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Covalent modification of proteins by ligands of steroid hormone receptors

(HL-60 cells/retinoic acid)

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ABSTRACT **Retinoylation, acylation with retinoic acid (RA), is a covalent modification of proteins occurring in a variety of eukaryotic cell lines. In this study, we found that proteins in HL-60 cells were labeled by 17β-[^{3}H]estradiol (E2), [^{3}H]progesterone (Pg), 1α,25-dihydroxy[^{3}H]vitamin D_3 [1,25$(OH)_2D_3$], [^{125}I]triiodothyronine (T_3), [^{125}I]thyroxine (T_4), and [^{3}H]prostaglandin E_2 (PGE_2). All of these hormones, except PGE_2, are ligands of the steroid hormone receptor family. Addition to the growth medium of 5 μM ketoconazole, an inhibitor of cytochrome P450-dependent enzymes, increased about 2-fold the labeling of proteins by T_3, T_4, 1,25$(OH)_2D_3$, and PGE_2. In contrast, ketoconazole did not change markedly the extent of labeling by RA, E2, or Pg. Alkaline methanolysis, which cleaves ester bonds, released variable percentages of the radioactive ligands bound to protein. These values were about 80% for RA and PGE_2; 50% for T_3, T_4, and Pg; and 20% for E2 and 1,25$(OH)_2D_3$. Treatment with thioether-cleavage reagents, iodomethane or Raney nickel catalyst, released <2% of the covalently bound ligands. Two-dimensional polyacrylamide gel electrophoresis patterns of labeled proteins were unique for each ligand. Proteins of M_r 47,000 and 51,000 were labeled by RA, E2, T_3, and T_4. These proteins had the same mobilities as RI and RII, the cAMP-binding regulatory subunits of type I and type II cAMP-dependent protein kinases. 1,25$(OH)_2D_3$ also bound to proteins of M_r 47,000 and 51,000. However, these proteins had pI values different from those of RI or RII. These results suggest that some activities of ligands of the steroid hormone receptor family and of PGE_2 may be mediated by their covalent modification of proteins.**

All-*trans*-retinoic acid (RA) induces terminal differentiation of the human acute myeloid leukemia cell line HL-60 (1) and of cells from patients with acute promyelocytic leukemia (2). As a sole agent, RA induces complete remissions of patients with acute promyelocytic leukemia (3–5).

Recently, we reported evidence that a nuclear protein in HL-60 cells is retinoylated via a thioester bond in a dose-dependent manner (6). Furthermore, the dose–response curves for RA-induced differentiation and for retinoylation are similar (6, 7). Retinoylation is not restricted to HL-60 cells and occurs in other cell types (7–9). These results showed that retinoylation is widespread and that the response to RA of different cell types may depend on the retinoylation of specific proteins. Thus, some effects of RA on cells may be independent of RA nuclear receptors (10–12).

The RA receptors are members of the steroid hormone receptor multigene family (10). Some biologic effects of ligands for the steroid hormone receptor family may not involve activation of a nuclear receptor and its interaction with specific DNA sequences (13–25).

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The covalent attachment to proteins may be a mechanism for the action of molecules that also bind to specific receptors. In experiments described almost 40 years ago, enzymes in rat liver homogenates catalyzed the covalent binding of estrogens to protein (26, 27). The activation of 17β-estradiol (E2) to a quinone, which can damage or form an adduct with either protein or DNA, may be involved in kidney tumor formation (28). The proestrogen/proantiestrogen activities of tamoxifen and chlorotriansene may entail activation by cytochrome P450-dependent enzymes to intermediates that covalently attach to the E2 receptor and the activating enzymes (29, 30). In MCF7 human breast cancer cells, the E2 metabolite 16α-hydroxyestrone covalently binds to nuclear matrix proteins and the E2 receptor (31, 32). We showed that E2 and RA bind covalently to proteins in MCF7 cells (9).

These results prompted us to see whether other ligands of the steroid hormone receptor family also bind covalently to cellular proteins. We have examined this question with HL-60 cells, which respond to many ligands of the steroid hormone receptor family, including RA (1), 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (33), 3,3′,5-triiodo-L-thyronine (T_3) (34), E2 (34–36), and dexamethasone (35, 37–42). HL-60 cells contain receptors for RA (43), 1,25$(OH)_2D_3$ (44, 45), dexamethasone (38), and E2 (46). They do not have androgen receptors (46) and we are unaware of any reports that they contain receptors for T_3 or progesterone (Pg).

