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Receptor Activator of NF-κB Ligand Protein Expression in UMR-106 Cells Is Differentially Regulated by Parathyroid Hormone and Calcitriol

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Abstract Expression of the cytokine, receptor activator of NF-κB ligand (RANKL), is stimulated by both parathyroid hormone (PTH) and calcitriol in osteoblasts. Most studies have examined the effects on RANKL mRNA, and less information is available on the protein products. We have determined the effects of PTH, the adenylate cyclase stimulator forskolin, and calcitriol, alone and in combination, on endogenous RANKL protein expression in UMR-106 rat osteoblastic osteosarcoma cells by Western blotting and enzyme immunoassay (EIA). PTH and forskolin dose dependently increased a ~52 kDa band in whole cell lysates that was detected by both C- and N-terminal directed RANKL antibodies. Calcitriol treatment produced little or no expression of this ~52 kDa band, but markedly increased the expression of a ~32 kDa band that was only detected with an antibody directed to the N-terminus of RANKL. An EIA based on RANKL binding to OPG detected a large increase in RANKL expression following calcitriol treatment, and much smaller increases with PTH or forskolin. The combination of PTH and calcitriol or forskolin and calcitriol elicited effects similar to those of PTH and forskolin alone, as detected by both Western blotting and EIA. In contrast to the effects on protein, all agents increased RANKL mRNA expression, with the greatest effects seen with the co-treatments. The results indicate that PTH, likely through effects on cyclic AMP, has a different effect on RANKL processing than calcitriol. The ~52 and ~32 kDa RANKL products appear to interact differently with OPG, which could affect responses to the agents in target cells. J. Cell. Biochem. 95: 1029–1041, 2005.

Key words: receptor activator of NF-κB ligand; osteoblastic cell; parathyroid hormone; calcitriol

Receptor activator of NF-κB ligand (RANKL) is an important regulator of osteoclast maturation and function [Fuller et al., 1998; Lacey et al., 1998; Jimi et al., 1999; Kong et al., 1999; Suda et al., 1999; Takahashi et al., 1999; Hofbauer et al., 2000]. Osteoblasts, bone marrow stromal cells and lymphoid tissues express RANKL [Hofbauer, 1999]. The expression of RANKL has also been detected in several stromal and osteoblast like cell lines [Hofbauer, 1999]. The binding of RANKL to receptor activator of NF-κB (RANK) on preosteoclasts and osteoclasts promotes osteoclastogenesis, osteoclast activity, and osteoclast survival [Nakagawa et al., 1998; Hsu et al., 1999].

RANKL is a type II transmembrane protein that lacks a signal peptide [Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998]. The protein consists of a cytoplasmic domain located at the amino terminus, a transmembrane domain, and an extracellular domain located at the carboxy terminus [Anderson et al., 1997; Lacey et al., 1998]. The extracellular domain consists of two functional domains, a stalk region and the active ligand site [Lacey et al.,

Abbreviations used: Ab, antibody; Con, control; EIA, enzyme immunoassay; Fsk, forskolin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RANKL, receptor activator of NF-κB ligand; RT-PCR, reverse transcription polymerase chain reaction; sRANKL, soluble RANKL; TACE, TNF-κ convertase; Vit.D, calcitriol.

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1998]. The extracellular domain also contains two potential N-glycosylation sites [Wong et al., 1997; Willard et al., 2000]. The crystal structure of the extracellular domain has been determined and found to form trimers [Lam et al., 2001; Ito et al., 2002].

RANKL has been described as being present in two forms, a membrane-bound, cell associated form and a soluble form [Lacey et al., 1998; Yasuda et al., 1998]. The soluble form of the protein is generated by post-translational processing of the cell-associated form by a metalloprotease [Lacey et al., 1998; Xu et al., 2000]. The metalloprotease-disintegrin tumor necrosis factor-α convertase (TACE) has been implicated in this post-translational processing [Lum et al., 1999]. Both the membrane-associated and the soluble form of the protein can bind to the RANKL receptor, RANK, on preosteoclasts and osteoclasts, and to the soluble decoy receptor osteoprotegerin (OPG) [Nakagawa et al., 1998; Nagai and Sato, 1999; Udagawa et al., 2000].

RANKL expression is increased in osteoblasts by a number of cytokines and hormones, including parathyroid hormone (PTH), and calcitriol (1,25-dihydroxyvitamin D_3) [Hofbauer, 1999; Lee and Lorenzo, 1999; Itoh et al., 2000; Kitazawa and Kitazawa, 2001; Ma et al., 2001; Fu et al., 2002; Kondo et al., 2002; Lee and Lorenzo, 2002; Kitazawa et al., 2003; Huang et al., 2004; Notoya et al., 2004]. It is believed that RANKL is the major mediator of the effects of PTH and calcitriol to induce bone resorption [Horwood et al., 1998; Tsukii et al., 1998; Kitazawa and Kitazawa, 2001; Kitazawa et al., 2003; Lerner, 2004]. The decoy receptor for RANKL, OPG, can block the bone resorptive effects of PTH and calcitriol, demonstrating the importance of RANKL in the bone resorptive effects of these factors [Fuller et al., 1998; Tsukii et al., 1998; Itoh et al., 2000; Udagawa et al., 2000; Huang et al., 2004]. PTH and calcitriol not only act on bone independently but also interact to regulate each other's actions. The interaction of calcitriol and PTH can enhance the mobilization of calcium and phosphate from bone [Broadus, 1999].

While the regulation of the expression of RANKL by these hormones has been extensively studied at the mRNA level, investigation of the regulation of endogenous RANKL protein is much more limited [Nakashima et al., 2000; Buckley et al., 2002; Miyazaki et al., 2004]. There are studies that have examined the effects of various factors on recombinant RANKL protein overexpressed in various cell lines [Wong et al., 1997; Nakashima et al., 2000; Kanamaru et al., 2004]. However expressed recombinant proteins do not necessarily reflect the behavior of the protein in the native system. In the current study we have examined the responses of endogenous RANKL protein to PTH and calcitriol in the UMR-106 rat osteosarcoma cell line. The results indicate that both hormones increase the expression of endogenous RANKL protein. However the size of the RANKL protein band in cells stimulated by calcitriol alone was smaller than that of the RANKL protein band in cells stimulated by PTH or forskolin, alone or in combination with calcitriol.

