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Control of insulin receptor level in 3T3 cells: Effect of insulin-induced down-regulation and dexamethasone-induced up-regulation on rate of receptor inactivation

(heavy-isotope density-shift technique)

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ABSTRACT **Chronic exposure of 3T3 mouse fibroblasts to insulin or to the glucocorticoid dexamethasone induces down-regulation and up-regulation, respectively, of cell-surface and total cellular insulin binding capacity. Both processes are reversed upon withdrawal of the inducer. Scatchard analysis of insulin binding for receptors in the down- and up-regulated states indicates that the changes in binding capacity result primarily from alterations in insulin receptor level. That these alterations in total receptor level are due to changes in cell-surface receptor level is indicated by the fact that the level of trypsin-insensitive, presumably intracellular, insulin binding sites does not change appreciably upon down- and up-regulation. The effects of insulin-induced down-regulation and dexamethasone-induced up-regulation on the rates of insulin receptor synthesis and decay were assessed by the heavy-isotope density-shift technique. Cells were shifted to medium containing heavy (^{2}H, ^{13}C, ^{15}N) amino acids and, at various times after the shift, light and heavy receptors solubilized from total cellular membranes were resolved by isopycnic banding on density gradients and then quantitated. It was demonstrated that the insulin- and dexamethasone-induced alterations in insulin receptor level were due entirely to changes in the rate constant for receptor inactivation. The decrease in the first-order rate constant for receptor decay caused by dexamethasone is unexpected in view of the known action of steroid hormones in the induction of the synthesis of specific proteins.**

The insulin receptor is a dynamic cellular protein whose equilibrium concentration in the plasma membrane can be altered by physiological perturbants. The effect of chronic treatment of cells with insulin on cell surface insulin receptor level is well documented. This phenomenon, known as ligand-induced receptor down-regulation, has been demonstrated *in vivo* with circulating monocytes (1) and fat cells (2) from obese humans and with cultured human IM-9 lymphocytes (3–6), rat hepatocytes (7), and chicken hepatocytes (8). The reciprocal effect—i.e., an increase in cell surface insulin binding capacity—is observed upon removal of insulin from a down-regulated system (3) or by induction of differentiation of 3T3-L1 preadipocytes into adipocytes (9–11).

Changes in the level or affinity of hormone receptors can also be induced by heterologous hormones (6). Glucocorticoids have been shown to affect cellular levels of prolactin receptors (12), epidermal growth factor receptors (13), and insulin receptors (14–18). It was of interest, therefore, to determine the mechanism by which glucocorticoids induce an increased insulin binding capacity of cells.

In this report the effects of two antagonistic perturbants of insulin receptor level in 3T3 cells are described: the effect of insulin, which causes down-regulation, and that of dexamethasone, which causes up-regulation of insulin receptor level. The heavy isotope density-shift technique (8, 19, 20) was employed to determine whether down- and up-regulation induced by these agents results from changes in the rates of receptor synthesis or inactivation.

EXPERIMENTAL PROCEDURES

3T3-C2 cells were cultured without insulin as previously described (20), except that at 2 days after confluence fetal calf serum (GIBCO) was used in place of calf serum for 4 days (two feedings). At 6 days after confluence, insulin (Elanco, Indianapolis, IN) or dexamethasone (Sigma) was added at the concentration and for the period of time noted in the figures.

Insulin was iodinated by the chloramine-T method and purified as described by Gavin *et al.* (3), to an average specific activity of ≈ 1 μCi/pmol (1 Ci = 3.7×10^{10} becquerels); the ^{125}I-labeled insulin (^{125}I-insulin) was 97–99% precipitable in 10% (wt/vol) trichloroacetic acid. Immediately before use, an aliquot of the ^{125}I-insulin was further purified by gel filtration on Sephadex G-50 (21). Prior to determination of insulin binding capacity, cell monolayers in 6-cm culture dishes were subjected to the ligand debinding protocol (21); ^{125}I-insulin binding to cell-surface or total cellular insulin receptors was then determined (21). The debinding protocol was judged efficient in removing insulin from cell monolayers by demonstrating that no cell-associated, trichloroacetic acid-precipitable radioactivity was detectable after imposing this procedure on cell monolayers previously exposed to 1.7 μM ^{125}I-insulin (3×10^{6} cpm/nmol) for 24 hr. Total cellular insulin receptor represents receptor quantitatively extracted with Triton X-100 from total cellular membranes and isopycnically banded on CsCl density gradients (21).

