Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 207-214

THE BOVINE INSULIN-LIKE GROWTH FACTOR (IGF) BINDING PROTEIN PURIFIED FROM CONDITIONED MEDIUM REQUIRES THE N-TERMINAL TRIPEPTIDE IN IGF-1 FOR BINDING

Laszlo Szabo, David G. Mottershead, F. John Ballard* and John C. Wallace

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

Received January 4, 1988

Summary: The insulin-like growth factor binding protein (BP) secreted by bovine kidney (MDBK) cells has been purified by affinity chromatography on a rat IGF-2 Sepharose column. Purified BP migrated as a single band of M_r 40,000 upon SDS polyacrylamide gel electrophoresis. An N-terminal sequence of 53 residues was obtained which was very similar up to residue 21 to the corresponding rat BRL-3A BP sequence. In competitive binding experiments with bovine IGF-1 and IGF-2, and recombinant human IGF-1, BP had a similar affinity for these ligands when IGF-1 tracer was used, but a higher affinity for IGF-2 with IGF-2 as radioligand. The N-terminal destripeptide truncated form of bovine IGF-1, which has enhanced biological activity, was found to have a markedly reduced affinity for BP compared to intact IGF-1. The increased bioactivity of destripeptide IGF-1 can be explained by this reduced affinity for BP.

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are growth stimulating polypeptides that circulate in plasma bound to two classes of binding proteins (BP), with M_r of 30-45,000 and 125-150,000. The small BP has been detected in a variety of body fluids, including plasma, amniotic fluid and cerebrospinal fluid, and is secreted by several cell lines growing in serum-free media. It has been purified from human amniotic fluid (1,2, see also 3) as well as from serum-free medium conditioned by the human hepatoma cell line HEP G2 (4) or rat BRL-3A cells (5,6,7). N-terminal amino acid sequencing indicates that the small BP from the two human sources are identical, but shows only limited similarity to the rat sequence. The aim of the present study was to purify the small BP present in the serum-free conditioned medium of the MDBK cell line, and examine the structure and properties of this small bovine BP, especially the specificity of its binding to IGF-1 and IGF-2 and to the more potent destripeptide form of IGF-1 (-3N:IGF-1).

MATERIALS AND METHODS

Materials: Bovine IGF-1, IGF-2 and -3N:IGF-1 were purified and iodinated with ^{125}I using the Chloramine T method, to a specific activity of between 20 and 80 Ci/g, as previously described (8). Synthetic -3N:IGF-1 was prepared as previously described (9). Recombinant human IGF-1,

Abbreviations: IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2; -3N:IGF-1, a truncated form of IGF-1 missing the first three N-terminal residues; BP, IGF binding protein; SDS, sodium dodecyl sulphate; HPLC, high performance liquid chromatography.

0006-291X/88 $1.50

which is identical to bovine IGF-1 (8), was a gift from Ciba-Geigy. For the purpose of standardising growth factor preparations it was assumed that the absorbance at 280nm of similar weights of IGF-1, IGF-2, -3N:IGF-1 and porcine insulin were equivalent. Bovine IGF-1 and IGF-2 N-terminal peptides were obtained from Biotechnology Research Enterprises of South Australia, and represent, respectively, the first 14 residues plus an Asp of IGF-1 and the first 9 residues of IGF-2. An IGF-2 affinity column was made by coupling 0.2mg rat IGF-2, purified by the method of Marquardt et al. (10), to 0.6ml of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

BP Assay: BP activity was measured using the charcoal binding assay essentially as described by Martin and Baxter (11), except that the incubation time with charcoal solution (which contained 0.25% bovine serum albumin) was increased to 30 min to improve assay precision, and the charcoal was removed by centrifugation at 10,000g for 3 min. BP assays with [^{125}I]IGF-1 or [^{125}I]IGF-2 had approximately 4000 dpm per tube, while 6000 dpm of [^{125}I]-3N:IGF-1 was used.

