Journal of Cellular Biochemistry 59:246–257 (1995)

Identification of Functional Insulin-Like Growth Factor-II/Mannose-6-Phosphate Receptors in Isolated Bone Cells

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Abstract The role of the IGF-II/cation independent mannose-6-phosphate (IGF-II/M6P) receptor in the transduction of cellular effects evoked by IGF-II has been extensively debated in the literature. Many reports suggest that IGF-II transduces its effects through the IGF-I receptor, while others show that IGF-II utilizes the type II receptor to affect cellular activity. This study 1) verifies the presence of the IGF-II/M6P receptor in rat calvarial osteoblasts, and 2) evaluates the ability of the receptor to initiate intracellular signals. Using conventional receptor binding assays, it was found that osteoblasts bind IGF-II with high affinity. Scatchard analyses indicated that there are 5.08×10^4 IGF-II/M6P receptors per osteoblast with a K_d near 2.0 nM). The receptor protein was further identified by cross-linking with ^{125}I-IGF-II. Northern analysis was used to identify an mRNA transcript for the IGF-II/M6P receptor protein. To examine if the IGF-II/M6P receptor can initiate second messenger signals, the ability of IGF-II to evoke Ca^{2+} transients was evaluated. Osteoblasts pretreated with IGF-I did not lose their ability to respond to IGF-II. Further, a polyclonal antibody against the rat IGF-II/M6P receptor (R-II-PAB1) 1) was able to evoke its own Ca^{2+} response, and 2) was able to block the generation of Ca^{2+} transients caused by IGF-II. The data in this report show that the osteoblastic Ca^{2+} response to IGF-II appears to be caused by an intracellular release of Ca^{2+} which is mediated by the IGF-II/M6P receptor making it possible to envision how the receptor may be an important modulator of osteoblast mediated osteogenesis.

Key words: osteoblasts, insulin-like growth factor-I, calcium signaling, fura 2, digital imaging, receptor crosslinking, Northern analysis, Scatchard analysis

A number of autocrine/paracrine effectors have been shown to mediate bone turnover and growth by influencing the differentiated state and cellular activity of osteoblasts. One such effector, insulin-like growth factor-II (IGF-II), has been found to stimulate DNA synthesis [Canalis and Raisz, 1979], collagen synthesis [McCarthy et al., 1989], alkaline phosphatase activity [Ishibe et al., 1991], and secretion of IGF-I [Tremollieres et al., 1991] in these cells. Furthermore, IGF-II has been identified in bone matrix [Frolik et al., 1988; Mohan and Baylink, 1994] and is synthesized by osteoblasts [McCarthy et al., 1992]. These observations collectively suggest that IGF-II receptor [Centrella et al., 1990; Mohan et al., 1989] may be intimately involved in the initiation of signals required for osteogenesis.

The IGF-II receptor, which shares identity with the cation-independent M6P receptor [Kiess et al., 1988; MacDonald et al., 1988; Nolan et al., 1990; Tong et al., 1988], is a 250–300 kDa integral membrane glycoprotein that specifically binds IGF-II, as well as proteins that have phosphomannosyl recognition markers [Kornfeld, 1992] (e.g., lysosomal enzymes). The mature protein is composed of 15 extracellular domains (of approximately 147 amino acids each) and a fibronectin type II domain. There is a single IGF-II binding site and two sites for bind-

Abbreviations: BCS, bovine calf serum; BSA, bovine serum albumin; dCTP, deoxycytidine 5′-triphosphate; DSS, dissuccinimidyl suberate; fura 2AM, fura 2 acetoxymethyl ester; IGF-I, insulin-like growth factor-I; IGF-II, insulin-like growth factor II; IGF-II/M6P receptor, insulin-like growth factor-II/cation-independent mannose-6-phosphate receptor; M6P, mannose-6-phosphate; R-II-PAB1, polyclonal anti-rat IGF-II/M6P receptor antibody; SDS, sodium dodecyl sulfate.

Received December 20, 1994; revised March 6, 1995; accepted March 10, 1995.

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ing of M6P [Westlund et al., 1991]. The receptor has a 23 residue transmembrane region and a 163 residue cytoplasmic region [Kornfeld, 1992] that includes several potential tyrosine, threonine, and serine residue phosphorylation sites [Giudice, 1992].

To understand how IGF-II evokes its effects in osteoblasts, it is important to identify the role of the IGF-II/M6P receptor in the transduction of second messenger signals. Paradoxically, since IGF-II binds to both the type I and type II IGF receptor with similar affinity, it has been difficult to determine which receptor, if not both, mediates the observed effects of IGF-II. As a result, the role of the IGF-II/M6P receptor in the initiation of intracellular signals has been extensively debated in the literature. A large body of data has shown that the IGF-I receptor, which is believed to utilize tyrosine kinase activity [Czech, 1989; Izumi et al., 1987; Kenner and Heidenreich, 1991; Oemar et al., 1991; Yamori et al., 1991], may account for all of the cellular effects evoked by IGF-II [reviewed in Cohick and Clemmons 1993; Gammeltoft et al., 1991]. Specifically, several reports suggest that the IGF-I receptor mediates IGF-II stimulated DNA synthesis in H-35 hepatoma cells [Mottola and Czech, 1984], human fibroblasts [Furlanetto et al., 1987] and human osteosarcoma cells [Raile et al., 1994], protein synthesis in rat pituitary tumor cells [Weber et al., 1992], and glucose uptake in L6 myoblasts [Ewton et al., 1987; Kiess et al., 1987]. In agreement with these observations is the purported role of the IGF-II/M6P receptor as an intracellular entity that binds and delivers newly synthesized phosphomannosylated enzymes from the *trans*-golgi to lysosomes [reviewed in Kornfeld, 1992]. In contrast, there have also been a large number of reports ascribing cellular responses to IGF-II acting through the type II receptor. These responses include amino acid uptake in human myoblasts [Shimizu et al., 1986], DNA synthesis in a human erythroleukemia cell line [Tally et al., 1987], and glycogen synthesis in human hepatoma cells [Hari et al., 1987]. It has been suggested that the IGF-II/M6P receptor may evoke these effects by controlling Ca^{2+} influx across the plasma membrane [Kojima et al., 1988; Nishimoto et al., 1987a,b], phosphoinositide-dependent protein kinase C activation, and intracellular Ca^{2+} release [Allen and Dawidowicz 1990; Rogers and Hammerman, 1989]. These signaling events may even be G protein mediated [Murayama et al., 1990; Nishimoto, 1993; Okamoto et al., 1990]. Since the debate about the potential signaling role of the IGF-II/M6P receptor has not been resolved, it is important to establish the action of the receptor in the osteoblastic control of bone formation.