In this study we show that some ligands of the steroid hormone receptor family covalently bind to HL-60 cellular proteins.*

MATERIALS AND METHODS

Cells. We maintained the human myeloid leukemia cell line HL-60 in RPMI 1640 medium (GIBCO) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonate (pH 7.3) and 10% fetal bovine serum (GIBCO). Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. We estimated cell number with an electronic particle counter (Coulter) and assessed cell viability by trypan blue dye exclusion.

Labeling of HL-60 Proteins. Exponentially growing cells were harvested by centrifugation and suspended at a concentration of 2×10^6 cells per ml in serum-free medium consisting of RPMI 1640 supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonate (pH 7.3), 5 μg of insulin per ml, and 5 μg of transferrin per ml (47). All

Abbreviations: RA, all-*trans*-retinoic acid; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; RI and RII, cAMP-binding regulatory subunits of type I and type II cAMP-dependent protein kinase; E2, 17β-estradiol; Pg, progesterone; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; T_3, 3,3′,5-triiodo-L-thyronine; T_4, L-thyroxine; PGE_2, prostaglandin E_2.

*The data in this paper apply to the overall formation of protein-bound radioactive ligands. Since the structure of the bound moiety is not known for all ligands, binding merely reflects an approximation of ligand equivalents.

radioactive compounds were purchased from DuPont/NEN. Unlabeled RA (Sigma) and radioactive RA ([11,12-^{3}H], 51 Ci/mmol; 1 Ci = 37 GBq), 1,25(OH)$_2$D$_3$ ([26,27-^{3}H], 160 Ci/mmol), prostaglandin E$_2$ (PGE$_2$) ([5,6,8,11,14,15-^{3}H], 200 Ci/mmol), E2 ([2,4,6,7,16,17-^{3}H(N)], 150 Ci/mmol), Pg ([1,2,6,7,21-^{3}H(N)], 193 Ci/mmol), L-thyroxine (T$_4$) ([^{125}I], 4400 Ci/mmol), and T$_3$ ([3,3',5-^{125}I], 648 Ci/mmol) were dissolved in absolute ethanol and diluted into the growth medium. The final concentration of ethanol was ≤0.1%. After incubation for 24 hr, cells were harvested by centrifugation (200 × *g*, 5 min) and washed with phosphate-buffered saline (1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4/136.9 mM NaCl, pH 7.2). A dried delipidated cell pellet was prepared by the Bligh–Dyer procedure (48) as described (49) and dissolved in various solutions depending on the test system.

Stability of Bound Ligands. Alkaline methanolysis to cleave ester bonds was as described (49). Treatment of labeled proteins to detect thioethers was carried out either with Raney nickel catalyst (Sigma) (50) or with CH_3I (51–53).

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE). 2D-PAGE was according to O'Farrell (54) with minor modifications (7). Gels were fixed, stained with Coomassie blue R-250, and prepared for fluorography with Entensify (DuPont/NEN) according to the manufacturer's instructions.

RESULTS

Labeling of Total HL-60 Proteins by Hormones. We saw radioactivity in the delipidated residues prepared by the Bligh–Dyer extraction procedure after incubation of HL-60 cells with [^{3}H]RA, [^{3}H]1,25(OH)$_2$D$_3$, [^{3}H]E2, [^{3}H]Pg, [^{125}I]T$_3$, and [^{125}I]T$_4$ (Table 1). Hydroxylated steroids could be intermediates in the binding of steroids to proteins. Ketoconazole inhibits cytochrome P450-dependent enzymes that catalyze many of these hydroxylations (55–57). We measured the labeling of HL-60 proteins in the presence of 5 μM ketoconazole, which effectively inhibits cytochrome P450-dependent enzymes in intact cells (57, 58). In the presence of ketoconazole the labeling of total proteins by T$_3$, T$_4$, and 1,25(OH)$_2$D$_3$ increased about 2-fold (Table 1) and the labeling by RA, E2, and Pg was about ±20% of the control. In the presence of ketoconazole there was a slight decrease of cell growth and cell viability was >95% (data not shown).

