Proc. Natl. Acad. Sci. USA
Vol. 77, No. 1, pp. 285–289, January 1980
Biochemistry

Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes

(insulin receptor half-life/receptor density shift/isopycnic banding on CsCl gradients/3T3-L1 adipocytes)

BRENT C. REED AND M. DANIEL LANE

Department of Physiological Chemistry, Johns Hopkins University Medical School, Baltimore, Maryland 21205

Communicated by Albert L. Lehninger, October 22, 1979

ABSTRACT **A density-shift method is described for analyzing insulin receptor synthesis and turnover in cultured cells labeled with "heavy" amino acids (^{2}H, ^{13}C, and ^{15}N). Solubilized newly synthesized heavy and old "light" receptors are separated by isopycnic banding on CsCl gradients and then quantitated. Insulin receptor synthesis and turnover were studied by this technique in 3T3-L1 preadipocytes which undergo an increase in insulin binding capacity during differentiation. The results indicate that the increase in insulin binding capacity is a consequence of new receptor synthesis, that the insulin receptor has a relatively short half-life (6.7 hr), and that an increased rate of receptor synthesis contributes to the increase of insulin receptor level during differentiation.**

The regulation of hormone receptor number or affinity in response to ligand are well-documented mechanisms for modulating a cell's sensitivity to homologous hormone (1, 2). Such hormone-induced "down regulation" of insulin receptor has been demonstrated in culture (1, 2); the inverse relationship of receptor level to circulating insulin concentration appears to hold *in vivo* (1, 2). An unequivocal approach has not been available to determine whether down regulation of insulin receptor occurs through an increased rate of receptor degradation or decreased rate of receptor synthesis. Nor is it known how these mechanisms differ in cells that do not exhibit receptor down regulation or that show an increase in receptor level.

To investigate these aspects of insulin receptor regulation, we have modified the approach developed in Fambrough's laboratory to analyze acetylcholine receptor metabolism (3–5). This method permitted the identification of newly synthesized insulin receptor after exposing cells to amino acids that were >90% enriched in carbon-13, nitrogen-15, and hydrogen-2. The incorporation of these amino acids into receptor protein substantially increased the density of newly synthesized insulin receptor. Hence, "new heavy" and "old light" receptor, solubilized with detergent, could be resolved by density-gradient centrifugation. Their relative amounts and positions in the gradient were then determined by their capacity to bind ^{125}I-labeled insulin (^{125}I-insulin).

This method was applied to the study of insulin receptor regulation in the 3T3-L1 and 3T3-C2 cell lines cloned by Green and Kehinde (6–8). 3T3-L1 cells, derived from mouse embryo fibroblasts, differentiate in culture to form cells possessing the morphological and enzymatic characteristics of mature adipocytes (6–16). We and other investigators (13, 17–21) have demonstrated that, during differentiation, 3T3-L1 cells increase their capacity to bind insulin. This change appears to be receptor specific and has been attributed to a differentiation-related increase in receptor number (18–21). Unlike nondifferentiating 3T3-C2 fibroblasts and other cell types (1, 2, 18–20), 3T3-L1 cells do not appear to down regulate insulin receptor upon exposure to insulin (19–21). Hence, the 3T3-L1/C2 cell system should be useful for investigating the differentiation-induced increase in receptor number, insulin-induced receptor down-regulation, and the apparent down regulation defect.

The present paper reports the successful application of the density-shift approach to investigate the turnover rate and differentiation-linked increase in level of the insulin receptor in 3T3-L1 preadipocytes.

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MATERIALS AND METHODS

Culture Conditions. 3T3-L1 and 3T3-C2 cells, provided by Howard Green of the Massachusetts Institute of Technology, were cultured in 6-cm dishes as described (9) but with fetal calf serum instead of calf serum. Differentiated 3T3-L1 adipocytes were generated by a modification of the method of Rubin *et al.* (19). Two to 3 days after confluence, medium (3.5 ml) supplemented with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 10 μg of insulin per ml was added (day 0) to induce differentiation. On day 3, isobutylmethylxanthine and dexamethasone were removed and the cells were fed every 2 days with medium containing 10 μg of insulin per ml. The monolayers contained 80–90% adipocytes by day 6.

