The heavy isotope density shift method, in combination with a procedure for labeling cell surface insulin receptors, was used to determine the rate of transit of receptor to the cell surface from their site of synthesis and to follow the net rate of receptor removal from the plasma membrane in 3T3-L1 adipocytes. To label surface receptors, $^{125}$I-insulin was bound to cells at 4°C and then covalently cross-linked to the receptors with disuccinimidyl suberate. The identity of the surface-labeled product as insulin receptor was evidenced by immunoprecipitation with antireceptor antibody and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Figuately differentiated 3T3-L1 adipocytes were shifted to medium containing heavy (>95% $^{15}$N, $^{13}$C and $^2$H) amino acids. The rates of appearance of newly synthesized heavy receptor at the cell surface and the loss of previously synthesized light receptor from the cell surface were followed by resolving labeled heavy and light surface receptors in CsCl density gradients and quantitating labeled receptor subunits by gel electrophoresis. It was shown that 2.5-3.0 h are required for newly synthesized insulin receptor to reach and become functional in the plasma membrane. Insulin-induced down-regulation of cellular insulin receptor level had no effect on the time required for the newly synthesized receptors to reach the cell surface. Down-regulation, however, increased the first order rate constants for the inactivation of cell surface insulin receptors from 0.046 to 0.10 h$^{-1}$. The fact that the rate constants for inactivation of cell surface and total cellular insulin receptor were identical in the up-regulated state (0.046 and 0.044 h$^{-1}$, respectively) or in the down-regulated state (0.10 and 0.096 h$^{-1}$, respectively) suggests that the rate-limiting step in the receptor inactivation pathway occurs at the cell surface.

The first site at which insulin can interact with, and thereby alter the metabolism of, a target cell is at its specific receptors on the plasma membrane. The responsiveness of a cell to insulin will, therefore, depend upon both the ambient insulin concentration and the number of functional insulin receptors the cell possesses. A large body of recent evidence (1-10) indicates that cells have the capacity to modulate the number of receptors they possess in response to a variety of physiological perturbants.

Insulin itself is an important modulator of the cellular level of its own receptor. Gavin et al. (1) were the first to show that chronic exposure of lymphocytes to insulin in vitro caused a decrease, or down-regulation, in the level of cell surface insulin receptors. This inverse correlation between ambient insulin concentration and surface insulin receptor level extends to other cell types studied either in culture or in vivo (1, 2, 5, 8). Moreover, in certain pathological states, cellular resistance to insulin has been attributed to the decreased reduction in the level of cell surface insulin receptors (11-13).

The number of active insulin receptors in a cell in the steady state depends upon both the rates of synthesis and inactivation of the receptor. To determine which of these parameters is affected by physiological perturbations that alter the level of cellular insulin receptors, we have made extensive use of the heavy isotope density shift technique (4, 14). With this method, newly synthesized and "old" receptor populations can be distinguished after shifting cells to medium containing "heavy" (>95% $^{15}$N, $^{13}$C, and $^2$H) amino acids. Thus, newly synthesized heavy and previously synthesized "light" receptors, solubilized in Triton X-100, can be separated by isopycnic banding on CsCl density gradients and then quantitated. By following changes in the relative amounts of heavy and light receptors after the shift to heavy amino acids, rates of synthesis and inactivation/decay of active receptor can be determined. It should be stressed that the density shift method follows the rate at which insulin-binding activity of the receptor is produced or lost and, thus, measures the physiologically relevant rate-limiting steps in the formation or inactivation of functional receptor.

By using this method, it was established that the dramatic rise in insulin receptor level, which accompanies the differentiation of 3T3-L1 preadipocytes into adipocytes, results from a comparable increase in the rate of receptor synthesis with little change in the rate of receptor inactivation (4). In contrast, the reduction in insulin receptor level which occurs during insulin-induced receptor down-regulation in 3T3-L1 adipocytes and 3T3-C2 fibroblasts was found to be due entirely to an increased rate of receptor inactivation with no change in the rate of receptor synthesis (5, 15). Glucocorticoid-induced up-regulation of receptor level in 3T3-C2 fibroblasts was shown to result from a decreased rate of receptor inactivation (15).

