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Isolation of an inhibitory insulin-like growth factor (IGF) binding protein from bone cell-conditioned medium: A potential local regulator of IGF action

(bone cell proliferation)

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ABSTRACT **Inhibitory insulin-like growth factor binding protein (In-IGF-BP) has been purified to homogeneity from medium conditioned by TE89 human osteosarcoma cells by two different methods using Sephadex G-100 gel filtration, FPLC Mono Q ion-exchange, HPLC C_4 reverse-phase, HPLC CN reverse-phase, and affinity chromatographies. In-IGF-BP thus purified appeared to be homogeneous and unique by the following criteria. (*i*) N-terminal sequence analysis yielded a unique sequence (Asp-Glu-Ala-Ile-His-Cys-Pro-Pro-Glu-Ser-Glu-Ala-Lys-Leu-Ala). (*ii*) Amino acid composition of In-IGF-BP revealed marked differences with the amino acid compositions of other known BPs. (*iii*) In-IGF-BP exhibited a single band with a molecular mass of 25 kDa under reducing conditions on sodium dodecyl sulfate/polyacrylamide gels. IGF-I and IGF-II but not insulin displaced the binding of ^{125}I-labeled IGF-I or ^{125}I-labeled IGF-II binding to In-IGF-BP. In-IGF-BP inhibited basal, IGF-stimulated bone cell proliferation and serum-stimulated bone cell proliferation. Forskolin increased synthesis of In-IGF-BP in TE85 human osteosarcoma cells in a dose-dependent manner. Based on these findings, we conclude that In-IGF-BP is a protein that has a unique sequence and significant biological actions on bone cells.**

Insulin-like growth factor binding proteins (IGF-BPs) are present in many tissues (1, 2). Two main classes of IGF-BP exist in human serum: the growth hormone-dependent 150-kDa species and the smaller non-growth hormone-dependent 30- to 40-kDa species. The 150-kDa species consists of an acid-labile subunit and a 53-kDa acid-stable binding subunit (IGF-BP III) bound to IGF-I or IGF-II (3). Full-length cDNA clones of human IGF-BP III have been isolated, and the complete deduced sequence of the protein has been determined (4).

In addition to the serum BPs, IGF-BPs have been found in tissue extracts and culture media conditioned by cells (1, 2). At least two forms, IGF-BP I and IGF-BP II, have been identified. The N-terminal sequences of IGF-BP I derived from several human sources (amniotic fluid, placental membranes, decidua, and HEP G2 hepatoma cells) are identical (5–8). The cloning and complete sequence of cDNA encoding IGF-BP I from HEP G2, human uterus, and human placental cDNA libraries have been recently reported (9–12). IGF-BP II is present in the conditioned medium (CM) from rat BRL-3A cells (13) and Madin–Darby bovine kidney (MDBK) cells (14). The gene encoding IGF-BP II has recently been cloned from BRL-3A cDNA library (15). Thus, a group of three BPs has been identified that bind IGF-I and IGF-II and have limited similarity in their N-terminal sequences.

The role of IGF-BPs in regulating the actions of IGFs is only now emerging. IGF-BP I and IGF-BP III have been shown to inhibit or enhance the action of the IGFs depending on the culture conditions (16–18). These findings are consistent with a role for BPs in regulating the responsiveness of cells to IGFs.

We have recently shown that skeletal growth factor isolated from human bones is very similar, if not identical, to IGF-II (19). The findings that human bone cell CM contains a large molecular weight form of IGF-II suggested that human bone cells in culture produce IGF-II and its BP (20). We report here the isolation and characterization of a protein from human bone cell CM that binds IGF-I and IGF-II and has limited N-terminal sequence similarity with the other known BPs. IGF-BP isolated from bone cell CM was inhibitory to bone cell proliferation under all culture conditions tested. Hence, we have arbitrarily assigned a functional name to this binding protein, inhibitory IGF-BP (In-IGF-BP).