Density-Shift Experiments. Monolayers were incubated at 37°C in 10% CO_2/90% air with 3.5 ml of "heavy" media, each 100 ml containing: 10 ml of 10% dialyzed (against phosphate-buffered saline) fetal calf serum, 0.8 mg of tryptophan, 3 mg of cystine, 6 mg of glutamine, 100 mg of a dense amino acid mixture (95% enriched in ^{13}C, ^{15}N, and ^{2}H; Merck Sharp and Dohme, Canada), 1 mg of insulin, and 90 ml of Dulbecco's modification of Eagle's minimal essential medium (without amino acids). Dense amino acids were dissolved in phosphate-buffered saline, passed through a UM-2 filter (Amicon, Lexington, MA), and filter-sterilized prior to use.

Isopycnic Banding of Soluble Insulin Receptor in CsCl Gradients. To remove bound unlabeled insulin, cell monolayers were washed three times with Krebs–Ringer phosphate buffer (pH 7.4) containing one-half the usual calcium level, 2% bovine serum albumin, and 25 mM glucose; they were incubated for 20 min at 37°C between washes. Cell monolayers were scraped into 50 mM Tris·HCl at pH 7.4 containing 400 units of Trasylol (FBA Pharmaceuticals, New York) per ml, homogenized in a Teflon glass homogenizer, and centrifuged for 1 hr at 35,000 rpm in a Beckman SW 56 Ti rotor at 4°C. Pellets were extracted with 0.6 ml of 4% Triton X-100 in Tris buffer as described by Cuatrecasas (22), centrifuged as above, and stored at −50°C. One-half milliliter of the supernatant fraction was mixed with

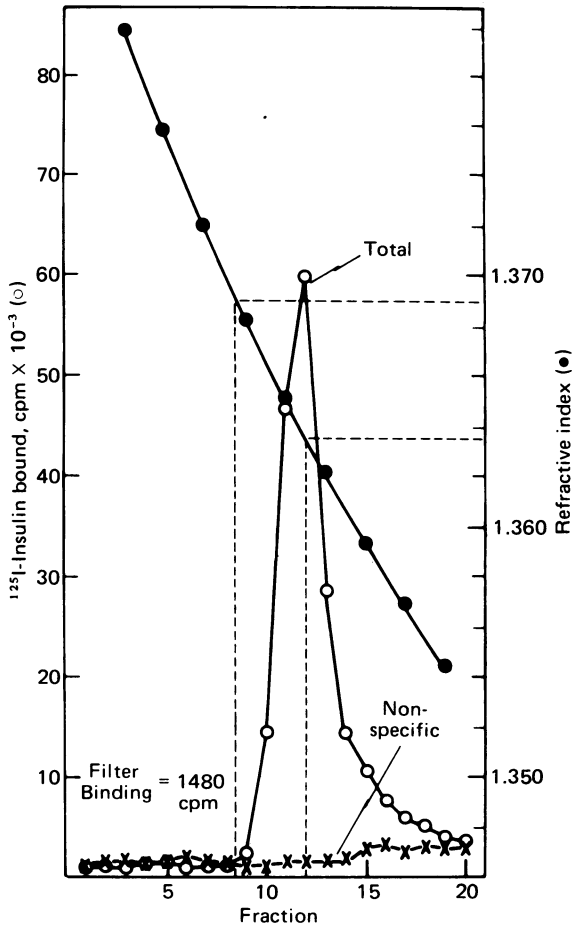

FIG. 1. Insulin binding to Triton-solubilized receptor from 2.2 × 10^6 differentiated 3T3-L1 cells after isopycnic banding in a CsCl density gradient. After centrifugation, 100-μl fractions were collected and assayed for ^{125}I-insulin (9.8 × 10^5 cpm/pmol) binding activity. Filter binding was 1480 cpm.

