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Insulin-Like Growth Factor Binding Proteins Localize to Discrete Cell Culture Compartments in Periosteal and Osteoblast Cultures From Fetal Rat Bone

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Abstract Insulin-like growth factor (IGF)-I and IGF-II are expressed at biologically effective levels by bone cells. Their stability and activity are modulated by coexpression of IGF binding proteins (IGFBPs). Secreted IGFBPs may partition to soluble, cell-associated, and matrix-bound compartments. Extracellular localization may sequester, store, or present IGFs to appropriate receptors. Of the six IGFBPs known, rat osteoblasts synthesize all but IGFBP-1. Of these, IGFBP-3, -4, and -5 mRNAs are induced by an increase in cAMP. Little is known about extracellular IGFBP localization in bone and nothing about IGFBP expression by nonosteoblastic periosteal bone cells. We compared basal IGFBP expression in periosteal and osteoblast bone cell cultures and assessed the effects of changes in cAMP-dependent protein kinase A or protein kinase C. Basal IGFBP gene expression differed principally in that more IGFBP-2 and -5 occurred in osteoblast cultures, and more IGFBP-3 and -6 occurred in periosteal cultures. An increase in cAMP enhanced IGFBP-3, -4, and -5 mRNA and accordingly increased soluble IGFBP-3, -4, and -5 and matrix-bound IGFBP-3 and -5 in both bone cell populations. In contrast, protein kinase C activators suppressed IGFBP-5 mRNA, and its basal protein levels remained very low. We also detected low M_r bands reactive with antisera to IGFBP-2, -3, and -5, suggesting proteolytic processing or degradation. Our studies reveal that various bone cell populations secrete and bind IGFBPs in selective ways. Importantly, inhibitory IGFBP-4 does not significantly accumulate in cell-associated compartments, even though its secretion is enhanced by cAMP. Because IGFBPs bind IGFs less tightly in cell-bound compartments, they may prolong anabolic effects by agents that increase bone cell cAMP. J. Cell. Biochem. 71:351–362, 1998.

Key words: IGFBP; cAMP; PKA; prostaglandin; bone

Insulin-like growth factor I (IGF-I) and IGF-II are among the more abundant growth factors isolated from bone tissue and are synthesized by various bone cells including osteoblasts [McCarthy et al., 1989a, 1990; Linkhart and Mohan, 1989]. The synthesis of IGF-I by osteoblasts is stimulated by the principal calciotropic hormone parathyroid hormone (PTH) and by locally produced and biologically active prostaglandin E_2 (PGE_2) [McCarthy et al., 1989a, 1991], while steroid hormones such as estrogen and cortisol may have counterregulatory or complex effects [McCarthy et al., 1990; Ernst et al., 1989; McCarthy et al., 1997]. Bone cells also synthesize significant levels of IGF-II, although it is less potent and its synthesis appears less sensitive to regulation [Mohan et al., 1988; McCarthy et al., 1989b, 1992]. IGFs enhance bone cell replication and type I collagen synthesis, among other activities. In intact bone explants, the IGFs increase DNA synthesis predominantly in the periosteal region, whereas collagen synthesis and bone matrix apposition are increased predominantly in the central bone, enriched with differentiated osteoblasts [Hock et al., 1988]. Stimulatory effects on collagen synthesis also occur in primary osteoblast-enriched cultures prepared from this tissue [McCarthy et al., 1989b; Schmid et al., 1989].

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Because IGF-I also is abundant in the circulation primarily from hepatic synthesis, it can act as an endocrine, paracrine, and autocrine regulator of skeletal growth and differentiation. Bone contains various cells that may represent osteoblasts at discrete stages of development or osteoblasts that have differentiated to perform a specific function. Cell populations can be obtained from fetal rat calvariae (intramembranous bone) that appear to express, to varying degrees, features associated with the differentiated osteoblast phenotype [McCarthy et al., 1988; Wong and Ng, 1992]. Little is known about less differentiated, periosteal bone cells. They may constitute a population that expands and differentiates in response to select growth regulators and may replenish, when necessary, the pool of more differentiated osteoblasts. In intramembraneous bone osteoblast precursors may develop from the periosteum. Alternately, periosteal cells may constitute a separate population that in some ways supports the activities of more differentiated osteoblasts. Towards this end, periosteal cells could synthesize and release factors that indirectly influence osteoblast function. Periosteal and osteoblast-enriched cultures derived from fetal rat parietal bone exhibit very different responses to certain osteotropic agents, including the prostaglandins [Centrella et al., 1994]. We also recently found selective changes in IGF receptors in these two populations [McCarthy et al., 1998]. Therefore prostaglandins may influence bone cell metabolism either directly or by altering IGF activity. Because both periosteal and osteoblast-enriched cell populations from fetal rats express abundant levels of IGF-I, IGF-II, and signal transducing type 1 IGF receptor, several processes can regulate IGF actions.

