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Structure of the M_r 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: Determination by reconstitution and affinity-labeling

(somatomedin/carrier protein/cross-linking/electrophoresis)

ROBERT C. BAXTER* AND JANET L. MARTIN

Department of Endocrinology, Royal Prince Alfred Hospital, and Department of Medicine, University of Sydney, Sydney, NSW, Australia

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ABSTRACT To determine the structure of the high molecular weight, growth hormone-dependent complex between the insulin-like growth factors (IGF-I and IGF-II) and their binding proteins in human serum, we have reconstituted the complex from its purified component proteins and analyzed it by gel electrophoresis and autoradiography after covalent cross-linking. The proteins tested in reconstitution mixtures were an acid-labile M_r 84,000–86,000 glycoprotein doublet (α subunit), an acid-stable M_r 47,000–53,000 glycoprotein doublet with IGF-binding activity (BP-53 or β subunit), and IGF-I or IGF-II (γ subunit). In incubations containing any one of the three subunits ^{125}I-labeled and the other two unlabeled, identical ^{125}I-labeled α–β–γ complexes of M_r 140,000 were formed. Minor bands of M_r 120,000 and 90,000 were also seen, thought to represent a partially deglycosylated form of the α–β–γ complex, and an α–γ complex arising as a cross-linking artifact. When serum samples from subjects of various growth hormone status were affinity-labeled with IGF-II tracer, a growth hormone-dependent M_r 140,000 band was seen, corresponding to the reconstituted α–β–γ complex. Other growth hormone-dependent labeled bands, of M_r 90,000 (corresponding to α–γ), M_r 55,000–60,000 (corresponding to labeled β-subunit doublet), and smaller bands of M_r 38,000, 28,000, and 23,000–25,000 (corresponding to labeled β-subunit degradation products), were also seen in the affinity-labeled serum samples and in the complex reconstituted from pure proteins. All were immunoprecipitable with an anti-BP-53 antiserum. We conclude that the growth hormone-dependent M_r 140,000 IGF-binding protein complex in human serum has three components: the α (acid-labile) subunit, the β (binding) subunit, and the γ (growth factor) subunit.

The insulin-like growth factors IGF-I and IGF-II circulate predominantly in growth hormone-dependent complexes of M_r 125,000–150,000, which dissociate irreversibly on acidification to yield free IGFs and one or more acid-stable IGF-binding protein (BP) species (1–7). Two hypotheses have been proposed to explain the structure of the circulating complexes: (*i*) they contains only IGFs and several identical or different binding subunits (8, 9) or (*ii*) in addition to IGF and BP there exists a non-IGF-binding subunit that is acid-labile (2, 10).

Consistent with the latter hypothesis, we have purified two components of the complex from human serum: an acid-stable glycoprotein of approximate M_r 50,000 (nonreduced) with IGF-binding activity (11), which we term BP-53 (12), and an acid-labile glycoprotein of approximate M_r 85,000, which does not bind IGFs (13). When fractionated by NaDodSO$_4$/PAGE and stained for protein, both appear as doublets (M_r 47,000–53,000 for the acid-stable protein, M_r 84,000–86,000 for the acid-labile protein); in each case the two bands are thought to represent glycosylation variants. The M_r 47,000 and 53,000 components of the BP-53 preparation are each able to bind both IGFs (14), with slightly higher affinity for IGF-II than for IGF-I (11). Sequencing of the cloned cDNA for BP-53 indicates a molecular weight for the nonglycosylated protein of 28,700 (15).

We have recently demonstrated that in the presence of IGF-I or IGF-II, the acid-stable and acid-labile proteins interact, resulting in the formation of one or more complexes of approximate M_r 150,000, as determined by gel chromatography (13, 16). We now describe the reconstitution and covalent cross-linking of radioactively labeled high molecular weight ternary complexes from pure components and demonstrate that the major reconstituted complex of M_r 140,000 is similar to the growth hormone-dependent complex previously identified in human serum by affinity-labeling. For convenience we abbreviate the components of these complexes as α (acid-labile subunit), β (binding subunit), and γ (growth factor subunit: γ_I and γ_{II} for IGF-I and IGF-II, respectively).

