Immunological Identification of 1,25-Dihydroxyvitamin D3 Receptors in Human Promyelocytic Leukemic Cells (HL-60) during Homologous Regulation*

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The actions of 1,25-dihydroxyvitamin D3 \((1,25-(OH)_{2}D_3)\)† in target cells are believed to be mediated by the interaction of the hormone with a specific intracellular receptor protein \((1,2)\). The receptor-hormone complex is postulated to regulate the expression of specific hormone-dependent genes, possibly by binding to promoter regions of genes, in a manner similar to that hypothesized for other steroid hormones \((3,4)\). Receptors for \(1,25-(OH)_{2}D_3\) have been identified recently in hematopoietic cells, including the human promyelocytic leukemic cell line, HL-60 \((11-16)\).

Evidence suggests that the magnitude of \(1,25-(OH)_{2}D_3\) biological responses in several cultured cell lines, including HL-60 cells, is dependent on receptor presence and the degree of ligand occupancy \((8, 17-20)\). Moreover, in HL-60 cells, the rank order of vitamin D3 metabolites inducing monocyctic differentiation is similar to that affinity to the \(1,25-(OH)_{2}D_3\) receptor \((7, 21)\). Both homologous and heterologous regulation of \(1,25-(OH)_{2}D_3\) receptor in several target cells have been reported in vivo and in vitro by using radiolabeled hormone-receptor binding techniques \((22-25)\). Therefore, alteration of receptor level is an important factor in modulating cell responsiveness to hormone.

We have previously reported \((26)\) that the level of \(1,25-(OH)_{2}D_3\) receptors in intact HL-60 cells increased (up-regulation) and then decreased (down-regulation) upon continuous exposure to \(1,25-(OH)_{2}D_3\). Since these findings were based on receptor quantification by high specific activity tritiated ligand, potentially nonhormone-binding or unoccupied forms of the receptor cannot be characterized from these studies. In this study, we have employed a monoclonal antibody to the porcine \(1,25-(OH)_{2}D_3\) receptor which has high cross-reactivity with other mammalian \(1,25-(OH)_{2}D_3\) receptors \((27-29)\). Evidence is provided to support the hypothesis that receptor autoregulation in the presence of \(1,25-(OH)_{2}D_3\) leads to up- and down-regulated receptors which have the same electrophoretic mobility as that of basal receptors and that these three receptor groups exist as one antigenically related species of 53,000 Da.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

To characterize the \(1,25-(OH)_{2}D_3\) receptors in the HL-60 cells during homologous regulation, monoclonal antibodies to the \(1,25-(OH)_{2}D_3\) receptor were used in immunoprecipitation and immunoblotting. The monoclonal antibody \((IVG8C11)\) raised against the porcine intestinal receptor for \(1,25-(OH)_{2}D_3\) was used for immunoprecipitation to concentrate \(1,25-(OH)_{2}D_3\) receptors in HL-60 cells. To assess the ability of this monoclonal antibody to bind the receptor in the crude nuclear fraction of HL-60 cells, the immunoprecipitation efficiency for the HL-60 receptor was determined and compared to that of the porcine intestinal receptor (Fig. 1). To a constant amount of pig or HL-60 cell nuclear extracts, increasing...
amounts of IVG8C11 were used to immunoprecipitate receptors. To precipitate the antibody-receptor complex, goat anti-mouse IgG coupled to Sepharose was added. HL-60 cell receptors, occupied in vivo, were immunoprecipitated quantitatively by IVG8C11 in a similar manner to the immunoprecipitation efficiency of the pig intestinal receptor. These titration experiments showed that 10 ng of IVG8C11 was sufficient to precipitate 1 fmol of $[^{3}H]1,25-(OH)_{2}D_{3}$-bound receptor. This ratio was used for all immunoprecipitations in preparation for immunoblotting. The hormone binding activity was determined by hydroxylapatite assay as described under “Materials and Methods.”