Table 1. Modulation by ketoconazole of labeling of HL-60 proteins

Labeled compound	Total bound, fmol per 10^6 cells		Ratio*
	− ketoconazole	+ ketoconazole	
RA (100 nM)	330	250	0.76
E2 (100 nM)	250	300	1.2
Pg (100 nM)	27	31	1.2
T$_4$ (0.75 nM)	0.17	0.33	2
T$_3$ (10 nM)	6	12.8	2.1
1,25(OH)$_2$D$_3$ (2.5 nM)	40	94	2.4

HL-60 cells were grown for 24 hr in medium containing the radiolabeled ligand, without and with 5 μM ketoconazole. Cells were harvested by centrifugation and extracted by the Bligh–Dyer procedure. *Total bound with ketoconazole divided by the total bound without ketoconazole.

Labeling of Individual HL-60 Proteins. With 2D-PAGE and fluorography we saw individual proteins labeled by these hormones (Figs. 1–4). We assigned a number to each labeled protein on the basis of its mobility compared with proteins that were stained by Coomassie blue R-250. We saw some proteins labeled by only one hormone and some proteins labeled by more than one hormone. Table 2 summarizes these observations. All hormones labeled protein *7* (arrows) and protein *22* and, except for T$_4$, also labeled proteins *18* and *23* (Figs. 1–4). Each hormone except Pg labeled protein *17*. RA, T$_3$, T$_4$, and E2 labeled protein *11*.

