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Modulation of 1α,25-dihydroxyvitamin D_3-Membrane Associated, Rapid Response Steroid Binding Protein Expression in Mouse Odontoblasts by 1α,25-$(OH)_2D_3$

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Abstract The rapid, nongenomic effects of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) have been related to a 1,25D_3-membrane associated, rapid response steroid binding protein or 1,25D_3-[MARRS]bp, with a molecular weight of 65 kDa, in several tissues and species. Currently, no information is available concerning the nongenomic responses to 1α,25-$(OH)_2D_3$ in dental tissues. In order to investigate the expression of 1,25D_3-[MARRS]bp in dental cells, in the presence or absence of 1α,25-$(OH)_2D_3$, we have used rabbit polyclonal antibodies directed against the N-terminus of the 1,25D_3-[MARRS]bp (Ab099) that recognizes the 1α,25-$(OH)_2D_3$ binding protein in chick intestinal basolateral membranes and a mouse odontoblast-like cell line (MO6-G3). Western blotting and flow cytometric analyses with Ab099 specifically detected 1,25D_3-[MARRS]bp in MO6-G3 cells. Moreover, 1,25D_3-[MARRS]bp was up-regulated, in vivo, in differentiated dental cells. Electron microscopic analysis confirmed the plasma membrane localization of this binding protein and also showed its intracellular presence. Incubation of MO6-G3 cells with different doses of 1α,25-$(OH)_2D_3$ for 36 h resulted in an inhibition of 1,25D_3-[MARRS]bp expression with a maximal effect at 50 nM steroid. In addition, the culture media of MO6-G3 cells contains immunoreactive 1,25D_3-[MARRS]bp. Immunogold positive membrane vesicle-like structures are present in the extracellular matrix of MO6-G3 cells. Altogether, these results indicate that the 1,25D_3-[MARRS]bp expression in MO6-G3 cells is modulated by 1α,25-$(OH)_2D_3$. In conclusion, this 1α,25-$(OH)_2D_3$ binding protein could play an important role in the rapid, nongenomic responses to 1α,25-$(OH)_2D_3$ in dental cells. J. Cell. Biochem. 94: 139–152, 2005.

Key words: 1α,25-dihydroxyvitamin D_3; dental cells; 1,25D_3-[MARRS]bp

The seco-steroid hormone 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) exerts a wide variety of biological responses by genomic mechanisms via nuclear receptors that result in transcriptional regulation of selected genes [for review see Norman, 1998; Pike, 1997]. Thus 1α,25-$(OH)_2D_3$ can act in different tissues and cells including kidney, intestine, osteoblasts [reviewed in Haussler et al., 1997], chondrocytes [for review see Boyan et al., 1997], and dental cells [Berdal et al., 1991a,b, 1993, 1997]. The genomic responses to 1α,25-$(OH)_2D_3$ in dental cells are related, at least in part, to the nuclear high affinity receptor for 1α,25-$(OH)_2D_3$ (nVDR) resulting in the modulation of gene expression such as that observed for calbindins and amelogenin genes [Berdal et al., 1991b, 1993; Berdal, 1997; Papagerakis et al., 1999]. Rapid, nongenomic actions of 1α,25-$(OH)_2D_3$

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have been described in vitro including changes in membrane fluidity [Swain et al., 1993], Ca^{2+} flux [Langston et al., 1990], turnover of phospholipids [Lieberherr et al., 1989; Wali et al., 1990; Swain et al., 1992], increase of intracellular calcium uptake [Nemere and Szego, 1981; Lieberherr, 1987; Sugimoto et al., 1992], and the activation of protein kinase C [de Boland and Norman, 1990a; Bissonnette et al., 1994, 1995; Slater et al., 1995; Sylvia et al., 1998; Boyan et al., 1999] in various cells such as chondrocytes [Schwartz et al., 1988; Swain et al., 1993; Sylvia et al., 1996, 1998; Pedrozo et al., 1999], osteoblasts [Baran et al., 1991; Zanello and Norman, 1997], and intestinal epithelial cells [de Boland and Norman, 1990a,b; Bissonnette et al., 1994, 1995; Norman, 1997; Nemere et al., 1998]. These direct and rapid effects have been related to a membrane binding protein for $1\alpha,25\text{-}(OH)_2D_3$: $1,25D_3$-membrane associated, rapid response steroid binding protein or $1,25D_3$-[MARRS]bp with a molecular weight of 64,500–65,000 [Baran et al., 1994; Nemere et al., 1994, 1996, 1998, 2000a,b; Lieberherr et al., 1997; Pedrozo et al., 1999; Nemere and Campbell, 2000]. It has been characterized at biochemical and functional levels in chick intestinal epithelial cells [Nemere et al., 1994, 2000b] and rat chondrocytes [Pedrozo et al., 1999] using rabbit polyclonal antibodies (Ab099) raised against the N-terminal sequence of the putative membrane receptor for $1\alpha,25\text{-}(OH)_2D_3$ [Nemere et al., 1996, 1997, 1998]. The complete cDNA sequence for the $1,25D_3$-[MARRS]bp was recently reported [Nemere et al., 2004]. This protein belongs to a superfamily of multifunctional proteins involved in the binding of thyroid hormones and estrogens and playing a role in immune response [Nemere et al., 2004]. Dental cells (odontoblasts and ameloblasts) which form mineralized tissues (dentin and enamel, respectively) express the nVDR [Bailleul-Forestier et al., 1996; Davideau et al., 1996]. The expression pattern of nVDR in dental tissues is well documented, however in contrast, expression of $1,25D_3$-[MARRS]bp reflecting the nongenomic pathway of $1\alpha,25\text{-}(OH)_2D_3$ and its potential roles in tooth development are still largely unknown. The aim of this study was to analyze the expression of $1,25D_3$-[MARRS]bp, in vivo, in mouse dental cells and, in vitro, in the stable mouse odontoblast-like cell line MO6-G3 [MacDougall et al., 1995] for further biochemical and functional investigations. We have also investigated the capacity of the ligand to induce regulation of $1,25D_3$-[MARRS]bp in this cell line.

MATERIALS AND METHODS

Antibodies

Ab099 is a polyclonal rabbit antibody directed against chicken $1,25D_3$-[MARRS]bp [Nemere et al., 1996, 1997, 2000b]. It was produced against the NH_2-terminal peptide sequence of the putative membrane vitamin D receptor [Nemere et al., 1994, 1996, 1997, 2004]. Nonimmune rabbit IgGs (Immunotech, Marseille, France) were used as a negative control. Horseradish peroxidase (HRP)-conjugated $F(ab')_2$ goat anti-rabbit IgG (H + L) antibodies (Immunotech) were used as secondary antibodies for Western and dot blotting analyses. For immunohistochemistry, biotinylated-conjugated $F(ab')_2$ goat anti-rabbit IgG antibodies were used as secondary antibodies (Sigma, Saint-Quentin Fallavier, France) and HRP-conjugated streptavidin (Extravidin Peroxidase conjugate, Sigma) as the detection reagent. For electron microscopy, colloidal gold particle-conjugated (10-nm-diameter) $F(ab')_2$ goat anti-rabbit IgG antibodies were used as secondary antibodies (Amersham, Les Ulis, France). For flow cytometry, fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) was used.

Chemical Reagents

$1\alpha,25$-dihydroxyvitamin D_3 (Sigma, la Verpillière, France) was prepared according to the manufacturer's instructions in ethanol at a stock solution of 10^{-4} M and used at a variety of concentrations.

