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HORMONE-DEPENDENT PHOSPHORYLATION OF THE 1,25-DIHYDROXYVITAMIN D_3 RECEPTOR IN MOUSE FIBROBLASTS

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Experimental results, employing several immunologic techniques, suggest that the mouse receptor for 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) undergoes hormone-dependent phosphorylation in intact cells. Treatment of monolayer cultures of mouse 3T6 fibroblasts with $1,25(OH)_2D_3$ reveals that the occupied $1,25(OH)_2D_3$ receptor displays a minor reduction in electrophoretic mobility as compared to its unoccupied 54,500 dalton counterpart, a change consistent with covalent modification. Similar results were obtained by immunoprecipitation of metabolically-labeled receptors after incubation of 3T6 cells with [^{35}S]methionine. This technique also provided greater insight into the precursor-product relationship between the two receptor forms. [^{32}P]Orthophosphate-labeling of 3T6 cells, followed by immunoprecipitation indicated that only the form exhibiting covalent modification was phosphorylated. The temporal correspondence between the binding of $1,25(OH)_2D_3$ to its cellular receptor and its phosphorylation suggests that the biochemical role of $1,25(OH)_2D_3$ may be to induce a conformational change susceptible to phosphorylation and possibly functional activation.

Mammalian receptors for 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are labile intracellular proteins of 52,000-55,000 daltons which occur in a variety of tissues responsive to $1,25(OH)_2D_3$ (1-3). They are considered to mediate the multiple actions of the vitamin D_3 hormone through their putative gene-regulating capacity in the nucleus of target cells (4). While this transcriptional modifying activity has not been directly demonstrated, the receptor's characteristic behavior in binding to nuclei, chromatin, and DNA remain suggestive of this particular role (5-7). As a result of the recent cloning of cDNA's for $1,25(OH)_2D_3$-sensitive calcium-binding protein (8) as well as osteocalcin (9), it is likely that more definitive proof will soon be forthcoming.

Limited insight has similarly accrued with respect to the mechanism whereby $1,25(OH)_2D_3$ is able to regulate the functional state of its receptor

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protein. Occupied receptors are, however, more stable and display an increase in affinity for DNA in vitro (10,11) suggesting that the hormone is responsible for initiating conformational changes in the macromolecule. In this report, we provide experimental evidence to suggest that the receptor undergoes $1,25(OH)_2D_3$-dependent post-translational modification in intact cells, and that this covalent alteration is due to phosphorylation. The fact that the binding of $1,25(OH)_2D_3$ to receptor and its phosphorylation are temporally related provides a role for $1,25(OH)_2D_3$ and suggests that the modification may represent a key event in the functional activation of the $1,25(OH)_2D_3$ receptor.

MATERIALS AND METHODS

CULTURED CELLS: Mouse 3T6 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/ml penicillin and 500 U/ml streptomycin. The cells were incubated in plastic 150 mm culture dishes in an humidified atmosphere of 95% air/5% CO_2.

LABELING OF 3T6 FIBROBLASTS: Incubation of 3T6 cells with $1,25(OH)_2[^3H]D_3$ (158 Ci/mmol) was carried out as previously described (11). Cells were also metabolically labeled in monolayer culture with [^{35}S]methionine or [^{32}P]orthophosphate. Confluent cultures of 3T6 cells (10^6-10^7 cells) were first washed twice under sterile conditions with either methionine-free or phosphate-free DMEM, respectively. Cells were then incubated for periods up to 8 h in methionine-free medium supplemented with 2% fetal bovine serum and [^{35}S]methionine (250 µCi/ml; 1100 Ci/mmol, Amersham International) or for periods up to 4 hours with phosphate-free DMEM supplemented with 2% fetal bovine serum and [^{32}P]orthophosphate (0.5 mCi/ml, carrier-free, Amersham International) and the cells harvested as below.

