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Potential Involvement of the Interaction Between Insulin-Like Growth Factor Binding Protein (IGFBP)-6 and LIM Mineralization Protein (LMP)-1 in Regulating Osteoblast Differentiation

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Abstract Insulin-like growth factor binding protein (IGFBP)-6 has been reported to inhibit differentiation of myoblasts and osteoblasts. In the current study, we explored the mechanisms underlying IGFBP-6 effects on osteoblast differentiation. During MC3T3-E1 osteoblast differentiation, we found that IGFBP-6 protein was down-regulated. Overexpression of IGFBP-6 in MC3T3-E1 and human bone cells inhibited nodule formation, osteocalcin mRNA expression and ALP activity. Furthermore, accumulation of IGFBP-6 in the culture media was not required for any of these effects suggesting that IGFBP-6 suppressed osteoblast differentiation by an intracellular mechanism. A yeast two-hybrid screen of an osteosarcoma library was conducted to identify intracellular binding partners to account for IGFBP-6 inhibitory effects on osteoblast differentiation. LIM mineralizing protein (LMP-1) was identified as a high affinity IGFBP-6 binding partner. Physical interaction between IGFBP-6 and LMP-1 was confirmed by co-immunoprecipitation. Fluorescent protein fusion constructs for LMP-1 and IGFBP-6 were transiently transfected into osteoblasts to provide evidence of subcellular locations for each protein. Coexpression of LMP-1-GFP and IGFBP-6-RFP resulted in overlapping subcellular localization of LMP-1 and IGFBP-6. To determine if there was a functional association of IGFBP-6 and LMP-1 as well as a physical association, we studied the effect of IGFBP-6, LMP-1 and their combination on type I procollagen promoter activity. LMP-1 increased promoter activity while IGFBP-6 reduced promoter activity, and coexpression of LMP-1 with IGFBP-6 abrogated IGFBP-6 suppression. These studies provide evidence that overexpression of IGFBP-6 suppresses human and murine osteoblast differentiation, that IGFBP-6 and LMP-1 physically interact, and supports the conclusion that this interaction may be functionally relevant. J. Cell. Biochem. 104: 1890–1905, 2008.

Key words: insulin-like growth factor binding protein; Enigma/LMP-1; osteoblast differentiation; transcription

Insulin-like growth factor binding protein (IGFBP)-6 is a member of a family of six widely expressed high affinity IGFBPs that were first identified as regulators of IGF activity to affect cell growth, survival, and differentiation [Clemmons, 1998; Firth and Baxter, 2002; Govoni et al., 2005; Cohen, 2006; Holly and Perks, 2006]. IGFBP-6 is distinguished from the other IGFBPs by its IGF-II binding preference, and effects of IGFBP-6 in a number of human and murine in vitro systems have been inhibitory for either growth or differentiation (reviewed in Bach [2005]). Studies have provided evidence not only for IGF-dependent inhibitory effects but also for IGF-independent effects on human neuroblastoma cells [Grellier et al., 2002], rat oligodendrocytes [Kuhl et al., 2003] and human small cell lung cancer cells [Sueoka et al., 2000] that could be mediated by either extracellular or intracellular actions. A recent study provided the first definitive evidence for IGF-independent IGFBP-6 actions, in which an IGF-binding deficient IGFBP-6 mutant protein stimulated cell migration when added to culture media of human RD rhabdomyosarcoma cells [Fu et al., 2007]. However, the underlying mechanism by which exogenously added IGF-binding deficient IGFBP-6 stimulated cell migration was not addressed.

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Previously, our laboratory found that stable SaOS-2 clones with reduced levels of IGFBP-6 created by transfection with a plasmid antisense mRNA vector had increased ALP activity [Yan et al., 2001]. Likewise, we transfected SaOS-2 cells with an IGFBP-6 expression vector and found that stable clones of SaOS-2 cells with increased IGFBP-6 mRNA and protein levels had reduced ALP activity (unpublished data), suggesting that IGFBP-6 inhibits osteoblast differentiation. We also found that exogenous addition of IGFBP-6 protein blocked IGF stimulated DNA synthesis in MC3T3-E1 cells but did not affect basal ALP activity [Srinivasan et al., 1996]. When we developed retroviral constructs to express non-secreted IGFBP-6 in human and murine osteoblasts, we found that osteoblast marker gene expression was suppressed. These findings, some of which are reported in this study, inferred that IGFBP-6 effects on cell differentiation in both human and murine osteoblasts might occur by an intracellular mechanism.

To begin to define mechanism(s) for IGFBP-6 intracellular actions on osteoblast differentiation, we proposed that IGFBP-6 could bind to a highly conserved intracellular protein(s) that played important roles in differentiation. In support of this proposal, intracellular binding partners of IGFBP-3 and IGFBP-5 have been identified and were reported to affect important cellular functions [Amaar et al., 2002, 2005, 2006a,b; Govoni et al., 2005; Lee et al., 2005]. IGFBP-5 binds to the multi-compartmental signaling protein, four and a half LIM domains 2, *Fhl2* [Amaar et al., 2002], and to two growth promoting proteins, RASSFIC [Amaar et al., 2005] and IGFBP-5 IP [Amaar et al., 2006b] in osteoblasts. IGFBP-5 also interacts with nuclear vitamin D receptor and attenuates the $1,25(OH)_2D_3$-induced expression of bone differentiation markers and $1,25(OH)_2D_3$-mediated inhibition of cell cycle progression in bone cells [Schedlich et al., 2007]. IGFBP-3 binds to retinoid X receptor-α (RXRα) and Nur77 in fibroblasts and prostate cancer cells [Liu et al., 2000; Lee et al., 2005]. The interaction of IGFBP-3 with RXRα was shown to alter the traditional role of the RXRα nuclear receptor as a transcription factor by relocation of the proapoptotic RXRα/Nur77/IGFBP-3 multi-component complex to the mitochondria. IGFBP-5 and IGFBP-3 intracellular binding partners were initially identified using the yeast two-hybrid screening assay. Therefore, we used a similar approach and screened a yeast two-hybrid osteoblast-like osteosarcoma library with IGFBP-6 bait to identify novel intracellular IGFBP-6-interacting proteins whose function could be associated with osteoblast differentiation in both human and murine osteoblasts. We identified Enigma, a PDZ-LIM domain protein identical to LIM Mineralization Protein (LMP)-1, as a highly conserved, osteoinductive IGFBP-6 binding partner and present evidence suggesting that IGFBP-6 inhibits osteoblast differentiation possibly through a mechanism involving interaction with LMP-1.

MATERIALS AND METHODS

Cell Culture

Early passage human osteoblasts were isolated as described [Chevalley et al., 1996] from bone specimens obtained from the Cooperative Human Tissue Network (supported by the National Cancer Institute) and maintained in DMEM supplemented with 10% calf serum. The human osteoblasts were part of a tissue bank approved by the VA Loma Linda HealthCare System human studies subcommittee. Because human osteoblasts are in limited supply and extremely difficult to induce to mineralize in vitro, the MC3T3-E1 mouse osteoblast cell line, which differentiates in a reproducible, defined time frame and forms mineralized nodules in vitro, was used to test the effect of IGFBP-6 overexpression on osteoblast mineralization. MC3T3-E1 cells (a gift from B. Frenkel, University of Southern California [Luppen et al., 2003]) were maintained in αMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. To induce differentiation, the media was supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate (osteogenic media). Media was changed every 2–3 days and cells were maintained in osteogenic media for 14–21 days to induce differentiation. ROS 17/2.8 cells, maintained DMEM with 10% CS, were used to assess type I procollagen promoter activity because these murine osteoblast-like cells are transfected at high efficiency unlike either MC3T3-E1 cells or primary human osteoblasts [Majeska et al., 1985]. SaOS-2 cells maintained in DMEM/10% CS were transduced with retroviral vectors to express IGFBP-6 or LMP-1, and ROS 17/2.8 cells were transfected with plasmid vectors to

express IGFBP-6 and LMP-1. Cell lysates and conditioned medium were collected for co-immunoprecipitation studies. All cell cultures were maintained in a 5% CO_2 air humidified incubator at 37°C.

