Receptors for B-Melanocyte-Stimulating Hormone Exhibit Positive Cooperativity in Synchronized Melanoma Cells†

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ABSTRACT: Cloudman S91 mouse melanoma cells respond in culture to B-melanocyte-stimulating hormone (B-MSH) with changes in morphology, growth rates, and melanin production. The effects of MSH appear to be mediated through a stimulation of the cyclic AMP system. It was reported earlier that at least some of the responses to MSH (increased cyclic AMP production and tyrosinase activity) occur in the G2 phase of the cell cycle [Wong, G., Pawelek, J., Sansone, M., & Morowitz, J. (1974) *Nature (London) 248*, 351–354] and that the apparent reason for this cell cycle restriction is that receptors for MSH are most active in the G2 phase [Varga, J. M., DiPasquale, A., Pawelek, J., McGuire, J., & Lerner, A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1590–1593]. In this report, we found that by two separate methods of obtaining populations of cells in the G2 phase of their cycle—centrifugal elutriation or synchronization with thymidine—we observed increased binding of MSH by cells in the G2 and possibly late S phases of their cycle. However, cultures of cells passing through their cycle in synchrony were quite different from nonsynchronized (random) cultures. Both synchronized and random cultures expressed receptors for MSH in the G2 and possibly late S phases of their cycle, but synchronized cultures bound severalfold more MSH per cell than random cultures. This increased binding of MSH by synchronized cells was accompanied by an increase in tyrosinase activity and pigment production. Analyses by Scatchard and Hill methods revealed a potential basis for the increased binding capacity and responsiveness to MSH by synchronized cells: receptors from synchronized cells exhibited positive cooperativity, while receptors from random populations of cells exhibited no cooperativity. Understanding the mechanisms regulating cooperative interactions in the MSH receptor system could be of fundamental value in understanding the regulation of proliferation and pigmentation by MSH.

Cooperative molecular interactions in biological systems have been studied for many years, a well-known example being the positive cooperative interactions between oxygen and hemoglobin (Hill, 1910; Imai, 1974). Cooperative phenomena have also been observed in hormone–receptor interactions. Although most peptide hormone–receptor interactions show no cooperativity, mathematical analyses of the interactions between insulin and insulin receptors, for example, are generally compatible with a model of negative cooperativity (DeMeyts et al., 1976; Gammeltoft et al., 1978). There are several examples in peptide hormone–receptor interactions where positive cooperativity is observed: gonadotropin releasing hormone on anterior pituitary cells (Zolman & Theodoropoulos, 1984); insulin receptors on rat hepatoma cells (Marsh et al., 1984; Gammeltoft, 1984; Taylor & Leventhal, 1983); oxytocin receptors in rat brains (Ferrier et al., 1983). Positive cooperativity could not be observed for the insulin receptors in fibroblasts from a human patient with a genetic form of extreme insulin resistance (leprechaunism), whereas positive cooperativity could be observed in fibroblasts from normal volunteers (Gammeltoft, 1984). The biologial significance of cooperativity in hormone–receptor interactions is not understood; however, regulation of receptor activity is a possibility.

We report here an example of such regulation. Melanocyte-stimulating hormone (MSH) receptors on synchronized Cloudman melanoma cells exhibit positive cooperativity while those on nonsynchronized cells show no cooperativity, even though both synchronized and nonsynchronized populations express MSH receptors in the late S and G2 phases of the cell cycle. The positive cooperativity for MSH–receptor interactions in synchronized cells is accompanied by increased responsiveness of the cells to MSH. The results provide evidence, at least for MSH, that receptor cooperativity may be a regulatory mechanism for amplification of a hormonal signal.

