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Cell Biology

Dexamethasone modulates binding and action of epidermal growth factor in serum-free cell culture

(permissive effects/human diploid fibroblasts/cell proliferation/insulin/thrombin)

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ABSTRACT **Experiments probing the mechanism by which glucocorticoids modulate cell proliferation were carried out on serum-free cell cultures of quiescent human diploid foreskin (HF) cells. Added alone, the synthetic glucocorticoid dexamethasone had no effect on cell number. However, dexamethasone enhanced the mitogenic response of HF cells to epidermal growth factor (EGF) by 50% at all EGF concentrations. The mitogenic action of EGF was maximally promoted by a dexamethasone concentration of 100 ng/ml (0.25 μM).**

Binding studies with ^{125}I-labeled EGF (^{125}I-EGF) suggested that dexamethasone caused this "permissive" effect by modulating cell surface receptors for EGF. Paralleling their increased responsiveness to EGF growth stimulation, dexamethasone-treated cells exhibited a 50–100% increased ability to bind physiological concentrations of ^{125}I-EGF. A binding increase was apparent after a 4-hr dexamethasone treatment. The dexamethasone-treated cells maintained an increased ability to bind ^{125}I-EGF during the prolonged exposure to EGF that was required to stimulate cell division. Moreover, the increase in ^{125}I-EGF binding exhibited a dexamethasone dose-dependence similar to that for the enhancement of EGF mitogenesis, suggesting a relationship between the dexamethasone effects on binding and growth.

An investigation of the binding increase showed that it was specific for glucocorticoids, and required protein synthesis. The enhancement of ^{125}I-EGF binding diminished with increasing concentrations of ^{125}I-EGF, indicating that dexamethasone caused a qualitative change in the EGF receptors (possibly a change in receptor affinity or cooperativity). The alteration in ^{125}I-EGF binding may occur as part of a far-reaching dexamethasone-mediated change in the cell surface, because dexamethasone treatment slightly increased the ability of HF cells to bind ^{125}I-insulin, and decreased by half their ability to bind ^{125}I-thrombin.

There are numerous reports that glucocorticoid steroid hormones affect the proliferation of animal cells in culture—in some cell types stimulating (1–3), and in others inhibiting cell division (4, 5). An intriguing property of these glucocorticoid actions is that they occur only in the presence of polypeptide growth factors or serum. Thus, it appears that glucocorticoids modulate cell growth indirectly by altering cell responsiveness to growth factors. Glucocorticoids might cause these effects by altering growth factor interaction with the cells as measured by growth factor binding (2). Alternatively, glucocorticoids could bring about these effects by altering key biochemical events subsequent to the binding of growth factors to cell surface receptors (6). Here we report that the synthetic glucocorticoid dexamethasone (dex) enhances the mitogenic action of epidermal growth factor (EGF) on human diploid foreskin (HF) cells. Moreover, we have discovered that dex also increases the specific cellular binding of EGF, suggesting that dex promotes EGF initiation of cell division by altering the cellular receptors for EGF. The dex modifications of EGF binding and mitogenesis occur in cultures without serum, allowing us to study these effects under chemically defined conditions.

MATERIALS AND METHODS

Materials. We purchased Dulbecco–Vogt modified Eagle's medium (DV medium) from Gibco, serum and other media products from Irvine Scientific, and tissue culture dishes from Falcon Plastics. EGF was purified from male mouse submaxillary glands by the procedure of Savage and Cohen (7). Highly purified human thrombin (3000 NIH units/mg, ref. 8) was generously provided by John Fenton, II. Mono[^{125}I]iodoinsulin (9) was a gift from E. Arquilla. Insulin used in growth experiments and crystalline bovine serum albumin were purchased from Sigma. All other chemicals were reagent grade.

Cells and Cell Culture. Stock cultures of human fibroblasts prepared from neonatal foreskin explants (HF cells) were grown in DV medium containing 10% calf serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml. The cells were between passages 7 and 18, and were maintained at subconfluent densities. The cultures were grown at 37° in a humidified atmosphere containing 5% CO_2 in air. HF cells were shown to be free of mycoplasma by the assay of Schneider *et al.* (10).