One aspect of insulin receptor metabolism not previously

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Kinetics of Insulin Receptor Transit to and Removal from the Plasma Membrane

EFFECT OF INSULIN-INDUCED DOWN-REGULATION IN 3T3-L1 ADIPOCYTES* (Received for publication, July 26, 1982)

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clarified by the heavy isotope density shift method is the identification of the cellular location at which the inactivation of functional receptor occurs. To localize this potential regulatory step and to characterize the kinetics of transit of the receptor to and its net removal from the plasma membrane, we have combined the heavy isotope density shift method with the procedure of Pilch and Czech (16, 17) for specifically and covalently labeling cell surface insulin receptors with $^{125}$I-insulin. This report describes the application of this method to study the metabolism of cell surface insulin receptors and the effect of insulin-induced receptor down-regulation on these processes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** 3T3-L1 cells were cultured as previously described (4) and differentiated by a modification of the methods of Reed and Lane (14) and Rubin et al. (15). Two days postconfluence, cells were fed with medium containing 10% fetal calf serum, 0.5% isobutylmethylxanthine, 1 mM dexamethasone, and 10 nM mouse insulin (Elanco) per ml of medium. By day 7, more than 90% of the cells expressed the adipocyte phenotype and the total number of insulin receptors/cell had reached a steady state. Cell monolayers were used for experiments between days 7 and 10.

**Covalent Cross-linking of $^{125}$I-insulin to Insulin Receptors—** For all experiments, insulin was iodinated by the chloramine-$T$ method, purified by the method of Gavin et al. (1), and was found to be >97% precipitable by 10% (w/v) trichloroacetic acid. Prior to use, $^{125}$I-insulin was further purified by gel filtration on Sephadex G-50 (9).

To covalently cross-link $^{125}$I-insulin to cell surface insulin receptors, the bifunctional chemical cross-linking reagent DSS', originally used by Pilch and Czech (16, 17) to label insulin receptors, was adapted for use with 3T3-LI adipocytes. Cell monolayers were first washed three times with Krebs-Ringer phosphate buffer, pH 7.4, containing 1% bovine serum albumin, 25 mM glucose and then incubated at 37°C for 20 min. Monolayers were subjected to two additional washes separated by 20-min incubations. This procedure effectively removes all cell-associated insulin (5).

Cell monolayers were then cooled to 4°C and incubated with 5 ml of Krebs-Ringer phosphate buffer containing 0.1% (w/v) bovine serum albumin, 5 nM $^{125}$I-insulin $\pm$ 5 nM unlabeled insulin for 6 h at 4°C. Monolayers were quickly rinsed six times with 8 ml of cold PBS, pH 7.4. A freshly made stock solution of 50 mM DSS (Pierce) in dimethyl sulfoxide was diluted immediately prior to use in cold PBS to a final concentration of 0.5 mM. Cross-linking of cell monolayers was carried out by incubating cell monolayers with 5 ml of 0.5 mM DSS for 15 min at 4°C, after which the solution was aspirated and the reaction was quenched by the addition of 8 ml of 50 mM Tris-HCl, pH 7.4, to each dish. Triton X-100-solubilized extracts were prepared from total cellular membrane pellets as described below. The protocol employed for the isolation, separation, and quantitation of heavy/light cell surface receptors with covalently linked $^{125}$I-insulin is illustrated in Fig. 1.

**Heavy Isotope-labeling Procedure, Preparation of Solubilized Insulin Receptor, and Isopycnic Binding of the Receptor in CsCl Density Gradients—** Heavy amino acids containing >95% $^{15}$N, $^{13}$C, and $^3$H were isolated after acid hydrolysis of protein derived from $^3$H-labeled algae (Chlorella pyrenoidosa) provided by the Stable Isotope Resource (Dr. Thomas Whaley), Los Alamos Scientific Laboratory, Los Alamos, NM. Cell monolayers were incubated at 37°C for 20 min. After washing and resuspending in Krebs-Ringer phosphate buffer, pH 7.4, containing 1% bovine serum albumin, 50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100 in a total volume of 250 ml, solubilized insulin receptor-binding assays were carried out by a modification of the polyethylene glycol precipitation method (19, 20).