Purification of BP: MDBK cells (Flow Laboratories ATCC CCL22) were grown to confluence using a cell factory (Nunc) in Dulbecco's modified Eagle's Minimal Essential Medium supplemented with 10% fetal bovine serum and subsequently maintained in the same medium but without serum. Conditioned medium was collected every 72 hours and stored at -15°C until required. Four litre batches were thawed and filtered through Whatman No.1 paper. All further steps were at room temperature. The filtrate was acidified to 50mM acetic acid with glacial acetic acid and the pH adjusted to 3.0 with concentrated HCl. After filtration through Whatman No.1 paper, the clear filtrate was pumped on to a S-Sepharose Fast Flow column (Pharmacia), dimensions 3.2 x 25cm, at a flow rate of 10 ml/min. The column was washed with 1 litre of 50mM acetic acid, followed by 1 litre of 50mM ammonium acetate pH 6.5. BP was eluted with a 1.8 litre linear gradient of 50mM ammonium acetate pH6.5 to 2.0M ammonium acetate pH8.0 at a flow rate of 2.5ml/min. The 0.05-0.5M ammonium acetate pool containing all detectable BP activity was freeze-dried.

The dried powder was resuspended in 40ml of 0.5M acetic acid and centrifuged at 30,000g for 30 min to remove insoluble proteins. The supernatant was freeze-dried, resuspended in 4.0ml of 0.5M acetic acid and further purified by size exclusion chromatography on a column (2.5 x 33 cm) of TSK-Fractogel (HW 55S, Merck), equilibrated in 0.5M acetic acid. The flow rate was 0.5ml/min. Fractions (6ml) were collected and those containing BP activity were pooled and freeze-dried.

The dried protein was resuspended in 5.0ml of 50mM Tris HCl containing 0.5M NaCl at pH 7.8 and pumped at 0.25ml/min onto the IGF-2 affinity column, equilibrated in the same buffer. The unabsorbed material was collected. The column was washed with at least 10ml of equilibration buffer after which the BP was eluted with 0.5M acetic acid at a flow rate of 0.5ml/min. The first 3.0ml of eluant, containing the BP activity, was collected. The column was re-equilibrated with the initial buffer until the pH returned to 7.8. The unadsorbed material from the first passage was recycled through the column. This procedure was repeated until the HPLC analysis showed no further binding of BP to the affinity column.

The BP in the eluant from each passage of material through the affinity column was further purified by HPLC, using an Aquapore RP-300 reverse phase cartridge (0.46 x 3.0cm, Brownlee Labs) equilibrated with 20% acetonitrile in 0.1% trifluoroacetic acid. BP was eluted with a linear gradient of 20-60% acetonitrile in 0.1% trifluoroacetic acid, over 30 min at 0.5ml/min. The major protein peak eluted at 31% acetonitrile. The BP peak from several HPLC runs was pooled and rerun on the HPLC column giving a single peak of BP, which was dried and stored at -15°C. BP was quantified on the HPLC column by comparison with the peak areas of increasing amounts of bovine serum albumin, using the absorbance of these proteins at a wavelength of 215nm. The final yield was approximately 100μg.

SDS polyacrylamide gel electrophoresis: This was performed using the discontinuous buffer system of Laemmli (12). The samples were solubilised in Laemmli loading buffer containing 5% 2-mercaptoethanol at 100°C for 5 min, loaded onto a 15% polyacrylamide slab gel and electrophoresed at 150V for 3h. The proteins were fixed and stained for 3h in 10% acetic acid and 50% methanol containing 0.1% Coomassie Brilliant Blue R250, after which the gels were destained overnight in a solution of 10% acetic acid and 5% methanol. The following marker proteins were used: phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000) and myoglobin (M_r 17,800).