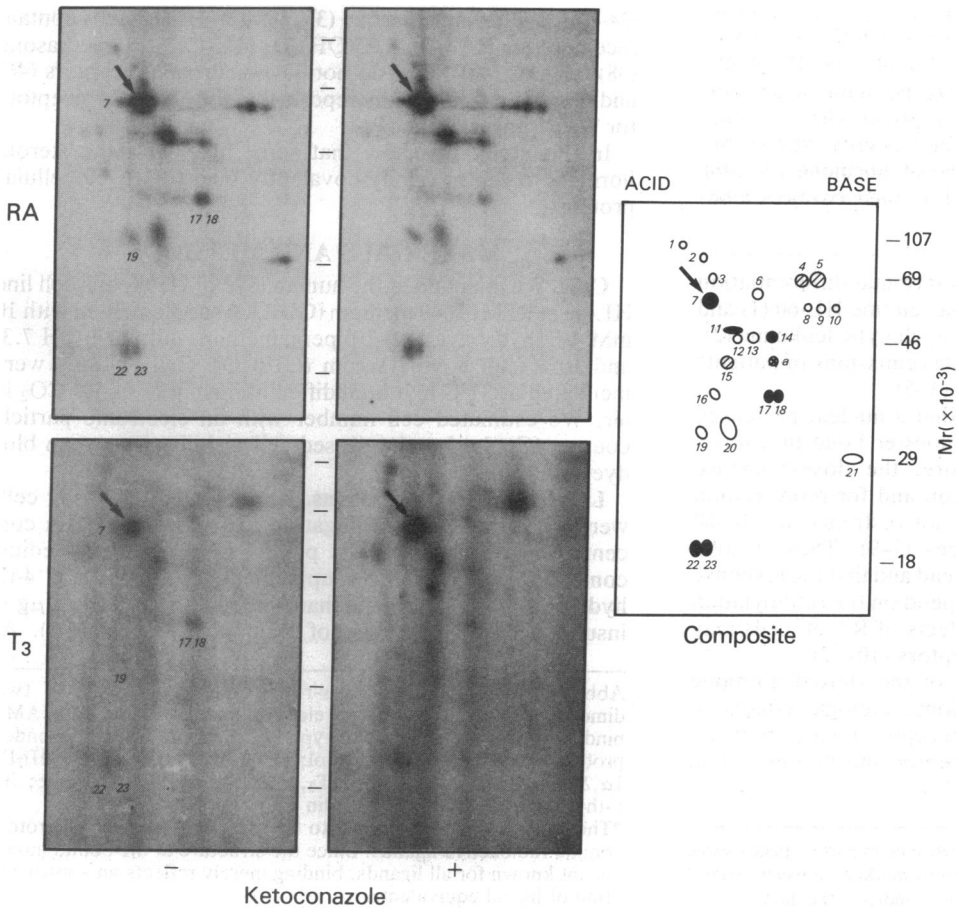

FIG. 1. Labeling of HL-60 proteins by [^{3}H]RA and [^{125}I]T$_3$ in the absence or presence of ketoconazole. HL-60 cells (2 × 10^6 per ml) were grown for 24 hr in serum-free medium containing 100 nM [^{3}H]RA or 10 nM [^{125}I]T$_3$ in the absence or presence of 5 μM ketoconazole. Proteins were separated by 2D-PAGE. The dried gels were exposed to the film for 118 days. The composite was drawn manually and the position of each numbered protein was assigned based on its mobility compared with proteins that were stained by Coomassie blue R-250. The position of actin (*a*) is shown (⊗). Actin was not labeled. The other protein spots in the composite are identified by the same numbers in the other figures and in the text and are solid for proteins labeled both by RA and T$_3$, hatched for proteins labeled only by T$_3$, and open for proteins labeled only by RA. For the RA panels, we analyzed the protein from 3.4 × 10^6 cells grown without ketoconazole and containing 54,500 cpm and the protein from 3.2 × 10^6 cells grown with 5 μM ketoconazole and containing 38,528 cpm. For the T$_3$ panels, we analyzed the protein from 3.8 × 10^6 cells grown without ketoconazole and containing 18,130 cpm and the protein from 2.94 × 10^6 cells grown with ketoconazole and containing 30,500 cpm.

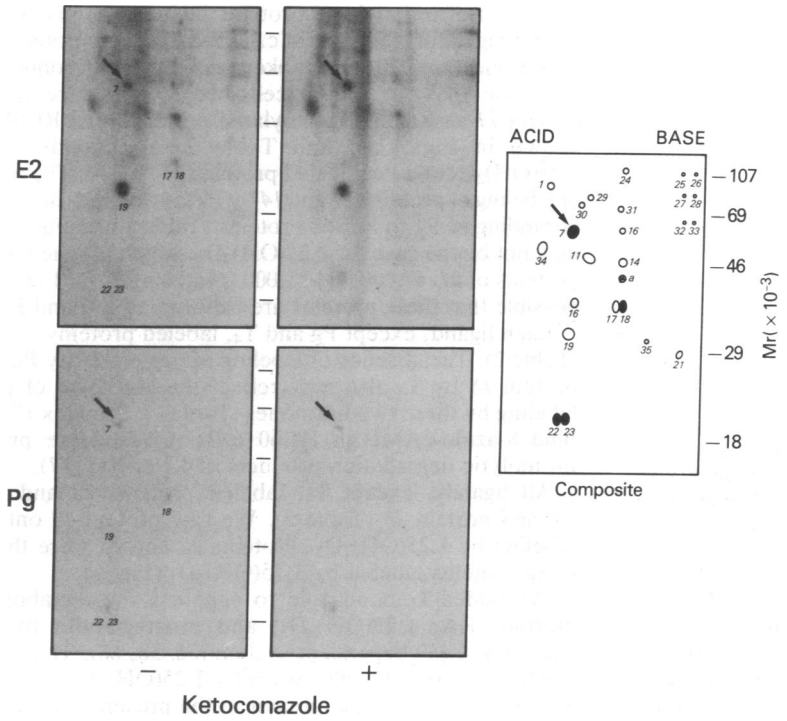

FIG. 2. Labeling of HL-60 proteins by [^{3}H]E2 and [^{3}H]Pg in the absence or presence of ketoconazole. HL-60 cells (2×10^6 per ml) were grown for 24 hr in serum-free medium containing 100 nM [^{3}H]E2 or 100 nM [^{3}H]Pg in the absence or presence of 5 μM ketoconazole. Proteins were separated by 2D-PAGE. The dried gels were exposed to the film for 118 days. The composite was drawn manually and the position of each numbered protein was assigned based on its mobility compared with proteins that were stained by Coomassie blue R-250. Actin (*a*, ⊗) was not labeled. The other protein spots in the composite are identified by the same numbers in the other figures and in the text and are solid for proteins labeled both by E2 and by Pg and open for proteins labeled only by E2. For the E2 panels, we analyzed the protein from 3.24×10^6 cells grown without ketoconazole and containing 97,300 cpm and the protein from 2.91×10^6 cells grown with 5 μM ketoconazole and containing 105,000 cpm. For the Pg panels, we analyzed the protein from 3.4×10^6 cells grown without ketoconazole and containing 14,000 cpm and the protein from 3×10^6 cells grown with ketoconazole and containing 14,300 cpm.