The amount of $^{125}$I-insulin binding activity, which accurately approximates the quantity of receptor, in the light receptor and heavy receptor peaks was determined as previously described (4, 14). Briefly, it has been established (14) that the leading edge of the light receptor peak (Fig. 4A, fraction 18, 0 h) does not contribute to the $^{125}$I-insulin-binding activity in the peak fraction (fraction 16) of the heavy receptor peak. In contrast, the trailing edge of the heavy receptor peak contribution significantly to the peak fraction (fraction 23 or 24) of the light receptor. Using a previously determined (4) ratio of peak area to peak height (ratio = 6.5), the height of the heavy peak at its maximum was used to calculate heavy peak area. Light receptor peak area was then determined to subtract the amount of peak heavy area from both peaks. As previously demonstrated, recovery of insulin-binding activity on CsCl gradients, based on Scatchard analysis (4), is about 85% without preferential loss of any class of binding activity. The results are not
corrected for losses incurred during CsCl density gradient centrifugation.

To verify that the solubilized receptor-binding assays were specific for insulin, competitive binding assays were conducted using unlabeled bovine insulin and desoctapeptide insulin. The concentrations of bovine insulin and desoctapeptide insulin required for half-maximal inhibition of binding of bovine 125I-insulin were 0.8 × 10⁻⁹ and 4.5 × 10⁻⁹ M, respectively; these values agree with previously published results (21). All data are presented as 125I-insulin bound specifically, i.e. total minus nonspecific.

A modified procedure was used to determine the amount of 125I-insulin covalently cross-linked to cell surface receptors. As these cell monolayers had already been exposed to 125I-insulin in the presence or absence of excess unlabeled insulin prior to cross-linking with DSS and solubilization, the 50-µl fractions from the CsCl gradients were directly counted to locate and quantitate heavy and light receptor peaks; the 125I-label associated with these peaks comprised 125I-insulin bound to receptor either covalently or noncovalently. To determine the amount of 125I-insulin which had become covalently linked to cell surface receptor subunits, fractions from the CsCl gradient were treated with SDS and applied to 7.5% SDS-polyacrylamide gels for electrophoresis according to the method of Laemmli (22). Labeled bands on each gel were quantitated both by counting the 125I-labeled gel bands directly and by densitometric scanning of autoradiograms of the gels using a Kontes Model 800 fiber optic scanner equipped with a Hewlett-Packard 3109A reporting integrator.

As with the solubilized native heavy and light receptors subjected to CsCl density gradient centrifugation, there is no contribution of light receptor covalently cross-linked with 125I-insulin to the peak fraction of heavy receptor cross-linked with 125I-insulin (compare Fig. 3, A and B). However, the trailing edge of the peak of heavy receptors cross-linked with 125I-insulin does contribute to a small extent to the peak fraction of the light cross-linked receptor peak (Fig. 3B). An identical overlap occurs for the native soluble heavy and light receptor peaks. Therefore, the experimentally determined percentage contribution, i.e. 10% of the height of the heavy cross-linked peak fraction, is deducted from the height of the cross-linked light peak fraction. Since there is a linear relationship between peak area and peak height for both native (4) and cross-linked (results not shown) receptors, the amount of 125I-label in receptor subunits in the peak fraction is proportional to the total amount of cross-linked receptor in the entire peak.

**Immunoprecipitation of Solubilized Insulin Receptor Cross-linked to 125I-Insulin**—The identity of the protein subunit to which cell surface-bound 125I-insulin became covalently cross-linked was established by immunoprecipitation of the solubilized receptor with anti-insulin receptor antibody. Serum from a patient with the autoimmune disease acanthosis nigricans, which contained a high titer of anti-insulin receptor antibody, was obtained from Dr. Phillip Gorden at the National Institutes of Health. In all assays, a 250-µl aliquot of detergent-solubilized extract of total cellular membranes which represents 6 × 10⁶ 3T3-L1 adipocytes was incubated with 1 µl of immune or normal serum at 4 °C for 12 h. The precipitation of immune complexes was performed by the use of Pansorbin (Calbiochem-Behring Corp.), a preparation of protein A-bearing cross-linked Staphylococcus aureus (23). An aliquot of a stock solution of 10% (w/v) Pansorbin in PBS was first pelleted at 3000 × g for 25 min in a Sorvall HS-4 rotor at 4 °C. The pellet was resuspended in 100 mM Tris-HCl, pH 7.4, containing 0.1% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100 (subsequently referred to as “wash buffer”). A 100-µl aliquot of Pansorbin prepared in this manner was added to each assay and incubated for 30 min at 4 °C with constant agitation. The Pansorbin, along with the bound immune complexes, was again pelleted and resuspended three times in 3 ml of wash buffer. After the final spin, the pellet was resuspended in Fairbanks’ reducing sample buffer (24) and subjected to polyacrylamide gel electrophoresis.