Protease digestion of BP: Samples of BP were reduced and S-carboxymethylated as described previously (8) after which 30 µg was dissolved in 0.20ml of 0.1M ammonium bicarbonate pH 7.8 containing 0.5 µg endoproteinase-Glu-C (Boehringer Mannheim) and incubated for 18h at 37°C. The digest was adjusted to 0.1% with trifluoroacetic acid after which the peptides were separated by HPLC, using an Aquapore RP-300 reverse phase cartridge (0.46 x 3.0cm, Brownlee Labs), with a linear gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid, over 30 min at 0.5ml/min.

Protein sequencing: The N-terminal sequence of intact BP and the sequence of S-carboxymethylated peptide fragments were determined by Edman degradation using an Applied Biosystems gas phase sequencer (13) as described previously (8).

RESULTS

SDS polyacrylamide gel electrophoresis of BP showed a single band of M_r 40,000 after staining with Coomassie Blue (Fig. 1A). N-terminal sequence analysis of 30 µg of unreduced BP identified the first thirty residues of this protein (Fig. 2). To extend the sequence, 30 µg of ^{3}H-labelled, S-carboxymethylated BP was digested with endoproteinase-Glu-C and the peptide fragments separated by HPLC (Fig. 1B). Incorporation of tritium into each peak was estimated and the highest level of incorporation was found in peptide B, which was sequenced. This sequence overlapped exactly with residues 13 to 30 of the N-terminal sequence of intact BP and confirmed cysteine residues at positions 13 and 17. The peptide sequence continued to residue

