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Homologous down-regulation of the insulin receptor is associated with increased receptor biosynthesis in cultured human lymphocytes (IM-9 line)

(proreceptor/translation/degradation)

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ABSTRACT **Cultured IM-9 lymphocytes were preincubated with 1 μM insulin, a condition resulting in a 56% reduction in cell surface insulin receptors. Cellular proteins were then metabolically labeled, and the radioactivity incorporated into the insulin proreceptor and receptor mature subunits was measured over a 4-hr chase period. As early as 30 min of chase, incorporation into the proreceptor was 28 ± 6% higher in down-regulated cells than in control cells (mean ± SEM, $P < 0.05$). By 1 hr of chase, the difference reached 41 ± 14% for the proreceptor and 84 ± 28% for the α subunit ($P < 0.01$); values returned to normal by 2 hr. At 4 hr of chase, labeling of the α subunit of down-regulated cells was diminished 36 ± 9% below control ($P < 0.05$). The increased biosynthetic rate of the proreceptor was more prominent when the chase medium contained 25 μM monensin, an inhibitor of processing of the proreceptor into mature subunits. Similar effects occurred whether [^{3}H]mannose or [^{3}H]lysine was used as biosynthetic marker. The effect was specific for the insulin receptor. These data demonstrate that insulin receptor homologous down-regulation is associated with increased proreceptor biosynthesis and processing into mature subunits. This might represent a cellular mechanism compensating for insulin-induced receptor loss.**

The insulin receptor is a highly regulated cell membrane glycoprotein. Homologous receptor down-regulation was initially described for insulin in IM-9 cultured lymphocytes by Gavin *et al.* (1) and has subsequently been reported in most cell types for insulin, other polypeptide hormones, and growth factors (2–4). Kasuga *et al.* (5) demonstrated that in IM-9 cells, the insulin receptor lifetime was greatly shortened under conditions of down-regulation; the decrease in biosynthetic labeling seen over an 8-hr incubation with 1 μM insulin was explained entirely by the increased receptor degradation. Heavy-isotope density-shift studies done on down-regulated chicken hepatocytes (6) and 3T3-C2 mouse fibroblasts (7) did not show changes in receptor biosynthesis. Similar results were obtained with insulin-treated 3T3-L1 adipocytes (8), though the ability of these cells to down-regulate insulin receptors in response to insulin is still controversial (9).

Receptor-mediated endocytosis is a mechanism to remove cell surface receptors from the plasma membrane and has been suggested to be the primary mechanism by which homologous hormone down-regulation occurs (3, 4). Though receptor-mediated endocytosis of cholesterol induces down-regulation by decreasing transcription of the low density lipoprotein receptor gene and biosynthesis of the receptor (10), there is no evidence that the endocytosis of the insulin receptor and its biosynthesis are linked. IM-9 cells have been shown to internalize insulin to a limited extent and to exhibit little (if any) insulin receptor recycling (11). Receptor homeostasis, therefore, is primarily a function of receptor degradation and synthesis. The mature α and β subunits (apparent molecular mass 135 kDa and 95 kDa, respectively) of the insulin receptor are synthesized from a single-chain precursor of 190 kDa (12–15). This proreceptor is believed to be the earliest product of mRNA translation (12–15) and is processed prior to insertion into the plasma membrane (16). Its measurement, therefore, should primarily reflect receptor biosynthesis and not be influenced by changes in cell surface receptor degradation. We recently described (17) a technique to study the kinetics of biosynthesis and processing of this component and were able to show that at least one agent, hydrocortisone, accelerated proreceptor biosynthesis. In the present study we have examined directly the effect of insulin-induced down-regulation on proreceptor synthesis and processing. The results show that homologous insulin receptor down-regulation is associated with a 40–60% increase in proreceptor biosynthesis and an increased processing of the proreceptor, resulting in an increased synthesis of mature receptor.

METHODS

Cell Culture and Insulin Treatment. Cultured human IM-9 lymphocytes were grown at 37°C in RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 25 mM Hepes (Sigma) and 10% (vol/vol) fetal bovine serum (Biofluids). Once they reached their plateau of growth (1.5–2 × 10^6 cells per ml), cells were resuspended at 2.3 × 10^6 per ml in fresh medium containing 5 mM glucose (sugar labeling) or 150 μM lysine (amino acid labeling) and preincubated for 6 hr unless noted otherwise, in the presence or absence of 1 μM pork insulin (Elanco, Indianapolis, IN).