In the present study, we have documented the presence of the IGF-II/M6P receptor in rat calvarial osteoblasts both by Northern analysis and by characterization of specific binding. We then examined the ability of that receptor to generate Ca^{2+} signals upon stimulation with IGF-II. Aside from verifying the presence of the receptor through conventional receptor binding analysis, we show for the first time that 1) osteoblasts express the IGF-II/M6P receptor and its mRNA message, and 2) the IGF-II/M6P receptor can mediate the generation of intracellular Ca^{2+} signals.

MATERIALS AND METHODS

Materials

$Na^{125}I$ was purchased from Amersham (Arlington Heights, IL). Recombinant human IGF-I and IGF-II were purchased from Bachem Inc. (Torrance, CA). All media components were purchased from Gibco (Grand Island, NY). BCS was purchased from Hyclone Lab (Logan, UT). Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO). DSS was purchased from Pierce Chemical Co. (Rockford, IL). Polyclonal anti-rat IGF-II/M6P receptor antibody, R-II-PAB1, was kindly provided by Dr. Ron Rosenfeld of Oregon Health Sciences University (Portland, OR). RNAzol was purchased from Tel-Test, Inc. (Friendswood, TX). The cDNA probe for the IGF-II/M6P receptor was made using the Megaprime DNA labeling system from Amersham. α-^{32}P-dCTP was also purchased from Amersham. All other chemicals were from Sigma.

Media

Isolation buffer was composed of 25 mM Hepes, 10 mM $NaHCO_3$, 100 mM NaCl, 3 mM K_2HPO_4, 12 mM mannitol, 24 mM KCl, 1 mM $CaCl_2$, 5 mg/ml glucose, 2 mg/ml bovine serum albumin, 100 U/ml penicillin, 100 μg/ml streptomycin, pH 7.4. Cell culture medium (DMEM) was a Dulbecco's modified Eagle base that was supplemented with 44 mM $NaHCO_3$, 50 μM L-ascorbic acid, 100 IU Pen-Strep (Gibco, Grand Island, NY), and 10% BCS, pH 7.6. Binding

buffer consisted of DMEM supplemented with 25 mM Hepes and 1 mg/ml BSA. Phosphate buffered saline (PBS) was composed of 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4. F12 isolation medium (F12I) was a Dulbecco's modified Eagle/F12 ham mixture (1:1) that was Ca^{2+}- and Mg^{2+}-free. It was supplemented with 14 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 100 IU Pen-Strep, pH 7.35. Fluorescence medium (F medium) was composed of 125 mM NaCl, 5 mM KCl, 1.7 mM $CaCl_2$, 0.7 mM NaH_2PO_4, 0.8 mM Na_2SO_4, 0.5 mM $MgCl_2$, 15 mM Hepes, 2 mM L-glutamine, 10 mM glucose, pH 7.4. Trypsin digest was a 308 mM Hepes base that was supplemented with 0.05% trypsin (w/v) and 0.002% disodium ethylenediamine tetraacetate (EDTA) (w/v). Transfer buffer sodium chloride sodium citrate (SSC) was composed of 150 mM NaCl and 15 mM sodium citrate, pH 7.0.

Cell Isolation

Calvariae were removed from 1–2-day-old rat pups. Segments of true intramembranous bone, free of adjacent suture lines and chondrocranium, were dissected from the parietal bones and were subjected to five sequential 20 min collagenase digestions in an isolation buffer (2.5 calvariae/ml) containing bacterial collagenase (0.5 mg/ml). The five cell fractions were designated in order of their release from the parietal bones: fraction 1, fibroblasts; fraction 2, fibroblasts plus osteoprogenitor cells; fraction 3, osteoprogenitor cells plus osteoblasts; fraction 4, osteoprogenitor cells plus osteoblasts; and fraction 5, surface lining osteoblasts. These cells have been previously characterized on the basis of alkaline phosphatase content, osteocalcin production, ability to synthesize type I collagen, proliferation rate, response to target hormones, and growth factors in bone and their localization in the tissue [Puzas and Ishibe 1992].

Cell Culture

Cells were cultured in a monolayer in multiwell culture plates (Corning, NY) at a density of 200 cells/mm^2 in DMEM. For binding studies, cells were grown to subconfluence in DMEM and then maintained in binding buffer until assay. For single cell fluorescence work, freshly isolated osteoblasts were cultured in DMEM in a monolayer on 25 mm diameter, round, No. 1 glass cover slips as described elsewhere [Zuscik et al., 1994].

Receptor Binding Assays

IGF-II was iodinated using a previously published chloramine-T method resulting in a specific activity of 13 μCi/μg IGF-II [Hunter and Greenwood, 1962]. Monolayers were washed three times with binding buffer and then bathed in 250 μl of fresh binding buffer containing ^{125}I-IGF-II. Incubations were gently agitated on a rocker platform for various lengths of time at 4°C in a room atmosphere. For certain experiments, the incubations were performed at 37°C in a 5% CO_2 atmosphere. After incubation, the cells were rinsed three times with 1 ml of ice-cold binding buffer, detached with 0.8 ml of 0.05% trypsin (15 min at room temperature), transferred to scintillation vials, and counted using a gamma counter. The binding was similar to that reported in other studies [Nishimoto et al., 1987a].