Light and heavy receptors in the Triton X-100 extracts of total cellular membranes were isopycnically banded on CsCl density gradients and quantitated as described earlier (20, 21), except that insulin binding capacities of the gradient fractions were measured with 0.6 nM ^{125}I-insulin by using the polyethylene glycol precipitation method (22) as modified by Krupp and Livingston (23). Nonspecific insulin binding was determined by using 3 μM unlabeled insulin. Isopycnic banding of the insulin receptor was performed as described (20, 21).

The density-shift experiments were performed as previously reported (20, 21) with some modification. Heavy amino acids were isolated by ion-exchange chromatography after acid hydrolysis of delipidated *Chorella pyrenoidosa* cells that had been isotopically labeled and 99 mol % enriched in ^{13}C, ^{15}N, and ^{2}H (Los Alamos Scientific Laboratory, Los Alamos, NM). To 90 ml of amino acid-free culture media were added 150 mg of heavy

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amino acids, 10 ml of undialyzed fetal calf serum, 0.8 mg of tryptophan, 3 mg of cystine, 6 mg of glutamine, and insulin or dexamethasone as dictated by the experiment. The dense amino acids were dissolved in phosphate-buffered saline and sterilized by filtration prior to use.

To inactivate cell-surface insulin receptors, cell monolayers were subjected to the ligand debinding protocol to remove insulin and serum factors and were then incubated for 20 min at 37°C with trypsin (twice recrystallized, Worthington) at 20 μg/ml in phosphate-buffered saline, pH 7.4. After incubation, the trypsin solution was aspirated and replaced with 2 ml of ice-cold soybean trypsin inhibitor (60 μg/ml, Sigma) in Krebs–Ringer phosphate buffer, pH 7.4, containing 1% bovine serum albumin. Monolayers were then washed four times with 3 ml of the Krebs–Ringer buffer/serum albumin at 4°C and surface or total soluble receptor binding assays were performed as described above.

RESULTS AND DISCUSSION

Insulin-Induced Down-Regulation and Dexamethasone-Induced Up-Regulation of Insulin Receptor Level. Like certain other cell types, 3T3-C2 mouse cells undergo insulin-induced down-regulation of insulin receptor level. In addition, 3T3-C2 cells can be induced to up-regulate insulin receptor level by exposure to dexamethasone. The concentration dependences of these changes in cell-surface ^{125}I-insulin binding capacity by cell monolayers exposed to insulin for 12 hr or dexamethasone for 24 hr are shown in Fig. 1. Chronic treatment with insulin produced maximal down-regulation at between 1 and 10 nM, whereas chronic exposure to dexamethasone caused maximal up-regulation at 10–100 nM.

The kinetics of insulin-induced down-regulation and dexamethasone-induced up-regulation are shown in Fig. 2. After the addition of insulin, cell-surface insulin binding capacity decreases to a new equilibrium level, 50% lower than that of untreated cells, in 10–12 hr (Fig. 2*A*); the half-life of this process is approximately 2 hr. Up-regulation induced by dexamethasone is a much slower process (Fig. 2*C*); after a lag of 3–6 hr, surface binding rises, reaching a plateau level about 48 hr after addition of dexamethasone. Cell-surface insulin binding capacity doubles with a half-time of about 24 hr.

