Parathyroid hormone induces rat interstitial collagenase mRNA through Ets-1 facilitated by cyclic AMP response element-binding protein and Ca^{2+} /calmodulin-dependent protein kinase II in osteoblastic cells

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ABSTRACT

Parathyroid hormone (PTH), a powerful bone-resorbing agent, is capable of stimulating interstitial collagenase (MMP-13) mRNA production in osteoblastic cells. In this study, a PEA3 consensus binding sequence (–80; AGGAAGT) in addition to a 'TRE-like' sequence (–89; CGACTCA) in the 5' upstream regulatory region of the rat MMP-13 gene were examined. In response to PTH, there was a time-dependent increase in binding of nuclear factors to an oligonucleotide containing the PEA3 region (–95 to –71). This increase in binding was first observed at 0.5 h, peaked at 4 h (7.6-fold) then returned to basal levels by 24 h. Mutagenesis of the PEA3 site in a chloramphenicol acetyl transferase (CAT) construct containing 5' upstream regulatory sequence of the rat MMP-13 gene significantly decreased activation by PTH. PTH-mediated binding of nuclear factors to an oligonucleotide containing the mutant PEA3 sequence was decreased as compared with the wild type. Mutation

or deletion of the TRE-like sequence affected basal as well as PTH-mediated induction of corresponding CAT constructs. Treatment with KN93, a Ca^{2+} /calmodulin-dependent protein kinase II specific inhibitor, greatly reduced the amount of protein binding to the PEA3 region in response to PTH which correlated to a notable decrease in the amount of MMP-13 mRNA produced in response to PTH. Antibodies against Ets-1, cyclic AMP response element (CREB)-binding protein (CBP) and CREB were capable of supershifting proteins binding to the oligonucleotide containing the PEA3 region. These data suggest a possible co-operative interaction of factors binding to the PEA3 and TRE-like sequences and provide the first indication of a role for a calcium-mediated pathway in the PTH induction of MMP-13 mRNA in osteoblastic cells.

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INTRODUCTION

Osteoblasts produce type I collagen, the major protein of bone (Simmons & Grynblas 1990). These cells are also capable of secreting collagen-degrading enzymes (Heath *et al.* 1984, Otsuka *et al.* 1984, Partridge *et al.* 1987). Secretion of interstitial collagenase (MMP-13), a matrix metalloproteinase, is thought to be a crucial step in the initiation of bone remodeling (Kahn & Partridge 1987). Bone remodeling, a tightly coupled process of resorption

and formation, is necessary to accommodate growth and allow fractures to heal. Bone, the major storehouse for calcium in the body, is also remodeled to maintain calcium homeostasis.

Our laboratory has utilized the UMR 106–01 osteoblastic cell line, derived from a rat osteosarcoma, to investigate the role of MMP-13 in bone remodeling. UMR 106–01 cells produce type I collagen, MMP-13 and tissue inhibitors of metalloproteinases (Partridge *et al.* 1981, 1987); the rat MMP-13 is virtually identical to the mouse

MMP-13 and highly homologous to the human collagenase-3 (Quinn *et al.* 1990, Henriot *et al.* 1992, Freije *et al.* 1994). Parathyroid hormone (PTH), the strongest stimulator in UMR 106-01 cells, has previously been reported to induce or augment the secretion of MMP-13 protein by normal and transformed rat osteoblastic cells (Heath *et al.* 1984, Otsuka *et al.* 1984, Cowen *et al.* 1985, Partridge *et al.* 1987) and mouse calvaria (Cowen *et al.* 1985, Eeckhout *et al.* 1986, Delaisse *et al.* 1988). Other agents which stimulate secretion of MMP-13 activity in UMR 106-01 cells include 1,25(OH)₂ vitamin D₃, prostaglandin E₂, and retinoic acid (Partridge *et al.* 1987).

It has previously been determined that MMP-13 induction by PTH in UMR 106-01 cells is, in part, transcriptional (Quinn *et al.* 1990, Scott *et al.* 1992). MMP-13 mRNA has also been shown to be regulated transcriptionally by prostaglandin E₂ and transcriptionally as well as post-transcriptionally by retinoic acid and platelet-derived growth factor BB in osteoblastic cells, the latter effect through mRNA stabilization (Clohisey *et al.* 1994, Connolly *et al.* 1994, Varghese *et al.* 1994, 1996). In osteoblastic cells, bone morphogenetic protein-2 and insulin-like growth factors-I and -II caused down-regulation of MMP-13 mRNA (Delany *et al.* 1996, Varghese & Canalis 1997). In rat uterine smooth muscle cells, the expression of this enzyme was positively and negatively regulated by serotonin and progesterone respectively (Wilcox *et al.* 1992).