Pulse–Chase Labeling. Pulse–chase labeling studies were performed as described (13, 17) and were carried out in parallel in treated and control cells. At the end of the preincubation period, cells were washed twice with phosphate-buffered saline (pH 7.4) (PBS; Biofluids), incubated for 30 min in medium depleted of glucose or lysine, and concentrated 10-fold to 2.5 × 10^7 cells per ml. Then either D-[2-^{3}H(N)]mannose (specific activity, 25 Ci/mmol) or L-[4,5-^{3}H(N)]lysine hydrochloride (specific activity, 97 Ci/mmol) (New England Nuclear) was added (0.5 mCi/ml of medium; 1 Ci = 37 GBq). After a 20-min incubation (the pulse), the cells were quickly washed three times with PBS, resuspended at 1.5–2 × 10^6 per ml in complete medium containing either 2 mM mannose or 4 mM lysine hydrochloride; equal fractions were incubated (chased) for 0.5, 1, 2, and 4 hr unless noted otherwise. In some experiments, 25 μM monensin (Calbiochem) was added to the chase medium. Until completion of the chase, all the washing buffers and culture media of the cells preincubated with insulin also contained 1 μM insulin. All incubations were carried out at 37°C.

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Cell Solubilization. At the end of each chase period, cells were counted using a Levy–Hausser chamber. Their viability was estimated by trypan blue exclusion to be always >90%. They were washed twice with cold PBS, collected by centrifugation, and solubilized in 150 mM NaCl/50 mM Hepes buffer (pH 7.6) containing 1% (vol/vol) Triton X-100, 2 mM phenylmethylsulfonyl fluoride (Sigma), and 0.05% α_2-macroglobulin (Boehringer Mannheim), for 35 min on ice with continuous stirring. Soluble material was separated by ultracentrifugation (200,000 × g, 4°C, 1 hr). Aliquots (10 μl) were taken both before ultracentrifugation and from the soluble extract for measurement of total and trichloroacetic acid-precipitable radioactivity.

Receptor Immunoprecipitation and Electrophoresis. The solubilized insulin receptors were immunoprecipitated as described (5, 17): the soluble extract was divided in two equal aliquots and incubated overnight at 4°C with human serum (titer 1:100) derived either from a patient (Bd) with spontaneous antireceptor autoantibodies or from a normal individual (24). The immune complexes were adsorbed with 40% Pansorbin (Calbiochem), washed, and dissociated by boiling for 5 min in 2% $NaDodSO_4$/0.1 M dithiothreitol/0.5 M mercaptoethanol/0.02% bromophenol blue/10 mM phosphate buffer, pH 7. The receptors were recovered as the supernatant of a 5-min centrifugation at 15,000 × g and kept frozen at −70°C until electrophoresis.

$NaDodSO_4$ (0.1%)/polyacrylamide (7.5%) gel electrophoresis was performed by a modification of the procedure of Laemmli (18), as described (5, 17). Gels were treated with an autoradiographic enhancer (EN^{3}HANCE, New England Nuclear), dried, and exposed for 2–3 weeks at −70°C on Kodak X-Omat AR film (Eastman Kodak). The radioactivity in the bands of interest was quantitated by elution of protein from the sliced bands overnight at 37°C in Econofluor (New England Nuclear) containing 3% Protosol (New England Nuclear) and measurement of eluted radioactivity in a scintillation counter. Results represent receptor-specific radioactivity—i.e., counts in the insulin receptor band minus counts in the same area from the control (nonimmune serum) lane.

