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Baculovirus-Expressed Vitamin D-Binding Protein-Macrophage Activating Factor (DBP-*maf*) Activates Osteoclasts and Binding of 25-Hydroxyvitamin D_3 Does not Influence This Activity

Narasimha Swamy,[1]* Sujoy Ghosh,[2] Gary B. Schneider,[3] and Rahul Ray[1]*

[1]Bioorganic Chemistry and Structural Biology Group, Vitamin D Laboratory, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118
[2]Department of Functional Genetics, GlaxoWellcome R&D, 5 Moore Drive, RTP, North Carolina 27709
[3]Divison of Basic Medical Sciences Northeastern Ohio Universities College of Medicine, Rootstown, Ohio 44272

Abstract Vitamin D-binding protein (DBP) is a multi-functional serum protein that is converted to vitamin D-binding protein-macrophage activating factor (DBP-*maf*) by post-translational modification. DBP-*maf* is a new cytokine that mediates bone resorption by activating osteoclasts, which are responsible for resorption of bone. Defective osteoclast activation leads to disorders like osteopetrosis, characterized by excessive accumulation of bone mass. Previous studies demonstrated that two nonallelic mutations in the rat with osteopetrosis have independent defects in the cascade involved in the conversion of DBP to DBP-*maf*. The skeletal defects associated with osteopetrosis are corrected in these mutants with in vivo DBP-*maf* treatment. This study evaluates the effects of various forms of DBP-*maf* (native, recombinant, and 25-hydroxyvitamin D_3 bound) on osteoclast function in vitro in order to determine some of the structural requirements of this protein that relate to bone resorbing activities. Osteoclast activity was determined by evaluating pit formation using osteoclasts, isolated from the long bones of newborn rats, incubated on calcium phosphate coated, thin film, Ostologic MultiTest Slides. Incubation of osteoclasts with ex vivo generated native DBP-*maf* resulted in a dose dependent, statistically significant, activation of the osteoclasts. The activation was similar whether or not the vitamin D binding site of the DBP-*maf* was occupied. The level of activity in response to DBP-*maf* was greater than that elicited by optimal doses of other known stimulators (PTH and $1,25(OH)_2D_3$) of osteoclast function. Furthermore, another potent macrophage activating factor, interferon–gamma, had no effect on osteoclast activity. The activated form of a full length recombinant DBP, expressed in *E. coli* showed no activity in the in vitro assay. Contrary to this finding, baculovirus-expressed recombinant DBP-*maf* demonstrated significant osteoclast activating activity. The normal conversion of DBP to DBP-*maf* requires the selective removal of galactose and sialic acid from the third domain of the protein. Hence, the differential effects of the two recombinant forms of DBP-*maf* is most likely related to glycosylation; *E. coli* expressed recombinant DBP is non-glycosylated, whereas the baculovirus expressed form is glycosylated. These data support the essential role of glycosylation for the osteoclast activating property of DBP-*maf*. J. Cell. Biochem. 81:535–546, 2001.

Key words: vitamin D–binding protein (DBP); vitamin D–binding protein macrophage activating factor (DBP-*maf*); recombinant DBP-*maf*; osteoclast activation; bone resorption

INTRODUCTION

Vitamin D-binding protein (DBP) is a novel serum protein that exhibits multiple functions. These include (i) binding and transportation of vitamin D metabolites, (ii) sequesteration of G-actin to prevent the formation of F-actin, (iii) binding of fatty acids and chemotactic agents

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*Correspondence to: Narasimha Swamy and Rahul Ray, Boston University School of Medicine, 715 Albany Street, M-1010 Boston, MA 02118.

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such as C5desARG, and (iv) activation of macrophages and osteoclasts [reviewed in Cooke and Haddad, 1989; Haddad, 1995; Ray, 1996]. DBP has been recently shown to be a ligand for megalin playing a crucial role in sustaining plasma 25-hydroxyvitamin D_3 (25-OH-D_3) by re-uptake in the kidney [Nykjaer et al., 1999].

DBP (also called Group specific component, Gc) is an evolutionarily conserved but genetically polymorphic glycoprotein. Gc1 and Gc2 are the major phenotypes. The nucleotide sequence of Gc1 and Gc2 genes and deduced amino acid sequences have been reported [Yang et al., 1985; Cooke and David, 1985; Cooke, 1986; Schoentgen et al., 1986; Ray et al., 1991]. The two phenotypes differ in only four amino acids (152, 311, 416, and 420). Gc1 is further divided into two subtypes, Gc1s and Gc1f (based on electrophoretic mobility, 's' for slow moving and 'f' for fast moving). Structurally DBP is highly homologous to serum albumin and has a triple domained modular structure (the three domains termed domain I, II, and III) (Fig. 1A) [Brown, 1979].

DBP has 28 Cys residues, which form 14 disulfide bonds leading to the formation of these domains. In the N-terminus, domain I spans about 200 amino acids and is shown to be responsible for vitamin D sterol-binding [reviewed in Ray, 1996]. Domain I is stabilized by five disulfide bonds and contains the only Trp (145) residue which is involved in vitamin D sterol-binding [Swamy et al., 1995a]. In the C-terminus, domain III spans about 85 amino acid residues (starting from amino acid residue 375)