MATERIALS AND METHODS

Collection of CM. Normal human bone cells (HBC) were isolated and cultured as described (20). HBC and human osteosarcoma cells TE89 and TE85 (obtained from American Type Culture Collection) were grown in monolayer cultures in Petri dishes or in roller bottles in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% calf serum. Upon 60–80% confluence, the cells were rinsed once with serum-free DMEM and maintained in serum-free medium for CM collections. The CM samples (2-day collections) were pooled and clarified at 500 × *g* to remove cellular debris.

Purification of BP. CM was acidified with acetic acid (1 M), concentrated using a YM5 membrane (Amicon), and used for purification of BPs by two different procedures.

Procedure 1. (*i*) Gel filtration: The CM concentrate was lyophilized, reconstituted in 2.5 ml of 1 M acetic acid, and applied to a Sephadex G-100 column (2.5 × 100 cm). The proteins were eluted with 1 M acetic acid and 2-ml fractions were collected. The fractions containing BP activity were pooled and lyophilized. (*ii*) FPLC: Sephadex G-100 pool was reconstituted with 20 mM Tris·HCl (pH 8.0) and applied to a Mono Q ion-exchange column. The proteins were eluted with a gradient of NaCl from 0 mM to 500 mM NaCl in 60 min, and 2-min fractions were collected. (*iii*) HPLC C_4: The FPLC Mono Q peak containing BP activity was concentrated and then subjected to HPLC reverse-phase chromatography in

Abbreviations: BP, binding protein; IGF, insulin-like growth factor; In-IGF-BP, inhibitory IGF-BP; CM, conditioned medium; TFA, trifluoroacetic acid; MDBK, Madin–Darby bovine kidney; HBC, human bone cells.

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0.1% trifluoroacetic acid (TFA) using a 4.6 × 250 mm C_4 column (Bio-Rad RP304). The unbound proteins were eluted for 5 min with 15% acetonitrile in 0.1% TFA. Bound proteins were eluted by a linear 15–40% acetonitrile gradient in 125 min. Two-minute fractions were collected. (*iv*) HPLC CN: Final purification of BP was achieved by HPLC reverse-phase chromatography in 0.1% TFA using a 3.9 × 150 mm μBondapak CN column (Waters) with a 1-propanol gradient (20–40% in 100 min).

Procedure 2. (*i*) IGF-I affinity: TE89 CM concentrate was dialyzed against phosphate-buffered saline (PBS) (pH 7.4) and applied to the affinity column (composed of recombinant human IGF-I coupled to CNBr-activated Sepharose 4B). The unbound proteins were eluted with PBS and the bound proteins were eluted with 4 M guanidine hydrochloride (pH 7.4). (*ii*) HPLC C_4: IGF-I affinity bound fraction was dialyzed against 0.1% TFA and separated on HPLC reverse-phase chromatography as described above using a gradient of acetonitrile from 15% to 40% in 125 min.

Assays. At each step during the purification of BP, aliquots of fractions were diluted with DMEM containing 1 mg of bovine serum albumin per ml and used for the following assays. (*i*) BP assay: This assay was based on the principle that polyethylene glycol (PEG) precipitated the large molecular weight BP-IGF complex and not the small molecular weight IGF (18). Briefly, 50 μl of sample was incubated with 25,000–50,000 cpm of ^{125}I-labeled IGF-I or IGF-II (^{125}I-IGF-I or ^{125}I-IGF-II) for 60 min at room temperature in 0.1 M Hepes/0.1% bovine serum albumin/0.1% Triton X-100/44 mM Na_2CO_3/0.02% NaN_3, pH 6.0 (250-μl volume). Bound and free ^{125}I-IGF-I or ^{125}I-IGF-II were separated by adding 100 μl of 2% immune serum globulin and 500 μl of 25% PEG (M_r 8000) and centrifuging. Specific binding was determined by subtracting nonspecific binding (in the presence of excess of unlabeled IGF-II) from total binding. IGF-I and IGF-II were radiolabeled with Na^{125}I by a modification of the chloramine-T method as described (19). (*ii*) Radioreceptor assay for IGF-II: H-35 rat hepatoma cells, which contain abundant IGF-II but not IGF-I receptors, were used as the receptor source (21). IGF-II purified from bone matrix extract was used as standard and tracer. The assay was carried out as described (19). (*iii*) Cell proliferation assay: The proliferation of bone cells in serum-free culture was assayed by the incorporation of [^{3}H]thymidine into tricholoroacetic acid-precipitable material (22).