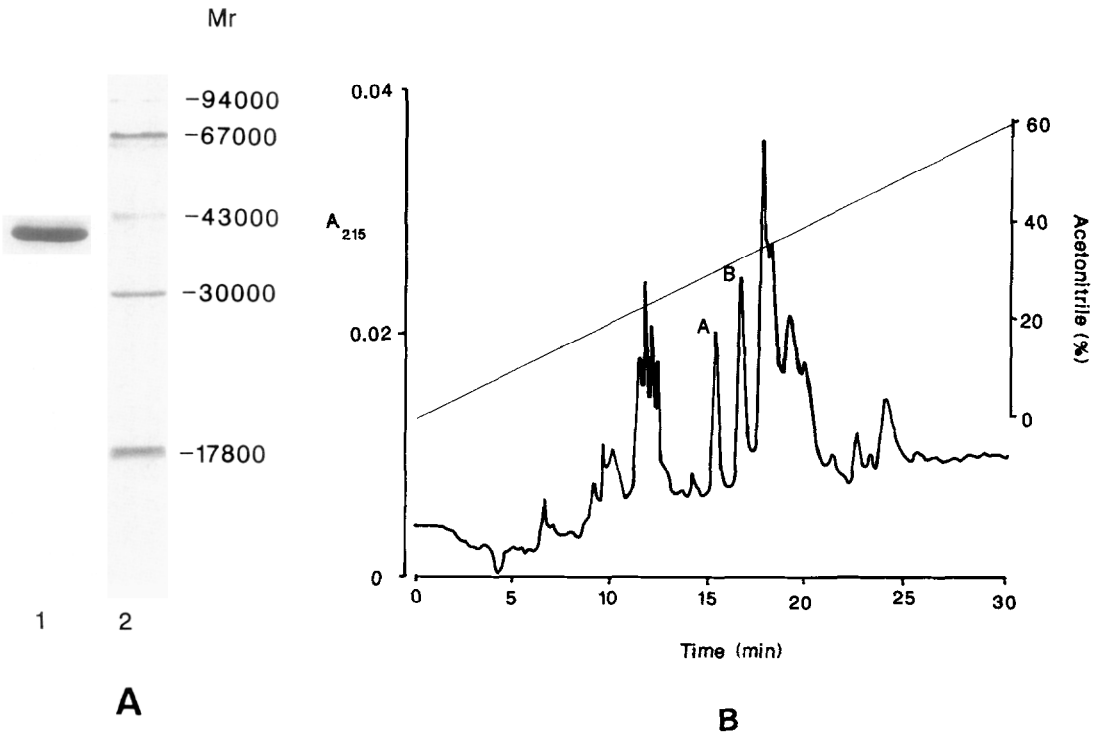

Figure 1. (A) 15µg of purified BP (track 1) was analysed by SDS polyacrylamide gel electrophoresis under reducing conditions on a 15% gel and the M_r estimated by comparison to marker proteins (track 2). (B) Separation of BP peptides by reverse phase HPLC. 30µg of reduced and S-carboxymethylated BP was digested with endoproteinase-Glu-C after which the peptides were separated by reverse phase HPLC. Peptides A and B were sequenced.

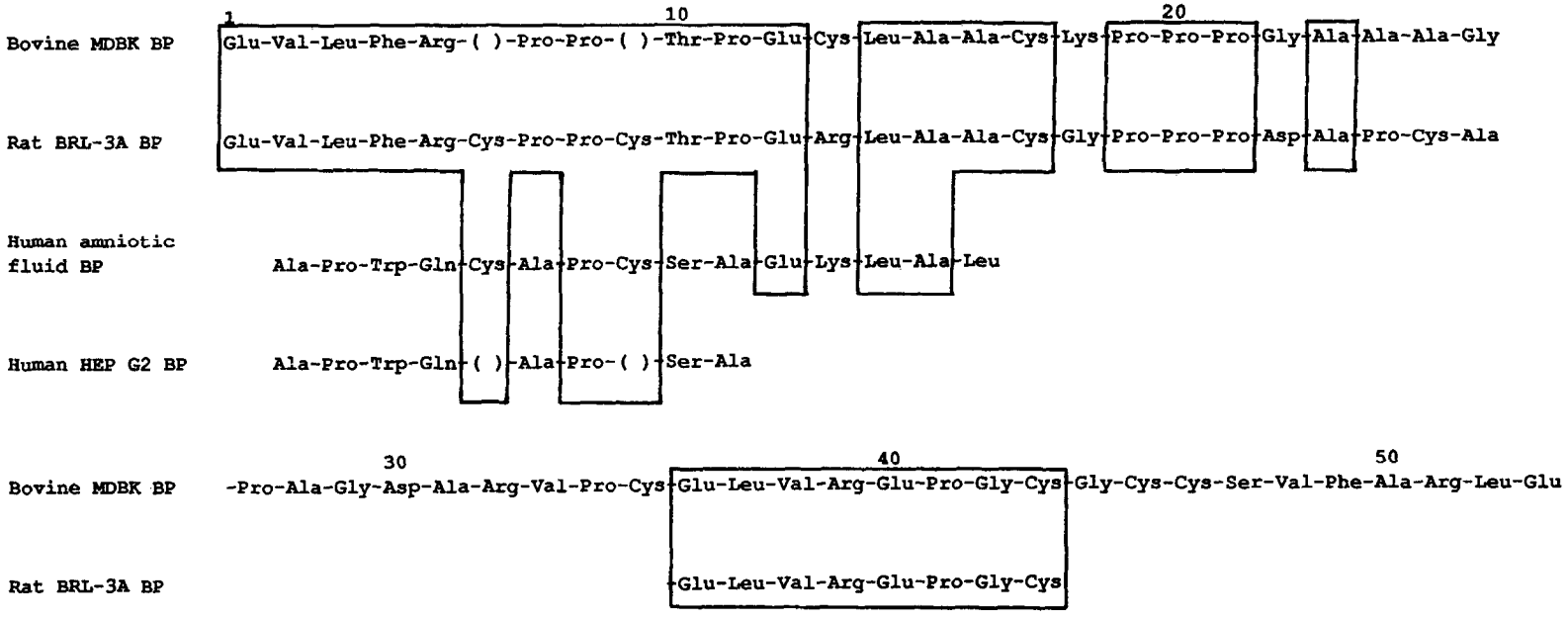

Figure 2. Comparison of the N-terminal sequence of MDBK BP with other BP sequences. The MDBK BP sequence is derived from the N-terminal sequence of unreduced BP (residues 1-30) and the overlapping sequence of peptide B (Fig. 1B) (residues 13 to 53). The gaps at residues 6 and 9 indicate no PTH derivatives were detected, and presumably represent Cys residues. The other sequences were obtained from published data (1,4,6,7,20). Regions of interspecies sequence identity are shown by boxes.