Cross-Linking of ^{125}I-IGF-II to the Type II Receptor

Isolated bone cells were incubated with 0.6 nM ^{125}I-IGF-II in the presence or absence of excess unlabeled IGF-II for 20 h at 4°C. After washing, the cells were incubated in binding buffer containing 0.5 mM DSS for 15 min at 4°C. The cells were then washed in cold PBS, scraped from the dishes, transferred to 1.5-ml microfuge tubes and centrifuged at 10,000*g* for 10 min. The pellets were solubilized in concentrated electrophoresis sample buffer and run on 5% polyacrylamide gels as previously described [Laemmli, 1970]. The electrophoresis gel was then stained for protein, dried, and autoradiographed on Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) with enhancing screens.

RNA Isolation and Northern Blot Analysis

Cultured osteoblasts were detached from culture vessels after 7 days with a trypsin/EDTA digest, centrifuged at 300*g* for 3 min, resuspended in PBS (for counting), and centrifuged to pellet again. Total RNA was extracted according to the method of Chomczynski and Sacci [1987]. Briefly, the cells were lysed with RNAzol and RNA was extracted twice with phenol/chloroform:isoamyl alcohol. The RNA was precipitated with isopropanol at −20°C, centrifuged at 14,000 rpm for 10 min in a microcentrifuge (4°C), and dried under vacuum. Samples of RNA (10 μg) were heated for 10 min at 65°C in a solution containing 0.2 M MOPS

(morpholinopropanesulfonic acid), 50 mM sodium acetate, 5 mM edetate (EDTA), and 12.3 M deionized formaldehyde. Electrophoresis was performed on a 0.7% agarose gel containing 1.5 M formaldehyde. The RNA was blotted onto a GeneScreen Plus membrane (Biotechnology Systems, Boston, MA) in 10× SSC overnight by capillary transfer. The membrane was then dried for 2 h at 80°C to immobilize the RNA. Prehybridization and all hybridizations were performed in 10% deionized formamide containing 100 μg/ml salmon sperm DNA. The hybridization probe was made by adding α-^{32}P-dCTP and the partial cDNA fragment (2.4 kb) of the rat IGF-II/M6P receptor (from the plasmid pBSK3) to the Megaprime DNA labeling kit (Amersham, Arlington Heights, IL). Synthesis of the probe was carried out over night at room temperature. Newly synthesized probe was added to the hybridization solution at 1×10^7 cpm and allowed to hybridize overnight at 60°C. The blot was washed twice with 2× SSC + 1% SDS for 10 min at 60°C, once with 1× SSC + 1% SDS for 20 min at 60°C, and then autoradiographed at −70°C using Kodak X-Omat AR film.

Detection of Ca^{2+} Responses in Single Cells

Osteoblasts that were plated onto glass coverslips were exposed to 2.5 μM fura 2 AM for 50–60 min in the cell incubator. After three washes with F12I, the slips were covered with fresh F12I until assay. A coverslip was secured to the stage of a Nikon Diaphot fluorescence microscope; covered with F medium; and a single cell was chosen, masked, and monitored using the ratio technique [Grynkiewicz et al., 1985; Gunter et al., 1990] during treatment with effectors. Since out-of-plane fluorescence due to dye that had leaked from the cells was shown to be negligible (< 1% of cellular fluorescence) no corrections were made for it when measuring single cell fluorescence ratios.

Digital Imaging Microscopy

To observe the Ca^{2+} responses of groups of osteoblasts that were plated on glass coverslips, fluorescence from 5–10 fura-2-loaded cells was simultaneously visualized by an intensified CCD camera (Photonic Microscopy, Inc., Oak Brook, IL). Cells were alternately excited with 340 nm and 380 nm light and 510 nm emission images were collected by the camera, displayed on a computer screen and saved on a pixel by pixel basis every 5 s. After correcting for any camera detection nonuniformity and background fluorescence, the ratio technique [Grynkiewicz et al., 1985; Gunter et al., 1990] was employed to quantitate intracellular Ca^{2+}. Ratios were calculated using pixel intensities of the 340 and 380 excitation images using software from Photon Technology International (New Brunswick, NJ).

Statistical Analysis

Data are expressed as the mean ± SE. An unpaired two-tailed Student's *t*-test was used to detect differences between experimental and control treatments. Statistical significance was set at the $P < 0.05$ probability level. In some cases the data were analyzed using an analysis of variance (ANOVA). Post hoc analysis was used to examine differences between groups when the ANOVA was significant. $P < 0.05$ was considered statistically significant.

RESULTS

Receptor Binding Characteristics

The time course of specific binding of ^{125}I-IGF-II (0.1 nM) to bone cells (pooled cell fractions 3–5) at 4°C is shown in Figure 1. Specific binding of ^{125}I-IGF-II achieved an equilibrium between 10 and 20 h of incubation and maintained a plateau thereafter. Specific binding of ^{125}I-IGF-II averaged 11.5 ± 0.8%/well (mean ± SE, n = 3) of the total radioactivity under these conditions. A demonstration of the dissociation constant (K_d) and number of binding sites for the different cellular phenotypes isolated from

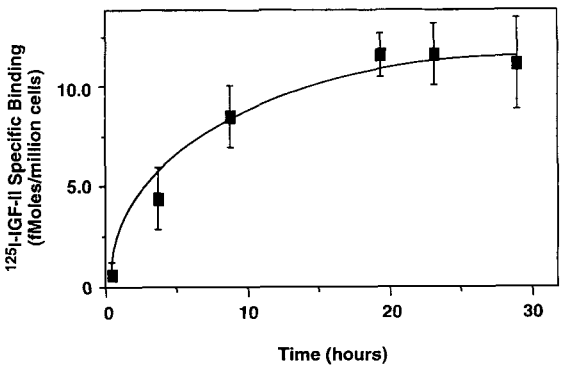

Fig. 1. Time course of ^{125}I-IGF-II binding to osteoblasts. Cell fractions 3–5 were pooled and incubated with ^{125}I-IGF-II (0.1 nM) at 4°C. At various time intervals, the amount of total cell-associated radioactivity was measured. Specific IGF-II binding to the cells was determined by subtracting nonspecific IGF-II binding from the total bound (in the presence of a 500-fold excess of unlabeled IGF-II). Each point represents the mean ± SEM of two independent experiments (n = 3).