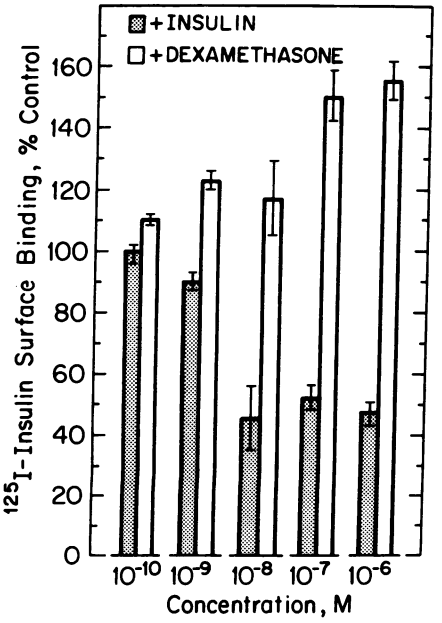

FIG. 1. Dependence of down-regulation and up-regulation of cell-surface insulin binding capacity on insulin and dexamethasone concentration, respectively. Confluent cell monolayers were incubated for 12 hr in insulin-containing medium or 24 hr in dexamethasone-containing medium. Cell monolayers were subjected to the ligand-debinding procedure, after which specific ^{125}I-insulin binding capacity at 0.6 nM ligand was determined. Untreated control cell monolayers had a specific insulin binding capacity of 10 fmol per 6-cm dish.

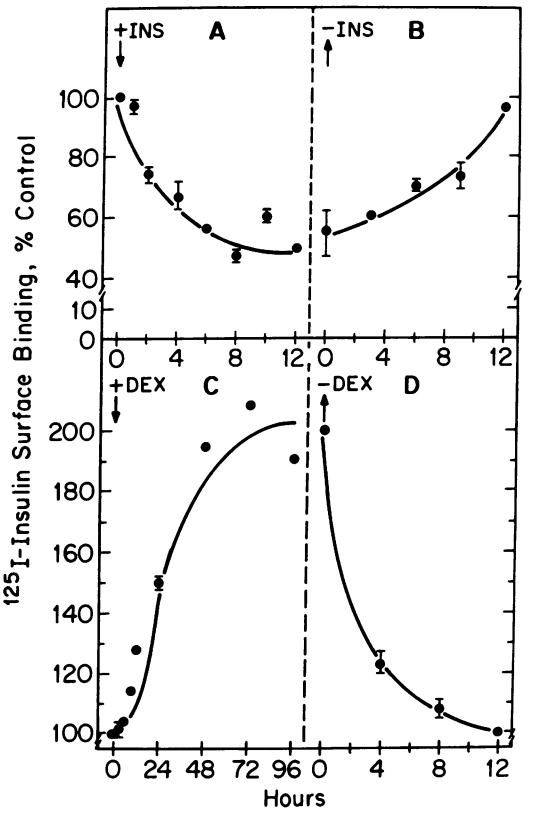

FIG. 2. Kinetics of insulin-induced down-regulation and dexamethasone-induced up-regulation and reversal after inducer withdrawal. ^{125}I-Insulin binding (at 0.6 nM) was determined at various times after the addition (*A*) and removal (*B*) of 1.7 μM insulin or after the addition (*C*) and removal (*D*) of 1.3 μM dexamethasone.

The effects of both insulin and dexamethasone on cell-surface binding are reversible. When insulin-treated monolayers were washed five times with insulin-free culture medium (20-min incubations at 37°C between the last three washes), 12 hr after the initial exposure to insulin (Fig. 2*A*), surface insulin binding capacity returned to initial control levels within 12 hr (Fig. 2*B*). The half-life for recovery is approximately 8 hr. Surface binding of dexamethasone-treated cells, subjected to the same debinding protocol as above (but with dexamethasone-free medium) 48 hr after the initial exposure to dexamethasone, also returned to control levels within 12 hr (Fig. 2*D*) but with a half-life of only 2 hr.