MATERIALS AND METHODS

Cell Line and Cell Culture

UMR-106 rat osteoblastic osteosarcoma cells were obtained from American Type Culture Collection (Manassas, VA) and used between passages 9 and 16. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 15% heat inactivated horse serum (GIBCO) and 100 U/ml penicillin (Sigma, St. Louis, MO), and kept in a humidified incubator at 37°C and 5.0% carbon dioxide.

Treatments

The UMR-106 cells were serum starved for 24 h before the addition of treatments by incubating the cells in DMEM containing 0.1% bovine serum albumin (BSA) (Calbiochem, La Jolla, CA) and 100 U/ml penicillin. Following the 24 h serum starvation the medium was removed and PTH (Bachem, San Carlos, CA), forskolin (Sigma), or calcitriol (gift from Dr. Milan Uskokovic) were added to the cells, and incubations carried out for predetermined lengths of time.

Whole Cell Lysates

To collect whole cell lysates the cells were scraped from the tissue culture plates in RIPA buffer [phosphate-buffered saline (PBS), 0.1% Nonidet P-40, 0.5% sodium deoxycholate (Sigma), 0.1% sodium dodecyl sulfate (SDS) (Fisher, Pittsburgh, PA), 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma), 0.15–

0.3 TIU/ml aprotinin (Calbiochem), 1 mM sodium orthovanadate (Sigma)] and passed through a 21-gauge needle approximately 10 times to shear the DNA. Additional PMSF (0.1 mg/ml) was added to each lysate. The lysates were incubated on ice for 30–60 min and then centrifuged at 13000 rpm for 20 min at 4°C in a refrigerated microcentrifuge. The supernatant was collected as the whole cell lysate. An aliquot of each sample was collected for determination of protein concentration using the Lowry et al. [1951] method. One half volume of electrophoresis sample buffer [50 mM Tris, 2 mM EDTA, 2% SDS (Fisher), 10% glycerol, 5% β-mercaptoethanol (Sigma), 0.001% bromophenol blue (BioRad, Hercules, CA)] was added to the remainder of the sample, and the sample was boiled for 5 min. Samples were used immediately for electrophoresis or stored at −70°C for later use.

Western Blotting

Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS–PAGE). A 4% stacking gel and a separating gel of 10% or 12.5% were used to separate the proteins. Following electrophoresis the proteins were electrophoretically transferred to nitrocellulose (0.2 μm; Schleicher and Schuell, Keene, NH) overnight at 30 V. To insure complete transfer of the proteins the voltage was increased to 100 V for 30 min following the overnight transfer. To confirm equal loading of the protein samples, the membranes were stained with Ponceau Red (Sigma). The membranes were washed, and blocked with blocking buffer [Tris-buffered saline (TBS), 0.05% Tween 20 {(Tween), Fisher}, 2% (w/v) BSA and 2% (w/v) Carnation powdered milk], and then incubated in RANKL specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in blocking buffer. After incubation with the primary antibody the membranes were washed in TBS-Tween two times for a total wash time of 15 min, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Sigma). The membranes were then washed three times in TBS-Tween for a total wash time of 15 min followed by a wash in TBS (no Tween). Immunecomplexes were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) using Kodak BioMax film (VWR, West Chester, PA). As an internal control, levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein were measured by Western blotting as described above using a GAPDH antibody (Novus Biologicals, Littleton, CO) and the appropriate horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech). Quantification of the immunecomplexes was carried out with densitometry using the Scion Image Analyzer program. RANKL values were normalized to GAPDH values. Prestained SDS–PAGE molecular weight standards (BioRad) were electrophoresed on all gels to determine the approximate molecular weights of the protein bands detected in the whole cell lysates by Western blotting.

Dot Blot

Recombinant soluble RANKL [sRANKL, (Peprotech, Rocky Hill, NJ)] (50–400 ng, in a volume of 20 μl of 5 mM Tris-HCl, pH 8.0) was spotted onto a nitrocellulose membrane and allowed to dry. The membrane was cut into strips and subjected to Western blotting as described above using the antibodies to the C- and N-terminus of RANKL.

Enzyme Immunoassay (EIA)

An EIA kit that measures murine soluble RANKL (sRANKL) based upon binding to the decoy RANKL receptor, OPG, was obtained from American Laboratory Products, Inc. (Windham, NH). The assay kit consists of a 96-well plate coated with OPG. Samples together with a biotinylated antibody to the C-terminal domain of RANKL, i.e., the domain that constitutes the soluble form of the molecule, were added to the wells, followed by a conjugated secondary antibody that recognizes the RANKL antibody. An enzymatic reaction leading to a colorimetric change is used to quantitate the levels of RANKL present in the samples.

For the assay, whole cell lysates were prepared as described above except that electrophoresis stop buffer was not added and the samples were not boiled before being stored at −70°C. Conditioned medium was prepared by collecting the medium from the tissue culture wells into 1.5 ml microfuge tubes, and centrifuging the samples at 13,000 rpm for 10 min at 4°C. The pre-cleared conditioned medium was stored at −70°C. The EIA was carried out following the manufacturer's protocol. The colorimetric change was measured at 450 nm

with a reference wavelength of 630 nm in a Dynatech MR5000 plate reader.

Deglycosylation

Deglycosylation of the RANKL protein was carried out using N-glycosidase F (Calbiochem). Whole cell lysates were collected and the protein concentration determined as described above. Assay buffer (5×) [250 mM sodium phosphate (NaH_2PO_4) (Sigma), pH 7.5] was added to 75 μg total protein followed by the addition of denaturation buffer [2% SDS, 1M β-mercaptoethanol (Sigma)]. Samples were heated to 100°C for 5 min and then cooled on ice. Triton-X 100 (10%) and N-glycosidase F were added, and the mixture was incubated for 3 h at 37°C, after which the samples were heated to 100°C for 5 min to stop the reaction. Electrophoresis sample buffer was added, and the samples were heated in a boiling water bath for 5 min. Samples were electrophoresed on a polyacrylamide gel followed by Western blotting.

As a positive control for the deglycosylation reaction, ribonuclease B (Sigma) was used. After deglycosylation of the ribonuclease B following the protocol described above, the samples were electrophoresed on a 12.5% polyacrylamide gel followed by staining with Coomassie blue (BioRad). A shift in the molecular weight of the detected band indicated deglycosylation of the protein.

Inhibition of glycosylation was carried out using tunicamycin (Sigma). Cells were treated with 30 nM PTH for a total of 48 h, with tunicamycin (5μg/ml) added for the final 8 h. Whole cell lysates were collected and subjected to SDS–PAGE and Western blotting as described above.