Materials and Methods

Cell Culture. Cells were cultured in monolayer in Ham's F10 medium supplemented with 10% horse serum as described (Pawelek, 1984). The cell line used for these studies, PS-1-mel-1, is a variant which synthesizes very little melanin unless MSH, or other agents which raise cyclic AMP levels, is added to the culture medium (Pawelek et al., 1975). MSH has little or no effect on the rate of proliferation of this line. The effects of MSH were potentiated by the cyclic nucleotide phosphodiesterase inhibitor methylisobutylxanthine (MIX). At a concentration of 5×10^{-6} M, MIX potentiated the MSH-mediated increase in tyrosinase activity 3-fold, but by itself, it had no effect on tyrosinase activity (Pawelek et al., 1985). In the studies concerning effects of MSH on tyrosinase activity (Figure 2), MIX was supplemented to the culture medium along wth MSH.

Tyrosinase Assays. Cells were lysed by exposure to Triton X-100 (0.5%) in sodium phosphate (10 mM, pH 6.8). Tyrosinase activity was measured in lysed cell preparations by a modified (Korner & Pawelek, 1977) method of Pomerantz (Pomerantz & Chuang, 1970).

Centrifugal Elutriation. Cells in the logarithmic phase of growth were harvested from the culture flasks with Joklik's medium containing ethylenediaminetetraacetic acid (EDTA,

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1 mM) (Pawelek, 1984), pelleted by low-speed centrifugation at 4 °C, and resuspended in sodium phosphate (10 mM, pH 7.9) containing NaCl (150 mM), bovine serum albumin (0.5%), and EDTA (1 mM) at a concentration of 10^7 cells/mL. Approximately 3×10^8 cells were injected into a JE-6 elutriator rotor (Beckman Instruments, Palo Alto, CA). Elutriation was carried out at 4 °C, at a rotor speed of 1600 rpm. Cells were injected into the elutriator at a rate of 8 mL/min and collected in 150-mL fractions with a Cole-Parmer Masterflex Pump (Model 7565-10) at rates increasing in increments of 2 mL/min to a final flow rate of 32-36 mL/min. Complete recovery of all the cells from the elutriator required collection of 13-15 fractions. Cells from each fraction were pelleted by centrifugation. Cell numbers and volumes were determined with a Coulter Counter (Model ZBI) and a Coulter Channelyzer (Coulter Electronics, Hialeah, FL). Polystyrene beads (11.4- and 17.1-μm diameter) were used as standards for volume measurements.

Synchronization of Cells by Treatment with Thymidine. Cells in the logarithmic phase of growth were synchronized with a double-thymidine block by a modification of the method of Puck (1964). The cells were exposed to culture medium containing thymidine (1 mM) for 48 h, thymidine-free medium for 18 h, thymidine-supplemented medium for an additional 8 h, and finally thymidine-free medium. DNA analyses (see below) indicated that 18-24 h after the final removal of thymidine, more than 70% of the cells were in the S-G2+M phase.

Analyses of DNA Content. The DNA content of cells was measured with the use of a fluorescence-activated cell sorter (FACS IV, Becton-Dickinson Corp. Sunnyvale, CA). Cells were removed from flasks by incubation with Joklik's medium containing EDTA (1 mM) and washed by centrifugation in isotonic phosphate-buffered saline solution. Cells were fixed for at least 12 h in formalin (10%, 4 °C) and then stained using Acriflavin-Feulgen dyes (Gill & Jotz, 1974), or cells were fixed by stepwise increase in ethanol to a final concentration of 70% and then stained with mithramycin (100 μg/mL) (Crissman & Tobey, 1974). Following either procedure, cells were applied to a FACS IV instrument, and the percentage of cells in G1, S, and G2+M phases of the cycle was estimated by computer analysis of the DNA histograms.

Analyses of Protein Content. Protein content was determined by the method of Bradford (1976).

Synthesis of ^{125}I-B-MSH. Iodination of B-MSH and isolation of the iodinated peptide were carried out in this laboratory by a modification (Pawelek et al., 1987) of the method of Lambert and Lerner (1983).