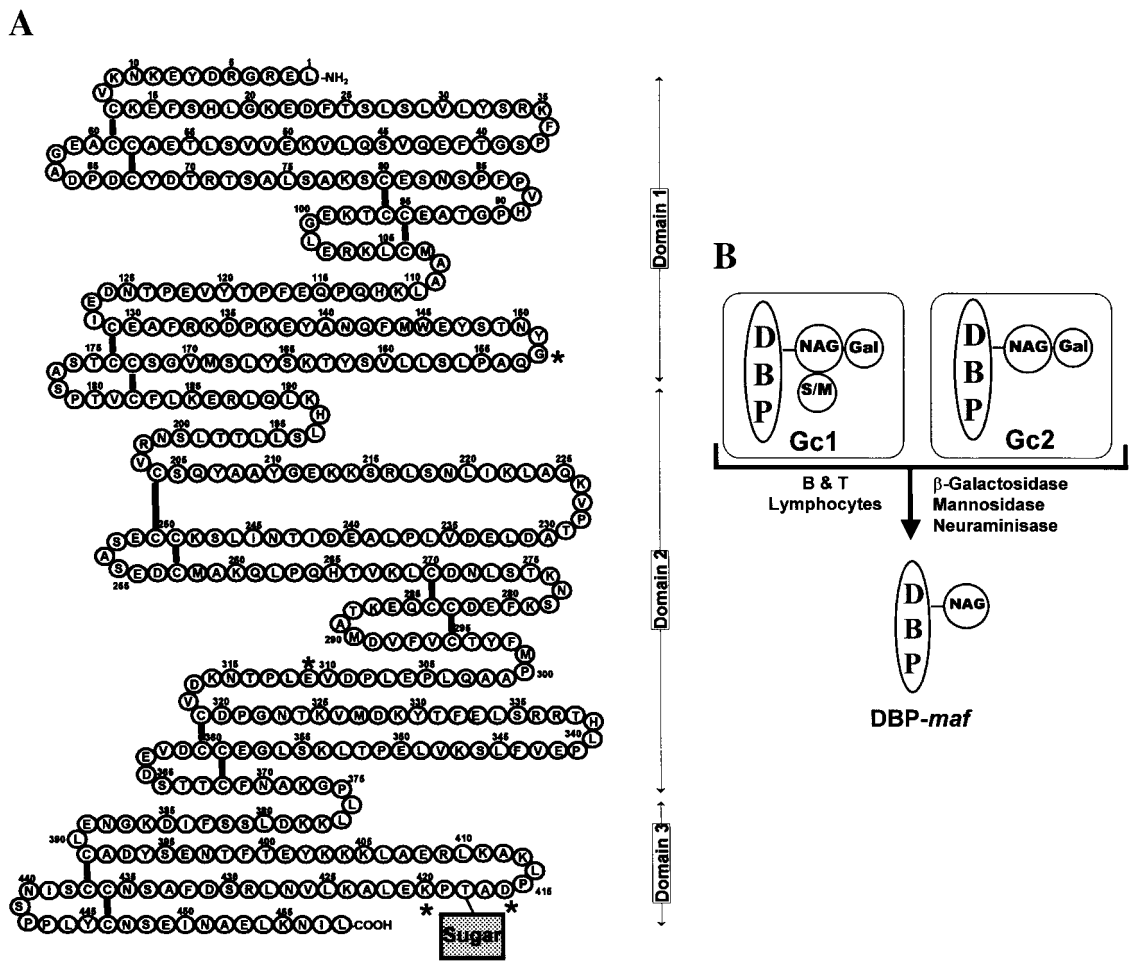

Fig. 1. **A:** Amino acid sequence of human DBP (Gc2) in albumin format. In case of Gc1 the amino acid sequence is same except for amino acid residues 152 is Glu, 311 is Arg, 416416 is Glu and 420 is Thr (marked with a star symbol). **B:** Schematic representation of conversion of DBP to DBP-*maf*. DBP is converted to DBP-*maf* by the action of β-galactosidase, mannosidase and sialidase of B-and T-lymphocytes. Gc1 and Gc2 are the genetic isomorphs of DBP. NAG: N-acetyl galactosamine, Gal: galactose, S: sialic acid, and M: mannose.

to the carboxy terminal residue and is stabilized by two disulfide bonds. Domain II is about 175 amino acids long and is stabilized by six disulfide bonds. Studies from many groups have revealed that Domains II and III are responsible for non-vitamin D-binding activities of DBP, like G-actin- and fatty acid binding and macrophage activation [reviewed in Haddad, 1995; Ray, 1996].

The C-terminal end of DBP (domain III) harbors a single glycosylation site in the vicinity of amino acids 416–420. In Gc1; the glycosylation site contains a trisaccharide with N-acetylgalactosamine attached to the core protein and a terminal galactosyl moiety; which branches with either a sialic acid (in Gc1f) or mannose (Gc1s). In Gc2 and rat DBP; the glycosylation pattern is simple with a core N-acetylgalactosamine linked to a terminal galactose moiety [Svasti et al., 1979; Viau et al., 1983; Cooke and Haddad, 1989; Yamamoto et al., 1991; Yamamoto et al., 1994]. The glycosylation of DBP imparts its newly discovered function of macrophage and osteoclast activation (illustrated in Fig. 1B).

The role of DBP in inflammation mediated activation of macrophages was established by Yamamoto et al. [1991] and Yamamoto and Homma [1991]. It was shown that DBP is converted to a potent macrophage-activating factor termed as DBP-*maf* by a cascade of reactions by membrane-bound inducible isoforms of β-galactosidase and sialidase of B- and T-lymphocytes. The events responsible for the conversion of DBP to DBP-*maf* by B- and T-lymphocytes were also demonstrated by an in vitro system utilizing peritoneal cells containing adherent macrophages and non-adherent B- and T-cells [Ngwenya and Yamamoto, 1990]. Furthermore, Yamamoto and coworkers treated purified DBP with β- galactosidase, α-mannosidase ,and sialidase to generate DBP-*maf* ex vivo [Yamamoto and Kumashiro, 1993].

Yamamoto and co-workers demonstrated that the treatment of peritoneal macrophages with sub-nano gram quantities of DBP-*maf* increased the superoxide production by macrophages and ingestion of erythrocyte ghosts [Yamamoto et al., 1991; Yamamoto and Homma, 1991]. In addition, DBP-*maf* was also shown to be a potent activator of osteoclasts [Schneider and Popoff, 1994; Schneider et al., 1995]. Although osteoclasts and macrophages are highly differentiated cell-types with distinct site specific functions, it is well known that they originate from a common progenitor cell and share common structural and functional characteristics [Marks and Popoff, 1988]. For example, oxygen radicals including superoxide radicals play an important role in the mechanism of action of macrophages and bone resorption by osteoclasts. It has been postulated that a defective superoxide production may be related to reduced bone resorption in osteopetrosis — a rare disorder of bone mass accumulation due to reduced osteoclastic activity [Garrett et al., 1990; Key et al., 1992].

It is postulated that glycosylation of DBP is imperative in its macrophage-and osteoclast-activating abilities (*maf*-activities). In order to test this hypothesis, we expressed DBP in bacteria to generate recombinant DBP (reDBP) which lacks glycosylation. We also developed a baculovirus expression system, which is well known to glycosylate the recombinant proteins [Altmann et al., 1999], for the expression of glycosylated reDBP and studied the requirement of the core-N-acetylgalactosamine moiety for osteoclast activation by DBP-*maf*.