Immunohistochemical and biochemical studies have shown that MMP-13 secretion in UMR 106-01 cells, in response to PTH, was mediated mainly by cAMP with a contributory effect by the calcium/protein kinase C system (Civitelli *et al.* 1989). In our previous study (Rajakumar & Quinn 1996), we proposed that induction of MMP-13 in UMR 106-01 cells by PTH occurred through phosphorylation of cyclic AMP response element binding protein (CREB) bound to a TRE consensus sequence (−51). Site-specific mutagenesis of this element eliminated protein binding as well as significantly decreasing the ability of PTH to activate a chloramphenicol acetyl transferase (CAT) promoter construct.

In this study, we focused on the PEA3 consensus binding sequence (−80; AGGAAGT). Site-specific mutagenesis of this sequence resulted in a significant decrease, but not total elimination of activation of a CAT promoter construct by PTH. In addition, adjacent to the PEA3 site, we identified a TRE-like sequence (−89; CGACTCA). Mutation or deletion of the latter affected both basal and PTH-induced expression of MMP-13 in UMR 106-01 cells, implying a co-operative interaction of factors

binding to the combined site. In our previous study, mutagenesis of the TRE (−51) also decreased but did not totally eliminate activation by PTH (Rajakumar & Quinn 1996). Here, we have demonstrated that when both the TRE (−51) and PEA3 (−80) elements were mutated there was a complete lack of stimulation of activity by PTH. There was a time-dependent increase in binding of nuclear proteins to the PEA3 region in response to PTH; no such change was detected with the TRE (Rajakumar & Quinn 1996). Gel supershift analysis indicated that Ets-1 as well as CREB-binding protein (CBP) and CREB were components of this bound complex. Inhibition of CaM kinase II activity significantly decreased protein binding to the PEA3 region as well as MMP-13 mRNA produced in response to PTH, suggesting the involvement of a calcium-mediated pathway in the PTH induction of MMP-13 mRNA in osteoblastic cells.

MATERIALS AND METHODS

Materials

PTH (rat, 1–34) and 8-bromo-cyclic AMP (8-Br-cAMP) were purchased from Sigma Chemical Company (St Louis, MO, USA). Radionucleotides were a product of NEN-Dupont (Boston, MA, USA). CAT assays were performed using the FLASH CAT kit from Stratagene (La Jolla, CA, USA). The Riboprobe kits for making RNA probes for the RNase protection assays were purchased from Ambion (Austin, TX, USA).

Tissue culture media and reagents were purchased from Washington University Tissue Culture Center (St Louis, MO, USA). Fetal bovine serum (FBS) was a product of JRH Biosciences (Lenexa, KS, USA). Antibodies against Fos, Jun, CREB, Ca²⁺/calmodulin-dependent protein kinase II (CaM kinase II) and CaM kinase IV were the products of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibodies against CBP and murine Ets-1 (p54) were a gift from Dr John Chrivia, Saint Louis University and Dr James Hagman, National Jewish Medical and Research Center, Denver, CO, USA, respectively.

Cell culture

UMR 106-01 cells (passage 15–25) were grown and maintained at 37 °C with 5% CO₂ in air, in Eagles' minimum essential medium supplemented with non-essential amino acids, penicillin (100 U/ml), streptomycin (100 µg/ml), and 5% FBS as previously described (Partridge *et al.* 1987). PTH

treatment was at 10^{-8} M in the growth medium described above but containing only 0.5% FBS. Cells were incubated in growth media containing 0.5% FBS for 16–20 h before treatment.

Stable transfectants

Transfections were carried out using the CaPO_4 precipitation method (Gorman *et al.* 1982). Ten micrograms of plasmid DNA in 2 M CaCl_2 and 0.01 M HEPES, pH 5.5, were allowed to form a fine precipitate at room temperature (20–30 min). This precipitate was added to cells that had been subcultured into 100 mm dishes the night before and had a media change 3 h before the transfection. The cells were glycerol shocked 4–5 h later, then incubated in growth media until confluent, at which time they were split 1:4 into selection media (growth media+400 mg/l G418 (Geneticin, Gibco BRL, Grand Island, NY, USA). When colonies were visible (approximately 2 weeks) the cells were harvested and pooled. Pools ranged in size from 500 to more than 1000 independent clones.

Cells were maintained in medium containing 200 mg/l G418. Confluent plates were treated with PTH (10^{-8} M; rat 1–34) for 16–24 h and CAT activity was determined.