MATERIALS AND METHODS

Pure Proteins. IGF-I and IGF-II (17) and BP-53 (11) were purified from Cohn fraction IV of human plasma. The BP-53 preparation appeared as a doublet on NaDodSO$_4$/PAGE, consisting of a major band of M_r 53,000 nonreduced and 43,000 reduced and a minor band of M_r 47,000 nonreduced and 40,000 reduced (11). The M_r 28,000 IGF-BP BP-28 was isolated from human amniotic fluid (12). The α subunit (i.e., acid-labile subunit) was purified from fresh human serum by ion-exchange chromatography, affinity chromatography on a column containing BP-53 noncovalently bound to agarose-IGF, and further high-performance ion-exchange chromatography, as recently described (13). The final preparation appeared on nonreduced and reduced NaDodSO$_4$/PAGE as a doublet of M_r 84,000–86,000 and yielded a unique amino-terminal sequence, different from that of known IGF-BPs or receptors (13).

IGF-I (18), IGF-II (17), and BP-53 (7) were iodinated using chloramine T and purified as in previous studies. Estimated specific activities were approximately 1500 Ci/mmol, 600 Ci/mmol, and 2500 Ci/mmol, respectively (1 Ci = 37 GBq). The covalently cross-linked complex between BP-53 and ^{125}I-labeled IGF-I was prepared as described to a specific activity of approximately 600 Ci/mmol (7). Pure α subunit was iodinated using chloramine T as briefly described (13); the estimated specific activity was 500 Ci/mmol.

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Abbreviations: IGF, insulin-like growth factor; BP, binding protein.

*To whom correspondence should be addressed at: Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, NSW 2050, Australia.

Binding and Cross-Linking Studies. For affinity-labeling studies with pure proteins, all binding incubations, in 1.5-ml Microfuge tubes, were in a final volume of 100–150 μl of 0.05 M sodium phosphate buffer (pH 6.5) containing 0.05% bovine albumin (Sigma; RIA grade). The concentrations of each component of the complex, when present in the incubation, were as follows: α subunit, 500 ng per tube, or 6 μg per tube when used as excess; β subunit (BP-53), 100 ng per tube, or 5 μg per tube when used as excess; BP-28, 100 ng per tube; γ subunit (IGF-I or IGF-II), 50 ng per tube, or 1 μg per tube when used as excess. ^{125}I-labeled tracers were added at approximately 0.1 μCi/tube. After a 2-hr incubation at 22°C, complexes were cross-linked by adding 0.05 vol of 5 mM disuccinimidyl suberate (Pierce) in dimethyl sulfoxide and, after a further 30 min at 22°C, the cross-linking reaction was terminated by adding 0.05 vol of 1 M Tris·HCl (pH 8.0).

To affinity-label the M_r 125,000–150,000 complexes in human serum, 0.2-ml serum samples from normal, growth hormone-deficient, and acromegalic adults were injected by way of a V-7 valve (Pharmacia) onto a Superose 12 HR 10/30 column (Pharmacia) equilibrated at 22°C in 0.15 M NaCl/0.05 M sodium phosphate, pH 6.5. Fractions of 0.5 ml were collected at a flow rate of 1 ml/min. Fractions corresponding to the M_r 125,000–150,000 complex, as determined in previous studies (16), were used for affinity-labeling. Binding incubations contained 200 μl of fractionated serum and approximately 0.2 μCi of ^{125}I-labeled IGF-II, made up to 400 μl with 0.05 M sodium phosphate buffer (pH 6.5) containing 0.05% bovine albumin. Parallel incubations in which complexes were reconstituted from pure components contained 1 μg of α subunit and 200 ng of β subunit in place of serum fractions, together with buffer and tracer as described above. When present, excess unlabeled IGF-II was added at 5 μg per tube. After 15-hr incubations at 22°C, the complexes were cross-linked with disuccinimidyl suberate as described above. Each incubation mixture was then divided into two 200-μl samples, and complexes reacting with anti-BP-53 antiserum were immunoprecipitated from one aliquot. Five microliters of undiluted antiserum R-7, a later bleed from the rabbit that produced antiserum R1-4 (7), was added, and the mixture was incubated 5 hr at 22°C. Immune complexes were precipitated by adding 10 μl of goat anti-rabbit immunoglobulin (Bioclone, Sydney, Australia), incubating 30 min at 22°C, adding 1 ml of ice-cold polyethylene glycol solution (60 g/liter in 0.15 M NaCl), and centrifuging in a Microfuge for 10 min at 2°C. Pellets were washed twice by adding 1 ml of ice-cold phosphate buffer and recentrifuging and then resuspended in 50 μl of phosphate buffer.

