Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 2908–2912, May 1981
Biochemistry

Role of glycosylation and protein synthesis in insulin receptor metabolism by 3T3-L1 mouse adipocytes

(receptor synthesis and turnover/receptor density shift)

BRENT C. REED*, GABRIELE V. RONNETT, AND M. DANIEL LANE

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

Communicated by Albert L. Lehninger, February 17, 1981

ABSTRACT The roles of glycosylation and protein synthesis in the maintenance of insulin receptor levels and turnover rates in 3T3-L1 adipocytes were investigated. The heavy isotope density-shift technique was employed to determine the effects of inhibitors of these processes on the rates of synthesis and degradation of cellular insulin receptors. Inhibitors of protein synthesis—i.e., cycloheximide and puromycin—markedly decreased the rate of degradation of the insulin receptor, the half-life for receptor decay increasing from 7.5 hr without to 25 hr with inhibitor. The continued synthesis of a short-lived protein appears to be necessary for normal insulin receptor turnover. Tunicamycin, a potent inhibitor of core oligosaccharide addition in the formation of *N*-glycosidically linked glycoproteins, caused the depletion of cell-surface and total cellular detergent-extractable insulin receptors. This inhibitor totally prevented the formation of functional newly synthesized insulin receptor, yet receptor degradation was affected minimally. Thus, glycosylation of the receptor appears to be required for its activation after translation.

The level of insulin receptor on the surface of target cells appears to be an important factor governing the sensitivity of cells to insulin. Several well-documented examples of this relationship exist. In certain disease states, for example, the resistance of tissues to insulin correlates inversely with the number of receptors per cell (1–3). During the differentiation of 3T3-L1 mouse preadipocytes into adipocytes, cells acquire both an increased level of insulin receptor and an increased sensitivity to insulin (4–7). To understand the mechanism(s) by which the level of functional insulin receptors is regulated, it will be necessary to delineate the steps in the normal pathway of receptor synthesis, processing, and turnover.

One of the crucial processing steps in the synthesis of the insulin receptors appears to be glycosylation. Compelling evidence (8) now indicates that this receptor is a membrane glycoprotein. The importance of the oligosaccharide moiety to insulin receptor function became apparent when Cuatrecasas's group (9) showed that the insulin binding capacity of the receptor was markedly decreased by sequential treatment with neuraminidase and β-galactosidase. Thus, the presence of the capping sugars—i.e., sialyl and β-galactosyl residues—appears to be essential for insulin binding. Nevertheless, a contradictory report has appeared (10).

More recently, Rosen *et al.* (11) reported that treatment of 3T3-L1 adipocytes with tunicamycin, an inhibitor of protein *N*-glycosylation (12–14), caused a rapid depletion of cell-surface insulin receptors when compared with cells treated with cycloheximide to block the synthesis of new receptor protein. Hence, the half-life for the decay of surface receptor was reduced from 24 hr with cycloheximide present to ≈9 hr with tunicamycin present. These findings were of particular interest, because we subsequently observed (15, 16) that the half-life for the insulin receptor of 3T3-L1 adipocytes, determined without inhibitors by the heavy isotope density-shift technique, was only 7–8 hr. The possibility was considered, therefore, that by inhibiting glycosylation, tunicamycin might block formation of active receptor rather than accelerate receptor turnover. In addition, the long half-life of the surface receptor of cells treated with cycloheximide suggested that protein synthesis may be required for normal insulin receptor turnover. In the present investigation the heavy isotope density-shift technique was employed to characterize the effects of these inhibitors on the steady-state rates of insulin receptor synthesis and degradation in 3T3-L1 adipocytes. It is demonstrated that tunicamycin totally inhibits the formation of functional receptor and that protein synthesis is required for normal receptor degradation.

EXPERIMENTAL PROCEDURE

Culture Conditions. Undifferentiated 3T3-L1 preadipocytes were cultured in 6-cm dishes (Falcon) with Dulbecco's modified Eagle's medium as described (17), but with fetal calf serum replacing calf serum. Differentiated 3T3-L1 adipocytes were generated from 2-day postconfluent preadipocytes by exposure for 3 days to medium (3.5 ml) supplemented with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 10 μg of insulin per ml. 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. By day 6, 80–90% of the cells expressed the adipocyte phenotype; the cell monolayers were used for experiments between days 7 and 10.

