CHARACTERIZATION OF THE CELL SURFACE RECEPTOR
FOR HUMAN GRANULOCYTE/MACROPHAGE
COLONY-STIMULATING FACTOR

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Granulocyte/macrophage colony-stimulating factor (GM-CSF) is one of a set
of specific glycoproteins collectively known as colony-stimulating factors, which
interact to control the growth and differentiation of hematopoietic cells (1–3).
This polypeptide hormone stimulates the proliferation of granulocyte/macrophage
precursor cells, can induce differentiation commitment in these precursors, and can, as well, modulate the functional activity of mature granulocytes
and macrophages (4–9).

Recently, the cloning of the genes encoding murine GM-CSF (10–12) and
human GM-CSF (12–14) and the subsequent expression in yeast of these recom-
binant proteins (12) has made possible a more detailed study of the mode of
action of these factors. As with other polypeptide hormones, GM-CSF appears
to initiate its activity by binding to specific receptors on the plasma membrane
of responding cells (15, 16). We have recently reported (16) the use of purified
recombinant murine GM-CSF, radiolabeled with $^{125}$I, to characterize the kinetic
parameters, cellular distribution, and molecular size of the receptor for this
factor on murine cells of both myeloid and T cell lineage.

This paper describes the use of recombinant human GM-CSF, radiolabeled
with $^{125}$I to high specific activity, to identify and characterize the receptor for
this hormone on both a mature primary cell, human neutrophils, and on the
promyelocytic leukemia cell line, HL-60. We show that the properties of the
GM-CSF receptor exhibited by these two cell populations are very similar, in
both cases characterized by a strikingly low number of receptors expressed per
cell. This initial characterization of the receptor for human GM-CSF should
begin to lay the groundwork that will be necessary to fully understand the
complex interactions of the human colony-stimulating factors, as well as allow an
accurate assessment of their potential as therapeutic agents.

Materials and Methods

Cell Preparations. All cell lines except HL-60 were grown in RPMI 1640 (Gibco
Laboratories, Grand Island, NY) containing 10% FCS, penicillin (50 U/ml), streptomycin

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Abbreviations used in this paper: GM-CSF, granulocyte/macrophage colony-stimulating factor;
CSF-2a, colony-stimulating factor 2a.
Human neutrophils were purified from leukocyte layers (Portland Red Cross, Portland, OR) with Neutrophil Isolation Medium (Packard Instrument Co., Inc., Downers Grove, IL), essentially following the manufacturer's specifications. Briefly, four leukocyte layers were diluted to 350 ml with PBS, and 35 ml was layered on 15 ml of Ficoll (Sigma Chemical Co., St. Louis, MO) and centrifuged at 1,600 rpm for 15 min. From each tube, the polymorphonuclear and erythrocyte layers were retained, diluted to 40 ml with PBS, and 20 ml was layered on 10 ml of Neutrophil Isolation Medium and centrifuged at 1,200 rpm for 30 min. The polymorphonuclear layers were retained, washed once with PBS, and contaminating erythrocytes were removed by a 1 min lysis with lysing buffer (Packard Instrument Co., Inc.). Remaining polymorphonuclear cells were washed twice with PBS, resuspended in RPMI 1640, and maintained at 25°C until use. Human monocytes were prepared as previously described (18).

Murine neutrophils were purified from whole blood on Neutrophil Isolation Medium, following the manufacturer’s specifications.

Hormone Preparations. Nerve growth factor, fibroblast growth factor, and epidermal growth factor were obtained from Bethesda Research Labs (Gaithersburg, MD). Human follicle-stimulating hormone, human luteinizing hormone, human thyroid-stimulating hormone, human growth hormone, and bovine insulin were obtained from Calbiochem-Behring (La Jolla, CA).

Human IL-2 was expressed in, and purified from Escherichia coli and provided via a collaborative research agreement between Immunex Corporation and Hoffman-La Roche Inc., Nutley, NJ. Colony-stimulating factor 2α (CSF-2α) (IL-3) was purified to homogeneity from medium conditioned by PHA-stimulated LBRM-33-5A4 cells, as previously described (19). Human IL-1 was purified to homogeneity, as previously described (20), from medium conditioned by activated human macrophages (20). Recombinant murine GM-CSF was produced in a yeast expression system and purified to homogeneity from yeast-conditioned medium by reverse-phase HPLC as previously described (12, 16).