NaDodSO$_4$/PAGE. Samples were prepared for NaDodSO$_4$/PAGE by addition of 0.33 vol of 4× concentrated sample buffer to give final concentrations of 15.5 mM Tris·HCl, 3% NaDodSO$_4$, 10% glycerol, 0.02% bromphenol blue (pH 6.8) without reducing agent. Samples were boiled for 5 min and allowed to cool before being loaded onto gels. Standards, prepared in the same way, were as follows: myosin (M_r 200,000) from Bio-Rad; cross-linked bovine albumin dimer (M_r 132,000) from Sigma; phosphorylase B (M_r 94,000), bovine albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,000), and lysozyme (M_r 14,000) from Pharmacia. Linear 10–15% gradient polyacrylamide slab gels, overlaid with 4% stacking gels, were prepared according to the method of Laemmli (19). Samples and standards were concentrated in the stacking gel at 50 V; then the voltage was increased to 125 V and electrophoresis was continued for a further 12–16 hr. Gels were fixed and stained in 25% isopropyl alcohol/10% acetic acid containing 0.1% Coomassie brilliant blue G-250 and destained in 25% methanol/10% acetic acid. Destained gels were dried under vacuum and placed in contact with Hyperfilm MP autoradiography film (Amersham) in a light-tight cassette with intensifying screens at −70°C for various times from 16 hr to 25 days.

RESULTS

The autoradiograph in Fig. 1 illustrates the formation of affinity-labeled complexes when α subunit was ^{125}I-labeled and β and γ were unlabeled. Neither IGF-I nor IGF-II alone formed a complex with α subunit (lanes 3 and 4), but a weak α–β complex of approximate M_r 135,000 was seen in the absence of IGFs (lane 5). When all three components were present, strong α–β–γ_I and α–β–γ_{II} complexes of M_r 140,000 were seen (lanes 6 and 8), which were abolished in the presence of excess unlabeled α subunit (lanes 7 and 9). Very weak complexes of M_r 120,000 can also be seen in lanes 6 and 8, which were also abolished by excess α subunit. The weak band at M_r 170,000 is a cross-linking artifact (presumably α dimer), as indicated by its absence in lane 1, containing α tracer alone that was not cross-linked, and its presence in lane 2 (α tracer, cross-linked).

Affinity-labeled α–β–γ complexes of M_r 140,000 were again seen clearly when the β-subunit doublet (M_r 47,000–53,000) was ^{125}I-labeled and the other components were unlabeled (Fig. 2). Both α–β–γ_I and α–β–γ_{II} complexes (seen in lanes 1 and 3, respectively) were absent in the presence of excess unlabeled β subunit (lanes 2 and 4). Lanes 5 and 6 illustrate the β–γ_I and β–γ_{II} doublet complexes, formed in the absence of α subunit (also seen in lanes 1 and 3), whereas lane 7 shows that there was barely detectable formation of a labeled α–β complex in the absence of IGFs. The band at approximate M_r 90,000, also seen in lane 8 (β tracer alone, cross-linked) is a cross-linking artifact, presumably β dimer, and was greatly reduced in the presence of excess unlabeled β (lane 9) and absent when β tracer was run without cross-linking (not shown). As seen in Fig. 1, the M_r 140,000 α–β–γ complexes (lanes 1 and 3) are accompanied by fainter complexes of M_r 120,000, thought to be a partially deglycosylated form of the M_r 140,000 complex. Also visible in each lane of Fig. 2 is a radioactive band of M_r 30,000, increasing to approximately 38,000 in the presence of IGFs.