IGFBP-6 Expression Vector Construction

Clone pHBP6-513 with human IGFBP-6 cDNA [Shimasaki et al., 1991] and the human pcDNA3-Enigma cDNA [Wu et al., 1996] were obtained for expression vector preparation.

hIGFBP6 (Met^1-Gly^{240}) and (Ala^{25}-Gly^{240}) cDNAs were prepared by PCR with 2.5 U of *Pfu* turbo (Stratagene, La Jolla, CA), 125 ng of the pHBP6-513 plasmid template, 10 nmol dNTPs and 10 pmol each of the forward and reverse primers. The forward primers introduced a SalI site, and were 5′-gcgcgtcgacgccgccatgacccccacagg or 5′-gcgcgtcgacgccgccatggccttggcgcggtgcccaggc and a single reverse primer to introduce a BamHI site, was 5′-gcgcggatccttagccgctactcccagtggggca. PCR was performed in a MJ Research/Biorad DNA Engine (Hercules, CA) under the following conditions: 5 min 94°C hot start followed by 30 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 1 min. The PCR fragments were ligated directly into pCR-Blunt (Invitrogen, Carlsbad, CA), removed with SalI and BamHI digestion and inserted into the VR1012 plasmid vector [Hartikka et al., 1996] or the CLSA MFG-based retroviral vector [Peng et al., 2001] for expression of an IGFBP-6 molecule (Met^1-Gly^{240}) with the natural secretory signal sequence, or a shorter form (Ala^{25}-Gly^{240}) similar to a naturally occurring intracellular form without a secretory signal sequence [Bach, 2005; Bach et al., 2005].

Replication-defective, MLV-based vector hIGFBP-6 (Met^1-Gly^{240}) and (Ala^{25}-Gly^{240}) were prepared and harvested from 293T cells and osteoblast-like cells were transduced as described [Peng et al., 2001]. Western immunoblotting of cell extracts with a polyclonal anti-hIGFBP-6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; AbCam, Inc., Cambridge, MA) confirmed expression of a glycosylated 34–36 kDa protein product, corresponding to IGFBP-6 proteins with and without the signal sequence.

IGFBP-6 (Met^1-Gly^{240}) was inserted in-frame into the pDSRed1-N1 (Clontech, Palo Alto, CA) vector at EcoRI and BamHI sites to produce an IGFBP-6-RFP fusion protein after transient transfection.

For the yeast 2-hybrid screen, a human IGFBP-6 (Ala^{25}-Gly^{240}) PCR product was prepared with Native *Pfu*, the pHBP-6-513 template DNA and forward and reverse primers, (5′-cgcgaattcgccttggcgcggtgcccagg and 5′-gcgcggatccttagccgctactcccagtc) to include EcoRI and BamHI restriction sites for cloning into the multiple cloning site of the pGBKT7 two-hybrid bait vector (Clontech).

LMP-1 Expression Vector Construction

The coding region of LMP-1 with an amino-terminal HA (tyr-pro-tyr-asp-val-pro-asp-tyr-ala) minimal sequence tag was removed from the pcDNA3 vector [Wu et al., 1996] with SalI and EcoRV and was subcloned into VR1012 for retroviral vector production. The cDNA was removed from VR1012 with SalI and BamHI and sublconed into the CLSA-MLV vector at compatible sites. Expression of a 52 kDa HA-LMP-1 protein was confirmed by Western immunoblotting with a polyclonal anti-Hemaglutinin (HA) antibody (Covance, Berkeley, CA). The human Enigma (Met^1-Val^{457})/LMP-1 cDNA was inserted in frame in the multiple cloning site of the peGFP-C3 vector at SalI and BamHI sites to produce a GFP-Enigma/LMP-1 fusion protein after transient transfection.

Yeast Two-Hybrid Screening

A human osteosarcoma Matchmaker cDNA library in the pACT2 vector (Clontech) was screened with the pGBKT7-IGFBP-6 (Ala^{25}-Gly^{240}) bait vector in the yeast reporter strain, AH109. Yeast extracts assessed by Western blot indicated that cMyc-tagged hIGFBP-6 fusion protein was expressed in yeast cells (data not shown). In addition, the IGFBP-6-GAL4-BD bait did not autoactivate the reporter gene promoter linked to the GAL4AD:U-2 cDNA library (data not shown). After transforming AH109 cells expressing hIGFBP-6 with the cDNA osteosarcoma library as described [Amaar et al., 2002], the cells were plated at high stringency in minimal selection medium with X-α-gal (Clontech) and incubated for 4–21 days at 30°C. Positive colonies were picked, screened again on minimal selection medium, and plasmid DNA was isolated from the resulting colonies. After transformation of HB101 cells with plasmid DNA isolated from the yeast clones and preparation of purified DNA, sequences were determined for each clone.

Promoter Analysis

Human genomic DNA was isolated from anonymous blood samples with a Puregene Kit (Gentra systems, Inc. Minneapolis, MN) and stored in an approved VA tissue bank. The human type I α2 procollagen promoter (containing 267 bp of 5′flanking region and 45 bp of 5′UTR) was prepared by PCR from 125 ng of genomic DNA, 2.5 U *Pfu* Turbo (Stratagene) 10 nmol dNTPs and 10 pmoles each of the forward and reverse primers 5′-ctgcagacaacgagtcagagt and 5′-gccagtacctccaacttagc. The PCR conditions were a 5 min 94°C hot start followed by 30 cycles of 94°C for 1 min, 58°C 1 min, 72°C 1 min. The PCR fragment was ligated directly into pCR-Blunt (Promega, Madison, WI), and subcloned with Kpn I and Xho I into the pGL3Basic luciferase reporter vector (Promega) at compatible sites.

In six-well plates, 100,000 cells per well were plated and the next day transiently cotransfected with the hCol1α2-pGL3 promoter construct, and either the LMP-1-pcDNA3 expression vector, the IGFBP-6-VR1012 vector or both using 5 μl of Effectene and 4 μl of Enhancer per well (Qiagen, Chatsworth, CA). VR1012 plasmid DNA was used to adjust total DNA concentrations to 3 μg. Cells were maintained in DMEM/10% CS. Media was changed 24 h after transfection and 48 h after transfection cells were extracted with 5× reporter lysis buffer (Promega) for luciferase and total protein assays. Promoter activity and total protein concentration was determined as previously described [Dailly et al., 2001]. Promoter activities from six replicate samples were expressed as luciferase activity (light units)/total cellular protein. Experiments were repeated two to four times.

Real-Time Quantitative PCR (qRT-PCR)

Total RNA was isolated from cells using a Versagene RNA Purification Kit (Gentra, Minneapolis, MN). Contaminating DNA was removed from the RNA with DNAse I treatment during the purification process. cDNA was synthesized using the Superscript III first strand synthesis system for RT-PCR (Invitrogen), with 250 pmol oligo dT (Invitrogen), and 1 μg of total RNA in a 20 μl volume. Real-time PCR was performed with 2 μl of cDNA, 10 pmol of each primer, and HotStarTaq DNA Polymerase using a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) in an Opticon DNA Engine (MJ Research/Biorad, Hercules, CA). PCR amplification conditions were 95°C for 15 min, 30 cycles with 95°C 30 s, annealing at 56.4–57.2 for 30 s and extension at 72°C for 30 s. Primer sets used to create amplicons from human or mouse mRNA are described in Table I. Relative mRNA abundances were quantified as Ct (cycle threshold value) relative to the Ct of cyclophilin A, a housekeeping gene, based on the assumption that cell cyclophilin A mRNA levels are constant and RT and PCR reaction efficiencies are constant. Expression levels are shown as fold or percentage increases or decreases in mRNA levels as calculated by the $2^{-\Delta\Delta C_t}$ method [Livak and Schmittgen, 2001]. Values of $\Delta\Delta C_t$ are the mean ± SEM of 4–8 replicates with one cycle representing a twofold difference in relative mRNA abundance.

Fluorescent Microscopy

Cells were seeded onto six-well plates and transiently transfected with 2 μg per well of LMP-1-GFP, IGFBP-6-RFP or 1 μg of each construct using 3 μl per well of TurboFectin

TABLE I. Primer Sets for Real-Time Quantitative PCR

Gene	Forward primer sequence	Reverse primer sequence	Accession #
Mouse			
BSP	aggtgcagaaggaaccacag	cgtcctcataagctcggtaa	NM_008318
Osteocalcin	gctaccttggagcctcagtc	atgcgtttgtaggcggtctt	NM_001032298/MUSOGC
Osterix	cacttgcctgctctgttcca	caagtggtcgcttctggtaa	NM_130458
Runx2	ctctgatcgcctcagtgatt	ggacttggtgcagagttcag	NM_009820
Cyclophilin A	gcatacaggtcctggcatct	gctctcctgagctacagaag	NM_008907
Human			
IGFBP-6	gaggaatccaggcacctcta	ggagcttccattgccatctg	NM_002178
Osteocalcin	ccaccgagacaccatgagag	tcgtcacagtccggattgac	NM_199173
Cyclophilin A	gcatacaggtcctggcatct	gctctcctgagctacagaag	NM_021130

8.0 (OriGene, Rockville, MD). Cells were maintained in αMEM with 10% FBS. Cells were viewed by fluorescent microscopy 24–48 h post-transfection for expression of LMP-1-GFP and IGFBP-6-RFP. Cell nuclei were labeled using Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA).