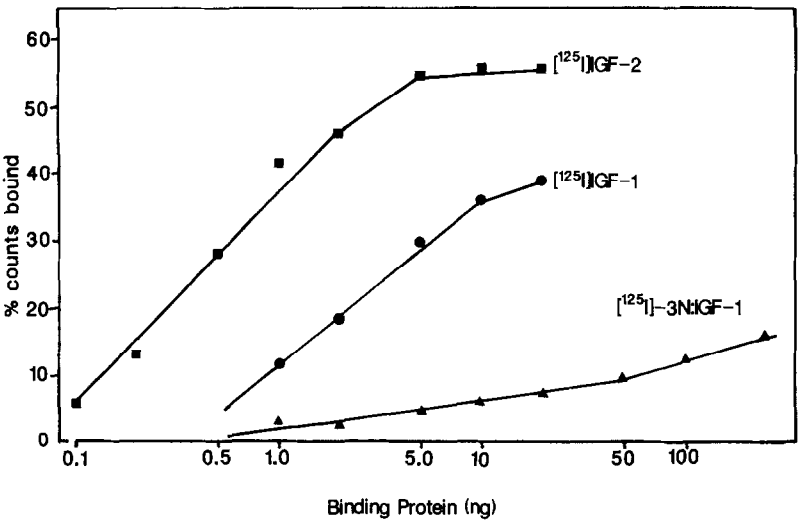

Figure 3. Binding of tracers to BP. Three tracers were used: [125I]IGF-1 (●), [125I]IGF-2 (■) and [125I]-3N:IGF-1 (▲). Increasing amounts of BP were added to tracer in a final volume of 300µl and the % counts bound to BP determined.

53, ending at a Glu as expected (Fig. 2). We note that the sequence continued past Glu residues at positions 36 and 40.

Peptide A, the other well separated peptide from the above HPLC run (Fig. 1B), was also sequenced fully, to give a presumably internal sequence of 23 residues, as follows: Cys-Trp-Cys-Val-Asn-Pro-Asn-Thr-Gly-Lys-Leu-Ile-Gln-Gly-Ala-Pro-Thr-Ile-Arg-Gly-Asp-Pro-Glu.

We examined the ability of increasing amounts of BP to bind 125I-labelled IGF-1, IGF-2 and -3N:IGF-1 (Fig 3). A ten fold higher concentration of BP was needed to achieve 30% binding of IGF-1 as compared with IGF-2. The truncated growth factor, -3N:IGF-1, bound very poorly. These results were confirmed and extended by competition studies using IGF-1 and IGF-2 tracers with increasing amounts of unlabelled ligands (Fig. 4). With IGF-1 tracer, the abilities of IGF-1, IGF-2, and recombinant IGF-1 to compete for tracer were similar. As expected from the measurements reported in Fig.3, -3N:IGF-1 was relatively ineffective in competing for tracer, with 20 fold more ligand required to achieve 50% inhibition of binding as compared with IGF-1 or IGF-2. When IGF-2 was used as radioligand (Fig. 4), the competition curves were very different. Thus IGF-2 was found to be 10 fold more effective in competing for tracer than either of the IGF-1 ligands, which in turn were 6 fold more effective than purified -3N:IGF-1. Synthetic -3N:IGF-1, although equal in potency and receptor binding to the purified -3N:IGF-1 (9), did not compete significantly for the binding of either tracer to the BP (Fig. 4). This result can be explained by a 2% contamination of IGF-2 in the preparation of purified -3N:IGF-1 used in the binding experiments.