Quiescent HF cultures for use in experiments were prepared as follows. Cells were plated at a density of 3×10^5 cells per cm^2 (or 1.5×10^5 cells per cm^2 in Fig. 2) in 35-mm diameter dishes containing 2 ml of DV medium with 10% calf serum. After 4 or 5 days, the medium was then changed to 2 ml of serum-free medium containing 2 parts DV medium and 1 part Waymouth's medium. [Waymouth's medium contains ascorbic acid and vitamin B_{12}, which reportedly enhance the mitogenic activity of EGF in serum-free medium (11, 12)]. Cell number in these quiescent cultures remained constant for more than 10 days.

Iodination of EGF and Thrombin. EGF was iodinated by the chloramine-T procedure of Carpenter and Cohen (13). Human thrombin was iodinated as described by Martin *et al.* (14). The ^{125}I-labeled thrombin (^{125}I-thrombin) had a specific activity of $2–5 \times 10^6$ cpm/μg, comigrated as a single band with nonlabeled thrombin on sodium dodecyl sulfate/polyacrylamide gels, and retained both its proteolytic and mitogenic activities.

Binding Assays. To measure ^{125}I-EGF binding, the medium on cultures was changed to DV medium (pH 7.4) containing 0.1% bovine serum albumin and the indicated concentration of ^{125}I-EGF. Incubations were carried out at 0° or 37° in 5% CO_2/95% air for the indicated times and were terminated by

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Abbreviations: dex, dexamethasone; EGF, epidermal growth factor; HF cells, human foreskin cells; DV medium, Dulbecco–Vogt modified Eagle's medium.

rinsing the cultures with seven 1-ml aliquots of ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin. The cells were then dissolved in 1 ml of 0.3 M NaOH. Aliquots were removed for protein determinations (15) and for measurements of radioactivity in a γ counter. Nonspecific binding, measured as the radioactivity bound to cultures containing nonlabeled EGF at 2 μg/ml in addition to the ^{125}I-EGF, was subtracted from the radioactivity bound to cultures that had been incubated with ^{125}I-EGF alone. Nonspecific binding was proportional to ^{125}I-EGF concentration, and was only about 2% of the total binding observed in a 60-min incubation using ^{125}I-EGF at 10 ng/ml.

^{125}I-Insulin binding was measured in DV medium containing 0.1% bovine serum albumin buffered with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.9 (the optimal pH for ^{125}I-insulin binding). ^{125}I-Insulin was added to a final concentration of 0.5 ng/ml (2–5 $\times$ 10^5 cpm/ml), and the cells were incubated at room temperature for 45 min. Insulin binding reached a steady-state level during this time and remained constant for more than 2 hr. Incubations were terminated by quickly rinsing the cells four times with cold phosphate-buffered saline. The plates were then processed as described in the ^{125}I-EGF binding protocol. Nonspecific binding, determined with monocomponent insulin (16) at 5 μg/ml, generally comprised 40–50% of the total binding.

^{125}I-Thrombin binding was measured using ^{125}I-thrombin at 50 ng/ml to measure total binding and nonlabeled thrombin at 2 μg/ml to measure nonspecific binding. The experimental procedures were the same as those used to measure ^{125}I-insulin binding except that the binding medium was buffered at pH 7.4. Nonspecific binding accounted for about 25% of the total ^{125}I-thrombin binding.

Dex did not significantly alter the amount of nonspecific binding of ^{125}I-EGF, ^{125}I-insulin, or ^{125}I-thrombin.