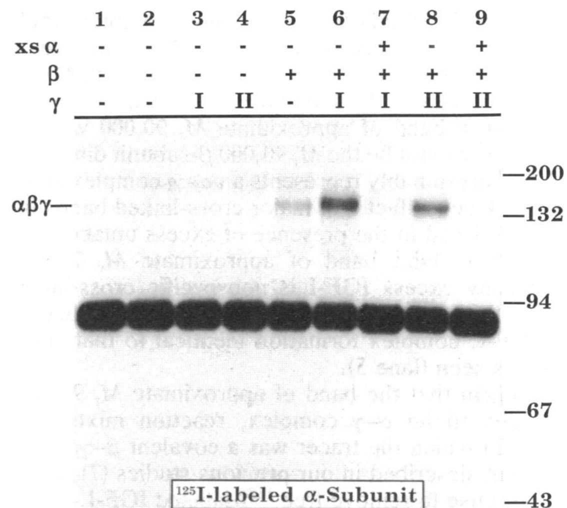

FIG. 1. Radioactive complexes formed by cross-linking ^{125}I-labeled α subunit and unlabeled β and γ subunits, analyzed by gel electrophoresis and autoradiography. Lane 1 shows α tracer alone, not cross-linked; the mixtures in lanes 2–9 were cross-linked. Where indicated above the autoradiograph, the 100-μl incubation volumes contained, in addition to α tracer, unlabeled α subunit [excess (xs) α, 6 μg], β subunit (100 ng), and γ subunit (IGF-I or IGF-II as indicated, 50 ng). Molecular weights (× 10^{-3}) of marker proteins are shown to the right of lane 9; the position of the M_r 140,000 α–β–γ complex is shown to the left of lane 1. The exposure time was 18 hr.

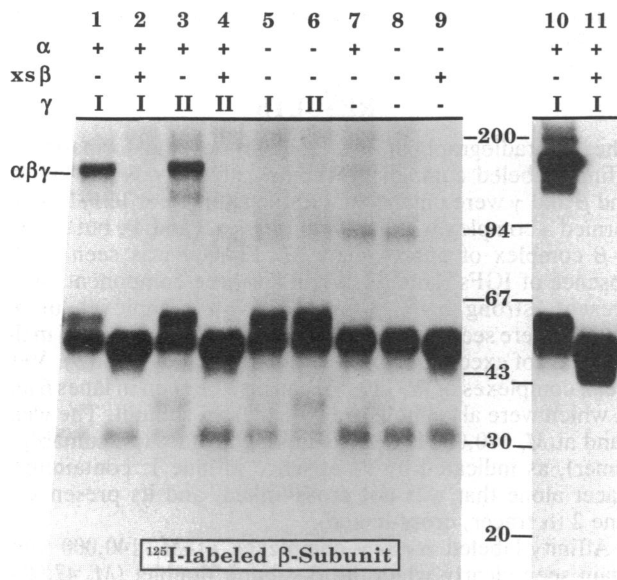

FIG. 2. Radioactive complexes formed by cross-linking ^{125}I-labeled β subunit and unlabeled α and γ subunits. Where indicated above the autoradiograph, the incubation volumes (150 μl for lanes 1–9, 100 μl for the separate experiment shown in lanes 10 and 11) contained, in addition to β tracer, unlabeled α subunit (500 ng), β subunit [excess (xs) β, 5 μg], and γ subunit (IGF-I or IGF-II as indicated, 50 ng). The position of the M_r 140,000 α–β–γ complex is shown to the left of lane 1. The exposure time was 72 hr for lanes 1–9 and 16 hr for lanes 10 and 11.

