Insulin-like growth factor (IGF)-binding proteins inhibit the biological activities of IGF-1 and IGF-2 but not des-(1–3)-IGF-1

Marina ROSS,* Geoffrey L. FRANCIS,* Laszlo SZABO,† John C. WALLACE† and F. John BALLARD*†

*CSIRO Division of Human Nutrition, Kintore Avenue, Adelaide SA 5000, and †Biochemistry Department, University of Adelaide, SA 5000, Australia

(1) Many cell types secrete insulin-like growth factor (IGF)-binding proteins that can be expected to sequester free IGF and modify the biological activities of the growth factors. (2) A binding protein purified from bovine kidney (MDBK) cells potentially inhibited the ability of IGF-2 to stimulate DNA synthesis or protein accumulation as well as to reduce rates of protein breakdown in chick embryo fibroblasts. The binding protein did not influence the biological activities of des-(1–3)-IGF-1, while effects on IGF-1 were intermediate. Since the chick embryo fibroblasts contain only the type 1 IGF receptor, the MDBK-cell binding protein must have reduced the accessibility of IGF-2 and IGF-1 to that receptor. Binding to the type 2 receptor on L6 myoblasts was also inhibited. (3) Inhibiting effects on both protein breakdown responsiveness to IGF and IGF binding to cell receptors were also observed with human amniotic fluid binding protein, although here IGF-1 and IGF-2 were equipotent. These results contrast with stimulatory responses on different IGF-1 actions of the same binding protein reported previously [Elgin, Busby & Clemmons (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3254–3258]. (4) The biological potencies of IGF-1, IGF-2 and des-(1–3)-IGF-1 correlate inversely with their binding to proteins released into the medium by cells, so that the enhanced potency of des-(1–3)-IGF-1 is a consequence of it not binding to purified binding proteins or those released by cultured cells.

INTRODUCTION

We have recently isolated and synthesized chemically an amino terminus truncated derivative of insulin-like growth factor 1 (IGF-1), known as des-(1–3)-IGF-1, that stimulates protein or DNA synthesis and inhibits protein breakdown at concentrations between 10 % and 20 % of those required by IGF-1 (Francis *et al.*, 1986, 1988; Ballard *et al.*, 1987). This increased potency was not associated with a comparable increase in affinity for the type 1 IGF receptor (Ballard *et al.*, 1987, 1988). Instead, des-(1–3)-IGF-1 bound relatively poorly to proteins in media conditioned by the L6 myoblasts used for the bioassays (Bagley *et al.*, 1989) and to a pure binding protein produced by MDBK cells (Szabo *et al.*, 1988). We proposed that the higher potency of des-(1–3)-IGF-1 was a result of increased concentrations of the free peptide being available for interaction with cell receptors (Szabo *et al.*, 1988).

In the present investigation we have examined directly the effect of the MDBK-cell binding protein on IGF activity in a cell type, chick embryo fibroblasts, that does not secrete a binding protein. Moreover, since Elgin *et al.* (1987) have recently reported that a binding protein purified from human amniotic fluid enhances rather than inhibits IGF action, we have evaluated responses produced by this binding protein in parallel experiments.

MATERIALS AND METHODS

Materials

Recombinant human IGF-1 (hIGF-1) was kindly provided by Drs. H. H. Peters and K. Scheibli, CIBA-

GEIGY, Basel, Switzerland. Des-(1–3)-IGF-1, with a sequence identical to human or bovine IGF-1 but with the amino terminal tripeptide Gly-Pro-Glu omitted, was prepared by chemical synthesis (Ballard *et al.*, 1987). Ovine IGF-2 and bovine IGF-2 were purified to homogeneity from ovine serum or bovine serum respectively by methods similar to those developed for bovine colostrum (Francis *et al.*, 1988). The peptides were dissolved in 10 mM-HCl and diluted in 0.15 M-NaCl/20 mM-KH₂PO₄/0.5 % bovine serum albumin, pH 7.4, for bioassay measurements, 0.1 M-Hepes/0.12 M-NaCl/5 mM-KCl/1.2 mM-MgSO₄/8 mM-glucose/0.5 % bovine serum albumin, pH 7.6, for receptor binding measurements, or 50 mM-NaH₂PO₄/0.25 % bovine serum albumin, pH 6.5, for binding protein assays.