Cross-Linking Studies. One nanogram of purified BP was incubated with 40,000 cpm of ^{125}I-IGF-II in the presence (10 ng of unlabeled IGF-II) or absence of competitor. The binding was performed in 0.1 ml of 50 mM sodium phosphate buffer/0.1% bovine serum albumin, pH 6.5, for 60 min at room temperature. Two microliters of 25 mM disuccinimidyl suberate (freshly prepared in dimethyl sulfoxide) was added and the incubation was continued for an additional 20 min. At the end of the incubation, 100 μl of 2× electrophoresis sample buffer was added and the samples were boiled for 3 min. BP–^{125}I-IGF-II complex was separated from free ^{125}I-IGF-II by electrophoresis using a 15% slab gel. The gel was air dried and exposed to Kodak X-Omat AR film with the use of an intensifying screen for 7 days at −70°C.

Amino Acid Analysis. Amino acid composition was analyzed with an Applied Biosystems model 420 instrument after hydrolysis in evacuated tubes for 24 hr at 110°C with 6 M HCl/0.5% phenol. N-terminal sequences were determined as described (19).

RESULTS

Isolation of In-IGF-BP. Previous studies have shown that human bone cells in culture produce IGF-II *in vitro* (19, 20). IGF-II levels between 10 and 50 ng/ml were detectable in the CM of HBC and TE89 cells when CM were assayed for their ability to compete with ^{125}I-IGF-II for binding to H-35 rat hepatoma cells. However, samples contained little if any IGF-II when assayed by their ability to stimulate chicken bone cell proliferation. This observation suggested that IGF-II BPs were present in the CM and were inhibiting the biological actions of IGF-II. An assay of BP activity in the CM demonstrated the presence of BPs. In order to identify and isolate the inhibitory BP, CM was fractionated on Sephadex G-100 using 1 M acetic acid to dissociate the BP–IGF complex. The fractions were then assayed by (*i*) a BP assay in which the binding of ^{125}I-IGF-II to BP is detected by precipitating the complex with PEG (this assay detects the BP and not the IGF-II); (*ii*) an IGF-II radioreceptor assay using H-35 cells in which either IGF-II or a BP (that competes with the receptors for IGF-II) would lower the binding of ^{125}I-IGF-II to IGF-II receptors; and (*iii*) a cell proliferation assay in which IGF-II but not inhibitory BP stimulates bone cell replication.

Fig. 1 shows the profile for BP activity in CM from TE89 cells that were separated in 1 M acetic acid on a Sephadex G-100 column. There were two major peaks of BP activity that eluted between fractions 37 and 53. When tested for inhibitory activity, peak 1 (fractions 39–43) but not peak 2 (fractions 47–51) strongly inhibited the binding of ^{125}I-IGF-II to H-35 cells in the radioreceptor assay. There was no measurable ^{125}I-IGF-II competing activity in fractions 71–78 where authentic IGF-II eluted. Fractions 39 and 41 from peak 1 also inhibited basal as well as IGF-II-induced chicken bone cell proliferation (data not shown). Thus, peak 1 appeared to contain an IGF-BP that is inhibitory to bone cell proliferation and has been termed In-IGF-BP.

As with TE89 CM, when the medium from HBC was subjected to gel filtration in 1 M acetic acid, we observed two peaks of BP activity that eluted between fractions 37 and 53 with inhibitory activity in peak 1. However, in contrast to TE89 CM, HBC CM showed an additional peak of ^{125}I-IGF-II competing activity between fractions 71 and 78 where authentic IGF-II eluted (data not shown). Thus, normal HBC produce BP and IGF-II, whereas TE89 cells produce only BP under basal conditions. Binding proteins have also been found in the CM of the TE85 human osteosarcoma cell line.