RESULTS

Enhancement by Dex of EGF Growth Initiation. Consistent with a previous report (11), the addition of EGF to growth-arrested cultures of HF cells caused an increase in cell number, which occurred in serum-free medium (Fig. 1*A*) as well as in serum-containing medium (Fig. 1*B*). Addition of dex by itself (100 ng/ml; 0.25 μM) to the quiescent HF cultures in serum-free medium had no effect on cell number (arrow in Fig. 1*A*). However, the addition of this concentration of dex along with EGF increased the response to EGF by about 50% (Fig. 1). In other experiments, dex enhanced EGF growth initiation by as little as 20% and by as much as 100%. This variability was not reduced by increasing the medium volume (to minimize medium depletion) or by altering the length of EGF or dex incubation.

Fig. 1 shows that dex increased EGF growth initiation by about the same proportion whether or not the culture medium contained serum. The same enhancement by dex was also observed with cultures that were rinsed, incubated for 30 min in serum-free medium, and then placed in serum-free medium for the growth experiments. These results suggest that this effect of dex did not require the participation of serum.

Fig. 2 shows the effect of various concentrations of dex on the mitogenic responsiveness of HF cells to EGF. The enhancement of EGF action by dex increased from an undetectable influence at a dex concentration of 5 ng/ml to a maximum effect at 100 ng/ml. Further increasing the dex concentration to 1 μg/ml did not change its ability to enhance EGF mitogenesis.

Increased ^{125}I-EGF Binding to Dex-Treated Cells. Dex could potentiate EGF growth stimulation by increasing the ability of HF cells to bind EGF. To test this possibility, we added dex to HF cells arrested in serum-free medium and examined the capacity of the cells to bind a mitogenic concentration (0.5 ng/ml) of ^{125}I-EGF. Dex increased the ability of the cells to bind ^{125}I-EGF by 50–100% (Fig. 3). As shown, this increase was maximal after a 24-hour dex incubation. Protein synthesis appeared to be required for the increase in ^{125}I-EGF binding capacity, because cycloheximide (10 μg/ml) completely prevented it (data not shown). In addition, the enhancement of ^{125}I-EGF binding was specific for glucocorticoid

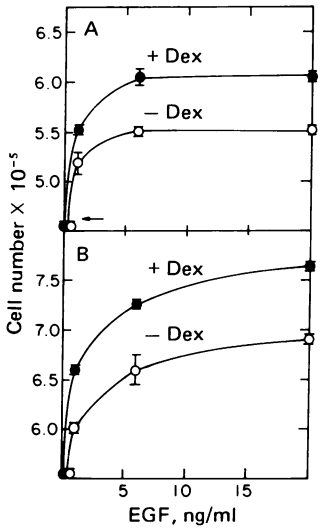

FIG. 1. Potentiation by dex of EGF-stimulated HF cell proliferation. Quiescent HF cells were placed in medium containing no serum (*A*) or 1% calf-serum (*B*). After 2 days the cultures were given 0.1% bovine serum albumin and EGF was added to yield the concentrations shown. Half the plates at each EGF concentration were then given 100 ng of dex per ml. The number of attached cells was measured with a Coulter electronic particle counter either at the time of EGF addition (arrow in *A*) or three days later. In all cases, less than 2% of the cells were found floating in the medium. The points represent the averages of duplicate measurements ± 1 SD from the mean.

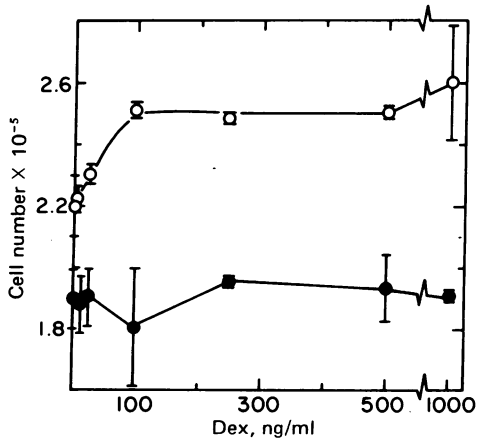

FIG. 2. Effect of dex concentration on EGF stimulation of cell division. Quiescent HF cells, incubated in serum-free medium for 1 day, were given the concentrations of dex shown. After 24 hr all the plates were given 0.1% bovine serum albumin and EGF (10 ng/ml) was added to the indicated plates. Three days later the number of cells was monitored as described in Fig. 1. The points represent the average of duplicate measurements ± 1 SD from the mean. ○, With EGF; ●, without EGF.