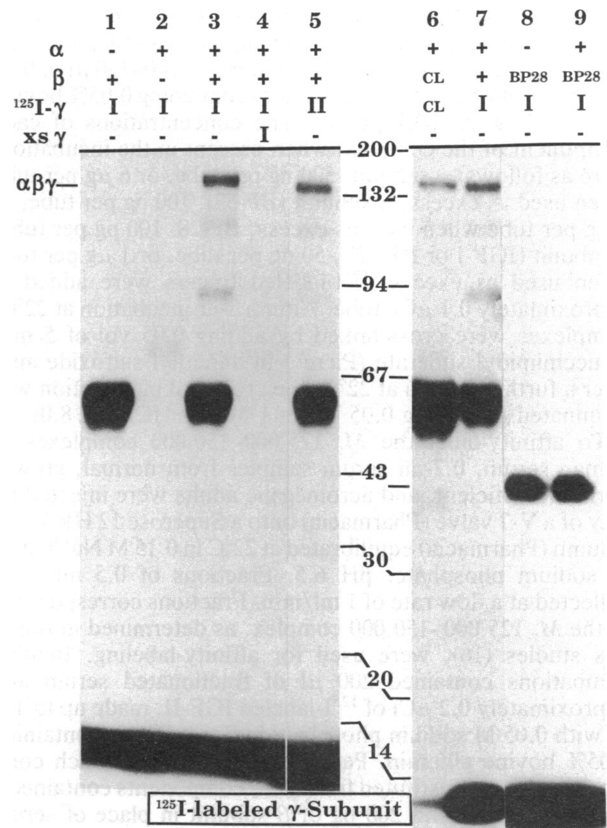

FIG. 3. Radioactive complexes formed by cross-linking ^{125}I-labeled γ subunit and unlabeled α and β subunits. Where indicated above the autoradiograph, the tracer (^{125}I-γ) was IGF-I, IGF-II, or a cross-linked complex of IGF-I tracer and β subunit (CL, lane 6). In addition to tracer, the 100-μl incubation volumes contained, where indicated, unlabeled α subunit (500 ng), β subunit (100 ng), and γ subunit [excess (xs) γ, 1 μg of IGF-I]. In lanes 8 and 9 the amniotic fluid BP BP-28 (100 ng) was substituted for β subunit. The position of the M_r 140,000 α–β–γ complex is shown to the left of lane 1. The exposure time was 65 hr.

This band represents a spontaneous breakdown product of BP-53, possibly a deglycosylated form, which was seen in some preparations. For comparison, lanes 10 and 11 show reaction mixtures identical to those in lanes 1 and 2, respectively, except that a different preparation of β tracer was used, in which this breakdown product was not visible.

Complexes affinity-labeled with ^{125}I-labeled γ-subunit (IGF-I or IGF-II) are shown in the autoradiographs in Fig. 3. IGF-I tracer readily formed β–γ_I complexes with the β-subunit doublet (lane 1), but no α–γ complex could be detected in the absence of β (lane 2). When all three components were present (lane 3), the strong M_r 140,000 α–β–γ_I band and the weaker M_r 120,000 band were once again seen. In addition, a band of approximate M_r 90,000 was clearly visible. This cannot be the M_r 90,000 β-subunit dimer seen in Fig. 2, but presumably represents an α–γ_I complex arising as a cross-linking artifact. All major cross-linked bands in lane 3 were abolished in the presence of excess unlabeled IGF-I (lane 4). The faint band of approximate M_r 75,000 not abolished by excess IGF-I is nonspecific cross-linking to albumin. When ^{125}I-labeled IGF-II was used instead of labeled IGF-I, complex formation identical to that shown in lane 3 was seen (lane 5).

To confirm that the band of approximate M_r 90,000 was indeed due to an α–γ complex, reaction mixtures were prepared in which the tracer was a covalent β–γ_I complex. This tracer, described in our previous studies (7), was purified before use to remove free ^{125}I-labeled IGF-I. As shown in Fig. 3, lane 6, this tracer, affinity cross-linked to unlabeled α subunit, resulted in the formation of the M_r 140,000 and 120,000 bands but not the 90,000 band. The latter band was, however, present when free IGF-I tracer was cross-linked to α and β (lane 7, similar to lane 3). Thus, whereas there is no interaction between α and γ subunits in the absence of β (Fig. 1, lanes 3 and 4; Fig. 3, lane 2), α and γ appear to interact when β is present. Fig. 3 also shows IGF-I tracer cross-linked to pure amniotic fluid BP-28, in the absence (lane 8) and presence (lane 9) of α subunit. The absence of any complexes above M_r 40,000 demonstrates that BP-28 cannot function as the β subunit in a ternary complex.

Fig. 4 compares the complexes reconstituted from pure proteins, affinity-labeled with IGF-II, with those formed when the growth hormone-dependent IGF-BP complex from human serum was affinity-labeled with IGF-II. The complexes from serum were obtained by subjecting serum samples from growth hormone-deficient, normal, and acromegalic adults to high-resolution gel chromatography on a Superose 12 column at neutral pH and selecting fractions corresponding to M_r 125,000–150,000. We have previously shown that the great majority of total serum immunoreactive BP-53 is found in this region (7).