Density-Shift Experiments. Cell monolayers were incubated at 37°C with 10% CO_2/90% air in Dulbecco's modified Eagle's medium (minus amino acids) containing 1 mg of heavy amino acids (>90% enriched in ^{13}C, ^{15}N, and ^{2}H) per ml and supplemented with tryptophan, cystine, glutamine, 10% fetal calf serum, and 10 μg of insulin per ml as described (15, 16).

Isopycnic Banding of Soluble Insulin Receptor in CsCl Gradients. A detailed description of this method has been published (15, 16). Briefly, bound unlabeled insulin was removed by successive incubation of cell monolayers at 37°C in insulin-free medium. After homogenization of the cells and centrifugation at 100,000 × *g* for 1 hr at 4°C, the total cellular membrane pellet was extracted with buffered 4% (vol/vol) Triton X-100. The supernate after centrifugation at 100,000 × *g* for 1 hr at 4°C, which contained >90% of the ^{125}I-labeled insulin (^{125}I-insulin) binding activity of the total cellular membrane fraction, was stored at −50°C. The thawed extract was mixed with a CsCl solution and the receptor was banded isopycnically by centrifugation for 18 hr at 50,000 rpm in a Beckman SW 56Ti rotor at 4°C. The gra-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* Present address: Department of Biochemistry, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235.

dient was fractionated into 50-μl fractions, which were used for ^{125}I-insulin binding assays to locate and quantitate the receptor. The cells from two 6-cm culture dishes yielded sufficient receptor for one gradient.

Total and nonspecific ^{125}I-insulin binding were determined on intact monolayers of 3T3-L1 adipocytes as described (5). ^{125}I-Insulin binding to soluble receptor in the CsCl gradient was measured by incubating each 50-μl fraction with ^{125}I-insulin (2 nM) 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. The amount of receptor-bound ^{125}I-insulin was measured after precipitation of the complex with polyethylene glycol (18) and centrifugation at 6000 rpm for 30 min at 4°C in a Sorvall HS-4 rotor (19). Nonspecific binding was determined identically in the presence of 4.6 μM insulin. Light receptor banded at a position centered at fraction 22 or 23 (refractive index η_D^{25} = 1.3635) (15). The integrated areas of peaks of light and heavy insulin receptor were quantitated as previously reported (15).

For Scatchard analysis of insulin binding to total soluble light receptor isolated from CsCl gradients, six cell monolayers were processed as above and fractions 16–34 from two identical gradients were combined. Aliquots (50 μl) of the combined fractions were incubated with different concentrations of ^{125}I-insulin (with or without 4.6 μM unlabeled insulin) and processed as above to determine total and nonspecific ^{125}I-insulin binding. ^{125}I-insulin (10^6 cpm/pmol) was prepared as described (5) and further purified by gel filtration on Sephadex G-50.

Measurement of [^{3}H]Leucine and [^{3}H]Glucosamine Incorporation into Total Cellular Protein. Duplicate cell monolayers were incubated at 37°C with 200 μCi of [4,5-^{3}H]leucine or 35 μCi of [6-^{3}H]glucosamine (1 Ci = 3.7 × 10^{10} becquerels) in 3.5 ml of standard medium for 0, 4, 9, and 13 hr in the presence or absence of tunicamycin (Merck), cycloheximide, or puromycin. After washing, cells were homogenized and the incorporation of ^{3}H into trichloroacetic acid-precipitable protein was determined. To determine the fraction of the [^{3}H]glucosamine-derived ^{3}H-labeled protein that was N-glycosidically linked, cell homogenates were incubated for 18 hr at pH 13 and 37°C prior to precipitation with trichloroacetic acid to remove O-linked oligosaccharide chains.

