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Binding properties and biological potencies of insulin-like growth factors in L6 myoblasts

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1. Protein synthesis in rat L6 myoblasts is stimulated and protein breakdown inhibited in a co-ordinate manner by insulin-like growth factors (IGF) or insulin. For both processes, bovine IGF-1 was somewhat more potent than human IGF-1, which was effective at a tenth the concentration of insulin, rat IGF-2 or human IGF-2. 2. A similar order of potency is noted when DNA synthesis or protein accumulation is monitored over a 24 h period, but between 20- and 50-fold higher concentrations of each growth factor are required than those needed to produce effects in the 4 h protein-synthesis or -breakdown measurements. 3. Binding experiments with labelled human or bovine IGF-1 as ligand demonstrated competition at concentrations of IGF-2, especially human IGF-2, lower than that of either IGF-1 preparation. This pattern was much more pronounced when the radioligand was either human IGF-2 or rat IGF-2. Insulin competed 10–15% for the binding of labelled IGF-1, but not at all with labelled IGF-2. 4. Ligand–receptor cross-linking experiments showed that labelled bovine IGF-1 bound approximately equally to the type 1 IGF receptor (M_r 130000 after reduction) and to the type 2 IGF receptor (M_r 270000 after reduction), and that unlabelled IGF-1 competed equally with radioligand binding to both receptors. On the other hand, rat IGF-2 competed more effectively for binding to the type-2 receptor, and insulin competed only for binding to the type-1 receptor. 5. Further cross-linking experiments with rat IGF-2 as radioligand demonstrated binding only to the type-2 receptor and to proteins with M_r values after reduction of 230000 and 200000. This binding was prevented by high rat IGF-2 concentrations, less effectively by bovine IGF-1 and not at all by insulin. 6. The apparently conflicting biological potencies and receptor binding of the different growth factors can be explained if all the biological actions are mediated via the type-1 IGF receptor, rather than through the abundant type-2 receptor.

INTRODUCTION

The administration of bovine growth hormone to normal rats results in greater weight gain and improved food-conversion efficiency that is observed in pair-fed littermates. The treated animals deposit less fat and more protein as compared with the controls (Lee & Schaffer, 1934). Growth hormone has been shown to produce similar anabolic effects in a range of species, with a selective increase in the proportion of body weight as muscle (Machlin, 1976). There is considerable evidence that these growth-promoting effects are mediated not directly by growth hormone but via the secondary production of somatomedins/insulin-like growth factors after the stimulation of liver and probably other tissues by growth hormone [see Laron (1982) and Hall & Sara (1983) for reviews]. Furthermore, a direct involvement of IGF in growth has been established by the finding that injection of pure IGF-1 into hypophysectomized rats stimulates growth to the same extent as growth hormone (Schoenle *et al.*, 1982).

The trophic action of growth hormone has been clearly established with both isolated muscles and myoblasts, model systems in which amino acid uptake and incorporation into protein are poorly responsive to growth hormone but are stimulated by IGF (Salmon & Du Vall, 1970; Ewton & Florini, 1980; Pfeifle *et al.*, 1982; Monier *et al.*, 1983). Rat L6 myoblasts represent a rapidly growing perpetual cell line that can form multinucleated muscle fibres concomitant with the cessation of DNA synthesis (Yaffe, 1968). In common with myogenic cells from other sources (Pfeifle *et al.*, 1982; Schmid *et al.*, 1983; De Vroede *et al.*, 1984), they bind substantial amounts of IGF and can be stimulated to grow (Richman *et al.*, 1980) and to differentiate (Ewton & Florini, 1981) by IGF.

We have utilized the stimulation of protein synthesis in L6 myoblasts as a bioassay for the purification of IGF-1 from bovine colostrum [one of the accompanying papers (Francis *et al.*, 1986)]. In the present paper the binding and responsiveness of bIGF-1, hIGF-1, rIGF-2, hIGF-2 and insulin have been compared to determine whether differences in biological potency occur between the preparations of IGF and to provide information on the receptor interactions required to elicit cellular events in L6 myobasts.

MATERIALS AND METHODS

Materials

Foetal-bovine serum (batch 29106909) was purchased from Flow Laboratories, Stanmore, N.S.W., Australia;

Abbreviations used: IGF; insulin-like growth factor; rIGF-2, rat IGF-2; hIGF-2, human IGF-2; hIGF-1, human IGF-1; bIGF-1, bovine IGF-1.
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bovine serum albumin (fraction V), amino acids and vitamins for the preparation of media were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; L-[4,5-^{3}H]leucine (40–60 Ci/mmol) and [*methyl*-^{3}H]thymidine (20 Ci/mmol) were from New England Nuclear, Boston, MA, U.S.A.; disuccinimidyl suberate was obtained from BRESA, Adelaide, South Australia, Australia; protein M_r standards were from Pharmacia, Uppsala, Sweden. Sources for the antibiotics used in media are given by Ballard & Francis (1983) and for the unlabelled and ^{125}I-labelled growth factors in the second of the accompanying papers (Read *et al.*, 1986). All dilutions of growth factors were made in the presence of 0.1% bovine serum albumin. Albumin used for this purpose and in the binding media was treated as described by Chen (1967).