Reverse Transcription-Polymerase Chain Reaction (PCR)

UMR-106 cells were cultured in 6-well tissue culture plates and treated as described for the indicated lengths of time. Total RNA was extracted using the RNeasy Mini kit from Qiagen (Valencia, CA). Single stranded cDNA was transcribed from the total RNA using Promega's (Madison, WI) Reverse Transcription System. PCR amplification of the cDNA was carried out in a reaction mixture containing 10× PCR buffer, 25 mM magnesium chloride ($MgCl_2$), dNTPs, primers, and Taq polymerase (Invitrogen, Carlsbad, CA). Amplification of RANKL was done using a rat specific RANKL primer pair from published sequences [Xu et al., 2000] (Invitrogen). The sequences of the RANKL primers were, forward 5′-CTT TGG ATC CTA ACA GAA TAT CAG-3′ and reverse 5′-AGG CTT CAG TCT ATG TCT TGA ACT TT-3′. Cyclophilin was used as a control, the primer pair for cyclophilin was purchased from Continental Lab Products (San Diego, CA). The sequences of the cyclophilin primers were, forward 5′-TGG CAC AGG AGG AAA GAG CAT C-3′ and reverse 5′- AAA GGG CTT CTC CAC CTC GAT C-3′. For amplification of RANKL, 5 μl of the cDNA reactions were subjected to PCR under the following conditions: an initial denaturing step of 95°C for 4 min, followed by 30 cycles of 94°C for 40 s, 54°C for 40 s, and 72°C for 40 s. A final extension was carried out at 72°C for 7 min, after which the reactions were kept at 4°C. For amplification of cyclophilin, 5 μl of the cDNA reactions were subjected to an initial denaturing step of 94°C for 5 min and then a step at 60°C for 5 min followed by 30 cycles of 72°C for 2 min, 94°C for 1 min, 60°C for 1 min. A final extension was carried out at 72°C for 10 min, after which the reactions were kept at 4°C. PCR reactions containing no cDNA were used as negative controls.

PCR products were electrophoresed on a 2% agarose (Shelton Scientific, Shelton, CT) gel [in 1× TAE (Tris, sodium actetate, EDTA) buffer] containing 0.005% (V/V) ethidium bromide (Sigma). The PCR products were visualized on a UV light box, and the gel was photographed using Polaroid 667 film (VWR, West Chester, PA). The bands were quantitated by densitometry using the Scion Image Analyzer program. RANKL values were normalized to cyclophilin values.

Statistics

Statistical significance was determined by one-way ANOVA and the Newman-Keuls post-test (Graph Pad Prism Software). $P < 0.05$ was considered significant.

RESULTS

Detection of RANKL Protein by Western Blotting

In initial experiments several antibodies to RANKL, including antibodies to full length RANKL as well as those specifically raised

against the C- and N-terminus of the molecule were tested to determine which gave the most reproducible results in our system. Of the antibodies tested it was found that the antibodies (Santa Cruz Biotechnology) raised against the C- and N-terminal regions of the RANKL protein were able to detect RANKL in our system without detecting multiple nonspecific bands. Therefore, these antibodies were used for the Western blotting studies. The C-terminal antibody was raised against a peptide in the extracellular, C-terminal region of the RANKL protein. Therefore, this antibody would be expected to recognize the full length form of RANKL as well as any fragments of the protein containing the peptide the antibody was raised against (Fig. 1), including the soluble form of RANKL. The N-terminal antibody was raised against a peptide in the intracellular, N-terminal region of the RANKL protein. Therefore, this antibody should also recognize the full length form of RANKL as well as any fragments containing the peptide that the antibody was raised against (Fig. 1).

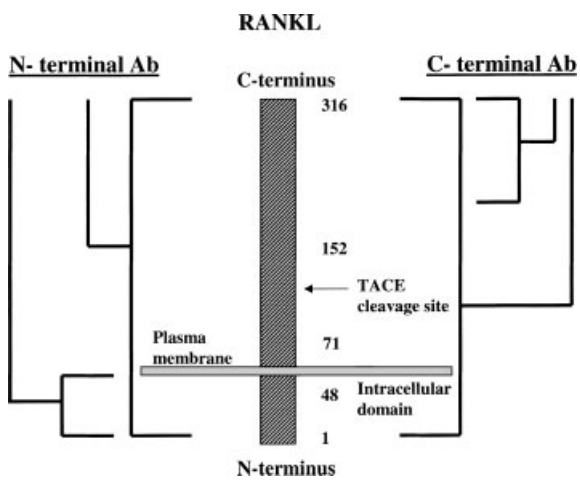

Fig. 1. Diagram of the RANKL protein and the portions of the protein detected by the RANKL antibodies used in this study. Brackets indicate the portions of the RANKL molecule expected to be recognized by each of the antibodies. The C-terminal antibody, according to the product information, was generated to a peptide in the extracellular domain and should recognize the full length form of RANKL as well as any fragments of the protein containing the peptide that the antibody was raised against. The N-terminal antibody, according to the product information, was generated to a peptide in the intracellular domain and should also recognize the full length form of RANKL as well as any fragments containing the peptide that the antibody was raised against. The TACE (TNF-α convertase) cleavage site is indicated by the arrow.

Regulation of RANKL Protein Expression by PTH, Forskolin, and Calcitriol as Detected by Western Blotting

The effects of PTH, forskolin, and calcitriol on endogenous RANKL protein expression in UMR-106 cells were examined by Western blotting. Preliminary time course experiments revealed that 48 h treatment with PTH, forskolin, or calcitriol consistently resulted in an increase in RANKL protein expression, whereas responses were variable at 24 h (data not shown). Therefore all treatments were carried out for 48 h. The cells were treated with PTH (3–100 nM), forskolin (3–10 μM), or calcitriol (1–10 nM) for 48 h, whole cell lysates were collected and then subjected to SDS–PAGE.