Immunoblotting experiments were performed to characterize the basal and apparent up- and down-regulated receptors for 1,25-(OH)$_{2}$D$_{3}$ in HL-60 cells. HL-60 cells were incubated with 2 nM $[^{3}H]1,25-(OH)_{2}D_{3}$ for 12, 36 h to prepare the basal and up- and down-regulated 1,25-(OH)$_{2}$D$_{3}$ receptors, respectively. The 1,25-(OH)$_{2}$D$_{3}$ receptors in nuclear extracts were immunoprecipitated and then electrophoresed on 9% discontinuous SDS-polyacrylamide gels. After the proteins in the gel were transferred to a polyvinylidene difluoride membrane, 1,25-(OH)$_{2}$D$_{3}$ receptors were detected by probing the membrane with $^{125}$I-labeled IVG8C11. As shown in Fig. 2, the basal receptor, occupied in vivo, was detected as a single form of 53,000 Da (lane 1). The up-regulated (lane 3) and down-regulated (lane 5) receptors were detected as a single form of 53,000 Da. The ratio of density of the three bands was 1:0.3:2.1:2 for basal and up- and down-regulated receptors, respectively, as measured by a Soft Laser scanning densitometer (Model SL-504-XL, Biomed Instruments Inc., Fullerton, CA). These data indicated clearly that there was a 3-fold increase in the amount of protein detected by IVG8C11 in lane 3 compared to the basal receptor level (lane 1). This phase was followed by the decrease in the level of receptor to the basal receptor level by incubating cells with 1,25-(OH)$_{2}$D$_{3}$ for 36 h (lane 5). The increase in the amount of receptor during up-regulation was blocked completely in the presence of 10 µM cycloheximide (lane 4). The cell viability was greater than 90% throughout the experiments as determined by trypan blue exclusion. To determine the immunoprecipitation efficiency, parallel flasks of cells were incubated with 2 nM $[^{3}H]1,25-(OH)_{2}D_{3}$. The immunoprecipitation efficiencies ranged from 85 to 90%. The regulation of $[^{3}H]1,25-(OH)_{2}D_{3}$ uptake into intact cells in the presence or absence of cycloheximide is shown in Fig. 3. Receptors for 1,25-(OH)$_{2}$D$_{3}$ became saturated within 1 h upon incubation of HL-60 cells with concentrations of $[^{3}H]1,25-(OH)_{2}D_{3}$ greater than 0.8 nM, as previously demonstrated (30). The amount of specific binding increased and became maximal at about 12 h. This up-regulated phase was followed by apparent down-regulation observed at 24 h. Up-regulation was prevented in HL-60 cells incubated in the presence of $[^{3}H]1,25-(OH)_{2}D_{3}$ and the protein synthesis inhibitor, cycloheximide. Moreover, receptors were barely detectable by either immunodetection (Fig. 2) or $[^{3}H]$1,25-(OH)$_{2}$D$_{3}$ binding at 36 h in the cycloheximide-treated cells. This concentration of cycloheximide inhibited protein synthesis by 92% as measured by $[^{3}H]$leucine incorporation and had no deleterious effect on cell viability (data not shown). Throughout these studies, the intensity of band from the Western blot was proportional to the amount of immunoprecipitated receptor protein loaded on the SDS-polyacrylamide gel (data not shown). However, the immunoblotting assay is not generally sensitive enough for the accurate quantitative determination of receptor protein. The broad diffuse bands observed just above the receptor band or at 26 kDa may correspond to the heavy and light chains of goat anti-mouse IgG used for the immunoprecipitation since two broad diffuse bands were observed at the same position when a blank sample, containing monoclonal antibody and goat anti-mouse IgG without receptor preparation, was immunoblotted (data not shown).