As shown in lane 1, almost no labeling was apparent in the growth hormone-deficient serum sample, whereas samples from normal and acromegalic subjects showed increasing labeling patterns (lanes 2 and 3). The α–β–γ complex of M_r 140,000 and the α–γ complex of M_r 90,000 were weakly labeled, but clearly growth hormone-dependent, together with another, diffuse band of M_r 110,000, possibly dimerized β–γ complexes. More strongly labeled were growth hormone-dependent complexes between γ tracer and the β-subunit doublet at M_r 55,000–60,000, a band of around M_r 38,000 similar to that described in Fig. 2 as a complex with a M_r 30,000 BP-53 breakdown or deglycosylation product, and smaller complexes of approximate M_r 28,000 and 23,000–25,000 (doublet). Each of the bands seen in the affinity-labeled serum samples was also apparent when a reconsti-

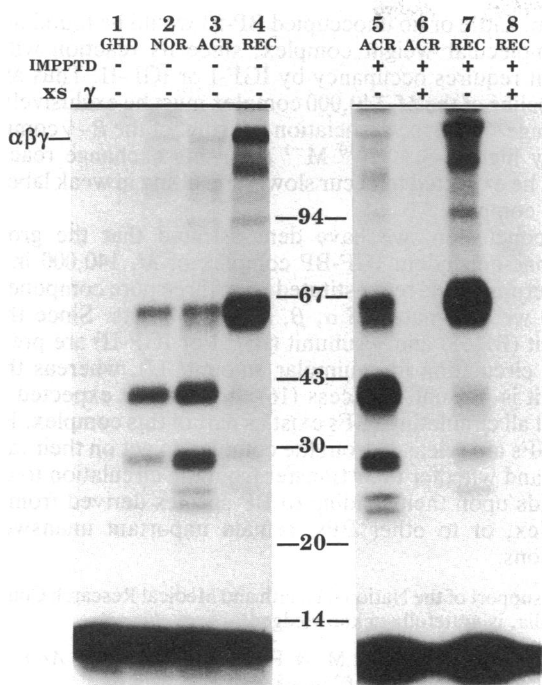

FIG. 4. Lanes 1, 2, 3, 5, and 6, radioactive complexes formed by cross-linking ^{125}I-labeled γ subunit (IGF-II) to high molecular weight IGF-BP complexes prepared from serum samples from growth hormone-deficient (GHD, lane 1), normal (NOR, lane 2), and acromegalic (ACR, lanes 3, 5, and 6) subjects. Lanes 4, 7, and 8, reconstituted complexes (REC) formed by cross-linking a mixture of ^{125}I-labeled γ subunit (IGF-II) and unlabeled α subunit (1 μg), and β subunit (200 ng). Incubations, in 400 μl, were for 15 hr at 22°C. Where indicated, excess unlabeled IGF-II [excess (xs) γ, 5 μg] was present in the incubation. Samples in lanes 5–8 were immunoprecipitated (IMPPTD) with anti-BP-53 antiserum before electrophoresis. The position of the M_r 140,000 α–β–γ complex is shown to the left of lane 1. The exposure time was 7 days (lanes 1–4) or 25 days (lanes 5–8).

tuted complex similar to that shown in Fig. 3, lane 5, was examined (lane 4), with the exception of the diffuse M_r 110,000 band and the smaller band of the M_r 23,000–25,000 doublet. In contrast, the M_r 120,000 band seen in the reconstituted complex was not apparent in the labeled serum complexes. It should be noted that in this experiment the binding incubations were for 15 hr at 22°C, in contrast to the 2-hr incubations used in the experiments illustrated in Figs. 1–3. This presumably accounts for the presence of IGF-labeled bands in the M_r 20,000–40,000 range seen in lane 4 but not visible in Fig. 3, lanes 3 and 5. This indicates that breakdown products derived from pure BP-53 can generate several small affinity-labeled complexes similar to those identified in affinity-labeled serum.