Recombinant Human GM-CSF Purification and Radiolabeling. Recombinant human GM-CSF was produced in a yeast expression system and purified to homogeneity from yeast-conditioned medium by reverse-phase HPLC (12, D. Urdal, unpublished data). Briefly, yeast cells transformed with a plasmid containing the cDNA sequence encoding GM-CSF (12) under the control of the alcohol dehydrogenase 2 promoter and alpha factor leader, secrete GM-CSF into the medium at levels of 5 mg/liter. Medium containing secreted recombinant GM-CSF was pumped directly onto a Vydac C4 reverse-phase column and proteins were eluted from the column with a gradient of acetonitrile in 0.1% trifluoroacetic acid. GM-CSF concentrations were determined by fluorescamine analysis (21) of the purified protein, with BSA as a standard. GM-CSF activity was measured in a human bone marrow colony assay, as previously described (12).

Human recombinant GM-CSF was radiolabeled using the Enzymobead radiiodination reagent (Bio-Rad Laboratories, Richmond, CA), essentially as previously described for murine recombinant GM-CSF (16). Bioactivity of 125I-GM-CSF was determined in the human bone marrow colony assay (12). Labeled GM-CSF preparations were analyzed by gel filtration chromatography on Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, NJ) to test for the presence of high molecular weight aggregates. The specific activity of the radiolabeled GM-CSF (generally 0.5–2 × 10^15 cpm/mmol) was based on protein concentration, as determined by fluorescamine analysis and by assuming an 80% yield of 125I-GM-CSF after separation on the G-25 column.

Assay for Binding of 125I-GM-CSF to Intact Cells. Binding assays were performed by a phthalate oil separation method (22) essentially as described previously for murine 125I-GM-CSF (16). Sodium azide (0.2%) was included in all binding assays to inhibit internalization and degradation of 125I-GM-CSF by cells at 37°C. To verify that no degradation of ligand was occurring, aliquots were removed from incubation mixtures maintained at 37°C for up to 1 h and precipitated on Whatman 3 MM paper with 10% TCA. No
FIGURE 1. Characterization of $^{125}$I-GM-CSF by SDS-PAGE. $^{125}$I-GM-CSF (specific radioactivity, $10^{15}$ cpm/mmol) was boiled for 3 min in sample buffer containing 2% SDS and 5% 2-ME and 10,000 cpm were applied to a linear 10–20% gradient gel. Electrophoresis and autoradiography were then conducted as described in Materials and Methods.

decrease in TCA precipitable counts was detected over this time period. Association and dissociation kinetic experiments were also conducted as previously described (16).

SDS-PAGE. Samples were boiled for 3 min in sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME), and analyzed on 10–20% gradient gels according to the stacking gel procedure of Laemmli (23). Relative molecular weight markers (Mr) cytochrome c, 12,300; lactoglobulin A, 18,367; carbonic anhydrase, 31,000; OVA, 46,000; BSA, 69,000; phosphorylase B, 97,400; and myosin, 200,000 methylated with [14C]methyl label were from New England Nuclear (Boston, MA). After electrophoresis, gels were stained with Coomassie blue (0.25% in 25% isopropanol and 10% acetic acid), dried, and then exposed to X-omat AR film (Eastman Kodak Co., Rochester, NY) at $-70^\circ$C.

Data Analysis. Curve fitting of binding and kinetic data was done using RS/1 (Bolt, Beranek and Newman Software Products Corporation, Boston, MA), a commercially available data processing package running on a VAX 11/750 under the VMS operating system. Kinetic data were analyzed with functions that are single or sums of exponential terms as described elsewhere (24, 25), binding data were analyzed using an equation describing simple bimolecular binding (22), and inhibition data were analyzed with an equation for competitive inhibition between two ligands for one type of site (26).