Previously, it has been reported (22) that vitamin D metabolites up-regulated the number of 1,25-(OH)$_{2}$D$_{3}$ receptors via a receptor-mediated induction mechanism in a cultured pig kidney cell line. Mutant skin fibroblasts from patients with vitamin D-dependent rickets type II, containing nonresponsive D$_{3}$ receptors, failed to exhibit characteristic up-regulation observed in normal cells (22, 35). More recently it has been demonstrated that 1,25-(OH)$_{2}$D$_{3}$ can increase mRNA activity for its receptor in mouse fibroblasts (3T6), indicative of receptor autoregulation (36). These data support our hypothesis that the presence of 1,25-(OH)$_{2}$D$_{3}$ leads to a homologous up-regulation of 1,25-(OH)$_{2}$D$_{3}$ receptors in HL-60 cells.

In order to examine whether the level of receptor decreased to below that of basal levels upon prolonged incubation, immunoblot and ligand binding assays were performed for the mutants cultured with hormone up to 72 h (Fig. 4). Cells were seeded at a lower density ($5 \times 10^5$ cells/ml) to exclude the possibility of the lack of nutrients in the medium with the extended incubation periods. Fresh hormone was added to the cells with 48- and 72-h incubations 24 h prior to the harvest. The ratio of density of the immunolabeled receptor bands was 1:0.4:2.3:9:1:2:0.9 for 2-, 12-, 24-, 48-, and 72-h treatments with hormone, respectively (Fig. 4). Whereas both the immunodetection and ligand binding methods estimated a similar increase in the amount of 1,25-(OH)$_{2}$D$_{3}$ receptor induction during up-regulation, they differed quantitatively for estimating the degree of down-regulation. Utilizing Western blotting, the level of the up-regulated receptor was observed to decline to basal levels by 36-48 h and remained at that level up to 72 h during continuous hormone incubation (Fig. 4). Conversely, $[^{3}H]1,25-(OH)_{2}D_{3}$ binding data showed that at 48 and 72 h,
up-regulated receptors had declined to 46 and 66% of the basal level, respectively (Fig. 5). This difference may be interpreted to mean that although receptor protein is present during down-regulation as detected by Western blots, the loss of specific [3H]1,25-(OH)2D3-binding sites is lost preferentially or at least is incapable of being largely due to a loss of hormone-binding capacity of steroid hormone (processing) proteins (37, 38).

The control of the expression of the steroid hormone receptor is not well understood. The data presented here suggest that the homologous up-regulation followed by down-regulation of the 1,25-(OH)2D3 receptor may represent a nuclear mechanism for the control of cellular responsiveness to hormone in the intact human target cells. In addition, these alterations might be components of the cellular differentiation process since 1,25-(OH)2D3 is the most potent inducing agent for monocytic differentiation of HL-60 cells (8). HL-60 cells have been shown to regulate other types of receptors during monocytic and granulocytic differentiation, including receptors for insulin and phorbol esters (39–41).

The immunological detection system for the 1,25-(OH)2D3 receptor employed in this study was specific for identifying the 1,25-(OH)2D3 receptors. Antibody X-VI-E6E6G10 recognized only the porcine 1,25-(OH)2D3 receptor (Fig. 6) and clearly bound to an epitope distinct from antibody IVG8C11 (27). Antibody X-VI-E6E6G10 precipitated receptors from the porcine intestinal nuclear extract, whereas it did not recognize the receptors from nuclear extracts of HL-60 cells (Fig. 6, lane 2 versus lane 5). The 1,25-(OH)2D3 receptors in HL-60 cells are a single form of protein species of 53 kDa. The pig intestinal receptors are detected at 55 kDa as described previously (28). The immunoprecipitation procedure itself did not affect the electrophoretic mobility of the receptor protein since the pig intestinal receptors immunoprecipitated with either IVG8C11 or X-VI-E6E6G10 have the same electrophoretic mobility as those without immunoprecipitation. These results are consistent with the conclusions that 1,25-(OH)2D3 receptors are protein species ranging from 52 to 60 kDa; and although their functional and immunological domains have been evolutionarily conserved, an inverse relationship apparently exists between phylogenetic status and receptor mass (36). The data presented here clearly demonstrate that the level of 1,25-(OH)2D3 receptors in intact human target cells undergoes up-regulation, followed by down-regulation upon continuous exposure to 1,25-(OH)2D3.