Binding of ^{125}I-B-MSH. The assay for binding of ^{125}I-B-MSH to cells was that of Lambert and Lerner (1983). For Scatchard and Hill analyses, concentrations of carrier-free ^{125}I-B-MSH were used ranging from 0.1 to 5.0 nM.. Specific binding was determined by subtracting the cpm bound in the presence of a 10000-fold excess of nonradioactive MSH from those obtained with the carrier-free ^{125}I-B-MSH. The specific binding was determined for each concentration of labeled MSH and ranged between 80% and 95% of the total counts bound to the cells. The kinetics of binding of ^{125}I-B-MSH to synchronized and random cultures were identical, reaching a plateau between 120 and 180 min at 10 °C. Scatchard analyses were performed at 120 min. Although the differences between the Scatchard and Hill plots for synchronized and random cultures were qualitatively consistent between experiments, the absolute level of binding to the cells varied between experiments, probably because of different batches of horse serum used in the culture media. To compensate for this variability, the data shown in Figure 3 (from which the Scatchard and Hill plots were derived) were normalized by adjusting four of the experiments on a percentage basis to the fifth experiment in which the absolute binding was highest, with the data being expressed as femtomoles bound. All binding studies were carried out at 10 °C, to prevent receptor-hormone internalization, in the presence of the protease inhibitors benzamidine and phenylmethanesulfonyl fluoride (1 mM each).

Table I: Effects of Lowered pH on Bound ^{125}I-MSH[a]

culture conditions	cpm of ^{125}I-MSH bound/10^6 cells	
	-HCl	+HCl
nonsynchronized	5131 ± 1589	0
synchronized	13230 ± 1598	0

[a] ^{125}I-B-MSH was bound to nonsynchronized cultures or cultures released from a double-thymidine block for 18 h. After 2 h of incubation with ^{125}I-MSH at 10 °C, some aliquots of cells were placed directly on ice with no further treatment, while some were exposed to pH 3.5 (adjusted with 1 N HCl) for 1 min on ice (Marshall, 1985). The amount of bound ^{125}I-MSH was then determined by pelleting the cells through 0.3 M sucrose and counting the pellets in a γ counter (Lambert & Lerner, 1983). Shown above are average specific counts ± the standard deviation for triplicate determinations. Nonspecific counts were subtracted and equalled less than 5% of the total counts bound. Counts obtained in the presence of HCl were equal to or less than the nonspecific counts. Trypan blue exclusion tests showed that greater than 90% of the cells maintained their integrity to dye exclusion at the end of the experiment, whether or not they had been treated with HCl.

Results

Cell Cycle and Tyrosinase Activity in Response to MSH. Previous work from this laboratory showed that when Cloudman melanoma cells are synchronized by treatment with colchine they respond to MSH with increased cyclic AMP content and tyrosinase activity in the G2 phase of the cell cycle and that this is the phase when receptors for MSH are expressed (Wong et al., 1974; Varga et al., 1974). Later, it was demonstrated that nonsynchronized cells exposed to MSH also show increased tyrosinase activity in the G2 phase when physically separated by centrifugal elutriation (Pawelek et al., 1985). However, we recently observed that nonsynchronized cells in the G2 phase were less responsive to MSH than cells passing synchronously into the G2 phase. Detailed analyses of MSH responsiveness in synchronized and nonsynchronized cells are presented below.

Binding of ^{125}I-B-MSH to Cells following Synchronization with Thymidine. Cells were arrested in the S phase of their cycle with a double-thymidine block as described under Materials and Methods. At various times following removal of thymidine, the ability of the cells to bind ^{125}I-B-MSH was assessed. Maximum binding of MSH was observed 18-24 h after release from the second thymidine block, at which time FACS analyses indicated that more than 70% of the cells were in late S and G2+M. In a typical experiment, cells released from thymidine for 18-24 h (when most of the population was in G2) bound 4 times more ^{125}I-MSH than those released from thymidine for 1 h, whereas cells released from thymidine for 70 h (when most of the population had cycled back to G1) bound about half as much ^{125}I-MSH as those released for 1 h (not shown).