No competition for the binding of labelled IGF-1 or labelled IGF-2 to BP could be detected when porcine insulin, an N-terminal peptide comprised of 14 residues plus Asp of IGF-1 or the N-terminal nonapeptide of bovine IGF-2 were tested at amounts up to 300 ng.

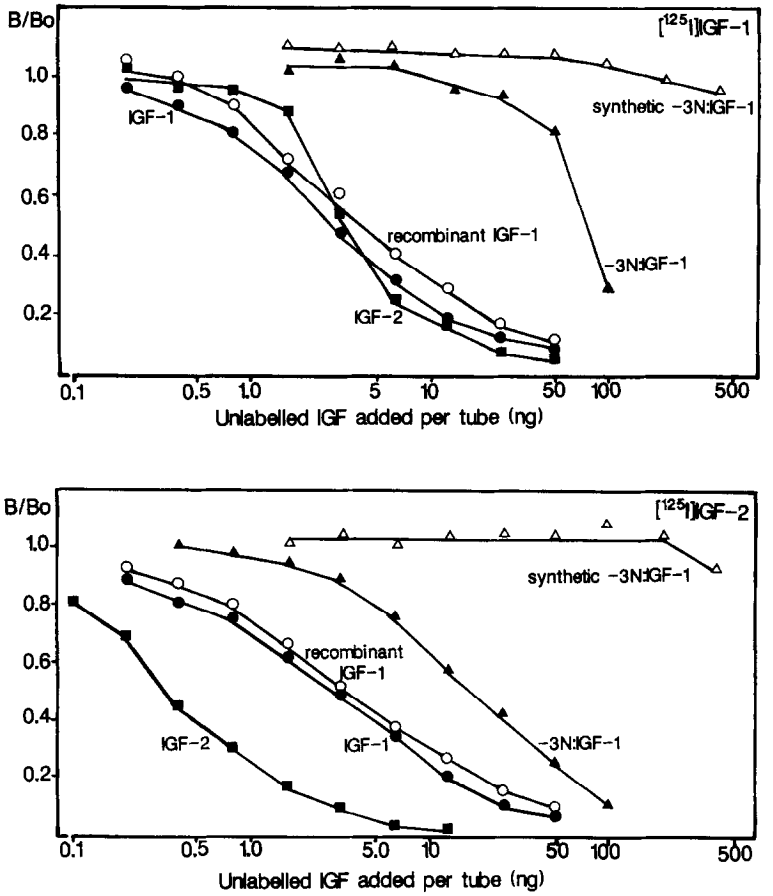

Figure 4. Competitive binding of peptides to BP. Two tracers were used: [^{125}I]IGF-1 (top) and [^{125}I]IGF-2 (bottom). Increasing amounts of bovine IGF-1 (●), IGF-2 (■), -3N:IGF-1 (▲) synthetic -3N:IGF-1 (Δ) and recombinant human IGF-1 (○) were added to each tube, followed by 4000dpm tracer and then 4.0ng BP with IGF-1 tracer (B_O=26% counts bound) or 1.0ng BP with IGF-2 tracer (B_O=40% counts bound), in a final volume of 300 µl. B/B_O was calculated after BP assay.

DISCUSSION

The IGF BP from rat BRL-3A conditioned medium has been characterised recently by three groups and appears to have a different N-terminal amino acid sequence to the small human BP isolated from amniotic fluid or HEP G2 conditioned medium (1,4,6,7). To examine further the structural relationship between various mammalian BP, the bovine BP secreted by MDBK cells was purified and partially sequenced. This revealed a region at the N-terminus of the protein that was highly conserved between rat and bovine BP, but showed less similarity to the limited sequence available for the human BP (Fig. 2). The strong similarity between the rat and bovine sequence continued beyond an insertion of nine residues in the bovine sequence. The MDBK BP, with a M_r of 40,000, has a higher M_r than the BP purified from human amniotic fluid, human decidua, human HEP G2 and rat BRL-3A conditioned media (M_r range of 33,000 to 36,300), perhaps due to the presence of insertions in the MDBK BP sequence. It should be noted that

these differences in BP M_r, although small, are credible because similar M_r standards have been used by the various research groups.