PTH. Treatment of UMR-106 cells with PTH resulted in a dose dependent increase in RANKL protein expression. As shown in Figures 2 and 3 both the antibody to the C-terminus of RANKL (Fig. 2) as well as the antibody to the N-terminus (Fig. 3) detected a

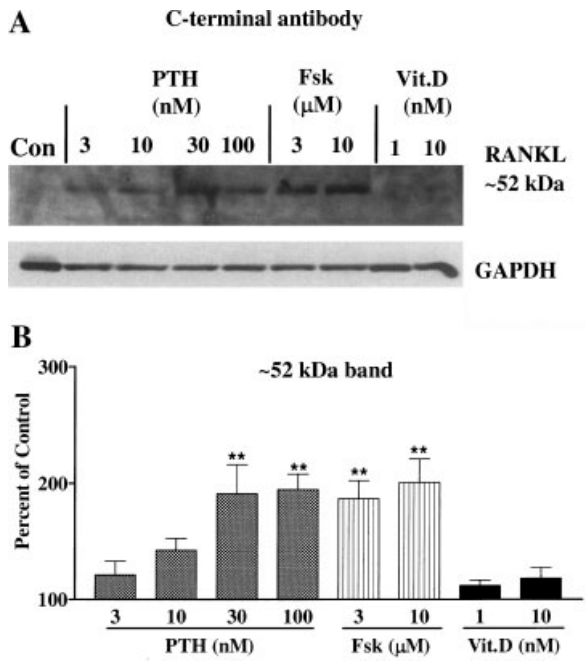

Fig. 2. PTH, forskolin, and calcitriol (Vit.D) increase RANKL protein expression in a dose dependent manner: Detection with the antibody to the C-terminus of RANKL. UMR-106 cells were treated with PTH (3–100 nM), forskolin (3–10 μM), or calcitriol (1–10 nM) for 48 h. Whole cell lysates were collected and subjected to SDS–PAGE on a 10% gel followed by Western blotting with an antibody generated to the C-terminus of RANKL. **A**: Representative Western blot. **B**: Quantitation of the ~52 kDa RANKL band. n = 3. RANKL values were normalized to GAPDH values. Data are presented as percent of control. $^{**}P < 0.01$ vs. control.

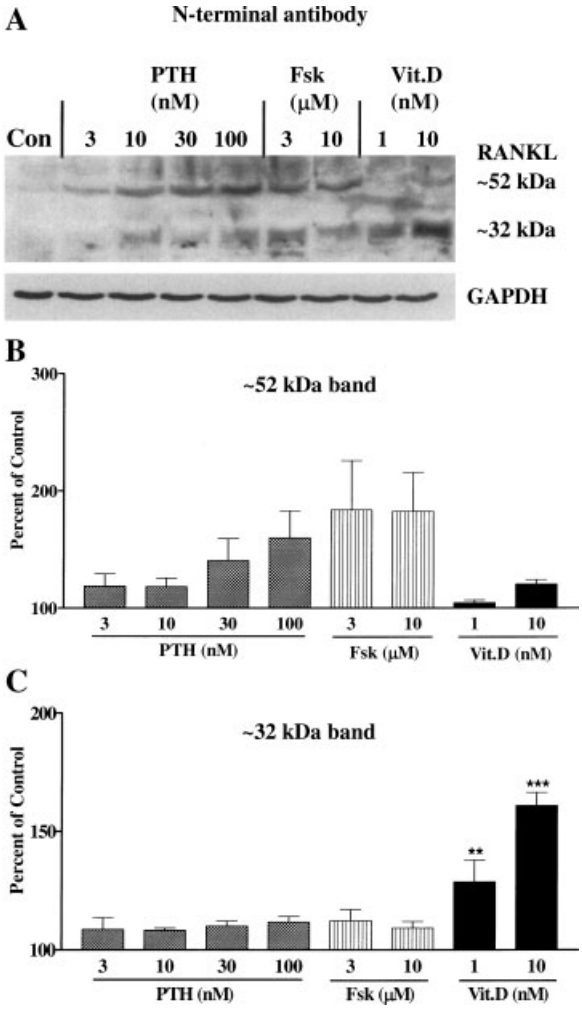

Fig. 3. PTH, forskolin, and calcitriol (Vit.D) increase RANKL protein expression in a dose dependent manner: Detection with the antibody to the N-terminus of RANKL. UMR-106 cells were treated with PTH (3–100 nM), forskolin (3–10 μM), or calcitriol (1–10 nM) for 48 h. Whole cell lysates were collected and subjected to SDS–PAGE on a 10% gel followed by Western blotting with an antibody generated to the N-terminus of RANKL. **A**: Representative Western blot showing that the antibody to the N-terminus of RANKL detects two bands. **B**: Quantitation of the ~52 kDa RANKL band. n = 3. **C**: Quantitation of the ~32 kDa band. n = 3. RANKL values were normalized to GAPDH values. Data are presented as percent of control. $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. control.

~52 kDa (estimated size was between 50 and 54 kDa in different experiments) RANKL band that was dose dependently increased by PTH. The Western blot using the antibody to the C-terminus of RANKL (Fig. 2) showed that PTH at concentrations of 30 and 100 nM significantly increased endogenous RANKL protein expression. The comparable size band detected by the antibody to the N-terminus of RANKL (Fig. 3) showed a similar trend in response to PTH, although the differences were not statistically significant.

The molecular weight of this band was higher than the previously reported molecular weight of 40–45 kDa [Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998] and could be due to post-translational modification of the RANKL protein. This higher than expected molecular weight of RANKL could result from post-translational glycosylation of the RANKL. To examine this possibility whole cell lysates from PTH treated cells were deglycosylated with N-glycosidase F followed by SDS–PAGE. As shown in Figure 4A, deglycosylation using N-glycosidase F did not alter the molecular weight of the RANKL band, suggesting that the increased molecular weight was not due to glycosylation. As a positive control, ribonuclease B

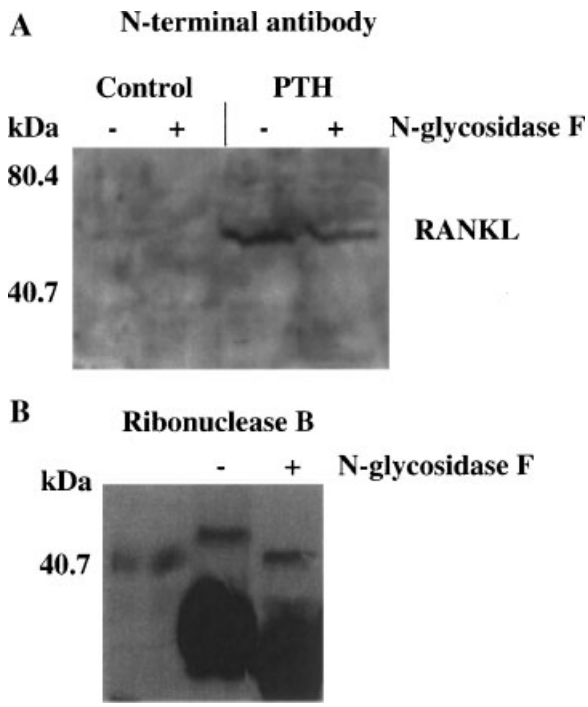

Fig. 4. Deglycosylation of RANKL with N-glycosidase F does not alter the molecular weight of RANKL protein. UMR-106 cells were treated with 30 nM PTH for 48 h. Whole cell lysates were collected and treated with N-glycosidase F. The samples were subjected to SDS–PAGE on a 12.5% gel followed by Western blotting using the antibody to the N-terminus of RANKL. As a positive control ribonuclease B was treated with N-glycosidase F under the same conditions and subjected to SDS–PAGE on a 12.5% gel followed by staining with Coomassie blue. **A**: Western blot showing that treatment with N-glycosidase F did not alter the molecular weight of RANKL protein. **B**: Coomassie stained gel showing the decrease in molecular weight of ribonuclease B after treatment with N-glycosidase F.