The changes in surface binding accompanying insulin or dexamethasone treatment are due primarily to alterations in the number of insulin binding sites and not to changes in the affinity of the receptor for insulin. Scatchard analysis of the binding isotherms of receptors from control and dexamethasone- and insulin-treated cells (Fig. 3) reveals similar high-affinity dissociation constants (0.41–0.45 nM) but appreciable changes in the number of insulin binding sites per cell. The increase in total surface sites expressed after exposure of cells to dexamethasone is consistent with the 2-fold increase in surface binding observed at 48 hr in Fig. 2*C*. Similarly, the insulin-induced decrease in cell-surface insulin binding (Fig. 2*A*) at 12 hr is consistent with

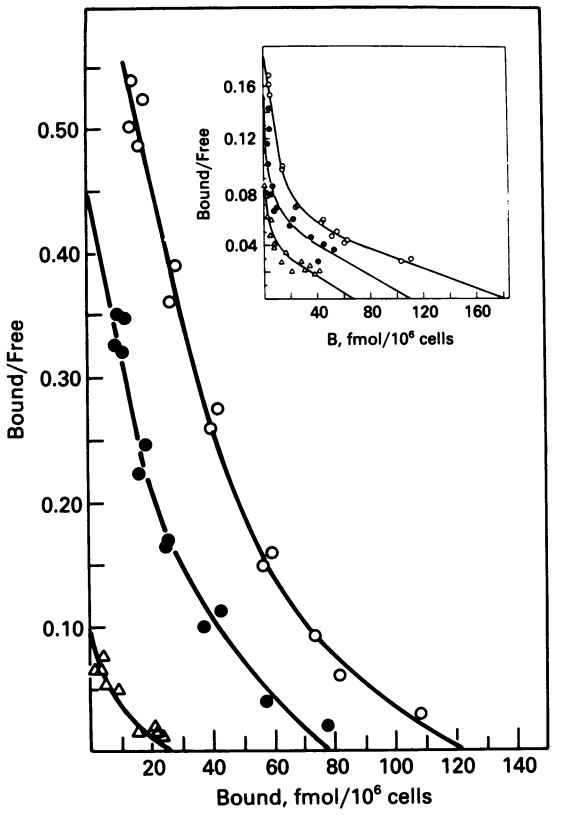

FIG. 3. Scatchard plots of cell-surface and total detergent-extractable isopycnically banded (*Inset*) insulin receptors from insulin-induced down-regulated (△), dexamethasone-induced up-regulated (○), and untreated control (●) cells. Confluent monolayers of 3T3-C2 cells were down-regulated for 12 hr with 1.7 μM insulin or were up-regulated for 24 hr with 1.3 μM dexamethasone. After the cell monolayers were subjected to the ligand-debinding procedure, ^{125}I-insulin binding by cell monolayers or isopycnically banded receptor solubilized with Triton X-100 from total cellular membranes was determined.

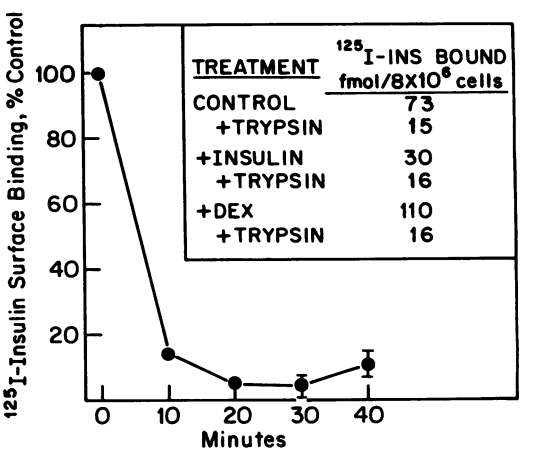

FIG. 4. Loss of cell-surface insulin binding capacity by treatment of cell monolayers with trypsin. 3T3-C2 cell monolayers were treated with trypsin at 20 μg/ml and 37°C for the times indicated (or *Inset*, 20 min); the reaction was terminated with trypsin inhibitor at 60 μg/ml followed by washing and subjecting the monolayers to the ligand-debinding protocol. (*Inset*) After insulin-induced down-regulation or dexamethasone-induced up-regulation (see Fig. 2), cell monolayers were either treated or not treated with trypsin; the remaining insulin binding capacity of solubilized total cellular receptors, extractable from total cellular membranes with Triton X-100 and isopycnically banded on CsCl gradients, was determined.

the decrease in the number of total surface receptors per cell derived from Scatchard plots (Fig. 3). Scatchard analysis (Fig. 3 *Inset*) of total cellular insulin binding capacity—i.e., receptors extracted from total cellular membranes with Triton X-100—like cell-surface insulin binding capacity, showed that the changes induced by insulin and dexamethasone were due to changes in number of receptors per cell rather than to alterations in their affinity for insulin. Thus, it can be concluded that the total number of receptors as well as the number of cell-surface receptors decrease during insulin-induced down-regulation and increase upon dexamethasone-activated up-regulation.