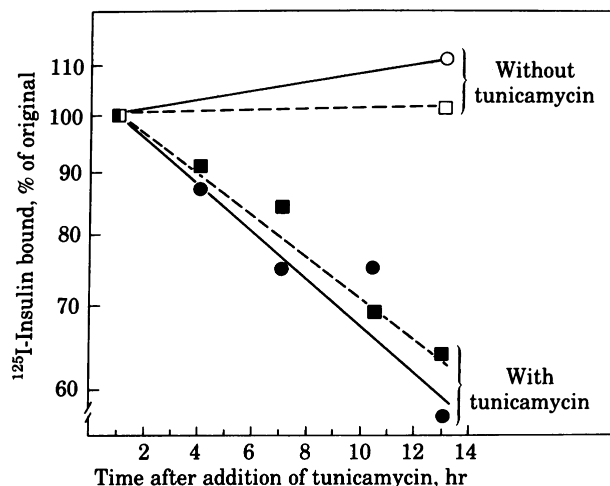

FIG. 1. Time course of tunicamycin-induced depletion of cell-surface and total cellular detergent-extractable insulin receptor. 3T3-L1 adipocytes were maintained under normal culture conditions in the absence (□, ○) or presence (■, ●) of tunicamycin at 1.0 μg/ml. ^{125}I-Insulin binding to cell-surface receptors (□, ■) and total cellular Triton X-100-extracted receptors after isopycnic banding on CsCl gradients (○, ●) were determined at 2 nM insulin and 4°C.

RESULTS

Tunicamycin-Induced Depletion of Cell-Surface and Total Cellular Detergent-Extractable Insulin Receptor. To compare