Six IGF binding proteins (IGFBPs) have been well characterized and serve various functions. They may inhibit or potentiate IGF activity, form storage complexes with IGFs, or stabilize IGFs in the circulation for slow release into peripheral tissue, thus avoiding the insulin-like effects [Clemmons, 1991; Jones and Clemmons, 1995]. Therefore, IGF activity appears to be influenced not only by the level of expression of IGF polypeptide but also by the type and concentration of IGFBPs present locally. IGFBPs bind the IGFs with an affinity that often exceeds that of IGF receptors [Clemmons, 1991]. Changes in IGFBP expression by bone cells may well contribute to the effectiveness of IGFs in this tissue. Bone cells synthesize several IGFBPs. Primary osteoblast-enriched cultures from neonatal rats produce IGFBP-3 under growth hormone (GH) and PGE_2 control, while glucocorticoid, 17β-estradiol and IGF-I may regulate IGFBP-2 expression in early passage fetal rat osteoblast cultures [Ernst and Rodan, 1990; Chen et al., 1991a,b; Tørring et al., 1991; Hassager et al., 1992; Schmid et al., 1992; McCarthy et al., 1994]. Our studies in primary osteoblast-enriched cultures from fetal rat bone showed unequal basal expression of IGFBP-2, -3, -4, -5, and -6 and differential regulation by PGE_2, GH, IGF-I, and IGF-II [McCarthy et al., 1994]. However, very little is known about IGFBPs produced by periosteal bone cells that express less differentiated characteristics.

Most IGFBP studies focus on material secreted into the culture medium. Complexes formed between IGFs and some IGFBPs are thought to prevent receptor binding. This is best exemplified by the inhibitory effects of IGFBP-4 [Mohan et al., 1989]. However, IGFBPs may also accumulate on the cell surface or in the extracellular matrix, and in so doing concentrate IGFs in close proximity to IGF receptors [Jones et al., 1993; Arai et al., 1996]. In this context, localization of IGFBP-3 and -5 on the cell membrane or cell matrix appears to potentiate IGF activity [McCusker et al., 1991; Andress and Birnbaum, 1992; Conover, 1992; Mohan et al., 1995]. Furthermore, posttranslational modifications, including phosphorylation of IGFBP-1, -3, and -5 [Coverley and Baxter, 1997] and proteolytic processing of IGFBP-3 and IGFBP-5, may alter IGF binding and activity [Jones et al., 1991; McCusker et al., 1991; Conover, 1992, 1995; Camacho-Hubner et al., 1992; Clemmons, 1993; Andress, 1995; Booth et al., 1995]. Therefore, bone-derived IGF-I and IGF-II activity may vary in positive and negative ways through regulated expression, localization, and modification of locally produced IGFBPs. More thorough characterization of the expression of IGFBPs, with attention to their localization on the cell membrane or within the extracellular matrix, is needed to complement our current understanding of the IGF system in bone.

Accordingly, IGF-I, IGF-II, IGFBP expression, and IGF receptor binding are regulated by a variety of factors in bone cell cultures. Among the notable changes are those produced by factors that induce cAMP synthesis or protein

kinase C (PKC) activity, and some may selectively occur in discrete bone cell populations. To understand the role of these signaling systems on IGFBP expression and localization in bone, we examined the influence of various prostanglandins (PGs) or other agents that selectively activate protein kinase A (PKA) or PKC in primary periosteal and osteoblast-enriched cultures from fetal rat parietal bone, guided by results from our earlier studies in these culture models.

MATERIALS AND METHODS

Cell Cultures

Cell cultures were prepared from parietal bones of 22-day-old Sprague-Dawley rat fetuses (Charles River Breeding Laboratories, Raleigh, NC). Rats were handled and sacrificed by methods approved by Yale Animal Care and Use Committee. Sutures were eliminated by dissection, and cells were released from parietal bones by five 20 min sequential collagenase digestions, as described previously [Centrella et al., 1987; McCarthy et al., 1988]. Cells from the first digestion (periosteal cells) appear less differentiated by biochemical characterization [McCarthy et al., 1988]. They respond poorly to PTH and do not make detectable amounts of osteocalcin, even when treated with dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Cells released during the last three digestions exhibit biochemical characteristics associated with differentiated osteoblasts, including an increased amount of PTH receptor, high levels of type I collagen synthesis, and a rise in osteocalcin expression in response to $1,25(OH)_2D_3$ [McCarthy et al., 1988; Centrella et al., 1987; Carpenter et al., 1998]. Histochemical staining demonstrates that approximately 80% of the cells express alkaline phosphatase [T.L.M., M.C., unpublished data], although this itself is not entirely specific for osteoblasts. By these criteria, differential sensitivity to transforming growth factor β (TGF-β), bone morphogenetic protein-2 (BMP-2), and various PGs and the ability to form mineralized nodules *in vitro* [Pockwinse et al., 1992; Centrella et al., 1987, 1994, 1995, 1996; Hughes et al., 1995], osteoblast-enriched cultures are well distinguished from the less differentiated periosteal cells from the first collagenase digestion. Cells from the first or the last three digestions were plated at 5,000/cm^2 or 15,000/cm^2, respectively, in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES (pH 7.2), 100 μg/ml ascorbic acid, penicillin, and streptomycin (Life Technologies, Grand Island, NY), and 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO).