PREPARATION OF CELLULAR EXTRACTS: Total cellular extracts of 3T6 fibroblasts were prepared by sonication in KETD-0.3 (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM dithiothreitol, and 0.3 M KCl) followed by ultracentrifugation as previously described (11). High-salt nuclear extracts of 3T6 cells preincubated with $1,25(OH)_2D_3$ (2 nM) were also obtained as detailed earlier (11). 3T6 fibroblasts which were metabolically labeled in monolayer with [^{35}S]methionine or [^{32}P]orthophosphate were washed 3 times in phosphate-buffered saline, and then lysed on the plates in KETD-0.3 containing 0.5% Triton X-100 (approximately 10^7 cells/ml). The lysates were removed, centrifuged at 165,000 x g for 30 min, and the extract subjected to immunoprecipitation.

IMMUNOBLOT ANALYSIS: Cellular extracts were electrophoresed on 11% SDS-polyacrylamide gels as described by Laemmli (12). Following electrophoresis, the protein species were transferred electrophoretically to nitrocellulose and the membrane sheets probed as described earlier utilizing rat anti-receptor monoclonal antibody (13,14). Conditions of immunoblot analysis were modified such that incubations were carried out with primary antibody (4 µg/ml), secondary anti-rat IgG antibody (1:10,000), and finally, iodinated protein A (100,000 cpm/ml; ICN). Autoradiograms were obtained at -70°C for 24-48 h utilizing Kodak Omat XAR film and a Cronex HiPlus intensifying screen.

IMMUNOPRECIPITATION: Lysates of metabolically-labeled 3T6 cells were subjected to immunoprecipitation by the addition of anti-receptor monoclonal antibody coupled directly to Sepharose-4B via a CNBr-activated intermediate. The derivitized beads (~10 µl) were incubated with the lysate (0.1-0.2 ml) overnight at 4°C with gentle shaking. Following incubation, the derivatized beads were washed by repeated resuspension in KETD-0.5 (0.5 M KCl) containing 0.5% Tween 20, 0.1% SDS, 0.5% NP-40, and 1% sodium deoxycholate. After sedimenting the beads through 0.5 ml of the above buffer containing 1 M sucrose and following a final wash in KETD (without KCl), the beads were boiled for 3 min in SDS-denaturing buffer and electrophoresed as above. The polyacrylamide gels were fixed in 40% methanol-10% acetic acid, treated with Enhance (New England Nuclear), and fluorographed overnight at -70°C on Kodak Omat XAR film.

RESULTS

Fig. 1 illustrates the immunoblot detection of both the unoccupied and *in vivo* occupied $1,25(OH)_2D_3$ receptor from mouse 3T6 fibroblasts. Although several crossreactive epitopes are present in Fig. 1, lane C, only the species at 54,500 daltons which represents the unoccupied $1,25(OH)_2D_3$ receptor, displays receptor-like properties such as nuclear retention and DNA-binding, and can be identified after *in vitro* translation of poly(A)$^+$ RNA and immunoprecipitation (14,15). Following treatment of 3T6 cells with 2 nM $1,25(OH)_2D_3$,

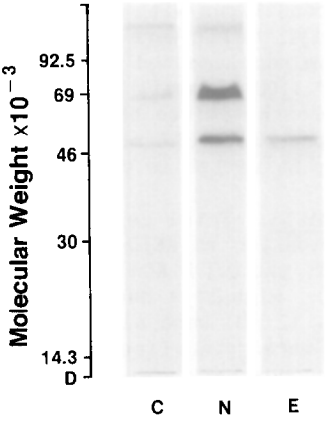

Figure 1. **Immunoblot analysis of $1,25(OH)_2D_3$ receptor in mouse 3T6 fibroblast cell fractions.** Lane C: 3T6 cells were sonicated in KETD-0.3 (25 x 10^6/ml) and after ultracentrifugation extract equivalent to 5 x 10^5 cells was subjected to "Western" immunoblot analysis as described in "Methods". Lane N: 3T6 cells (10^7/ml) were incubated with $1,25(OH)_2D_3$ (2 nM) for 90 min at 37°C, the nuclear fraction isolated and the $1,25(OH)_2D_3$-receptor complex extracted as described previously (11). Sample equivalent to 10^7 cell nuclei was probed as above. Lane E: $1,25(OH)_2D_3$-receptor complexes obtained as in lane N were chromatographically enriched on DNA cellulose, and a receptor containing sample (0.1 pmol of receptor) obtained from the peak fraction analyzed as in lane C. Standard proteins are: phosphorylase (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa).