Cell cultures

L6 myoblasts, obtained from Dr J. M. Gunn, Texas A & M University, College Station TX, U.S.A., were grown by serial passaging of prefused cultures in Dulbecco-modified Eagle's Minimal Essential Medium containing 5% (v/v) foetal-bovine serum, together with 50 mg of gentamycin, 100 mg of streptomycin, 60 mg of penicillin and 1 mg of fungizone/litre of growth medium. The cells were grown and used for experiments as monolayers at 37 °C under an atmosphere of CO_2/humidified air (1 : 19). For experimental purposes the cells were subcultured into Linbro 24-place or 6-place multiwell dishes in growth medium and used during the 3 days after the monolayers first became confluent. At this stage myotubes were not evident.

Measurement of protein synthesis

The growth medium in each well of a 24-place dish was replaced with leucine-free Eagle's Minimal Essential Medium containing 1% foetal-bovine serum and antibiotics as listed above for growth medium. After 18 h the monolayers were washed twice with serum-free Eagle's Minimal Essential Medium plus antibiotics and with the leucine concentration adjusted to 2 mM (chase medium), and left for a further 3 h in the same medium. The measurement period began by replacement of this medium with 1 ml of fresh chase medium containing growth factors as indicated, together with 2 μCi of [^{3}H]leucine. After labelling for 4 h, each monolayer was washed at 0 °C, twice with Hanks' salts, twice with 5% trichloroacetic acid over a 10 min period, and once with water. The washed cells were dissolved by trituration in 0.5 M-NaOH containing 0.1% Triton X-100 for the measurement of protein (Dulley & Grieve, 1975) and radioactivity. Rates were calculated as the percentage of protein synthesized, $100A/BC$, where A is the incorporation of leucine in d.p.m./μg of cell protein, B the specific radioactivity of medium leucine (d.p.m./nmol) and C the leucine content of cell protein in L6 myoblasts (0.765 nmol/μg; Ballard, 1982).

Measurement of protein breakdown

Rates of protein breakdown were quantified by using the same procedures and identical media as those adopted for the measurement of protein synthesis, except that [^{3}H]lecuine was absent from the final 4 h incubation but present at 1 μCi/ml during the initial 18-h period. At the completion of the final incubation in chase medium with or without growth factors, the medium was removed and protein precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v). A 10 μg portion of carrier bovine serum albumin was added to each tube before the precipitation. The residual monolayer was dissolved without washing in 0.5 M-NaOH containing 0.1% Triton X-100. The percentage of protein degraded over the 4 h measurement period was calculated as $100D/(D+E+F)$, where D is the total d.p.m. in the medium trichloracetic acid-soluble fraction, E is the total d.p.m. in the medium trichloroacetic acid-insoluble fraction and F the total d.p.m. in the residual monolayer (Ballard *et al.*, 1980*a*).

DNA labelling and protein accumulation

Monolayers were washed for 2 h in chase medium, which was then replaced with a similar medium but containing growth factors at the indicated concentrations together with 5 μM-thymidine. After 18 h incubation, 1 μCi of [^{3}H]thymidine was added per well and the cells kept for a further 6 h. Harvesting of monolayers was carried out by using a procedure identical with that described for the protein-synthesis measurements. DNA synthesis was calculated as d.p.m. incorporated/monolayer and expressed as the percentage of values obtained in the absence of any growth factor. No correction was made for the changing protein or DNA content of monolayers. The amount of protein in the dissolved monolayers was also measured (Dulley & Grieve, 1975) as an index of the change in protein content produced by exposure to growth factors for 24 h.

IGF binding experiments

Confluent myoblast monolayers in 24-place multiwell dishes were washed twice with Hanks Minimal Essential Medium containing 20 mM-Tes and 0.5% bovine serum albumin at pH 7.5 (Tes binding medium) and incubated for 1 h in air at 23 °C in the same medium. Subsequently 480 μl of Tes binding medium with 5×10^{4} d.p.m. of radioligand was added to each well, together with 20 μl of different concentrations of unlabelled ligand in a solution containing 0.15 M-NaCl, 0.05 M-potassium phosphate and 0.1% bovine serum albumin at pH 7.6. The amounts of radioligand added were 0.8 ng of rIGF-2 or bIGF-1, 0.15 ng of hIGF-1 or 0.08 ng of hIGF-2. The plates were incubated in air at 23 °C for 90 min, after which they were washed five times with Hanks salts at 0 °C. Monolayers were dissolved by trituration in 0.5 M-NaOH containing 0.1% Triton X-100 for the determination of radioactivity and protein. Binding was corrected for the protein content of each well and expressed as the percentage of that found in the absence of unlabelled ligand. The procedure was modified where indicated by using a temperature of 4 °C and an incubation time of 18 h.