Results

Radiolabeling of Human GM-CSF. Recombinant human GM-CSF was iodinated to high specific activity with the use of the Enzymobead radioiodination reagent from Biorad Laboratories. Fig. 1 illustrates an autoradiograph of a typical iodinated GM-CSF preparation, where the major species after iodination had an apparent molecular weight of 21,000 on analysis by SDS-PAGE. GM-CSF protein concentrations were determined by the Fluorescamine protein assay and the radiolabeled preparations were calculated to have specific activities in the range of $10^{15}$ cpm/mmol. When tested in a human bone marrow colony assay, radiolabeled GM-CSF preparations were found to retain >50% of their biological activity. Preparations of $^{125}$I-GM-CSF were stable for at least 1 mo
**Figure 2.** Association and dissociation kinetics of $^{125}$I-GM-CSF with neutrophils at 37°C. (A) Neutrophils (6.67 x 10^7 cells/ml) were incubated with 7.28 x 10^{-10} M (△), 2.31 x 10^{-10} M (□), or 7.75 x 10^{-11} M (○) $^{125}$I-GM-CSF at 37°C. At the time points indicated, aliquots were removed and assayed for binding as described in Materials and Methods. Nonspecific binding was measured in the presence of a 100-fold molar excess of unlabeled GM-CSF at each concentration of $^{125}$I-GM-CSF. The continuous curves passing through the data were calculated from the best-fit parameter values using a single exponential term (14). Infinite time binding and association rate constants for each curve were, respectively: △, 117.5 ± 3.5 molecules/cell and 8.5 ± 0.7 x 10^{-2}/M.min; □, 84.4 ± 4.8 molecules/cell and 6.9 ± 1.0 x 10^{-2}/M.min; ○, 50.8 ± 3.3 molecules/cell and 4.5 ± 0.8 x 10^{-2}/M.min. A plot of the association rate constant calculated from each curve versus the molar concentration of $^{125}$I-GM-CSF initially present in the medium is shown in the inset (B). (B) Neutrophils (2 x 10^8 cells) were incubated with $^{125}$I-GM-CSF (8.2 x 10^{-10} M) in 1 ml of binding medium for 30 min at 37°C. The cells were then divided, harvested by centrifugation, and resuspended in either binding medium alone (○) or binding medium containing 5 x 10^{-7} M unlabeled GM-CSF (□). The cells were maintained at 37°C, and aliquots were removed at various time points and assayed for binding. Free $^{125}$I-GM-CSF concentration during the dissociation incubation was 1.7 x 10^{-11} M. Error bars on each data point did not exceed the symbol size.

When stored at 4°C in 0.05 M NaPO₄, pH 7.2, containing 0.01% BSA and 0.02% sodium azide, and they exhibited no changes in binding characteristics over that time period. In addition, gel filtration chromatography showed no evidence of formation of high molecular weight aggregates upon storage.

**Interaction of GM-CSF with Cell Surface Receptors on Neutrophils and HL-60 Cells.** Human $^{125}$I-GM-CSF was shown to exhibit specific binding to human neutrophils and to the promyelocytic leukemia cell line, HL-60. Fig. 2A shows...
the association kinetics at 37 °C of neutrophils with ¹²⁵I-GM-CSF at three different concentrations (corrected for nonspecific binding). The data in Fig. 2A show that both the final equilibrium amount bound to cells and the rate of approach to equilibrium are dependent on the initial concentration of ¹²⁵I-GM-CSF in the medium. The curves passing through the data in Fig. 2A are best-fit single exponential time-dependency curves (25), consistent with the presence of a single population of receptors on these cells. In contrast to the rapid binding seen at 37 °C, binding at 4 °C was slow and required >12 h to reach equilibrium, behavior reminiscent of that seen with murine GM-CSF (16, data not shown).

The inset (Fig. 2B) shows the dependence of the pseudo first-order forward rate constant (determined by curve fitting the data of Fig. 2A) on the concentration of ¹²⁵I-GM-CSF in the medium. For a bimolecular reaction such data should fit a straight line, with the slope being the forward rate constant and the intercept on the ordinate being the reverse rate constant (25). The data shown in the inset generate values of 5.5 ± 2.3 × 10⁷/M·min for the forward rate constant and 4.7 ± 1.0 × 10⁻²/min for the reverse rate constant. The ratio of these parameters gives a range of values for the affinity constant of the radiolabeled GM-CSF preparation for its receptor of 0.6 - 2.1 × 10⁹/M.