CAT assay

CAT assays were performed using the FLASH CAT kit according to the manufacturer's instructions (Stratagene). The treated cells were washed with Ca/Mg-free phosphate-buffered saline (PBS), scraped into PBS and pelleted. Lysis was accomplished by three freeze–thaw cycles in an ethanol–dry ice bath. The supernatant was heated at 65 °C for 10 min and cleared by centrifugation. Protein concentration was determined by the Bio-Rad assay (Richmond, CA, USA). Lysates containing 5 µg protein were incubated with 1-deoxy CAM substrate reagent (fluorescent chloramphenicol) at 37 °C for 4 h or overnight. Products were separated by thin layer chromatography, eluted into methanol, and quantified in an Aminco-Bowman Series 2 luminescence spectrometer. The results were calculated as the percentage acetylated and standardized to control levels (fold induction).

Nuclear extract preparation

Nuclear extracts were prepared as previously described (Schreiber *et al.* 1989). Cells were scraped into media and pelleted by centrifugation. The cells were rinsed with cold PBS, resuspended in

approximately four times the volume of buffer I (20 mM Tris–HCl, pH 7.8, 5 mM MgCl_2 , 0.5 mM dithiothreitol (DTT), 0.3 M sucrose, 1 mM phenyl-methylsulfonyl fluoride (PMSF), 0.2 mM EGTA, 5 mM β -glycerol phosphate, and 1 ng/ml each of aprotinin, antipain, leupeptin and pepstatin A), then incubated on ice for 1 min. This mix was then adjusted to between 0.5 and 0.6% NP-40, vortexed for 5 s and centrifuged at 2000 *g* for 5 min at 4 °C to pellet nuclei. The nuclei were resuspended in an equal volume of buffer II (10 mM Tris–HCl, pH 7.8, 5 mM MgCl_2 , 350 mM NaCl, 0.2 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 10 mM β -glycerol phosphate, 25% glycerol, and 1 ng/ml each of aprotinin, antipain, leupeptin and pepstatin A). After incubation on ice for 15 min, the nuclei were pelleted by centrifugation at maximum speed. The supernatant was recovered and protein concentration determined by Bio-Rad assay. Samples were stored in aliquots at –80 °C.

Mobility shift assay

Gel shift assays were carried out as previously described (Singh *et al.* 1986) with modifications. End-labeled oligonucleotide (20 000 c.p.m.) and nuclear extract (10 µg protein) were incubated for 15 min at room temperature in 20 µl buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol) in the presence of polydeoxyinosine-deoxycytidylic acid or calf thymus DNA as competitor. The shifted bands were separated on a 4% polyacrylamide gel (28:1, acrylamide-bis), dried and exposed for autoradiography or phosphorimage analysis.

Supershifts were carried out as described for mobility shift assays except that specific antibodies (1 µl of appropriate dilutions) were added. The incubations were carried out at 4 °C, overnight.

RNA isolation

Total cellular RNA was isolated as previously described (Favaloro *et al.* 1980). Cells were rinsed twice in saline then scraped into saline. Cells were recovered by centrifugation and resuspended in TSM (10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM MgCl_2)+0.5% IGEPAL CA-630 ((octyl phenoxy) polyethoxyethanol, Sigma Chemical Company). After incubation on ice (2–3 min), cellular debris was pelleted. An equal volume of TSE+S (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 7.4, 0.2% SDS) was mixed with the supernatant. The samples were extracted twice with 50% phenol +50% CHCl_3 :isoamyl alcohol (24:1) and once with CHCl_3 :isoamyl alcohol (24:1). The supernatant was

adjusted to 0.3 M sodium acetate, pH, 5.2, two volumes of ethanol were added and precipitated overnight at -20°C . RNA was quantified by measuring absorbance at 260 nm and purity was assessed by calculating the absorbance ratio 260/280 nm.