Cross-linking experiments

The procedure is essentially that described by Kasuga *et al.* (1981). Confluent L6 myoblasts monolayers in 6-place multiwell dishes were incubated at 37 °C in serum-free growth medium for 3 h and then washed briefly at 0 °C with a solution containing 0.1 M-Hepes, 0.12 M-NaCl, 5 mM-KCl, 1.2 mM-$MgSO_4$, 8 mM-glucose and 10 mg of bovine serum albumin/ml at pH 7.6 (Hepes binding medium). A 1 μCi portion of ^{125}I-labelled bIGF-1 or ^{125}I-labelled rIGF-2 (50 ng) was added to each well with the indicated amounts of unlabelled ligands in a final volume of 1 ml of Hepes binding medium. After

incubation at 15 °C for 4 h in air, the medium was removed and the monolayers were washed twice rapidly at 0 °C with Hepes binding medium without albumin. To cross-link the bound ligand, disuccinimidyl suberate at a final concentration of 0.5 mM was added in 1 ml of Hepes binding medium without albumin and the monolayers incubated at 15 °C for 15 min. The reaction was quenched with 3 ml of 0.1 M-Tris containing 1 mM-EDTA at pH 7.4. After 5 min at 15 °C, this solution was aspirated and the cells solubilized in 0.25 ml of a dissociating solution composed of 2% (w/v) sodium dodecyl sulphate, 10% (w/v) glycerol, 100 mM-dithiothreitol, 0.01% Bromphenol Blue and 62.5 mM-Tris at pH 6.8. After being left for 30 min the cell lysate was transferred to a Microfuge tube and heated at 95 °C for 10 min.

Portions (25 μl) of the dissociated samples were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on 5% (w/v) acrylamide gels (Laemmli, 1970). The gels were stained for 18 h with 0.04% Coomassie Blue R-250 in 50% (v/v) methanol containing 7% acetic acid, and de-stained in a similar, but dye-free, solution. The gels were dried by using a Hoeffer slab-gel drier and exposed for autoradiography with Kodak X-Omat film in association with a du Pont Cronex Lightning Plus enhancing screen. The M_r values for the receptor–ligand complexes were obtained by comparison with a mixture of M_r marker proteins (thyroglobulin, 330000; ferritin half-unit, 220000; β-galactosidase, 116000; phosphorylase *b*, 94000; bovine serum albumin, 66200) that was electrophoresed in an adjacent lane.

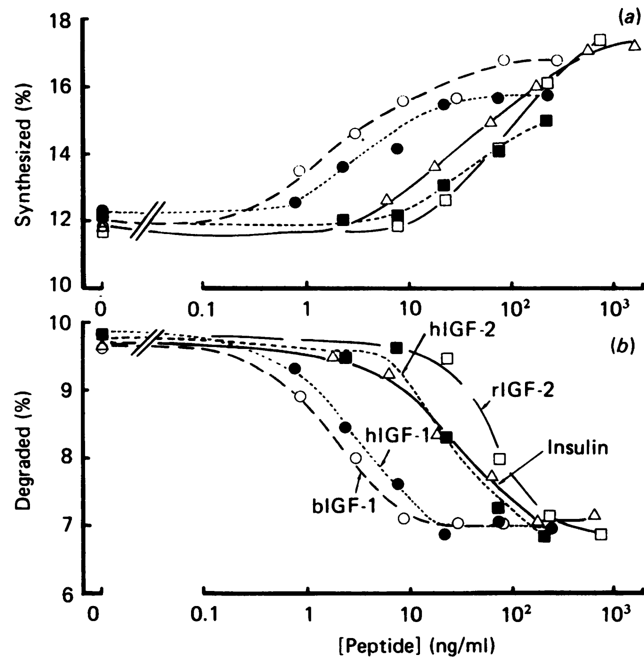

Fig. 1. Effects of different growth factors on the fractional rates of (*a*) protein synthesis and (*b*) protein breakdown in L6 myoblasts, measured over 4 h

The symbols used are: ○, bIGF-1; ●, hIGF-1; △, insulin; □, rat IGF-2; ■, hIGF-2; results represent triplicate measurements at each concentration.

RESULTS

For each of the measurements described below, we attempted to minimize variability by testing all five growth factors at the same time. The concentrations given in potency comparisons have not been corrected for differences in purity, even though we have indicated that the pool-3 bIGF-1 used was only 60–70% pure (Francis *et al.*, 1986).