Confluent cultures (approximately 50,000 cells/cm^2) were rinsed four times and serum-deprived for 20 h before treatment in order to maintain quiescence, to restrict responses to test agents, and to remove serum-derived IGFBPs. Medium was collected, cell layers were rinsed with phosphate buffered saline (PBS), and cell membrane–associated material was isolated by gentle lysis in 1% Triton X-100 in PBS. Residues were rinsed with 1% Triton X-100/PBS, and the matrix-associated compartment was harvested in nonreducing Laemmli sample buffer. All PGs (PGE_2, PGE_1, and $PGF_{2\alpha}$) and forskolin (Sigma Chemical Co.) were dissolved in 95% ethanol. Phorbol myristate acetate (Sigma) was dissolved in 50% ethanol/50% serum-free medium. Control treatments were the highest concentration of ethanol (vehicle) present in the treatment reagents.

Northern Analysis

Cells 9.6 cm^2 or 78.5 cm^2 cultures were dissolved in 5 M guanidine monothiocyanate, 25 mM trisodium citrate, 0.5% sarkosyl and 0.1 M 2-mercaptoethanol and extracted with phenol-chloroform-isoamyl alcohol (75:25:1) with 0.2 M sodium acetate [Chomczynski and Sacchi, 1987]. Total RNA was precipitated, ethanol-washed, dried, denatured with 2.2 M formaldehyde/12.5 M formamide at 65°C for 15 min, and fractionated on a 1.5% agarose/2.2 M formaldehyde gel. Co-electrophoresed RNA standards were excised and ethidium bromide–stained, and the remaining gel was blotted onto GeneScreen Plus charge modified nylon (DuPont NEN, Boston, MA). Restriction fragments containing rat IGFBP-1 cDNA clones provided by Dr. L. Murphy and Drs. S. Shimasaki and N. Ling [Murphy et al., 1990; Nakatani et al., 1991], rat IGFBP-2 cDNA clones provided by Drs. S. Shimasaki and N. Ling [Brown et al., 1989; Nakatani et al., 1991], and rat IGFBP-3, -4, -5, and -6 cDNA clones provided by Drs. S. Shimasaki and N. Ling [Shimasaki et al., 1989, 1990, 1991a,b] were agarose gel–purified and labeled with [α-^{32}P]deoxycytidine triphosphate and [α-^{32}P]-thymidine triphosphate by random hexanucleotide primed second strand synthesis [Feinberg and Vogelstein, 1984]. The blots were hybridized with individual cDNAs, and the filters

were washed under conditions of progressively increasing stringency. Final washes were with 0.2× SSC (20× SSC contains 3 M NaCl, 0.3 M trisodium citrate, pH 7.0) and 0.1% sodium dodecylsulfate for 1 h at 55°C. Bound radioactive material was visualized by autoradiography using Amersham (Arlington Heights, IL) Hyperfilm and a Dupont Cronex intensifying screen. Filters were eluted of specifically bound [^{32}P]-labeled cDNA by washing with diethyl pyrocarbonate–treated deionized water (100°C) for 5 min. Control probing for equivalent RNA blotting efficiency was with [^{32}P]-labeled antisense 18S rRNA probe (Ambion, Austin, TX) prepared using a T7-MEGAshortscript kit.

Western Ligand Blots

Medium and cell-associated compartments were acidified to a final concentration of 0.5 M acetic acid and supplemented with Tween-20 to 0.05%. IGFBPs were concentrated by precipitation using 2.5 volumes of 95% ethanol at -20°C overnight [Bachrach et al., 1991]. Proteins were collected by centrifugation (12,000*g* for 1 h), vacuum-dried, and resuspended in nonreducing Laemmli sample buffer. Protein was quantitated by the Pierce (Chicago, IL) BCA protein assay, and equal amounts of protein were fractionated on 7.5–15% polyacrylamide gradient gels under nonreducing conditions. Gels were electroblotted onto Immobilon P membranes (Millipore Corp., Bedford, MA), and membranes were air-dried, washed with Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, 150 mM NaCl), blocked with 1% bovine serum albumin (BSA) in TBS, and incubated with 0.1 × 10^6 cpm/ml ^{125}I-IGF-I or ^{125}I-IGF-II in TBS, 1% BSA, and 0.1% Tween-20 overnight at 4°C [Hossenlopp et al., 1986]. Filters were washed four times with TBS, and bound radioactive material was visualized by autoradiography using Amersham Hyperfilm and a Dupont Cronex intensifying screen.