Fig. 4, lanes 5 and 6, show identical reaction mixtures to those in lane 3, incubated in the absence (lane 5) or presence (lane 6) of excess unlabeled IGF-II before cross-linking and immunoprecipitated after cross-linking with antiserum R-7 against BP-53. This antibody, like R1-4, cross-reacts <0.1% with amniotic fluid BP-28 (12). Similarly, lanes 7 and 8, respectively, show the reconstituted complexes (similar to that in lane 4) formed in the absence and presence of excess IGF-II, then immunoprecipitated. The exposure of the autoradiograph shown in lanes 5–8 was for a prolonged period (25 days) to emphasize even weak radioactive bands. This experiment illustrates (*i*) that the serum and reconstituted complexes are specific (i.e., displaceable by excess IGF-II), (*ii*) that each radioactive band either contains a protein immunologically related to BP-53 or one that interacts with a protein related to BP-53, and (*iii*) that almost every band obtained when serum is affinity-labeled can be found in complexes reconstituted from purified proteins (and their breakdown products).

DISCUSSION

In these studies we have demonstrated that ^{125}I-labeled IGF-BP complexes of M_r 140,000 can be reconstituted from well-defined components: the α (acid-labile) subunit, β subunit (BP-53), and γ subunit (IGF). Since complexes of identical size are formed regardless of which component is ^{125}I-labeled, we interpret the data as indicating that these are simple ternary complexes requiring no other components apart from the α, β, and γ subunits. This interpretation is supported by two sets of observations. First, the calculated average molecular weight of the ternary complex is close to 140,000, if the average molecular weights of the three components are 85,000 (α), 50,000 (β), and 7500 (γ); it thus seems highly unlikely that either two α subunits or two β subunits exist in the complex. Second, direct binding studies indicate that β subunit has a single binding site for γ subunit (11) and a single binding site for α subunit (13). Some possible ambiguity arises because each of the subunits is known to exist in at least two forms: the α subunit is actually a M_r 84,000–86,000 doublet, the β subunit is a M_r 47,000–53,000 doublet, and the γ subunit can be IGF-I or IGF-II. It cannot be stated with certainty whether each component of the α-subunit doublet can take part in complex formation. However, it is clear that IGF-I and IGF-II can participate equally, and gel chromatography experiments with the separate M_r 47,000 and 53,000 components of the β-subunit doublet indicate that each can form a high molecular weight complex (unpublished data).

Weak formation of cross-linked α–β complex was detected in the absence of γ subunit. This is surprising since in direct binding studies no complex between ^{125}I-labeled α and β could be detected unless γ was also present (13). Therefore, if the α–β complex is not an artifact, it forms only with extremely low affinity. Complexes corresponding to α–γ were also seen when β subunit was present, although there was no α–γ formation when the two subunits were coincubated without β. This suggests that in the ternary complex, the α and γ subunits are in close proximity (≈10 Å) (20), though not specifically interacting with each other. Therefore we propose the model shown in Fig. 5 for the structure of the ternary complex rather than a "linear" model in which β subunit acts as a bridge between α and γ.

In addition to the reconstituted M_r 140,000 complexes, weaker complexes of M_r 120,000 were also seen in most reconstitution experiments. These are postulated to be ternary complexes between α and γ subunits and a smaller, M_r 30,000 form of β subunit. This smaller BP is seen clearly in Fig. 2 and is thought to represent a deglycosylated form of BP-53, since the predicted molecular weight of this protein without carbohydrate is 28,700 (15). However, in preliminary experiments with the isolated M_r 30,000 form of BP-53, we have been unable to generate a M_r 120,000 complex (unpublished data), suggesting that it may result from deglycosyla-

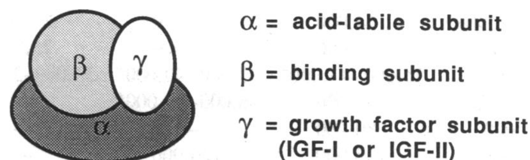

FIG. 5. Proposed model for the α–β–γ complex of M_r 140,000. When the γ-subunit binding site of the β subunit is occupied, the affinity of the β subunit for the α subunit increases and the ternary complex can form. In this complex the α and γ subunits are in close proximity, although there is no evidence that they interact. See text for experimental support for this model.

tion that occurs after covalent cross-linking of the three subunits.