TABLE I. K_d and Receptor Numbers for Each of the Isolated Cell Fractions*

Fraction No.	Cell type	K_d (nM)	Receptor number (sites/cell × 10^4)
1	Fibroblast	2.6 ± 0.6	3.04 ± 0.22
2	Fibroblast + osteoprogenitor	2.2 ± 0.8	4.20 ± 0.38
3	Osteoprogenitor + osteoblast	2.3 ± 1.0	5.39 ± 0.25
4	Osteoprogenitor + osteoblast	2.4 ± 1.2	7.34 ± 0.25
5	Osteoblast	2.0 ± 0.7	5.08 ± 0.69

*The K_d and receptor number per cell was determined by Scatchard analysis performed on binding data collected from distinct populations of calvarial cells at 4°C. Values are mean ± SE of two to three independent experiments (n = 3). There were no significant differences ($P > 0.05$) in the K_d (nM) of the different cell fractions.

the calvaria are found in Table I. There were no statistically significant differences in the K_d between any of the cell fractions; however, cells with a bony phenotype (i.e., osteoprogenitor cells and osteoblasts, fractions 3–5) contain the largest number of binding sites/cell.

Specificity of IGF-II Binding

As shown in Figure 2, approximately 70% of the bound ^{125}I-IGF-II was displaced by the highest dose of unlabeled IGF-II tested (a 500-fold molar excess). In contrast, 1, 10, and 100 nM of IGF-I and insulin had no effect on binding of ^{125}I-IGF-II to the cells. As has been previously shown in other cell models, d-mannose-6-phosphate (M6P) enhanced IGF-II binding to the bone cell receptors in a dose dependent manner at a concentration of 10 mM. Binding was 159 ± 2% of control levels (data not shown).

To further confirm that IGF-II binds specifically to the type II receptor in bone cells, we examined whether a polyclonal antibody to the rat IGF-II/M6P receptor, R-II-PAB1, could inhibit IGF-II binding. Figure 2 also demonstrates binding of ^{125}I-IGF-II in the presence and absence of R-II-PAB1. The antibody inhibited binding of ^{125}I-IGF-II in a dose-dependent manner. At 55 μg/ml the antibody inhibited specific ^{125}I-IGF-II binding by approximately 60%. Preimmune serum used as a control had no effect on the binding of ^{125}I-IGF-II.

Identification of IGF-II/M6P Receptor Protein and mRNA

The high affinity binding site on osteoblasts was visualized by cross-linking studies. Figure 3A demonstrates the covalent cross-linking of ^{125}I-IGF-II to a protein complex which migrates, under reducing conditions, at an apparent Mr of 250,000 (lane 1). The binding of ^{125}I-IGF-II to osteoblast membranes was effectively eliminated in the presence of an approximately 150-fold excess of unlabeled IGF-II (lane 2).

Northern blot analysis of the rat IGF-II/M6P receptor message using a 2.4 kb cDNA probe indicated that rat calvarial osteoblasts contain a transcript of 9.8 kb for the IGF-II/M6P receptor (Fig. 3B).

Calcium Responses Evoked by IGF-II

The ability of IGF-II to evoke Ca^{2+} signals was studied in monolayers of osteoblasts that were loaded with fura 2. Using the ratio technique, intracellular [Ca^{2+}] was determined in individual cells. No corrections were made for leaked dye because <1% of the total cellular fluorescence was found to be due extracellular fura 2FA (data not shown).

Initially, Ca^{2+} transients were observed in osteoblasts immediately upon exposure to 5 nM IGF-II (data not shown). Since a Ca^{2+} signal is known to occur through the type I receptor in a number of cell types, it was important to show that the osteoblastic responses to IGF-II were not generated through an activation of the IGF-I receptor. This was accomplished by either 1) sequentially pretreating cells with IGF-I until they lost their ability to respond, then treating the cells with IGF-II; or 2) rendering the cells unresponsive to IGF-I, blocking IGF-II binding with R-II-PAB1, then treating the cells with a final dose of IGF-II.

Initially, it was found that treatment of an individual osteoblast with two additions of IGF-I (final concentration of 20 nM IGF-I in the medium) resulted in a loss of IGF-I responsiveness, with no loss occurring in the ability of the cells to respond to a subsequent treatment with 5 nM IGF-II (Fig. 4). A summary of cellular Ca^{2+} responses seen after multiple (two or three) exposures of IGF-I followed by a final exposure of IGF-II can be found in Table II. As shown in the table, after two repetitive 10 nM treatments with IGF-I, only 3% of the cells responded to a final 10 nM dose. However, upon a subsequent