RESULTS

Pulse–Chase Labeling of the Insulin Proreceptor and Receptor Mature Subunits Under Conditions of Homologous Down-Regulation. Cells (≈2 × 10^6 per ml) were preincubated for 6 hr in the presence or absence of 1 μM insulin. After a 20-min pulse with [^{3}H]mannose, cells were "chased" for 0.5–4 hr by incubation with excess nonradioactive mannose. These conditions reliably down-regulate cell surface insulin receptors of IM-9 lymphocytes (1, 5); in the present study, we confirmed that these conditions of insulin preincubation lead to a 56% decrease in insulin binding. In cells preincubated with insulin, the hormone was present throughout the pulse–chase. Radioactivity incorporated into the 190-kDa proreceptor reached a peak at 1 hr and declined to almost undetectable levels thereafter (Fig. 1). At 30 min and 1 hr, the incorporation of [^{3}H]mannose into insulin-treated cells was modestly increased over control cells. The incorporation into the mature 135-kDa and 95-kDa subunits increased progressively throughout the chase. At 30 min and 1 hr, the incorporation was higher in insulin-treated than in control cells. By 2 hr, it was back to control levels, and it decreased below control by 4 hr. Quantitative measures of the changes were obtained by elution of the bands of interest in Protosol, followed by liquid scintillation counting. In insulin-treated cells, incorporation into the 190-kDa proreceptor was increased 48% over control at 0.5 hr and 38% at 1 hr and was back to control values at 2 hr (Fig. 2 *Upper*). The mature 135-kDa subunit of insulin-treated cells demonstrated higher labeling than in the control at the early time points (44% and 70% increase at 0.5 and 1 hr, respectively) and a 46%-decreased incorporation at 4 hr (Fig. 2 *Lower*). Radioactivity incorporated into the mature 95-kDa subunit was not quantitated because of unreliably low values.

In order to validate the modest increase in the insulin proreceptor and newly synthesized mature α subunit, a total of eight pulse–chase experiments similar to the one whose results are shown in Figs. 1 and 2 were carried out. In addition to the 6-hr insulin preincubation, 11- and 20-hr preincubations were studied. Since there was considerable variation in the incorporation of radioactivity into cells from one experiment to another, we found that the effect of treatment was best expressed as the mean percent of control (± SEM) for each time point. The incorporation into the 190-kDa precursor was significantly increased over control as early as 0.5 hr after the start of the chase (28 ± 6% over control, $P < 0.05$, $n = 6$) and also at 1 hr (41 ± 14%, $P < 0.01$, $n = 8$). There was no

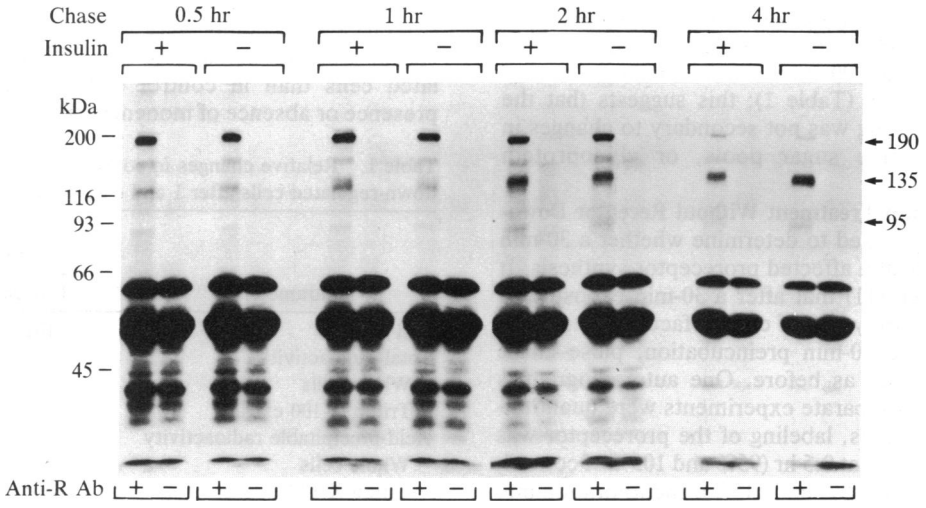

FIG. 1. $NaDodSO_4$/PAGE and autoradiography of insulin receptor components of cultured lymphocytes, pulse–chased with [^{3}H]mannose, in conditions of homologous down-regulation. Cells were preincubated for 6 hr with or without 1 μM insulin (+ or − above the lanes). Glycoproteins were pulse-labeled with [^{3}H]mannose for 20 min and "chased" by incubation in the presence of excess unlabeled mannose for various times. Solubilized extracts of the cells were treated with anti-insulin receptor antiserum (Anti-R Ab +) or normal patient serum (anti-R Ab −). Immunoprecipitates were analyzed by $NaDodSO_4$/PAGE under reducing conditions, followed by autoradiography. Positions and sizes of standards run in parallel are at left. Positions of the proreceptor (190 kDa), α subunit (135 kDa), and β subunit (95 kDa) are indicated at right.