The role of the small BP as a binding protein for IGF-1 and IGF-2 is not yet clear; for example, it has not been established whether BP acts as a carrier protein for both of these peptides *in vivo*, or whether BP is selective for one of them. Our measurements showed that the relative affinities of MDBK BP for IGF-1 and IGF-2 were indistinguishable when IGF-1 tracer was used, but a preference for IGF-2 was evident with IGF-2 as radioligand. These results are similar to those reported for the BP from cerebrospinal fluid (14), except in our hands native IGF-1 and recombinant human IGF-1 were equipotent. These two studies with recombinant IGF-1 raise the possibility that earlier experiments used to compare IGF-1 and IGF-2 binding to BP may have used IGF preparations cross contaminated with the other growth factor, a problem also seen in competitive binding experiments with type 1 and type 2 IGF receptors (15). In other competitive binding experiments, however, purified amniotic fluid BP showed a similar affinity for both IGF-1 and IGF-2 when either was used as tracer (16).

It would appear from our results that there may be two binding sites for IGF on the MDBK BP, one site for which IGF-1 and IGF-2 have a similar affinity (since IGF-1 and IGF-2 gave similar competitive binding curves with IGF-1 tracer), and another site which has a higher affinity for IGF-2 compared to IGF-1 (since IGF-2 is more potent than IGF-1 in displacing IGF-2 tracer). Cross-linking studies and analysis of IGF-BP complexes by size exclusion chromatography have, however, shown no more than one mole IGF bound per mole of BP (6,7). Accordingly, if separate sites do exist for IGF on the MDBK BP, they probably overlap so that they cannot be occupied simultaneously.

An IGF-1 related protein has been purified from bovine colostrum that after sequencing was found to be identical to IGF-1 except for the deletion of the three N-terminal residues (8). This -3N:IGF-1, either purified from bovine colostrum or chemically synthesised (9), has approximately ten times the biological activity of IGF-1 and a low affinity for MDBK BP in competition assays with either IGF-1 or IGF-2 tracer. Clearly the deletion of only three residues from the N-terminus of IGF-1 is enough to reduce drastically the affinity for BP, and is consistent with the results of others who have found that the B domain of IGF-1 is important for the binding of IGF-1 to BP (17). On the other hand, we also found that synthetic peptides comprised of part of the N-terminal sequence of the B domain of IGF-1 or IGF-2 failed to [illegible] binding of IGF-1 and IGF-2 tracer to MDBK BP. The two results argue that the N-terminal region is required but is not sufficient for binding to the BP, and that the first three residues of IGF-1 are especially critical for binding of IGF-1 to MDBK BP.

Recently Elgin *et al.* (18) reported that a purified human amniotic fluid BP could enhance, rather than inhibit, IGF-1 mediated cell replication in chick embryo fibroblasts. Knauer and Smith, however, reported an inhibition of DNA synthesis by purified rat BRL-3A BP in similar cells (19). The differences seen between small BP sequences, their varying affinities for IGF-1 and IGF-2, and their conflicting effects on IGF mediated activity in cultured cells, point to the existence of more than one type of mammalian small BP. Our examination of the binding of -3N:IGF-1 to MDBK BP is consistent with this particular BP acting as an inhibitor of IGF-1

activity in cells, by sequestering IGF-1 away from type 1 IGF receptors. Any endogenous MDBK-like BP secreted by cells in culture would be unable to prevent -3N:IGF-1 from binding to cell surface receptors, leading to increased DNA and protein synthesis in those cells. Hence the increased biological potency of -3N:IGF-1.