Furthermore, DBP is a multifunctional protein and different functions are known to be associated with its different structural domains, as stated earlier. However, the influence of the bound ligands (of DBP) on *maf*-activity is yet to be determined. In the present study, we investigated the influence of bound 25-OH-D_3 (to DBP-*maf*) on the osteoclast activation by DBP-maf.

MATERIALS AND METHODS

Sialidase, α-mannosidase, and β-galactosidase were obtained from Roche Molecular Biochemicals (Indianapolis, IN); CNBr-activated Sepharose 4B was from, Sigma chemical company (St. Louis MO). Bac-to-Bac baculovirus expression system, Bluo-gal, isopropylthiogalactoside (IPTG), serum free media (SFM) adapted Sf9 cells, SFM, culture media, and additives were from Life Technologies (Grand Island, NY). Sf9 cells were cultured and maintained in serum-free medium (SFM) as per manufacturer's recommendations. DBP was purified from pooled human serum according to a previously described procedure [Swamy et al., 1995], 25-OH-D_3-3-BE was synthesized as described previously [Haddad et al., 1992; Swamy and Ray, 1996]. Osteologic MultiTest Slides

were from Millenium Biologix Inc., (Kingston, Ontario, Canada).

Development of Baculovirus Expression System for Expression of Glycosylated Recombinant DBP (reDBP-bv)

Bac-to-Bac baculovirus expression system was used for the expression of full-length glycosylated DBP. The Bac-to-Bac baculovirus expression system is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*. The bacmid contains a low-copy-number mini-F replicon, a kanamycin resistance marker and a segment of DNA encoding the *LacZ*α peptide. A short segment of an attachment site for the bacterial transposon Tn7 (mini-*att*Tn7) is inserted into the N-terminus of the *LacZ*α gene in order to facilitate the blue–white selection of the recombinants by a chromogenic substrate such as Bluo-gal and the inducer, IPTG.

The baculovirus expression of DBP was carried out in four steps. In the first step, the coding sequence of Gc2 was cloned into pFast-Bac HTb baculovirus expression vector. The coding sequence of Gc2 was excised from pGEX-DBP [Swamy et al., 1997] using Bam HI and Not I restriction enzymes and cloned into pFastBac HTb digested similarly with Bam HI and Not I restriction enzymes to generate pHTb-Gc2. pFastBac HTb vector contains a transcription start site driven by the baculovirus polyhedrin promoter followed by the His-tag (a sequence of six histidyl residues) and a rTEV protease site. The presence of the His-tag aids in purification of the recombinant protein from the cell lysate by nickel affinity chromatography. The rTEV protease digestion site is positioned between the His-tag and the recombinant protein of interest and the digestion of the isolated recombinant protein with rTEV protease releases the His-tag from the protein of interest (in the present case reDBP-bv).

In the second step, engineered baculovirus (recombinant bacmid) carrying coding sequence of Gc2 was generated from pHTb-Gc2 construct by transposition with the wild type baculovirus shuttle vector, bacmid (bMON14272) in *E. coli* host DH10Bac according to the procedure developed by Luckow et al. [1993]. This step generated *E. coli* carrying recombinant bacmid-Gc2 DNA.

In the third step, bacmid-Gc2 DNA was converted to live recombinant virion by the lipid transfection procedure into Sf9 insect cells using CellFECTIN reagent according to the manufacturer's recommendations (during this post transfection period live recombinant virion was generated and found in culture media). After 72 h of transfection, the supernatant containing recombinant baculovirus was harvested by centrifugation (500 ×g at 4°C) for 5 min and stored at 4°C. At this stage the presence of the Gc2 cDNA was confirmed using PCR. Using this initial stock of re-baculovirus, a high titer stock was generated by infecting a 100 ml culture of Sf9 cells in SFM. Sf9 cells (growing in a mid log phase) in 100 ml of SFM was treated with 1.0 ml of primary viral stock of 20×10^6 pfu/ml (pfu-viral plaque forming unit) and grown for 48 h. The supernatant contained approximately 100 fold amplified viral preparation to generate high titer viral preparation or high multiplicity of infection (MOI) determined according to manufacturer's recommendations. In general, experimental procedures recommended by the manufacturer were followed.

In the fourth step, the high titer re-baculovirus generated in the previous step was used for expression of reDBP-bv. Growing Sf9 cells in SFM (25 ml) in mid log phase were infected with 5–10 MOI and grown at 27°C in a suspension culture. Samples of 1.0 ml were withdrawn at 24, 48, 72, and 96 h and cells were collected by centrifugation (500 × g at 4°C) for 5 minutes. The cells were resuspended in 0.1 ml PBS, treated with SDS-PAGE sample buffer, and boiled for 5 minutes. The samples were separated on 10% SDS-PAGE and immunoblotted using anti-DBP-antibody (Diasorin, Stillwater, MN). The intensity of the corresponding immunoreactive band indicated the expression level of reDBP-bv. Judging by the intensities of the bands on the immunoblot, 48–72 h of growth after transfection was found to be optimum period for harvesting (data not shown). Manufacturer's recommended procedures were used throughout the experiment.

Expression and Purification of reDBP-bv in Baculovirus Expression System

reDBP-bv was expressed in scaled up quantities. Sf9 cells growing in SFM (500 ml) in the mid-log phase was infected with 50–100 MOI of recombinant Gc2-baculovirus and grown for

48–60 h. The cells were harvested by centrifugation (500x*g* at 4°C) for 5 minutes. The cell pellet was washed once with TBS (50 mM Tris HCl, pH 8.0, 150 mM NaCl) and resuspended in lysis buffer (20 mM Tris HCl, pH 8.3, 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM PMSF, 1% NP40) at 4°C at a ratio of 5 ml/gm cells pellet. The cells were mixed gently on an end to end mixer and briefly sonicated in a cup-horn sonicator. All operations were carried out at 4°C. The cell debris was removed by centrifugation at 4°C at 10,000 × g for 10 min and the supernatant was loaded onto a nickel–agarose affinity column pre-equilibrated with 20 mM Tris HCl, pH 8.3, 5 mM 2-mercaptoethanol, 500 mM KCl, 20 mM imidazole, and 10% glycerol. The column was washed once with 20 mM Tris HCl, pH 8.3, 5 mM 2-mercaptoethanol, 100 mM KCl, 10% glycerol, followed by once with 20 mM Tris HCl, pH 8.3, 5 mM 2-mercaptoethanol, 500 mM KCl, 20 mM imidazole, and 10% glycerol, and finally with 20 mM Tris HCl, pH 8.3, 5 mM 2-mercaptoethanol, 100 mM KCl, 10% glycerol. The bound-protein was eluted with 20 mM Tris HCl, pH 8.3, 5 mM 2-mercaptoethanol, 100 mM KCl, 100 mM imidazole, and 10% glycerol. The purified reDBP-bv was dialyzed against PBS, and stored at −80°C in aliquots. The samples were analyzed by 10% SDS-PAGE alongside molecular weight markers, and native DBP (isolated from human serum by 25-OH-D_3 affinity chromatography).