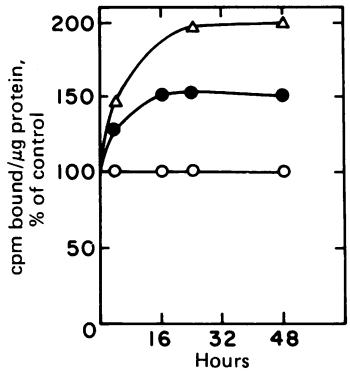

FIG. 3. Time course of the dex-mediated increase in ^{125}I-EGF binding to two strains of HF cells. Quiescent HF 17 and HF 8 cells were prepared in serum-free medium. At various intervals over the following 48 hr, dex was added to a final concentration of 100 ng/ml to triplicate cultures. All the cultures were then incubated at 37° for 60 min with medium containing ^{125}I-EGF (0.5 ng/ml) and were processed as described in *Materials and Methods*. ●, Dex-treated HF 17 cells; △, dex-treated HF 8 cells; ○, untreated control cells. The data for each strain are from separate experiments. The points represent the averages of duplicate measurements.

steroids, because cortisol was about 70% as active as dex on a weight basis, whereas cholesterol and estrogen were inactive over concentrations from 5 ng/ml to 1 μg/ml (Fig. 4). As shown, the glucocorticoid elevation of ^{125}I-EGF binding was noticeable at steroid concentrations of 5 ng/ml, half-maximal at 25 ng/ml, and maximal at 100–250 ng/ml. The dex dose–response curve for enhancement of EGF binding (Fig. 4) was similar to the dex dose–response curve for the enhancement of EGF mitogenesis (Fig. 2), suggesting a relationship between these effects.

It was possible that the increased ^{125}I-EGF binding was caused only by a change in the rate at which the cells bind ^{125}I-EGF. We found, however, that dex-treated cells bound more ^{125}I-EGF at steady state. Dex-treated cells displayed the same relative enhancement of binding whether the binding incubation lasted for 5, 45, or 90 min (Table 1).

As shown in Table 1, the increased binding of ^{125}I-EGF to dex-treated cells also occurred when the binding measurements were carried out at 0° for only 5 min, conditions that minimize EGF internalization. Thus, the dex alteration in EGF binding

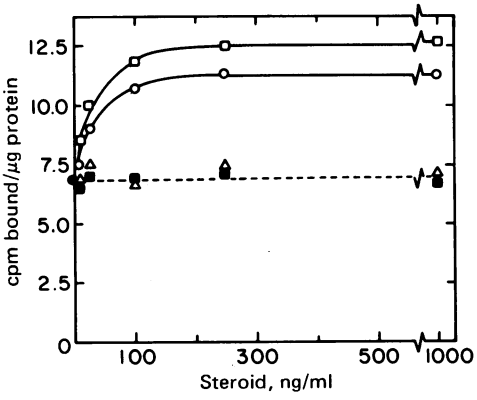

FIG. 4. Binding of ^{125}I-EGF to cells pretreated with various concentrations of dex (□), cortisol (○), estrogen (■), cholesterol (△), or no additions (⊗). Quiescent HF cells incubated in medium without serum for 1 day were given the various steroids at the concentrations shown. After 24 hr the medium was replaced with binding medium containing ^{125}I-EGF at 0.2 mg/ml, and the cultures were incubated at 37° for 90 min. Specific binding was measured; the points represent averages of duplicate measurements.