RNase protection assay

Radioactively labeled antisense transcripts were produced utilizing the appropriate RNA polymerase (SP6, T7 or T3) and α -[^{32}P]UTP according to the manufacturer's specifications (Ambion). The resulting transcripts were treated with RNase free DNase (1U/ μl), 37°C for 30 min, extracted with an equal volume of phenol and CHCl_3 :isoamyl alcohol (1:1). Ammonium acetate was added to 4 M and samples were precipitated with two volumes of ethanol and recovered by centrifugation (5 min, room temperature). Probes (1×10^6 c.p.m.) were annealed to 20 μg total RNA (85°C , 5 min; 54°C , overnight) in hybridization buffer (40 mM PIPES, pH 6.8, 0.4 M NaCl, 1 mM EDTA, 80% formamide). The annealed sample was diluted 1:10 in RNase buffer (0.3 M NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA). Digestion with RNase T1 (200 U) and RNase A (10 μg) was at 30°C for 1 h. The reaction was stopped by adding SDS to 0.6% and proteinase K to 0.15 $\mu\text{g}/\mu\text{l}$. Digested samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (1:1) and precipitated by adding ethanol (two volumes) and recovered by spinning for 15 min at room temperature. Samples were heated (90°C , 5 min) and run on 8% polyacrylamide sequencing gels (Maniatis *et al.* 1989). Protected fragments were visualized and quantified utilizing a Molecular Dynamics Phosphorimager and ImageQuant program (Amersham Pharmacia Biotech, Uppsala, Sweden).

The MMP-13 riboprobe was produced from the 130 bp internal EcoRI fragment of the rat MMP-13 cDNA (Quinn *et al.* 1990) subcloned into pGem7 (Promega, Madison, WI, USA). The 18S probe was produced according to the manufacturer's specifications utilizing the MEGAscript riboprobe kit (Ambion).

Western blotting

Samples were run on polyacrylamide gels and transferred to Hybond ECL nitrocellulose (Amersham, Arlington Heights, IL, USA) using a discontinuous transfer buffer system (Kyhse-Andersen 1984) in a semi-dry blotter as modified by Schleicher and Schuell (Keene, NH, USA). The discontinuous buffer system consisted of anode

buffer I (300 mM Tris, pH 10.4), anode buffer II (25 mM Tris, pH 10.4), and cathode buffer (40 mM aminocaproic acid, 25 mM Tris, pH 9.4). The transfer was performed at 10 volts for 1–1.5 h. Filters were blocked for 1 h (PBS, 0.05% Tween-20, 5% non-fat dry milk), incubated with primary antibody (PBS, 0.05% Tween-20, 2.5% non-fat dry milk) overnight and secondary antibody for 1 h. The ECL Western detection kit (Amersham) was used to visualize antibody binding.

RESULTS

A PEA3 consensus-binding site contributes to PTH induction of MMP-13

Previously we demonstrated, through 5' deletional analysis, that removal of the sequence from -201 to -102 in the MMP-13 gene regulatory region resulted in a small reduction in PTH-mediated induction of the corresponding CAT construct (Rajakumar & Quinn 1996, Fig. 1A). In this study, the role of the PEA3 consensus-binding site in the MMP-13 gene activation by PTH was examined. Initially, additional deletions were created followed by mutations in the -102 CAT construct, the latter having retained full PTH activation potential compared with the largest construct previously examined (-6500 CAT; Rajakumar & Quinn 1996). With removal of sequence to within two nucleotides of the PEA3 consensus-binding site (-82 CAT), CAT activity was reduced to near basal levels (1.13 ± 0.13 ; Fig. 1A). However, with removal of the PEA3 consensus-binding site, this response was almost fully restored (-73 CAT; Fig. 1A).

Mutation of the PEA3 site in the -102 CAT construct, a change from AGGAAGT to ATCAAGT, resulted in a statistically significant reduction in CAT activity produced in response to PTH ($P < 0.01$; Fig. 1C). In our previous study, we reported that mutation of the TRE (-51) in the -102 CAT construct also resulted in a statistically significant decrease in the amount of CAT produced in response to PTH as compared with the wild type (Rajakumar & Quinn 1996). Despite the fact that the CAT response was greatly reduced in the TRE mutant, the response to PTH was diminished to control levels only when all elements except the TATA box were eliminated. Similarly, mutation of the PEA3 site alone resulted in residual CAT activity. Only when UMR cells that were stably transfected with the -102 CAT construct containing both the PEA3 and TRE site mutations were treated with PTH, were the CAT levels obtained equivalent to control (Fig. 1A).

FIGURE 1. CAT analysis of the -102 to -46 region. CAT constructs were stably transfected into UMR 106-01 cells. Pooled clones were treated with PTH (10^{-8} M) for 16 to 24 h. Cell lysates were assayed for CAT activity. These are graphical representations of three to four independent experiments expressed as the mean $\pm$ s.e.m. Significance was determined by ANOVA. (A) Deletion mutants. Deletions were created as indicated. The lower case 'tre' refers to the TRE-like sequence. The results are expressed as fold induction normalized to control levels. The labels are a reminder of which motifs are present. * $P > 0.01$ not significantly different from control. (B) Basal activity of deletion mutants. CAT activity in the presence of media containing 5% serum was measured, normalized to control levels (0.05% serum) and expressed as percent of the -46 CAT construct (containing only TATA element). * $P < 0.01$ compared with -46 CAT levels. (C) Site-specific mutants. Point mutations were created in the -102 CAT construct as indicated. The results are expressed as fold induction normalized to control levels. * $P < 0.01$ compared with wild type. (D) Basal activity of site-specific mutants. Basal activity of the site-specific mutants were measured and normalized as described above. Values are expressed as percent of wild type.