Carrier-free Na¹²⁵I and [4,5-³H]leucine (specific activity 40–60 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K.

IGF-binding protein was either purified from medium conditioned by MDBK cells (Szabo *et al.*, 1988) or from human amniotic fluid. The latter was generously provided by Drs. W. H. Busby and D. R. Clemmons, University of North Carolina, Chapel Hill, NC 27514, U.S.A.

Tissue culture reagents, rat L6 myoblasts and HE(39)L human lung fibroblasts were obtained as described previously (Ballard *et al.*, 1988). Chick embryo fibroblasts were prepared from day 9 chick embryos (Rein & Reubin, 1968) and were used between the third and ninth subculture.

Collection of conditioned media

For initial screening purposes cells were grown in the presence of Dulbecco-modified Minimal Essential

Abbreviations used: IGF, insulin-like growth factor; des-(1–3)-IGF-1, des-Gly-Pro-Glu-IGF 1; DMEM, Dulbecco-modified Minimal Essential Medium.

† To whom correspondence should be addressed.

Medium (DMEM) containing 5% or 10% (v/v) fetal bovine serum in 24-place multiwell dishes until confluent. Subsequently the medium was replaced with serum-free DMEM and the cells washed twice over a 2 h period. Further portions of serum-free DMEM were added to the cells which were incubated for 18 h before the collection of the conditioned medium. After centrifugation (2000 g, 10 min) to remove cell debris, the medium was stored at -20°C .

Measurement of binding protein activity

The binding protein present in media conditioned by various cell lines or the pure MDBK-cell form was measured using the charcoal assay essentially as described by Martin & Baxter (1986). The modifications included the addition of 0.25% bovine serum albumin in the charcoal solution, an increase of the incubation time to 30 min and centrifugation of the mixtures at 10000 g for 3 min (Szabo *et al.*, 1988).

Effects of growth factors and binding proteins on intracellular protein breakdown

Confluent monolayers of cultured cells in 24-place multiwell dishes were prelabelled with [^3H]leucine after which rates of protein breakdown were measured over a 4 h period (Ballard *et al.*, 1986) with growth factors or binding proteins present. Breakdown rates were expressed as percentage inhibition relative to the rate in control medium, which was serum-free Eagle's Minimal Essential Medium containing 2 mM-leucine plus the diluent used for growth factors or binding proteins.

Effects of growth factors and binding protein on DNA synthesis and protein accumulation

Confluent monolayers of chick embryo fibroblasts were washed twice over 2 h with 1:1 mixture of Waymouth's MB 721/1 medium and DMEM. Each monolayer in a 24-place multiwell dish was subsequently incubated for 24 h in similar medium containing growth factors, binding protein or appropriate diluent. During the last 4 h of this period 1 μCi (5 nmol) of [^3H]thymidine was added per well (Gargosky *et al.*, 1987). Cells were harvested as described for protein synthesis measurements (Ballard *et al.*, 1986). DNA labelling in test wells was expressed as percentage incorporation relative to those with 10% fetal bovine serum added. The protein content per well was measured according to the method of Dulley & Grieve (1975).

IGF binding to cell receptors

Confluent monolayers in 24-place multiwell dishes were washed twice over 2 h in 0.1 M-Hepes/0.12 M-NaCl/5 mM-KCl/1.2 mM-MgSO₄/8 mM-glucose/0.5% bovine serum albumin, pH 7.6, and incubated for 18 h at 4°C in 0.5 ml of the same solution containing radioligand [(2-4) $\times 10^4$ d.p.m.], together with unlabelled peptides, binding protein or diluent. Subsequently the monolayers were washed and the bound radioactivity determined (Ballard *et al.*, 1986, 1988). Binding was expressed as a percentage of that bound in the absence of competing peptide.