Brief exposure of cells to pH 3.5 (adjusted with 1 N HCl) released virtually 100% of the bound ^{125}I-MSH whether or not the cells had been synchronized with thymidine (Table I). This result demonstrated that the radioactive hormone remained on the outer cell surface during the binding assay and

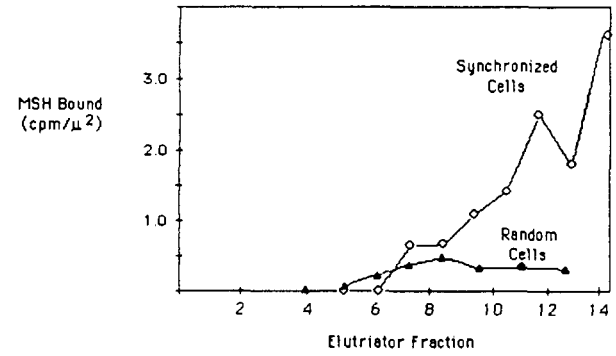

FIGURE 1: Binding of ^{125}I-B-MSH to cells following centrifugal elutriation. Cells were subjected to centrifugal elutriation as described under Materials and Methods. Following elutriation, cells were centrifuged and resuspended in binding buffer to assay binding of ^{125}I-B-MSH binding (Lambert & Lerner, 1983). Results represent averages of triplicate samples expressed as cpm of ^{125}I-B-MSH bound to 2.5×10^5 cells per unit surface area of the cells (u^2). Variation between triplicates was less than ±15%. The experiments were repeated 3 times with similar results. Nonsynchronized, random cultures (▲); cultures released from a double-thymidine block for 18 h (◇).

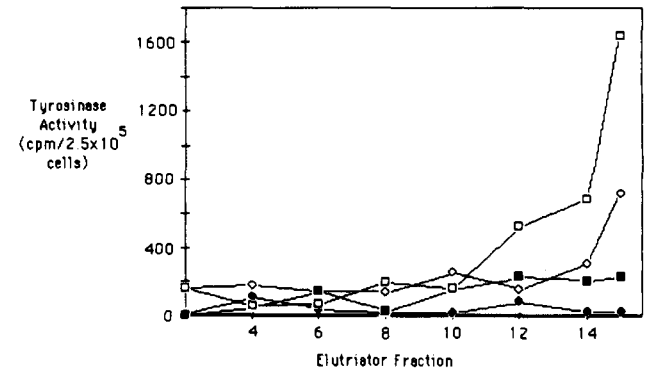

FIGURE 2: Tyrosinase activity following centrifugal elutriation of nonsynchronized cultures and synchronized cultures. Cultures were synchronized with thymidine. Immediately after the release from thymidine, half of the random cultures and half of the thymidine-synchronized cultures were exposed to MSH (2×10^{-7} M) and MIX (5×10^{-6} M) for 18 h prior to elutriation. Cells were isolated from the elutriator and lysed at a ratio of 10^6 cells/mL of Triton X-100 (0.5%) in sodium phosphate (10 mM, pH 6.8). Tyrosinase activity was measured in lysates from 2.5×10^5 cells by a modification of the method of Pomerantz. Results represent averages of duplicate determinations with variation between duplicates of less than ±10%. Random cultures (♦); random cultures treated with MSH/MIX (◇) synchronized cultures (■); synchronized cultures treated with MSH/MIX (□).

that no significant hormone internalization occurred at 10 °C.