REFERENCES


Continued on next page.
Supplementary Material to
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Immunological Identification of 1,25-(OH)2D3 Receptors

MATERIALS AND METHODS

Materials. 1,25-dihydroxyvitamin D3, radiolabeled with tritium at the 26,27-methyl groups [TH25,26,27]-1,25-(OH)2D3 (26 Ci/mmol, Cortex-Biosystems)], 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3, from DuPont, New England Nuclear Products, Boston, MA), anti-1,25-(OH)2D3, was a gift from D. U. Molkentin of Hoffmann-LaRoche, Inc. (Nutley, NJ). Determinations of purity and concentration of 1,25-(OH)2D3 were achieved by UV absorption spectrophotometry using an extinction coefficient (ε = 260) of 20,000 M-1 cm-1. Hydroxyapatite (HAP), acrylamide and bis-acrylamide were purchased from BIO-RAD (Richmond, CA). Sephadex G-10 (5-mm particle size 50-70 mm) from Pharmacia Inc. (Piscataway, NJ), gelatin and sodium dodecyl sulfate (SDS) from Sigma Chemical Co., (St. Louis, MO) were dissolved in 0.1 M acetate buffer, pH 7.0, 3.5 mg/mg SDS, pH 7.0, 0.1 M tris(hydroxymethyl)aminomethane, 0.1 M EDTA, 0.1 M dithiothreitol, 0.1 M sodium phosphate, TEDMK-63, TEDMK buffer with 0.1 M KCl.

Preparation of 1,25-(OH)2D3 Receptors. Receptors for 1,25-(OH)2D3 from HL-60 cells were prepared as described previously (39). Briefly, HL-60 cells cultured in SFM were incubated with 2,5H-1,25-(OH)2D3 or SFM in 37°C in a humidified 95% air-5% CO2 atmosphere for various times. The following procedures were done at 4°C. After cells were washed twice in FBS, they were resuspended and incubated for 20 min in TEDMK buffer with intermolecular crosslinking followed by immunoprecipitation using a Tosanimer type SIDE (Tekmar Co., Cincinnati, OH) for 3 h at a speed of setting of 6. The homogenate was centrifuged for 10 min at 600 g to obtain crude pellet fractions. The crude nuclear pellet was incubated in TEDMK-63 for 30 min followed by centrifugation at 10,000 g for 45 min in a Beckman L55 ultracentrifuge using a type 50 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was utilized as the source of occupied 1,25-(OH)2D3 receptors in HL-60 cells for electrophoresis experiments. The nuclear pellets were prepared from separate intact cells as described previously (20). The total cellular extracts of HL-60 cells were prepared essentially as described previously (31). All operations were performed at 4°C. After cells were washed 3 times with PBS, they were resuspended in TEDMK-63 with a protease inhibitor, 5 mM disopropylphosphorofluoridate (DFP), and sonicated with three 20sec cycles interrupted by 60sec pauses. The sonicate was centrifuged at 100,000 g for 60 min to yield the total cellular receptor preparation.

Measurement of Total Cellular Receptors. The amount of total cellular receptors for 1,25-(OH)2D3 was determined in HL-60 cells as described previously (30). Briefly, cells in SFM were incubated with various concentrations of [TH25,26,27]-1,25-(OH)2D3 in the presence or absence of a 20 fold excess of nonradioactive hormone. After each incubation period, cells were washed twice with PBS and resuspended in TEDMK buffer for 20 min at 4°C with intermolecular crosslinking. Aliquots of cells in TEDMK buffer were used for receptor-binding [TH25,26,27]-1,25-(OH)2D3, by HAF assay (32) with slight modification as described earlier. The specific binding was estimated as the difference between total binding ([TH25,26,27]-1,25-(OH)2D3, alone) and non specific binding (with a 200 fold excess of nonradioactive hormone). The radioactivity was measured in a liquid scintillation spectrometer (Packard Tri-Carb Spectrometer, model P-502D) using Opti- fluor scintillation fluid (Packard, Downers Grove, IL) with an efficiency of tritium of 90%.