Protein synthesis and breakdown

The fractional rate of protein synthesis in L6 myoblasts in the absence of growth factors is approx. 12% over the 4 h measurement period. This rate is increased to between 16 and 17.5% in the presence of optimal concentrations of each growth factor tested (Fig. 1). Since insufficient amounts of some growth factors were available to determine whether protein-synthesis rates had reached a plateau at high concentrations, the sensitivities of the different growth factors were assessed by comparing the concentrations needed to increase the fractional synthesis rate to 14.5%. By using this criterion, the decreasing order of sensitivity with concentrations (ng/ml) given in parentheses was:

bIGF-1 (2.3) > hIGF-1 (5.8) > insulin (55) > hIGF-2 = rIGF-2 (100)

Addition of 10% (v/v) foetal-bovine serum in the same series of experiments increased the fractional synthesis rate to 15.4%.

The fractional rate of protein breakdown was decreased from 9.7% in 4 h measured in the absence of growth factors to a plateau value of 6.8–7.2% at high concentrations of each growth factor (Fig. 1). The decreasing order of sensitivity of the growth factors, with the concentrations (ng/ml) required to decrease the fractional breakdown rate to 8.3% in 4 h given in parentheses was:

bIGF-1 (2.0) > hIGF-1 (2.6) > insulin = hIGF (22) > rIGF-2 (55)

DNA synthesis and protein accumulation

L6 myoblast cultures that had been incubated in serum-free media for a total time of 20 h incorporated 0.16 nmol of thymidine/well or 2.15 nmol/mg of protein over the subsequent 6 h. This control rate was increased substantially when 10% foetal-bovine serum was present for 18 h of the preincubation time as well as during the 6 h measurement period. When expressed per well, the stimulation was 9.5-fold.

None of the individual growth factors stimulated DNA synthesis as effectively as did 10% serum. Twofold increases in DNA labelling were produced by 50 ng of bIGF-1/ml and 100 ng of hIGF-1/ml, whereas this level of stimulation was not achieved at 1000 ng of hIGF-2, rIGF-2 or insulin/ml (Fig. 2). Extrapolation of the rIGF-2 and insulin dose–response curves suggests that a 2-fold increase in DNA labelling for these growth factors would occur at approx. 10 μg/ml.

Protein-accumulation measurements in the same wells as used for DNA synthesis showed that a protein content of 100 μg/well, 33% above that present in control monolayers, was attained with each of the growth factors (Fig. 2). The order of sensitivity, with the concentrations (ng/ml) required to achieve that level of stimulation given in parentheses, was:

bIGF-1 (7.5) > hIGF-1 (15) > hIGF-2 (220) > rIGF-2 (400) > insulin (1000)

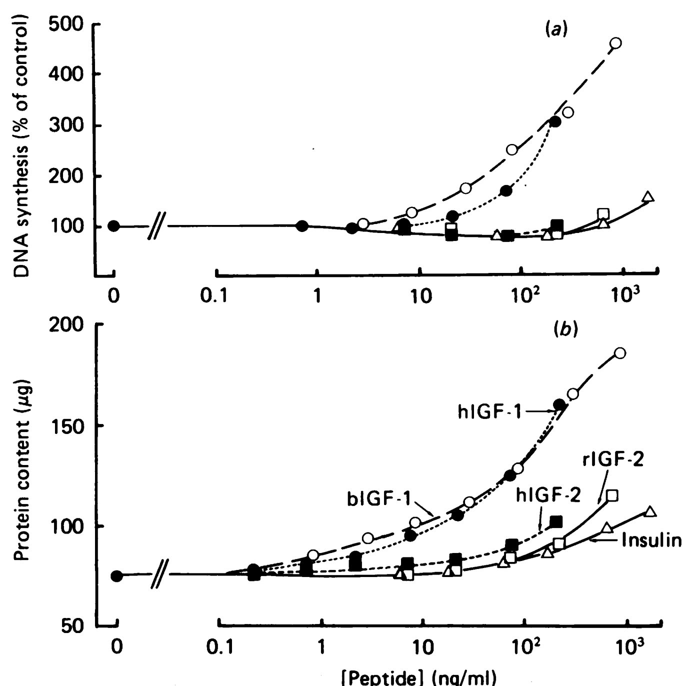

Fig. 2. Effects of different growth factors on (*a*) DNA synthesis and (*b*) protein accumulation in L6 myoblasts measured during 24 h exposure

The symbols used are: ○, bIGF-1; ●, hIGF-1; △, insulin; □, rat IGF-2; ■, hIGF-2; results represent triplicate measurements at each concentration.