Cells and Culture Conditions

The stable mouse odontoblast cell line MO6-G3, derived from Swiss Webster E-18 mouse first mandibular molars [MacDougall et al., 1995], was obtained by immortalization of odontoblasts using retrovirus transfection. Odontoblasts are highly specialized, polarized cells producing the dentin extracellular matrix. The MO6-G3 cell line showed high constitutive expression of dentin phosphoprotein, type I collagen and alkaline phosphatase, characteristic of a mature odontoblast phenotype [MacDougall et al., 1995]. MO6-G3 cells were

grown in α-MEM medium L-glutamine (Gibco Invitrogen, Cergy, France) supplemented with 15% fetal calf serum (HyClone, Logan, UT), 100 U/ml penicillin–streptomycin (Gibco Invitrogen), 50 μg/ml ascorbic acid (Sigma), 10 mM β-glycerophosphate (Sigma) at 33°C in a humidified atmosphere of 95% air and 5% CO_2. For 1,25D_3-[MARRS]bp expression variation experiments, cells were plated at initial densities of 5×10^5 cells/ml with 1α,25-$(OH)_2D_3$ or 0.1% ethanol as vehicle for 36 h. Details for individual experiments are provided in the figure legends.

Total Protein Preparation

MO6-G3 cells were directly lysed in 1% sodium dodecyl sulfate (SDS) (Sigma) containing a mixture of protease inhibitors (Protease inhibitor cocktail, Sigma) and then boiled for 5 min. Total protein concentration was determined using a mini-BCA microassay (Pierce, Rockford, IL) with a spectrophotometer (Dynatech MR 4000, UK) at 550 nm wavelength. Total proteins from the incisor mesenchyme of Cobs Dawley mice (Charles River France, Saint Aubain les Elbeuf) were prepared as described above. All the protein samples were stored at −80°C.

Extraction of Hydrophobic Membrane Proteins

MO6-G3 cells were plated in duplicate at initial densities of 10^7 cells/ml in 12-well plates (Costar, Corning, NY) with 50 nM 1α,25-$(OH)_2D_3$ or 0.1% ethanol as vehicle for 36 h as described above. The viability of the cells was monitored using a trypan blue exclusion test, with viability remaining up to 95%. Hydrophobic membrane proteins were extracted by phase separation [Bordier, 1980] (unless otherwise indicated, all chemicals were from Sigma). Briefly, cells were washed twice in phosphate-buffered saline (PBS) and lysed in 1 ml 1% (w/v) Triton X-114 (Serva Feinbiochemica, Heidelberg, Germany) in 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 [Tris-buffered saline (TBS)], containing 10 mM EDTA, 2 mM phenylmethyl sulfonyl fluoride (PMSF) and protease inhibitors (Protease inhibitor cocktail, Sigma). The detergent phase was washed in TBS and dissolved in 1 ml 25 mM Tris-HCl, pH 8.2, 50 mM NaCl, 10 mM NaN_3, 0.5% Nonidet-P40 (Fluka Biochemica, Buch, Switzerland), 0.5% deoxycholic acid, 2 mM PMSF, and protease inhibitors (Protease inhibitor cocktail, Sigma). The detergent phase obtained from solubilized membrane extracts was used for Western blotting (25 μl aliquots). The detergent phase contained the amphiphilic hydrophobic membrane proteins, whereas the hydrophilic proteins are recovered in the aqueous phase. All the protein samples were stored at −80°C.

One-Dimensional SDS-Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blotting Analysis

One-dimensional SDS–PAGE was performed on 10% polyacrylamide gels according to Laemmli [1970] with a minilab gel system (Mini-Protean II Electrophoresis cell; Bio-Rad SA, Marnes la Coquette, France). Total protein samples or hydrophobic membrane protein samples (see above Triton X-114 protein extraction) from vehicle- or 1α,25-$(OH)_2D_3$-treated MO6-G3 cells were boiled for 3 min in 80 mM Tris-HCl-buffer, pH 6.8 containing 100 mM dithiotreitol, 2% SDS, 10% glycerol, and 0.01% bromophenol blue (all chemical reagents were purchased from Sigma). SDS–PAGE was performed using 25 mM Tris-base-buffer, pH 8.3, 192 mM glycine, and 0.1% SDS (running buffer) under reducing conditions at room temperature for 45 min at 200 V. Gels were washed in running buffer containing 20% methanol and transferred onto nitrocellulose membranes (Hybond ECL, Amersham) for 1 h at 100 V using the transblot cell system (Bio-Rad SA). Ponceau Red (Sigma) staining of the filters allowed verification of the protein transfer efficiency and the homogeneity between the different lanes. Nitrocellulose membranes were washed in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 (Western buffer, WB) at room temperature for 5 min prior to blocking non-specific binding sites with 5% non-fat dry milk in WB for 45 min at 37°C. Membranes were incubated for 3 h at room temperature with Ab099 (1:4,000 final dilution) in 5% non-fat dry milk in WB. The membranes were then washed four times (5 min each) in WB/0.05% Tween-20 and incubated with HRP-conjugated secondary antibodies diluted at 1:40,000 in 5% non-fat dry milk in WB for 60 min at room temperature. After washing twice in WB/0.05% Tween 20 and twice in WB, the membranes were incubated for 1 min with Super Signal Substrate (Pierce) and chemiluminescence was detected by exposure of the membranes to a film (Hyperfilm ECL, Amersham) for 5 min.

Dot Blotting of Culture Supernatants

Culture media of confluent MO6-G3 monolayer cell cultures were harvested after 36 h incubated with 50 nM 1α,25-$(OH)_2D_3$ or 0.1% ethanol as vehicle. PMSF (1 mM) and 0.2 μg/ml aprotinin (both from Sigma) were added to the media supernatants, prior to centrifugation at 10,000*g* (4°C, 5 min). All the samples were stored at −80°C before use. Serial dilutions of supernatants were spotted onto nitrocellulose membranes (Hybond ECL, Amersham) under vacuum with a dot blot apparatus (SRC 96, Schleicher and Schuell, France). Membranes were then treated with Ab099 as described above.

Densitometric Analysis of the Autoradiographs

Band and spot areas on autoradiographs were quantified by scanning densitometry (Imager Appligen model scanning densitometer), using an image-analysis computer program (Image 1.61 Macintosh, Apple). The density of the spot or band areas was expressed as arbitrary units and plotted as a function of protein applied to the gels or directly onto the membranes. The variations of the relative amount of 1,25D_3-[MARRS]bp protein samples were expressed as a percentage of the density of the corresponding band or spot area obtained in the control samples, which were set at 100.