Affinity-labeling of serum proteins in the high molecular weight IGF-BP complex from subjects with different growth hormone status indicated several growth hormone-dependent proteins with IGF-binding activity. Most of the labeled bands were also seen when the complex was reconstituted from pure components incubated for 15 hr, although those in the M_r 20,000–40,000 range were not visible after 2-hr incubations. Table 1 compares these labeled bands to those reported in studies from other laboratories (8, 9, 21). The discrepancies may be due to different calibration of molecular weight markers and to methodological differences. Notably, the labeled bands representing IGF bound to the 47,000–53,000 BP-53 doublet, seen as a M_r 55,000–60,000 β–γ doublet in our study, appear about 10,000 lower in molecular weight in other studies (8, 9). The lower molecular weights correspond to IGF bound to the BP-53 doublet at its reduced molecular weight of 40,000–43,000 (11). This discrepancy may result from the practice of running reduced standards containing mercaptoethanol on the same gel as nonreduced samples (9).

Our hypothesis that the M_r 38,000 band represents labeling of nonglycosylated M_r 28,700 BP-53 is supported by the previous observation that this protein is unable to bind to an agarose-concanavalin A column (8). Although the amniotic fluid BP, BP-28, is also found in serum and forms an affinity-labeled complex of a similar size (22), this cannot account for the M_r 38,000 labeled band seen in our studies, since this band was immunoprecipitable with anti-BP-53 antiserum R-7, which, like antiserum R1-4, cross-reacts <0.1% with BP-28 (12). Furthermore, it seems unlikely that BP-28 forms part of the high molecular weight complex, since in our cross-linking experiments and a gel chromatography study (16), no reaction was seen between a BP-28-IGF-I complex and the α subunit. Thus if a high molecular weight form of BP-28 does exist, it must form by a different mechanism. This possibility cannot be excluded, since several studies have suggested that BP-28 can exist at M_r 150,000–200,000 (23, 24).

In our study, the M_r 140,000 band in serum, representing the α–β–γ complex, showed clear growth hormone dependence but was weakly affinity-labeled compared to the bands of lower molecular weight. This emphasizes the nonquantitative nature of the affinity-labeling technique. Even though the great majority of immunoreactive BP-53 in serum is in the high molecular weight complex, and very little appears in the M_r 20,000–40,000 range (7), the protein present in the complex binds added tracer relatively poorly because its binding site is already occupied by IGF-I or IGF-II. We have previously shown that BP-53 is present in serum at a concentration equimolar with that of total IGF-I plus IGF-II (7, 25), suggesting substantial occupancy of IGF-binding sites on this protein. Little or no unoccupied BP-53 would be found in the high molecular weight complex, since its reaction with α subunit requires occupancy by IGF-I or IGF-II. Thus affinity-labeling of the M_r 140,000 complex must be exclusively by exchange. Since the association constant of the β–γ complex is very high, 2–3 × 10^{10} M^{-1} (11), this exchange reaction would be expected to occur slowly, resulting in weak labeling of the complex.

In conclusion, we have demonstrated that the growth hormone-dependent IGF-BP complex of M_r 140,000 in human serum can be reconstituted from three pure components, which we designate the α, β, and γ subunits. Since the β subunit (BP-53) and γ subunit (IGF-I or IGF-II) are present in the circulation in equimolar amounts (7), whereas the α subunit is present in excess (16), it would be expected that almost all circulating IGFs exist as part of this complex. How the IGFs are released from the complex to act on their target cells, and whether their transfer from the circulation to cells depends upon their binding to BP species derived from the complex, or to other BPs, remain important unanswered questions.

Table 1. Comparison of estimated molecular weight of affinity-labeled IGF-BPs in human serum

This study	Ref. 8	Ref. 9	Ref. 21
23,000–25,000*	24,000	22,000	21,500
28,000	28,000	27,000	25,500
38,000	35,000–43,000*	37,000–40,000*	34,000–37,000*
55,000–60,000*	50,000	46,000–49,000*	—
90,000	80,000	—	—
110,000	110,000	120,000	—
140,000	160,000†	150,000	—

*These proteins appear as doublet bands on $NaDodSO_4$/PAGE.
†The M_r 135,000 band described in this study does not consistently appear growth hormone-dependent or displaceable by excess unlabeled IGF (8).

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