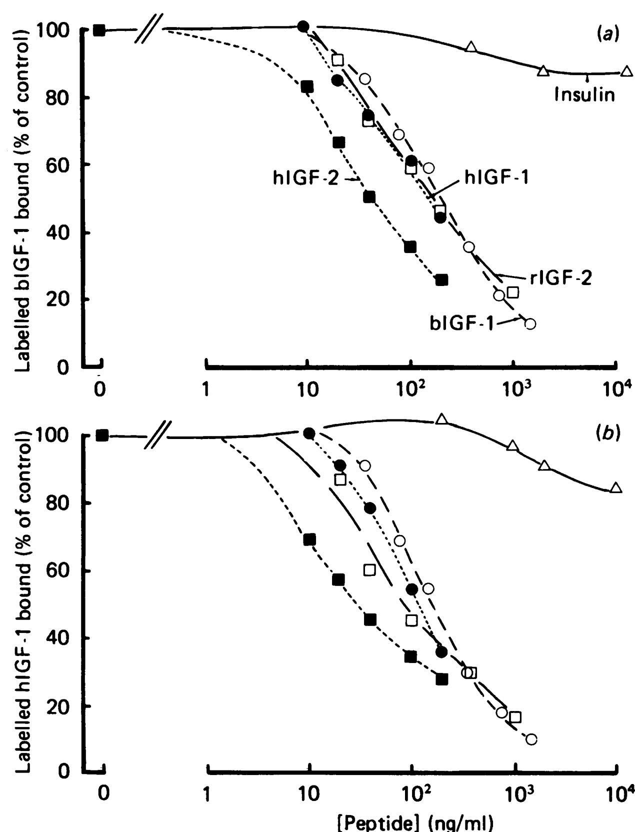

Fig. 3. Competitive binding of different growth factors to L6 myoblasts in the presence of ^{125}I-labelled (*a*) bIGF-1 and (*b*) hIGF-1

The symbols used are: ○, bIGF-1; ●, hIGF-1; △, insulin; □, rat IGF-2; ■, hIGF-2; results represent triplicate measurements at each concentration.

It should be noted that even with the highest concentration of bIGF-1 tested, 850 ng/ml, the amount of cell protein at the end of the 24 h incubation period (185 μg) was below that achieved when 10% foetal-bovine serum was present (200 μg).

Competitive-binding experiments

The L6 myoblasts bound 7.3% of the added ^{125}I-labelled bIGF-1 in the absence of unlabelled ligand. Substantial competition of binding occurred with all IGF preparations (Fig. 3). The concentrations (in ng/ml) that gave 50% inhibition of binding were:

hIGF-2 (40) > rIGF-2 = hIGF-1 (160) > bIGF-1 (210).

In competition experiments with labelled hIGF-1 as radioligand, the order of sensitivity for the different growth factors, with concentrations (ng/ml) needed to reduce binding of ^{125}I-labelled hIGF-1 by 50%, given in parentheses, was:

hIGF-2 (30) > rIGF-2 (80) > hIGF-1 (120) > bIGF-1 (160).

In experiments with labelled bIGF-1 or hIGF-1, insulin produced a 10–15% inhibition of binding at concentrations above 1 μg/ml. This competition was not increased at even higher insulin concentrations (Fig. 3).

Both hIGF-2 and rIGF-2 were far more effective in competing for receptors on L6 myoblasts when either hIGF-2 or rIGF-2 was the radioligand (Fig. 4). In the former series of experiments, 15% of added ^{125}I-labelled hIGF-2 was bound and the order of sensitivity of the different growth factors, with the concentrations (ng/ml) required to inhibit binding by 50% given in parentheses was:

hIGF-2 (45) > rIGF-2 (180) > bIGF-1 (1600).

The competition produced by hIGF-1 appeared to be slightly less than by bIGF-1, but high concentrations were not available for testing.

With rIGF-2 as radioligand, the same order of effectiveness was found as reported above for labelled hIGF-2. This experiment is not strictly comparable with the other three binding studies, because measurements were carried out over 18 h at 4 °C. The concentrations (ng/ml) of growth factors required to inhibit the binding of ^{125}I-labelled rIGF-2 by 50% were:

hIGF-2 (16) > rIGF-2 (35) > bIGF-1 (320).

Extrapolation of the competition curve with hIGF-1 to 50% inhibition would give a concentration of approx. 500 ng/ml.

Insulin does not decrease the binding of labelled IGF-2. Rather, an increase in the amount of radioligand bound is usually observed, as can be seen in Fig. 4.

Ligand–receptor cross-linking experiments

The biological-responsiveness studies shown in Figs. 1 and 2 indicate that L6 myoblasts are more sensitive to the two IGF-1 preparations than to IGF-2. However, the