A bacterial expression system was used to generate non-glycosylated version of DBP (reDBP-ec) in *E. coli* as described previously [Swamy et al., 1997].

25-OH-D_3-Binding Assays

Competitive radioligand binding assays were carried out according to the published procedure [Swamy et al., 1997a]. The solutions containing reDBP-bv or native DBP (400 ng each), ^{3}H-25-OH-D_3 (3,750 cpm, 0.17 pmol), and 25-OH-D_3 (0.99–63.8 pmol) in 10 μl of ethanol, and 490 μl of assay buffer (50 mM Tris.HCl, 150 mM sodium chloride, 1.5 mM ethylenediaminetetraacetic acid, and 0.1% Triton X 100) were incubated at 4°C for 16 h followed by treatment with ice-cold Dextran-coated charcoal, and centrifugation. Supernatants from centrifuged samples were mixed with scintillation cocktail and counted for radioactivity.

Preparation of 25-OH-D_3-DBP

DBP (2 nmol) was incubated with 25-OH-D_3-3-BE (4 nmol) spiked with ^{14}C-25-OH-D_3-3-BE [Swamy et al., 1997a] in 0.1 ml of 50 mM Tris HCl buffer pH 8.3 for 12 h at 4°C followed by 4 h at 25°C. The excess ligand was separated from the protein by hydroxylapatite chromatography as described previously [Swamy et al., 1995a]. The recovery of radioactivity showed that over 98% of the added ligand (25-OH-D_3-3-BE) was associated with DBP. The covalent attachment of 25-OH-D_3-3-BE to DBP was confirmed by SDS-PAGE [Swamy et al., 1997a]. This procedure resulted in 25-OH-D_3 covalently coupled to DBP (25-OH-D_3-DBP).

Preparation of DBP-*maf*, reDBP-bv–*maf*, reDBP-ec–*maf* and 25-OH-D_3-DBP-*maf*

DBP-*maf*, reDBP-*maf*s, and 25-OH-D_3-DBP-*maf* were prepared from DBP (isolated from human serum), reDBPs and 25-OH-D_3-DBP respectively by treatment with immobilized sialidase, α-mannosidase, and β-galactosidase by following the reported procedure with suitable modifications [Yamamoto and Kumashiro, 1993].

Briefly, sialidase, α-mannosidase, and β-galactosidase (two units each) were mixed with 0.6 g of CNBr activated Sepharose (prewashed with 1 mM HCl) in a coupling buffer (0.1 M $NaHCO_3$ pH, 8.3 containing 0.5 M NaCl) at 25°C in an end to end shaker for 4–6 h. The excess reactive sites (on CNBr activated Sepharose) were blocked by incubating with 0.2 M glycine in coupling buffer. The Sepharose immobilized enzymes were washed with coupling buffer to remove free proteins and glycine followed by 0.1 M acetate buffer, pH 4 containing 0.5 M NaCl. The immobilized enzymes were washed with coupling buffer and stored at 4°C until required.

Various DBP preparations (100 μg) were incubated with a mixture of immobilized sialidase, α-mannosidase, and β-galactosidase (0.2 units of activity each) in PBS-Mg (10 mM sodium phosphate buffer pH 5.5, 0.9% sodium chloride and 1mM $MgSO_4$) at 37°C in an end-to-end shaker for 4 h. The gel was sedimented by centrifugation at 600 × g at 4°C for 5 minutes. The supernatant (containing DBP-*maf* preparations) was collected and sterilized by filtering through a 0.22-μm filter.

Osteoclast Activation Analysis

Osteoclast activation by various DBP-*maf* preparations was carried out by measuring the pit resorption on osteologic discs by osteoclasts. The osteoclasts were isolated from long bones of newborn rats by using standardized procedure of Chambers and Dunn [1983] with suitable modification. The femurs, tibiae, and humeri from newborn rats were rinsed several times with α-MEM (minimal essential medium) supplemented with 10% fetal calf serum, penicillin G, Gentamicin, fungizone, ascorbic acid, and sodium β-glycero-phosphate. The harvesting of the bones was carried out by splitting them longitudinally (after the epiphyseal ends were cut off), followed by curretting with a scalpel and agitating the bone fragments using a wide-mouth pipette. After the large fragments settled (10–15 s) the remaining suspension was aspirated out and cells were concentrated by slow speed spinning in a microcentrifuge tube. The loose pellet was resuspended in 0.5 ml culture media (see above), pH reduced to 6.5–6.8, and plated onto Osteologic MultiTest Slides (quartz slides coated with hydroxylapatite thin film). The osteoclasts were allowed to attach to the slides for 30 min, the non-adherent cells were removed by gentle wash, and the adherent cells were incubated for 72 h in the presence of different concentrations of various DBP-*maf* preparations or interferon-γ (0.1, 0.2, 0.5, and 1.0 ng). PTH (1–34) and 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), known activators of osteoclasts, were used as controls at concentrations of 0.5 IU/ml and 10^{-8} M respectively.

Osteoclast activity was measured by quantitating the pit area generated by the action of osteoclasts [Davies et al., 1993]. The osteoclast mediated pit resorption was quantitated using an Osteologic analyzer (Microst). Briefly, the slides, after incubation with various DBP-*maf* preparations for 72 h, were washed thoroughly with distilled water, and incubated in distilled water for 3 h to remove all the cells. The pits were stained with 10% silver nitrate solution overnight. The discs were washed and analyzed by Osteologic analyzer (Microst) in order to quantitate the percent surface area resorbed on the slides. Multiple cultures ($n = 6$) of each dose was assessed and analyzed by one-way analysis of variance. The percent pit area resorbed was plotted against the amount of the osteoclast activating agent used under question.