was deglycosylated under the same conditions as the whole cell lysates. N-glycosidase F treatment decreased the molecular weight of ribonuclease B indicating that the control protein had been deglycosylated (Fig. 4B).

Tunicamycin, an antibiotic that inhibits the glycosylation of proteins, was also used to examine the effects of lack of glycosylation on the molecular weight of RANKL. This treatment at the concentration used (5 μg/ml) also did not alter the molecular weight of RANKL (data not shown). Higher concentrations of tunicamycin were found to be toxic to the cells.

The antibody to the N-terminus of RANKL also detected a second band with a molecular weight of ~32 kDa (estimated size was between 30 and 34 kDa in different experiments), however, this band was not regulated by PTH (Fig. 3).

Forskolin. Cyclic AMP (cAMP) signaling appears to be the major pathway mediating PTH-stimulated RANKL mRNA expression [Fu et al., 2002; Kondo et al., 2002; Lee and Lorenzo, 2002]. We, therefore, examined the effect of activating the cAMP/protein kinase A (PKA) pathway on RANKL protein expression using the adenylate cyclase activator forskolin. As shown in Figures 2 and 3, forskolin dose dependently increased the expression of the ~52 kDa RANKL, with a statistically significant increase detected with the antibody to the C-terminus of RANKL (Fig. 2). A similar trend was seen with the antibody to the N-terminus of RANKL (Fig. 3). The ~32 kDa band detected by the antibody to the N-terminus of RANKL was not regulated by forskolin. Thus the effects of forskolin mimicked those of PTH.

Calcitriol. The effects of calcitriol on endogenous RANKL differed from those elicited by either PTH or forskolin treatment. A 48 h treatment with calcitriol failed to significantly affect the ~52 kDa band (Figs. 2 and 3). However, the ~32 kDa band detected by the antibody to the N-terminus of RANKL (Fig. 3) was dose dependently increased by 1 and 10 nM calcitriol. These increases were statistically significant.

The possibility was considered that the smaller molecular weight form of RANKL is the soluble form of RANKL (sRANKL) generated after post-translational processing of the full length form [Lum et al., 1999]. To test this, a dot blot of sRANKL (Peprotech) was probed with the C- and N-terminal RANKL antibodies. The N-terminal antibody that detected the ~32 kDa band in the calcitriol treated cells did not detect the soluble form of RANKL (Fig. 5). The C-terminal antibody, which can detect the soluble form of RANKL (Fig. 5), did not detect the ~32 kDa form of RANKL (Fig. 2). These findings suggested that the ~32 kDa band was not soluble RANKL.

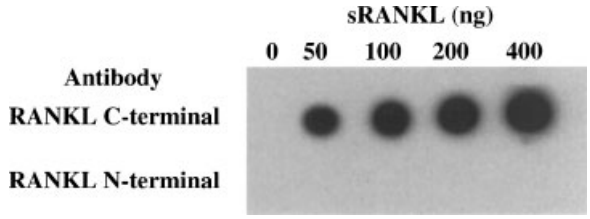

Fig. 5. Specificity of the RANKL antibodies. Recombinant sRANKL (50–400 ng in a volume of 20 μl) was spotted onto nitrocellulose. The membrane was cut into strips and blocked in blocking buffer. Each strip was incubated in either the antibody to the C-terminus of RANKL or the antibody to the N-terminus of RANKL. The strips were washed and incubated in the appropriate secondary antibody. Immunecomplexes were visualized with ECL.

Regulation of RANKL Protein Expression by PTH, Forskolin, and Calcitriol as Measured by sRANKL EIA

To further examine the effects of PTH, forskolin, and calcitriol on RANKL protein expression an enzyme immunoassay (EIA) that measures soluble RANKL (sRANKL), captured by binding to immobilized OPG was used. As stated in the manufacturer's protocol, this EIA was designed to measure levels of soluble RANKL in conditioned medium or serum. We attempted to measure the levels of RANKL in the conditioned medium following PTH, forskolin, and calcitriol treatment using the EIA. However, the concentration of sRANKL was below the lower detection limit of the kit (data not shown).

Since the C-terminus, which binds to OPG, is the same in both the soluble form of RANKL as well as the full length cell associated form, the EIA was used to evaluate endogenous RANKL levels in whole cell lysates following PTH, forskolin, or calcitriol treatment. PTH increased RANKL protein expression in a dose dependent manner with a significant increase measured following treatment with 100 nM PTH (Fig. 6). Forskolin also increased RANKL protein expression with a significant increase elicited by 3 μM forskolin (Fig. 6). One and 10 nM calcitriol elicited a large significant

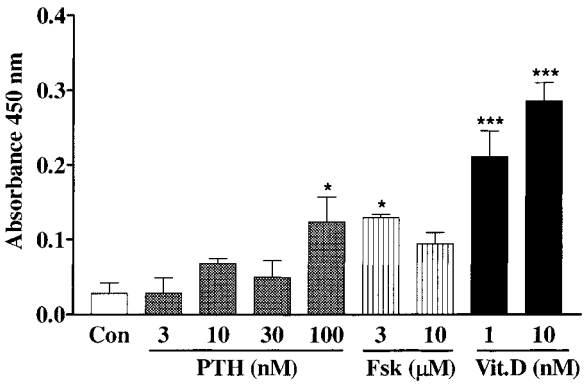

Fig. 6. PTH, forskolin, and calcitriol (Vit.D) increase RANKL protein expression in a dose dependent manner: sRANKL EIA. UMR-106 cells were treated with PTH (3–100 nM), forskolin (3–10 μM), or calcitriol (1–10 nM) for 48 h. Whole cell lysates were collected and RANKL protein levels were measured using a sRANKL EIA. Absorbance was measured at 450 nm. n = 3. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. control.

increase in RANKL protein expression as detected by the sRANKL EIA (Fig. 6).