Electron Microscopic Analysis and Indirect Immunogold Labeling Procedures

Samples were prepared as previously described [Raposo et al., 1997]. Briefly, 10^6 MO6-G3 cells were incubated in 100-mm culture-dishes (Becton Dickinson, Le Pont De Claix, France) and washed twice with serum-free α-MEM medium and fixed for 1 h at 4°C in 4% paraformaldehyde (PFA, Sigma), 0.1% glutaraldehyde (Sigma) in 100 mM phosphate buffer (PB), pH 7.4. Cells were washed by centrifugation with PB containing 0.1% glycine (Sigma) at 4°C overnight. Cells were centrifuged with 5% gelatin-PB (Sigma) at 37°C and were then incubated at 37°C for 15 min in the same buffer. After cooling at 4°C, samples were embedded in gelatin and then embedded in 2.3 M sucrose (Sigma) in PBS at 4°C overnight. Samples were then treated with azote. Ultrathin sections (50–80 nm) were prepared at −120 to −135°C with an ultracryomicrotome (Reichert FCS, Paris, France). Cryosections were incubated in 100 mM PB containing 2% gelatin at 37°C for 10 min. After rinsing in PBS containing 50 mM glycine, and then with PBS–0.1% BSA (Bovine Serum Albumin) (Sigma), sections were incubated with Ab099 diluted to 1:1,000 in PBS–1% BSA for 45 min. They were then washed in PBS–0.1% BSA and incubated with 10-nm colloidal gold conjugate particles (1:25 final dilution) for 20 min. After rinsing in PBS, and PBS–1% glutaraldehyde, samples were washed in distilled water. Sections were then stained with 2% uranyl-oxalate (Sigma) pH 7 for 5 min, washed with 2% methyl cellulose 0.4% uranyl acetate (Sigma) pH 4 at 4°C, and examined under a Philips CM12 (80-kV) transmission electron microscope.

Immunoperoxidase Localization of 1,25D_3-[MARRS]bp

Cobs Dawley mice (Charles River France) aged 2, 3, or 4 days were sacrificed and semi-mandibles dissected and fixed with 4% PFA (Sigma) in PBS pH 7, for 12 h at 4°C. Samples were rinsed in PBS containing 30% sucrose (Sigma), and the fixed mandibles dehydrated and embedded in paraffin. Sections (6–10 μm) were cut, placed on slides coated with poly-L-lysine (Sigma), and kept at −20°C. Sections were deparaffinized, rehydrated, and treated with trypsin (Sigma) in PBS for 5 min. Sections were then rinsed in PBS and endogenous peroxidases blocked for 10 min with 3% H_2O_2 in PBS at room temperature. After rinsing, sections were incubated for 30 min with 1:30 diluted normal goat serum (Vector Laboratories, AbCys SA, Paris, France) at room temperature in PBS–1% BSA. They were then incubated with Ab099 diluted 1:400 or 1:500 in PBS–1% BSA for 1 h in a humid atmosphere at room temperature. For control staining, Ab099 was omitted. After rinsing in PBS, sections were incubated with biotinylated conjugate for 1 h (1:800 dilution in PBS–1% BSA) at room temperature. They were then rinsed in PBS and incubated with HRP-conjugated-streptavidin for 30 min (1:300 dilution) at room temperature. After rinsing in PBS, immunoreactive sites were visualized with 3,3′-diaminobenzidine (5 mg/10 ml) (Sigma) in 100 mM Tris-HCl, pH 7.6 with 0.03% H_2O_2 for 5 min. The sections were finally rinsed with water, counterstained with hematoxilin, dehydrated, mounted in DePeX (Merkoglass, Merck, Germany), observ-

ed, and photographed with a Zeiss Orthoplan microscope.

Flow Cytometry

MO6-G3 cells (5×10^5 cells in 100 µl) were washed with PBS–1% BSA at 4°C and incubated with 50 µl of various dilutions of Ab099 or control rabbit IgGs for 30 min at 4°C in the same buffer. Cells were washed twice with PBS–0.5% BSA, and incubated with the FITC-conjugate diluted at 1:50 in the same buffer for 30 min at 4°C. After two washes, cells were suspended in PBS and 5,000 or 10,000 viable cells were analyzed using a FACScalibur (Becton Dickinson). The relative cell number was plotted against a logarithmic scale of fluorescence intensity, measured by flow cytometry. The data were stored and processed using the FACScalibur software. Results were expressed as the mean ± SD of the percentage positive cells of duplicate cultures. Inhibition experiments were performed by incubating 5×10^5 MO6-G3 cells in 12-well-plates (Costar) with various doses of 1α,25-$(OH)_2D_3$ or with 0.1% ethanol as a control for 36 h. Flow cytometric analysis was then performed as described above with the results expressed as the mean ± SD of the percentage inhibition of control cultures (ethanol as vehicle). The mean fluorescence index (MFI) was used to express the variation of immunofluorescence of the samples treated with 1α,25-$(OH)_2D_3$ or ethanol as vehicle.

RESULTS

Molecular Weight and Membrane Expression of 1,25D_3-[MARRS]bp by MO6-G3 Cells and Mouse Incisor Odontoblasts Cells

The Western blotting analysis showed a 65–66 kDa broad band, the expected size of 1,25D_3-[MARRS]bp, detected by Ab099 in 20 µg of total proteins extracted from MO6G-3 cells (Fig. 1A). No band was detectable in the sample with control rabbit IgGs at 2 µg/ml ascertaining the specificity of the reaction (Fig. 1B). Figure 1C shows that total proteins extracted from dental mesenchyme of mouse incisors also contained a major 65–66 kDa band corresponding to 1,25D_3-[MARRS]bp. However, additional bands of 116 and 45 kDa were also cross-reactive with Ab099 in this sample, probably corresponding to aggregates or degradation proteins, respectively (Fig. 1C). To test for the presence of 1,25D_3-[MARRS]bp, on the plasma membrane,

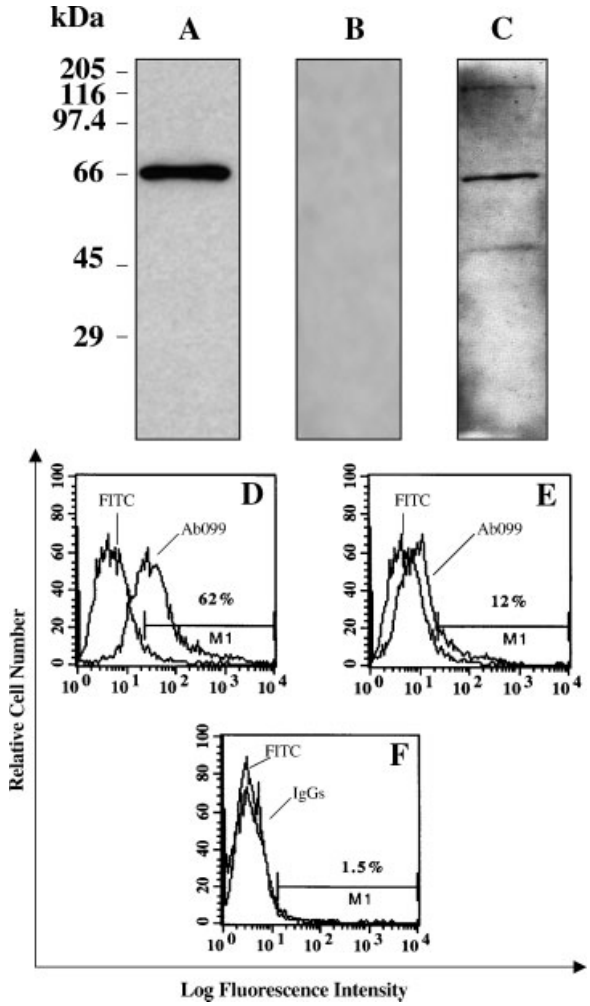

Fig. 1. 1,25D_3-[MARRS]bp expression by MO6-G3 cells and mouse dental tissues. **A–C**: Western blotting analysis of 1,25D_3-[MARRS]bp expression. Total proteins of MO6-G3 cells (20 µg) (**lanes A and B**) or dental mesenchymal tissues (10 µg) (**lane C**) were separated on a 10% SDS–PAGE and transferred onto a nitrocellulose membrane. Filters were then incubated with Ab099 diluted at 1:4,000 (**lanes A, C**) or control rabbit IgGs at 2 µg/ml (**lane B**) followed by HRP-conjugated goat anti-rabbit IgG (1:40,000). The reaction was visualized by chemiluminescence. **D–F**: Flow cytometric analysis of 1,25D_3-[MARRS]bp expression by MO6-G3 cells. 5×10^5 cells were harvested and stained with Ab099 diluted at 1:10 (**D**) or 1:200 (**E**) or with control rabbit IgGs at 1 µg/ml (**F**) followed by FITC-conjugated goat anti-rabbit IgG (1:50). 1,25D_3-[MARRS]bp expression was analyzed with a FACScalibur. Results are expressed as the percentage of positive cells as compared to the negative control (FITC-conjugate, background fluorescence). M1, area of Ab099 labeled cells.