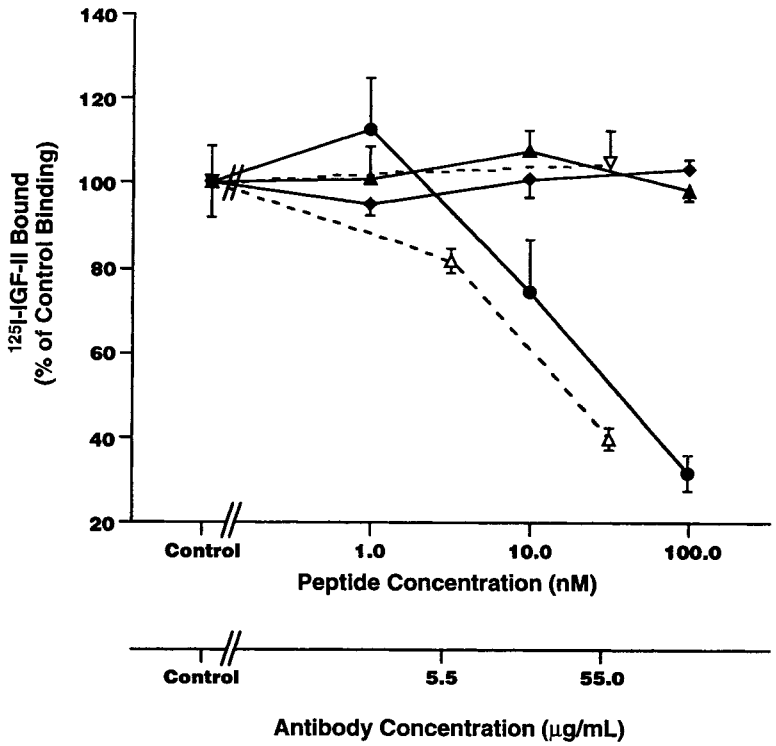

Fig. 2. Competitive inhibition of the binding of ^{125}I-IGF-II to osteoblasts. Of the following peptides, IGF-II (—●—), IGF-I (—▲—) and insulin (—◆—), only IGF-II was able to successfully compete with ^{125}I-IGF-II for binding to osteoblasts. Control specific binding was 356 ± 5 (mean ± SEM) dpm/well (n = 3). Further, binding of ^{125}I-IGF-II was determined in the presence of various concentrations of either the anti-IGF-II receptor antibody R-II-PAB1 (– –△– –) or preimmune serum (– –▽– –). Values are mean ± SEM (n = 3).

addition of 5 nM IGF-II, 40% of the cells responded.

In similar osteoblasts that were rendered unresponsive to IGF-I, R-II-PAB1 not only caused a Ca^{2+} response of its own, but a 220 μg/ml dose blocked the ability of 5 nM IGF-II to evoke a subsequent response (Fig. 5C and D). Comparatively, control cells that were treated with PBS (R-II-PAB1 vehicle) did not lose their ability to respond to 5 nM IGF-II (Fig. 5A and B). A summary of cellular Ca^{2+} responses observed after sequential treatment with R-II-PAB1 and IGF-II can be found in Table III. The table shows that ~ 70% of tested osteoblasts exhibited a Ca^{2+} response to 110 or 220 μg/ml antibody. Furthermore, while 60% of the cells responded to 5 nM IGF-II following pretreatment with 110 μg/ml R-II-PAB1, pretreatment with 220 μg/ml reduced the IGF-II response to 10%.

Other experiments were performed to determine if the IGF-II-evoked Ca^{2+} responses seen in osteoblasts were due to influx of Ca^{2+} across the plasma membrane (possibly through Ca^{2+} channels) or release of Ca^{2+} from intracellular stores. This was accomplished by assessing the ability of IGF-II to evoke a response in IGF-I saturated, fura 2-loaded osteoblasts that were in Ca^{2+}-free F medium ($[Ca^{2+}] < 1$ nM, buffered with ethyleglycol-bis-(β-amino ethyl ether) N,N,N′,N′,-tetracetic acid). IGF-II was found to be capable of stimulating Ca^{2+} transients in IGF-I-saturated osteoblasts that were either bathed in F medium that contained normal Ca^{2+} (Fig. 6A) or deprived of an extracellular Ca^{2+} source (Fig. 6B). These results suggest the utilization of intracellular stores of Ca^{2+} by IGF-II to evoke a Ca^{2+} response.

DISCUSSION

Identification of IGF-II/M6P Receptor in Osteoblasts

This study provides evidence for the IGF-II/M6P receptor in rat calvarial osteoblasts by Northern blot analysis and by conventional receptor binding assays. The IGF-II/M6P receptor message was identified using a 2.4 kb rat IGF-II receptor cDNA (Fig. 3B). The IGF-II/M6P receptor protein observed by affinity cross-linking (Fig. 3A) corresponds in molecular weight

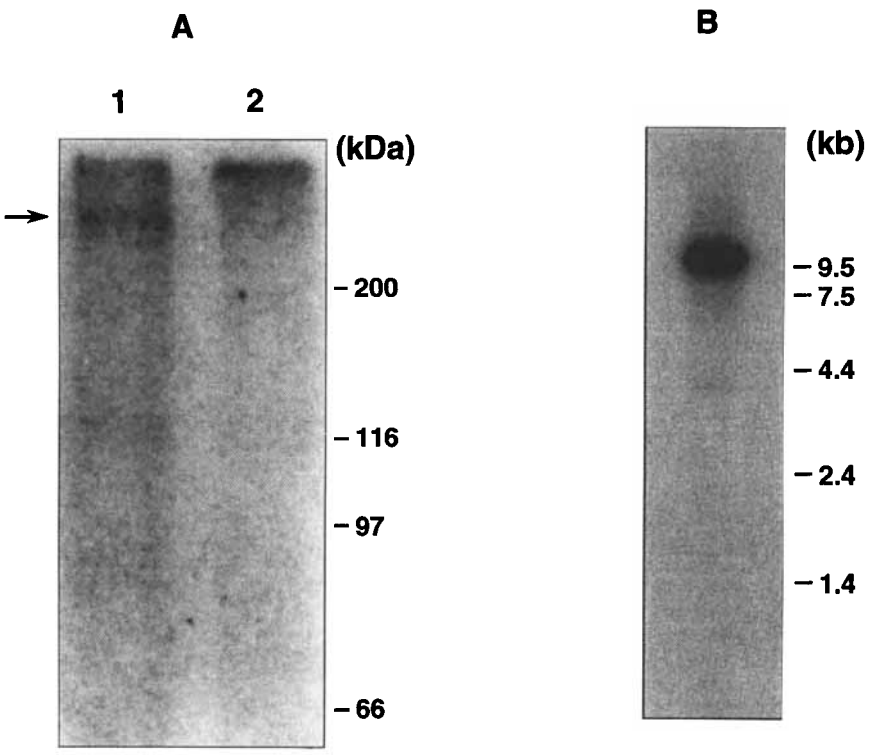

Fig. 3. Affinity cross-linking and Northern analysis of the IGF-II/M6P receptor. **A:** Cross-linking of ^{125}I-IGF-II to the osteoblastic IGF-II/M6P receptor in the absence (*lane 1*) or presence (*lane 2*) of 100 nM unlabeled IGF-II. The arrow indicates the cross-linked species. Osteoblastic RNA was extracted and message for the IGF-II/M6P receptor was probed using Northern analysis. **B:** The result of an autoradiograph made from a 5 day exposure of the blot at −70°C.