Effect of Co-treatment on RANKL Protein Expression

Since the effects of PTH and forskolin on the size of endogenous RANKL protein differed from that of calcitriol, the effects of combination treatment were examined. UMR-106 cells were co-treated with PTH (30 nM) and calcitriol (10 nM) or with forskolin (10 μM) and calcitriol (10 nM) for 48 h. Following the treatments whole cell lysates were collected and analyzed by Western blotting and EIA.

As shown in Figure 7, the antibody to the C-terminus of RANKL again detected a significant increase in a ~52 kDa form of RANKL following PTH or forskolin treatment but no significant effect on this band following calcitriol treatment. Expression of the ~52 kDa band was increased in samples co-treated with PTH and calcitriol or forskolin and calcitriol over that of either PTH or forskolin treatment alone. With the antibody to the N-terminus (Fig. 8), a significant increase in a ~52 kDa form of RANKL was detected following PTH or forskolin treatment, and the smaller ~32 kDa form of RANKL was significantly increased by calcitriol treatment. Similar to what was seen with the antibody to the C-terminus, the antibody to the N-terminus detected an increase in the expression of the ~52 kDa form of RANKL in the co-treated samples over that elicited by PTH or

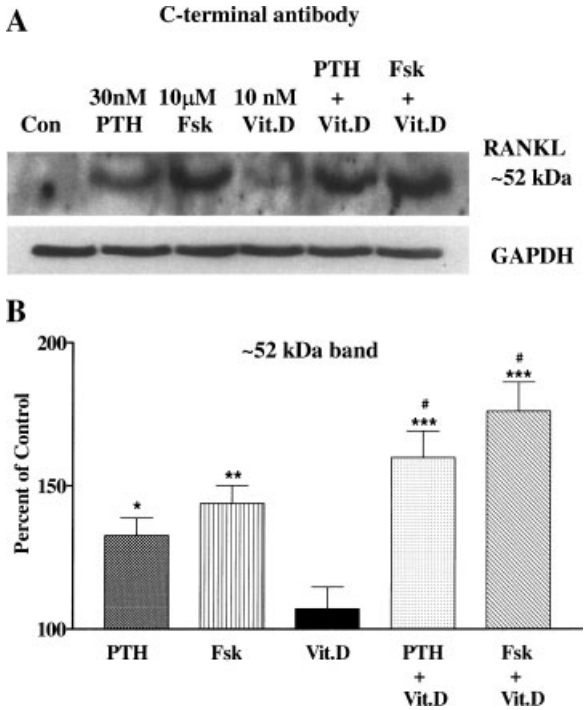

Fig. 7. Co-treatment of UMR-106 cells with PTH and calcitriol (Vit.D) or forskolin and calcitriol (Vit.D) increases the expression level of a ~52 kDa RANKL band detected by the antibody to the C-terminus of RANKL. UMR-106 cells were co-treated with 30 nM PTH and 10 nM calcitriol or 10 μM forskolin and 10 nM calcitriol for 48 h, cells were also treated for 48 h with each of the factors alone for comparison. Whole cell lysates were collected and subjected to SDS–PAGE on a 10% gel followed by Western blotting using the antibody to the C-terminus of RANKL. **A**: Representative western blot. **B**: Quantitation of the ~52 kDa band. RANKL values were normalized to GAPDH values. Data are presented as percent of control. n = 5. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. control, $^{\#}P < 0.05$ vs. PTH or forskolin alone.

forskolin alone (Fig. 8B). Interestingly, co-treatment decreased the level of expression of the ~32 kDa form of RANKL as compared to the response seen with calcitriol alone (Fig. 8C).

The sRANKL EIA was also used to assess the effects of co-treatment on endogenous RANKL protein expression (Fig. 9). As was seen previously (Fig. 6) 30 nM PTH and 10 μM forskolin failed to significantly affect RANKL protein as measured by the EIA, while calcitriol treatment elicited a large response. Co-treatment resulted in a decrease in RANKL protein levels compared to the response to calcitriol alone.

Effect of Co-treatment on RANKL mRNA Expression

To determine the effects of co-treatment on RANKL mRNA expression, RT-PCR was

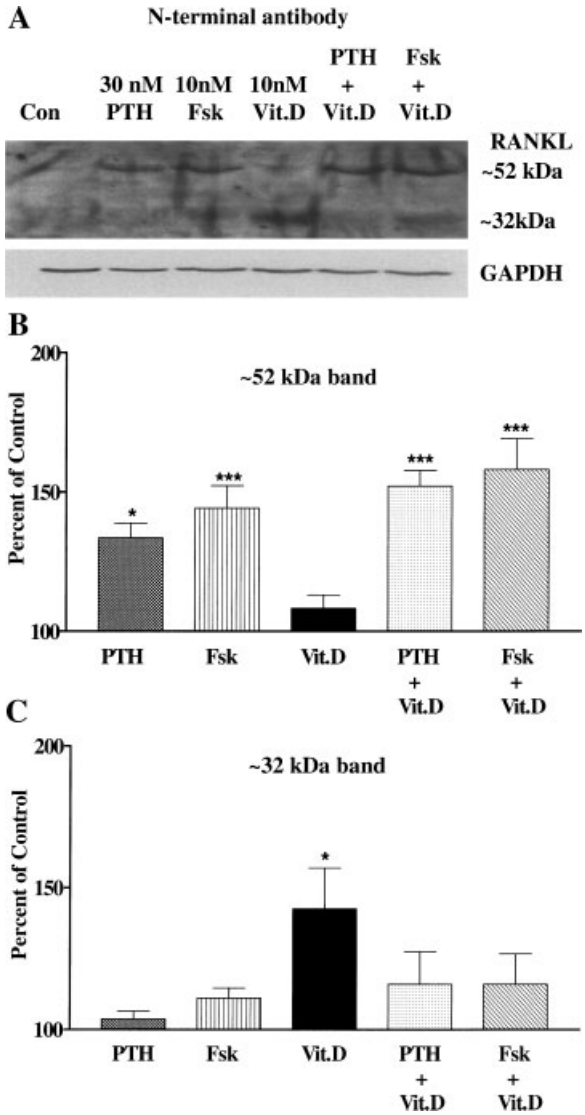

Fig. 8. Co-treatment of UMR-106 cells with PTH and calcitriol (Vit.D) or forskolin and calcitriol prevents the effect of calcitriol on the ~32 kDa RANKL band detected by the antibody to the N-terminus of RANKL. UMR-106 cells were co-treated with 30 nM PTH and 10 nM calcitriol or 10 μM forskolin and 10 nM calcitriol for 48 h, cells were also treated for 48 h with each of the factors alone for comparison. Whole cell lysates were collected and subjected to SDS–PAGE on a 10% gel followed by Western blotting using the antibody to the N-terminus of RANKL. **A**: Representative Western blot. **B**: Quantitation of the ~52 kDa RANKL band. n = 7. **C**: Quantitation of the ~32 kDa RANKL band n = 4. RANKL values were normalized to GAPDH values. Data are presented as percent of control. $^{*}P < 0.05$ vs. control, $^{***}P < 0.001$ vs. control.