Centrifugal Elutriation of Cells Synchronized with Thymidine: Binding of ^{125}I-B-MSH and Measurement of Tyrosinase Activity. Cells were released from a double-thymidine block as above. Eighteen hours following the release, cells were harvested from the culture flasks and applied to the centrifugal elutriator. Specific binding of ^{125}I-B-MSH to the thymidine-treated cells was observed beginning in fraction 7 of the elutriator run which corresponded to late S and early G2 phase cells (Figure 1). Data for ^{125}I-B-MSH binding to control and synchronized cells are superimposed in Figure 1. Although both random and synchronized cultures bound MSH in the late S-G2+M phase of the cycle, synchronized cultures bound considerably more MSH per cell than random cultures. The data for ^{125}I-B-MSH binding are expressed as cpm per surface area (u^2), demonstrating that the increased binding of the hormone was not simply due to the increase in surface area of the cells which occurred as they progressed through their cycle.

In parallel experiments, we measured the effects of MSH on tyrosinase activity in random cultures and synchronized cultures. Cells were grown in plain medium, or they were subjected to a double-thymidine block. Following release from the second thymidine treatment, both the synchronized and random cells were cultured for an additional 18 h in either plain medium or medium supplemented with B-MSH (2×10^{-7} M) and MIX (5×10^{-6} M). The cells were then harvested and subjected to centrifugal elutriation. Fractions were collected, and tyrosinase was assayed (Figure 2). Tyrosinase activity in both random and synchronized cultures was highest in the fractions which corresponded to the G2 region of elutriation. However, synchronized cultures had higher tyrosinase activity per cell than random cultures, and synchronized cultures treated with MSH/MIX had about 4 times more tyrosinase activity per cell than random cultures treated with MSH/MIX. Together, the results from Figures 1 and 2 indicate that the increased binding of MSH in synchronized cultures correlated with an increased responsiveness of the cells to MSH.

Scatchard and Hill Analysis of MSH–Receptor Interactions. The above results indicated that cells passing through their cycle synchronously have increased MSH binding compared to nonsynchronized cells ,even though both populations bind MSH preferentially in the late S and G2 phases of their

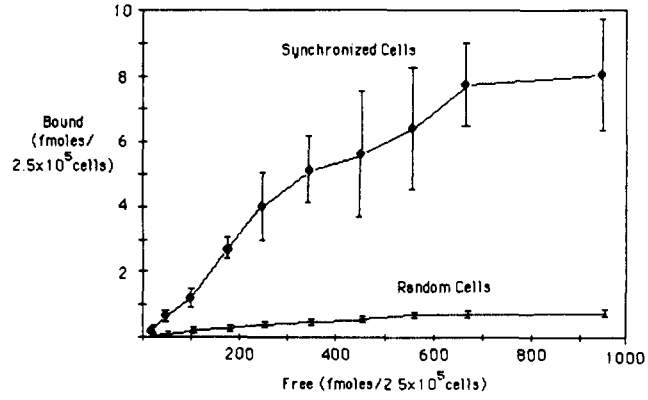

FIGURE 3: Specific binding of B-MSH to synchronized and random cells. The cells were synchronized by exposure to thymidine. The amount of MSH bound was plotted as a functin of the amount of MSH added to the binding assay. The data are expressed as femtomoles, but they are normalized as described under Materials and Methods. The results represent the average and standard deviation for five separate experiments.

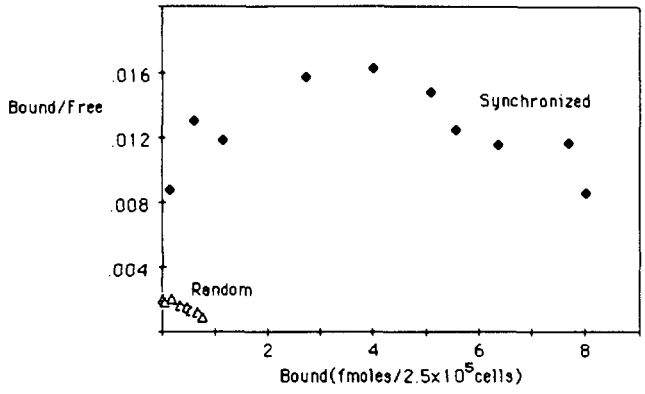

FIGURE 4: Scatchard analyses of the data in Figure 3.