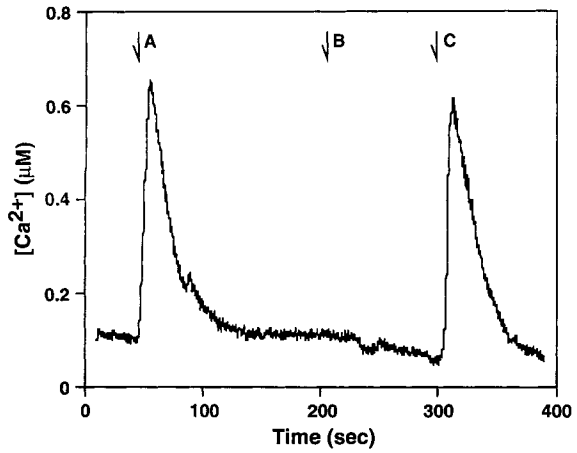

Fig. 4. Ca^{2+} transients observed in a single osteoblast treated with sequential additions of IGF-I and IGF-II. Osteoblasts were plated onto glass coverslips, cultured overnight in an incubator and then loaded with fura 2. The above representative trace shows the Ca^{2+} responses evoked by 10 nM IGF-I (added at arrows A and B, for a final bath concentration of 20 nM IGF-I) and 5 nM IGF-II (added at arrow C) in a single osteoblast.

TABLE II. Summary of Ca^{2+} Responses by Individual Osteoblasts Exposed to Sequential Treatments With IGF-I and IGF-II*

Percentage cells that responded to 3 sequential treatments with 10 nM IGF-I			Percentage of IGF-I pretreated cells that responded to a final treatment with 5 nM IGF-II: final addition
1st addition	2nd addition	3rd addition	
73%	14%	2.5%	40%

*Values represent the percentage of cells (out of 290 cells tested) that responded with Ca^{2+} transients to sequential additions of IGF-I (at time = 0, 150, and 200 s) and IGF-II (at time = 250 s).

to the IGF-II/M6P receptor studied in a number of tissues [Kornfeld, 1992; Nielsen, 1992]. Through conventional Scatchard analyses, we verified that 1) mature bone cells possess a larger number of IGF-II/M6P receptors than fibroblastic cells, and 2) the K_d of those receptors for IGF-II is near 2.0 nM (Table I). The osteoblastic IGF-II/M6P receptor has a much higher affinity for IGF-II than IGF-I or insulin (Fig. 2) and the kinetics of IGF-II binding to the IGF-II/M6P receptor is qualitatively similar to that in a number of cell types [Beguinot et al., 1985; Centrella et al., 1990; Mohan et al., 1989; Oppenheimer and Czech 1983; Rechler et al., 1980; Weber et al., 1994]. M6P has been shown to potentiate the binding of IGF-II to the receptor