Table 1. Effect of incubation time and temperature upon ^{125}I-EGF binding to dex-treated and control cells

Binding incubation conditions		^{125}I-EGF bound, cpm/μg protein	
Temperature, °C	Time, min	+Dex	−Dex
37	5	2.66 ± 0.20	1.71 ± 0.12
37	45	14.31 ± 1.01	7.80 ± 0.88
37	90	15.06 ± 1.81	8.08 ± 0.70
0	5	0.80 ± 0.09	0.50 ± 0.06
0	45	5.02 ± 0.23	3.22 ± 0.34

Dex-treated and untreated HF cells were prepared as described in Fig. 3, and the binding measurements were made with duplicate plates as outlined in *Materials and Methods*.

probably resulted from a dex modification of cell surface receptors for EGF.

The amount of enhancement of EGF binding was dependent on the concentration of ^{125}I-EGF in the binding medium (Fig. 5). Dex elevated the cellular capacity to bind ^{125}I-EGF at a low concentration (0.2 ng/ml) by 80%, and the enhancement of binding steadily decreased with increasing concentrations of ^{125}I-EGF in the binding medium (Fig. 5 *A* and *B*).* At ^{125}I-EGF levels that saturated the specific binding capacity of the cells for ^{125}I-EGF, dex-treated cells had only a 10–20% increased ^{125}I-EGF binding capacity. This concentration dependence is most apparent when the percent increase in ^{125}I-EGF binding caused by dex is plotted as a function of ^{125}I-EGF concentration in the binding medium (Fig. 5*C*). These data suggest that dex qualitatively altered the EGF receptors. Dex did not simply increase the number of EGF receptors, because that would have increased the binding of ^{125}I-EGF at all concentrations by a constant proportion. This result is consistent with a model in which dex alters the affinity of the EGF receptors, although other interpretations are also possible (18).

Because this dex alteration of EGF binding might be causally related to the enhanced responsiveness of dex-treated HF cells to EGF stimulation, it was important to determine whether dex altered EGF binding under the actual conditions in which EGF stimulates growth. Stimulation of HF cell division by EGF requires prolonged exposure of the cells to the hormone (11). Therefore, we incubated HF cultures for prolonged periods with EGF with or without dex. We then rinsed the cultures free of EGF and measured their capacity to bind ^{125}I-EGF at 0.5 ng/ml. In agreement with the previous findings of Carpenter *et al.* (17), Fig. 6 shows that within an 8-hr exposure to EGF alone, the HF cells lost ("down-regulated") about 90% of their capacity to bind ^{125}I-EGF. Significantly, cells additionally treated with dex reduced their ^{125}I-EGF binding capacity by only 75%, giving a final capacity twice that of cells treated only with EGF. It is noteworthy that the dex-treated cells maintained this 2-fold greater ^{125}I-EGF binding capacity over a several-day incubation with EGF and dex. Thus, dex caused a significant and stable alteration in HF cell receptors for EGF which occurred coordinately with the dex-mediated increase in cell responsiveness to growth stimulation by EGF.

Modulation by Dex of Insulin and Thrombin Binding. The finding that dex-treated HF cells exhibited an increased ability to bind ^{125}I-EGF led us to examine the effect of dex on HF cell binding of ^{125}I-insulin and ^{125}I-thrombin, proteins that are

* A Scatchard plot analysis of EGF binding to HF cells at physiological temperature is inappropriate, because the amount of ^{125}I-EGF bound to the cells is affected by processes in addition to the association and dissociation reactions between EGF and its receptor (17).