MO6-G3 cells were incubated with various dilutions of the antiserum Ab099. After labeling for 30 min, flow cytometric analysis detected a significant increase in fluorescence intensity compared with unlabeled control cells (Fig. 1D). Analysis by FACScalibur showed that 62%

(Fig. 1D) and 12% (Fig. 1E) of MO6-G3 cells expressed 1,25D_3-[MARRS]bp when Ab099 was used at a final dilution of 1:10 and 1:200, respectively. The specificity of the reaction was analyzed with control rabbit IgGs at 1 μg/ml. As shown in Figure 1F, fluorescence intensity was under 1.5% with control rabbit IgGs, corresponding to a nonspecific background labeling.

In Vivo Detection of 1,25D_3-[MARRS]bp in Mouse Dental Cells

Table I summarizes the results of 1,25D_3-[MARRS]bp localization obtained from 2–4 day old mice incisor and molar samples. Ab099 reacted with various cells of epithelial and mesenchymal origin which are involved in dental extracellular matrix mineralization, indicating that 1,25D_3-[MARRS]bp could be detected in vivo in dental cells. Ameloblasts cells, which produce the enamel matrix, reacted strongly with Ab099, as compared to the odontoblasts cells which produce the dentin. In general, the intensity of the reaction was weaker for mesenchymal cells as compared to epithelial cells. Immunoperoxidase staining showed a strong labeling of odontoblasts cells as compared to the undifferentiated preodontoblasts. Preameloblasts cells were also stained to a lesser extent than the more mature ameloblasts. Figure 2A is representative of the results obtained, when Ab099 was used at a final dilution of 1:400. Immunoreactive sites of 1,25D_3-[MARRS]bp were very intense in the ameloblast zone as shown in Figure 2A; in contrast no labeling was present in the supra-ameloblastic region, listed as stellate reticulum and stratum intermedium in Table I (Fig. 2A, arrow head). Odontoblast cells were positive for 1,25D_3-[MARRS]bp (Fig. 2A) whereas a low labeling was detected in pulp cells (Fig. 2A, asterisk) and mesenchymal cells were Ab099 negative (Fig. 2A, arrow). No significant difference was seen in the localization between the 2 and 3–4 days mouse samples (molars and incisors). At higher magnification (Fig. 2B), immunoreactivity between mesenchymal origin cells (odontoblasts) and epithelial origin cells

TABLE I. In Vivo 1,25D_3-[MARRS]bp Expression in Mouse Incisor and Molar Dental Cells

Cell type	Ab099	HRP-Control
Cells of epithelial origin		
Inner dental epithelium		
Cuboid undifferentiated cells	+/–	–
Preameloblasts	+/–	–
Ameloblasts	++++	–
Stratum intermedium	–	–
Stellate reticulum	–	–
External enamel epithelium	–	–
Cells of mesenchymal origin		
Undifferentiated pulpal cells	+/–	–
Preodontoblasts	+/–	–
Odontoblasts	+++	–
Reflexion zone cells	+	–

The intensity of immunoreactive sites was expressed as: –, no reaction; +/–, weak reaction; +, medium reaction; +++, strong reaction; ++++, very intense reaction. HRP: HorseRadishPeroxidase. Dental epithelium is formed of several layers: the inner dental epithelium is composed of cuboid undifferentiated cells, preameloblasts, and differentiated ameloblasts which form enamel. Stellate reticulum, stratum intermedium, and external enamel epithelium are composed of cells localized up the inner dental epithelium. In the mesenchyme, the odontoblasts form dentin. The reflexion zone is composed of undifferentiated cells.

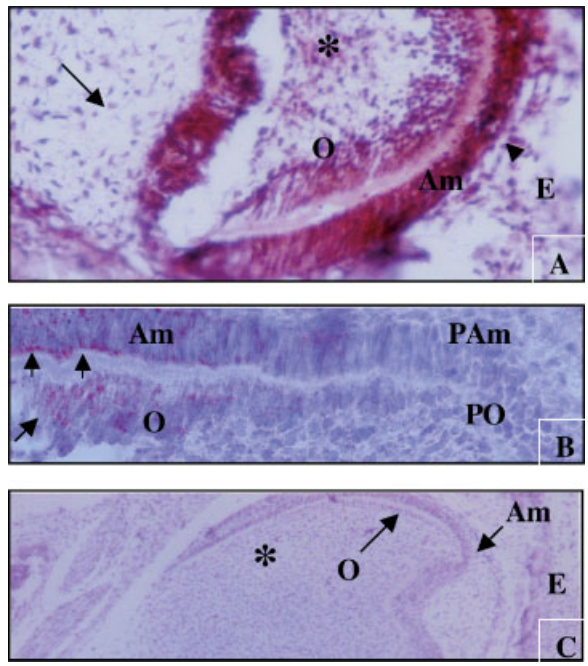

Fig. 2. In situ 1,25D_3-[MARRS]bp immunostaining of incisor and molar dental tissues. **A**: 1,25D_3-[MARRS]bp immunoreactivity is more intense in the epithelial cells (E) than the mesenchymal cells (arrow) of the tooth. Pulp cells (*) were slightly 1,25D_3-[MARRS]bp positive in 3-day-old incisor tooth germ as well as the supra-ameloblastic cell (arrow head) containing stratum intermedium and stellate reticulum. Odontoblasts (O) and ameloblasts (Am) are positively stained (Ab099 diluted 1:400; ×20). **B**: At high magnification, the intracellular staining of odontoblasts (O) and ameloblasts (Am) (arrows) was observed (left-side). The 1,25D_3-[MARRS]bp immunoreactivity increased with the cytodifferentiation of these cells. Neither the preameloblasts (PAm) nor the preodontoblasts (PO) were stained (Ab099 diluted 1:500; ×100) in 2-day-old incisor tooth germs. **C**: Negative control. Section was labeled only with the biotinylated conjugate. Neither epithelial (E) components including ameloblasts (Am) nor the mesenchymal tissues (*) including odontoblasts (O) were stained (2-day-old molar tooth germ).

(ameloblasts) was markedly different (Fig. 2B). 1,25D_3-[MARRS]bp was detected not only at the plasma membrane of ameloblasts but also in intracellular compartments (Fig. 2B). Furthemore, preodontoblasts were not very immunoreactive and immunostaining seemed related to the polarization of odontoblasts. Differentiated odontoblasts and ameloblasts were more labeled than undifferentiated preodontoblasts and preameloblasts, respectively, and the intensity of labeling increased with the differentiation stages of odontoblasts and ameloblasts (Fig. 2B). Negative control (Fig. 2C) with biotinylated-conjugate shows the low background levels.