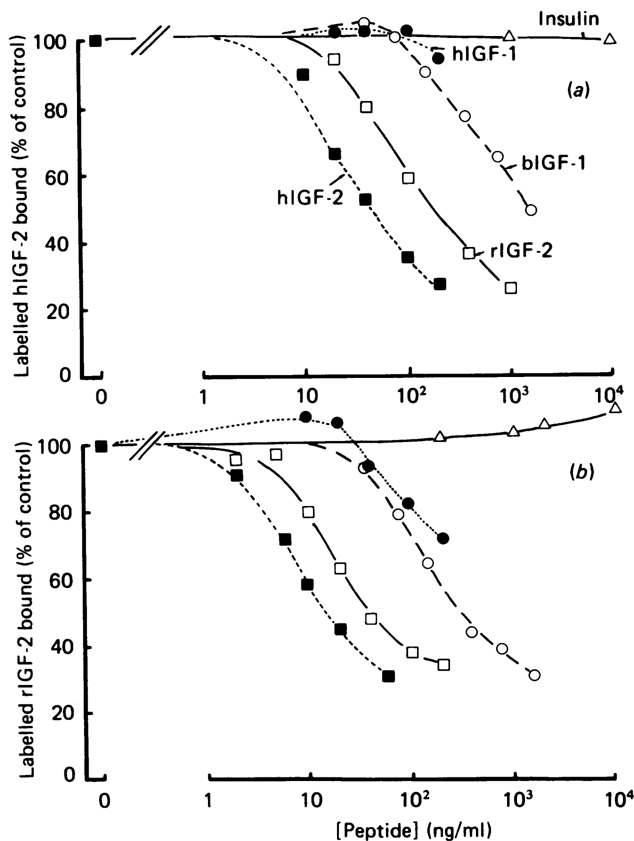

Fig. 4. Competitive binding of different growth factors to L6 myoblasts in the presence of ^{125}I-labelled (*a*) hIGF-2 and (*b*) rIGF-2

The symbols used are: ○, bIGF-1; ●, hIGF-1; △, insulin; □, rat IGF-2; ■, hIGF-2; results represent triplicate measurements at each concentration.

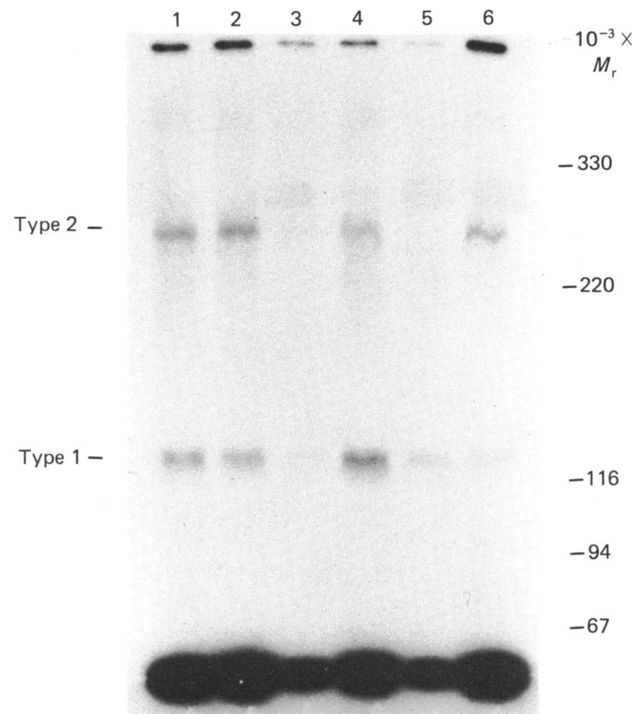

Fig. 5. Autoradiograph of gels after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions after cross-linking experiments with ^{125}I-labelled bIGF-1 to L6 myoblast monolayers

The concentrations of non-radioactive ligands added were: lane 1, no addition; lane 2, 50 ng of bIGF-1/ml; lane 3, 1000 ng of bIGF-1/ml; lane 4, 50 ng of rIGF-2/ml; lane 5, 1000 ng of rIGF-2/ml; lane 6, 10 μg of insulin/ml. The positions of M_r markers in adjacent lanes are indicated. The type 1 and type 2 IGF receptor bands are indicated. The autoradiography exposure period was 5 days.

binding experiments give the opposite result and imply a much greater affinity of L6 myoblasts for IGF-2. In an attempt to understand these apparently conflicting results, we have cross-linked bIGF-1 and rIGF-2 to the myoblasts, measured the subunit M_r of the receptor–ligand complexes and carried out competition experiments. With ^{125}I-labelled bIGF-1 (Fig. 5), 8.3% of the added radioligand was bound in control wells, although most of this radioactivity moved with the buffer front. Bands corresponding to M_r 130000 and 270000 are also detected in the autoradiograph, indicative of type-1 and type-2 IGF receptors respectively (Kasuga *et al.*, 1981). Addition of 1 μg of bIGF-1/ml during the binding experiment (lane 3) decreased the total amount of radioligand bound by 70% and showed approximately equal competition for both type-1 and type-2 receptors. A similar extent of competition was evident at 1 μg of rIGF-2/ml, but in this case radioligand binding to the type-2 receptor was decreased to a much greater extent than to the type-2 receptor (lane 5). Although 10 μg of insulin/ml did not decrease the total binding of ^{125}I-labelled bIGF-1 to the myoblasts, the radioactivity associated with the type-1 receptor was substantially decreased (lane 6).