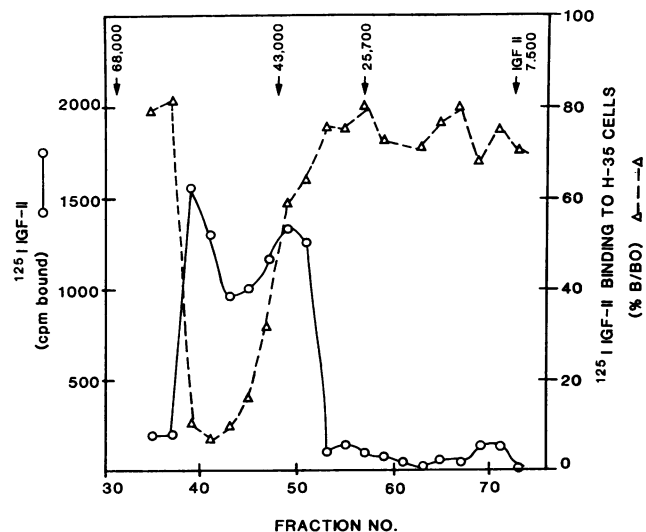

FIG. 1. Purification of IGF-BP from TE89 CM proteins by Sephadex G-100 chromatography. The solid line represents BP activity determined by using ^{125}I-IGF-II as a tracer; the broken line represents the relative amounts of ^{125}I-IGF-II bound to H-35 cells with and without competitor (% B/BO).

To further purify the BP, peaks 1 and 2 (fractions 37–51) from the TE89 CM were pooled and subjected to Mono Q anion-exchange chromatography using a gradient of NaCl (data not shown). Again, there were two peaks of BP activity. Peak 1 eluted at 0.1 M NaCl (fractions 9–15), whereas peak 2 eluted at 0.2 M NaCl (fractions 17–25). As was found using gel filtration chromatography, peak 1 was a strong competitor and peak 2 was a weak competitor for the binding of ^{125}I-IGF-II to H-35 cells. Fractions (9–15) containing peak 1 (In-IGF-BP) were pooled and analyzed further.

Upon HPLC reverse-phase chromatography (using a C_4 column), In-IGF-BP activity (as determined by the inhibition of ^{125}I-IGF-II binding to H-35 cells) eluted between 31% and 32% acetonitrile (fractions 45–48) (data not shown). Although fractions 49–54 exhibited some BP activity (by PEG precipitation of ^{125}I-IGF-II–BP complex), none of these fractions inhibited binding of ^{125}I-IGF-II to H-35 cells. Further purification of In-IGF-BP (fractions 45–48) was achieved by rechromatographing the active fractions on an HPLC CN column using a 1-propanol gradient. In-IGF-BP eluted at 36% 1-propanol (fractions 54–56). The fractions that contained BP activity also exhibited ^{125}I-IGF-II-competing activity. This preparation appeared to be homogeneous as it produced a single sequence signal on N-terminal analysis.

In order to confirm that the N-terminal sequence obtained was not from a major contaminating protein in the BP preparation, In-IGF-BP was purified by an alternate procedure involving affinity chromatography. The BPs were separated from the other proteins in TE89 CM by chromatography on IGF-I linked to Sepharose 4B. The In-IGF-BP was further purified from other BPs by HPLC reverse-phase chromatography using a C_4 column as described in procedure 1. Fractions 44 and 45, which contained the In-IGF-BP activity, were used for subsequent chemical and biological characterization studies. The recovery of In-IGF-BP during purification was 18% (Table 1).

Characterization of In-IGF-BP. *Chemical.* Fig. 2 compares the N-terminal amino acid sequence of In-IGF-BP to the known IGF-BPs purified from various sources. The N-terminal amino acid sequence of In-IGF-BP is unique with limited sequence similarity to other known BPs.