cycle. To investigate this phenomenon in more detail, we analyzed the interactions between MSH and its receptors by the methods of Scatchard and Hill (Boeynaems & Dumont, 1975). The amount of MSH bound to random and thymidine-treated cells was measured as a function of increasing

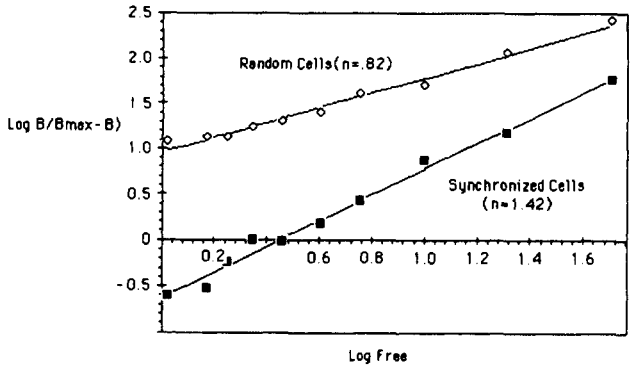

FIGURE 5: Hill analyses of the data in Figure 3.

^{125}I-MSH concentration (Figure 3). Analyses of these data revealed striking differences between receptors on synchronized and nonsynchronized cells. For nonsynchronized cultures, the Scatchard plot was linear (Figure 4), and the Hill coefficient was 0.824 ± 0.08 (Figure 5), indicating no cooperativity. For synchronized cultures, the Scatchard plot was convex, and the Hill coefficient was 1.42 ± 0.12, indicating positive cooperativity. Scatchard analyses of the nonsynchronized cultures indicated the presence of 2100 receptors per cell and a dissociation constant for MSH of 0.79 nM. We did not attempt to estimate the number of receptors or dissociation constant for MSH in synchronized cultures due to the inherent limitations of the analyses (Klotz, 1982).

Discussion

We have shown that although synchronized and nonsynchronized populations of cells each display MSH receptor activity in the G2 or late S phases of the cycle, receptors on synchronized cells show positive cooperativity for MSH while those on nonsynchronized cells show no cooperativity for MSH. The change in receptor activity in synchronized cells is accompanied by a corresponding change in cellular responsiveness to MSH, providing evidence that cooperativity in MSH–receptor interactions may be a regulatory mechanism for amplification of the hormonal signal.

One explanation for the differences in receptor populations between random and synchronized cultures could be that when cells pass through their cycle synchronously in a "wave", they might produce autocrine factors that alter the MSH–receptor interactions (Sporn & Roberts, 1985). Such factors might be produced or be active only at specific times in the cycle so that synchronized cultures would periodically exhibit high concentrations of the factors. Nonsynchronized cultures could also be producing such factors, but in amounts too low to result in receptor alterations. We have not, as yet, obtained evidence for such factors, but since there is precedent for autocrine regulation of proliferation, this possibility remains open to explain our findings of positive cooperativity in synchronized cells. An alternative explanation for differences in receptor populations could be that exposure to either colchicine or thymidine caused a generalized change in the plasma membrane, which could result in altered binding of MSH as well as other peptide hormones. An argument against this explanation is that ^{125}I-insulin binding to cells was constant throughout the cycle whether or not the cells were synchronized with thymidine (unpublished results). In addition, since the mechanisms of action of thymidine and colchicine are quite different, it seems unlikely that both agents would cause the same artifactual change in MSH receptors.