Western Antibody Blots

Western blots were prepared as described above, air-dried, washed with TBS, blocked, and then exposed to rabbit antisera to native bovine IGFBP-2 (1:1,000) (a gift of Dr. D. Clemmons), rat IGFBP-3 (1:400), rat IGFBP-4 (1:400), rat or human IGFBP-5 (1:200; antihuman IGFBP-5 cross-reactive with rat IGFBP-5 [Upstate Biotechnologies, Inc., Lake Placid, NY]), or rat IGFBP-6 (1:400). Anti-rat IGFBP antisera were a kind gift of Drs. N. Ling and S. Shimasaki [Liu et al., 1993]. Blots were blocked in TBS containing 0.1% Tween-20 and 5% defatted powdered milk and then incubated in this same solution containing a specific anti-IGFBP antisera for 20 h. Blots were washed with TBS containing 5% powdered milk, exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase, washed with TBS, and developed with ECL chemiluminesence reagent (Amersham).

RESULTS

IGFBP mRNA

We first examined mRNA levels for various IGFBPs in response to 6 h treatment with activators of PKA (PGE_2, PGE_1, forskolin) or PKC (PGE_2, PGE_1, $PGF_{2\alpha}$, phorbol myristate acetate (PMA)). As shown in Figure 1, steady-state mRNA transcripts for IGFBP-2 and IGFBP-6 were relatively unaffected by these agents. Periosteal cell cultures exhibited relatively higher basal mRNA levels for IGFBP-6 than osteoblast cultures, while osteoblast-enriched cultures contained higher levels of IGFBP-2 and IGFBP-5 mRNA. IGFBP-1 transcripts were not detected in control or treated cultures (data not shown). PKA activators each enhanced IGFBP-3, IGFBP-4, and IGFBP-5 mRNA levels in both cell models, although this effect was more pronounced in the osteoblast-enriched cultures. The PKC activator PMA suppressed IGFBP-5 mRNA in both cultures, although the effect by $PGF_{2\alpha}$ was evident only in the periosteal cell cultures. These results are summarized in Table 1.

Secreted IGFBP

Western ligand blots were prepared from 24 h conditioned medium and probed with ^{125}I-IGFs. Data are shown for ^{125}I-IGF-II, which tends to bind IGFBPs with equal or higher affinity compared to IGF-I [Clemmons, 1991]. Ligand binding patterns reflected the results of the Northern blot analyses; that is, agents that induced cAMP increased the expression of IGFBPs with relative molecular masses consistent with IGFBP-3, IGFBP-4, and IGFBP-5 (Fig. 2). Based on earlier evidence from our work and from other laboratories [Mohan et al., 1989; Clemmons, 1991, 1993; Conover, 1991; McCarthy et al., 1994; Andress, 1995; others] , the ^{125}I-IGF-II ligand bound material of M_r 46 kDa represents IGFBP-3 (heterogenous bands with different levels of glycosylation), the mate-

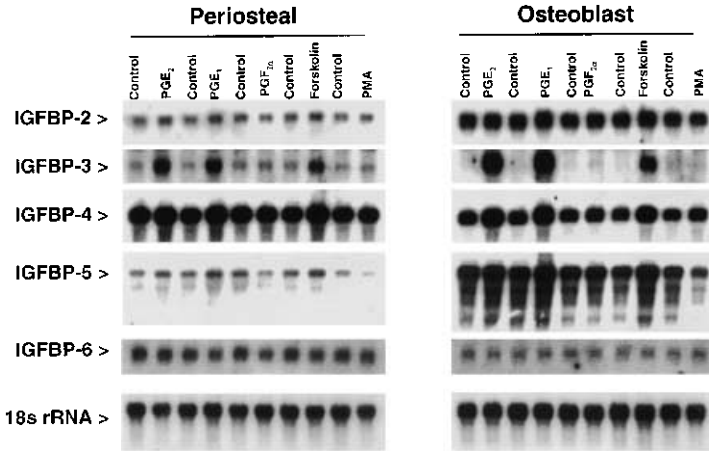

Fig. 1. Northern blot analysis of IGFBP-2, -3, -4, -5, and -6 mRNA levels after a 6 h treatment of periosteal and osteoblast-enriched cultures with control (ethanol vehicle), PGE_2, PGE_1, $PGF_{2\alpha}$ (each at 1 μM), forskolin (10 μM), or PMA (1 μM). Ten micrograms of total RNA was denatured in 50% formamide–2.2 M formaldehyde at 65°C, fractionated on a 1.5% agarose, 2.2 M formaldehyde gel, blotted onto GeneScreen charge-modified nylon membrane, and UV cross-linked, and replicate blots were hybridized to individual [^{32}P]-labeled IGFBP cDNAs. Representative autoradiograms are shown for each IGFBP. Blots were stripped and reprobed with 18S cRNA to assess loading and blotting efficiency.