Fig. 3*A* illustrates the $NaDodSO_4$/PAGE of purified In-IGF-BP at pH 8.8. Under reducing conditions, a major protein band (25 kDa) was seen on silver staining just above the 24-kDa marker protein. Chemical cross-linking of In-IGF-BP at different stages of its purification revealed its ability to specifically bind ^{125}I-IGF-I or ^{125}I-IGF-II. Fig. 3*B* shows the autoradiographic results of an experiment in which purified In-IGF-BP was cross-linked to ^{125}I-IGF-II. One band was diminished in intensity by the presence of unlabeled IGF-II during the incubation. The molecular mass of this band, calculated from several determinations, is 32 kDa, which corresponds to the combined molecular masses of the BP (25 kDa) and ligand (7.5 kDa). The other bands in the autoradiograph, not specifically diminished in intensity by the presence of unlabeled IGF-II, are likely to be derived from bovine serum albumin and ^{125}I-IGF-II present in the assay. Thus, the relative mobility of In-IGF-BP (25 kDa) in $NaDodSO_4$/PAGE appears to be different from that of IGF-BP I (30–35 kDa), IGF-BP II (33 kDa), and IGF-BP III (40–53 kDa).

The specificity of IGF binding was examined in detail by performing competitive binding experiments in liquid phase.

Table 1. Purification of In-IGF-BP from medium conditioned by TE89 cells

Purification step	BP, μg/ml	Volume, ml	Total BP, μg	Recovery, %
Serum-free medium	0.041	4890	200.5	100
Concentrate	1.28	75	96.0	48
IGF-I affinity	1.09	50	54.5	27
HPLC reverse-phase	9.22	4	36.9	18

The relative inhibition of ^{125}I-IGF-II tracer to H-35 cells in a radioreceptor assay was used to determine the concentration of BP at each step of purification. Purified In-IGF-BP was used as a standard.

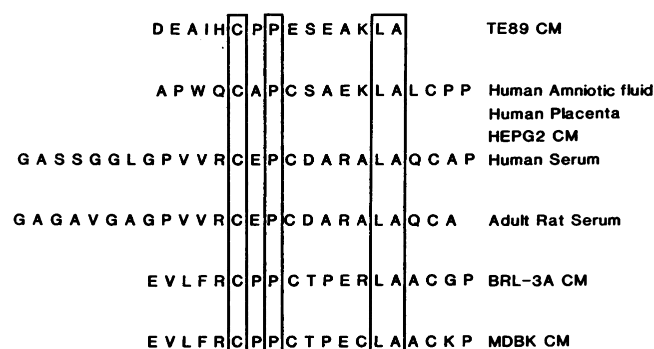

FIG. 2. Alignment of N-terminal amino acid sequence of In-IGF-BP with that of other known BPs. In-IGF-BP is compared with the N-terminal sequences of IGF-BP I (amniotic fluid, placenta, and liver), IGF-BP II (BRL-3A and MDBK), and IGF-BP III (human and rat serum). The amino acid composition of In-IGF-BP in % mol is D = 9.2, E = 12.5, S = 5.5, G = 10.0, H = 5.5, R = 9.0, T = 2.5, A = 6.4, P = 7.7, Y = 1.5, V = 3.8, M = 1.0, C = 6.9, I = 2.6, L = 8.0, F = 1.7, K = 6.4 (mean of two preparations).

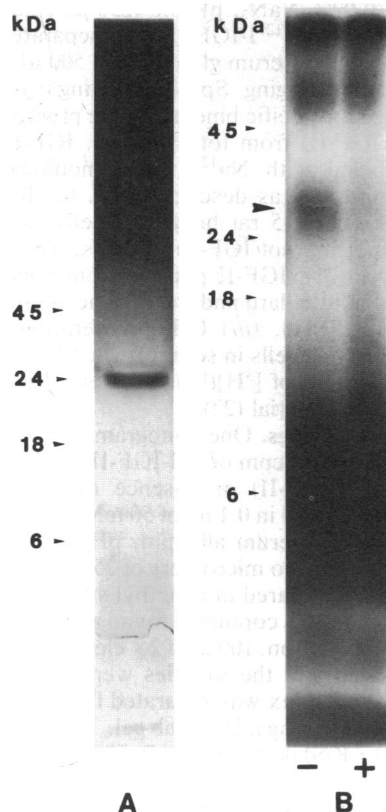

FIG. 3. Molecular mass estimate of In-IGF-BP by $NaDodSO_4$/PAGE: (*A*) One-half microgram of In-IGF-BP was applied to 3–27% polyacrylamide gradient slab gel according to Laemmli (23). The gel was silver stained (22) and the relative molecular masses of the standard proteins are indicated. (*B*) One nanogram of purified In-IGF-BP was cross-linked with ^{125}I-IGF-II in the presence (+) or absence (−) of unlabeled IGF-II. The samples were solubilized and analyzed electrophoretically on a 15% $NaDodSO_4$/polyacrylamide slab gel. An autoradiograph of the gel with the relative molecular mass of protein standards is shown.