Ketoconazole increased the extent of labeling of most proteins by T_3 and $1,25(OH)_2D_3$ (Figs. 1 and 3). This increase reflected the 2-fold increases in total labeling shown in Table 1. However, the labeling of proteins *17* and *18* by T_3 and $1,25(OH)_2D_3$ decreased in the presence of ketoconazole.

Labeling of Proteins by PGE_2. PGE_2, while not a ligand for the steroid receptor family, induces differentiation of HL-60 cells (59). Its effect on differentiation probably is via its increase of cAMP (59, 60). The PGE_2 molecule has a long-chain fatty acid portion that could bind covalently to HL-60 proteins as shown for RA, palmitic acid, and myristic acid (61). We found that after growth of HL-60 cells with 10 nM [^{3}H]PGE_2 for 24 hr, the delipidated residue contained 5.4 fmol per 10^6 cells grown without ketoconazole and 10.6 fmol per 10^6 cells grown with 5 μM ketoconazole. We could not get informative 2D-PAGE fluorograms after exposure of the gel to the film for 4 months, possibly because of low levels of incorporation distributed in many proteins. However, we saw labeling of proteins *22* and *23* by PGE_2 after exposure of the gel to the film for 11 months.

Chemical Stability of the Covalently Bound Ligand to Protein. Alkaline methanolysis releases as methyl retinoate more than 80% of RA covalently bound to protein in HL-60 cells (6). These and other results established that RA is bound primarily via an ester linkage that is most likely a thioester. The values for the percentage of total bound hormone released after alkaline methanolysis for 2 hr were 18% for both E2 and $1,25(OH)_2D_3$, 34% for both T_3 and T_4, 45% for Pg, and 80% for PGE_2. We saw slightly higher values after 14 hr of hydrolysis. These results show that most of the PGE_2 is bound to protein via an ester bond. In contrast, large percentages of radioactive moieties of E2, Pg, T_3, T_4, and $1,25(OH)_2D_3$ do not bind this way. We found that treatment with thioether-cleaving agents, (Raney nickel or CH_3I) released <2% of the total radioactivity in the delipidated residue prepared from cells labeled by either RA, E2, Pg, T_3, T_4, PGE_2, or $1,25(OH)_2D_3$.

DISCUSSION

In this study we extended our previous findings that RA covalently binds to HL-60 proteins and that RA and E2 covalently bind to proteins in the human breast tumor cell line MCF7 (9). Here we show that radioactive E2, T_3, T_4, $1,25(OH)_2D_3$, Pg, and PGE_2 also attach to proteins in HL-60

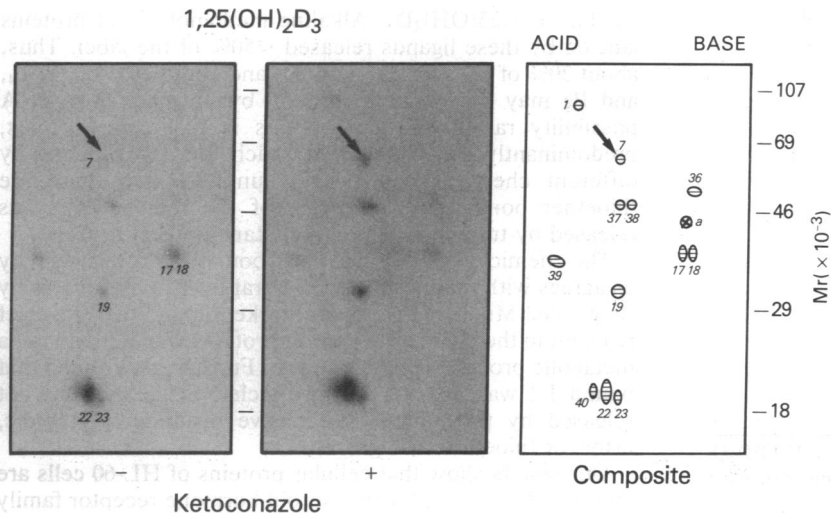

FIG. 3. Labeling of HL-60 proteins by [^{3}H]$1,25(OH)_2D_3$ in the absence or presence of ketoconazole. HL-60 cells (2×10^6 per ml) were grown for 24 hr in serum-free medium containing 2.5 nM [^{3}H]$1,25(OH)_2D_3$ in the absence or presence of 5 μM ketoconazole. Proteins were separated by 2D-PAGE. The dried gels were exposed to the film for 118 days. The composite was drawn manually and the position of each numbered protein was assigned based on its mobility compared with proteins that were stained by Coomassie blue R-250. Actin (*a*, ⊗) was not labeled. The other protein spots in the composite are identified by the same numbers in the other figures and in the text. We analyzed the protein from 3.54×10^6 cells grown without ketoconazole and containing 1980 cpm and the protein from 2.3×10^6 cells grown with ketoconazole and containing 1979 cpm.