From these results, it appeared that the number of intracellular (total minus surface) insulin binding sites remained relatively constant at about 20,000 sites per cell, despite the 2-fold increase or the 2-fold decrease in surface sites. To test this hypothesis, intact cell monolayers were subjected to mild trypsin treatment to proteolyze and inactivate cell-surface receptors. As shown in Fig. 4, >95% of the specific cell-surface insulin binding sites were inactivated within 20 min (Fig. 4). Under these conditions cells remained firmly attached to the culture dish and nonspecific binding did not change.

After a 12- or 24-hr exposure to insulin or dexamethasone, respectively, cell monolayers were treated with trypsin to inactivate cell-surface receptors and the remaining intracellular insulin binding sites were extracted with Triton X-100 and quantitated. While chronic exposure to insulin or dexamethasone had caused down- or up-regulation, respectively (Fig. 3), of both total cellular and cell-surface receptors, the number of total cellular insulin binding sites resistant to trypsin (presumably intracellular) remained constant (Fig. 4). These results indicate that the decrease or increase in total cellular receptor level during down- or up-regulation, respectively, was due primarily to changes in cell-surface insulin-receptor level.

Effect of Insulin-Induced Down-Regulation and Dexamethasone-Induced Up-Regulation on Rates of Insulin Receptor Synthesis and Decay. The heavy isotope density-shift method (8, 20, 21) was used to determine whether insulin-induced down-regulation and dexamethasone-induced up-regulation resulted from a change in rate of receptor synthesis or decay. After exposure to insulin for 12 hr or dexamethasone for 24 hr, cell monolayers were shifted from normal medium containing light (^{1}H, ^{12}C, ^{14}N) amino acids to medium containing heavy (>95% ^{2}H, ^{13}C, ^{15}N) amino acids. After the density shift, formation of new heavy receptors and concomitant decay of old light receptors were monitored. Light and heavy receptors extracted from total cellular membranes were isopycnically banded on CsCl density gradients and quantitated (see *Experimental Procedures*) at 0, 3, 6, 9, 13, and 18 hr after the density shift. Typical banding profiles for light and heavy insulin receptors from control, insulin down-regulated, and dexamethasone up-regulated cells before and 13 hr after the shift to "heavy" medium are shown in Fig. 5.

Comparison of the relative amounts of light receptor (represented by the peak areas in Fig. 5 *A*, *C*, and *E*) at 0 hr, just before the density shift, shows that chronic exposure of the cells to insulin or dexamethasone caused the expected down-regulation or up-regulation, respectively, of total cellular insulin receptor level. Thirteen hours after the density shift, however, the size of the light receptor peak, relative to the heavy receptor peak, was markedly lower in insulin down-regulated cells (Fig. 5*D*) and higher in dexamethasone up-regulated cells (Fig. 5*F*) than in control cells (Fig. 5*B*), suggesting that corresponding changes in receptor decay rate had occurred. From the integrated areas of the light and heavy receptor peaks in the density gradients shown in Fig. 5, progress curves for the decay of light