A TRE-like consensus-binding sequence is involved in PTH activation of MMP-13

The region just 5' of the PEA3 consensus-binding site, when deleted as described above, resulted in complete reduction of PTH-mediated induction of the corresponding CAT construct (−82 CAT). This region contains a TRE-like consensus-binding sequence (CGACTCA; −89). Since activator protein-1 binding activity has been noted to be in close proximity and to affect Ets binding activity at PEA3 consensus-binding sites (Gutman & Wasylyk 1990, Moulton *et al.* 1994, Wu *et al.* 1994, Logan *et al.* 1996), we created a mutation in this region in the −102 CAT construct. Mutating CGACTCA to CGTGTG actually caused an increase in CAT activity of 1.5-fold over wild-type levels (Fig. 1C).

Basal expression

In our previous study, we reported that removal of sequence from −201 to −102 resulted in a significant decrease in basal activity of the corresponding CAT construct (Rajakumar & Quinn 1996, Fig. 1B). Our current data indicated that deletion of the TRE-like sequence (−82 CAT) resulted in a statistically significant increase ($P < 0.01$) in the basal level CAT activity (Fig. 1B). This increased basal activity, however, was reduced to levels equivalent to that observed with the −102 CAT construct when the PEA3 site was removed (−73 CAT). The basal activity of the −102 CAT construct containing the mutation of the TRE-like site was increased twofold (Fig. 1D). Mutation of the PEA3 site in −102 CAT also increased basal CAT activity (3.5-fold; Fig. 1D). Previously we determined that mutation of the TRE (−51) reduced basal expression below that of the wild type (Rajakumar & Quinn 1996, Fig. 1D). Here we present data illustrating that in the TRE (−51) and PEA3 double mutant the basal level was further reduced as compared with the TRE mutation alone (Fig. 1D).

PTH induces a time-dependent increase in binding of nuclear factors to the −95 to −71 region of the MMP-13 enhancer

The amount of protein binding to the TRE (−51) was shown not to change in response to PTH (Rajakumar & Quinn 1996). Since it was determined that protein synthesis was necessary for MMP-13 mRNA induction by PTH (Scott *et al.* 1992), the level of protein binding to the PEA3 region in response to PTH was examined (Fig. 2). UMR 106–01 cells were treated with PTH (10^{-8} M) for the times indicated. Nuclear extracts were isolated and gel shift analysis performed utilizing an oligonucleotide-containing sequence of the −95 to −71 region of the rat MMP-13 gene.

Within this region is the PEA3 consensus binding site (−80; AGGAAGT). A time-dependent change in the amount of protein binding was observed (Fig. 2A). Phosphorimager or densitometric analysis of these data indicated a 3.5-fold increase in binding at 0.5 h, with a maximum amount of binding of 7.6-fold at 4 h, which returned to basal level by 24 h (Fig. 2B). In addition, protein binding to the −95 to −71 oligonucleotide containing the PEA3 mutation, described above, was also significantly reduced when compared with the wild-type sequence (Fig. 2B).

Supershift analysis indicated that Ets-1 and CBP are components of the binding activity at the −95 to −71 region

In order to ascertain which nuclear factors were binding to the PEA3 region, supershift analysis was performed. Protein binding activity was analyzed utilizing the oligonucleotide containing sequence of the −95 to −71 region and nuclear extracts from PTH (10^{-8} M, 4 h)-treated UMR 106–01 cells. These data demonstrated that antibodies against Ets-1 and CBP were capable of supershifting the band which was reported above to increase with PTH treatment (Fig. 3). In addition, an antibody against CREB produced a supershift, but to a lesser extent. Antibodies against Fos and Jun, however, did not interact (Fig. 3).

UMR 106–01 cells contain CaM kinase II

It was previously reported that modulation of Ets-1-mediated transcriptional effects could occur through calcium-activated pathways (Valentine *et al.* 1995). To determine which pathways may be functional in the UMR cells, Western analyses were performed on total cell lysates. The data demonstrated that the UMR 106–01 cells express CaM kinase II (Fig. 4) but not CaM kinase IV (data not shown).