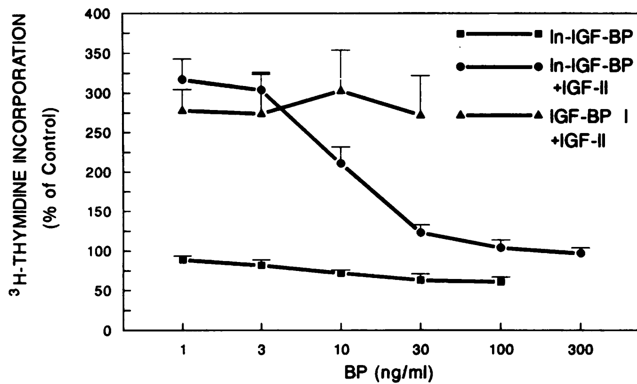

FIG. 4. [^{3}H]Thymidine incorporation into DNA of chicken calvarial cells in the presence or absence of 3 ng of IGF-II per ml and various In-IGF-BP or IGF-BP I concentrations. The values are mean ± SD of six replicate wells. Basal and IGF-II-stimulated [^{3}H]thymidine incorporation were significantly inhibited ($P < 0.001$) by In-IGF-BP at 10, 30, 100, and 300 ng/ml. In contrast to In-IGF-BP, IGF-BP I (from M. Rutanen, University of Helsinki, Finland) did not inhibit IGF-II-stimulated [^{3}H]thymidine incorporation.

IGF-I and IGF-II (0.2–1.0 ng) were equipotent in displacing the binding of ^{125}I-IGF-II to purified In-IGF-BP. Insulin up to 200 ng/ml did not displace binding of ^{125}I-IGF-II tracer to In-IGF-BP. Similar results were obtained using ^{125}I-IGF-I as tracer (data not shown).

Biological. Purified In-IGF-BP inhibited chicken bone cell proliferation in serum-free culture in a dose-dependent manner (Fig. 4). In addition, purified In-IGF-BP inhibited IGF-II-induced chicken bone cell proliferation at submaximal concentrations. For example, at 3 ng/ml, IGF-II stimulated [^{3}H]thymidine incorporation 2.2-fold over control in chicken calvarial cells. A dose-dependent inhibition of 3 ng of IGF-II-stimulated [^{3}H]thymidine incorporation per ml was observed with increasing concentrations of 25-kDa In-IGF-BP (Fig. 4). Although the IGF-BP III has been reported to enhance rather than inhibit the action of IGF-I when cells were pretreated with the BP, pretreatment of bone cells with In-IGF-BP did not alter the BP's inhibitory effect. In contrast to the effect of In-IGF-BP, IGF-BP I (from human placenta) at concentrations up to 30 ng did not inhibit IGF-II-induced cell proliferation. The inhibitory effect of a fixed concentration of In-IGF-BP could be overcome by increasing the concentrations of IGF-I (Fig. 5). In addition to inhibiting IGF-induced [^{3}H]thymidine incorporation, In-IGF-BP (5 ng) preincubated with fetal calf serum prior to addition to culture