**RESULTS**

**Characterization of Cell Surface Insulin Receptor Cross-linked Covalently to 125I-Insulin**—To characterize the 125I-labeled product(s) of the covalent cross-linking reaction between bound 125I-insulin and cell surface insulin receptors of 3T3-L1 adipocytes, fully differentiated cells to which 125I-insulin had been bound were cross-linked using the homobifunctional cross-linking agent DSS (16). Insulin binding and cross-linking were carried out at 4 °C to prevent endocytosis of 125I-insulin bound either covalently or noncovalently and to prevent exocytosis of intracellular receptors. Optimal conditions to obtain maximal yield of covalently cross-linked detergent-extractable receptor, established in preliminary experiments, were employed. Fig. 2 shows typical autoradiograms of gels of 125I-labeled cross-linked receptor extracted from total cellular membranes with Triton X-100 and subjected to SDS-polyacrylamide gel electrophoresis. The major 125I-labeled polypeptide which entered the gel has M₉ = 135,000 (Lane 1, Fig. 2) and corresponds in size to the insulin-binding component of the receptor described by others (25-27). In addition, another insulin-binding component(s) is evident at the top of the running gel. When applied to lower percentage acrylamide gels, this high molecular weight material migrated as two distinct components with mobilities corresponding to M₉ = 280,000 and M₉ = 400,000 (results not shown). These components appear to be insulin receptor subunits, which in addition to their covalent linkage to 125I-insulin, have become covalently linked to other receptor subunits or to other proteins with which the receptor is closely associated. In the absence of the cross-linking agent, no cellular components become covalently labeled with 125I-insulin (Lane 2, Fig. 2). When excess unlabeled insulin is added at the time of incubation of the monolayer with 125I-insulin, all radioactivity is competed away from the M₉ = 135,000 and higher molecular weight components (Lane 3, Fig. 2). Hence, those components to which 125I-insulin becomes covalently attached bind insulin specifically. To further characterize these insulin binding proteins, anti-insulin receptor antisera was added to detergent-solubilized extract of total cellular membranes from cells exposed to 125I-insulin and cross-linked with DSS. A duplicate sample of “cross-linked” extract was incubated with nonimmune serum. Labeled immune complexes were precipitated by the use of Pansorbin (Calbiochem-Behring Corp.), a preparation of protein A-bearing cross-linked Staphylococcus aureus (23). An aliquot of a stock solution of 10% (w/v) Pansorbin in PBS was first pelleted at 3000 × g for 25 min in a Sorvall HS-4 rotor at 4 °C. The pellet was resuspended in 100 mM Tris-HCl, pH 7.4, containing 0.1% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100 (subsequently referred to as “wash buffer”). A 100-µl aliquot of Pansorbin prepared in this manner was added to each assay and incubated for 30 min at 4 °C with constant agitation. The Pansorbin, along with the bound immune complexes, was again pelleted and resuspended three times in 3 ml of wash buffer. After the final spin, the pellet was resuspended in Fairbanks’ reducing sample buffer (24) and subjected to polyacrylamide gel electrophoresis.
plexes were adsorbed to Pansorbin (a cross-linked S. aureus protein A-containing preparation) and after extensive washing were eluted and subjected to SDS gel electrophoresis. The $^{125}$I-labeled antigens had the same mobilities as those covalently labeled by $^{125}$I-insulin with DSS in intact cells (Lane 4, Fig. 2); nonimmune serum did not, however, immunoprecipitate these labeled antigens (Lane 5, Fig. 2). Thus, by two criteria, namely the specific binding of radiolabeled insulin and the specific precipitation of those labeled proteins by anti-insulin receptor antibodies, the components covalently cross-linked to $^{125}$I-insulin by DSS treatment appear to be receptor subunits or cross-linked forms of the insulin receptor. Cellular components with the same mobilities are labeled when this method is applied to detergent-solubilized receptor or cellular membranes (results not shown).