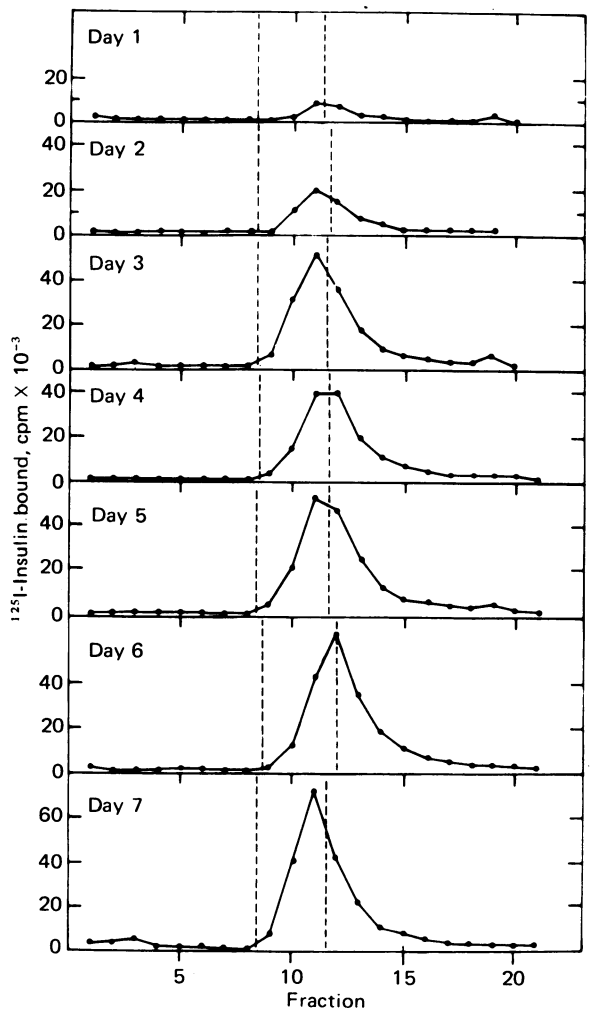

FIG. 2. Isopycnic banding of soluble insulin receptor from differentiating 3T3-L1 preadipocytes (2.5 culture dishes per gradient for days 1–3; 1.25 for days 4–7) cultured in light media. Density gradient centrifugation and ^{125}I-insulin (9.6 × 10^5 cpm/pmol) binding were determined as described in Fig. 1.

1.7 ml of CsCl (0.568 g/ml, η_D^{25} = 1.3735) in the Tris buffer and overlayered with paraffin oil. After 18 hr at 50,000 rpm in a Beckman SW 56 Ti rotor at 4°C, 50- or 100-μl fractions were collected from the bottom of the tube. Each fraction was incubated overnight with 2 nM ^{125}I-insulin in Krebs–Ringer phosphate buffer at pH 7.4, (without Ca^{2+} or Mg^{2+}) containing 0.1% Triton X-100 and 0.1% bovine serum albumin in a total volume of 250 μl.

Bound ^{125}I-insulin was determined by the polyethylene glycol precipitation method (23). Nonspecific binding was determined in the presence of 4.6 μM unlabeled insulin. Filter binding of ^{125}I-insulin in the absence of soluble receptor was similar to nonspecific binding; to obtain specific binding when nonspecific binding was not measured, filter binding was subtracted from total binding. Insulin degradation was <4% as determined by precipitation with trichloroacetic acid. Binding of ^{125}I-insulin (2 nM) to cell monolayers was measured as described (18). After iodination (18), insulin was further purified by gel filtration on Sephadex G-50.

RESULTS

Preliminary experiments were conducted to test the feasibility of banding and detecting the insulin receptor from 3T3-L1 cells after prolonged isopycnic centrifugation in CsCl gradients. A typical profile of the soluble insulin receptor in a CsCl gradient is shown in Fig. 1. A single sharp peak of total insulin binding activity* was evident; nonspecific binding was <0.2% of labeled insulin in the assay and <2% of the specific binding in the peak fractions. The dotted vertical lines are position markers for light and heavy receptor at refractive indices (η_D^{25}) of 1.3635 and 1.3690, respectively. Studies with [^{3}H]Triton X-100 indicated that levels of the detergent capable of inhibiting insulin binding (23) were confined to the top two or three fractions of the gradient.

In order to utilize the density-labeling approach for the analysis of insulin receptor turnover in differentiating 3T3-L1 cells, it was necessary to establish that changes in receptor level measured after extraction and isopycnic banding paralleled those measured in intact cell monolayers. Therefore, during the course of differentiation, insulin binding to intact 3T3-L1 cells was compared with insulin binding to soluble receptor banded in CsCl gradients. The gradient profiles of soluble receptor in Fig. 2 reveal that peak area increased with time and maintained a constant density distribution, indicating that differentiation does not cause detectable alteration of receptor density. The 10- to 12-fold increase in insulin binding to soluble light receptor during differentiation correlated well with that of insulin

* Insulin binding to receptor was measured at a subsaturating concentration of insulin (2 nM) in CsCl gradients. Comparisons of receptor level reflect relative rather than absolute receptor number. The amount of insulin bound to soluble receptor at subsaturating concentrations of insulin has been shown to be proportional to receptor concentration (23).