RESULTS

Comparisons between the effects of IGF-1, IGF-2 and des-(1-3)-IGF-1 on protein breakdown in different cell

Table 1. Effects of insulin-like growth factors on protein breakdown in different cell types in relation to the production of binding proteins by those cells

Concentrations giving half-maximal effects were obtained from dose-response curves for protein breakdown studies or from competition curves in binding-protein assays with IGF-2 as tracer. The precision of these curves is such that differences greater than two-fold in concentrations giving half-maximal effects would be reproducible. Abbreviation: n.d., no binding protein detected.

Measurement	L6 myoblasts	HE(39)L fibroblasts	Chick embryo fibroblasts
Concentrations giving 50% inhibition of protein breakdown (nM)			
IGF-1	0.15	0.8	0.15
des-(1-3)-IGF-1	0.02	0.02	0.15
IGF-2	1.0	1.2	0.15
Concentrations giving 50% inhibition of IGF-2 binding (nM)			
IGF-1	0.7	0.7	n.d.
des-(1-3)-IGF-1	> 100	25	n.d.
IGF-2	0.1	0.3	n.d.

types have demonstrated four dose-response patterns. In rat L6 myoblasts the order of potency for the three peptides is des-(1-3)-IGF-1 > IGF-1 > IGF-2 (Table 1) while with either IMR-90 or HE(39)L human lung fibroblasts a second dose-response pattern occurred with des-(1-3)-IGF-1 much more potent than either IGF-1 or IGF-2, which have approximately equal activities. The third pattern, where all three peptides are equipotent, is observed with chick embryo fibroblasts (Table 1). A fourth pattern, in which IGF-1 and des-(1-3)-IGF-1 are equipotent and IGF-II less potent, was found with HTC hepatoma cells and human skin fibroblasts. These differences have not been evaluated further because the main purpose of the present investigation was to account for the increased potency of des-(1-3)-IGF-1 in some cell types.

Since the differences in biological potencies of IGF-1 and des-(1-3)-IGF-1 demonstrated in the first and second of these patterns could not be attributed to altered cell receptor binding (Ballard *et al.*, 1987, 1988), a plausible explanation was that some cells secreted a protein into the medium that selectively modified one of the two growth factors. Accordingly, media conditioned by the cell types were tested for the presence and specificities of any secreted binding proteins (Table 1). The L6 myoblasts secreted a binding protein(s) to which IGF-2 competed at lower concentrations than IGF-1. Competition by des-(1-3)-IGF-1 for radioligand binding was not detectable. The human lung fibroblasts also produced binding protein(s), but with this conditioned medium IGF-1 was equipotent with IGF-2 as competing ligand. Des-(1-3)-IGF-1 did compete for binding, albeit at high concentrations. The third cell type tested, chick embryo fibroblasts, did not secrete any detectable binding protein activity (Table 1).

Fig. 1. Effects of added binding proteins on the inhibition of protein breakdown produced by insulin-like growth factors

IGF-1 (●, ○), IGF-2 (■, □) or des-(1-3)-IGF-1 (▲, △) were added to chick embryo fibroblasts in the absence (solid symbols) or presence (open symbols) of 100 ng of binding protein/ml. (a) MDBK-cell binding protein; (b) amniotic fluid binding protein. Points represent the means of determinations on triplicate wells. Variability is indicated by the inclusion of S.E.M. bars (where sufficiently large) in the IGF-2 experiments in the presence of binding protein.

The results shown in Table 1 can be explained by postulating that all three peptides are intrinsically equipotent in their abilities to inhibit protein breakdown, but that the observed potencies have been modified by the existence and specificities of IGF-binding proteins. Moreover, the observation that the highest biological potencies occur with IGF peptides that bind least to the secreted binding proteins, and *vice versa*, suggests that binding proteins lower the effective concentration of the growth factors.