CaM kinase II-specific inhibitor disrupts PTH induction of MMP-13 mRNA

To investigate whether CaM kinase II is involved in the production of MMP-13 mRNA in response to PTH, the effect of a CaM kinase II specific inhibitor was examined. UMR 106–01 cells were pretreated with the CaM kinase II specific inhibitor, KN93 (Sumi *et al.* 1991), followed by treatment with PTH (10^{-8} M, 4 h) or 8-Br-cAMP (10^{-3} M, 6 h); the latter was used as a control to monitor toxicity and specificity of effect. Pretreating the cells with KN93 (3.7 μ M; IC_{50} = 370 nM; Sumi *et al.* 1991), followed by PTH (10^{-8} M, 4 h) resulted in a statistically significant decrease in the amount of MMP-13

FIGURE 2. Time-course of nuclear factor binding to the -95 to -71 region. UMR 106-01 cells were treated with PTH (10^{-8} M) for the times indicated. Nuclear extracts were prepared as described in Materials and Methods. A [32 P]labeled oligonucleotide consisting of wild-type (WT) or mutant sequence encompassing the -95 to -71 region was incubated with nuclear extract equivalent to 10 μ g protein. Incubation was for 15 min at room temperature. (A) This is a representative gel of three independent experiments of PTH-induced protein binding to the wild-type sequence. The lower arrow indicates the major shifted band, the upper arrow a secondary band. (B) Phosphorimage or densitometric quantification of the lower band in each time-course was performed. These data are represented in graphical form as the mean $\pm$ S.E.M. of three independent experiments.

mRNA induced compared with treatment with PTH alone (16%, $P < 0.05$; Fig. 5). Upon treatment with 8-Br-cAMP (10^{-3} M, 6 h), after pretreatment with KN93 (3.7 μ M), a slight increase in MMP-13 mRNA was apparent when compared with 8-Br-cAMP treatment alone. Increasing the amount of KN93 to 18.5 μ M resulted in a further decrease in response to PTH (68%) in addition to a slight decrease, which was not statistically significant, in response to 8-Br-cAMP ($P > 0.05$; Fig. 5).

Inhibition of CaM kinase II activity disrupts PTH-mediated binding of nuclear factors to the -95 to -71 region of the MMP-13 enhancer

To correlate the effect of CaM kinase II inhibition on MMP-13 mRNA levels to promoter related activity,

UMR 106-01 cells were pretreated with the CaM kinase II specific inhibitor, KN93 (3.7 μ M), followed by treatment with PTH (10^{-8} M, 4 h). Nuclear extracts were isolated and used in gel shift analysis utilizing an oligonucleotide-containing sequence of the -95 to -71 region of the rat MMP-13 gene; above we reported a time-dependent increase in binding of nuclear factors to this oligonucleotide in response to PTH treatment. Phosphorimager analysis of these data indicated a statistically significant decrease (40%, $P < 0.01$) in protein binding to this region (Fig. 6).

DISCUSSION

The purpose of this study was to continue the characterization of 5' upstream elements involved in

FIGURE 3. Supershift analysis of proteins binding to the -95 to -71 region. UMR 106-01 cells were treated with PTH (10^{-8} M, 4 h). Nuclear extracts were prepared as described in Materials and Methods. A [32 P]labeled oligonucleotide encompassing the -95 to -71 region was incubated with nuclear extract equivalent to 10 μ g protein. Incubation was overnight at 4 °C or 1 h at room temperature. Antibodies were added prior to incubation, as indicated. Broad-spectrum antibodies against Fos and Jun along with specific antibodies against Ets-1 and CBP were utilized.

FIGURE 4. Western analysis for CaM kinase II. UMR 106-01 cells were grown to confluence and total cellular lysates obtained. Protein samples (10 μ g) were run on 4–20% gradient polyacrylamide/SDS minigels and transferred to Hybond ECL nitrocellulose as described in Materials and Methods. Hybridization was to an anti CaM kinase II antibody (Sc1542 diluted 1:400). The position of molecular mass markers is as indicated.

the PTH transcriptional induction of MMP-13 in rat osteoblastic cells. Previously we reported that removal of sequence from -201 to -102, which contains the Cbfa1 consensus binding site (-135), decreased the basal activity by approximately 50% but had minimal effect on PTH induction of the corresponding CAT construct (Rajakumar & Quinn 1996); osF2/Cbfa1, a member of the AML/CBF/PEBP2/Runtdomain transcription factor family, has been shown to be a transcription activator of osteoblast differentiation (Ducy *et al.* 1997). These data were confirmed by Selvamurugan *et al.* (1998) in which they demonstrated that not only deletion but mutation of this site produced similar results. They also reported that mutation of the TRE affected PTH induction of the gene only in the