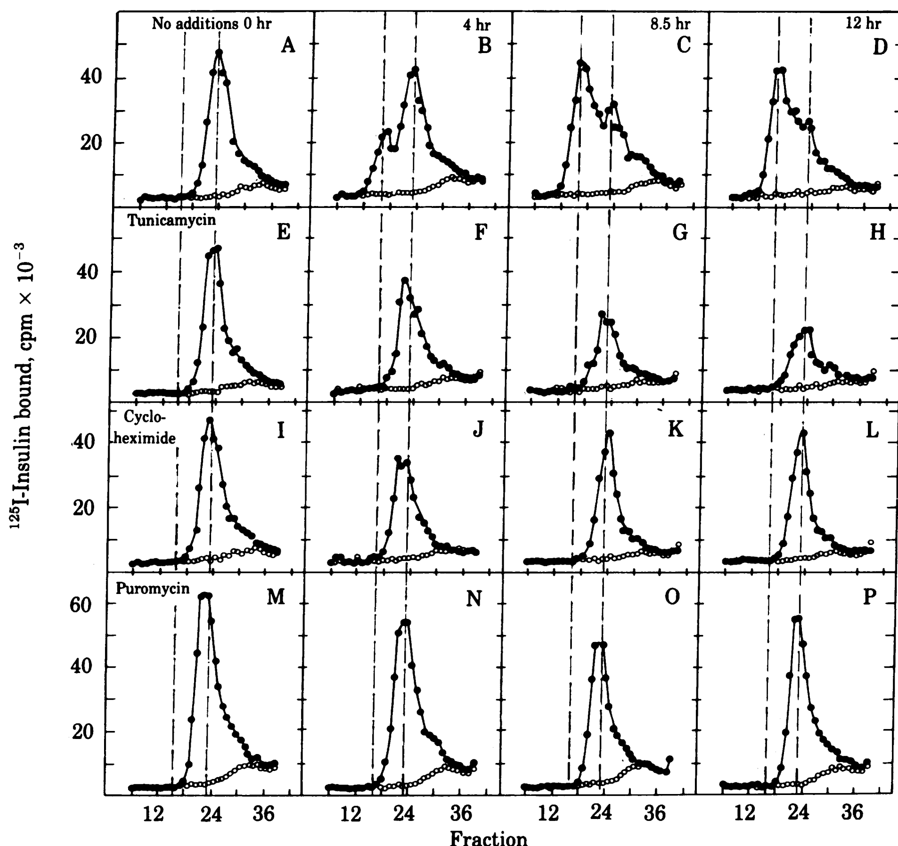

FIG. 2. Altered isopycnic banding patterns of light and heavy insulin receptors from 3T3-L1 adipocytes incubated with heavy amino acids in the presence and absence of tunicamycin, cycloheximide, or puromycin. 3T3-L1 adipocytes were incubated in normal medium containing tunicamycin (1.3 μg/ml; E–H), cycloheximide (5 μg/ml; I–L), puromycin (100 μg/ml; M–P), or no additions (A–D). After 1 hr the cells were shifted to medium containing heavy (>90% ^{15}N, ^{13}C, ^{2}H) amino acids and the same inhibitor concentration as above. At 0, 4, 8.5, and 12 hr after the shift to heavy amino acids, insulin receptor extracted from total cellular membranes was isopycnically banded on CsCl density gradients; 50-μl fractions were collected and assayed for ^{125}I-insulin binding activity (at 2 nM insulin, 10^6 cpm/pmol). Total (●) and nonspecific (○) binding were determined on separate but identical gradients. The vertical broken lines show the normal banding positions of heavy (left) and light (right) receptor.

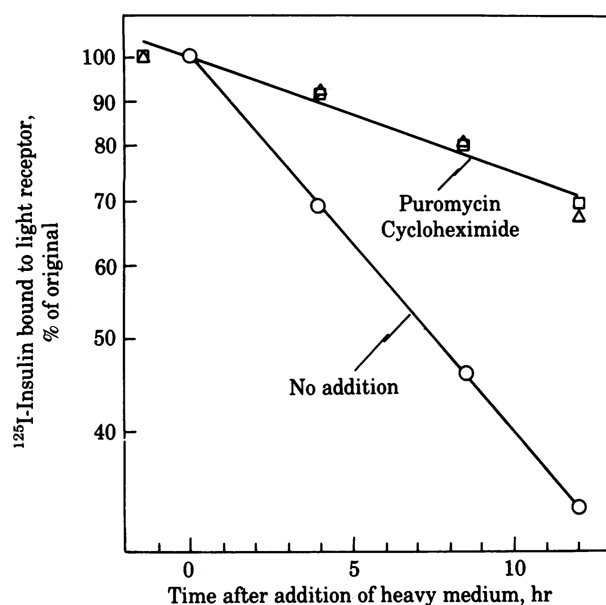

FIG. 3. Semilogarithmic plot of light receptor degradation in 3T3-L1 adipocytes cultured in the absence (○) and presence of cycloheximide (△) or puromycin (□). The amount of light receptor remaining after each interval of time was quantitated by using the data from the CsCl gradients in Fig. 2. For those experiments performed in the presence of tunicamycin, cycloheximide, and puromycin, dense receptor does not contribute to insulin binding in the gradient; thus, total specific insulin binding activity in the gradient represents the amount of light receptor remaining. In the absence of inhibitors (Fig. 2 *A–D*), incomplete resolution of the newly synthesized peak of heavy receptor and the previously synthesized peak of light receptor requires a different quantitative analysis for light receptor. An empirically derived linear relationship exists between the peak height of a single density class of receptor and its peak area (15, 20). The constant ratio of peak area to peak height derived from a large number of experiments was used to compute the peak area of the heavy receptor peak from the measured value for the peak height of the partially resolved dense receptor peak; the leading edge of the light receptor peak does not contribute to the binding in the peak fractions—i.e., fractions 16 and 17, of the dense receptor peak. The value for insulin binding to light receptor was then obtained by subtracting the calculated binding contribution of dense receptor from the total insulin binding on the gradient.

the effect of tunicamycin on the rates of loss of cell-surface and total cellular Triton X-100-extractable insulin receptor, cell monolayers of differentiated 3T3-L1 adipocytes were exposed to medium with or without tunicamycin (1 μg/ml). At intervals after the addition of tunicamycin, the ^{125}I-insulin binding capacity (at 2 nM insulin) of both cell surface and isopycnically-banded soluble receptor extracted from total cellular membranes was determined. The ^{125}I-insulin binding capacity of isopycnically-banded Triton X-100-extracted receptor has been shown to be a reliable measure of total cellular receptor (15, 16).