TABLE I. IGFBP mRNA as a Function of Bone Cell Population and Treatment

	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Periosteal					
Control	<Osteoblast	≈Osteoblast	≈Osteoblast	≪Osteoblast	>Osteoblast
PGE_2	⇔	⇑	⇑	⇑	⇔
PGE_1	⇔	⇑	⇑	⇑	⇔
$PGF_{2\alpha}$	⇔	⇔	⇔	⇓	⇔
Forskolin	⇔	⇑	⇑	⇑	⇔
PMA	⇔	⇔	⇔	⇓	⇔
Osteoblast					
Control	>Periosteal	≈Periosteal	≈Periosteal	≫Periosteal	<Periosteal
PGE_2	⇔	⇑	⇑	⇑	⇔
PGE_1	⇔	⇑	⇑	⇑	⇔
$PGF_{2\alpha}$	⇔	⇔	⇔	⇔	⇔
Forskolin	⇔	⇑	⇑	⇑	⇔
PMA	⇔	⇔	⇔	⇓	⇔

rial of M_r 30 kDa is comprised of IGFBP-2 and IGFBP-5 [McCarthy et al., 1994], and the low M_r material at 24 kDa is consistent with IGFBP-4.

Cell-Associated IGFBP

While many of the IGFBPs produced by cells in culture accumulates in the medium in a soluble form, they also bind to the cell membrane or to the matrix surrounding the cell. Protein extracts derived from these compartments were also assessed by Western ligand probing with ^{125}I-IGF-II. Proteins released by 1% Triton X-100 in PBS comprise cell membrane–associated material and perhaps to a small extent newly synthesized IGFBPs not yet secreted. IGFBPs of M_r 46 kDa and 30 kDa occurred in cell-associated extracts from both cultures, although M_r 30 kDa material was more evident in osteoblast-enriched cultures and the 46 kDa material was more evident in the periosteal extracts. A very small amount of M_r 24 kDa material was detected in periosteal membrane extracts, but none was found in extracts from osteoblast cultures (Fig. 3). Similar arrays of IGFBPs occurred in matrix-associated extracts from both cultures (Fig. 4). This compartment from periosteal cell cultures was highly enriched with the M_r 46 kDa IGFBP (IGFBP-3), and matrix from both cultures contained high levels of IGFBP(s) at M_r 30 kDa (consistent

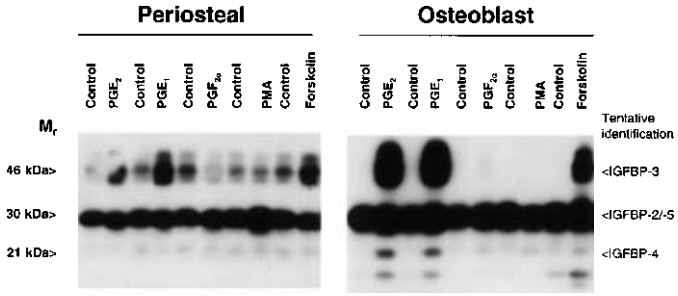

Fig. 2. Western ligand blot analysis of conditioned medium from periosteal and osteoblast-enriched cultures treated 24 h with control (ethanol vehicle), PGE_2, PGE_1, $PGF_{2\alpha}$ (each at 1 μM), forskolin (10 μM), or PMA (1 μM). Culture medium was collected, acidified to release bound IGF, concentrated, and size-fractionated on an SDS-PAGE gel (nonreducing). Numbers on the left (M_r) indicate position of migration of prestained molecular weight standards (Amersham). Tentative identification of IGFBPs is based upon known relative migration. Gels were electroblotted onto Immobilon P membranes (Millipore), blocked with nonfat powdered milk in Tris-buffered saline (TBS) containing Tween-20, and then probed with ^{125}I-IGF-II. Representative autoradiograms are shown.

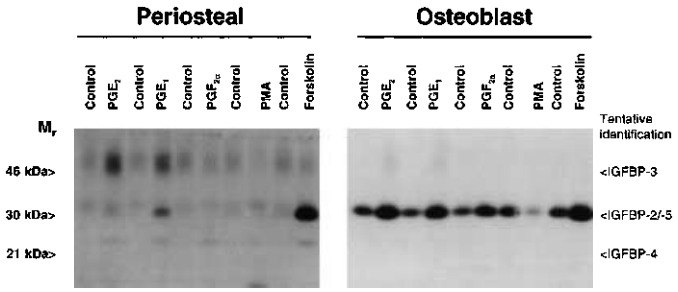

Fig. 3. Western ligand blot analysis of cell membrane extract (1% Triton X-100) from periosteal and osteoblast-enriched cultures treated 24 h with control (ethanol vehicle), PGE_2, PGE_1, $PGF_{2\alpha}$ (each at 1 μM), forskolin (10 μM), or PMA (1 μM). Culture medium was collected and used in the preparation of samples shown in Fig. 2. The cell layer was rinsed twice with PBS. The cell membrane fraction was released by detergent lysis (1% Triton X-100), collected, acidified to release bound IGF, concentrated, and size-fractionated on an SDS-PAGE gel (nonreducing). Numbers on the left (M_r) indicate position of migration of prestained molecular weight standards (Amersham). Tentative identification of IGFBPs is based upon known relative migration. Gels were electroblotted onto Immobilon P membranes (Millipore), blocked with nonfat powdered milk in TBS containing Tween-20, and then probed with ^{125}I-IGF-II. Representative autoradiograms are shown.

with IGFBP-2 and/or IGFBP-5). PGE_2 and PGE_1 enhanced the M_r 46 kDa IGFBP in cell-associated and matrix-associated compartments from both cultures, while PGE_2, PGE_1, and forskolin weakly enhanced M_r 30 kDa IGFBPs in cell-associated compartments. In contrast, M_r 30 kDa IGFBP was suppressed by PMA in cell-associated material in osteoblast-enriched cultures, where it is initially in relatively high concentration.