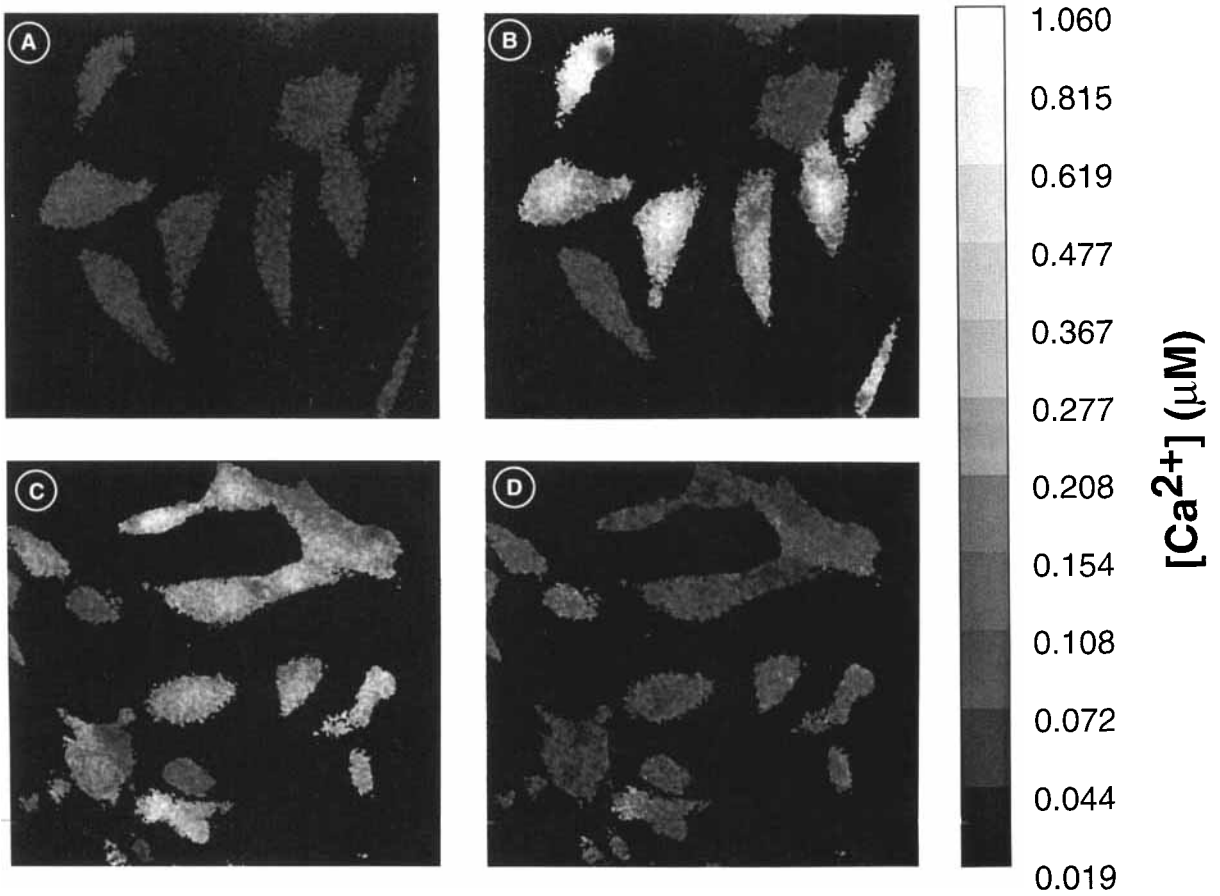

Fig. 5. Affect of anti-IGF-II/M6P receptor antibody on Ca^{2+} transients evoked by IGF-II in IGF-I pretreated osteoblasts. Osteoblasts were plated onto glass coverslips, cultured overnight in an incubator, and then loaded with fura 2. The panels show fluorescence digital images of representative fields of cells responding to two different experimental protocols. The control experiment tested for Ca^{2+} responses by IGF-I-saturated cells exposed to sequential additions of PBS (**A**, 114 s) and 5 nM IGF-II (**B**, 161 s). The second experiment tested for Ca^{2+} responses by IGF-I-saturated cells exposed to a sequential treatment with 220 μg/ml R-II-PAB1 (**C**, 96 s) and 5 nM IGF-II (**D**, 157 s).

in human fetal brain [Roth et al., 1987] and rat liver [Polychronakos et al., 1988]. Similar to these findings, IGF-II binding to osteoblasts is enhanced by pretreatment with mannose-6-phosphate (data not shown). Finally, in agreement with earlier reports [Centrella et al., 1990; Mohan et al., 1989], an anti-IGF-II/M6P receptor antibody (R-II-PAB1) inhibited IGF-II specific binding in a dose-dependent manner (Fig. 2). Collectively, these data show that 1) rat calvarial osteoblasts possess IGF-II/M6P receptor message, and 2) the message codes for a protein that behaves similarly to the IGF-II/M6P receptor that has been characterized in a number of tissues.

Ca^{2+} Signaling Through the Osteoblastic IGF-II/M6P Receptor

The ability of the IGF-II/M6P receptor to transduce signals initiated by interactions with IGF-II or M6P-containing proteins has been the focus of much attention in the literature. The well-documented effects of IGF-II on bone and other tissues [Kiess et al., 1994; Lowe 1991; Mohan and Baylink, 1994] must be mediated by an interaction with a plasma membrane receptor that leads to the generation of intracellular signals. Despite this requirement, many reports have suggested that the IGF-II/M6P receptor does not mediate the numerous effects of IGF-II on its target tissues [reviewed in Czech, 1989; Gammeltoft et al., 1991]. This idea stems from the observation that IGF-II/M6P receptor is known to mediate other cell functions, such as the trafficking of phosphomannosylated proteins to lysosomes [Czech, 1989; Dintzis et al., 1994; Kornfeld, 1992]. As such, it does not structurally resemble a plasma membrane receptor that can generate second messenger signals. Specifically, it does not have the classic 7 transmem-

TABLE III. Summary of Ca^{2+} Responses by Individual, IGF-I-Treated Osteoblasts Exposed to Sequential Treatments With IGF-II Receptor Antibody (R-II-PAB1) and IGF-II

Percentage of IGF-I-treated cells that responded to sequential additions of 110 μg/ml R-II PAB1 and 5 nM IGF-II (45 cells tested)		Percentage of IGF-I-treated cells that responded to sequential additions of 220 μg/ml R-II-PAB1 and 5 nM IGF-II (51 cells tested)	
R-II-PAB1 at ~100 s	IGF-II at ~165 s	R-II-PAB1 at ~75 s	IGF-II at ~145 s
71%	60%	69%	10%

*Values represent the percentage of cells that responded with Ca^{2+} transients to sequential additions of 110 μg/ml or 220 μg/ml doses of R-II-PAB1 and 5 nM IGF-II. Antibody and growth factor additions were made at the times indicated. Prior to the experiments, all cells tested were made unresponsive to IGF-I by pretreatment with 10–30 nM IGF-I.

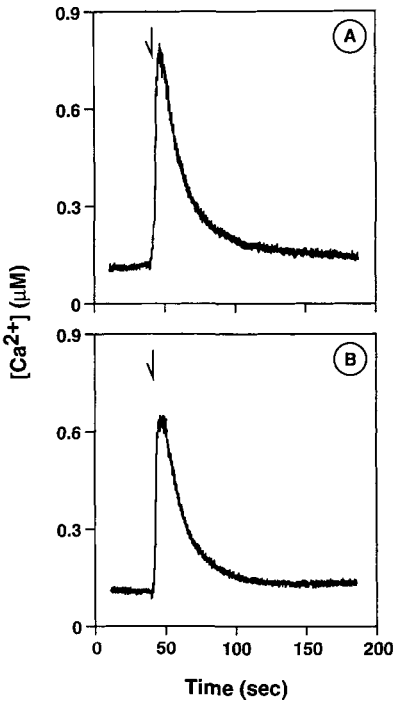

Fig. 6. Ca^{2+} transients observed in single osteoblasts bathing in either Ca^{2+}-containing or Ca^{2+}-free medium. Osteoblasts were plated onto glass coverslips, cultured overnight in an incubator, and then loaded with fura 2. **A:** The typical response of a single, IGF-I saturated cell (30 nM IGF-I final, after three additions), bathing in F medium, to 5 nM IGF-II. **B:** The typical response of a similar IGF-I-saturated cell, bathing in Ca^{2+}-free F medium, to 5 nM IGF-II. Additions were made at the times indicated by the arrows.

brane spanning domains of G protein-linked receptors [Kornfeld, 1992] and its cytoplasmic tail does not have homology to any known sequence that exhibits a kinase activity [Giudice, 1992]. Based on this reasoning, signaling through the IGF-I receptor, which is known to bind IGF-II with high affinity, could explain how IGF-II causes many of its effects. In this model, binding of IGF-II to the IGF-I receptor would lead to a tyrosine kinase activity that could transduce signals. Many investigators believe that all IGF-II action is mediated through the IGF-I receptor [Damke et al., 1994; Ewton et al., 1987; Furlanetto et al., 1987; Kiess et al., 1987; Mottola and Czech, 1984; Raile et al., 1994; Weber et al., 1992].