Immunoprecipitation. The monoclonal antibodies to the porcine intact 1,25-(OH)2D3 receptors used in this work, BVG4231 (IgG) and BVG4308 (IgM) were described previously (27). The nucle- ar extracts from HL-60 with or with 1,25-(OH)2D3, or SFM, were incubated overnight with monoclonal antibodies to immunoprecipitate 1 of 1,25-(OH)2D3 receptor with continuous shaking for 1 h at 4°C. The pig pig immunoprecipitated was washed in TEDMK-63 in the presence of 0.350 fold excess of nonradioactive hormone. The purified antigen was isolated in TEDMK-63, and the concentrated antigens were then used for immunoprecipitations. The determination of the protein in the immunoprecipitate was determined by a spectrophotometric method. The radioactivity was measured as described above. To electrophoreses the immunoprecipitated receptors, the Sephadex beads incubated with occupied 1,25-(OH)2D3-receptors were filtered for 3 min in 1 vol. of TEDMK-63, detaining buffer. After the tubes were centrifuged at 200 g for 10 min at 25°C, the supernatants were electrophoresed on 9% SDS-polyacrylamide gel (12.5 mm span) and were analyzed by Laemml (35).

Immunoblot. For use in immunoesternum experiments, purified monoclonal antibody, IGV, was isolated with "B" Stauromen-Hamer reagent (NE Research Products) as described previously (34). Briefly, 30 of IGV in 10 of PBS were reacted with "B" Stauromen-Hamer reagent (1 NG) for 2 h on ice. To stop the reaction, 6,25 fold of glycine in 0.1 M peptide (pH 8.5) was added for 0,25 min, then 0.25 M PBS containing 0,25% gelatin was added to the reaction mix. The monoclonal antibody coupled with "B" specific for "B" was separated for "B" using a Sepharose 4B column, which was bound with 1 of 3% (v/v) bovine serum albumin in PBS and then equilibrated with PBS containing 0.2% gelatin. Fractions were collected (0.15 ml each) and the radioactivity in the peak fractions determined in a Radioimmune assay. 1.25-(OH)2D3 was measured using the deoxycorticosterone-TCA precipitation method (80% of radioligand was precipitated) by incubating with 100 of 0.01% deoxycorticosterone and 100 of 1% TCA. After electrophoreses, the gels were equi- libriated in transfer buffer 15 Sm transfer electrophoresed to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA) at 14 mA for 1 h using Tris-borate electrophoresis unit (Hoefer Scientific Instrument, San Francisco, CA). The PVDF membranes were then washed in 0.1% methanol for 1 h at room temperature, and washed with 0.5% PBS for 2.5 min. The membranes were then incubated with 3 of 0.15% (v/v) IGV for 1 h. After the membranes were washed with 10 of PBS for 15 min to remove free ligand buffer, the membranes were stained with 3 of 0.5% (v/v) Coomassie Blue R-250 in DuPont Coomassie Quansel-III enhancing manner (DuPont, New England Nuclear) at 70°C.

Immunological Identification of 1,25-(OH)2D3 Receptors in Human Promyelocytic Leukemic Cells (HL-60) During Homogenezation Regulation

Figure 1. Immunoprecipitation of [TH25,26,27]-1,25-(OH)2D3 binding activity in the nuclear extract of pit tissue (C) and cellular extract of HL-60 (A). Each extract was labeled with 1.0 fmol [TH25,26,27]-1,25-(OH)2D3, in the presence or absence of an excess hormone. 200 ul of the labeled sample, containing 12 fold of specific binding activity, was then incubated with the indicated amounts of monoclonal antibody IVG for 16 hr. Following immunoprecipitation with 50 ul of goat anti-mouse IgG-Sepharose, the supernatant was removed by HAP assay. The remaining pelleted was counted as a 4 ml OptiFluor scintillation fluid in a liquid scintillation counter. 100% was defined as the sum of the specific binding activity in the supernatant and precipitate.