Fluorescent microscopy and data capture was conducted with an Olympus IX70 inverted microscope with U-MWIBA, U-MWIG, and U-MNUA excitation filters with barrier filters to selectively identify LMP-1-GFP, IGFBP-6-RFP, and 4′,6-diamidino-2-phenylindole (DAPI) respectively. A MagnaFire-SS9980 CCD camera with Lucis Version 4.0 software (Olympus America, Melville, NY) was used to capture and process fluorescent images.

Alkaline Phosphatase Assay

Cells were plated at a density of 20,000 cells/cm^2. Culture media was removed, and cells were rinsed with PBS and extracted with 0.5 ml of 0.1% Triton X-100. Alkaline phosphatase specific activity was calculated by normalizing to the cell extract protein concentration determined with the BCA assay kit (Pierce Biotechnology, Inc., Rockford, IL) as previously described [Kyeyune-Nyombi et al., 1991].

Alizarin Red-S Staining

Alizarin red staining was used to assay mineralized nodule formation at days 14 and 21. Briefly cells were fixed in 10% formalin for 30 min at 4°C, washed with PBS three times, stained with 40 mM Alizarin Red-S (Sigma–Aldrich, St. Louis, MO) pH 4.2 for 15 min at room temperature, washed with distilled water five times, rinsed in 1× PBS for 10 min to reduce nonspecific staining, and dried overnight. The intensity of red staining and nodule formation was evaluated by photography.

RIA

IGFBP-6 levels in the conditioned medium of cultured cells were assessed by RIA as described [Yan et al., 2001]. The inter-assay and intra-assay CV for the RIAs were less than 10%.

Western Immunoblotting

Total cell extracts were prepared using RIPA buffer with PMSF and protease inhibitors. Protein concentrations were determined using the BCA total protein assay (Pierce Biotechnology, Inc.). Extracts (20 μg) were run on a 10% SDS–PAGE gel and electrotransferred onto a nitrocellulose membrane. The membrane was blocked in Odyssey blocking buffer (Li-Cor, Lincoln, NE) at room temperature for 2 h. For detection of hIGFBP-6, the membrane was incubated with chicken anti-human IGFBP6 (Abcam, Inc.) for 1 h, washed in 1× PBS 0.1% Tween-20, and incubated with IRDye 800CW anti-chicken (Rockland, Gilbertsvillle, PA) for 1 h. For detection of HA-tagged LMP-1, the membrane was incubated with mouse anti-HA antibody (Covance) for 1 h, washed in 1× PBS 0.1% Tween-20 and incubated with IRDye 800CW anti-mouse (Li-Cor) for 1 h. For detection of β-actin, the membrane was incubated with rabbit anti-β-Actin (Santa Cruz Biotechnology, Inc.) for 1 h, washed PBS 0.1% Tween-20 and incubated with IRDye 700CW anti-rabbit (Li-Cor) for 1 h. Immunoreactive proteins were detected and quantitated using the Odyssey Infrared Imaging system (Li-Cor).

Co-Immunoprecipitation

SaOS-2 cells were transduced with MLV-GFP or MLV-HA-LMP-1 virus to overexpress GFP or HA-tagged LMP-1. In addition, ROS cells were transiently transfected with VR-1012-HA-LMP-1, VR1012-IGFBP-6 Met^{1}-Gly^{240}, VR1012-IGFBP-6 Ala^{25}-Gly^{240} or VR-1012 to overexpress protein in whole cell lysates or CM for co-immunoprecipitation studies. Concentrated whole cell lysates from 100 mm plates of virally transduced cells or cells harvested 48 h post transfection were prepared with lysis buffer: 150 mM NaCl/50 mM Tris–HCl pH 7/1% Triton X-100. Expression of HA-LMP-1 protein was verified by Western blot analysis with anti-HA antibodies (Covance), and expression of 34–36 kDa IGFBP-6 Ala^{25}-Gly^{240} and IGFBP-6 Met^{1}-Gly^{240} proteins and integrity of recombinant IGFBPs was verified with IGFBP specific polyclonal antibodies (Cell Sciences, Canton, MA).

For co-immunoprecipitation studies with recombinant IGFBP-5 and IGFBP-6 input protein, 0.25μg of anti-IGFBP-6 or anti-IGFBP-5 polyclonal antibodies (Cell Sciences), 0.5 μg of recombinant IGFBP-6 or IGFBP-5 protein and protease inhibitor cocktail (Sigma, St. Louis, MO) was incubated overnight at 4°C [Amaar et al., 2002]. One hundred microliters of Sepharose A Fast Flow (Upstate, St. Louis, MO) pre-equilibrated with binding buffer (cell lysis buffer adjusted to 0.1% Triton X-100) was

incubated with or without antibodies for 2 h at 4°C. The protein A-Sepharose was washed extensively with binding buffer. To identify protein–protein interactions between IGFBPs and LMP-1, 100 μl (50 μg) of HA-LMP-1 or GFP cell lysate was added to 100 μl of protein A-Sepharose and incubated for 2 h at 4°C. The beads were washed extensively with binding buffer.

Co-immunoprecipitation studies performed with overexpressed BP-6 Met^{1}-Gly^{240}, BP-6 Ala^{25}-Gly^{240}, and HA-LMP-1 input proteins produced by transfected ROS 17/2.8 cells were performed with the ProFound CoIP Kit #23600 (Pierce Biotechnology, Inc.). Twenty-five micrograms of whole cell lysate from VR1012-IGFBP-6 Met^{1}-Gly^{240}, VR1012-IGFBP-6 Ala^{25}-Gly^{240} or VR1012 vector transfected cells, and 25 μg of whole cell lysate from VR1012-HA-LMP-1 transfected cells was combined and incubated overnight at 4°C with protease inhibitor cocktail [Amaar et al., 2002]. 0.25 μg of polyclonal anti-IGFBP-6 antibody (Cell Sciences) in 200 μl of Coupling Buffer was immobilized to AminoLink Plus Gel and washed as described by the manufacturer. The IGFBP-6/HA-LMP-1 cell lysates were incubated with the anti-IGFBP-6-AminoLink Plus Gel overnight at 4°C and the beads were extensively washed.

After washing the protein A-Sepharose or AminoLink Plus Gel beads, immune complexes were dissociated by boiling and analyzed by Western blot analysis with anti-HA antibodies (Covance) as described [Amaar et al., 2002].

Statistical Analysis

Values are the mean ± SEM. Significance was evaluated by the Student's two tailed *t*-test, $P < 0.05$ was considered significant.

RESULTS

IGFBP-6 Suppresses Osteoblast Marker Gene Expression

MLV-based retroviral vectors expressing hIGFBP-6 Met^{1}-Gly^{240} and hIGFBP-6 Ala^{25}-Gly^{240} were prepared (Fig. 1A) and tested to determine the effect of increasing IGFBP-6 in the conditioned medium (extracellular) and intracellular IGFBP-6 on osteoblast differentiation. As expected the secretory signal sequence (Met^{1}-Gly^{240}) was required to increase the amount of IGFBP-6 in the conditioned medium (Fig. 1B). Increased expression of either form of IGFBP-6 in primary human osteoblasts and osteoblast-like osteosarcoma cells significantly reduced ALP activity compared to MLV-GFP transduced control cells (Fig. 1C), suggesting that extracellular IGFBP-6 was not required. To further test this hypothesis, recombinant human (h)IGFBP-6 protein (500 ng/ml) in excess of endogenous IGF-II concentration in the conditioned medium [Yan et al., 2001] was added to human osteoblast-like cell cultures not transduced with retroviral vector. Figure 1D shows that addition of excess exogenous IGFBP-6 did not significantly affect ALP activity.