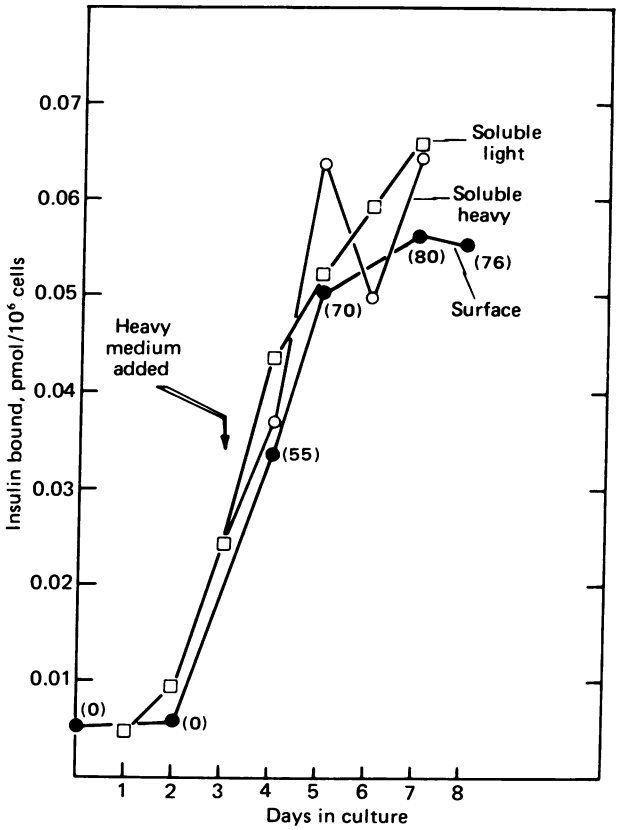

FIG. 3. Insulin binding to cell monolayers and soluble receptor from cells cultured in light or heavy medium. Heavy medium was added on day 3. Values for soluble receptor were calculated from the results shown in Figs. 2 and 4. Numbers in parentheses are percentage of adipocytes.

binding to intact cell monolayers (Fig. 3). These changes parallel the increase in the percentage of adipocytes (Fig. 3) in the cell population.

Binding isotherms for soluble receptor isolated from CsCl gradients retain the same nonlinearity in Scatchard plots (data not shown) observed for insulin binding to cells.† Extrapolation to saturating insulin concentration indicates that 0.28 pmol of insulin is bound to soluble receptor from 10^6 cells, a value close to our reported value (18) for intact differentiated cells.

To assess the effect of dense amino acids on the increase of insulin receptor level during differentiation, on day 3 cells were switched from light medium to heavy medium containing dense amino acids. The gradient profiles of receptor extracted from cells incubated in heavy and light media were then compared during the next several days (Fig. 4). Within 16 hr, receptor from cells incubated in heavy medium shifted to a higher-density region of the gradient. No further change in density was observed. The increase in peak area with time (Fig. 4, heavy; also, Fig. 3) parallels that from cells incubated in light medium (Fig. 4, light; also, Fig. 3). Thus, the normal increase in insulin receptor level during differentiation is not appreciably affected by dense amino acids. Furthermore, when extracts of cells incubated in light or heavy medium (day 5, Fig. 4) were mixed (1 hr at 4 or 24°C) prior to centrifugation, heavy and light receptors were resolved and their respective positions in CsCl gradients were not altered (results not shown). The same results were obtained when cells were mixed prior to homogenization and extraction.

† This nonlinearity has been interpreted to arise either from multiple classes of independent binding sites or from negative cooperativity (24).