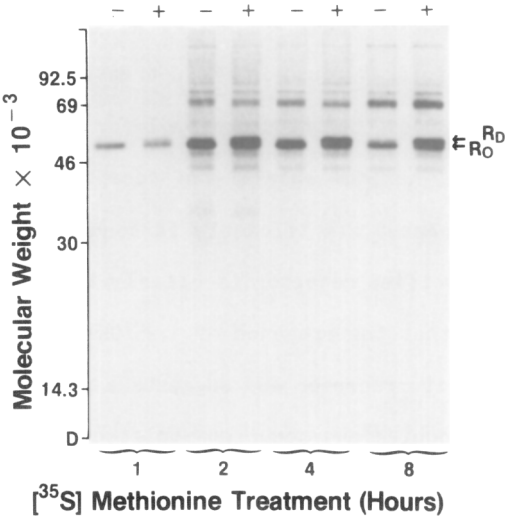

Figure 2. **Metabolic labeling of 3T6 fibroblasts with [^{35}S]methionine and immunoprecipitation of 1,25(OH)$_2$D$_3$ receptor.** Confluent 3T6 cells (~10^6) were labeled for increasing time periods with [^{35}S]methionine (see Methods) with or without 1,25(OH)$_2$D$_3$ (5 nM). Cells were lysed in KETD-0.3 plus 0.5% Triton X-100 (10^7/ml) and extract equivalent to 10^6 cells immunoprecipitated as described. R_O and R_D signify unoccupied and occupied receptor respectively. Standards are as in Fig. 1.

immunoblot of high-salt extracts obtained from the nuclear fraction of these cells reveals the detection of a similarly reactive species (Fig. 1, lane N). This species comigrates with a single protein detected after DNA cellulose chromatographic enrichment, a procedure which coincidently eliminates all nonreceptor epitopes (Fig. 1, lane E). Most importantly, neither the crude nor the DNA cellulose-enriched occupied 1,25(OH)$_2$D$_3$ receptor exactly comigrates with the unoccupied form--each displays a small but detectable and reproducible reduction in electrophoretic mobility as compared to the unoccupied receptor, suggestive of hormone-induced covalent modification.

[^{35}S]Methionine-labeling of 3T6 cells was utilized to confirm this observation. 3T6 cells were incubated for periods up to 8 h with [^{35}S]methionine in the presence or absence of 1,25(OH)$_2$D$_3$ in order to assess the latter's effects on the 1,25(OH)$_2$D$_3$ receptor. As illustrated in Fig. 2, the unoccupied receptor is detectable by metabolic labeling as early as 1 h, appears to maximize at 2 h and then decreases thereafter. After a 1 h treatment with 1,25(OH)$_2$D$_3$, however, the 54,500 dalton form as well as the larger modified

form are both evident. At this time, the 54,500 dalton species exhibits a lower intensity relative to that found in the untreated cells, suggesting that this form most likely provides the precursor pool for the detected level of modified receptor. At 2 h, maximum labeling of receptor is achieved, although the modified form has increased significantly in concentration. Finally, at 4 and 8 h the covalently modified receptor is clearly the predominant species. This experiment confirms that the presence of $1,25(OH)_2D_3$ in 3T6 cells induces covalent modification of its receptor and suggests a precursor-product relationship between the unoccupied and occupied-modified forms. Whether occupied but unmodified receptor is present in the faster migrating form remains to be determined.

Since phosphorylation is known to cause anomalous migration of proteins on electrophoretic gels (16), and because of the increasing evidence for phosphorylation of steroid receptors (17-19), we evaluated the possibility that the