RESULTS AND DISCUSSION

Osteoclast-Activation by DBP-*maf*

Growing bone is a dynamic tissue in which bone matrix is constantly laid down by osteoblasts and resorbed by osteoclasts. Osteoclasts are responsible for bone resorption and maintenance of skeletal health. It has been demonstrated that the production of oxygen-derived free radicals (superoxide radical) by osteoclasts plays an important role in the mechanism of bone resorption [Garrett et al., 1990]. Furthermore, it was postulated that a defective superoxide production mechanism may be responsible for reduced bone resorption in osteopetrotic subjects and mutant animals [Key et al., 1992; Yamamoto et al., 1994; Schneider et al., 1995]. Osteopetrosis is a rare and often fatal-hereditary juvenile disorder that is manifested in the development of excessive bone-mass. Schneider et al., [1995] demonstrated that infusions of ex vivo generated DBP-*maf*, a post-translationally modified form of serum DBP, to *op* (osteopetrosis) and *ia* (incisor absent) mutant rats (with abnormal accumulation of bone mass due to a defect in osteoclast activity) resulted in significant increase in osteoclast activity and decrease in bone mass [Schneider et al., 1995]. Thus, DBP-*maf* was implicated in vivo-activation of osteoclasts.

In the present study we employed three forms of DBP-*maf* (DBP-*maf*: from human serum DBP, reDBP-*maf*-ec: from bacterially expressed reDBP, and reDBP-*maf*-bv: from baculovirus-expressed reDBP) to address the importance of core N-acetyl galactosamine (GalNAc) in DBP-*maf* for its osteoclast-activating property. It is well-known that an *E. coli*-expressed protein is devoid of any carbohydrate; and the reverse is true for a baculovirus-expressed protein. Thus we had two carbohydrate-containing DBPs (natural DBP and reDBP-bv) and a non-carbohydrate-containing DBP (reDBP-ec) at our disposal, which were activated to corresponding DBP-*maf* preparations (DBP-*maf*, reDBP-*maf* -ec and DBP-*maf* -bv) by treatment with immobilized β-galactosidase, α-mannosidase and sialidase [Yamamoto and Kumashiro, 1993].

We studied the activation of osteoclasts (isolated from long bones of newborn rats) by the above mentioned DBP-*maf*s using an Osteologic multitest slide assay. The use of osteologic slides to study osteoclast activity is a highly

reproducible and one of the newly established procedures that is designed to simplify and standardize the in vitro bone resorption analysis [Davies et al., 1993, Misener et al., 1999; Sindrey et al., 1999]. At the end of the incubation period of osteoclasts on the Osteologic multiTest slides, random slides were selected for analysis by scanning electron microscopy (SEM). Some slides were fixed in 1% glutaraldehyde, dehydrated through a graded series of ethanol and prepared for SEM. Other slides were treated with distilled water to lyse the attached cells to expose the underlying surface and subsequently prepared for SEM. Figure 2A illustrates a typical osteoclast attached to the Osteologic slide surface. The fact that these cells were multinucleated was confirmed by light microscopy prior to preparation for SEM. Figure 2B is a scanning electron micrograph of the surface of a slide from which the attached cells were removed prior to preparation for SEM. The circular structure represents a resorption pit in the Osteologic substrate created by an osteoclast during the incubation period. Figure 2C represents the effect of increasing dose of native DBP-*maf* on osteoclast mediated pit resorption when compared to the untreated osteoclasts. There was a dose dependent increase in pit resorption when osteoclasts were treated with DBP-*maf*. For example, there was a 211% increase in the pit resorption by 0.5 ng of DBP-*maf* when compared to untreated control. Known osteoclast activators PTH (1–34) and $1,25(OH)_2D_3$ were used as positive controls and compared with the osteoclast activating ability of DBP-*maf*. PTH (1–34) and $1,25(OH)_2D_3$ showed a 123% and 145% increase in pit resorption respectively at their optimal in vitro doses when compared to control. It is important to note that osteoclasts showed over 200% increase in pit resorption activity upon treatment with DBP-*maf* at femto molar level (10 fM) when compared to $1,25(OH)_2D_3$ (a well known osteoclast activator) which was used at sub- micro molar level (0.1 μM) stimulated osteoclasts by about 150 %. IFN-γ is a known activator of macrophages. Since both DBP-*maf* and IFN-γ activate macrophages, we tested the ability of IFN-γ to activate osteoclasts parallel to DBP-*maf*. As shown in Figure 2D, treatment of osteoclasts with increasing concentration of IFN-γ did not alter pit resorption significantly from the control levels at any dose, in contrast with DBP-*maf*. Although both IFN-γ and DBP-*maf* are considered to be macrophage activators, the above results strongly suggested that these cytokines do not act in a similar manner with respect to osteoclast activation.

Heterologous expression of DBP in a baculovirus expression system. The expression of functional reDBP-bv was achieved in Sf9 insect cells grown in SFM. The reDBP-bv was designed to carry a six residue long histidine tag (His-tag) to facilitate purification of the recombinant protein from the cell lysate using nickel affinity chromatography. reDBP-bv co-migrated with native DBP (Fig. 3A, Lane 3), isolated from human plasma, and showed an apparent molecular weight of 55 kDa on SDS-PAGE (Fig. 3A, Lane 2).

Functional characterization of reDBP-bv was carried out by determining its ability to bind 25-OH-D_3. Competitive binding assay of reDBP-bv with a fixed amount of ^{3}H-25-OH-D_3 and increasing amounts of 25-OH-D_3 indicated that ^{3}H-25-OH-D_3 was displaced by 25-OH-D_3 in a dose-dependent manner (Fig. 3B). The displacement curve was similar to that of native DBP indicating that the reDBP-bv is functionally similar to native DBP in terms of vitamin D sterol binding.

Osteoclast activation by recombinant DBP expressed in *E. coli* and baculovirus expression systems. The reDBP preparations from *E. coli* and baculovirus expression systems were converted to respective *maf*s by incubating them with immobilized glycosidases, similar to native DBP; and their osteoclast activating abilities were tested in the usual fashion. While native DBP-*maf* activated osteoclasts in a dose-dependent manner, reDBP-maf-ec failed to do so (Fig. 4A). On the other hand, reDBP-maf-bv activated osteoclasts, but its precursor i.e., reDBP-bv did not (Fig. 4B). Since reDBP-maf-ec lacks the sugar moiety, but reDBP-maf-bv contains such moiety, these results clearly emphasized and further supported the essential role of glycosylation (the presence of core-NAG moiety) for its osteoclast activating property.