The present study shows that IGF-II can evoke Ca^{2+} signals in rat calvarial osteoblasts. Since these signals could be caused by the aforementioned interaction of IGF-II with the IGF-I receptor, the ability of IGF-II to stimulate Ca^{2+} transients was assessed in cells that had lost their ability to respond to IGF-I. Most importantly, it was found that IGF-I-saturated osteoblasts exhibited the ability to respond to IGF-II with a Ca^{2+} transient (Fig. 4, Table II). Interestingly, these Ca^{2+} responses were observed in cells that were bathing in either a Ca^{2+}-containing or a Ca^{2+}-free medium, suggesting at least a partial dependence of the event on the release of Ca^{2+} from an intracellular store. Since IGF-II-evoked transients seen in Ca^{2+}-deprived cells were always smaller than similar transients seen in cells with an extracellular Ca^{2+} source (Fig. 6), influx of Ca^{2+}, possibly through Ca^{2+} channels [Nishimoto et al., 1987a,b], may accompany intracellular Ca^{2+} release. Since part of the IGF-II-evoked Ca^{2+} response appears to have an intracellular origin, it is possible to envision the involvement of Ca^{2+} stores that are either inositol-1,4,5-trisphosphate-sensitive [Putney and Bird, 1993] or cyclic-ADP-ribose-sensitive [Galione et al., 1993]. However, irrespective of the source(s) of IGF-II-dependent Ca^{2+} signals and in contrast to the model described above, these findings provide the first evidence suggesting a signaling role for the IGF-II/M6P receptor in rat calvarial osteoblasts.

As stated earlier, R-II-PAB1 was found to effectively compete with IGF-II for binding to the IGF-II/M6P receptor (Fig. 2). Using this competitive binding action of R-II-PAB1 as a tool, IGF-II-mediated signaling through its own receptor was examined further. IGF-I-pretreated

osteoblasts that were exposed to R-II-PAB1 not only lost their ability to respond to a subsequent challenge with IGF-II, but also exhibited the ability to respond to the antibody directly (Fig. 5, Table III). These R-II-PAB1-evoked Ca^{2+} responses are not surprising since the antibody interacts with and possibly activates the IGF-II/M6P receptor. In fact, biological responses to R-II-PAB1 in bone cells have been previously identified [Ishibe et al., 1991; Mohan et al., 1989]. These findings substantially strengthen the emerging hypothesis suggesting an important signaling role for the IGF-II/M6P receptor.

Our observation of Ca^{2+} signals mediated by the IGF-II/M6P receptor agrees with an emerging role for the receptor in the transduction of IGF-II effects [Hari et al., 1987; Shimizu et al., 1986; Tally et al., 1987]. Several reports identify receptor-dependent stimulation of inositol metabolism in kidney cells [Rogers and Hammerman, 1989] and generation of Ca^{2+} signals that are caused by an influx of Ca^{2+} in Balb/c 3T3 fibroblasts [Kojima et al., 1988; Nishimoto et al., 1987a,b]. The activation of these signaling events may be due to a coupling of the IGF-II/M6P receptor to a pertussis toxin-sensitive G protein [Murayama et al., 1990; Nishimoto, 1993; Okamoto et al., 1990]. An interaction of the IGF-II/M6P receptor with a G protein is a novel finding that is strongly defended by Nishimoto in a 1993 review [Nishimoto, 1993]. Nishimoto's hypothesis raises the possibility that G proteins may interact with growth factor or hormone receptors that do not exhibit the 7 transmembrane spanning domain motif. In summary, our observation of IGF-II/M6P receptor-stimulated Ca^{2+} transients in rat calvarial osteoblasts agrees well with a growing body of data that identifies how the receptor can mediate IGF-II effects in a number of tissues.

CONCLUSIONS

The expression of the IGF-II/M6P receptor varies considerably and is dependent upon the species, tissue, and level of development of the organism [Kiess et al., 1994; Nielsen, 1992; Nissley et al., 1993]. IGF-II/M6P receptor mRNA concentrations have been studied using Northern analysis, RNase protection assay [Nissley et al., 1993], and in situ hybridization [Senior et al., 1990]. These studies showed that receptor expression was high in fetal tissues (heart > muscle > liver > brain) and declined after birth. Those authors suggest that IGF-II may play a critical role in many of the earliest developmental events that accompany rapid growth. This hypothesis can be extended to include bone development. Since neonatal rat calvarial osteoblasts have abundant IGF-II/M6P receptors, a critical role for IGF-II in early bone formation can be envisioned. Through its stimulation of osteoblastic matrix accretion and proliferative activity [Canalis and Raisz 1979; McCarthy et al., 1989, 1992], IGF-II present in bone matrix may directly influence the ultimate formation and growth of the skeleton. Therefore, to delineate a role for IGF-II in bone, further study is required 1) to identify whether the IGF-II/M6P receptor is expressed in growing or aging bone and, if so, 2) to determine if the receptor has a functional role in bone formation throughout life.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Ron G. Rosenfeld at Oregon Health Sciences University for the anti-rat IGF-II/M6P receptor antibody, to Dr. David Morgan at the University of California at San Francisco for the partial cDNA of the rat IGF-II receptor, to Dr. Yuko Ishibe at the University of Rochester for providing preimmune control serum, and to Mary Ellen Felter for her excellent technical assistance. We also are grateful to Dr. Thomas E. Gunter at the University of Rochester for critiquing this manuscript. This work was supported in part by NIH AR 28420, NIH AR 38618, and Endocrine Training Grant 5T32-DK07092.

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