Cross-linking experiments with labelled rIGF-2 (Fig. 6) gave substantial binding to the type-2 receptor, as well as to proteins with M_r 200000 and 230000. No significant radioactivity was associated with the type 1 receptor. Binding to the type-2 receptor was almost completely prevented at 1000 ng of bIGF-1 (lane 3) or of rIGF-2 (lane 5)/ml. In all three cases where binding was decreased, less radioactivity occurred in the 200000- and 230000-M_r bands as well as in the decreased type-2 receptor, suggesting that these bands may represent proteolytic cleavage products. Addition of 10 μg of insulin/ml increased the binding of labelled rIGF-2 by 30%, an increase that was associated with the type-2 receptor and its putative breakdown products (lane 6).

DISCUSSION

IGF preparations produce a co-ordinate anabolic effect on protein metabolism in L6 myoblasts by stimulating protein synthesis and inhibiting protein breakdown. Half-maximal effects on both pathways are produced at concentrations between 2 and 6 ng/ml, with bovine IGF-1 being somewhat more potent than human IGF-1. Approx. 10 times higher concentrations of rat IGF-2, human IGF-2 or insulin are required to achieve the same biological responses. The similar biological potency within each IGF class reflects the very considerable structural homology, both between human and bovine IGF-1 (Rinderknecht & Humbel, 1978*a*; Francis *et al.*, 1986) and between human and rat IGF-2

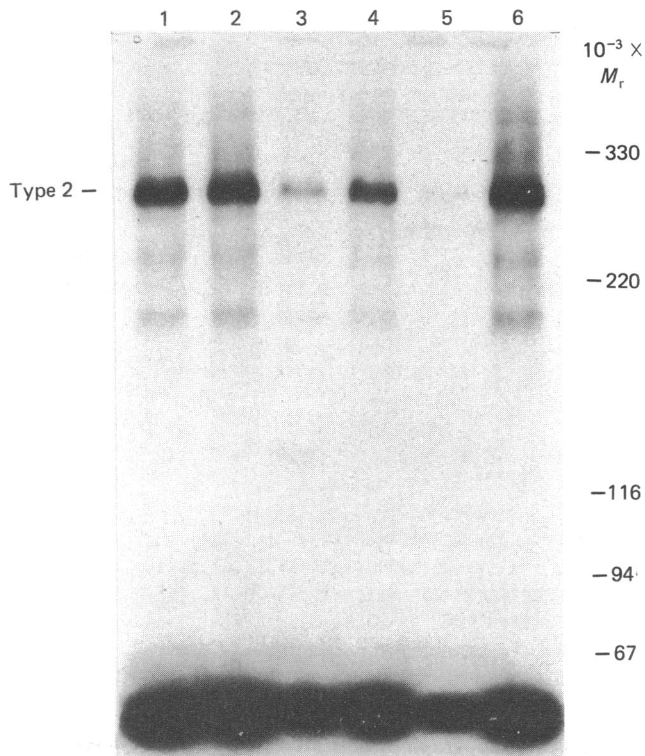

Fig. 6. Autoradiograph of gels after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions after cross-linking with ^{125}I-labelled rIGF-2 to L6 myoblast monolayers.

Details are given in the legend to Fig. 5. The exposure time was 1 day.

(Rinderknecht & Humbel, 1978*b*; Marquardt *et al.*, 1981).

Essentially similar results, with IGF-1 being approximately an order of magnitude more potent than IGF-2 in eliciting biological responses, have been reported for many cell types (Ballard *et al.*, 1980*a,b*, 1981; King *et al.*, 1980; Ewton & Florini, 1981; De Vroede *et al.*, 1984; Conover *et al.*, 1985). On the other hand, insulin may be more potent than IGF-1 in some hepatomas and isolated muscles (Gunn *et al.*, 1977; Poggi *et al.*, 1979; Ballard *et al.*, 1980*b*, 1981; Monier *et al.*, 1983; Monier & Le Marchand-Brustel, 1984; Yu & Czech, 1984), but usually substantially higher insulin than IGF-1 concentrations are needed to elicit similar biological responses (Zapf *et al.*, 1978; Ballard *et al.*, 1980*b*; 1981; King *et al.*, 1980; Ewton & Florini, 1981; Pfeifle *et al.*, 1982; Conover *et al.*, 1985; Borland *et al.*, 1984; De Vroede *et al.*, 1984).

Although DNA synthesis was stimulated by all the growth factors in essentially the same order of potency as for protein metabolism, we found that 20–50-fold higher concentrations were required. A large difference between DNA- and protein-synthesis sensitivity to hIGF-1 was not observed by Pfeifle *et al.* (1982) in a study of human smooth-muscle cells, but the effects on DNA synthesis were very small compared with those described here. A number of investigations have shown that pure IGF preparations have only a weak mitogenic activity when tested alone. Rather, the addition of a competence factor such as platelet-derived growth factor or fibroblast growth factor is necessary to achieve rates of DNA synthesis comparable with those found with serum (Scher *et al.*, 1979).