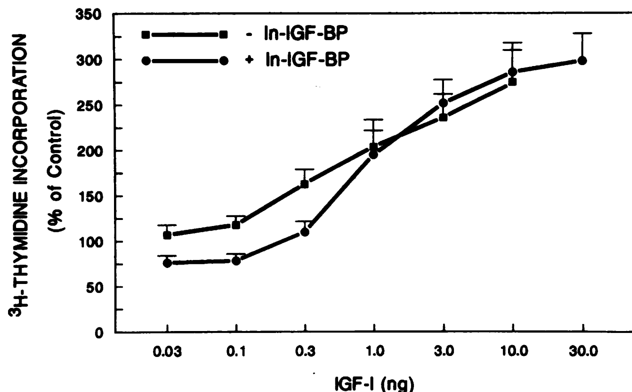

FIG. 5. [^{3}H]Thymidine incorporation into DNA of chicken calvarial cells in the presence or absence of In-IGF-BP (5 ng per well) and various concentrations of IGF-I. The values are mean ± SD of six replicate wells. [^{3}H]Thymidine incorporation was significantly decreased ($P < 0.001$) in the presence of In-IGF-BP at 0.03, 0.1, and 0.3 ng per well of IGF-I.

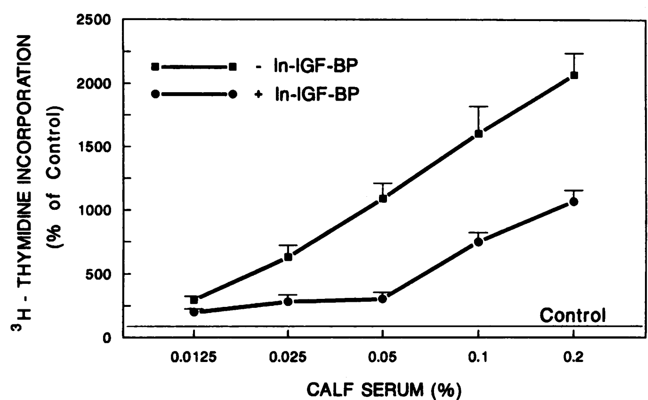

FIG. 6. Effect of In-IGF-BP on [^{3}H]thymidine incorporation in MC3T3-E1 cells (24) stimulated by fetal calf serum. Various concentrations of fetal calf serum were incubated with In-IGF-BP (5 ng) for 60 min at room temperature prior to addition to culture wells. [^{3}H]Thymidine incorporation was significantly decreased ($P < 0.001$) in the presence of In-IGF-BP at all concentrations of serum tested.

wells inhibited serum-stimulated [^{3}H]thymidine incorporation by about 50–70% (Fig. 6).

Regulation of In-IGF-BP Production. Forskolin, a stimulator of cAMP production, increased production of In-IGF-BP in TE85 cells in a dose-dependent manner (Table 2). The maximum effect of >8-fold increase in In-IGF-BP production was seen at 10 μM forskolin. 1,9-Dideoxyforskolin, an analog of forskolin that does not stimulate cAMP production had no effect on In-IGF-BP production by TE85 cells. Consistent with these results, N^{6},$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate stimulated In-IGF-BP production in TE85 cells (data not shown).

DISCUSSION

In this study, we have discovered a unique human IGF-BP produced by human TE89 osteosarcoma cells and probably by normal HBC as well. This IGF-BP is a strong inhibitor of IGF-induced cell proliferation and is different from the other known IGF-BPs in size, N-terminal sequence, and amino acid composition. Comparison of the N-terminal amino acid sequence of In-IGF-BP to those purified from human amniotic fluid, serum, BRL-3A CM, and MDBK CM (4, 5, 9, 13, 14) reveals that these sequences are different for each BP but have some similarities. Interestingly, amino acid residues at positions 6 (Cys), 8 (Pro), 14 (Leu), and 15 (Ala) of the In-IGF-BP are conserved in all members of the IGF-BP

Table 2. Effects of forskolin on In-IGF-BP production in cultured TE85 human osteosarcoma cells

	In-IGF-BP, ng of IGF-II equiv/μg of DNA	
Concentration, μM	Forskolin	1,9-Dideoxyforskolin
Vehicle control	<5.0	<5.0
0.1	14.4 ± 2.8	<5.0
1.0	20.6 ± 5.6	<5.0
10.0	38.1 ± 11.0	<5.0
100.0	40.8 ± 4.8	<5.0