To explore the effect of increased IGFBP-6 expression on osteoblast marker gene expression in human osteoblasts further, primary human osteoblasts were transduced with MLV-hIGFBP-6 Met^{1}-Gly^{240} or MLV-GFP and grown to confluence. Total RNA was isolated and mRNA levels of IGFBP-6, osteocalcin, and cyclophilin A were determined by real-time quantitative PCR (qRT-PCR) with human primer sets listed in Table I. IGFBP-6 mRNA levels were increased 72 h after transduction of cells with MLV-hIGFBP-6 compared to MLV-GFP (Table II). MLV-hIGFBP6 transduction also resulted in a 18.5-fold reduction of osteocalcin mRNA expression compared to MLV-GFP transduced control cells (Table II). Furthermore transduction of MC3T3-E1 cells with MLV-hIGFBP-6 also resulted in a 4.3-fold reduction of osteocalcin mRNA expression compared to MLV-GFP transduced control cells (Table II). These data supported our previous antisense DNA studies [Yan et al., 2001] showing an inverse correlation of osteoblast marker gene expression with IGFBP-6 mRNA levels.

Intracellular IGFBP-6 Protein Is Reduced in Mineralizing MC3T3-E1 Cells

To assess endogenous IGFBP-6 expression during osteoblast differentiation and mineralization, the MC3T3-E1 model of osteoblast differentiation was utilized because these cells differentiate in a reproducible defined time frame, unlike primary human osteoblasts [Luppen et al., 2003]. MC3T3-E1 cells were plated in six-well plates at 25,000 cells/cm^2 in differentiation medium (αMEM, 10% FBS, 50 μg/ml ascorbate, 10 mM β-glycerophosphate) and cultured for up to 21 days for analysis of intracellular IGFBP-6 protein by Western

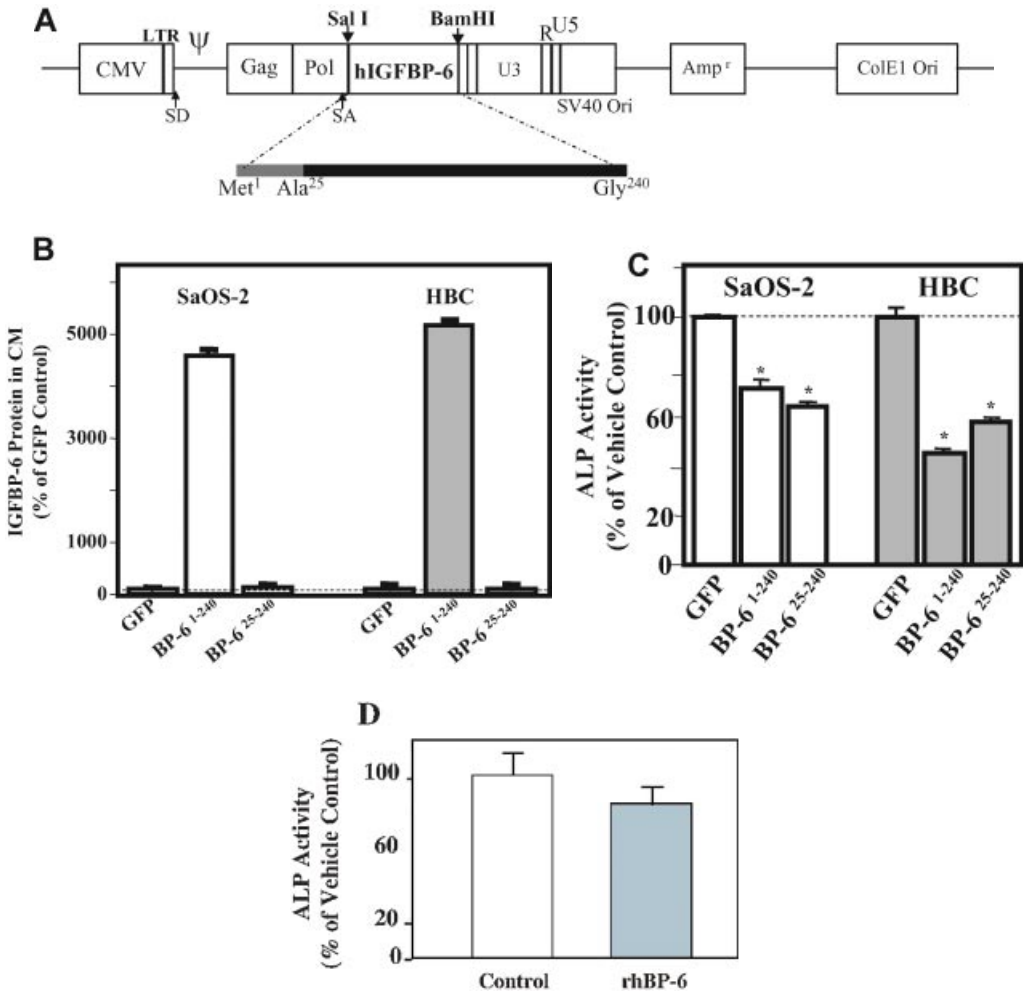


Fig. 1. Retroviral overexpression of IGFBP-6 in human osteoblasts inhibits ALP activity without a requirement for extracellular IGFBP-6 accumulation. (**A**) Structure of the hIGFBP-6 (Met^{1}-Gly^{240}, secreted) and hIGFBP-6 (Ala^{25}-Gly^{240}, not secreted) retroviral vectors that place constitutive expression under the control of the viral LTR. (**B**) SaOS-2 osteoblast-like osteosarcoma cells and low passage normal human bone cells (HBCs) and were transduced (45–60%) with MLV-GFP control, MLV-hIGFBP-6Met^{1}-Gly^{240} or MLV-hIGFBP-6Ala^{25}-Gly^{240} retroviral vectors, harvested 72 h after transduction, and IGFBP-6 protein levels in the CM were measured by RIA (ng/ml). (**C**) ALP activity (mU/mg protein) was measured in lysates of the transduced cells and is expressed as % of GFP control. Values represent the mean ± SEM (n = 5). (**D**) Recombinant hIGFBP-6 (500 ng/ml) or vehicle was added to cell media for 72 h under serum free conditions with 1% BSA/DMEM. ALP activity and total protein were determined as in (C). Values represent the mean ± SEM (n = 5).

blot analyses. Mineralized nodule formation assessed in parallel cultures by Alizarin Red-S staining was evident at days 14 and 21 (Fig. 2). Western blot analysis of IGFBP-6 in MC3T3-E1 cell lysates isolated at days 3, 7, 14, and 21 run concurrently on SDS–PAGE gels revealed that endogenous IGFBP-6 protein decreased as differentiation progressed (Fig. 2).

TABLE II. Effect of Increased IGFBP-6 Expression on Osteocalcin mRNA Levels

	hIGFBP-6 mRNA (fold change)	Osteocalcin mRNA (fold change)
Primary human osteoblasts	891.4	−18.5
MC3T3-E1 pre-osteoblasts	N/A	−4.3

Fold change is calculated by the formula where $\Delta C_t = C_t$(gene of interest)$-C_t$(cyclophilin) and $\Delta\Delta C_t = \text{mean}\Delta C_t$(MLV-BP6 cells)$-\text{mean}\Delta C_t$(MLV-GFP cells) for the mRNA expression of the gene of interest. Because osteocalcin mRNA was suppressed by IGFBP6, values are presented as negative numbers compared to expression in MLV-GFP transduced cells. Four to six replicate samples were averaged. hIGFBP6 was detected at high levels in MC3T3-E1 cells transduced with MLV-BP6, however a fold change calculation is not possible since MC3T3-E1 are a mouse cell line and therefore do not express human IGFBP-6. All values were significant ($P < 0.05$).

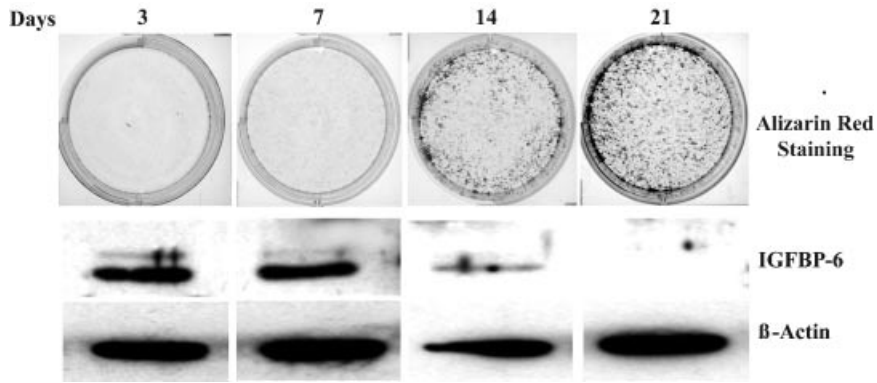


Fig. 2. Analysis of intracellular IGFBP-6 protein levels in differentiating MC3T3-E1 osteoblasts. Cells were plated in differentiation medium. Cell protein was extracted at 3, 7, 14, and 21 days and IGFBP-6 protein (34–36 kDa) levels in 20 μg of cell lysates were assessed with anti-mouse IGFBP-6 antibody using Western Immunoblot analysis. Anti-β-actin antibody was used to confirm equal loading and the 42 kDa band was observed in all samples. All protein samples were run in duplicate on gels, transferred to membranes and developed with anti-mouse IGFBP-6 antibodies, concurrently. Representative single protein samples of each duplicate are presented for comparison. Parallel cultures of MC3T3-E1 cells were stained with Alizarine Red-S to assess mineralization. One representative of three independent experiments is presented.