A more direct way to examine the effects of binding proteins on the biological activities of IGF peptides is to add pure binding proteins to the assays. Chick embryo fibroblasts are particularly useful for such an evaluation because the results will not be complicated by the presence of endogenously-produced binding protein. The inclusion of a binding protein purified from MDBK cells (Szabo *et al.*, 1988) to a protein breakdown assay with chick embryo fibroblasts (Fig. 1a) had no effect on the dose-response curve for des-(1-3)-IGF-1. However, the equivalent curves for IGF-1 and especially IGF-2 were displaced so that higher added concentrations of these

Fig. 2. Effect of added binding proteins on the binding of labelled growth factors to L6 myoblast cultures

Radiolabelled IGF-1 (●), IGF-2 (■) or des-(1-3)-IGF-1 (▲) was added to the cell monolayers and binding measured in the presence of (a) MDBK-cell binding protein, or (b) amniotic fluid binding protein. The plotted values are means of determinations ($\pm$ S.E.M.) on triplicate wells and are expressed as percentages of the cell radioactivity in the absence of added binding protein.

two growth factors were needed to inhibit protein breakdown.

Somewhat different results were obtained when purified human amniotic fluid binding protein was added to the cells (Fig. 1b). In this experiment the dose-response curves for IGF-1 and IGF-2 were displaced equally and to a large extent in the presence of binding protein. As with the MDBK-cell binding protein, the effect on the biological activity of des-(1-3)-IGF-1 was minimal. A second similarity was the increased slope of the dose-response curves in the presence of both binding proteins. Indeed, when 100 ng of binding protein per ml was added, no significant effect of IGF-2 was observed until the same molarity was reached (Fig. 1). This concentration of binding protein was chosen because it had been used previously by Elgin *et al.* (1987) and because a large response was observed in dose-response studies (see Fig. 2).

Both the shifts in the IGF-1 and IGF-2 dose-response curves and their altered slopes in the presence of either

Fig. 3. Stimulation of DNA synthesis in chick embryo fibroblasts by growth factors

Thymidine incorporation into DNA was measured at the indicated concentrations of (a) IGF-1, (b) IGF-2 or (c) des-(1-3)-IGF-1, and expressed as the percentage incorporation relative to wells containing 10% fetal bovine serum as a source of growth factors (control). Measurements were taken in the presence (open symbols) or absence (closed symbols) of MDBK-cell binding protein (100 ng/ml). Values are the means ($\pm$ S.E.M.) of six determinations.

binding protein are consistent with the growth factors being sequestered as inactive complexes. This proposal has been tested by measuring the effect of increasing amounts of binding protein on the receptor binding of the three radiolabelled peptides (Fig. 2). The MDBK-cell binding protein inhibited the binding of ¹²⁵I-labelled IGF-2 and ¹²⁵I-labelled IGF-1 to L6 myoblasts, giving 50% inhibition at 4 ng/ml and 30 ng/ml respectively. The results obtained with the amniotic fluid binding protein in this receptor assay reflected its effect on the growth factor dose-response curves, since receptor binding was inhibited approximately equally with both IGF-1 and IGF-2 (Fig. 2b). Much higher concentrations of each type of binding protein were needed to inhibit the binding of labelled des-(1-3)-IGF-1 to the myoblast monolayers.

Measurements of protein breakdown are particularly useful in the present investigations because they can be accomplished with considerable precision over a relatively short 4 h incubation period. Thus the production of any endogenous binding proteins or growth factors will be less than for the longer time

Fig. 4. Changes in protein content in chick embryo fibroblasts over a 24 h incubation period in the presence of different growth factors

Values are the means ($\pm$ S.E.M.) of determinations on six wells at each concentration of (a) IGF-1, (b) IGF-2 or (c) des-(1-3)-IGF-1, and are expressed relative to the protein content of cells incubated in media containing 10% fetal bovine serum. Measurements were taken in the presence (open symbols) and absence (closed symbols) of MDBK-cell binding protein (100 ng/ml).