**Quantitation of Cell Surface Receptors Covalently Cross-linked with $^{125}$I-Insulin**—Before the procedure to cross-link $^{125}$I-insulin to cell surface insulin receptors could be used reliably in heavy isotope density shift experiments, it was necessary to validate the precision, efficiency, and proportionality of the procedure for 3T3-L1 adipocytes. To assess the variability of labeling, four identical cell monolayers were extracted from total cellular membranes with Triton X-100 and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Comparison of the amounts of covalently linked $^{125}$I-ligand in the gel bands identified above (Fig. 2, lanes 1 and 4) as insulin receptor subunits revealed 5% variation among gels from the four cell monolayers (results not shown). The amount of $^{125}$I-labeled receptor subunits detected on polyacrylamide gels was shown to be linearly proportional to the quantity of SDS-solubilized labeled membrane applied to the gel (results not shown). The overall efficiency of cross-linking, i.e., counts/min of $^{125}$I-insulin covalently linked to receptor subunits/counts/min of $^{125}$I-insulin specifically bound to receptor $\times 100$, in this and other experiments was approximately 10%. Although unlikely, it is possible that the cross-linking procedure labels a particular population of receptors. The efficiency of cross-linking probably underestimates the true value since a significant fraction of the $^{125}$I label, which is located in the A chain (A-16 and A-14 tyrosyl residues) of insulin, would be lost under reducing conditions from molecules cross-linked to receptor through the B-chain (B-1 and/or B-29 amino groups) of insulin.

To monitor the rates of appearance of newly synthesized heavy receptors at the cell surface and the disappearance of previously synthesized light receptors from the cell surface, it was necessary to separate and quantitate solubilized heavy and light receptors with cross-linked $^{125}$I-insulin by CsCl density gradient centrifugation. It was previously shown (4) that heavy and light receptors without covalently linked $^{125}$I-ligand attached could be resolved and detected on CsCl density gradients. Detergent-solubilized cross-linked (to $^{125}$I-insulin) receptor or receptor from cells not cross-linked was prepared from cells maintained in medium containing light amino acids or from cells which had been shifted to heavy amino acid-containing medium for a period of 9-12 h. Solubilized receptor extracts from the equivalent of $6 \times 10^6$ cells were applied to each gradient. $^{125}$I-Insulin-binding assays were performed on fractions from gradients to which noncross-linked receptor was added; gradients to which cross-linked receptor was added were subjected to SDS-polyacrylamide gel electrophoresis for quantitation of labeled cross-linked receptor subunits. As shown in Fig. 3, heavy and light receptors to which $^{125}$I-insulin had been covalently cross-linked were resolved and banded at positions in CsCl density gradients identical with those of heavy and light native insulin receptors.

**Kinetics of Appearance of Heavy Receptors at and Loss of Light Receptors from the Plasma Membrane following the Shift of Cells to Heavy Amino Acids**—The heavy isotope density shift method and DSS cross-linking to label (with $^{125}$I-insulin) cell surface insulin receptors were used to measure the rate of transit of newly translated receptor to and the rate of loss of previously synthesized receptor from the plasma membrane. Fully differentiated 3T3-L1 adipocytes maintained in the presence of insulin were shifted to medium containing heavy (>95%, $^{14}$N, $^{13}$C, and $^3$H) amino acids. At time intervals following the density shift, cell monolayers were subjected to the ligand-debinding protocol to remove cell-associated insulin and then cooled to 4°C. At this temperature, the processes of exocytosis and endocytosis are blocked (28).

To determine the amounts of heavy and light receptors on the cell surface, $^{125}$I-insulin was bound to the cell monolayers at 4°C, after which bound insulin was covalently cross-linked to surface receptor labels with DSS. The $^{125}$I-insulin-labeled receptors were then extracted from total cellular membranes with Triton X-100 and the extract was applied to CsCl gradients to resolve and quantitate light and heavy receptors. The gradients were fractionated and the heavy and light peak fractions (e.g. fractions 16 and 22, respectively, in Fig. 3B) were subjected to SDS-polyacrylamide gel electrophoresis to quantitate $^{125}$I-labeled surface insulin receptor subunits as described above.
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Fig. 4. Kinetic progress curves for the transit of newly synthesized heavy receptor in up-regulated cells. 3T3-L1 adipocytes were shifted to medium containing heavy (>95% ¹³C, ¹⁵N, and ²H) amino acids, and at various times thereafter (1) the amounts of active (□) and light (●) receptor present on the cell surface (solid lines) and (2) the amounts of heavy receptor synthesis (▵) and light receptor decay (▲) (broken lines) were determined by the heavy isotope density shift method as described under “Experimental Procedures” and outlined in Fig. 1. Each data point represents 1.2 × 10⁸ cells.