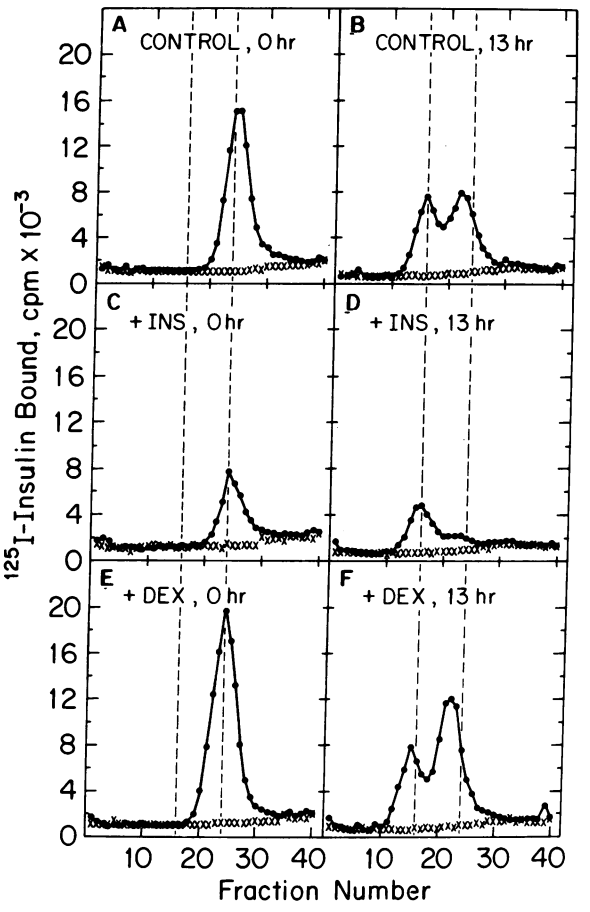

FIG. 5. Isopycnic banding of solubilized receptors from control (*A*, *B*), insulin-induced down-regulated (*C*, *D*), and dexamethasone-induced up-regulated (*E*, *F*) cells before (*A*, *C*, and *E*) and 13 hr after (*B*, *D*, and *F*) the addition of medium containing heavy amino acids. 3T3-C2 cells were down-regulated for 12 hr or up-regulated for 48 hr as in Fig. 2 prior to the shift to heavy amino acids. At 0, 3, 6, 9, 13, and 17 hr after the shift, cellular receptors were extracted from total cellular membrane and banded isopycnically on CsCl density gradients, after which light and heavy receptors were quantitated; only the gradients at 0 and 13 hr are shown. Light receptor bands at fractions 23–24 and heavy receptor bands at fractions 15–16.

receptor and the formation of newly synthesized heavy receptor were generated (results not shown). These kinetic plots showed that chronic exposure of 3T3-C2 cells to insulin promotes more rapid decay of total cellular insulin receptors, whereas dexamethasone slows this process.

The reciprocal effects of insulin and dexamethasone on total cellular insulin receptor level in 3T3-C2 cells can be accounted for almost entirely by changes in the rate constant for receptor decay. As shown in Fig. 6, the $t_{1/2}$ for insulin receptor decay in control cells of 10.2 hr was shortened to 4.2 hr in cells down-regulated by insulin and lengthened to 18 hr in cells up-regulated by dexamethasone. From the results summarized in Table 1, it is evident that the rate of insulin receptor synthesis is essentially the same whether experimentally determined from the limiting slopes of the progress curves for heavy receptor synthesis (190–220 sites per cell per hr at 0.6 nM insulin) or calculated from $R_t = k_S/k_D$ (220–260 sites per cell per hr at 0.6 nM insulin, Table 1). It appears, therefore, that the control of insulin receptor level by insulin and dexamethasone in 3T3-C2 cells is exerted at the level of receptor decay, rather than receptor synthesis. With respect to dexamethasone, this is an unexpected mechanism of action for a steroid hormone. Steroid–receptor interactions with nuclear chromatin are known to result in concomitant increases in specific protein synthesis (24), not inactivation as demonstrated here. It is possible, however, that dexamethasone induces the synthesis of a protein that inhibits the insulin receptor inactivation process.