periods required for DNA synthesis. However, DNA labelling is more generally used to evaluate growth factor preparations. Accordingly, we have tested the effects of added MDBK-cell binding protein on DNA synthesis in chick embryo fibroblasts (Fig. 3). In the absence of binding protein, thymidine incorporation was increased 2-fold by each of the IGF peptides, from approx. 10% to approx. 20% of the incorporation achieved with 10% fetal bovine serum. The basal rate was depressed by addition of the binding protein, presumably due to the endogenous production of an IGF by the cells. The IGF-2 dose-response curve (Fig. 3b) was shifted about 10-fold towards higher concentrations, while the response with des-(1-3)-IGF-1 (Fig. 3c) was minimal, except at the lowest concentration tested. The pattern with IGF-1 was intermediate (Fig. 3a), similar to that reported above for the protein breakdown measurements.

The protein content of chick embryo fibroblast monolayers was increased markedly by insulin-like growth factors, with the responses over 24 h more than 50% of those obtained when 10% fetal bovine serum was present (Fig. 4). In the absence of added binding protein the potencies of IGF-1, IGF-2 and des-(1-3)-

IGF-1 were approximately equal, although the truncated peptide was slightly more active at low concentrations and slightly less active at high concentrations (compare Fig. 4c with Fig. 4a or 4b). The MDBK-cell binding proteins reduced the basal protein content, a result that, as with the thymidine incorporation study, presumably reflects endogenous secretion of an IGF by the cells. The dose-response curves for IGF-1 (Fig. 4a) and especially IGF-2 (Fig. 4b) were displaced by the binding protein towards higher concentrations. The des-(1-3)-IGF-1 response was not altered other than as would be expected to account for the differences in endogenous IGF activity (Fig. 4c).

DISCUSSION

Both IGF-binding proteins evaluated in this investigation markedly inhibited the biological activities of IGF-2. Thus the protein purified from media conditioned by MDBK cells displaced the dose-response curves for IGF-2 action towards higher concentrations when DNA synthesis, protein accumulation or the inhibition of protein breakdown was measured in chick embryo fibroblasts. A similar result was obtained when the amniotic fluid binding protein was evaluated in the protein breakdown assay. Moreover, the inhibitor effects of the two binding proteins on IGF-2 action were accompanied by marked reductions in the binding of labelled IGF-2 to rat L6 myoblast cultures. It seems clear from these results that each binding protein must form a complex with IGF-2 that is either partially or totally inactive. Evidence supporting a totally inactive complex comes from the observation that the slopes of the dose-response curves became very steep in the presence of the binding proteins, with IGF-2 activity only evident when the molar ratio of IGF-2 to binding protein approached unity. Inhibitory effects of a binding protein isolated from BRL-3A-cell-conditioned medium on the ability of rat IGF-2 to stimulate DNA synthesis or glucose transport in chick embryo fibroblasts have been reported previously (Knauer & Smith, 1980).

There is good evidence that the biological actions produced by IGF-2 occur following binding of the peptide to the type 1 IGF receptor or perhaps the insulin receptor rather than to the type 2 receptor. Although this generalization applies to several cell types tested (Mottola & Czech, 1984; Yu & Czech, 1984; Furlanetto *et al.*, 1987), it is particularly apt for chick embryo fibroblasts because these cells do not contain the type 2 receptor (Rechler *et al.*, 1980; Mottola & Czech, 1984). Accordingly, we can be confident that the ability of each binding protein to blunt IGF-2 action results from a blocking of IGF-2 binding to the type 1 receptors on the chick embryo fibroblasts. On the other hand, we have established that labelled IGF-2 binds only to the type 2 IGF receptor on L6 myoblasts, since competition by IGF-1 for IGF-2 binding is extremely weak and all crosslinking is to the type II receptor (Ballard *et al.*, 1988). Interestingly, in the L6 myoblasts, the two binding proteins inhibited IGF-2 binding very effectively. Moreover, inspection of the binding data (Fig. 2) and the effects on protein breakdown (Fig. 1) for the amniotic fluid binding protein indicates virtually superimposable curves for IGF-1 and IGF-2. Taken together, these results imply that the two binding proteins prevent IGF peptides binding to both type 1 and type 2 IGF receptors on the surface of cells.