When compared on the basis of per cent of cell-surface or total cellular receptors, the progress curves for surface and total heavy receptor formation and light receptor decay closely parallel each other following their respective lag periods. A 1.5-h lag after the density shift in the appearance of active (capable of binding insulin) total heavy cellular receptor has been observed previously (4, 5). This lag has been attributed to a post-translational processing step(s), perhaps glycosylation or subunit assembly, required for receptor activation. The rates of loss of receptor from the cell surface and the decay of total cellular receptor are identical (kₐᵦ = 0.10 and 0.096 h⁻¹, respectively). The rate constants for inactivation of total cellular and cell surface insulin receptors were determined as previously described (4, 5, 14). Briefly, the amount of light receptor present at each time point is plotted semilogarithmically and the rate of receptor decay is described by a first order rate equation. First order rate constants are calculated from the slopes of the plots. This suggests that receptor inactivation occurs at the cell surface or that removal of active receptor from the cell surface is rate-limiting for receptor inactivation.

Effect of Insulin on the Rates of Appearance of Newly Synthesized Receptor and the Net Removal of Previously Synthesized Receptors from the Cell Surface—In earlier studies (5, 15), it was established that chronic exposure of 3T3-L1 adipocytes or 3T3-C2 fibroblasts to insulin causes down-regulation of both cell surface and detergent-extractable (total cellular) insulin receptors. These effects were the result of an increase in the rate constant for inactivation of total cellular receptor. To locate the site of the rate-limiting process(es) in the receptor pathway responsible for the increased rate of receptor inactivation, the effect of chronic insulin treatment on the rate of transit of newly synthesized receptor to the cell surface and the rate of inactivation of cell surface receptor were determined. The combined heavy isotope density shift and cell surface receptor-labeling method described above was employed.

Fully differentiated 3T3-L1 cells (day 7), which had been maintained in the presence of insulin throughout differentiation, were subjected to the insulin-debinding protocol and then transferred either to medium with or without insulin for 24 h, a time sufficient to allow insulin receptor to up-regulate in the absence of insulin (5). Both up-regulated and down-regulated cells were then shifted to medium containing heavy (>95% ¹³C, ¹⁵N, and ²H) amino acids. Immediately after the density shift and at intervals thereafter, cell monolayers were freed of bound insulin and cooled to 4°C to prevent further endo- or exocytosis of receptors. ¹³C-I (%) was then bound and covalently cross-linked to cell surface receptors with DSS and the solubilized heavy and light receptors were separated by isopycnic banding on CsCl density gradients. The peak heavy and light receptor fractions from the gradients were subjected to polyacrylamide gel electrophoresis.

In addition, the rates of synthesis and decay of total cellular receptor in up-regulated cells were determined by the heavy isotope density shift method for comparison to the rates of transit of receptors to and removal from the cell surface in up-regulated cells. The progress curves for the comparison of the turnover of total cellular and cell surface receptors in up-regulated cells are shown in Fig. 5. It is evident that the kinetics of the loss of surface receptors and the kinetics of total cellular receptors are parallel, as in the down-regulated state. When plotted semilogarithmically, the rates of loss of receptor from the cell surface and the decay of total cellular receptor were first order and identical (kₐᵦ = 0.046 and 0.044 h⁻¹, respectively).

The autoradiograms of the gels for the peak heavy and light receptor fractions from up- and down-regulated cells which
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Fig. 6. Effect of insulin-induced down-regulation on the rate of appearance of heavy receptor at and the rate of loss of light receptor from the cell surface following a shift to heavy amino acids. 3T3-L1 adipocytes were maintained in medium with or without 1 μM insulin for 24 h and then shifted to similar medium containing heavy (>95% 15N, 13C, and 3H) amino acids. The 24-h treatment with insulin caused a 55 and 60% down-regulation of cell surface and total (Triton X-100-extractable) cellular insulin receptor number (5). At various intervals after the density shift, cell surface insulin receptors were covalently labeled with 125I-insulin by the DSS cross-linking procedure and heavy- and light-labeled receptors were separated using the protocol shown in Fig. 1 and described in detail under “Experimental Procedures.” Heavy and light 125I-labeled surface receptors were then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 2).

had been subjected to the cell surface cross-linking procedure are shown in Fig. 6. The amounts of newly synthesized heavy receptor on the cell surface increase with time after a lag in both the up- and down-regulated cells. Following a similar lag, the previously synthesized light receptor decayed from the surface, although more rapidly in the down-regulated cells.