Western Immunoblots

To assist identification of IGFBP-2 and IGFBP-5 which comigrate by ligand blot analysis, we also evaluated samples with IGFBP-specific antisera by Western immunoblot analysis. As shown in Figure 5A, while IGFBP-2 and several smaller immunoreactive fragments were detected in the medium and matrix extracts, no large changes occurred in response to PMA or forskolin (activators of PKC and PKA, respectively). No IGFBP-2 was detected in cell membrane–associated Triton X-100 extracts from either periosteal or osteoblast culture (data not shown). In constrast, as shown in Figure 5B, IGFBP-5 occurred in all three culture compartments. Nearly one-quarter of the secreted IGFBP-5 (medium compartment) from osteoblast-enriched cultures migrated at M_r 30 kDa, while the remainder from osteoblast-enriched and essentially all from periosteal cell cultures migrated with the 21.5 kDa molecular weight standard (similar to our previous observation for osteoblasts [McCarthy et al., 1994]). Consistent with changes in steady-state mRNA levels seen by Northern blot analysis, soluble IGFBP-5 was suppressed by PMA treatment and enhanced by forskolin. The immunoreactive band-

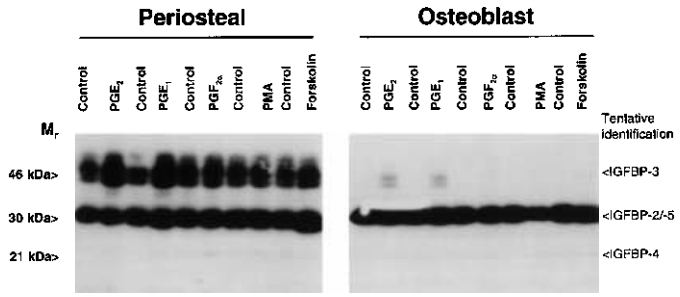

Fig. 4. Western ligand blot analysis of cell matrix extract from periosteal and osteoblast-enriched cultures treated 24 h with control (ethanol vehicle), PGE_2, PGE_1, $PGF_{2\alpha}$ (each at 1 μM), forskolin (10 μM), or PMA (1 μM). Cellular matrix remaining after collecting culture medium and membrane fractions was collected in standard nonreducing Laemmli sample buffer and size-fractionated on an SDS-PAGE gel (nonreducing). Numbers on the left (M_r) indicate position of migration of prestained molecular weight standards (Amersham). Tentative identification of IGFBPs is based upon known relative migration. Gels were electroblotted onto Immobilon P membranes (Millipore), blocked with nonfat powdered milk in TBS containing Tween-20, and then probed with ^{125}I-IGF-II. Representative autoradiograms are shown.

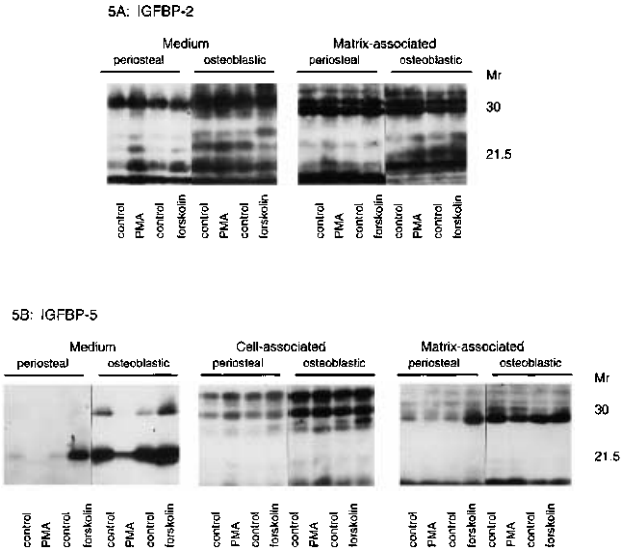

Fig. 5. Western immunoblot analysis of medium, membrane, and matrix fractions of periosteal and osteoblast-enriched cultures following 24 h treatments with control (vehicle), phorbol myristate acetate (PMA, 1 μM), or forskolin (10 μM). Culture medium was collected and concentrated and the membrane extracts prepared by lysing cells with 1% Triton X-100 in PBS. The matrix compartment consists of remaining nuclei and matrix collected in nonreducing Laemmli sample buffer. The membrane fraction was concentrated and resuspended to a final 1× concentration of Laemmli sample buffer. Medium, membrane, and matrix samples were electrophorsed on a 7.5–15% PAGE and electroblotted onto Immonbilon P, and replicate blots were probed with antisera to IGFBP-2 (**A**) or IGFBP-5 (**B**). Immune complexes were visualized with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase, employing chemiluminescence (ECL) (Amersham). The 24 h culture medium was positive for IGFBP-2 and IGFBP-5 (A,B) and IGFBP-3 and IGFBP-4 (data not shown). IGFBP-6 was undetectable in any cellular compartment. Numbers on the right (M_r) indicate position of migration of prestained molecular weight standards (Amersham).