Acknowledgements: This work was supported by National Health and Medical Research Council Research Grant #860416. We wish to thank Mr. Stephen Rogers and Ms. Denise Turner for carrying out the sequence analyses and Mr. Geoffrey Francis and Mr. Peter McNamara for the supply of the bovine growth factors, as well as Mr. Christopher Bagley and Mr. Bruce May for providing the synthetic -3N:IGF-1. Recombinant human IGF-1 was kindly provided by Drs. H. Peter and K. Scheibli, CIBA-GEIGY, Basel, Switzerland.

REFERENCES

1. Povoa, G., Enberg, G., Jornvall, H. & Hall, K.(1984) *Eur. J. Biochem. 144*, 199-204.
2. Drop, S.L.S., Kortleve, D.J. & Guyda, H.J. (1984) *J. Clin. Endocrinol. Metab. 59*, 899-907.
3. Koistinen, R., Kalkkinen, N., Huhtala, M.-L., Seppala, M., Bohn, H. & Rutanen E.-V. (1986). *Endocrinology 118*, 1375-1378.
4. Povoa, G., Isaksson, M., Jornvall, H. & Hall, K. (1985) *Biochem. Biophys. Res. Commun. 128*, 1071-1078.
5. Romanus, J.A., Terrell, J.E., Yang, Y.W.H., Nissley, S.P., & Rechler, M.M. (1986) *Endocrinology 118*, 1743-1757.
6. Mottola, C., MacDonald, R.G., Brackett, J.L., Mole, J.E., Anderson, J.K. & Czech, M.P. (1986) *J. Biol. Chem. 261*, 11180-11188.
7. Lyons, R.M. & Smith, G.L. (1986) *Mol. Cellular Endocrin. 45*, 263-270.
8. Francis, G.L., Upton, F.M., Ballard, F.J., McNeil, K.A. and Wallace, J.C. (1988) *Biochem. J.* in press.
9. Ballard, F.J., Francis, G.L., Ross, M., Bagley, C.J., May, B. & Wallace, J.C. (1988) *Biochem. Biophys. Res. Commun.* in press.
10. Marquardt, H., Todaro, G.J., Henderson, L.E., Oroszlan, S. (1981) *J. Biol. Chem. 256*, 6859-6865.
11. Martin, J.L. & Baxter R.C. (1986) *J. Biol. Chem. 261*, 8754-8760.
12. Laemmli, U.K. (1970) *Nature 227*, 680-685.
13. Hunkapiller, M.W., Hewick, R.M., Dreyer, W.J. & Hood, L.E. (1985) *Methods. Enzymol. 91*, 399-412.
14. Hossenlopp, P., Seurin, D., Segovia-Quinson, B. & Binoux, M. (1986) *FEBS Lett., 208*, 439-444.
15. Rosenfeld, R.G., Conover, C.A., Hodges, D., Lee, P.D.K., Misra, P., Hintz, R.L. & Li, C.H. (1987). *Biochem. Biophys. Res. Commun. 143*, 199-205.
16. Baxter, R.C., Martin, J.L. & Wood, M.H. (1987) *J. Clin. Endocrinol. Metab. 65*, 423-431.
17. De Vroede, M.A., Rechler, M.M., Nissley, S.P., Joshi, S., Burke, G.T. & Katsoyannis, P.G. (1985). *Proc. Natl. Acad. Sci. USA 82*, 3010-3014.
18. Elgin, R.G., Busby, W.H. & Clemmons, D.R. (1987) *Proc. Natl. Acad. Sci. USA 84*, 3254-3258.
19. Knauer, D.J. & Smith, G.L. (1980) *Proc. Natl. Acad. Sci. USA 77*, 7252-7256.
20. Baxter, R.C. & Martin, J.L. (1987) *Biochem. Biophys. Res. Commun. 147*, 408-415.