IGFBP-6 Interferes With MC3T3-E1 Osteoblast Differentiation and Mineralization

To determine the cause and effect relationship between decreased intracellular IGFBP-6 protein levels and nodule formation, we produced sustained hIGFBP-6 expression with retroviral transduction of MLV-hIGFBP-6 Met^{1}-Gly^{240} or MLV-hIGFBP-6 Ala^{25}-Gly^{240} in MC3T3-E1 cells and determined the effect on osteoblast differentiation and mineralization. After transduction, cells were cultured under osteoinductive conditions for 21 days. IGFBP-6 protein production and the extent of mineralization were assessed at day 21 by Western blot and Alizarin Red staining, respectively. Mineralization in MC3T3-E1 cultures expressing hIGFBP-6 (Ala^{25}-Gly^{240}) or hIGFBP-6 (Met^{1}-Gly^{240}) was significantly reduced compared to control cultures transduced with MLV-GFP control cultures (Fig. 3). Inhibition of differentiation by hIGFBP6 overexpression observed in cells transduced with either MLV-hIGFBP-6 Met^{1}-Gly^{240} or MLV-hIGFBP-6 Ala^{25}-Gly^{240} again suggests that the inhibitory effect of IGFBP-6 on osteoblast differentiation is through an intracellular mechanism.

In support of an early inhibitory effect of IGFBP-6 on osteoblast differentiation, mRNA levels for osterix did not significantly increase in IGFBP-6 transduced cultures compared to GFP control or untransduced cultures, which demonstrated a four to fivefold increase in osterix mRNA level between days 1 and 7 (Table III). However, Runx2 mRNA levels were not affected by IGFBP-6 overexpression (Table III).

Identification of Intracellular Proteins That Interact With hIGFBP-6

A high complexity MatchMaker (Clontech) human osteosarcoma cDNA library was amplified in the AH109 yeast reporter strain carrying the hIGFBP-6 (Ala^{25}-Gly^{240})-bait vector. The cDNA library was screened as described [Amaar et al., 2002] and six colonies representing IGFBP-6 interacting partners were identified under high stringency conditions. By sequence analysis, four distinct high affinity binding partners were identified. These included a mitochondrial translocase subunit, a structural protein in basement membranes and vessel walls, a type I transmembrane protein and Enigma. Enigma has become better known in the bone field as LIM Mineralization Protein (LMP)-1 and more recently as PDLIM7 (accession no. NM_203353). Because LMP-1 has been reported to stimulate osteoblast differentiation in vitro [Boden et al., 1998], LMP-1 was the best candidate to begin to examine as a mediator for the inhibitory actions of IGFBP-6 on osteoblast differentiation. The LMP-1 fragment (His^{354}-Val^{457}) that bound to IGFBP-6 is illustrated in Figure 4. This fragment contained a portion of the second and the complete third LIM domain, suggesting that IGFBP-6 bound to the LIM domains in LMP-1. Real-time PCR indicated that LMP-1 was expressed in both primary human osteoblasts and MC3T3-E1 cells (data not shown).

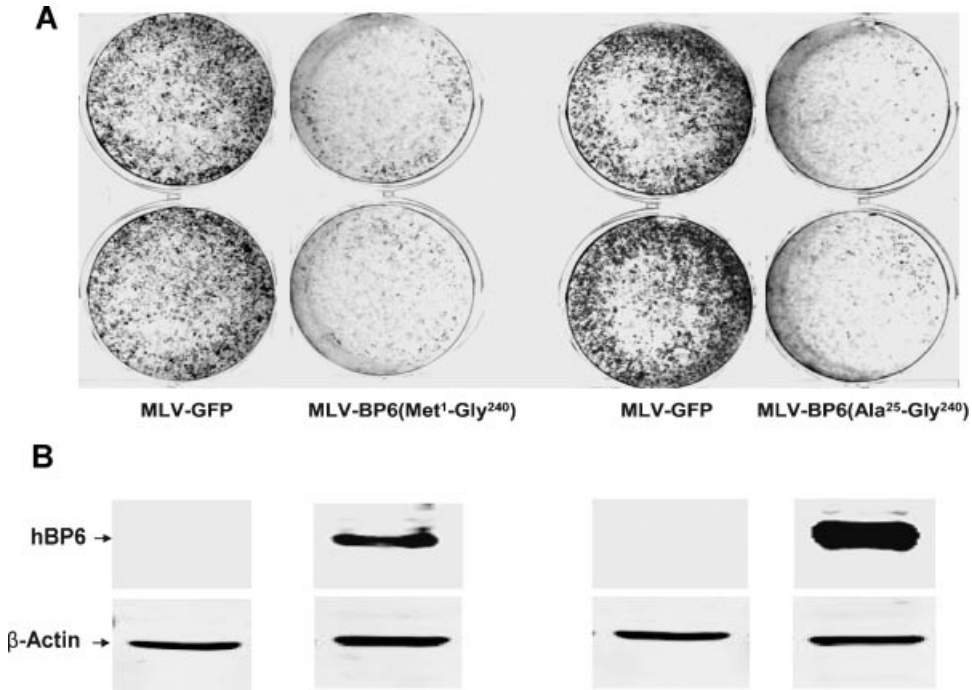


Fig. 3. IGFBP-6 reduces mineralized nodule formation in MC3T3-E1 osteoblasts. (**A**) Cells were transduced with MLV-GFP, MLV-hIGFBP-6 (Met^{1}-Gly^{240}) or MLV-hIGFBP-6 (Ala^{25}-Gly^{240}) retrovirus and plated in differentiation medium for 21 days. Media was removed, cells were rinsed and stained with Alizarin Red S and nodule formation was compared. (**B**) Expression of hIGFBP-6 transgene protein, as detected by Western blot, was observed throughout differentiation. Western blots of 21-day samples developed with anti-human IGFBP-6 antibody are presented. Table III describes qRT-PCR analysis of mRNA levels in parallel transduced cultures.

LMP-1 Is an IGFBP-6 Binding Partner

To further confirm interaction between IGFBP-6 and LMP-1 observed in the yeast two-hybrid assay, cell lysates from SaOS-2 cells overexpressing HA-tagged LMP-1 or GFP after retroviral transduction were combined with either recombinant human IGFBP-5 or IGFBP-6, immunoprecipitated with the corresponding IGFBP-specific antibody, and Western blots probed with anti-HA antiserum (Fig. 5A,B). Western blots developed with anti-IGFBP-6 (hBP6 ab), anti-IGFBP-5 (hBP5 ab) and anti-HA (HA ab) were used to assess the integrity of input protein samples (Fig. 5A). HA-tagged LMP-1 was immunoprecipitated and present in immune complexes released from protein A-Sepharose bound to anti-hIGFBP-6/IGFBP-6 (Fig. 5B). By contrast protein A-Sepharose bound to anti-hIGFBP-5/

TABLE III. Effect of Increased IGFBP-6 Expression on mRNA Levels in MC3T3-E1 Cells During Osteoblast Differentiation

	Day 1	Day 7	Days 1–7	Days 1–7
Cells (mRNA)	ΔC_t	ΔC_t	$\Delta\Delta C_t$	Fold change
Untransduced				
Runx2	4.30 ± 0.38	4.11 ± 0.18	-0.19	1.1
Osx	3.44 ± 0.42	1.17 ± 0.67	-2.27	4.8*
GFP				
Runx2	4.27 ± 0.46	3.39 ± 0.42	-0.88	1.8
Osx	3.72 ± 0.29	1.39 ± 0.96	-2.33	5.0*
hIGFBP-6				
Runx2	4.63 ± 0.36	4.34 ± 0.39	-0.29	1.2
Osx	2.66 ± 0.53	2.03 ± 0.58	-0.63	1.55

Fold change ($2^{(-\Delta\Delta C_t)}$) reflects the change in mRNA levels from days 1 to 7.
*$P < 0.05$.