In the presence of tunicamycin, ^{125}I-insulin binding to cell-surface and detergent-extractable receptor decreased with apparent first-order kinetics and a half-life of 15–16 hr (Fig. 1). Under the same conditions, tunicamycin caused an 80% inhibition of [^{3}H]glucosamine incorporation into cellular protein in *N*-glycosidically linked form[†] and a 10% inhibition of [^{3}H]leucine incorporation into protein. In the absence of tunicamycin the levels of both cell-surface and detergent-extractable receptor remained relatively constant (Fig. 1). These results are similar to those of Rosen *et al.* (11) but show in addition that the tunicamycin-induced loss of surface receptor is not merely due to its translocation to an intracellular compartment. This possibility is ruled out by the fact that both surface and total cellular detergent-extractable receptor levels decrease in parallel.

Analysis of Inhibitor Effects on Insulin Receptor Turnover by the Heavy Isotope Density-Shift Technique. The heavy isotope density-shift technique was employed to determine whether changes in the level of functional insulin receptor, such as the tunicamycin-induced loss of receptors, result from a decreased rate of receptor synthesis or an increased rate of receptor degradation. This method permits the quantitation of newly synthesized insulin receptor after cells have been exposed to medium containing amino acids enriched in nitrogen-15, carbon-13, and deuterium (>90% ^{15}N, ^{13}C, ^{2}H). Because the incorporation of heavy amino acids into receptor protein increases the density of newly synthesized receptor, "new heavy" and "old light" receptor, solubilized in detergent, can be resolved by isopycnic banding on CsCl density gradient and then quantitated (15, 16). Validation of this procedure for measuring simultaneously the rates of insulin receptor synthesis and degradation has been documented (15, 16, 20, 21). Typical isopycnic CsCl density gradients of insulin receptor, extracted from 3T3-L1 adipocytes cultured in the absence of inhibitor, are shown in Fig. 2 *A–D*. A rise in the level of newly synthesized dense receptor occurs with a concomitant fall in the level of previously synthesized light receptor.

A semilogarithmic plot of the amount of light receptor remaining, calculated from the integrated areas of light receptor peaks (15) in Fig. 2, versus time after the shift to heavy medium is shown in Fig. 3. Light receptor was degraded in an apparent first-order decay process with a half-life of approximately 7.5 hr. In several experiments the half-lives observed ranged between 6.7 and 9.4 hr.

This degradation rate for total cellular insulin receptors, determined by the density-shift method, is considerably faster than that ($t_{1/2}$ = 24–40 hr) for surface receptor determined by methods (7, 11, ‡) requiring the use of inhibitors, such as cycloheximide, to prevent receptor synthesis. Because these inhibitors are not required in density-shift experiments and, therefore, were not used, the possibility was considered that inhibition of protein synthesis interferes with normal receptor turnover. To test this hypothesis, density-shift experiments were conducted in the presence of cycloheximide or puromycin. Under conditions in which protein synthesis (results not shown) and the appearance of newly synthesized dense receptor (Fig. 2) were inhibited >90% by these agents, degradation rates of previously synthesized light receptor were drastically reduced (Fig. 2), the half-life for receptor decay increasing from 7.5 hr without to 25 hr with inhibitor (Fig. 3). Thus, it is evident that protein synthesis is required to maintain a normal rate of degradation of the insulin receptor, suggesting that the continued synthesis of a protein of very short half-life is required for maintenance of normal receptor turnover rate.

Effect of Tunicamycin on the Rate of New Receptor Formation and Old Receptor Degradation. The density-shift technique was employed to determine whether the tunicamycin-induced depletion of surface and total cellular insulin receptor level was due to an altered rate of receptor formation or to a change in the rate of receptor degradation. Treatment of 3T3-L1 adipocytes with tunicamycin at a level (1.3 μg/ml) that in-

† Approximately 60% of total [^{3}H]glucosamine incorporated into trichloroacetic acid-precipitable protein is in *N*-glycosidically linked form as judged by its stability in alkali.

‡ Kosamakos, F. C. & Roth, J. (1976) *58th Annual Meeting of the Endocrine Society*, San Francisco, CA, June 23–25, abstr. 26.