carried out (Fig. 10). There was an increase in RANKL mRNA expression following treatment with PTH, forskolin, or calcitriol alone. An additive increase was seen following co-treatment with PTH and calcitriol or forskolin and calcitriol.

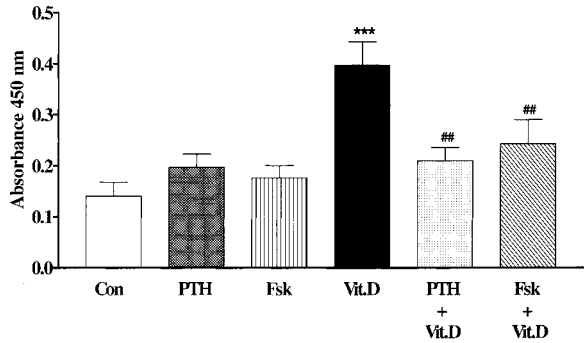

Fig. 9. Co-treatment of UMR-106 cells with PTH and calcitriol (Vit.D) or forskolin and calcitriol (Vit.D) prevents the effect of calcitriol on RANKL protein levels as measured by sRANKL EIA. UMR-106 cells were co-treated with 30 nM PTH and 10 nM calcitriol or 10 μM forskolin and 10 nM calcitriol for 48 h, cells were also treated with each of the factors alone for comparison. Whole cell lysates were collected and RANKL protein levels were measured using a sRANKL EIA. Absorbance was measured at 450 nm. n = 7. $^{***}P < 0.001$ vs. control, $^{\#\#}P < 0.01$ vs. calcitriol alone.

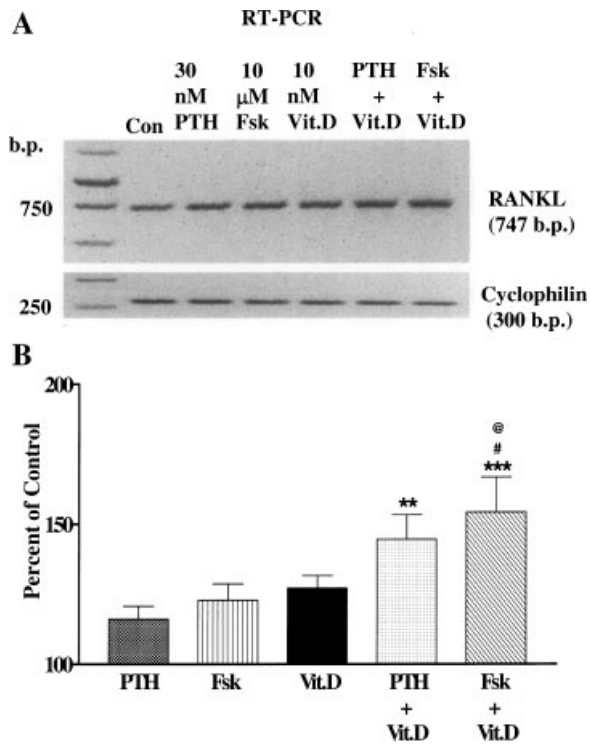

Fig. 10. PTH, forskolin and calcitriol (Vit.D) treatment increase RANKL mRNA expression, and co-treatment with PTH and calcitriol or forskolin and calcitriol elicits an additive effect. UMR-106 cells were treated with 10 nM PTH, 10 μM forskolin, 10 nM calcitriol, or cotreated with PTH and calcitriol or forskolin and calcitriol for 16 h. Total RNA was extracted and subjected to first strand cDNA synthesis followed by RT-PCR using RANKL and cyclophilin specific primers. **A**: Representative DNA gel. **B**: Quantitation of the RANKL PCR product. RANKL values were normalized to cyclophilin values. Data are presented as percent of control. n = 4. $^{**}P < 0.01$ vs. control, $^{***}P < 0.001$ vs. control, $^{\#}P < 0.05$ vs. forskolin alone, $^{@}P < 0.05$ vs. calcitriol alone.

DISCUSSION

RANKL is an essential regulator of bone resorption, and as such it is crucial to understand how the endogenous protein is regulated by bone resorptive factors. In this study we used Western blotting and a sRANKL EIA to examine the effects of PTH and calcitriol treatment on endogenous RANKL protein expression. Our studies reveal that endogenous RANKL is expressed as a larger size protein when UMR-106 cells are stimulated by PTH or forskolin compared to when they are stimulated by calcitriol (Fig. 3). Co-treatment of the cells with calcitriol and PTH or with calcitriol and forskolin antagonized the effect of calcitriol on the generation of the smaller molecular weight form of RANKL (Fig. 8).

For Western blotting, endogenous RANKL protein in UMR-106 whole cell lysates was detected using antibodies generated to either the C-terminus or N-terminus of the RANKL protein. While both of the antibodies used in this study were able to detect RANKL, they gave different patterns of expression. The N-terminal antibody detected both a ~52 and ~32 kDa band. The C-terminal antibody also detected the ~52 kDa band but did not detect the ~32 kDa band. The detection of different molecular weight bands suggests the presence of isoforms of RANKL in the UMR-106 cells.