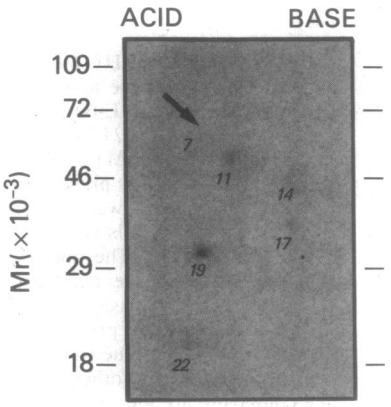

FIG. 4. Labeling of HL-60 proteins by [^{125}I]T_4. HL-60 cells (2×10^6 per ml) were grown for 24 hr in serum-free medium containing 2.5 nM [^{125}I]T_4. Proteins were separated by 2D-PAGE. The dried gel was exposed to the film for 30 days. The protein spots are identified by the same numbers in the other figures and in the text. We analyzed the protein from 1×10^7 cells containing 6660 cpm.

cells. All of these hormones, except PGE_2, are ligands for members of the steroid hormone receptor family.

The steroid hormone receptors function as regulators of gene expression (10). It is likely that these receptors mediate most of the biological effects of their ligands. However, some effects of T_3 (13), $1,25(OH)_2D_3$ (14, 25, 62), RA (20, 24), E2 (22, 23), and Pg (23) may be non-receptor-mediated or nongenomic or both. The mechanisms for these effects are unknown.

Our results raise the possibility that some effects of T_3, T_4, $1,25(OH)_2D_3$, RA, E2, and Pg are mediated by their covalent binding to proteins. We saw 40 proteins of HL-60 cells labeled by at least one of these six ligands (Figs. 1–4; Table 2). Twelve of these proteins were labeled by more than one ligand. More than 600 HL-60 proteins (silver-stained or labeled with [^{35}S]methionine) are seen by 2D-PAGE under conditions similar to those we used (63, 64). Thus, the probability is very high that some proteins are modified by more than one hormone. It will be of interest to determine whether more than one hormone can bind to one protein molecule.

Table 2. Summary of HL-60 proteins labeled by RA, T_3, T_4, E2,

No.	RA	T_3	T_4	E2	Pg	D_3	No.	RA	T_3	T_4	E2	Pg	D_3
1	●	●		●			*21*	●			●		
2	○						*22*	●	●	●	●	●	●
3	○						*23*	●	●		●	●	●
4		○					*24*				○		
5		○					*25*		●		●		
6		○					*26*		●		●		
7	●	●	●	●	●	●	*27*				○		
8	○						*28*				○		
9	○						*29*				○		
10	○						*30*				○		
11	●	●	●	●			*31*				○		
12	○						*32*				○		
13	○						*33*				○		
14	●	●	●	●			*34*				○		
15		○					*35*				○		
16	○						*36*						○
17	●	●	●	●		●	*37*						○
18	●	●		●	●	●	*38*						○
19	●		●	●		●	*39*						○
20	○						*40*						○

Proteins are identified by number as in Figs. 1–4. D_3, $1,25(OH)_2D_3$; ●, protein labeled by more than one ligand; ○, protein labeled by only one ligand.

The list of retinoylated proteins includes RI and RII, the cAMP-regulatory subunits of cAMP-dependent protein kinase type I and type II (65); cytokeratins (8); and ribonucleotide reductase (66). In HL-60 cells we identified retinoylated protein *11* as RII and retinoylated protein *14* as RI (65). As shown in Figs. 1–4 and Table 2, all ligands, except $1,25(OH)_2D_3$ and Pg, labeled proteins *11* and *14*. The absence of labeling of proteins *11* and *14* by Pg may reflect the low level of binding of Pg to HL-60 proteins (Table 1 and Fig. 2). This may not be the case for $1,25(OH)_2D_3$, which uniquely labeled proteins of M_r 47,000 and 51,000 (proteins *36–38*, Fig. 3). It is possible that these proteins are isoforms of RII and RI.