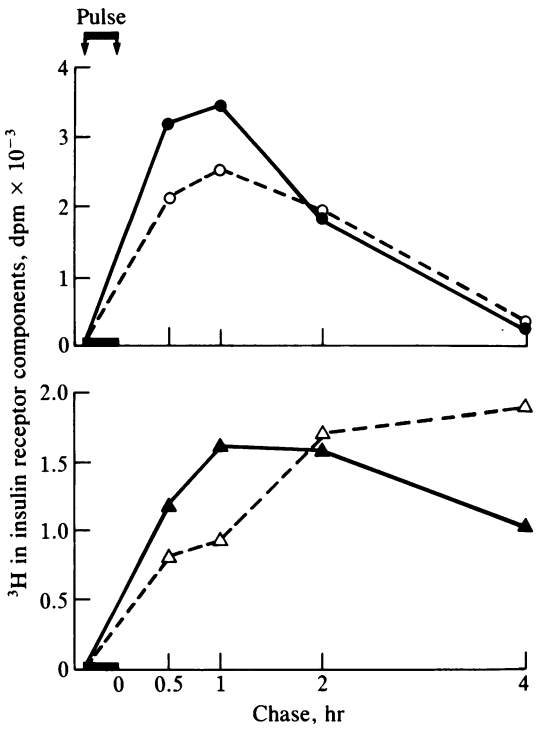

FIG. 2. Quantitative representation of the kinetics of labeling with [^{3}H]mannose of the insulin proreceptor (*Upper*) and the α subunit (*Lower*) of down-regulated (solid line, filled symbols) or control (broken line, open symbols) IM-9 cells. Data were obtained by excising the bands of interest from the gel shown in Fig. 1, eluting with Protosol, and measuring the eluted radioactivity in a scintillation counter.

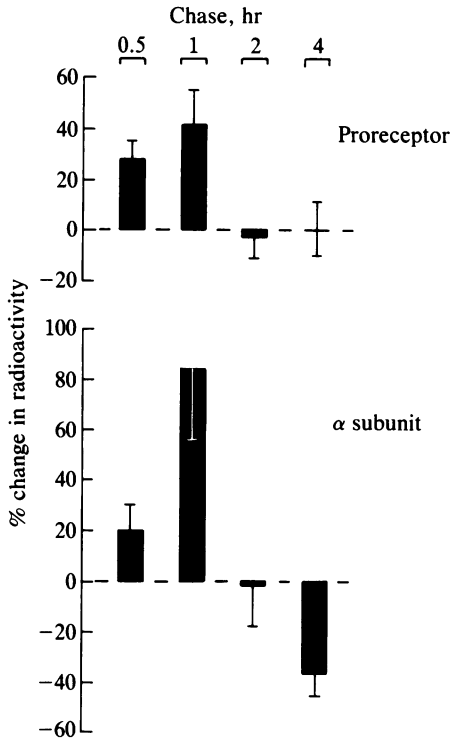

FIG. 3. Kinetics of changes in labeling of 190-kDa insulin proreceptor (*Upper*) and 135-kDa α subunit (*Lower*) during homologous down-regulation. A total of eight experiments similar to the one shown in Figs. 1 and 2 were carried out. Percent changes from control cells in conditions of down-regulation were averaged for each time point (mean ± SEM). The paired *t* test was used to determine statistical significance. For this analysis, absolute values of receptor-specific dpm were compared between control and down-regulated cells. Data are given in text.

difference from control by 2 hr (−3 ± 8%), suggesting an associated increased degradation or processing of the proreceptor in response to insulin (Fig. 3 *Upper*).