The autoradiograms were densitometrically scanned to quantitate the amounts of active cell surface receptor (M, = 135,000 and high molecular weight bands) present at each point. Fig. 7 shows the progress curves generated from the densitometric scans of the M, = 135,000 insulin receptor subunit on these autoradiograms. The initial rates of appearance of active heavy receptor at the plasma membrane are quite similar in up-regulated (control) and down-regulated cells (Fig. 7A). However, with time, the two curves splay due to an increased rate of receptor degradation which is particularly manifested at later times. When the loss of light cell surface receptor is plotted semilogarithmically (Fig. 7B), it is evident that after a 3-h lag insulin induces a 2-fold increase in the rate of loss of active receptor from the cell surface. Hence, the half-life of the receptor on the cell surface decreases from 15 to 7 h in the presence of insulin. Similar results were obtained when progress curves were generated for the receptor band at the gel origin (Fig. 6), shown to be immunoprecipitable with antireceptor antibody. Thus, insulin does not affect the rate of appearance of newly synthesized active receptor at the plasma membrane; rather, the ligand increases the net rate of loss of receptor from the cell surface.

Fig. 7. Kinetic progress curves for the appearance of heavy receptor at and the rate of loss of light receptor from the cell surface in control and down-regulated 3T3-L1 adipocytes. Results are calculated from the data of the experiment described in Fig. 6.

DISCUSSION

By combining the heavy isotope density shift technique with covalent labeling of cell surface receptors with 125I-insulin, it was possible to measure the rates of transit of newly synthesized receptor to and previously synthesized receptor from the plasma membrane. It had been determined earlier (4, 5) and was confirmed in the present investigation (Figs. 4 and 5) that about 1.5 h are required for processing of newly translated “proreceptor” into an active form, i.e. a form capable of binding insulin. An additional 1.5 h are needed for the active receptor to be inserted into the plasma membrane and thereby to become functional (Fig. 8). Overall, the time for processing/transit from the site of translation to the cell surface is about 3 h. This unexpectedly long period greatly exceeds the 30-min processing/transit time of many secretory glycoproteins, e.g. very low density lipoproteins (29), which also traverse the Golgi system en route to the plasma membrane. The 1.5-h requirement for activation of newly trans-

Fig. 8. Proposed pathway and regulation of insulin receptor metabolism in 3T3-L1 adipocytes. After translation of insulin proreceptor polypeptide(s) (Step 1) in the rough endoplasmic reticulum, further processing (Step 2), which requires 1.5 h, gives rise to active receptor capable of binding insulin. A total time of 3 h is required (Steps 1–3) for newly synthesized receptor to reach the plasma membrane; these steps are not affected by the presence of insulin in the medium. Cell surface receptors, including those to which insulin has become bound (Step 5), undergo inactivation/internalization (Step 6, a and b) at rates dependent upon the presence of bound ligand; inactivated receptors (and/or ligand) are degraded to amino acids (Step 8). A pathway for internalization of ligand-receptor complex leading to ligand degradation (Step 8) and receptor recycling (Step 7) appears to be independent of the receptor inactivation/degradation pathway (Steps 6, a and b, and 8). The size of the arrows is intended to indicate relative rate: □, inactive proreceptor; □, active receptor capable of binding insulin; ○, inactivated receptor; V, insulin.

Fig. 8. Proposed pathway and regulation of insulin receptor metabolism in 3T3-L1 adipocytes. After translation of insulin proreceptor polypeptide(s) (Step 1) in the rough endoplasmic reticulum, further processing (Step 2), which requires 1.5 h, gives rise to active receptor capable of binding insulin. A total time of 3 h is required (Steps 1–3) for newly synthesized receptor to reach the plasma membrane; these steps are not affected by the presence of insulin in the medium. Cell surface receptors, including those to which insulin has become bound (Step 5), undergo inactivation/internalization (Step 6, a and b) at rates dependent upon the presence of bound ligand; inactivated receptors (and/or ligand) are degraded to amino acids (Step 8). A pathway for internalization of ligand-receptor complex leading to ligand degradation (Step 8) and receptor recycling (Step 7) appears to be independent of the receptor inactivation/degradation pathway (Steps 6, a and b, and 8). The size of the arrows is intended to indicate relative rate: □, inactive proreceptor; □, active receptor capable of binding insulin; ○, inactivated receptor; V, insulin.
lated prorreceptor could be needed for glycosylation, polypeptide modification, and/or subunit assembly. Earlier studies (30, 31) showed that glycosylation is essential for the formation of active receptor. Thus, glycosylation per se or a glycosylation-dependent processing step is necessary for the maturation of the receptor polypeptide(s).