Each ligand, except Pg and T_4, labeled proteins *17* and *18* (Table 2). The absence of labeling of protein *17* by Pg and of protein *18* by T_4 also may reflect the low level of protein labeling by these two hormones (Table 1). Proteins *17* and *18* bind 8-azido-cAMP in HL60 cells (65) and are probably proteolytic degradation products of RI or RII (67).

All ligands, except T_4, labeled proteins *22* and *23*. T_4 labeled protein *23* (Table 2). We saw protein *40* only after labeling by $1,25(OH)_2D_3$. Proteins *22* and *23* were the principal proteins labeled by $1,25(OH)_2D_3$ (Fig. 3).

We added ketoconazole to suppress the metabolism of steroid, RA, $1,25(OH)_2D_3$, and prostaglandin by a cytochrome P450-dependent system (56–58, 68). The covalent binding to total HL-60 protein by $1,25(OH)_2D_3$, T_3, T_4, and PGE_2 was about 2-fold greater in the presence of ketoconazole (Table 1). In comparison, ketoconazole affected slightly the covalent binding to protein of RA, E2, and Pg. These results show that the covalent labeling of proteins by RA, E2, and Pg may not require their conversion to an intermediate of a cytochrome P450-dependent enzyme reaction. We do not have an explanation for the increased binding of T_3 and T_4 in the presence of ketoconazole.

We showed that about 80% of RA covalently binds to HL-60 protein via a thioester linkage (6). However, this linkage of RA to protein is not universal. RA is not attached by a thioester bond to cytokeratins of normal keratinocytes (8). PGE_2 is the only other ligand we investigated that may bind to HL-60 protein primarily via an ester bond. Alkaline methanolysis released about 80% of the PGE_2 bound to protein. This procedure hydrolyzes thioesters and most oxoesters. Therefore, it is possible that PGE_2 binds by an ester bond between its carboxyl group and either a hydroxy amino acid or a cysteine of a protein. Our results with PGE_2 may be similar to those showing an alkali-labile covalent binding of Δ^{12}-prostaglandin J_2 to proteins in the nuclear matrix and chromatin of mouse leukemia L1210 cells (69).

Compared to our knowledge of the chemical bond between protein and either RA or PGE_2 we have less information about the chemical bond between protein and either E2, T_3, T_4, Pg, or $1,25(OH)_2D_3$. Alkaline methanolysis of proteins labeled by these ligands released <50% of the label. Thus, about 20% of E2 and $1,25(OH)_2D_3$ and about 40% of T_3, T_4, and Pg may be bound to protein by an ester linkage. A possibility raised by these results is that some ligands, predominantly Pg, T_3, and T_4, each bind to proteins by different chemical bonds. It is unlikely that there are thioether bonds, because <2% of the radioactivity was released by treatment with either Raney nickel or CH_3I.

The chemical stability of the E2 bound to protein seen by us agrees with findings reported in rat liver homogenates by Riegel and Mueller (27). Those workers found that the chief reactant in the binding process to proteins was E2, and not a metabolic product such as estrone. Further, they found that bound E2 was not attached to nucleic acids and was not released by procedures that cleave disulfide, glycosidic, ester, or thioether linkages.

Our results show that cellular proteins of HL-60 cells are modified by ligands for the steroid hormone receptor family

and by PGE_2. HL-60 cells respond to many of these ligands. Thus, RA or $1,25(OH)_2D_3$ alone (1, 33) and T_3 or PGE_2 in combination with low concentrations of RA (34, 59) induce differentiation of HL-60 cells. E2 has variable effects on proliferation of HL-60 cells (34–36). It is possible that the covalent attachment to specific proteins is a mechanism for the action of many biologically active molecules that also bind to specific receptors. Some effects may be receptor-dependent and other effects may be receptor-independent. Our finding that RI and RII may be acceptor proteins for these covalent modifications provides well-characterized proteins for studying functional alterations.

Of immediate interest is the identification of the other proteins covalently bound with these active biological agents. This information should aid in clarifying the physiologic function of these covalent modifications of proteins.

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