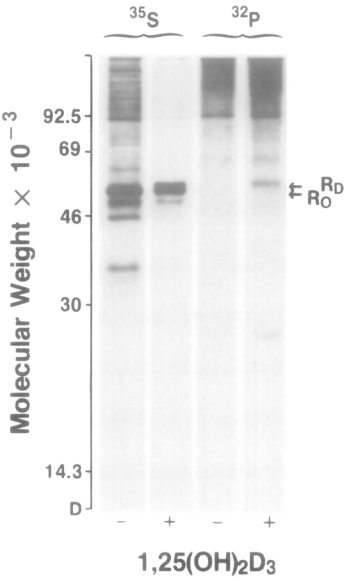

Figure 3. **Metabolic labeling of 3T6 fibroblasts with [^{32}P]orthophosphate and immunoprecipitation of phosphorylated $1,25(OH)_2D_3$ receptor.** Confluent 3T6 cells (~10^7 cells) were incubated with [^{32}P]orthophosphate in the presence or absence of $1,25(OH)_2D_3$ (5 nM) for 4 h at 37°C and the cells lysed and immunoprecipitated as in Fig. 2. Lanes designated "^{35}S" represents a parallel labeling of receptor in the presence (+) or absence (-) of $1,25(OH)_2D_3$ as in Fig. 2. Lanes designated "^{32}P" represent immunoprecipitation of $1,25(OH)_2D_3$ receptor obtained from cells labeled with [^{32}P]orthophosphate in the absence (-) or presence (+) of $1,25(OH)_2D_3$. R_o, R_D and molecular weight standards are as in Fig. 2.

$1,25(OH)_2D_3$ receptor was undergoing hormone-dependent phosphorylation. Cells were labeled with [^{32}P]orthophosphate in the presence or absence of $1,25(OH)_2D_3$, the cells lysed and the receptor immunoprecipitated via immobilized anti-receptor antibody as above. As depicted in Fig. 3, only in cells treated with $1,25(OH)_2D_3$ (lane 4) is there evidence of a phosphorylation signal in the $1,25(OH)_2D_3$ receptor region. This signal is not present in cells which remain free of $1,25(OH)_2D_3$ (lane 3), and exactly comigrates with the covalently modified form of the receptor identified after immunoprecipitation in a parallel labeling with [^{35}S]methionine (lane 2). These results would suggest that as a function of $1,25(OH)_2D_3$, its receptor protein becomes covalently modified via phosphorylation.

DISCUSSION

The experimental results described here suggest that the $1,25(OH)_2D_3$ receptor in mouse 3T6 fibroblasts undergoes rapid phosphorylation in response to cellular treatment with $1,25(OH)_2D_3$. This modification is temporally coincident with the formation of $1,25(OH)_2D_3$-receptor complexes (11), and thus, implies that the binding of hormone to its receptor protein facilitates phosphorylation, perhaps by altering the receptor's conformation such that modification can occur. Since binding of $1,25(OH)_2D_3$ is known to increase the stability of the receptor and increase its affinity for heterologous DNA (10,11), these altered characteristics could reflect such a $1,25(OH)_2D_3$-induced conformational change. Alternatively, $1,25(OH)_2D_3$ could stimulate receptor protein kinase activity. However, at present, no evidence has been obtained to suggest that $1,25(OH)_2D_3$ receptor is a phosphokinase. Thus, while it is evident that the receptor undergoes phosphorylation, the mechanism of this modification and the particular amino acid residues which are phosphorylated remain to be elucidated.

The speculative functions of receptor phosphorylation are multiple. Although modification is not required for increased receptor affinity for general DNA *in vitro* (the $1,25(OH)_2D_3$-receptor complex formed *in vitro* does not undergo modification (14), but displays increased affinity for calf thymus

DNA (10,11)), it is possible that phosphorylation plays an essential role in the receptor's interaction with specific gene sequences. Studies of this nature are feasible and may soon be forthcoming with the recent cloning of vitamin D-sensitive cDNA's (8,9). Phosphorylation may serve to thermodynamically "fix" the conformational state of the receptor initially achieved as a function of $1,25(OH)_2D_3$ and may, therefore, be essential for continued activity. Alternatively, modification may stimulate an unidentified enzymatic activity or reflect a signal for eventual receptor inactivation by nuclear proteases. The latter seems less likely in view of the rapidity with which phosphorylation is achieved. Clearly, the function of this $1,25(OH)_2D_3$-dependent post-translational modification remains to be clarified.

Regardless of the role of phosphorylation, this modification may prove to be a key reaction regulating $1,25(OH)_2D_3$ receptor function. Certainly, these data provide the first evidence for a direct involvement of $1,25(OH)_2D_3$ in this hormone's own mechanism of action. Whether steroid receptor phosphorylation represents an essential event in steroid hormone action remains to be defined. Recent evidence, however, suggests that this may be the case (17-19).

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