Electron Microscopic Observation of 1,25D_3-[MARRS]bp Expression in MO6-G3 Cells

To investigate the cellular localization of 1,25D_3-[MARRS]bp, MO6-G3 cells were examined by transmission electron microscopy. The morphological aspects of MO6-G3 cells are illustrated in Figure 3A, indicating the overall preservation of the plasma membrane and intracellular structures such as mitochondria, endoplasmic reticulum cisternae, Golgi apparatus saccules, as well as nuclei. At higher magnification, immunogold labeling, performed on ultrathin cryosections, showed that 1,25D_3-[MARRS]bp was localized in the Golgi apparatus and the endoplasmic reticulum (Fig. 3B arrow heads). At very high magnification, we observed the immunogold labeling of the Golgi apparatus saccules as well as the plasma membrane (Fig. 3C). As shown in Figure 4A, 1,25D_3-[MARRS]bp was clearly detected in the inner and outer aspects of plasma membrane. As shown in Figure 4A,B, in addition to the cell surface labeling, immunogold staining was localized in the cytoplasmic region of the cells.

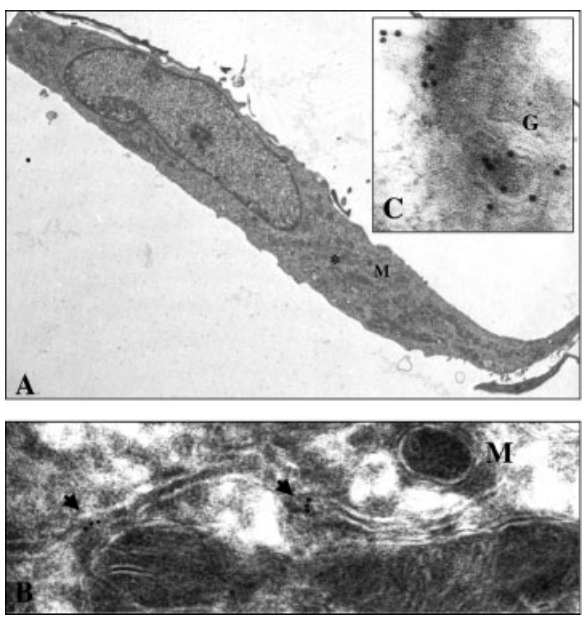

Fig. 3. Transmission electron micrographs of MO6-G3 cells stained with Ab099. Ultrathin cryosections (50–80 nm) were incubated with Ab099 (1:1,000 final dilution) and immunogold labeling performed. Photomicrographs of three representative sections are shown. **A**: Transmission electron micrograph of MO6-G3 cells prepared by the Epon inclusion technique showing that the overall structures were preserved. The presence of numerous mitochondria (M), the plasma membrane, and the Golgi apparatus (*) are clearly visible (×6,000 magnification). **B**: A high magnification (×112,000) showing gold labeling near the Golgi apparatus (arrow heads). **C**: Gold labeling near a Golgi apparatus (G) is clearly visible (×110,000 magnification).

1α,25-Dihydroxyvitamin D_3 Effects on 1,25D_3-[MARRS]bp Expression in MO6-G3 Cells

In a first series of experiments, MO6-G3 cells were incubated with 50 nM 1α,25-$(OH)_2D_3$ or with vehicle for 36 h. A representative Western blotting is shown in Figure 5A. Hydrophobic membrane protein extracts prepared from MO6-G3 cells incubated with vehicle contained the 65–66 kDa band (Fig. 5A, lane 1), as did the total protein extracts (Fig. 5A, lane 3). Triton X-114 membrane protein extracts prepared from the same number of cells after the 36 h incubation period with 50 nM 1α,25-$(OH)_2D_3$, contained

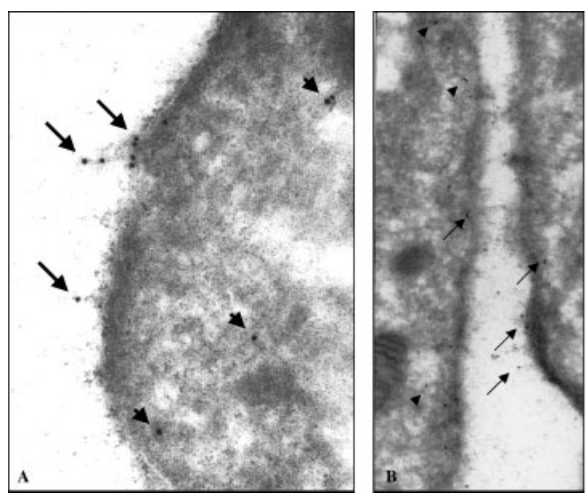

Fig. 4. Transmission electron micrographs of MO6-G3 cells (described in Fig. 3). **A**: High magnification (×65,000) showing gold particles in the plasma membrane (arrows) and in the intracytoplasmic compartment (arrow heads). **B**: 1,25D_3-[MARRS]bp is present in the inner and the outer membrane (arrows). Note the presence of labeling in the intracytoplasmic region (arrow heads) (×52,000 magnification).

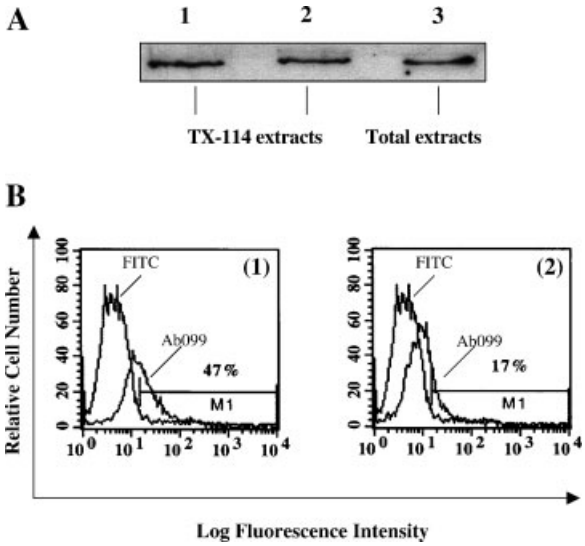

Fig. 5. Effect of 50 nM 1α,25-$(OH)_2D_3$ for 36 h on 1,25D_3-[MARRS]bp expression. **A**: Western blotting analysis of 1,25D_3-[MARRS]bp expression in Triton X-114 protein extract of MO6-G3. MO6-G3 cells were cultured for 36 h with the vehicle ethanol (**lane 1**) or with 50 nM 1α,25-$(OH)_2D_3$ (**lane 2**). Total proteins (10 μg) of MO6-G3 cells were used as internal positive control (**lane 3**). Western blotting was performed as described in Figure 1A, with a representative autoradiograph shown. **B**: Flow cytometric analysis of 1,25D_3-[MARRS]bp expression by MO6-G3. MO6-G3 cells were cultured for 36 h with the vehicle ethanol (**1**) or with 50 nM 1α,25-$(OH)_2D_3$ (**2**). 1,25D_3-[MARRS]bp expression was analyzed by flow cytometry with Ab099 diluted at 1:20 followed by FITC-conjugated goat anti-rabbit IgG (final dilution 1:50). In this representative experiment, 10,000 cells per sample were analyzed by FACScalibur. Results are expressed as percentage of positive cells as compared to the negative control (FITC-conjugate). M1, area of Ab099 labeled cells.

lesser amounts of 1,25D_3-[MARRS]bp (Fig. 5A, lane 2). Densitometric analysis of three independent autoradiographs showed a 40% decrease of the 65–66 kDa band intensity of the membrane extracts prepared from cells incubated with 50 nM 1α,25-$(OH)_2D_3$.