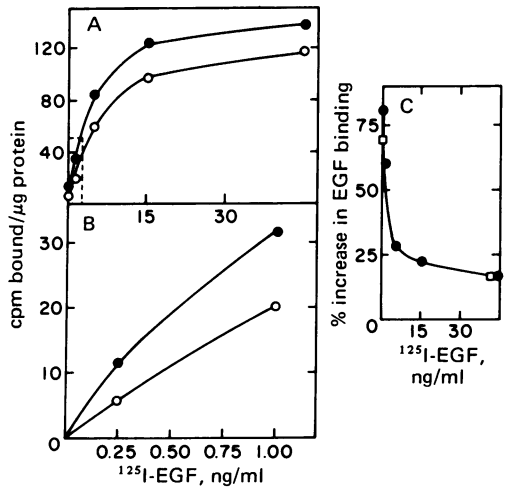

FIG. 5. (*A*) Effect of dex upon the concentration dependence of ^{125}I-EGF binding to HF cells. Quiescent HF cultures were incubated in serum-free medium for 48 hr with (●) or without (○) dex (100 ng/ml). The cultures then were incubated for 60 min at 37° in medium containing the concentrations of ^{125}I-EGF shown, and were processed for radioactivity and protein measurements. Each point represents the average of triplicate measurements. (*B*) Enlargement of low-concentration portion of *A*. (*C*) Percentage increase in ^{125}I-EGF binding capacity caused by dex, represented as a function of ^{125}I-EGF concentration. The closed symbols represent data from the experiment in *A* averaged with another identical experiment. The open symbols represent data from binding measurements carried out for 60 min at 0°.

structurally different from EGF. Table 2 shows that the dex-treated cells exhibited altered abilities to bind ^{125}I-insulin and ^{125}I-thrombin. The dex-treated cells displayed an increased capacity to bind a physiological concentration of ^{125}I-insulin (Table 2). On the other hand, the ability of the dex-treated cells to bind ^{125}I-thrombin was reduced by half (Table 2). These results suggest that the effect of dex on ^{125}I-EGF binding occurs as part of a far-reaching dex-mediated change in the cell surface which alters the receptors for a number of polypeptides (see *Discussion*).

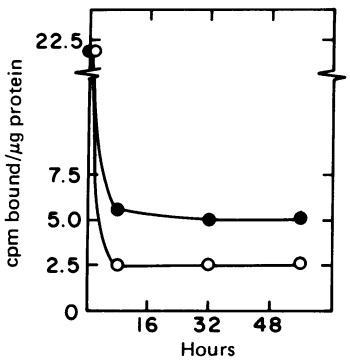

FIG. 6. Time courses of the loss of EGF binding capacity of HF cells exposed to EGF (○), or EGF plus dex (●). At various intervals over a 56-hr period, quiescent HF cells in medium containing 0.1% bovine serum albumin but no serum were given EGF at 20 ng/ml with or without dex at 100 ng/ml. All of the cultures were then washed three times in phosphate-buffered saline to remove the nonlabeled EGF. Washing the cells this way, or additionally incubating them in phosphate-buffered saline at 37° for 20 min, allowed the cells to bind equivalent amounts of ^{125}I-EGF. The cultures were incubated in medium containing ^{125}I-EGF (0.2 ng/ml) for 90 min at 37°, and the bound radioactivity was determined. The points represent averages of triplicate measurements.

Table 2. Effect of dex on the ability of HF cells to specifically bind ^{125}I-insulin and ^{125}I-thrombin

^{125}I-Protein	^{125}I-Protein bound, fmol/mg cell protein	
	+Dex	−Dex
^{125}I-Insulin	1.21 ± 0.15	0.63 ± 0.14
^{125}I-Thrombin	2.00 ± 0.33	4.27 ± 0.43

Quiescent HF cells were either incubated with dex at 10 ng/ml for 2 days and measured for ^{125}I-insulin binding, or incubated with dex at 100 ng/ml for 2 days and measured for ^{125}I-thrombin binding. The specific binding ^{125}I-insulin or ^{125}I-thrombin was measured with duplicate plates.

Both insulin and thrombin initiate the division of some cells (19, 20). This prompted us to determine if dex modulated the action of these molecules on HF cells. We found that HF cell division was not stimulated by insulin, and was only moderately initiated by thrombin. A maximally effective dose of 4 μg of thrombin per ml increased HF cell number by only 20% after 5 days. With this small stimulation, it was not possible to determine if dex modulated thrombin action.