Influence of 25-OH-D_3-Binding by DBP-*maf* on the Osteoclast Activation Property of the Latter

It has been demonstrated that DBP exerts its multitude of functions via its various structural domains. While vitamin D sterol-binding activity is restricted to N-terminal domain I, actin- and fatty acid-binding sites are located in

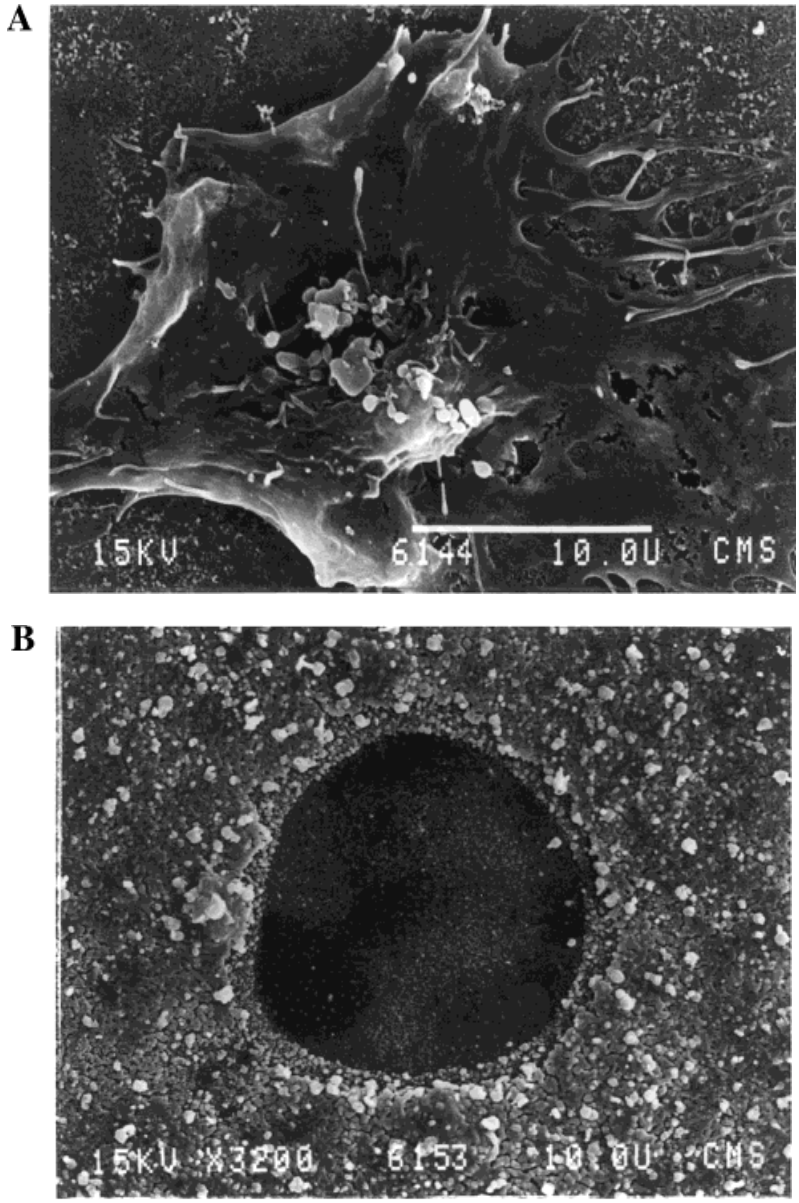

Fig. 2. Osteoclast activation by DBP-*maf*. Approximately 50 osteoclasts were allowed to adhere on to Osteologic MultiTest slides in α-MEM (minimal essential medium) supplemented with 10% fetal calf serum, penicillin G, Gentamicin, fungizone, ascorbic acid, and sodium β-glycerophosphate in 24 well tissue culture plates. **A:** Multinucleated single osteoclast cultured on an osteologic disc (×3000 magnification). **B:** High power scanning electron micrograph of a resorption pit created by an osteoclast such as seen above. Cells were removed to visualize the pit. (×2650 magnification). **C:** The osteoclasts were incubated for 72 h in the presence of different amount of various DBP-*maf* preparations (0.1, 0.2, 0.5 and 1.0 ng). PTH (1–34) and $1,25(OH)_2$ vitamin D_3 were used as controls at a concentration of 0.5 IU/ml and 10^{-8} M respectively as controls. The osteoclast mediated pit resorption was quantitated by Osteologic analyzer (Microst). The slides after incubation with various DBP-*maf* preparations were washed thoroughly with distilled water. The pits were stained with 10% silver nitrate solution overnight. The slides were washed in distilled water and analyzed by Osteologic analyzer to determine the surface area resorbed in each well. Multiple cultures (n=6) of each dose were assessed and analyzed by one-way analysis of variance. The percent pit area resorbed was plotted against the amount of the osteoclast activating agent used under question. Statistically significant osteoclastic activity above control levels ($P < 0.05$). **D:** Osteoclasts were incubated with interferon-γ (0.1, 0.2, 0.5, and 1.0 ng) for 72 h. The rest of the procedure is same as described above. None of the responses were statistically significant compared to controls.

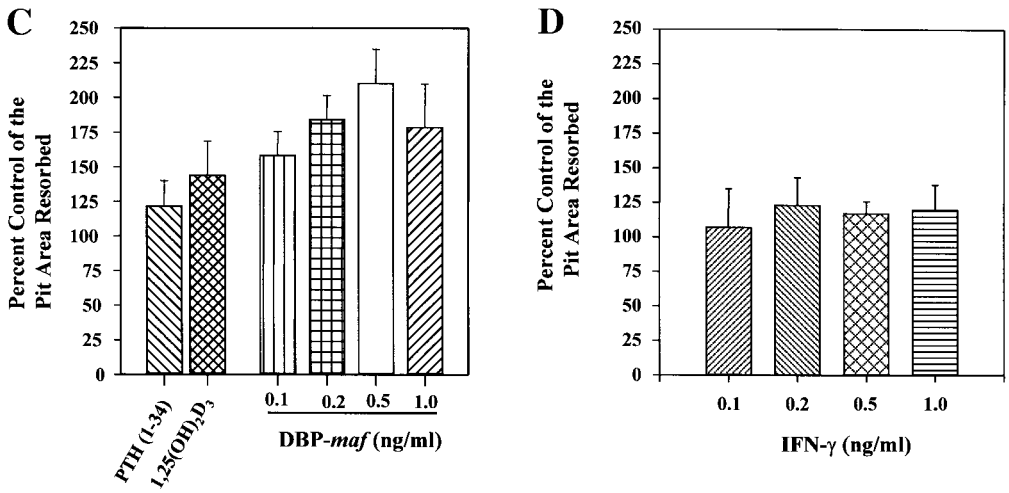

Fig. 2. (*Continued*)

the C-terminal domain III of the protein. The macrophage/osteoclast activating activity is also related to domain III [reviewed in Haddad, 1995; Ray, 1996]. However, little is known about the influence of binding of one ligand on the binding of other/s. For example, Ena et al., [1989] showed that binding of palmitic acid by DBP did not influence the binding of 25-OH-D_3, but binding of arachidonic acid decreased the binding of 25-OH-D_3 to the protein [Ena et al., 1989; Calvo and Ena, 1989].