To confirm and extend these observations, 1,25D_3-[MARRS]bp expression by MO6-G3 cells was investigated by flow cytometry. After a 36 h incubation with or without 50 nM 1α,25-$(OH)_2D_3$, we observed that this dose induced a strong decrease of 1,25D_3-[MARRS]bp expression by MO6-G3 cells. As shown in one representative experiment, illustrated in Figure 5B, a 2.7 fold-decrease of fluorescence intensity in the presence of 50 nM 1α,25-$(OH)_2D_3$ was observed. Statistical analysis by FACScalibur software showed that only 17% of 50 nM treated MO6-G3 cells were 1,25D_3-[MARRS]bp positive (Figs. 5B(2)) versus 47% for vehicle control treated cells (Figs. 5B(1)). Results obtained from four independent experiments demonstrated that 1α,25-$(OH)_2D_3$ had an inhibitory effect on 1,25D_3-[MARRS]bp expression. The mean ± SD of the percentage inhibition was: 51.46 ± 8.68%, n = 4 (Table II). Table II shows that the expression of 1,25D_3-[MARRS]bp was decreased when MO6-G3 cells were exposed to 50 nM 1α,25-$(OH)_2D_3$ for 36 h when considering both the percentage positive cells and the MFI. For example, in experiment number 2, the MFI was 4 for background fluorescence (FITC control), 37 for cells exposed to 0.1% ethanol as vehicle, and 16 for cells incubated with 50 nM 1α,25-$(OH)_2D_3$, thus indicating a down-regulation of 1,25D_3-[MARRS]bp expression by MO6-G3 cells when the ligand 1α,25-$(OH)_2D_3$ interacts with this binding protein. In another set of experiments (Table III), MO6-G3 cells were treated for 36 h with lower or higher doses of 1α,25-$(OH)_2D_3$ and 1,25D_3-[MARRS]bp expression was investigated by flow cytometry as described above. As shown in Table III, the addition of lower doses of 1α,25-$(OH)_2D_3$ (25 through 0.5 nM) led in most experiments (number 1, 2, 4, and 5), to a partial inhibition of 1,25D_3-[MARRS]bp expression (the mean ± SD of the percentage inhibition is: 29 ± 11%, n = 10) when considering both the percentage positive cells and the MFI. In experiment number 1, the MFI was 24 for vehicle treated cells, 17 for 12.5 nM treated cells, 22 for 6.25 nM treated cells, and 19 for 50 nM treated cells (Table III). Higher doses of 1α,25-$(OH)_2D_3$ (100–500 nM) led also to a decrease of 1,25D_3-[MARRS]bp expression (Table III) but surprisingly this decrease was less than that obtained with 50 nM 1α,25-$(OH)_2D_3$. In three independent experiments (number 2, 3, and 4), the mean ± SD of the percentage inhibition was 27 ± 10.34%, n = 5, for this range of doses (Table III). In experiment number 4, the MFI was 37 for control cells, 28 when cells were incubated with 200 nM 1α,25-$(OH)_2D_3$, and 34 for 100 nM 1α,25-$(OH)_2D_3$ thus indicating a slight inhibitory effect of 1α,25-$(OH)_2D_3$ on 1,25D_3-[MARRS]bp expression by MO6-G3 cells. Altogether the results from Figure 5 and Tables II and III indicate that 1,25D_3-[MARRS]bp expression by MO6-G3 is down-regulated by 1α,25-$(OH)_2D_3$, to a greater extent at an optimal concentration of 50 nM steroid.

TABLE II. Inhibitory Effects of 1α,25-$(OH)_2D_3$ on 1,25D_3-[MARRS]bp Expression

Experiment number	Control or +: 1α,25-$(OH)_2D_3$[a]	1,25D_3-[MARRS]bp expression[b]		Background fluorescence[c]		Inhibition of 1,25D_3-[MARRS]bp expression[d]
		%	MFI	%	MFI	%
1	Control	52.23 ± 2.80	143	4.21 ± 0.26	55	—
	+	20.28 ± 0.33	96			61.17
2	Control	71.16 ± 3.80	37	0.98 ± 0.59	4	—
	+	31.94 ± 0.01	16			55.11
3	Control	50.69 ± 0.01	24	2.70 ± 0.05	9	—
	+	26.03 ± 1.50	19			48.64
4	Control	34.61 ± 3.90	25	0.77 ± 0.01	8	—
	+	20.44 ± 3.70	17			40.94

MFI, mean fluorescence index.
[a]50 nM 1α,25-$(OH)_2D_3$ or 0.1% ethanol as vehicle (control) were added to duplicate cultures of MO6-G3 cells for 36 h.
[b]The 1,25D_3-[MARRS]bp membrane expression was measured after 36 h of duplicate cultures of MO6-G3 cells treated with 50 nM 1α,25-$(OH)_2D_3$ or 0.1% ethanol (vehicle) by indirect immunofluorescence with Ab099. Data shown as mean ± SD of the percentage positive cells of duplicate cultures. FACScalibur software give percentage positive cells (M1 zone in dot figure).
[c]The background immunofluorescence was measured after 36 h of duplicate cultures of MO6-G3 cells treated with 0.1% ethanol (vehicle) with FITC-conjugate. Data shown as mean ± SD of the percentage positive cells of duplicate cultures. FACScalibur software give percentage positive cells (M1 zone in dot figure).
[d]Percentage inhibition. Results are expressed as percentage inhibition of 1,25D_3-[MARRS]bp expression between the 50 nM 1α,25-$(OH)_2D_3$ treated cells compared to 0.1% ethanol treated cells. Mean ± SD of the percentage inhibition (four experiments): 51.46 ± 8.68%.

TABLE III. Modulation of 1,25D_3-[MARRS]bp Expression at Various Doses of 1α,25-$(OH)_2D_3$

Experiment number	Control or 1α,25-$(OH)_2D_3$[a]	1,25D_3-[MARRS]bp expression[b]		Background fluorescence[c]		Inhibition of 1,25D_3-[MARRS]bp expression[d]
		%	MFI	%	MFI	%
1	Control	50.69 ± 0.01	24	2.70 ± 0.05	9	—
	50	26.03 ± 1.50	19			48.64
	25	33.73 ± 9.90	21			33.45
	12.5	26.44 ± 1.85	17			47.83
	6.25	42.35 ± 5.60	22			16.09
2	Control	34.61 ± 3.90	25	0.77 ± 0.01	8	—
	500	24.11 ± 1.70	20			30.34
	50	20.44 ± 3.70	17			40.94
	5	22.65 ± 2.00	17			34.55
	0.5	26.23 ± 1.80	22			24.21
3	Control	52.23 ± 2.80	143	4.21 ± 0.26	55	—
	200	36.46 ± 0.07	104			30.19
	100	38.24 ± 1.40	113			26.78
	50	20.28 ± 0.33	96			61.17
4	Control	71.16 ± 3.80	37	0.98 ± 0.59	4	—
	200	44.34 ± 7.30	28			37.69
	100	64.07 ± 0.01	34			9.9
	50	31.94 ± 0.01	16			55.11
	25	61.30 ± 4.00	37			13.86
5	Control	79.50 ± 0.01	49	0.89 ± 0.72	8	—
	25	45.50 ± 14.8	23			42.77
	12.5	36.50 ± 2.10	25			54
	6.25	48.50 ± 3.50	31			38.99
	3.1	65.50 ± 5.60	33			17.61