The incorporation of radioactivity into the mature α subunit of treated cells reached significance over control at 1 hr of chase (84 ± 28%, $P < 0.01$, $n = 8$), was back to control values by 2 hr, and was significantly below control at 4 hr (−36 ± 9%, $P < 0.05$, $n = 8$) (Fig. 3 *Lower*). Preincubation with insulin for 6, 11, or 20 hr gave essentially identical results.

Under the conditions of these experiments, cell number, viability, and total and acid-precipitable radioactivity in whole cells and Triton X-100 extract were similar in control and down-regulated cells (Table 1); this suggests that the effect on receptor labeling was not secondary to changes in cell growth, radioactive sugar pools, or glycoprotein sugar/protein ratio.

Effect of Brief Insulin Treatment Without Receptor Down-Regulation. We next wished to determine whether a 30-min preincubation with insulin affected proreceptor synthesis. It was previously shown (11) that after a 30-min exposure of IM-9 cells to insulin, only 3% of cell surface receptors are internalized. After the 30-min preincubation, pulse–chase labeling was carried out as before. One autoradiogram is shown on Fig. 4. Two separate experiments were quantitated. In insulin-treated cells, labeling of the proreceptor was not different from control at 0.5 hr (95% and 105% of control; $n = 2$) and 1 hr of chase (88% and 115%). Incorporation into the mature subunit was also unchanged in response to insulin at 0.5 hr (75% and 117%) and 1 hr (78% and 119%). A modest decrease in labeling occurred in insulin-treated cells at 2 hr (69% and 89% of control) and 4 hr (78% and 74%). These results argue against an acute effect of insulin on proreceptor synthesis.

Effect of Monensin. The ionophore monensin significantly inhibits both the processing of the 190-kDa proreceptor (12, 13) and the degradation of mature receptor–insulin complexes (19). If, as suggested above, insulin acts by stimulating both biosynthesis and processing of the proreceptor, the relative increase in response to insulin should be greater when cells are pulsed in the presence of monensin.

Cells were preincubated for 6 hr with or without insulin; two chase time points were studied (1 and 4 hr). At 1 hr of chase, proreceptor labeling was 25% higher in down-regulated cells than in control cells, whether chased in the presence or absence of monensin (Fig. 5). By 4 hr of chase in

Table 1. Relative changes in control parameters in down-regulated cells after 1 and 4 hr of chase

Parameter	% change* (mean ± SEM, $n = 8$) 1-hr chase	4-hr chase
Cell number	101 ± 3	102 ± 4
Total radioactivity		
Whole cells	100 ± 7	104 ± 4
Triton X-100 extract	102 ± 4	102 ± 4
Acid-precipitable radioactivity		
Whole cells	113 ± 6	108 ± 8
Triton X-100 extract	105 ± 6	102 ± 5
60-kDa band	98 ± 5	106 ± 4
Scanning densitometry†		
Anti-receptor serum	110 ± 8	
Nonimmune serum	98 ± 8	

*Changes are expressed as percent of control; none is significant.
†Of non-receptor bands.

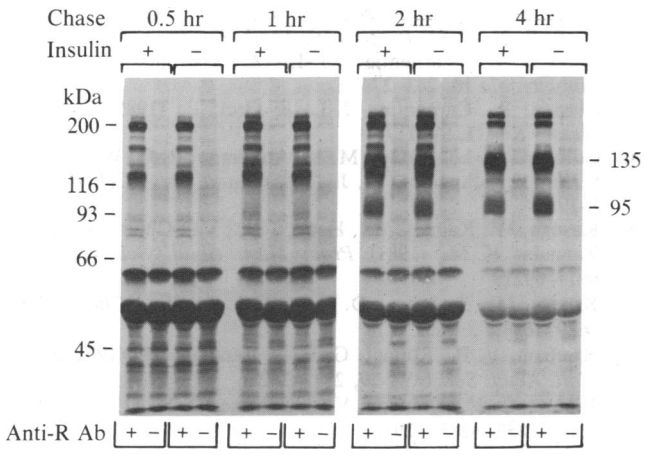

FIG. 4. Pulse–chase labeling with [^{3}H]mannose of insulin receptor components of cultured lymphocytes under conditions of brief insulin exposure without down-regulation. Cells were preincubated for 30 min with or without 1 μM insulin (+ or − above the lanes). Glycoproteins were pulse-labeled with [^{3}H]mannose for 20 min and chased with excess unlabeled mannose for various times. Solubilized extracts of the cells were treated with anti-insulin receptor antiserum (Anti-R Ab +) or normal patient serum (anti-R ab −). Immunoprecipitates were analyzed by NaDodSO$_4$/PAGE under reducing conditions, followed by autoradiography. The 210-kDa band represents the uncleaved $\alpha\beta$ complex.