FIGURE 5. Effect of inhibition of CaM kinase II activity on interstitial collagenase mRNA production. UMR 106-01 cells were pretreated with KN93 (3.7 μM) followed by treatment with PTH (10^{-8} M, 4 h) or 8-Br-cAMP (10^{-3} M, 6 h). RNase protection was performed on 20 μg total RNA. (A) A representative gel of three independent experiments. (B) A graphical representation of phosphorimager analysis presented as the mean $\pm$ s.e.m. of three independent experiments. * $P < 0.05$ compared with control, ** $P > 0.05$ not statistically different from control (ANOVA).

absence of a functional Cbfa1-binding site. Our previous data are in agreement with this, where mutation of the TRE in the -102 CAT construct also significantly decreased PTH responsiveness.

In the present study, we examined the PEA3 consensus binding sequence (-80; AGGAAGT). This site has been characterized as binding the Ets family of transcription factors that have been implicated in the regulation of other matrix metalloproteinase genes (Wasylyk *et al.* 1991, Buttice *et al.* 1996, Iwasaka *et al.* 1996, Schneikert *et al.* 1996, Westermarck *et al.* 1997). The study by Selvamurugan *et al.* (1998) concluded that this region did not affect PTH induction of MMP-13; however, their studies were performed in the presence of a functional Cbfa1-binding site. The data we present here indicate that, similar to the mutation of the TRE site in the -102 CAT construct, mutation of the PEA3 site also significantly decreased the PTH response and that mutation of both sites totally eliminated what residual stimulation remained. These data suggest that Cbfa1 is involved in basal expression of the MMP-13 gene in the osteoblast, whereas the TRE and PEA3 sites contribute to PTH activation of the gene. In support of this theory are data presented by

Selvamurugan *et al.* (1998) that show a decrease in PTH responsiveness when both the TRE and PEA3 sites were mutated in the presence of a functional Cbfa1-binding site; this decrease was not observed with mutation of either site alone. In addition, in the Cbfa1 knockout mouse no bone was formed, implying that Cbfa1 is necessary for osteoblast activity as a prerequisite for PTH stimulation of osteoblastic genes (Komori *et al.* 1997).

When examining the PEA3 site, we identified an adjacent region that, when mutated or deleted, resulted in increased basal level expression of the corresponding CAT construct. This TRE-like site (-89; CGACTCA) appeared to co-operate with the PEA3 site, since mutation of the PEA3 site in the presence of the TRE-like site increased basal expression but decreased PTH responsiveness of the -102 CAT construct. In addition, mutation of the TRE-like site in -102 CAT, if anything, caused an increase in PTH responsiveness, while deletion resulted in a significantly decreased response. In the double mutant, where both the PEA3 and TRE-like sites were mutated in -102 CAT, the PEA3 mutation dominated, demonstrating a decreased PTH response with increased basal activity (data not shown). Co-operativity of PEA3

FIGURE 6. Effect of inhibition of CaM kinase II activity on binding of nuclear factors to the -95 to -71 region of the interstitial collagenase enhancer. UMR 106-01 cells were pretreated with KN93 ($3.7 \mu\text{M}$) followed by treatment with PTH (10^{-8} M , 4 h). Nuclear extracts were prepared as described in Materials and Methods. A [^{32}P]labeled oligonucleotide encompassing the -95 to -71 region was incubated with nuclear extract equivalent to $10 \mu\text{g}$ protein and gelshift analysis performed. This is a graphical representation of phosphorimager analysis of the resulting gelshift. Results are presented as the mean $\pm$ S.E.M. for three independent experiments.

* $P < 0.01$ compared with control (ANOVA).

sites with adjacent consensus binding sequence elements has previously been noted to be of importance in the regulation of other genes, including matrix metalloproteinases (Gutman & Wasylyk 1990, Moulton *et al.* 1994, Wu *et al.* 1994, Logan *et al.* 1996). These interactions have been noted to result in positive as well as negative responsiveness of the corresponding gene. Our data imply that such an interaction is occurring in the MMP-13 regulatory region and could be a repressor type of interaction, since deletion or mutation of the TRE-like site caused an increase in basal activity. In addition, mutation of the TRE-like site resulted in increased PTH responsiveness.