MFI, mean fluorescence index.
[a]1α,25-$(OH)_2D_3$ or 0.1% ethanol as vehicle (control) were added to duplicate cultures of MO6-G3 cells for 36 h.
[b]The 1,25D_3-[MARRS]bp membrane expression was measured after 36 h of duplicate cultures of MO6-G3 cells treated with 1α,25-$(OH)_2D_3$ or 0.1% ethanol (vehicle) by indirect immunofluorescence with Ab099. Data shown as mean ± SD of the percentage positive cells of duplicate cultures. FACScalibur software give percentage positive cells (M1 zone in dot figure).
[c]The background immunofluorescence was measured after 36 h of duplicate cultures of MO6-G3 cells treated with 0.1% ethanol (vehicle) with FITC-conjugate. Data shown as mean ± SD of the percentage positive cells of duplicate cultures. FACScalibur software give percentage positive cells (M1 zone in dot figure).
[d]Percentage inhibition. Results are expressed as percentage inhibition of 1,25D_3-[MARRS]bp expression between the 1α,25-$(OH)_2D_3$ treated cells compared to 0.1% ethanol treated cells.

Detection of 1,25D_3-[MARRS]bp in the Supernatant of MO6-G3 Cells

We then investigated whether the variations of 1,25D_3-[MARRS]bp expression induced by 1α,25$(OH)_2D_3$ at the surface of MO6-G3 cells are consistent with the presence of 1,25D_3-[MARRS]bp in the culture media. Serial dilutions of supernatants of MO6-G3 cells treated or not treated with 50 nM 1α,25-$(OH)_2D_3$ (the dose inducing a maximal inhibitory effect on 1,25D_3-[MARRS]bp expression) were tested for the presence of 1,25D_3-[MARRS]bp by an immunodot assay with Ab099. A representative autoradiograph illustrated in Figure 6A shows that the two samples of supernatants obtained from the control or treated MO6-G3 cells reacted with Ab099 (Fig. 6A). The 1,25D_3-[MARRS]bp was specifically detected in these supernatants because no spots were detected with a control antibody (data not shown). Nevertheless the densitometric analysis of the dots showed no difference between the two supernatants with or without addition of 50 nM 1α,25-$(OH)_2D_3$. Thus, the amounts of immunoreactive 1,25D_3-[MARRS]bp in the culture media seems identical between the treated and untreated groups. Then, we investigated by transmission electron microscopy the presence of the extracellular form of 1,25D_3-[MARRS]bp produced by MO6-G3 cells. As shown in Figure 6B, immunogold staining was detected near the plasma membrane of MO6-G3 cells in the extracellular matrix area and associated with electron dense structures. At higher magnification, the gold labeling clearly was co-localized with these membrane vesicle-like structures (Fig. 6C,D). Altogether, these results support the existence of an extracellular form of 1,25D_3-[MARRS]bp produced by MO6-G3 cells.

DISCUSSION

The present data show that mouse dental cells express 1,25D_3-[MARRS]bp, corresponding to that previously described in multiple cell types from different species [Nemere et al., 1994, 1996, 1997, 1998, 2000a,b; Pedrozo et al., 1999; Nemere and Campbell, 2000; Mesbah et al., 2002]. Furthermore 1α,25-$(OH)_2D_3$ appeared to down-regulate the expression of 1,25D_3-[MARRS]bp with variable intensity. We have shown, for the first time, that the mouse incisor dental mesenchyme and the mouse odontoblast-like cell line MO6-G3 specifically express the membrane receptor for 1α,25-$(OH)_2D_3$ with a molecular weight of 65,000–66,000. The presence of the additional bands in dental mesenchyme could be explained by a higher protease activity in heterogenous mouse incisor preparations, although protease inhibitors were added to all samples prepared at 4°C, or by incomplete denaturation of 1,25D_3-[MARRS]bp to account for higher Mr bands. Moreover, flow cytometric analysis confirmed the specific expression of 1,25D_3-[MARRS]bp by MO6-G3 cells. Furthermore, immunostaining of mouse tooth tissues showed that both ameloblasts and odontoblasts express the 1,25D_3-[MARRS]bp with variable intensity depending on their stage of maturation. The 1,25D_3-[MARRS]bp was strongly expressed by differentiated matrix producing cells by contrast to undifferentiated dental cells. It is in agreement with recent results also showing a modulation of 1,25D_3-[MARRS]bp expression in human bone cells [Mesbah et al., 2002]. Thus, 1,25D_3-[MARRS]bp could play a role in tooth morphogenesis and dental cell differentiation. Electron microscopic analysis showed a plasma membrane localization of 1,25D_3-[MARRS]bp, as well as a 1,25D_3-[MARRS]bp detection in subcellular compartments. These results are in agreement with the detection of 1,25D_3-[MARRS]bp in the nucleus, and Golgi apparatus of chick intestinal epithelial cells [Nemere et al., 2000b]. The endoplasmic reticulum and Golgi apparatus detection of 1,25D_3-[MARRS]bp could correspond to the classical anterograde biosynthetic pathway of proteins. Nevertheless a retrograde transport of 1,25D_3-[MARRS]bp to the Golgi apparatus and endoplasmic reticulum could also explain the presence of 1,25D_3-[MARRS]bp in these compartments. Retrograde transport exists for various proteins [Johannes and Goud, 2000]. In accordance, plasma membrane-associated estrogen receptors have been detected in the intracellular compartments of mouse macrophages [Benten et al., 2001]. This G-protein-coupled receptor mediates Ca^{2+} mobilization and Ca^{2+} influx and is sequestrable upon 17β-estradiol stimulation [Benten et al., 2001].

The cDNA sequence of 1,25D_3-[MARRS]bp appears to be identical to that of the multifunctional thiol-dependent oxidoreductase ERp57 [Nemere et al., 2004]. These data strengthen our observation of an endoplasmic reticulum and Golgi localization of 1,25D_3-