TE85 cells were plated in 24-well culture dishes in serum-free DMEM. Twenty-four hours after plating, forskolin was added to the TE85 cells, which were incubated for 48 hr. CM samples were subjected to filtration with Amicon membrane (YM 10) under acidic conditions to separate IGFs from In-IGF-BP (retentate). The relative inhibition of ^{125}I-IGF-II tracer binding to H-35 cells in a radioreceptor assay was used to determine the concentration of In-IGF-BP using purified In-IGF-BP as standard. The values are mean ± SD of four wells.

family (Fig. 2). In-IGF-BP has the most identity with IGF-BP I but even when aligned to give maximum sequence identity, 9 of 15 residues are different. In addition, comparison of the amino acid composition of the two proteins reveals marked differences. Similarly, comparison of the In-IGF-BP with those of IGF-BP II and IGF-BP III demonstrates marked differences in N-terminal amino acid sequences and in amino acid composition. Moreover, based on the sequence data, it is unlikely that In-IGF-BP is a truncated form of an IGF receptor. Together, these observations suggest that In-IGF-BP originates from a gene that is yet to be isolated.

The idea of IGF BPs being inhibitory has been advanced by other workers (1, 2). Our report shows that bone cells in culture produce an inhibitory IGF-BP in addition to IGFs; there is no doubt about the existence of this protein. The question is: What is its physiological function? Several studies were conducted to evaluate the functional significance of In-IGF-BP. (*i*) Because chicken bone cells make IGF-I and IGF-II, we added In-IGF-BP to these cells under basal conditions and found that basal proliferation was inhibited by 40%, which emphasizes the importance of the IGFs in the local regulation of bone cell proliferation and the importance of In-IGF-BP in the IGF action. (*ii*) Not surprisingly, In-IGF-BP completely inhibited the proliferative action of added IGF-I or IGF-II. (*iii*) Serum is the strongest stimulator of bone cell proliferation yet discovered and the addition of In-IGF-BP (10 ng/ml) inhibited 50% of this effect. This again emphasizes the importance of the IGFs in the regulation of bone cell proliferation and the importance of In-IGF-BP in IGF action. (*iv*) The amount of In-IGF-BP found in the CM of HBC was relatively high (10–50 ng/ml). Moreover this concentration produced maximum inhibition in our chicken bone cell proliferation assay system. Thus adequate amounts are present in the CM for In-IGF-BP to have a strong physiological action. (*v*) Our In-IGF-BP was very potent in inhibiting chicken bone cell proliferation, severalfold more than another IGF BP reported to be an inhibitor of cell proliferation, IGF-BP I. In addition to inhibiting cell proliferation, IGF-BP I has been shown to increase IGF-I-induced cell proliferation in muscle cells (18). Similarly, De Mellow and Baxter (17) have shown that IGF-BP III can either inhibit or potentiate IGF-stimulated [^{3}H]thymidine incorporation in neonatal skin fibroblasts, depending on the incubation conditions used. In contrast to other IGF BPs, our In-IGF-BP did not stimulate cell proliferation under any *in vitro* condition. (*vi*) If In-IGF-BP has a regulatory function, it should be possible to alter its secretion rate. In this regard we found that forskolin stimulated the production of In-IGF-BP in a dose-dependent manner. Based on these observations it follows that In-IGF-BP could be an important physiological regulator of IGF action in bone cells.

A major unresolved issue with respect to the function of In-IGF-BP is whether other cells produce the same or a similar inhibitory BP. Various other cell types have been shown to produce BPs [liver (13, 18), kidney (14), muscle (25), skin fibroblasts (26, 27), granulosa (28), breast cancer (29), neuroblastoma (30)], including low molecular mass 24-kDa forms (26, 29, 30). In addition, low molecular mass inhibitory BPs have been reported in serum (2). Whether these BPs are potent inhibitors of IGF action or are related to the bone In-IGF-BP has not been determined. If, like bone, other tissues produce potent inhibitory IGF BPs, such proteins could function throughout the body to provide tissue specificity for the actions of the IGFs. Tissue specificity would seem to be essential, given the diverse actions of the IGFs on different tissues and the fact that the IGFs function as hormones as well as local factors.

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