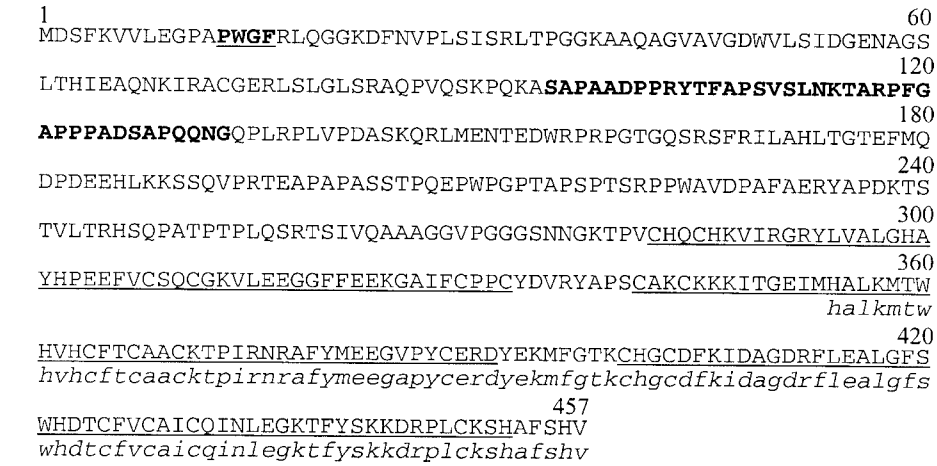


Fig. 4. Alignment of the human Enigma/LMP-1 sequence with sequence from clone-3 isolated from the yeast two-hybrid screen. The sequence from clone 3 identified in the yeast two-hybrid screen (lower case and italicized) was 99% identical to the sequence of Enigma (NCBI # NM_203353). The only variation was a Val^{385} to Ala^{385} change in the second LIM-domain. This sequence is identical to mouse LMP-1 (NCBI# NM_026131). Sequence in bold is the osteoinductive domain, the underlined sequence in bold is the PDZ domain and the three underlined sequences at the carboxy-terminal are the LIM domains.

IGFBP-5 or unconjugated protein A-Sepharose did not immunoprecipitate HA-LMP-1. GFP containing retroviral input protein did not immunoprecipitate HA-LMP-1.

We further evaluated the IGFBP-6/LMP-1 interaction by incubating ROS 17/2.8 cell lysates containing overexpressed BP6Met^{1}-Gly^{240} or the CM containing BP6Ala^{25}-Gly^{240} with overexpressed HA-LMP-1, followed by incubation with anti-IGFBP-6 immobilized to AminoLink Plus Gel. The integrity of the input proteins was confirmed by Western blot analysis with anti-IGFBP-6 (hBP6 ab) and anti-HA (HA ab) (Fig. 5C). Each of the immunoprecipitated complexes was analyzed on western immunoblots developed with anti-HA antibodies (Fig. 5D). HA-LMP-1 was immunoprecipitated by BP6Met^{1}-Gly^{240} and BP6Ala^{25}-Gly^{240} but not with unconjugated AminoLink Plus Gel.

The co-immunoprecipitation data together with the yeast two-hybrid data provide compelling evidence that IGFBP-6 and LMP-1 physically interact.

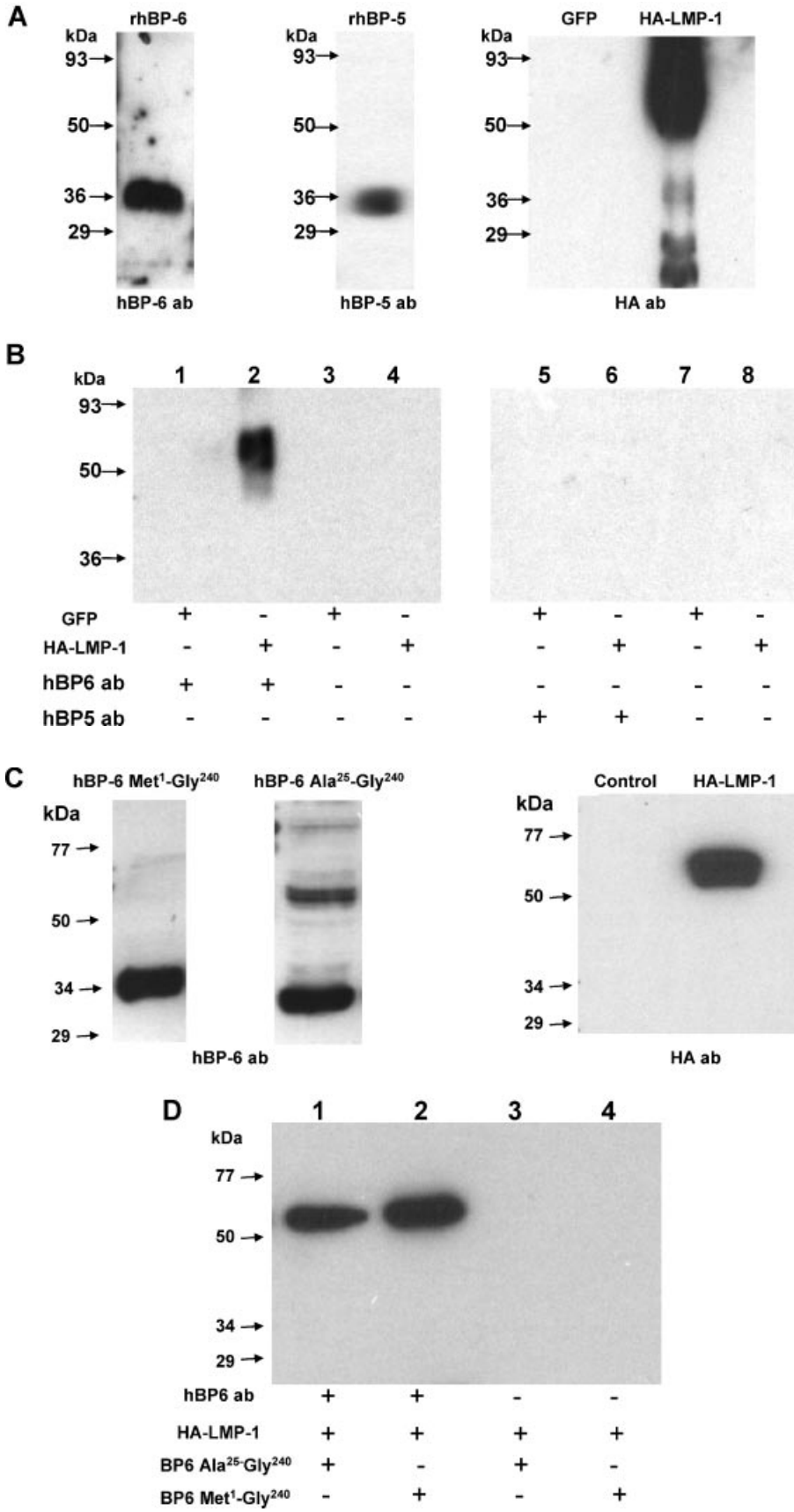
IGFBP-6 Alters the Subcellular Location of LMP-1

Intracellular localization of IGFBP-6 and LMP-1 was assessed in living and fixed cells using fluorescent microscopy. To determine the subcellular location of IGFBP-6 and LMP-1 in living osteoblasts, plasmid expression vectors were prepared that produced IGFBP-6 Met^{1}-Gly^{240}-RFP (BP6-RFP) and LMP-1 Met^{1}-Val^{457}-GFP (LMP-1-GFP) fluorescent fusion proteins. Low passage human osteoblasts and MC3T3-E1 cells were transiently transfected with either BP6-RFP, LMP-1-GFP or both and living cells were examined by fluorescent microscopy 24–48 h post-transfection. Cells were stained with DAPI to indicate nuclear boundaries. The DAPI stained nuclei outnumbered fluorescent cells because transfection efficiencies in MC3T3-E1 and normal human bone cells was 1–2%. LMP-1-GFP was observed dispersed throughout the cytoplasm in murine (Fig. 6A) and human osteoblasts (Fig. 6B). BP6-RFP was found in and around the periphery of the nucleus, in murine (Fig. 6A) and human osteoblasts (Fig. 6B) which is consistent with recent publications [Kuhl et al., 2003; Iosef et al., 2007]. Co-expression of BP6-RFP and LMP-1-GFP resulted in co-localization of LMP-1 and IGFBP-6 with the expression of LMP-1-GFP appearing to be confined to the areas of BP-6-RFP expression (Fig. 6A,B, lower panel).