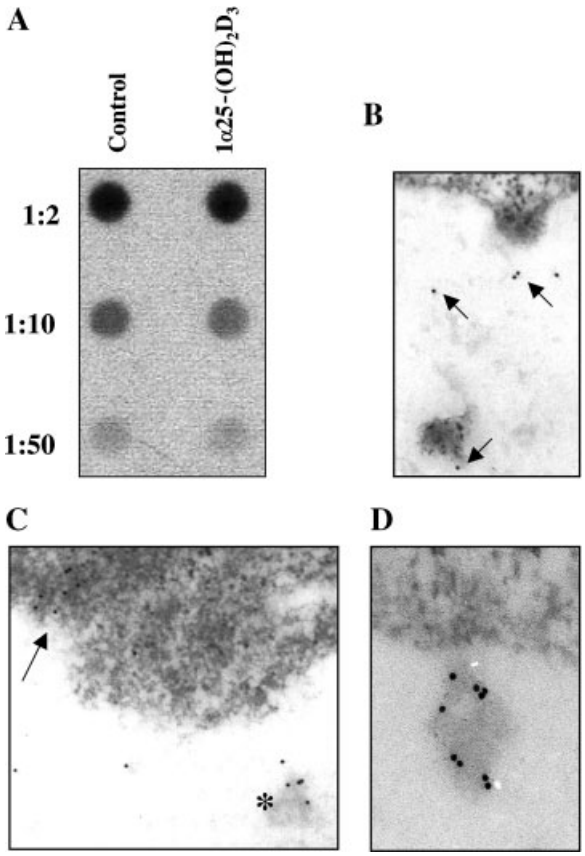

Fig. 6. Cell culture supernatants of MO6-G3 cells contain immunoreactive 1,25D_3-[MARRS]bp. **A**: Culture medium harvested at 36 h from MO6-G3 incubated with ethanol vehicle (control) or with 50 nM 1α,25-$(OH)_2D_3$ were tested for the presence of 1,25D_3-[MARRS]bp by immunodot assay with Ab099 (diluted at 1:4,000) followed by HRP-conjugated secondary antibody (1:40,000). Serial dilutions of medium were deposited under vacuum on a nitrocellulose membrane. The autoradiograph was then analyzed with a scanning densitomer. **B–D**: Transmission electron micrographs of MO6-G3 cells. Ultrathin cryosections (50–80 nm) were incubated with Ab099 (1:1,000 final dilution) and immunogold labeling was performed as described in Figure 3. **B**: The extracellular matrix of MO6-G3 cells contains 1,25D_3-[MARRS]bp gold staining [arrows] (×110,000 magnification). **C**: An immunogold labeling is showed near the plasma membrane (arrow) and on a vesicle-like structure (*) (×105,000 magnification). Note that in **D**, at higher magnification (×140,000 magnification), a vesicle-like structure contained numerous gold particles.

[MARRS]bp in dental cells, since ERp57, a member of the protein disulphide isomerases, plays a role in the folding of glycoproteins in the endoplasmic reticulum [for review see High et al., 2000]. Moreover the protein disulfide isomerase binds estradiol and 3,3′,5-triiodo-L-thyronine inside the endoplasmic reticulum that is considered as a major hormone reservoir [Primm and Gilbert, 2001]. Thus, 1,25D_3-[MARRS]bp might have multifunctional activities such as controlling 1α,25-$(OH)_2D_3$ levels in the microenvironment of dental cells. The expression of receptors are often modulated by their own ligand. Nuclear receptors of seco-steroid hormones and cytokine receptors are known to be modulated at the mRNA or protein levels. Other membrane receptors of steroid hormones are modulated at the expression and functional levels by their own ligand, depending on the dosage of hormone. A biphasic response of intracellular Ca^{2+} and phosphoinositide metabolism inhibition induced by 17β-estradiol has been described in osteoblasts and related to membrane receptors coupled to a phospholipase C via a *Pertussis* toxin-sensitive G protein [Lieberherr et al., 1993]. Agonist-sequestrable receptors for testosterone and for 17β-estradiol also have been described in the membrane of mouse macrophages and T cells [Benten et al., 1999a,b, 2001]. Here, we tested the possibility that 1α,25-$(OH)_2D_3$ regulates 1,25D_3-[MARRS]bp expression. We showed by both flow cytometry and biochemical analysis, that 1α,25-$(OH)_2D_3$ modulates 1,25D_3-[MARRS]bp expression in MO6-G3 cells with a maximal decrease of the expression at 50 nM steroid. Analysis of cell viability 36 h after addition of 1α,25-$(OH)_2D_3$ by trypan blue exclusion assay showed that the modulation of 1,25D_3-[MARRS]bp expression is not due to a direct cellular toxicity exerted by 1α,25-$(OH)_2D_3$. In cultures with 50 nM 1α,25-$(OH)_2D_3$, a dose that led to a maximal inhibition of 1,25D_3-[MARRS]bp expression, cell viability was not significantly affected as compared with the control cultures (90% cell viability for each type of treatment). Furthermore, when propidium iodide was used during the flow cytometric analysis, it showed that the viability of MO6-G3 cells was around 95% after 1α,25-$(OH)_2D_3$ treatment (data not shown). Moreover, ponceau red staining of the blots obtained with Triton X-114 extracts indicated that the lanes were equally loaded, thus excluding protein degradation occurring in the ligand treated samples (data not shown). Thus it seems that the 51% inhibition of 1,25D_3-[MARRS]bp expression observed after addition of the ligand at day 0 is a consequence of an effective decrease of 1,25D_3-[MARRS]bp expression by MO6-G3 cells. Such hormone-related down regulation is also seen in chick intestinal basal lateral membranes, but

not kidney or brain [Nemere and Campbell, 2000]. In addition, the responses to 1α,25-$(OH)_2D_3$ seems biphasic since lower inhibition of 1,25D_3-[MARRS]bp was observed at higher and lower doses of 1α,25-$(OH)_2D_3$. Altogether, these two procedures allow a relative quantitation of these variations in the presence or in the absence of ligand. Furthermore, addition of 1α,25-$(OH)_2D_3$ did not affect the level of extracellular 1,25D_3-[MARRS]bp. These data are in line with previous studies showing a ligand-induced intracellular translocation of the 1,25D_3-[MARRS]bp and a recompartmentalization of this binding protein [Nemere et al., 2000b]. Several hypotheses can be raised to decipher the molecular mechanism(s) through which 1α,25-$(OH)_2D_3$ exerts its modulation activity. Our results could be explained by an internalization process similar to those described for membrane estradiol receptor and for testosterone surface receptors [Benten et al., 1999b, 2001] or by a translational process at the mRNA level or a post-translational process at the protein level. The modulation of 1,25D_3-[MARRS]bp expression could also reflect changes in the conformation and/or accessibility of the 1,25D_3-[MARRS]bp epitope. Another possibility is a proteolytic cleavage of 1,25D_3-[MARRS]bp induced by 1α,25-$(OH)_2D_3$. In addition, the immunopositive dots obtained by dot blotting MO6-G3 cell culture supernatants with Ab099 could be related to the presence of membrane vesicle-like structures in the extracellular matrix of MO6-G3. However, we cannot totally exclude the presence of a soluble form of 1,25D_3-[MARRS]bp, due to a proteolytic cleavage of 1,25D_3-[MARRS]bp expressed by MO6-G3 cells and/or the production of a soluble form of 1,25D_3-[MARRS]bp. The membrane vesicle-like structures detected in the extracellular matrix of MO6-G3 cells could correspond to the well-described plasma membrane sloughing process described for osteoblasts [for review, see Anderson, 2003] and chondrocytes [for review, see Boyan et al., 1997]. Similarly, it has been shown that a number of cells from different lineages, including lymphocytes and tumor cells, produce exosomes [Blanchard et al., 2002; Riteau et al., 2003]. They may play a role in ligand transport by biological fluids in a manner similar to the vitamin D binding protein detected in mouse and human serum [Cooke and Haddad, 1989; Haddad, 1995] to target cells [for review see Christakos et al., 2003] or in the storage of the molecules in the dentin extracellular matrix to act on target cells. It is tempting to speculate that the presence of 1,25D_3-[MARRS]bp at the membrane and in the intracellular compartment of dental cells may confer important properties of this protein in tooth development and matrix biomineralization.

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