Fig. 2B shows the dissociation of ¹²⁵I-GM-CSF from neutrophils at 37 °C. The experiment is designed, as originally described by Demeyts et al. (27), to test whether the GM-CSF receptor exhibits any cooperative properties. Comparison of the rate of dissociation of ¹²⁵I-GM-CSF from cells in medium alone when a fraction of the receptors are occupied, with that in the presence of 5 × 10⁻⁷ M unlabeled GM-CSF where almost all the receptors are occupied, revealed no significant sensitivity to receptor occupancy, suggesting that GM-CSF receptors on neutrophils are noncooperative. The dissociation rate constants measured in this experiment were 2.8 ± 0.5 × 10⁻²/min in medium alone and 1.8 ± 0.6 × 10⁻²/min in the presence of unlabeled GM-CSF. Since neither curve is a simple first-order process, these values are averages from the sum of two exponential terms. While complex dissociation kinetics appear to be generally observed for cell surface receptor systems (22, 25, 27-29), the cause(s) for such behavior remain unclear and cannot necessarily be interpreted as indicating site heterogeneity.

Kinetic experiments with the promyelocytic leukemia cell line HL-60 showed human GM-CSF binding characteristics very similar to those exhibited by human neutrophils. Association and dissociation kinetic experiments generated a forward rate constant of 3.05 ± 0.43 × 10⁸/M·min and dissociation rate constants of 5.0 ± 1.3 × 10⁻²/min in medium alone and 4.1 ± 2.1 × 10⁻²/min in the presence of unlabeled GM-CSF. For both HL-60 and neutrophils, we used results of association kinetic experiments to ensure that, in all subsequent binding experiments, the system had reached equilibrium. These experiments also revealed a strikingly low number of GM-CSF molecules bound to the cells at equilibrium, results that were confirmed by equilibrium binding experiments.

Fig. 3 illustrates typical equilibrium binding data for ¹²⁵I-GM-CSF at 37 °C to neutrophils (Fig. 3A) and HL-60 cells (B). In both cases, display of the data in the Scatchard coordinate system (30) yielded a straight line, indicating a single class of binding sites for GM-CSF. Only linear Scatchard curves were observed...
for binding measured over a concentration range of 10 pM to 10 nM. Nonspecific binding increased linearly with increasing concentration and did not exceed 2% of the total cpm added. For neutrophils, the calculated apparent $K_a$ was $2.47 \pm 1.31 \times 10^9$/M with $260 \pm 70$ specific binding sites per cell (average from seven binding experiments), and for HL-60 cells the calculated apparent $K_a$ was $3.70 \pm 1.44 \times 10^9$/M with $167 \pm 87$ specific binding sites per cell (average from six binding experiments). Similar experiments with neutrophils carried out at 4°C (allowing 18 h to reach equilibrium) showed an apparent $K_a$ of $2.18 \pm 0.19 \times 10^9$/M with $240 \pm 60$ specific binding sites per cell, indicating that when given sufficient time to reach equilibrium the parameters for human GM-CSF binding were the same at 37°C as at 4°C.

To assess whether the affinities of labeled and unlabeled GM-CSF for the receptor were significantly different, the inhibition of binding of $^{125}$I-GM-CSF to neutrophils at 37°C by unlabeled GM-CSF was determined (data not shown). Analysis of the data with a single-site competitive inhibition equation (26) yielded an inhibition constant of $1.48 \pm 0.53 \times 10^{10}$/M, indicating that while not substantial, radiolabeling of GM-CSF produced some loss in affinity for the receptor.

The specificity of $^{125}$I-GM-CSF binding was examined by testing a number of...
purified lymphokines and other polypeptide hormones for their ability to compete with $^{125}$I-GM-CSF for binding to its receptor on both neutrophils and HL-60 cells. As shown in Fig. 4, a 10-fold excess of recombinant human GM-CSF eliminated 65% of human $^{125}$I-GM-CSF binding (Fig. 4b), while none of the other lymphokines or hormones tested, including murine GM-CSF, exhibited any ability to compete, even when present at concentrations that were 100-fold greater (on a molar basis) than those of $^{125}$I-GM-CSF (Fig. 4, c–n). Identical results were found for competition of $^{125}$I-GM-CSF binding to HL-60 cells (data not shown).