These experiments demonstrate that IGFBP-6 and LMP-1 proteins are located in distinct subcellular locations in living cells and that when co-expressed in human or murine osteoblasts, LMP-1 and IGFBP-6 fusion proteins coincide.

LMP-1 Rescues IGFBP-6 Inhibition of Type I Procollagen Promoter Activity

To provide evidence of a functional IGFBP-6 and LMP-1 interaction in osteoblasts, we tested


A
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GFP + - + - + - + -
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hBP5 ab - - - - + + - -
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hBP-6 Met1-Gly240
hBP-6 Ala25-Gly240
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hBP-6 ab
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BP6 Met1-Gly240 - + - +

Fig. 5.

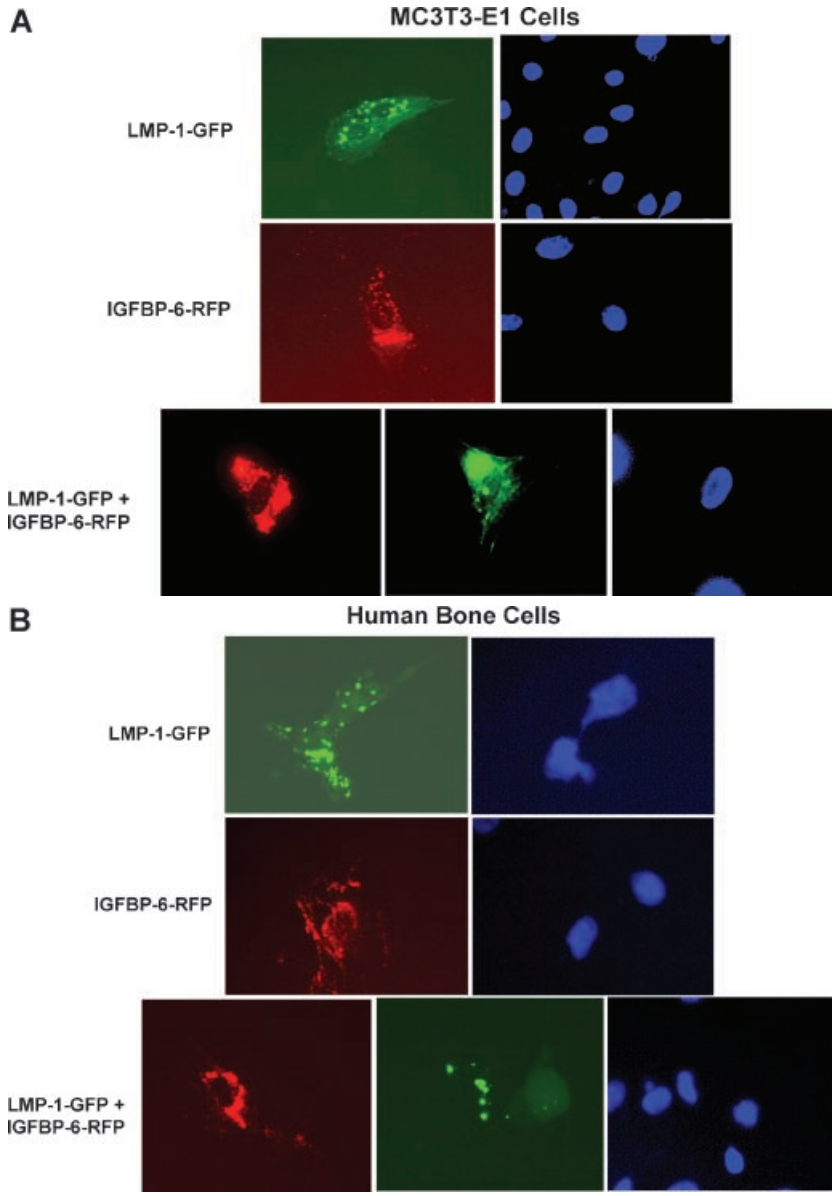

Fig. 6. IGFBP-6 and LMP-1 colocalize in human osteoblasts and MC3T3-E1 cells. Transient transfection with LMP-1-GFP plasmid DNA produced a fluorescent fusion protein that was observed in MC3T3-E1 cells (**A**) and low passage normal human osteoblasts (**B**) **upper panels**. Transient transfection with IGFBP-6-RFP plasmid DNA, stimulated expression of fluorescent fusion protein in living MC3T3-E1 (A) and human bone cells (B) **middle panels**. Co-transfection and expression of IGFBP-6-RFP and LMP-1-GFP in the same cell resulted in co-localization of the RFP and GFP fusion protein in MC3T3-E1 (A) and human osteoblasts (B) **lower panels**. The cells were fixed with methanol and stained with DAPI to identify the fluorescent fusion proteins in relation to nuclear boundaries.

Fig. 5. Co-immunoprecipitation studies of IGFBP-6/LMP-1. (**A**) Western immunoblots of input recombinant (IGFBP-5, IGFBP-6) proteins and HA-tagged -LMP-1 (HA-LMP-1) or GFP transgenes in whole cell extracts from retroviral transduced SaOS-2 cells developed with anti-IGFBP-6 (hBP6 ab), anti-IGFBP-5 (hBP6 ab), or anti-HA (HA ab) antibodies. (**B**) Analysis of HA-LMP-1 in immune complexes formed with input proteins (IGFBP-6, IGFBP-5, GFP, HA-LMP-1), and anti-IGFBP-6 (hBP6 ab) or anti-IGFBP-5 (hBP5 ab) conjugated to protein A-Sepharose or unconjugated protein A-Sepharose. HA-LMP-1 in the immune complexes that bound to protein A-Sepharose was detected by anti-HA antibody on Western immunoblots. **Lanes 1**, **3**, **5**, **7**: GFP transduced cell extract, input protein; **Lanes 2**, **4**, **6**, **8**: HA-LMP-1 transduced cell extract, input protein. Lanes 1–4: recombinant IGFBP-6 input protein. Lanes 5–8: recombinant IGFBP-5 input protein. An immune complex with HA-LMP-1 formed only with A-Sepharose conjugated to anti-IGFBP-6 antibody. (**C**) Western immunoblots of input proteins from ROS 17/2.8 cells overexpressing HA-LMP-1, BP6Ala^{25}-Gly^{240}, BP6Met^{1}-Gly^{240} or from VR1012-based plasmid vectors developed with anti-IGFBP-6 (BP6 ab) or anti-HA antibodies. Control is an aliquot of whole cell lysate from VR1012 transfected cells. (**D**) Immune complexes containing HA-LMP-1 were identified on Western immunoblots with anti-HA (HA ab) antibody. **Lanes 1**–**4**: HA-LMP-1 input protein; Lanes 1 and 3, BP6Ala^{25}-Gly^{240} input protein; Lanes 2 and 4, BP6Met^{1}-Gly^{240} input protein.

the effect IGFBP-6, LMP-1 and both IGFBP-6 and LMP-1 on type I procollagen promoter activity in ROS 17/2.8 cells. We tested the effect of IGFBP-6 on basal hCol1α2 promoter activity after transient co-transfection with the hCol1α2-pGL3basic plasmid vector and increasing doses of IGFBP-6-VR1012 plasmid. IGFBP-6 produced from the plasmid vector dose-dependently reduced basal procollagen promoter activity down to 35% of vector control (Fig. 7A).

We next determined if LMP-1-VR1012 plasmid vectors stimulated hCol1α2 transcription. When hCol1α2-pGL3Basic and LMP-1-VR1012 vectors were transiently co-transfected, LMP-1 expression from the plasmid vector dose-dependently stimulated promoter activity up to threefold (Fig. 7B). To determine if hCol1α2 promoter activity could be affected by interaction between IGFBP-6 and LMP-1, we co-expressed hCol1α2-pGL3basic, a dose of IGFBP-6 that inhibited promoter activity and a series of increasing doses of the LMP-1 plasmid vector to rescue IGFBP-6 suppression of basal promoter activity. Significantly, LMP-1 dose-dependently abrogated suppression of hCol1α2 promoter activity by hIGFBP-6 when they were co-expressed (Fig. 7C).