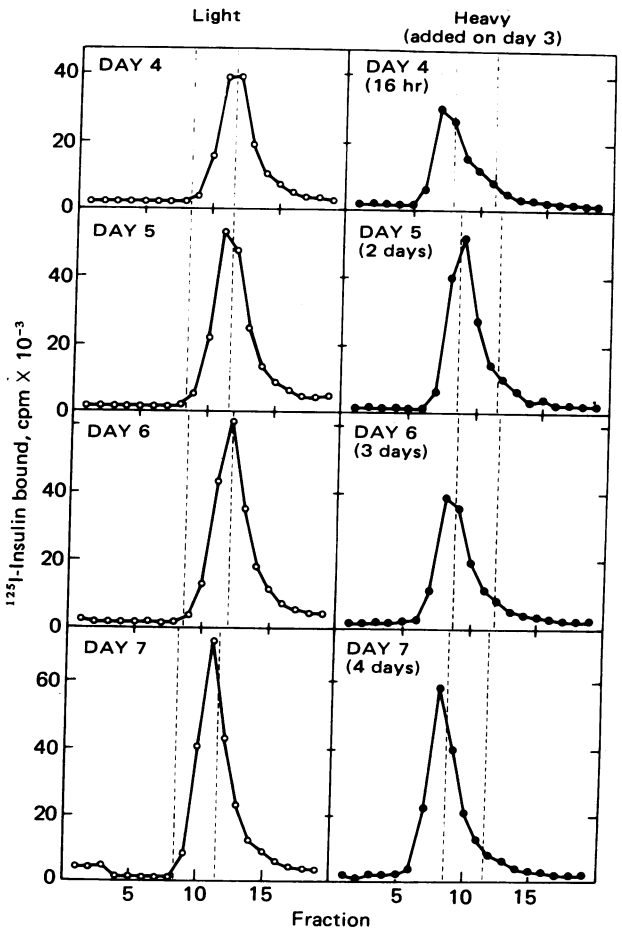

FIG. 4. Isopycnic banding of soluble insulin receptor from differentiating 3T3-L1 preadipocytes (1.25 culture dishes per gradient) cultured in light (*Left*) or heavy (*Right*) medium. On day 3, some cells cultured in light medium were switched to heavy medium. Density gradient centrifugation and ^{125}I-insulin (9.9×10^5 cpm/pmol) binding were determined as described in Fig. 1.

To measure the kinetics of receptor synthesis and degradation, 3T3-L1 cells were shifted to heavy medium late in differentiation (day 6) and the change in distribution of light and heavy receptors was followed (Fig. 5). As early as 2–3 hr after the shift to heavy medium, newly synthesized heavy receptor was detected in CsCl gradients. During the ensuing 24-hr period the peak of heavy receptor continued to increase concomitantly with a decrease in light receptor. By 27.5 hr, light receptor was almost totally replaced by newly synthesized heavy receptor.

Because light receptor contributes little to ^{125}I-insulin binding at the peak for heavy receptor (refractive index = 1.3690 for the heavy position marker of CsCl gradients in Figs. 1, 2, and 4), the relative amount of heavy receptor can be determined from the height of the heavy receptor peak. Analysis of the data from 28 CsCl gradients revealed an excellent linear correlation ($r^2 = 0.976$) between peak area and peak height. By using the average peak area-to-height ratio, 7.4, obtained for the 0- and 27.5-hr time points (Fig. 5), the relative amount of heavy receptor in each gradient in Fig. 5 was determined. The amount of light receptor was calculated by difference (total minus heavy). From these data, the relative rates of synthesis of heavy (new) receptor and degradation of light (old) receptor were evaluated (Fig. 6). It is evident (Fig. 6 *Inset*) that the decay of light receptor follows first-order kinetics. The kinetics of receptor synthesis and degradation can be described by:

$$R_t = \frac{k_s}{k_d}(1 - e^{-k_d t}) + R_0 e^{-k_d t} \qquad [1]$$

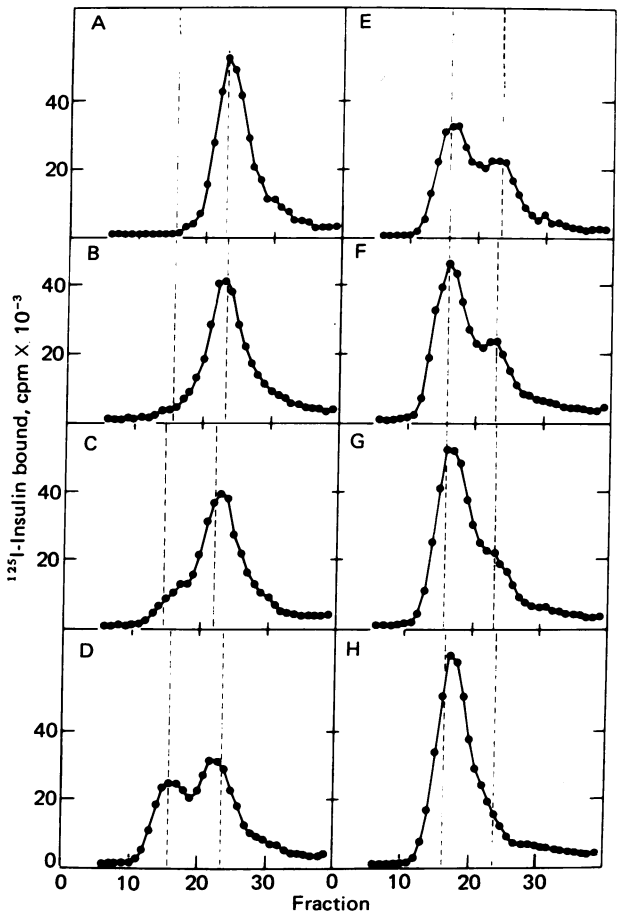

FIG. 5. Isopycnic banding of soluble insulin receptor from differentiated 3T3-L1 adipocytes (2.5 culture dishes per gradient) switched to heavy medium on day 6. After centrifugation, 50-μl fractions were collected and assayed for ^{125}I-insulin (8.1 X 10^5 cpm/pmol) binding activity. Time after switch and total insulin binding capacity in each gradient (as pmol of insulin bound per 10^6 cells): *A*, 0 hr, 0.053; *B*, 2 hr, 0.049; *C*, 3 hr, 0.053; *D*, 6 hr, 0.058; *E*, 9 hr, 0.055; *F*, 12 hr, 0.066; *G*, 16 hr, 0.071; *H*, 27.5 hr, 0.072.

in which receptor level, R_t, at time t is described by a constant synthetic rate k_s, a first-order degradation constant k_d, and an initial receptor level R_0 at zero-time. The half-life of the insulin receptor, calculated from the rate constant of degradation, k_d (Fig. 6), is 6.7 hr. Thus, the rate of insulin receptor degradation is approximately 10 times faster than the mean rate of turnover of total cellular protein in differentiated 3T3-L1 cells (unpublished data).

There appears to be a lag of 1.5 hr between the addition of heavy medium and the first appearance of heavy receptor (Fig. 6). This lag probably represents the time between initiation of receptor synthesis and formation of active receptor—i.e., the time required for processing and assembly.

DISCUSSION

The density-labeling of receptor provides an unambiguous basis for distinguishing between "old" and newly synthesized membrane receptor without isolating the receptor in pure form (3–5). This approach, used first to study the acetylcholine receptor (3–5), has been successfully applied in modified form to determine rates of synthesis and degradation of the insulin receptor in 3T3-L1 adipocytes. In principle, this technique should be applicable to any receptor. Importantly, the method avoids the use of inhibitors of protein synthesis, as usually used to follow receptor degradation when ligand-binding activity alone serves as the basis for estimating receptor decay. The use of inhibitors such as cycloheximide may inhibit or prevent the synthesis of short-lived proteins involved in the turnover of the receptor. Indeed, this may account for the far shorter half-life of the insulin receptor determined in the present investigation compared to that estimated in previous studies. The $t_{1/2}$ for receptor degradation in 3T3-L1 adipocytes found in this study was 6.7 hr (Fig. 6 *Inset*).

Insulin receptor decay in 3T3-L1 cells and other cell lines (21, 25, 26‡) measured in the presence of cycloheximide had a $t_{1/2}$ of 20–40 hr. Thus, it appears that the synthesis of a short-lived protein(s) is required for the normal turnover of the receptor. Alternatively, our low value for the half-life of the insulin receptor could be due to an increased rate of receptor degradation induced by insulin present in the medium. This does not seem likely, however, because insulin receptor down regulation does not appear to occur in 3T3-L1 adipocytes (19–21).

Exposure of 3T3-L1 cells to tunicamycin, an inhibitor of protein *N*-glycosylation, also leads to an apparent short half-life (9 hr) of the receptor (25). This suggests that tunicamycin may inhibit the normal processing of the insulin receptor (a glycoprotein) to yield inactive receptor without affecting receptor degradation.

Compelling evidence indicates that Triton-solubilized insulin receptor exists in a multimeric form consisting of several subunits (26–28). If this is the state of the soluble receptor from 3T3-L1 cells in CsCl gradients, the complex is very stable, indicating that there is little, if any, subunit interchange in solution or in the cell membrane. This is supported by the finding that intermediate density forms were not observed when homogenous heavy and light receptors were mixed prior to centrifugation. Furthermore, only one major density class of receptor was detected in CsCl gradients and the density of this receptor was unaltered by differentiation (Fig. 2). During the shift from light to heavy receptor, the increasing heavy peak and the decreasing light peak showed no evidence of subunit interchange because no variation in their respective peak densities occurred (Fig. 5). Although unlikely, it would be possible for a receptor to form a stable complex with another membrane protein. Were this to occur, the apparent half-life of the receptor would be biased toward that of the membrane protein.