Although we previously demonstrated that chronic exposure of 3T3-L1 adipocytes to insulin had no effect on the rate of formation of labeled receptor (6), the possibility was not ruled out that this treatment altered the fraction of receptors reaching the cell surface. It is conceivable that insulin treatment of the cells increased the fraction of newly synthesized receptor shunted directly into an intracellular degradation pathway (Step 4, Fig. 8). This possibility was ruled out by the finding (Fig. 7) that the time required for newly synthesized receptor to reach the cell surface is not affected by insulin. It can be concluded, therefore, that the insulin-activated step(s) in the receptor pathway (Fig. 8) responsible for down-regulation must lie beyond the point (Step 3, Fig. 8) at which newly synthesized receptor is inserted into the plasma membrane. This supposition is supported by the finding (Fig. 7) that insulin markedly increases the net rate of removal and/or inactivation of active insulin receptors from the plasma membrane.

While it has not yet been possible to locate the precise site of insulin receptor inactivation, i.e. whether at the cell surface or in an intracellular compartment, the rate-limiting step in this process in 3T3-L1 adipocytes is either the net removal of receptor from the plasma membrane or the inactivation of the receptor at the cell surface prior to internalization. It is suggested that inactivation occurs at the cell surface since receptor would be expected to accumulate intracellularly in the down-regulated state were the rate-limiting step a postendocytotic event. In certain other cell types, an apparent accumulation of intracellular receptors has been reported (8, 34). In 3T3-L1 adipocytes, however, such an insulin-induced build-up of intracellular receptor does not occur (5). This and the fact that clearance of active receptor from the cell surface limits the rate of cellular receptor decay supports the view that receptor inactivation is a cell surface event.

Endocytosis of the receptor (Step 6, a and b, Fig. 8) appears to be too rapid to be implicated as the regulated rate-limiting step in the receptor inactivation process. The minimum rate of receptor endocytosis in the presence of insulin (5 × 10⁻⁶ receptors/cell/h) was estimated from the rate of receptor-mediated ¹²⁵I-insulin degradation⁴ and the rate of receptor inactivation (1.5 × 10⁻⁶ receptors/cell/h) from the first order rate constant for receptor inactivation in the presence of insulin (Fig. 7B). If it is assumed that a receptor complex accompanies each molecule of ¹²⁵I-insulin into the cell where the ligand is degraded to [¹²⁵I]monoiodotyrosine, then the receptor itself must rapidly recycle back to the cell surface; otherwise, cell surface receptors would be quickly depleted since the rate of receptor synthesis is orders of magnitude slower. This has previously been demonstrated for the insulin receptor in the liver cell (32). Hence, insulin is degraded at a rate ≥300-fold faster than its receptor.

The processes of receptor inactivation and insulin-induced down-regulation (Step 6, a and b, Fig. 8) can be dissociated from the internalization of ligand and receptor internalized for

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⁴ Two lines of evidence indicate that insulin receptors mediate the internalization of insulin prerequisite to its lysosomal degradation. First, the dependence of ¹²⁵I-insulin degradation upon insulin concentration closely approximates the insulin-binding isotherm for cell surface receptors. Second, down-regulation of cell surface insulin-binding capacity causes a similar decrease in ¹²⁵I-insulin degradation rate (G. V. Ronnett and M. D. Lane, unpublished results).

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The purpose of ligand degradation (Step 7, Fig. 8). This is indicated by the fact that receptor inactivation (31) and down-regulation (33) are quickly (60 min) blocked by inhibitors of protein synthesis, such as cycloheximide and puromycin, whereas receptor-dependent ¹²⁵I-insulin degradation is not. A short-lived protein(s) whose synthesis is blocked by these inhibitors appears to be required for a step common to the receptor inactivation and down-regulation processes, but not for ligand/receptor endocytosis. Thus, the step(s) activated by chronic exposure of cells to insulin and which is responsible for the down-regulation of cell-surface receptor level is no doubt distinct from the ligand entry-degradation/receptor recycling pathway (Steps 4 and 6, Fig. 8). Further investigations utilizing the approaches developed in this paper should prove useful in distinguishing these pathways and delineating the mechanism of insulin-induced receptor inactivation.

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