the presence of monensin, however, the increase in proreceptor labeling of down-regulated cells reached 64% over control cells. In the absence of monensin, incorporation of [^{3}H]mannose into the α subunit of down-regulated cells was increased 135% over control at 1 hr and decreased 36% below control at 4 hr. When cells were chased in the presence of monensin, radioactivity in α-subunit bands was very low, as expected. By 4 hr, however, the levels were high enough to allow quantitation. At this time there was a 67% increase in labeling of down-regulated cells, contrasting with the 36% decrease observed in the absence of monensin.

In an additional experiment, cells were preincubated for 6 hr with 1 μM insulin, pulsed with [^{3}H]lysine instead of [^{3}H]mannose, and chased in the presence or absence of monensin for 1 and 4 hr. Proreceptor labeling was modestly increased at 1 hr, though we could not quantitate the effect due to high background radioactivity; at 4 hr, however, proreceptor labeling in down-regulated monensin-treated cells was increased 214% over monensin-treated control cells (data not shown).

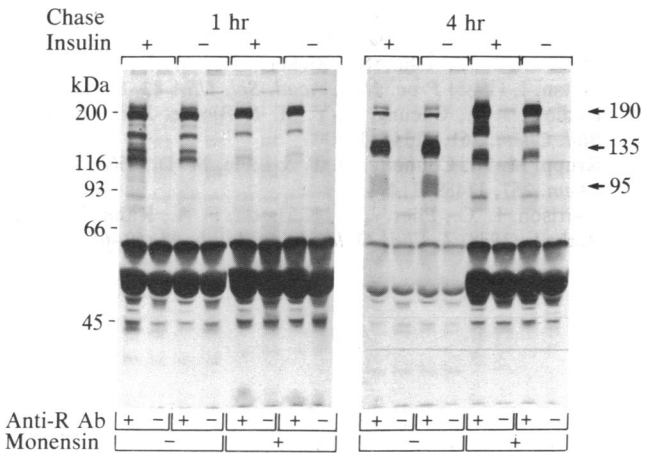

FIG. 5. Insulin receptor components of cultured lymphocytes, pulse-labeled with [^{3}H]mannose and chased in the presence or absence of monensin, in conditions of down-regulation. Cells were preincubated for 6 hr with or without 1 μM insulin (+ or − above the lanes). Glycoproteins were pulse-labeled with [^{3}H]mannose for 20 min and chased with excess unlabeled mannose for various times, in the presence (+) or the absence (−) of 25 μM monensin. Solubilized extracts of the cells were treated with anti-insulin receptor antiserum (Anti-R Ab +) or normal patient serum (anti-R Ab −). Immunoprecipitates were analyzed by NaDodSO$_4$/PAGE under reducing conditions, followed by autoradiography.

Specificity of the Insulin Effect on Receptor Biosynthesis. To determine whether homologous insulin receptor down-regulation affects other glycoproteins, several experiments were carried out. Incorporation into a 60-kDa non-insulin-receptor glycoprotein was measured and shown not to be significantly modified by insulin treatment (Table 1). Likewise, scanning densitometry showed that the intensities of the autoradiographic bands below the insulin receptor-specific bands were not significantly altered by insulin treatment at the time of the peak insulin effect on proreceptor levels (Table 1). In addition, on one occasion, an aliquot from the nonimmunoprecipitated Triton X-100 extract was applied to a gel and run under nonreducing conditions. The autoradiogram (not shown) revealed that glycoprotein labeling was not altered by insulin, as estimated by scanning densitometry (108% of control). This provides further evidence for a specific effect of down-regulation on insulin receptor biosynthesis.