DISCUSSION

Glucocorticoids cause many of their diverse effects by modulating cell responses to other hormones (21–23). The mechanism of these "permissive" actions is not understood. The present results have shown that the synthetic glucocorticoid dex sensitizes quiescent HF cells to the mitogenic action of EGF. Additionally, we have discovered that dex increases the ability of HF cells to bind ^{125}I-EGF. Dex elevated the binding of physiological levels (24) of EGF under the same conditions in which dex increased the mitogenic action of EGF on the cells. The dex dose–response curve for enhancement of EGF binding was similar to the dex dose–response curve for the enhancement of EGF mitogenesis, suggesting a relationship between the two effects. The kinetics of the dex alteration of EGF binding and the persistence of this effect in cells in which binding was down-regulated by long-term exposure to EGF are both consistent with the possibility that the alteration in EGF receptors is related to the enhancement of EGF action. It is noteworthy that Gospodarowicz has reported that dex markedly potentiates the action of fibroblast growth factor (FGF) on 3T3 cells (2). It will be of interest to learn whether the binding of EGF to 3T3 cells is enhanced by dex treatment.

Our finding that dex increased the ability of HF cells to respond to the mitogenic action of EGF at first seemed inconsistent with a report by Carpenter and Cohen (11) that dex had no effect on EGF stimulation of [^{3}H]thymidine incorporation. However, measurement of thymidine incorporation appears not to provide a valid measure of cell proliferation in this case, because dex in the absence of EGF inhibits thymidine incorporation into HF cells (ref. 25, and our unpublished results). In agreement with the observation of Carpenter and Cohen, we found that in the presence of EGF, dex-treated and untreated cells incorporated [^{3}H]thymidine similarly.

HF cells treated with dex exhibited altered abilities to bind not only ^{125}I-EGF, but also ^{125}I-thrombin and ^{125}I-insulin. In the case of ^{125}I-EGF the dex alteration of binding probably reflected an alteration in the cell surface receptors for EGF, because the increased binding of ^{125}I-EGF was observed in measurements carried out at 0° for short times to inhibit EGF internalization. It is likely that the altered binding of ^{125}I-insulin and ^{125}I-thrombin was also caused by changes in cell surface receptors, because these measurements were carried out at room

temperature for only 30 min. (Our unpublished results indicate that these growth factors bind predominately to the cell surface under these conditions.) Thrombin, insulin, and EGF do not compete with each other for binding (ref. 26, and our unpublished results), indicating that they bind to different receptors. Together, these observations suggest that dex causes a far-reaching change in the surfaces of HF cells. It is interesting to consider the possibility that a glucocorticoid modification of the cell surface receptors for a variety of proteins causes the large number of permissive actions of glucocorticoids. There are other indications that glucocorticoids affect cell surfaces. Glucocorticoids increase cell adhesiveness (27), sialic acid content (28), and agglutinability by concanavalin A (1).

Although little is understood about how growth factor signals are transmitted, it is worth considering how the dex alteration in EGF receptors described in *Results* might be involved in increasing the responsiveness of the cells to EGF. There are two ways that dex could modulate cell growth by altering growth factor receptors. One is by changing the ability of the cell to interact with the growth factor. Alternatively, dex could cause a qualitative change in the receptors that alters their mitogenic potency when occupied by the growth factor. Some of the present observations seem more consistent with the latter possibility. The effect of dex on the concentration dependence of EGF binding suggests that dex does not simply increase the number of EGF receptors, but causes a qualitative change in the receptors (possibly involving a change in affinity or cooperativity). Moreover, dex increased the mitogenic response of HF cells to EGF by the same percentage regardless of the EGF dose, but substantially increased the binding of EGF only at the lower mitogenic levels of the growth factor. Consequently, it seems plausible that dex could potentiate EGF action by changing the mitogenic activity of the EGF–receptor complexes, rather than by increasing EGF binding. Thus, it will be important in future studies to examine the effects of dex on other EGF receptor properties that might influence the ability of the receptors to transmit a hormonal signal. These include receptor mobility, turnover, processing, and second messenger production.

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