One of the most well characterized functions of DBP is its capability to bind vitamin D and its metabolites with high affinity (Kd = $10^{8-11}M^{-1}$) and to transport them to target organs [Ray, 1996]. Among all the metabolites of vitamin D, 25-OH-D_3 has the highest binding affinity for DBP. However, influence of 25-OH-D_3-binding on DBP-*maf* activities (osteoclast and macrophage-activation) is yet to be explored.

Interaction between 25-OH-D_3 and DBP is an equilibrium process. Hence at any given time liganded protein (*holo*-DBP) will be contaminated with the unlignaded protein (*apo*-DBP) due to the dissociation of the ligand depending on its affinity constant (Kd). This would introduce heterogeniety in the system. Furthermore, 25-OH-D_3 is the immediate metabolic precursor for 1,25$(OH)_2D_3$ which is a potent activator of osteoclasts. Thus, dissociation of 25-OH-D_3, bound to DBP, could potentially generate 1,25$(OH)_2D_3$ upon hydroxylation by 25-hydroxyvitamin D_3-1α-hydroxylase, a cytochrome P450-containing enzyme. Such a situation would also complicate the studies on the effect of 25-OH-D_3-binding by DBP-*maf* on the activation of osteoclasts. In an ideal situation there should be no contamination of the *holo*-DBP (and holo-DBP-*maf*) with the apo-varieties.

In the present study we chose an analog of 25-OH-D_3 (25-hydroxyvitamin D_3-3-bromoacetate, 25-OH-D_3-3-BE) that gets 'covalently' attached to DBP via a covalent bond with an amino acid residue in the vitamin D sterol-binding pocket [Swamy et al., 1997a]. We have carried out extensive studies to characterize the interaction between this analog and DBP. For example, we have observed that the DBP-binding affinity of 25-OH-D_3 is only marginally compromised upon derivatization; and this analog covalently and specifically modifies the 25-OH-D_3-binding pocket of DBP. Furthermore, we have shown that covalent modification of DBP by 25-OH-D_3-3-BE is restricted to the N-terminal domain I of DBP [Swamy et al., 1997a]. A schematic representation of irreversible binding of 25-OH-D_3-3-BE to DBP is depicted in Figure 5A. It should be noted that covalent modification of the vitamin D sterol-binding pocket of DBP by 25-OH-D_3-3-BE should exclusively produce the holo-protein, and such a process should also prevent the conversion of 25-OH-D_3 to 1,25$(OH)_2D_3$.

In order to study the effect of binding of 25-OH-D_3 on osteoclast activation by DBP-*maf*, DBP was covalently labeled with 25-OH-D_3-3-BE. In order to determine the extent of covalent attachment (by 25-OH-D_3-3-BE) it was spiked with its radioactive version [^{14}C-25-OH-D_3-3-BE]. Radioactive counting of the labeled protein

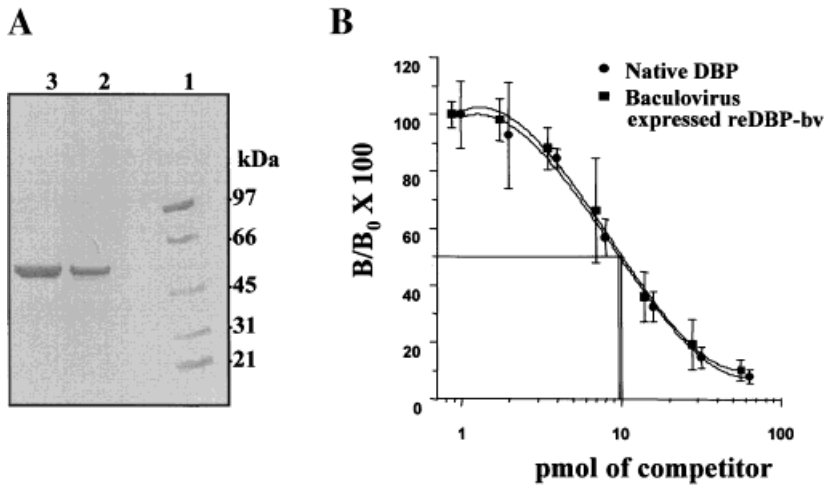

Fig. 3. Characterization of baculovirus-expressed reDBP (reDBP-bv). DBP was expressed in Sf-9 cells as described in Methods section and analyzed by SDS-PAGE for its purity and integrity, and its ability to specifically bind 25-OH-D_3. **A:** SDS-PAGE analysis of baculovirus expressed reDBP (reDBP-bv). Purified native DBP (10 μg) and reDBP-bv (5 μg) were boiled with SDS-PAGE sample buffer and analyzed by 10% SDS-PAGE. **Lane 1**, Molecular weight marker standards; **Lane 2**, baculovirus expressed reDBP (reDBP-bv); **Lane 3**, Native DBP isolated from human plasma. **B:** Competitive radioligand binding analysis of baculovirus expressed reDBP (reDBP-bv). Native DBP or reDBP-bv (400 ng each) were incubated with ^{3}H-25-OH-D_3 (3,750 cpm, 0.17 pmol) and 25-OH-D_3 (0.99–63.8 pmol) in 0.5 ml of assay buffer at 4°C for 16 h followed by treatment with ice-cold Dextran-coated charcoal and centrifugation. Supernatants from centrifuged samples were mixed with scintillation cocktail and counted for radioactivity.