Figure 3. Effect of cycloheximide on the time-dependent regulation of [TH25,26,27]-1,25-(OH)2D3 uptake into intact HL-60 cells. Cells cultured in SFM were incubated with 1 pm [TH25,26,27]-1,25-(OH)2D3, in the presence or absence of an excess hormone. Cells were placed in humidified 95% air-5% CO2 atmosphere at 37°C for indicated times at a seeding density of 5 x 105 cells per 1 ml of culture medium. Viability of cells was examined by trypan blue exclusion at the end of the incubation periods. Specifically bound [TH25,26,27]-1,25-(OH)2D3 was quantified by the HAP batch assay. Values represent the means for four replicate wells with duplicate measurements from each well. All values of SFM are similar than the diameters of symbols.

Figure 4. Examination of the down-regulated receptor level from HL-60 cells exposed to hormone for up to 72 hr. HL-60 cells in SFM were incubated with 2,5H-1,25-(OH)2D3 for 2 (lane 1), 24 (lane 2), 24 (lane 3), 48 (lane 4) and 72 hr (lane 5) at a seeding density of 5 x 105 cells per ml. To the cells with 48 and 72 h incubation, fresh hormone (2.5 mM) was added 24 h prior to the harvest. After each incubation period, nuclear extracts from HL-60 cells were immunoprecipitated with IVG, electrophoresed and then immunostained with "B" (IVG) as described in Fig. 3. Each lane represents the immunoprecipitated receptors from 200 x 106 HL-60 cells. Lane 6 represents 200 pm of porcine nuclear extract protein without immunoprecipitation. Molecular mass standards are shown to the left of the figure. The arrows indicate the positions of 1.25-(OH)2D3, receptor from HL-60 cells (33 kDa) and pig intestine (55 kDa).
Figure 5. The time-dependent regulation of [3H]1,25-(OH)_2D_3 uptake into intact HL-60 cells. HL-60 cells (3 x 10^6 cells/ml) were labeled with 2 nM [3H]1,25-(OH)_2D_3, in the presence or absence of a 200-fold excess of nonradioactive hormone for up to 72 h. Fresh hormone (2 nM [3H]1,25-(OH)_2D_3, with or without excess nonradioactive hormone) was added to the cells with 48 and 72 h incubation 24 h prior to harvest. After the incubation period, total cellular specific hormone binding activities were measured by HAP assay.

Figure 6. Immunoblotting of 1,25-(OH)_2D_3 receptor in the nuclear extracts of HL-60 cells and pig intestine. The nuclear extract from porcine intestine (300 µg of protein) was incubated with 2 nM [3H]1,25-(OH)_2D_3 in the presence of IVG (lane 1) or XVI (lane 2). After HL-60 cells were incubated with 2 nM 1,25-(OH)_2D_3 for 2 h at 37°C, nuclear extracts were immunoprecipitated with IVG (lane 4) or XVI (lane 5). These steps were followed by the incubation with goat anti-mouse IgG-Sepharose at 4°C. After the immunoprecipitated receptors were electrophoresed, they were transferred to PVDF membrane which was subsequently probed with 35S-labeled IVG. Lane 3 represents 200 µg of protein of porcine nuclear extract without immunoprecipitation; lanes 4 and 5 are immunoprecipitated receptors from 1 x 10^6 HL-60 cells. Molecular mass standards are shown to the left of the gel. The arrows indicate the position of 1,25-(OH)_2D_3 receptor from HL-60 (53kDa) and pig intestine (35 kDa).