The increase in level of insulin receptor on the cell surface during differentiation of 3T3-L1 preadipocytes could involve regulated changes in the rate of: (*i*) receptor synthesis, (*ii*) receptor degradation, (*iii*) insertion into the plasma membrane from an existing active receptor pool, or (*iv*) formation of active receptor from inactive receptor or receptor subunits. Our results are inconsistent with mechanisms *iii* and *iv*. During differentiation the increase of insulin binding to total soluble receptor closely paralleled that of binding to intact cells (Fig. 3). In contrast, mechanism *iii* predicts constant levels of binding to soluble receptor throughout differentiation. Contrary to mechanism *iv*, the increased insulin binding capacity of differentiated adipocytes can be accounted for by new receptor protein synthesis. When 3T3-L1 cells were incubated with heavy medium early in the differentiation process, subsequent increases in insulin binding were associated solely with newly synthesized heavy receptor (Figs. 3 and 4).

The relative contributions of mechanisms *i* and *ii* have not yet been definitely established; however, it appears that increased rate of receptor synthesis accounts for a major part of the increase of receptor level during differentiation. Undifferentiated 3T3-L1 cells and 3T3-C2 cells possess predominantly heavy receptor after 20–24 hr of exposure to medium

‡ Kosamakos, F. C. & Roth, J. (1976) *Endocrine Society, 58th Annual Meeting*, Abstr. 26.

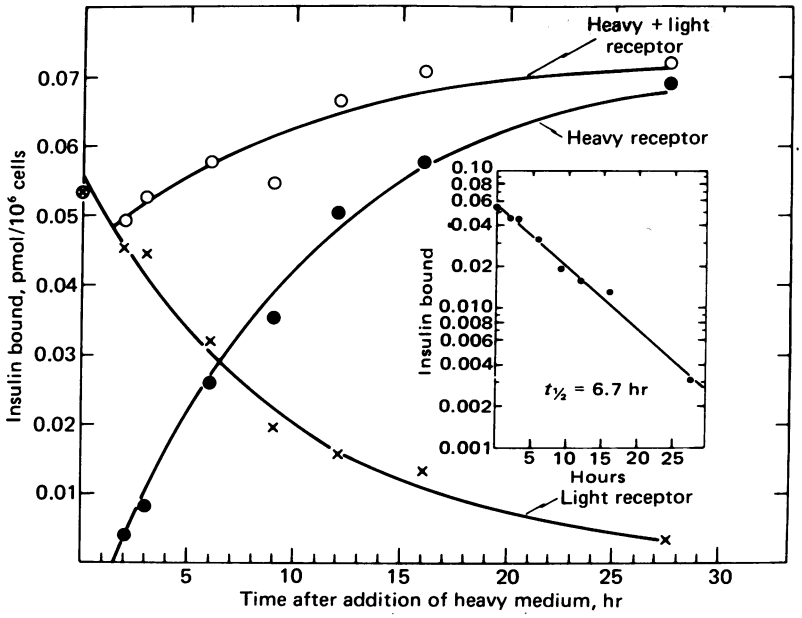

FIG. 6. Kinetics of heavy receptor synthesis and light receptor degradation in differentiated 3T3-L1 adipocytes. (*Inset*) Semilogarithmic plot of light receptor degradation. The data are from Fig. 5. The line through the data points for light receptor represents a least-squares fit ($r^2 = 0.987$) to Eq. 1, with k_s for synthesis of light receptor in heavy medium = 0. Values of 0.103 hr^{-1} for k_d and 6.7 hr for receptor half-life were obtained. The line through the data points for heavy receptor represents the values of R_t generated by Eq. 1, with $t_0 = 1.5$ hr, $k_s = 0.0075$ pmol of insulin binding activity (at 2nM) per hr per 10^6 cells (approximated by the limiting slope of heavy receptor synthesis at t_0), $k_d = 0.103\ hr^{-1}$, and heavy receptor $R_0 = 0$ at t_0. The line through the data points for total receptor was generated by using the above values for k_s and k_d and $R_0 = 0.048$ pmol of insulin bound per 10^6 cells at $t_0 = 1.5$ hr from the least-squares fit of the data for light receptor decay).

containing dense amino acids (results not shown), as do differentiated 3T3-L1 cells. Although changes in receptor binding upon differentiation of 3T3-L1 cells could occur by alteration of receptor half-life, preliminary experiments indicate that the shift from light receptor to heavy receptor is only about 50% complete after 10 hr. The >10-fold increase in receptor level during differentiation (Fig. 3) would require a >10-fold reduction of receptor half-life ($t_{1/2} < 1$ hr) in undifferentiated 3T3-L1 cells were only an alteration in k_d responsible. This definitely is not the case.