DISCUSSION

Early in the course of a pulse–chase experiment, labeling of the insulin proreceptor with [^{3}H]mannose was significantly increased in down-regulated IM-9 cells. This effect was not due to changes in marker pools, receptor sugar/protein ratio, or cell growth and showed specificity for the insulin receptor. By 2 hr of chase, labeling was back to control levels, suggesting that the proreceptor lifetime was shortened in down-regulated cells. If an increased processing of the proreceptor occurs in down-regulated cells, the 41% increase we measured at 1 hr is probably an underestimate of the actual proreceptor biosynthetic rate. When monensin was added to the chase medium to inhibit proreceptor processing of both down-regulated and control cells, we found a 64% increase in insulin proreceptor in down-regulated cells at 4 hr; this is 2.6 times the level obtained at 1 hr in the same experiment when processing was not blocked. Comparable results were obtained when [^{3}H]lysine was used as the biosynthetic marker. In that case, by 4 hr in the presence of monensin, proreceptor labeling in down-regulated cells was increased 214%.

The kinetics of labeling of the α subunit was also studied. In down-regulated cells, by 1 hr of chase, there was a 84% increase in [^{3}H]mannose incorporation, compared to control cells. At 2 hr, labeling was back to normal values and decreased a further 36% below control by 4 hr. Monensin has been shown to interfere also with degradation of mature receptor–insulin complexes (19). After a 4-hr chase in the presence of monensin, the mature subunit, which escaped blockade at the proreceptor step, was increased 67% in down-regulated cells. This confirms increased receptor biosynthesis, which is normally masked by accelerated degradation.

We assume that the early rise in proreceptor levels reflects increased receptor biosynthesis, whereas the late decrease in mature subunits is the consequence of shortened receptor lifetime previously described (5). Since IM-9 cells do not exhibit the classic insulin biologic effects, and since the biosynthetic effect could not be demonstrated with short insulin incubation, it is tempting to speculate that a cause–effect relationship exists between insulin-induced down-regulation and receptor biosynthesis. This hypothesis is supported by a significant positive correlation between

proreceptor synthesis and mature receptor degradation (data not shown). Increased receptor biosynthesis may then represent a mechanism to compensate for insulin-induced receptor loss.

This mechanism may be especially important in cells where *de novo* receptor recycling from the plasma membrane is extremely limited, as has been shown for IM-9 lymphocytes (11). Whether down-regulation is associated with enhanced receptor synthesis in cells with a more active recycling mechanism, such as primary rat hepatocytes (20), is unknown.

Previous studies (5) of IM-9 cells suggested that receptor down-regulation could be fully accounted for by increased receptor degradation. The present results are in accord with this view. The failure of Kasuga *et al.* (5) to detect an increase in receptor biosynthesis is most likely explained on methodologic grounds: (*i*) the pulse–chase design used in the present study is a more sensitive indicator of early biosynthetic events and (*ii*) in order to account for the differences that we see, it would have been necessary to directly measure degradation and biosynthesis in the same experiment, thus obviating interassay variation.

Previous studies (6–8) on down-regulation, using the heavy-isotope density-shift technique, did not show changes in insulin receptor biosynthesis. The discrepancy with our results may well be a cell line difference: certainly there are differences in human cell lines with respect to internalization and recycling (11). An alternative explanation for the discrepancy is that because the heavy-isotope density-shift technique relies on insulin binding to mature subunits, a 2- to 3-hr delay is necessary between heavy-isotope labeling and initial measurement. Our study demonstrates that at 2–3 hr of chase, because of the increased turnover, the effect can no longer be detected. The same discrepancy was noted in the case of epidermal growth factor (EGF) homologous down-regulation. Clark *et al.* (21) recently demonstrated an EGF-induced increase in mRNA for EGF receptors in KB cells (epidermoid carcinoma line), and Kudlow *et al.* (22) showed increased EGF proreceptor levels in a human breast carcinoma cell line. In contrast, Krupp *et al.* (23), who used the heavy-isotope density-shift method, did not detect a change in EGF receptor biosynthesis in another epidermoid carcinoma cell line, A431.

In conclusion, the data show that homologous insulin receptor down-regulation is associated with increased proreceptor biosynthesis and processing into mature subunits. This may represent a cellular mechanism compensating for insulin-induced receptor loss.

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