We observed a PTH-induced time-dependent increase in binding of nuclear factors to the PEA3 region encompassing the PEA3 and TRE-like sites. This is the first reported observation of a time-dependent change in protein binding to sequence in

the 5' regulatory region of the rat MMP-13 gene in response to PTH treatment. Protein binding to an oligonucleotide-containing sequence of this region (-95 to -71), shown through gel shift experiments, indicated a dramatic increase in binding, first evident at 0.5 h after PTH (10^{-8} M) treatment, with maximum levels observed at 4 h (7.6-fold). The peak binding at 4 h coincides with maximum MMP-13 mRNA production (Scott *et al.* 1992).

To determine the components of the protein complex induced to bind to the -95 to -71 region in response to PTH, supershift analysis was performed. Antibodies against Ets-1, CBP and CREB caused additional shifts to occur. CBP was originally identified by its ability to bind specifically to phosphorylated CREB to activate CREB-mediated transcription (Chrivia *et al.* 1993). CBP also has been shown to be a necessary component of Ets-1-mediated transcription (Yang *et al.* 1998). In addition, the Ets-1/CBP interaction was determined to be independent of DNA binding (Yang *et al.* 1998). Integrating these data into our model, we hypothesize that PTH-induced binding of Ets-1 to the -95 to -71 region, with recruitment of CBP to the complex and subsequent interaction with phosphorylated CREB, results in activation of transcription. The involvement of Ets-1 in this process is significant since the expression of Ets-1 has been associated with developing bone during murine embryogenesis (Kola *et al.* 1993).

The cAMP analogue, 8-Br-cAMP, was able to mimic the transcriptional induction of the rat MMP-13 gene by PTH in UMR 106-01 cells (Scott *et al.* 1992). However, previous data indicated that a calcium-mediated pathway may contribute to PTH-induced secretion of the enzyme in these cells (Civitelli *et al.* 1989). To inquire into the possibility that a calcium-mediated pathway may also be involved at the level of the mRNA, we used a CaM kinase II specific inhibitor and determined that inhibition of CaM kinase II activity did indeed interfere with the PTH-mediated induction of MMP-13 mRNA in response to PTH in the UMR cells. This decrease in mRNA was coincidental to a decrease in binding of nuclear factors to the -95 to -71 region containing the PEA3 consensus binding sequence (-80). Inhibition of CaM kinase II activity did not suppress cAMP-mediated induction through 8-Br-cAMP, which indicated that the results obtained were not due to a general toxicity effect. From these data we surmise that MMP-13 induction by PTH in these cells involves at least two pathways.

It was previously shown, in a lymphocyte system, that CaM kinase II is rapidly activated through an increase in intracellular calcium and that the

resulting phosphorylation events could regulate the activity of DNA-binding proteins (Valentine *et al.* 1995). PTH is known to cause a measurable increase in intracellular calcium levels in UMR 106–01 cells (Yamaguchi *et al.* 1987). The supposition is that PTH increase of intracellular calcium activates CaM kinase II. This would be supported by the fact that the use of a specific CaM kinase II inhibitor resulted in a disruption of the physiological responses to PTH that we were measuring. In the lymphocyte system, Ets-1 can be phosphorylated by CaM kinase II; however, it was proposed that this phosphorylation could result in down-modulation of transcription (Valentine *et al.* 1995). Other data have indicated that phosphorylation of Ets-1 through Ras enhances transcriptional activation (Yang *et al.* 1996). In our system, we have demonstrated that inhibition of CaM kinase II activity not only affected DNA–protein complex formation but was also detrimental to MMP-13 mRNA production in response to PTH. Therefore, we postulate that phosphorylation event(s) through PTH activation of CaM kinase II are an important component of the PTH-signaling process for gene activation.

Initially we demonstrated that CREB was bound to the TRE (–51) in the upstream region of the MMP-13 gene and that phosphorylation of the former in response to PTH in the UMR 106–01 osteoblastic osteosarcoma cells was a possible mechanism for activation of the gene (Rajakumar & Quinn 1996). Our current data extend our previous results by establishing that disruption of the PEA3 consensus binding sequence (–89) also resulted in loss of PTH activation of the gene. In addition, we have demonstrated that Ets-1 interacts with sequence in the 5' upstream regulatory region of the gene in response to PTH, and that CBP is part of this complex. Expanding our model to include these data, we speculate that upon stimulation by PTH, CREB bound to the TRE (–51) becomes phosphorylated by protein kinase A, resulting in tighter binding to the DNA and interaction with CBP. CBP may be recruited to the enhancer region through interaction with Ets-1, since previous data indicated that the Ets-1/CBP interaction is independent of DNA binding (Yang *et al.* 1998). In this case, the binding of CBP would be augmented through PTH action. Thus, we propose that induction of MMP-13 mRNA by PTH in these cells results through CBP-mediated activation of transcription.

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