As with IGF-2, the addition of binding protein blunted the biological responses produced by IGF-1 in chick-embryo fibroblasts. One difference between the two IGF peptides was the relatively weak effect seen on IGF-1 action when the MDBK-cell binding protein was tested. This result occurred in the DNA synthesis, protein accumulation and protein breakdown experiments with chick embryo fibroblasts and was also evident in the binding measurements with L6 myoblasts. Since the two binding proteins were investigated at the same concentrations and using the same batches of labelled and unlabelled IGF peptides, the results clearly demonstrate that the binding proteins have different IGF specificities. Moreover, although both the MDBK-cell and amniotic-fluid proteins can be classified as low molecular mass binding proteins, the MDBK-cell protein has a larger molecular mass and a very different amino-terminal sequence compared with the amniotic fluid binding protein (Szabo *et al.*, 1988).

Recently, Elgin *et al.* (1987) and Ritvos *et al.* (1988) have evaluated the effects of binding proteins on various biological activities in cells. Whilst the binding proteins used in the two studies have identical amino-terminal sequences, and are thus likely to be identical proteins, the results obtained were very different. Thus Elgin *et al.* (1987) observed substantial stimulatory effects on IGF-1-induced DNA synthesis and cell proliferation in several cell lines, whereas Ritvos *et al.* (1988) demonstrated inhibition of IGF-1 binding and inhibition of IGF-1-induced amino acid transport. The likelihood that the two groups were using the same binding protein and the certainty that the amniotic fluid binding protein used by us here is the same as that used by Elgin *et al.* (1987), eliminate the possibility that the different results were a consequence of different proteins being compared. Rather, it seems likely that assay differences must account for the conflicting results. Moreover, in the present investigation and the two other studies discussed in which inhibitory effects of binding protein were observed, IGF was added in the absence of proteins other than bovine serum albumin (Knauer & Smith, 1980; Ritvos *et al.*, 1988). Elgin *et al.* (1987), on the other hand, included in all incubations 1% platelet-poor plasma obtained from hypopituitary subjects. Perhaps this constituent contains a factor that influences IGF-1 binding.

The inclusion of des-(1-3)-IGF-1 with IGF-1 and IGF-2 in ligand specificity studies has provided an explanation for the different biological potencies of the IGF peptides. Since the truncated growth factor either does not bind or only binds weakly to the purified binding proteins or to the conditioned media examined in this report, the concentration of free des-(1-3)-IGF-1 remains approximately equal to the total concentration added. The free concentration of IGF-1, however, will be particularly reduced by the amniotic fluid binding protein or the one present in medium conditioned by HE(39)L human lung fibroblasts, and to a lesser extent by the MDBK-cell binding protein or medium conditioned by L6 myoblasts. Hence higher IGF-1 concentrations will be required to exert biological effects. Similar logic can be used to explain the poor potency of IGF-2, especially when an IGF-2-preferring binding protein is present. Indeed it could be argued that the presence or absence of binding proteins could totally account for the potency differences between IGF-1, des-(1-3)-IGF-1 and IGF-2. This hypothesis certainly fits the results obtained with

chick embryo fibroblasts, since the three growth factors are approximately equipotent in different assays and the cells do not produce any detectable binding protein. Furthermore, the occurrence of excess IGF-2-preferring binding proteins in fetal blood, partly as the extracellular domain of the type 2 receptor (Hey *et al.*, 1987; Kiess *et al.*, 1987), could explain why circulating IGF-2 does not seem to act as a growth factor for the young animal (Schoenle *et al.*, 1985; Gluckman *et al.*, 1987; Jones *et al.*, 1988).

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