and SDS-PAGE analysis showed that $>98\%$ of the label [25-OH-D_3-3-BE] was covalently associated with DBP. The modified protein, 25-OH-D_3-DBP was converted to its *maf*-version (25-OH-D_3-DBP-*maf*) by a procedure described earlier. Figure 5B shows that, both DBP-*maf* and 25-OH-D_3-DBP-*maf* displayed a similar increase in osteoclast activity in response to increase in dose (of the activating agent). These results strongly suggested that binding of 25-OH-D_3 to DBP-*maf* did not have any effect on the osteoclast activating ability of DBP-*maf*. Furthermore, because the 25-OH-D_3 remains bound to the protein, DBP-*maf* is not merely carrying the

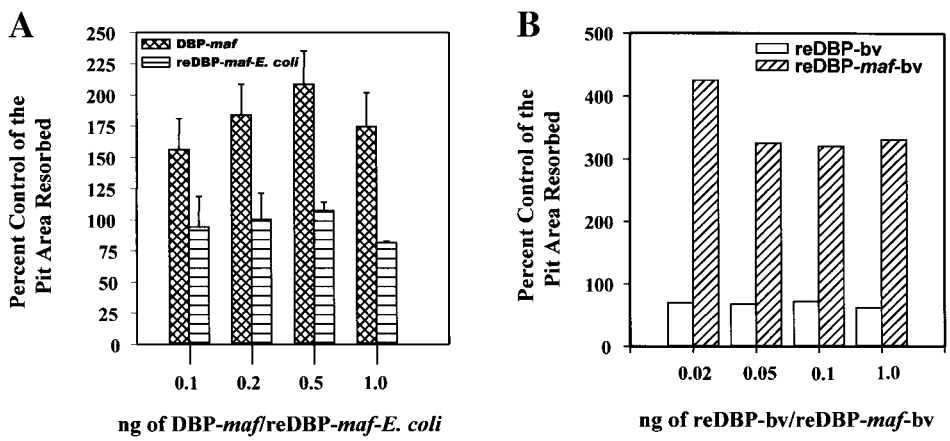

Fig. 4. Osteoclast activation by reDBP-*maf* expressed in *E. coli* and baculovirus expression system. DBP was expressed in *E. coli* and Sf-9 cells and purified as described in Methods section. The reDBP preparations were converted to respective *maf*s and used in osteoclast activation studies as described in Figure 2. **A:** Osteoclast activation by native DBP-*maf* and reDBP-*maf*-ec. All of the responses to DBP-*maf* were statistically greater than control values ($P<0.05$). The responses to reDBP-*maf-ec did not differ from control values.* **B:** Osteoclast activation by reDBP-*maf*-bv. All of the responses to reDBP-bv did not differ from control values. All of the responses to reDBP-*maf-bv were stastically greater than control values* ($P<0.05$).

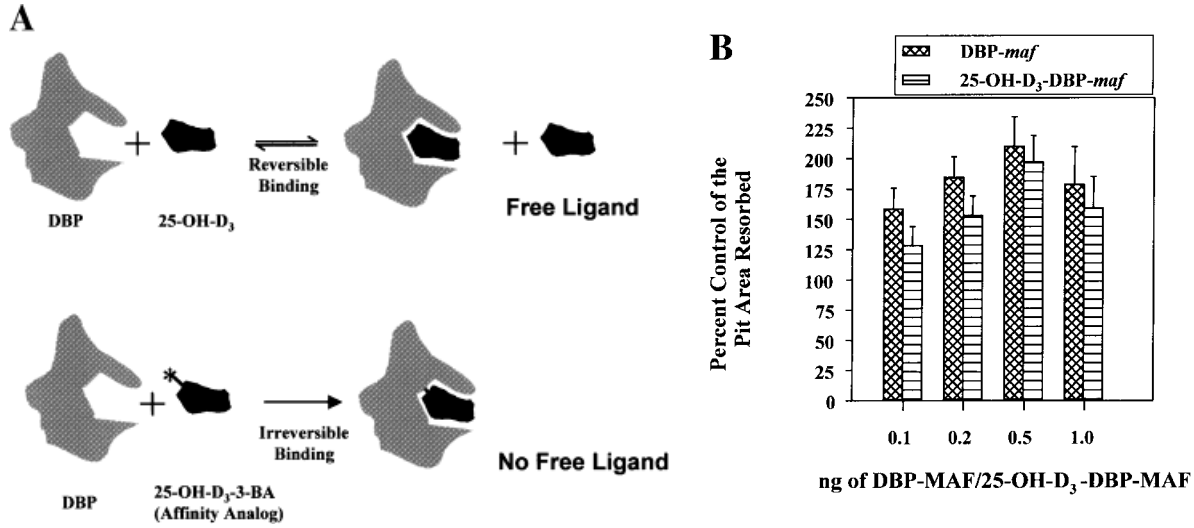

Fig. 5. **A:** Schematic representation of irreversible binding of 25-OH-D_3 to DBP. DBP binds to 25-OH-D_3 in a reversible fashion, which leads to dissociation of ligand and there is always free ligand in the system at any time. 25-OH-D_3-3-bromoacetate (25-OH-D_3-3-BE) covalently cross-links 25-OH-D_3 to vitamin D binding pocket of DBP to generate 25-OH-D_3-DBP and the bound ligand does not dissociate. Covalent attachment of 25-OH-D_3-3-BE to DBP also prevents the metabolism of the parent ligand (25-OH-D_3) to osteoclast-activating 1,25$(OH)_2D_3$. **B:** Effect of binding of 25-OH-D_3 to DBP-*maf* on osteoclast activation. 25-OH-D_3-3-BE was covalently bound to DBP and converted to 25-OH-D_3-DBP-*maf* as described in Methods section. Osteoclast activation studies were same as described in Figure 2. The responses to DBP-*maf* and 25-OH-D_3-DBP-*maf* did not differ significantly.

vitamin D metabolites to the osteoclasts to elicit activity; osteoclast activation is achieved by an alternate mechanism.

In summary, we evaluated the structural implications on the osteoclast-activating property of DBP-*maf*. We developed a baculovirus expression system for producing reDBP-*maf* containing a carbohydrate moiety. We used baculovirus and bacterial expression system to produce reDBP with and without any carbohydrate moieties. DBP-*maf*s generated ex vivo from these proteins were crucial in emphasizing the essential role of core NAG in DBP-*maf* in the activation of osteoclasts. We further showed that 25-OH-D_3-binding by DBP-*maf* had no significant effect on the DBP-*maf* -mediated activation of osteoclasts.

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