ing pattern of cell-associated IGFBP-5 was more complex. A higher M_r complex was observed, and little change was evident with treatments. Nearly all of the matrix-associated IGFBP-5 migrated just below the M_r 30 kDa complex detected in the culture medium, and additional immunoreactive material was detected below the 21.5 kDa molecular weight marker. The effects of PMA and forskolin on IGFBP-5 in the matrix-associated compartment were similar to those in the culture medium, although the stimulatory effect of forskolin was somewhat more pronounced in the periosteal cell cultures, where basal levels were initially lower. Effects of PGE_2, PGE_1, and $PGF_{2\alpha}$ were consistent with their abilities to regulatePKA and/or PKC path-

TABLE II. Summary of Western Ligand and Immunoblot Compartment Data for IGFBPs[a]

	IGFBP-2			IGFBP-3			IGFBP-4			IGFBP-5			IGFBP-6		
	CM	MEM	MTX	CM	MEM	MTX	CM	MEM	MTX	CM	MEM	MTX	CM	MEM	MTX
Periosteal															
Control	+	0	+	++	+	+	+	0	0	±	+	+	ND	ND	ND
Forskolin	+	0	+	+++	+	++	++	0	0	++	+	++	ND	ND	ND
PMA	+	0	+	++	+	++	+	0	0	±	+	+	ND	ND	ND
Osteoblast															
Control	++	0	+	±	+	+	+	0	0	±	+	+	ND	ND	ND
Forskolin	++	0	+	+++	++	++	++	0	0	++	+	++	ND	ND	ND
PMA	++	0	+	±	+	++	+	0	0	±	+	+	ND	ND	ND

[a]CM, conditioned medium; MEM, membrane; MTX, matrix; ND, not detected.

ways and the patterns established by forskolin and PMA (data not shown). As shown elsewhere in the current work and our earlier studies [Centrella et al., 1994], $PGF_{2\alpha}$ was less effective than PMA in the osteoblast-enriched cultures (data not shown). Consistent with previous reports from our laboratory and others, low molecular mass (less than 21.5 kDa) immunoreactive proteins thought to be processed or proteolytic fragments of intact IGFBPs were also detected with antisera to IGFBP-2 and IGFBP-5. Whereas IGFBP-6 mRNA was detected in periosteal and osteoblast-enriched cultures, no immunoreactive IGFBP-6 was detected with the rabbit antirat IGFBP-6 kindly provided by Drs. Ling and Shimasaki. Relative IGFBP abundance in periosteal and osteoblast-enriched cultures, derived from both ligand binding and immunoblots, is summarized in Table 2.

DISCUSSION

IGF-I and IGF-II are made by bone cells and accumulate in skeletal matrix. Bone cells also synthesize a variety of IGFBPs. Several hormones are known to modulate local growth factor activity in the skeleton. In this context, PTH increases bone resorption as well as local IGF-I synthesis, and in this way new bone formation may be coupled to previous resorption during the physiological bone remodeling process [McCarthy et al., 1989a; Canalis et al., 1989]. The abundance of IGFs in the skeletal environment predicts that they have an important function locally. While fetal rat bone cells secrete five of the six known IGFBPs [McCarthy et al., 1994; Birnbaum and Wiren, 1994], several may have more critical roles in the skeleton. As in other tissues, IGFBP-2 limits IGF activity for skeletal cells and may also accumulate IGFs within the extracellular matrix [Arai et al., 1996]. IGFBP-3 appears to be bifunctional, depending upon its association with cellular components, where it is potentiating [Conover 1992, Conover et al., 1996], whether it is in soluble form, where it is inhibitory [DeMellow and Baxter, 1988], or its state of degradation, where proteolysis releases IGF from its complex with IGFBP-3 [Schmid et al., 1991b]. IGFBP-3 also may have IGF-independent actions [Oh et al., 1993]. Cell surface adherence of IGFBP-3 is associated with a lower ligand affinity and in some instances increased biological activity [Jones and Clemmons, 1995]. IGFBP-4 is thought to inhibit IGF activity, while IGFBP-5 appears to be potentiating [Mohan et al., 1989; Andress and Birnbaum, 1991, 1992; Andress et al., 1993]. IGFBP-5 may also have IGF-independent effects. For example, it may directly enhance bone cell replication and regulate growth hormone receptor abundance [Bautista et al., 1991; Slootweg et al., 1996].