Cellular Distribution of Human GM-CSF Receptors. A number of primary cells and continuous cell lines of mouse or human origin were examined for their ability to bind $^{125}$I-GM-CSF. In all cases, complete binding curves were done over a range of $^{125}$I-GM-CSF concentrations, and affinity constants and receptor numbers per cell were generated by Scatchard analysis of the data. As shown in Table I, none of the murine cells tested bound any $^{125}$I-labeled human GM-CSF, although all had been previously shown to bind murine GM-CSF (16). In addition to neutrophils and HL-60 cells, only monocytes and the myelogenous leukemia line KG-1 of the human cells tested were found to bind detectable amounts of human GM-CSF. A similar low receptor number was found on all four cell types. Although two cells of T cell origin had been previously found to bind murine GM-CSF (16), none of the human T cell lines tested exhibited any binding of human GM-CSF.
TABLE I

Cellular Distribution of Human GM-CSF Receptors

| Cells          | Characteristics   | HuGM-CSF bound (molecules/cell)* |
|---------------|-------------------|----------------------------------|
| Human         |                   |                                  |
| HL-60         | Promyelocytic leukemia | 167 ± 87                        |
| KG-1          | Myelogenous leukemia | 170 ± 80                        |
| U937          | Monocytic tumor    | 0                                |
| K562          | Erythroleukemia    | 0                                |
| CEM           | T lymphoma         | 0                                |
| HSB-2         | T lymphoma         | 0                                |
| JMB-12        | T lymphoma         | 0                                |
| JA-1 (Jurkat) | T lymphoma         | 0                                |
| RPMI-8402     | T lymphoma         | 0                                |
| PEER          | T leukemia         | 0                                |
| A375          | Myeloma            | 0                                |
| BMB           | B lymphoma         | 0                                |
| Neutrophils   |                   | 260 ± 70                         |
| Monocytes     |                   | 450 ± 75                         |
| Mouse         |                   |                                  |
| P388D1        | Macrophage tumor   | 0                                |
| J774          | Macrophage tumor   | 0                                |
| P815          | Mastocytoma (ascites) | 0                              |
| Wehi 3        | Myelomonocyte tumor | 0                               |
| LSTRA         | T lymphocytic leukemia | 0                              |
| LBRM-33       | T lymphoma         | 0                                |
| Neutrophils   |                   | 0                                |

* Binding experiments were conducted as described in the legend to Fig. 3.

Discussion

Recombinant human GM-CSF was radiolabeled with $^{125}$I to high specific activity, and was used to characterize the specific receptor for this lymphokine on human neutrophils and on the promyelocytic leukemia cell line, HL-60. We also found that GM-CSF receptors were expressed on human monocytes and on the myelogenous leukemia cell line, KG-1. None of the murine cells tested, all of which express murine GM-CSF receptor (16), was found to bind detectable levels of human GM-CSF. This again confirms the lack of crossreactivity between the GM-CSFs from these two species.

As has been found for three of the murine colony-stimulating factors, GM-CSF (15, 16), G-CSF (31), and CSF-2α (32, 33), the level of expression of GM-CSF binding sites on the human cells tested was strikingly low, indicating that human GM-CSF can exert its biological effects with few molecules bound per cell. All the murine CSFs are active in stimulating cell proliferation and colony formation at $10^{-11}$–$10^{-13}$ M concentrations (3), and a similar concentration of recombinant human GM-CSF has been found to be sufficient to induce the formation of granulocyte and macrophage colonies in a human colony assay (unpublished observations). Binding studies showed that the affinity of human GM-CSF for its receptor was $10^9$–$10^{10}$/M, thus making the concentration of GM-CSF required to produce 50% maximal receptor occupancy $10^{-9}$–$10^{-10}$ M. These concentrations are two to three orders of magnitude higher than those required...
to produce biological effects, indicating that only a small component of the detectable binding is required for maximal biological activity. These results parallel those previously reported for murine GM-CSF (15, 16), IL-3 (32–34), and the lymphokine, human IL-1 (35), and are reminiscent of other systems in which only fractional receptor occupancy is required to produce a maximal biological effect (26).

