endothelial cells, and ultimately be transported to target cells; alternatively, bound IGFs might dissociate, perhaps via a proteolytic mechanism [19], and be taken up without their binding proteins. Uptake of IGFs by vascular endothelial cells has been demonstrated previously, although it is not known if binding proteins play a role in this process [28]. If binding proteins do participate in cellular IGF uptake, it will still remain to be demonstrated whether IGF and IGFBP-3, presented to target cells as the β - γ complex, act as stimulators or inhibitors of cellular processes [13,29–31].

This study was supported by a grant from the National Health and Medical Research Council, Australia.

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Received 1 May 1990/29 June 1990; accepted 9 July 1990