Our current studies to assess IGFBP expression confirm our earlier work showing IGFBP-2, -3, -4, -5, and -6 mRNA expression by osteoblast-enriched cultures [McCarthy et al., 1994] and reveal a similar array present in less differentiated periosteal bone cells. We focused our current work on agents that activate PKA and PKC pathways because both appear to be involved in the physiological response of bone cells to PTH and PGE_2. As in our earlier studies with osteoblast-enriched cultures, activation of PKA increased IGFBP-3, -4, and -5 mRNA levels in periosteal cultures that exhibit a less differentiated bone cell phenotype. Nonetheless, even with activation of PKA, their mRNA levels never achieved that found in osteoblast-

enriched cultures. In contrast, activation of PKC, particularly with PMA, reduced IGFBP-5 expression in both bone cell populations. We also found differential distributions of IGFBPs among cell-associated and matrix-associated compartments, sites in close proximity to cell surface IGF receptors. Notably, osteoblast-enriched cultures exhibited more M_r 30,000 IGFBP in a cell membrane–associated pool composed of IGFBP-5. Less differentiated periosteal cells accumulated more IGFBP-3 (M_r 46,000) in the matrix, and both populations accumulated large amounts of IGFBP-2 and IGFBP-5 (M_r 30,000) in the matrix. These results agree with earlier studies showing IGFBP-5 bound to the cell layers of osteoblast and fibroblast cultures [Andress and Birnbaum, 1992; Jones et al., 1993; Hakeda et al. 1996; Schmid et al., 1996]. We and others previously reported cAMP-dependent increases in IGFBP-3, -4 and -5 in osteoblast cultures [Tørring et al., 1991; Schmid et al., 1992, 1994; McCarthy et al., 1994]. Because IGFBP-3 can in some instances potentiate IGF activity, our observations of IGFBP-3 in cell membrane–associated and matrix-bound compartments suggest positive effects on endogenous IGF activity throughout intramembraneous bone. Whereas IGFBP-3 may directly activate cells perhaps through its own specific cell surface binding sites [Oh et al., 1993], this has not yet been studied in our culture models. Both cell populations that we examined expressed IGFBP-4 mRNA, but immunoreactive protein was detected only in culture medium (data not shown). IGFBP-4 consistently inhibits IGF activity *in vitro*, but in some instances it may retain functional IGF that can be subsequently released by limited proteolysis [Kanzaki et al., 1994; Durham et al., 1994].

Many components of the IGF axis are regulated by cAMP [McCarthy et al., 1989a, 1994, 1998; many others], including IGF binding to type 2 IGF receptors [McCarthy et al., 1998]. Interestingly, PKC activation by PMA appears to inhibit IGFBP-5 expression, while at the same time it enhances IGF-I binding to the type 1 IGF receptor [McCarthy et al., 1998]. Complicating our understanding of the IGF axis further in bone and other tissues is the expression or activation of IGFBP selective proteases with preference for IGFBP-3, -4, or -5 [Schmid et al., 1991; Kanzaki et al., 1994; Durham et al., 1994; Thrailkill et al., 1995; Conover, 1995; Nam et al., 1996]. We noted small immunoreactive protein bands with antisera to several IGFBPs, consistent with proteolysis. Intact IGFBPs have a higher affinity for IGFs than do cell surface receptors, and partial degradation of IGFBPs reduces their affinity and may help to release IGFs from these inactive complexes. When this occurs within the pericellular environment, the IGF ligand may become available to bind to cell surface IGF receptors. A serum protease that selectively cleaves IGFBP-3 within the 150 kDa acid labile protein complex is thought to be responsible for the release of IGF from this serum reservoir, allowing it cross the capillary-endothelial barrier into peripheral tissue [Lee and Rechler, 1996]. IGFBP-4 proteolysis appears to be regulated by IGF, phorbol ester, and IGFBP-3 [Conover et al., 1993; Kanzaki et al., 1994; Donnelly and Holly, 1996]. Several proteases that degrade IGFBP-5 occur in cell cultures, including a 92 kDa serine protease, and 52–72 kDa matrix metalloproteinases-1 and -2 [Thrailkill et al., 1995]. In addition, IGF-II itself appears to decrease IGFBP-5 proteolysis and to enhance IGFBP-4 degradation in primary and continuous human bone cell cultures [Kanzaki et al., 1994]. Again, release of IGFs from complexes with IGFBPs by selective proteases could release active ligand, but in some instances release might also increase the risk of degradation of free IGFs.

In summary, similar IGFBPs are expressed by less differentiated periosteal bone cells and osteoblast-enriched cultures from fetal rat bone. Expression of IGFBP-3, IGFBP-4, and IGFBP-5 was enhanced by activators of PKA, while potent activators of PKC inhibited IGFBP-5 expression. Although expression of IGFBP-2 and IGFBP-5 appears to predominate in the osteoblast-enriched cultures, significantly more IGFBP-3 accumulates in the matrix compartment of less differentiated bone cells. IGFBP-2, IGFBP-3, and IGFBP-5 are all found associated with the cell layer directly or with the cell matrix. Protein fragments reactive with IGFBP antisera also accumulate in these areas, suggesting local IGFBP degradation and potential release of IGFs within the pericellular region. These observations complement other studies showing regulation of expression of IGFs and IGF receptors or their binding affinity by bone cells. Therefore, many systems appear to inte-

grate a complex control of the IGF axis within bone.

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