Further investigations using the density-labeling approach should enable us to determine: (*i*) to what extent, if any, an altered rate of receptor degradation contributes to the increase of insulin receptor level in differentiating 3T3-L1 preadipocytes and (*ii*) whether the apparent "down regulation defect" in differentiated 3T3-L1 cells is due to the cells' lack of response to hormone or to insulin-induced alterations in both receptor synthesis and degradation rates which operate to maintain an unchanged level of receptor. Moreover, the density shift technique should provide a means to determine whether down regulation occurs through a change in synthesis or degradation rates in cells, like 3T3-C2 fibroblasts, which are known to exhibit down regulation of insulin receptor.

The authors express their appreciation to Drs. D. M. Fambrough, P. N. Devreotes, J. M. Gardner, J. Osborne, and P. Englund for their helpful discussions during the early phases of this work. This investigation was supported by Research Grants AM 14574-5 from the National Institutes of Health and 78822 from the American Heart Association, Inc. B.C.R. was supported by a Postdoctoral Fellowship (AM 05506) from the National Institutes of Health.

1. Catt, K. J., Harwood, J. P. Aguilera, G. & Dufau, M. L. (1979) *Nature (London)* **280**, 109–116.
2. Tell, G., Haour, F. & Saez, J. (1978) *Metabolism* **27**, 1566–1592.
3. Fambrough, D. M. & Devreotes, P. N. (1976) in *Biogenesis and Turnover of Membrane Macromolecules*, ed. Cook, J. S. (Raven, New York), pp. 124–144.
4. Devreotes, P. N., Gardner, J. M. & Fambrough, D. M. (1977) *Cell* **10**, 365–373.
5. Gardner, J. M. & Fambrough, D. M. (1979) *Cell* **16**, 661–674.
6. Green, H. & Kehinde, O. (1974) *Cell* **1**, 113–116.
7. Green, H. & Kehinde, O. (1975) *Cell* **5**, 19–27.
8. Green, H. & Kehinde, O. (1976) *Cell* **7**, 105–113.
9. Mackall, J. C., Student, A. K., Polakis, S. E. & Lane, M. D. (1976) *J. Biol. Chem.* **251**, 6462–6464.
10. Mackall, J. C. & Lane, M. D. (1977) *Biochem. Biophys. Res. Commun.* **79**, 720–725.
11. Eckel, R. H., Fujimoto, W. Y. & Brunzell, J. D. (1977) *Biochem. Biophys. Res. Commun.* **78**, 288–293.
12. Kuri-Harcuch, W. & Green, H. (1977) *J. Biol. Chem.* **252**, 2158–2160.
13. Rubin, C. S., Lai, E. & Rosen, O. M. (1977) *J. Biol. Chem.* **252**, 3554–3557.
14. Wise, L. S. & Green, H. (1978) *Cell* **13**, 233–242.
15. Spooner, P. M., Chernick, S. S., Garrison, M. M. & Scow, R. O. (1979) *J. Biol. Chem.* **254**, 1305–1311.
16. Coleman, R. A., Reed, B. C., Mackall, J. C., Student, A. K., Lane, M. D. & Bell, R. M. (1979) *J. Biol. Chem.* **253**, 7256–7261.
17. Hoffman, S. S. & Kolodny, G. M. (1977) *Exp. Cell Res.* **107**, 293–299.
18. Reed, B. C., Kaufmann, S. H., Mackall, J. C., Student, A. K. & Lane, M. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4876–4880.
19. Rubin, C. S., Hirsch, A., Fung, C. & Rosen, O. M. (1978) *J. Biol. Chem.* **253**, 7570–7578.
20. Chang, T. & Polakis, S. E. (1978) *J. Biol. Chem.* **253**, 4693–4696.
21. Karlsson, F. A., Grunfeld, C., Kahn, C. R. & Roth, J. (1979) *Endocrinology* **104**, 1383–1391.
22. Cuatrecasas, P. (1972) *J. Biol. Chem.* **247**, 1980–1991.
23. Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 318–322.
24. Cuatrecasas, P. & Hollenberg, M. (1976) *Adv. Protein Chem.* **30**, 251–450.
25. Rubin, C. S. & Rosen, O. M. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 306.
26. Ginsberg, B. H., Kahn, C. R., Roth, J. & DeMeyts, P. (1976) *Biochem. Biophys. Res. Commun.* **73**, 1068–1073.
27. Maturo, J. M. & Hollenberg, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3070–3074.
28. Krupp, M. N. & Livingston, J. N. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2593–2598.