DISCUSSION

Our previous antisense IGFBP-6 studies in human SaOS-2 cells indicated an inverse correlation between IGFBP-6 levels and ALP activity, a marker of osteoblast differentiation [Yan et al., 2001]. This association was not observed with the other IGFBPs (IGFBP-3, IGFBP-4, or IGFBP-5) produced by human osteoblasts. Experiments in low passage human osteoblasts and MC3T3-E1 osteoblasts expressing IGFBP-6 after retroviral transduction presented in this study support our previous conclusion that IGFBP-6 inhibits osteoblast differentiation and further expands our conclusion from human osteosarcoma cells to include normal human osteoblasts and murine osteoblasts. We found that retroviral transduction to increase IGFBP-6 levels in human and murine osteoblasts significantly reduced osteocalcin mRNA levels.

In human osteoblasts, ALP activity and osteocalcin mRNA expression was significantly decreased by both a secreted and non-secreted, intracellular form of IGFBP-6. In MC3T3-E1 murine cells, we found that IGFBP-6 was down-

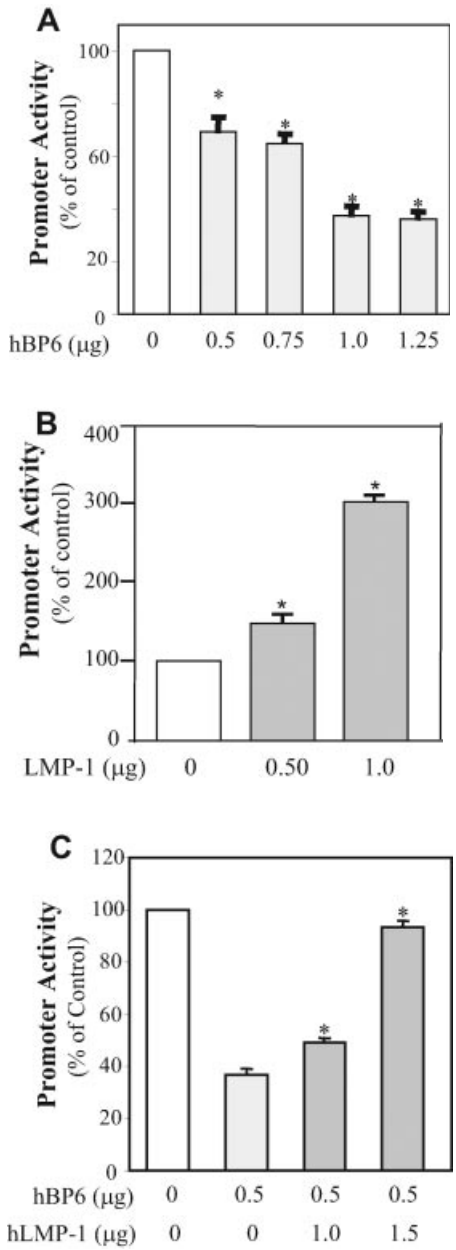

Fig. 7. LMP-1 rescues suppression of type I procollagen promoter activity by IGFBP-6. ROS17/2.8 murine osteoblast like cells were transiently transfected with 0.5 μg of the hCol1α2-pGL3Basic luciferase reporter vector and increasing amounts (0.5–1.5 μg) of hIGFBP-6 plasmid DNA (**A**), increasing amounts of LMP-1 (1–1.5 μg) plasmid DNA (**B**), or a fixed amount of IGFBP-6 plasmid DNA (1 μg) with increasing amounts of LMP-1 plasmid DNA (**C**). ROS17/2.8 cells were used due to their ability to be transfected at high efficiency levels. Total DNA concentrations were adjusted to 3 μg with empty VR1012 plasmid vector. Promoter activity was determined 48 h after DNA addition and is defined as light units/protein.

regulated during differentiation and mineralized nodule formation. Additionally, IGFBP-6 overexpression inhibited MC3T3-E1 differentiation and mineralized nodule formation. This was observed using both a secreted and non-secreted form of IGFBP-6 further supporting the conclusion that an intracellular mechanism mediates the inhibition of osteoblast differentiation by IGFBP-6.

It is noteworthy that IGFBP-6 suppressed differentiation in both human and murine osteoblast models because regulation and expression patterns of some IGF system components in human and murine cells is not identical [Bautista et al., 1990]. In rodents, IGF-II is expressed in embryos and neonates but not in adults. By contrast, in humans, IGF-II is expressed thoughout life, and more IGF-II than IGF-I is present in adult serum and bone tissues. Because IGFBP-6 is a preferential inhibitor of IGF-II actions and murine osteoblasts produce minimal amounts of IGF-II, this provided a clue that the mechanism by which IGFBP-6 suppressed osteoblast differentiation might not be dependent on extracellular IGF sequestration.

Because our findings in osteoblasts suggested that an intracellular process mediated the inhibitory effect of IGFBP-6, we screened a human osteosarcoma library with a IGFBP-6 bait expression vector and found that a LMP-1 fragment containing LIM-domain three and part of LIM-domain two, bound to IGFBP-6 with high affinity. Co-immunoprecipitation studies with recombinant IGFBPs and lysates expressing HA-tagged LMP-1, as well as, co-IP studies with IGFBP-6 Met^{1}-Gly^{240} or IGFBP-6 Ala^{25}-Gly^{240} and LMP-1 expressed after transfection with plasmid vectors again demonstrated a physical interaction between these two proteins.

LIM mineralization protein-1 (LMP-1) is one of a large diverse family of intracellular proteins with zinc-containing motifs called LIM-domains that shuttle between the cytoskeleton and nucleus with subcellular compartment movement dictated by interactions with unique binding partners [Kadrmas and Beckerle, 2004]. The LIM-domain proteins are involved in the regulation of development, cellular differentiation, cytoskeletal function, and carcinogenesis. LMP-1 was discovered in osteoblasts by Titus and Boden as a protein induced by glucocorticoids that was required for osteoblast differentiation and mineralization in vitro [Boden et al., 1998]. Because LMP-1 is a highly conserved, well-known stimulator of osteoblast differentiation and is expressed in both murine and human cells, we speculated that this novel protein–protein interaction might in part account for the species-independent, intracellular effect of IGFBP-6 to inhibit osteoblast differentiation. Our findings that LMP-1 rescues IGFBP-6 suppression of type I procollagen promoter activity further suggest that IGFBP-6 may act in part by inhibiting the effects of LMP-1 to stimulate osteoblast differentiation.

Fluorescent microscopy studies also suggest that LMP-1 and IGFBP-6 physically interact and that this interaction alters the subcellular location of LMP-1. A common feature of LIM domain protein interactions with intracellular proteins is altered subcellular compartmentialization of either or both proteins [Xu and Massague, 2004]. During limb bud development, Tbx4 and Tbx5 transcription factors bind to chicken LMP-4, a PDZ-LIM domain protein corresponding to LMP-1 or LMP-2 [Krause et al., 2004]. LMP-4, by association through its PDZ domain with the actin cytoskeleton was shown to inactivate transactivation mediated by Tbx genes [Krause et al., 2004]. The Enigma homolog (ENH, PDZLIM5), a PDZ-LIM domain protein associated with actin and closely related to Enigma/LMP-1, is proposed to be a cytoplasmic retention factor for transcription factor, Id2 [Lasorella et al., 2006]. ENH is up-regulated during neural differentiation and increasing ENH expression ectopicially in neuroblastoma cells results in translocation of Id2 from the nucleus to the cytoplasm to inactivate transactivation and cell cycle functions [Lasorella and Iavarone, 2006]. Our subcellular location studies suggest that IGFBP-6, by changing the location of LMP-1, could possibly alter the functionality of LMP-1.

Although our data clearly provides evidence for physical and functional interaction between IGFBP-6 and LMP-1 in osteoblasts, it is important to point out that our studies involved overexpression of IGFBP-6 and LMP-1. Future studies with dominant negative IGFBP-6 and with LMP-1 mutants are required to determine if such interactions occur physiologically and are key regulators of osteoblast differentiation.

In our studies on the IGFBP-6 mechanism of actions, we used different osteoblast cell models for three main regions: (1) different established cell lines offered some advantages that were

unique to each for mechanism studies, (2) to rule out that the data obtained using transformed cell lines is not a consequence of cell transformation by using both untransformed normal osteoblasts and transformed osteoblasts, and (3) to confirm the validity of the IGFBP-6 effect in both murine and human osteoblasts.

In summary, we have shown that IGFBP-6 is regulated during osteoblast differentiation, that it inhibits osteoblast differentiation in human and murine osteoblasts and that IGFBP-6 interacts physically and functionally with the osteoinductive protein, LMP-1. We have also presented evidence that the interaction of IGFBP-6 and LMP-1 may be functionally significant in the regulation of osteoblast differentiation. We propose that this intracellular action of IGFBP-6 may define a novel paradigm to regulate osteoblast differentiation.

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