The ~52 kDa form of RANKL that was detected by both antibodies was found to be regulated by PTH and forskolin (Figs. 2 and 3). A molecular weight of ~52 kDa is higher than the previously reported molecular weight for RANKL of 40–45 kDa [Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998]. Possible mechanisms through which RANKL could have a higher than expected molecular weight include post-translational processing, and the formation of dimers or trimers. RANKL contains two potential glycosylation sites in the C-terminal domain [Wong et al., 1997; Willard et al., 2000], therefore, it is possible that glycosylation of RANKL increased the molecular weight of the protein. Other groups have also detected RANKL at a higher than expected molecular weight and each group suggested that glycosylation of RANKL may be responsible for the increased molecular weight [Wong et al., 1997; Lum et al., 1999; Nagai et al., 2000]. One group reported that deglycosylation decreased the molecular weight of the RANKL band to the expected molecular weight [Lum et al., 1999]. However in our study (Fig. 4) deglycosylation of the RANKL protein from UMR-106 cells had no effect on the molecular weight of RANKL.

Another possible mechanism that could increase the apparent molecular weight of RANKL is the formation of dimers or trimers. The crystal structure of the extracellular domain of RANKL indicates that it can form trimers, however the interactions between these domains is non-covalent [Lam et al., 2001; Ito et al., 2002]. Since the Western blot experiments in this study were done under reducing conditions, which should dissociate such complexes, this would suggest that the increased molecular weight is not due to the presence of RANKL dimers or trimers.

The ~32 kDa form, detected in the UMR-106 cells by the N-terminal antibody, appears to be a novel product. Because of its molecular weight, it was considered that the ~32 kDa form of RANKL could be the soluble form of RANKL generated by post translational processing of the full length form of RANKL. However, since the N-terminus of the protein is intracellular, this seems unlikely. Also, the dot blot (Fig. 5) shows that the N-terminal antibody cannot detect the soluble form of RANKL, whereas C-terminal antibody that does not detect the ~32 kDa band was able to detect the soluble form of RANKL on the dot blot. These findings indicate that this band is not soluble RANKL, and support the possibility that the two forms of RANKL detected by the N-terminal antibody are two distinct cellular forms of RANKL.

The possibility that the ~32 kDa band was an artifact due to proteolytic degradation during preparation of the lysates appears unlikely, since the whole cell lysates are collected in a lysis buffer containing protease inhibitors to prevent the degradation of proteins. We also examined the effects of collecting the whole cell lysates directly in electrophoresis buffer immediately followed by SDS–PAGE, and found that this method of sample collection resulted in the same pattern of RANKL expression as in the samples collected as described in "Materials and Methods" (data not shown).

The presence of two forms of RANKL in UMR-106 cells that appear to be differentially regulated by PTH and calcitriol suggests that these cells can express more than one isoform of RANKL. The presence of RANKL isoforms in

mouse and human cells has been previously reported [Ikeda et al., 2001, 2003; Suzuki et al., 2004]. Although post translational processing seems the most likely mechanism for generation of the iosforms, protein isoforms can also arise through alternative splicing from distinct promoters [Zennaro et al., 1996; Catalano et al., 2003; Itani et al., 2003; Seth et al., 2003]. Although beyond the scope of this study, this possibility cannot be ruled out for RANKL.

PTH has been shown to regulate RANKL expression through the cAMP/PKA signaling pathway [Fu et al., 2002; Kondo et al., 2002; Lee and Lorenzo, 2002]. We previously found that PTH and forskolin increase RANKL mRNA expression in UMR-106 cells dose and time dependently [Dossing and Stern, 2003]. The results of the current study reveal that endogenous ~52 kDa RANKL protein expression is also dose dependently regulated by PTH, and that these effects are mirrored by forskolin, suggesting that this regulation involves the cAMP/PKA signaling pathway.

Calcitriol has been shown to increase RANKL mRNA expression in several cell lines [Horwood et al., 1998; Kitazawa and Kitazawa, 2001; Kitazawa et al., 2003; Lerner, 2004]. Our current study shows that calcitriol dose dependently regulates the expression of endogenous RANKL protein, specifically the ~32 kDa form of the RANKL protein. The decrease in the lower molecular weight band following co-treatment with calcitriol and PTH or forskolin, with a subsequent increase in the higher molecular weight band, suggests that the treatments affect processing of the RANKL protein. The ~32 kDa form of RANKL that is increased by calcitriol could be processed to the higher molecular weight form by a mechanism that requires cAMP. It is also possible that calcitriol is capable of processing the larger form into the smaller molecular weight form and that this mechanism is inhibited by cAMP. The RT-PCR measurements (Fig. 10), indicate that net changes in mRNA do not explain the interaction, since the co-treatments resulted in additive responses on RANKL mRNA.

An EIA to sRANKL that is based on RANKL binding to OPG was also used to examine the effects of PTH and calcitriol on the regulation of RANKL protein. Comparison of the results obtained using the EIA with those obtained by Western blotting suggest that results obtained using the sRANKL EIA reflect mainly responses of the ~32 kDa form of RANKL that was detected by the N-terminal antibody since both were markedly increased by calcitriol. The results obtained using the EIA in the co-treatment studies also seem to correspond mainly to changes in the ~32 kDa form of RANKL. Co-treatment with calcitriol together with PTH or forskolin reduced the effect of calcitriol as assayed by either the EIA or the amount of the ~32 kDa band. However, while it appeared that the sRANKL EIA primarily detected the changes elicited by calcitriol, it also revealed small but significant increases in RANKL protein expression in cells treated with 100 nM PTH or 3 μM forskolin (Fig. 6). These same concentrations of PTH and forskolin did not increase the ~32 kDa band, thus the EIA must be able to detect the ~52 kDa form. However the relative responses to PTH and forskolin compared to those elicited by calcitriol as detected by EIA were less than the sum of the protein bands detected by Western blotting. A possible explanation for the differences in the levels of RANKL detected by Western blotting and by the EIA is that the recombinant OPG bound to the plate does not recognize or bind as well to the higher molecular weight form of RANKL that is detected by the antibodies used for Western blotting. If the increased apparent molecular weight of RANKL is due to a post-translational modification of the protein, this modification could interfere with the ability of the EIA to detect the higher molecular weight form of RANKL in the whole cell lysates.

In summary, the studies presented demonstrate that, in UMR-106 cells, PTH and calcitriol induce the expression of different forms of endogenous RANKL protein. Cyclic AMP/PKA signaling and PTH induce the expression of a ~52 kDa form of endogenous RANKL protein while calcitriol induces the expression of a ~32 kDa form of RANKL. In the presence of calcitriol, together with either PTH or forskolin, the formation of the higher molecular weight form predominates and little or none of the lower molecular weight form is seen. The lower molecular weight form appears to interact more strongly with OPG. Since OPG is a physiological regulator of RANKL action, the findings could provide a mechanism by which different agonists could result in distinct effects in target cells.

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