One possibility for the lower sensitivity of cell growth factors in DNA-synthesis assays is the 24 h period involved. During this time the growth factors will be partially degraded after internalization into the myoblasts, so that the average concentration throughout the test period may be much lower than at the beginning. This situation would explain why protein-accumulation rates in the same cells used for DNA labelling were much less responsive to the growth factors than were protein synthesis or protein breakdown measured over 4 h, even though the difference between these last two rates is a measure of protein accumulation.

Evidence obtained from a range of cell types has established the existence of three distinct receptors that bind insulin and the insulin-like growth factors. With the specific insulin receptor and the type-1 IGF receptor, cross-linking of ligand was demonstrated to the 130000-M_r α-subunit (Pilch & Czech, 1980; Massague *et al.*, 1980; Kasuga *et al.*, 1981; Bhaumick *et al.*, 1981; Chernausek *et al.*, 1981; Massague & Czech, 1982). The third receptor, termed the type-2 IGF receptor, is a single polypeptide chain with an M_r of 260000 under reducing conditions (Kasuga *et al.*, 1981; Massague & Czech, 1982). Cell types show considerable divergence in the relative proportions of the three receptors (Massague & Czech, 1982). In most cells the type-1 IGF receptor shows a specificity towards IGF-1 and the type-2 receptor to IGF-2, although these specificities are only partial. In addition, insulin combines weakly with the structurally similar type-1 IGF receptor, but not with the type-2 IGF receptor.

The L6 myoblasts bound more IGF-2 than IGF-1, whereas hIGF-2 competed more effectively than either preparation of IGF-1, not only when the radioligand was hIGF-2 or rIGF-2, but also in the presence of labelled hIGF-1 or bIGF-1 (Figs. 3 and 4). We found this last result surprising, given the considerably greater biological potency of IGF-1 in L6 myoblasts. Similar binding results have been obtained by Adams *et al.* (1983) with rat-embryo fibroblasts, although in many other cell types IGF-1 competes at lower concentrations than IGF-2 for the binding of labelled IGF-1. This generalization applies to human fibroblasts, the rat liver line BRL3A2, hamster fibroblasts and HTC hepatoma cells (Rechler *et al.*, 1980; Rosenfeld & Dollar, 1982; Van Obberghen-Schilling & Pouyssegur, 1983; Heaton *et al.*, 1984).

The L6 myoblasts are alone amongst the cells tested in that IGF-1 is biologically more potent than IGF-2 or insulin, whereas the binding data show more effective competition by IGF-2 than IGF-1. The results obtained when insulin was added to the cells with labelled IGF-1 or labelled IGF-2 suggest that only 10–15% of the total IGF-1 binding is to a type-1 receptor, because only this proportion of the labelled hIGF-1 or bIGF-1 binding can be prevented by extremely high insulin concentrations (Fig. 3). Since our (L. C. Read & F. J. Ballard) unpublished work shows minimal binding of ^{125}I-labelled insulin to the myoblasts, we infer that the ability of insulin to modify rates of protein synthesis and protein breakdown is mediated via the type-1 IGF receptor. A further prediction is that occupancy of the type-2 receptor is not associated with the biological responses measured here. Rather, the relatively low biological potency of IGF-2 is

a result of its binding in very small amounts to the type-1 receptor and with a lower affinity than IGF-1.

The predictions from the competitive binding curves are supported by the receptor cross-linking experiments. Thus (*a*) bIGF-1 binds in approximately equal amounts to both types of IGF receptor, but non-radioactive bIGF-1 competes more effectively for the type 1 receptor; (*b*) unlabelled rIGF-2 competes for bIGF-1 binding to the type 1 receptor, but less effectively than bIGF-1; (*c*) insulin competes for labelled hIGF-1 binding, but only to the type-1 receptor; (*d*) virtually all the labelled rIGF-2 is bound to the type-2 receptor. Each of these findings can be explained if the biological actions of IGF-1, IGF-2 and insulin are exerted through the type-1 receptor. The function of the large numbers of type-2 receptors in L6 myoblasts is unknown, as is also the case in H35 cells, where IGF responses are mediated through the insulin receptor instead of through the abundant IGF-2 receptor (Mottola & Czech, 1984).

Some differences in potency and in binding properties in L6 myoblasts exist between human IGF-1 and bovine IGF-1 and between rat IGF-2 and human IGF-2. The difference is shown especially with bIGF-1, which is more potent in all the biological assays and slightly more effective in the receptor binding studies than hIGF-1. Moreover, the magnitude of these differences would be greater if allowance was made for our estimation that the pool-3 bIGF-1 tested here was only 60–70% pure (Francis *et al.*, 1986). It will be especially interesting to compare IGF-1 preparations from other species to determine whether even more marked differences occur. A species difference is also seen with the two IGF-2 preparations. rIGF-2 was approximately equipotent to hIGF-2 in biological responsiveness, but much less effective in the receptor competition assays. Again, an explanation of this situation must await more widespread testing of IGF preparations.

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