Our results on a G2 restriction for MSH receptors are in agreement with earlier reports from this laboratory (Varga et al., 1974; Wong et al., 1974; Pawelek et al., 1985; McLane & Pawelek, 1985). However, Fuller and co-workers (Fuller & Brooks, 1980; Shimizu et al., 1981) were unable to confirm a G2 restriction. They suggested that the use of colchicine in previous experiments might have produced artifacts and that the ^{125}I-B-MSH preparations might not have been biologically active. We believe our studies have satisfied these criticisms. We observed a G2 restriction of receptor activity in random cultures when we isolated cells in various phases of their cycle by physical separation on the centrifugal elutriator. We also observed a G2 restriction when the cells were synchronized either with thymidine or with colchicine. In addition, the ^{125}I-B-MSH we used retained full biological activity (Lambert & Lerner, 1983). The G2 restriction for ^{125}I-B-MSH binding was specific in that we saw no corresponding restriction for ^{125}I-insulin binding to the cells throughout their cycle, in agreement with the findings of Shimizu et al. (1981).

Our demonstration of a direct relationship between cooperativity of MSH receptors and cellular responsiveness to MSH indicates that MSH receptors are central to the regulation of phenotype. Thus, understanding the mechanism of action of the MSH-receptor complex is of primary importance to understanding the molecular basis of growth and the expression of differential functions in these cells.

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Identification of Cysteine-644 as the Covalent Site of Attachment of Dexamethasone 21-Mesylate to Murine Glucocorticoid Receptors in WEHI-7 Cells†

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ABSTRACT: Dexamethasone 21-mesylate is a highly specific synthetic glucocorticoid derivative that binds covalently to glucocorticoid receptors via sulfhydryl groups. We have identified the amino acid that reacts with the dexamethasone 21-mesylate by using enzymatic digestion and microsequencing for radiolabel. Nonactivated glucocorticoid receptors obtained from labeling intact WEHI-7 mouse thymoma cells with [^{3}H]dexamethasone 21-mesylate were immunopurified and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The purified ~100-kDa steroid-binding subunit was eluted from gel slices and subjected to enzymatic digestion. Trypsin digestion followed by reversed-phase high-performance liquid chromatography (reversed-phase HPLC) produced a single [^{3}H]dexamethasone 21-mesylate labeled peptide. Automated Edman degradation of this peptide revealed that the [^{3}H]dexamethasone 21-mesylate was located at position 5 from the amino terminus. Dual-isotope labeling studies with [^{3}H]dexamethasone 21-mesylate and [^{35}S]methionine demonstrated that this peptide contained methionine. *Staphylococcus aureus* V8 protease digestion of [^{3}H]dexamethasone 21-mesylate labeled steroid-binding subunits generated a different radiolabeled peptide containing label at position 7 from the amino terminus. On the basis of the published amino acid sequence of the murine glucocorticoid receptor, our data clearly identify cysteine-644 as the single residue in the steroid-binding domain that covalently binds dexamethasone 21-mesylate. We have confirmed this finding by demonstrating that a synthetic peptide representing the amino acid sequence 640–650 of the murine glucocorticoid receptor behaves in an identical manner on reversed-phase HPLC as the trypsin-generated peptide from intact cells.

Most effects of glucocorticoids appear to be mediated through specific cellular proteins known as glucocorticoid receptors. These receptors bind steroid and under physiological conditions undergo a process termed activation. The activated glucocorticoid receptor complex binds with affinity to certain consensus sequences on the DNA, altering transcription of genes thought to code for proteins that give rise to the observed physiological effects of glucocorticoids (Yamamoto, 1985).

Dexamethasone 21-mesylate (DM),[1] the α-keto mesylate derivative of dexamethasone, is a synthetic steroid that binds

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[1] Abbreviations: DM, dexamethasone 21-mesylate; dexamethasone, 9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; reversed-phase HPLC, reversed-phase high-performance liquid chromatography; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BIS, bis(acrylamide); BAC, bis(acrylylcystamine); TFA, trifluoroacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PTC, phenyl isothiocyanate; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; Boc, *tert*-butoxycarbonyl; Tos, tosyl; HTC, rat hepatoma cell line; GR, glucocorticoid receptor.

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