As previously discussed (16), these three factors also share a common complex pattern of ligand dissociation, in which a slowly exchanging component was observed which might reflect a subpopulation of receptors that can effectively bind ligand irreversibly. As shown in this paper, the dissociation of $^{125}$I-GM-CSF from human neutrophils exhibits a similar slowly exchanging (or irreversible) component. Whether or not this apparently irreversible interaction is primarily responsible for the capacity of these lymphokines to produce biological effects at such low levels of receptor expression, as has been previously suggested (16), is not known, but the similarities between them suggest that they may all share common mechanisms of receptor action.

GM-CSF can not only stimulate the proliferation and differentiation of granulocyte-macrophage precursor cells, but can modulate the functional activity of mature granulocytes and macrophages as well. Alterations in the binding characteristics of the GM-CSF receptor, possibly in a maturation-dependent manner, could explain the different biological effects mediated by this hormone. In their report on the murine GM-CSF receptor, Walker and Burgess (15) have in fact suggested that the small subclass of high-affinity binding sites that they observe may decline in concert with cell maturation. In our previous study with recombinant murine GM-CSF, we found only a single class of high affinity receptors on all cell types examined (16). In this study, using recombinant human GM-CSF, we have similarly found a single class of high-affinity receptors on human cells. In addition, we examined in detail the binding characteristics of the receptor on both a mature cell (neutrophils) and on an undifferentiated promyelocytic leukemia cell line (HL-60). No significant differences in the kinetic parameters of receptor-binding were seen between these two cell types, suggesting that maturation-specific responses to GM-CSF are not mediated by overt changes in the binding characteristics of the hormone for its receptor.

In addition to the multiple ways that GM-CSF may function in the control of the normal physiology of hematopoiesis, GM-CSF and its receptor may also play a role in the pathology of leukemia. Myeloid leukemia cells, for example, cannot proliferate autonomously in vitro, but will grow in the presence of exogenous colony-stimulating factors (2), suggesting that these cells grow in response to factors much as do normal hematopoietic cells. Current studies on the expression of GM receptors on human leukemias indeed suggest that many, but not all, myeloid leukemias express GM-CSF receptor. Receptor levels are low and some leukemia cells appear to respond to recombinant GM-CSF (L. S. Park, R. Andrews, I. Berstein, and D. L. Urdal, unpublished data). Alternatively, recent observations that some onc genes are homologous to growth factors or growth factor receptors, notably, that the erb b onc gene is homologous to the receptor for epidermal growth factor (36) and that the c-fms proto oncogene is related to the receptor for CSF-1 (37), intensify the interest in the GM-CSF receptor found
on malignant cells. Finally, radiolabeled GM-CSF represents a useful reagent for describing the surface phenotype of malignant cells, and the knowledge of the GM-CSF receptor gleaned from such studies will expand our understanding of hematopoiesis and may contribute to the diagnosis and potential treatment of cancer.

Summary

$^{125}$I-labeled recombinant human GM-CSF was used to identify and characterize receptors specific for this lymphokine on both a mature primary cell, human neutrophils, and on the undifferentiated promyelomonocytic leukemia cell line, HL-60. Human GM-CSF also bound to primary human monocytes and to the myelogenous leukemia cell line, KG-1, but not to any of the murine cells known to express the murine GM-CSF receptor. In addition, although some murine T lymphomas can express the GM-CSF receptor, none of the human cell lines of T cell lineage that we examined bound iodinated human GM-CSF. Binding to all cell types was specific and saturable. Equilibrium binding studies revealed that on all cell types examined, GM-CSF bound to a single class of high affinity receptor (100–500 receptors per cell) with a $K_a$ of $10^9$–$10^{10}$/M. More extensive characterization with neutrophils and HL-60 cells showed that in both cases, binding of GM-CSF was rapid at 37°C with a slow subsequent dissociation rate that exhibited marked biphasic kinetics. Among a panel of lymphokines and growth hormones, only human GM-CSF could compete for binding of human $^{125}$I-GM-CSF to these cells. GM-CSF can not only stimulate the proliferation and differentiation of granulocyte/macrophage precursor cells, but can modulate the functional activity of mature granulocytes and macrophages as well. No significant differences in the kinetic parameters of receptor binding were seen between mature neutrophils and the undifferentiated promyelocytic leukemia cell line HL-60, indicating that maturation-specific responses to GM-CSF are not mediated by overt changes in the binding characteristics of the hormone for its receptor.

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