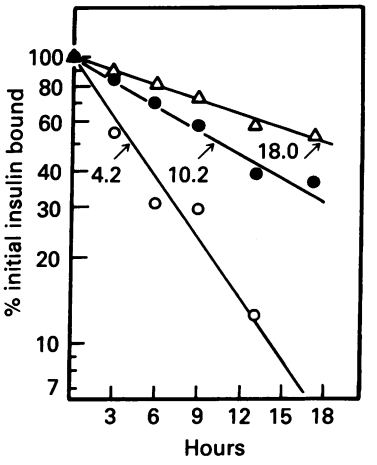

FIG. 6. Semilogarithmic plots for the decay of light receptor in control (●), insulin-induced down-regulated (○), and dexamethasone-induced up-regulated (△) 3T3-C2 cells. Cells were subjected to the heavy-isotope density-shift protocol (Fig. 5) and analyzed as described in *Experimental Procedures* and ref. 21; data points represent average values from two experiments. The $t_{1/2}$ values, in hr, are given for each curve.

An earlier study with cultured chicken hepatocytes (8) demonstrated that insulin-induced down-regulation of cell-surface receptors in this cell system resulted in conservation of the total number of cellular receptors, rather than a decrease as shown here, with no effect on the rate constant for receptor decay. This difference may be due to a cell-type-dependent change in a rate-determining step—i.e., a kinetic difference—in the pathway leading to receptor decay or in a competing pathway of receptor metabolism. Results are not yet available to distinguish between these possibilities.

Table 1. Effect of insulin-induced down-regulation and dexamethasone-induced up-regulation on the kinetic constants for insulin receptor synthesis and decay

Treatment	Insulin receptor level,* sites/cell	$t_{1/2}$,† hr	k_D,† hr^{-1}	k_S, sites/cell hr^{-1} Measured‡	k_S, sites/cell hr^{-1} Calculated§
Control	3,200	10.2	0.068	190	220
Down-regulated (+ insulin)	1,380	4.2	0.164	220	230
Up-regulated (+ dexamethasone)	6,800	18.0	0.038	210	260

* Sites per cell were measured at 0.6 nM ^{125}I-insulin with soluble receptor from total cellular membranes banded isopycnically on CsCl density gradients under conditions identical to those employed to determine the kinetic constant for synthesis, k_S, and the kinetic constant for decay, k_D. Because the binding isotherms on Scatchard plots for receptors from control, down-regulated, and up-regulated cells are parallel, sites per cell at 0.6 nM are a measure of the total number of high-affinity receptors.

† Results from Fig. 6.

‡ Determined from the limiting slopes of progress curves for heavy receptor formation and sites per cell at 0.6 nM insulin.

§ Calculated from $R_t = k_S/k_D$, in which R_t is sites per cell at 0.6 nM insulin.

The density-shift method used in this investigation has identified receptor inactivation as the rate-limiting step modulated by insulin-induced down-regulation and dexamethasone-induced up-regulation. This step is of key importance in the pathway leading to the degradation of receptor protein because it is the point at which receptor function is lost. Several lines of evidence suggest that the site of inactivation of the insulin receptor may be at the plasma membrane, rather than intracellularly as generally believed. In 3T3-C2 cells most of the receptor is located on the cell surface and is subject to inactivation by trypsin (Fig. 3 *Inset*). Whereas cell-surface receptor level differs widely (4-fold) in down- and up-regulated cells (Fig. 2), the insulin binding capacity of the trypsin-insensitive, presumably intracellular, compartment is invariant (Fig. 4 *Inset*). The constancy of the level of insulin binding in this pool suggests that receptor in this compartment is derived from newly synthesized receptor and, therefore, would not be affected by perturbants—e.g., insulin or dexamethasone, that act distally on the receptor inactivation step. Were the rate-limiting receptor inactivation step (affected by insulin and dexamethasone) to occur intracellularly, far greater fluctuations in the level of receptor in the trypsin-insensitive pool would have been expected in the down- versus up-regulated states. Consistent with this proposal, we have recently shown (25) that insulin receptor is inactivated in a chloroquine-insensitive nonlysosomal cell compartment, whereas, its ligand is degraded in the chloroquine-sensitive lysosomal compartment. Further work will be required to establish how insulin receptor inactivation is promoted by insulin and is attenuated by glucocorticoids.

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