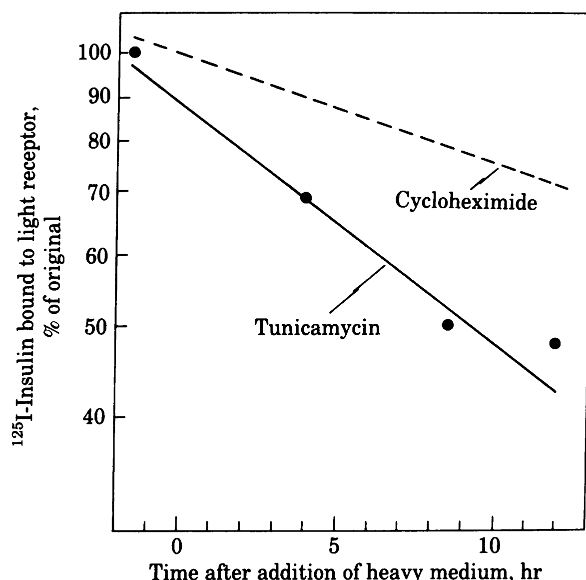

FIG. 4. Semilogarithmic plot of light receptor degradation in 3T3-L1 adipocytes cultured in the presence of tunicamycin (●). The broken line represents the degradation rate in the presence of cycloheximide and was taken from Fig. 3. The level of light receptor binding activity was determined by analyzing the gradient results in Fig. 2 as described for Fig. 3.

hibited [^{3}H]glucosamine incorporation into alkali-stable form† in cellular protein by >95% totally inhibited the formation of active newly synthesized dense receptor (fractions 16 and 17, Fig. 2 *E–H*). Nevertheless, degradation of previously synthesized light receptor continued, as evidenced by the decrease in size of the light receptor peak with time (Fig. 2 *E–H*), albeit at a somewhat reduced rate. Tunicamycin increased the half-life for receptor decay from 7.5 hr (Fig. 3) to 9–10 hr (Fig. 4). Under these conditions the rate of [^{3}H]leucine incorporation into cellular protein was maintained at 70–90% of control values.

Because the experiments above, showing that tunicamycin inhibits the appearance of newly synthesized dense insulin binding activity (Fig. 2 *E–H*), employed only a single subsaturating insulin concentration (2 nM), it was important to ascertain whether this effect was due to a reduced affinity of the receptor for insulin or to a reduced number of functional receptors. Differentiated 3T3-L1 cells were exposed to tunicamycin or cycloheximide for approximately one receptor half-life. Insulin receptor was then extracted from total cellular membranes and banded isopycnically, and the pooled receptor-containing fractions were used for ^{125}I-insulin binding analysis. Fig. 5 compares the insulin binding properties of receptor isolated from tunicamycin- and cycloheximide-treated and untreated cells. Receptor from control cells bound approximately 2-fold more insulin at all insulin concentrations than did receptor from cells treated with tunicamycin or cycloheximide for one half-life. The ratio of insulin bound to receptor from treated versus untreated cells ranged between 0.40 and 0.63, averaging 0.52 ± 0.09 (Fig. 5*B*). The binding isotherms in Scatchard plots (Fig. 5*A*) had similar slopes and when extrapolated to infinite insulin concentration gave values of 0.13 and 0.24 pmol of insulin binding sites per 10^6 for tunicamycin- or cycloheximide-treated and untreated cells, respectively. Thus, tunicamycin and cycloheximide reduced the total number of functional insulin receptors and had no detectable effect on the affinity of the receptor for insulin. On the basis of these findings, it is concluded that by blocking *N*-glycosylation with tunicamycin, activation of newly synthesized insulin receptor polypeptide(s) is prevented.

DISCUSSION

The use of the heavy isotope density-shift approach has made it possible to determine the rate of turnover of the insulin receptor without interrupting normal protein synthetic processes with inhibitors such as cycloheximide. That such inhibitors can give rise to erroneous receptor turnover rates is indicated by the fact that cycloheximide lengthened the half-life for degradation of the receptor of 3T3-L1 adipocytes from 7.5 hr to 25 hr (Fig. 3). Other investigators have reported half-lives of 24–40 hr for the insulin receptor in this (7, 11) and another‡ cell system that had been exposed to cycloheximide. As shown in this communication, the effect of cycloheximide is due to an inhibition of protein synthesis *per se* and not to a specific effect of the inhibitor on insulin receptor synthesis, because puromycin also increased the half-life of the receptor to 25 hr (Fig. 3).

These results suggest that the continued synthesis of a short-lived protein is required for a rate-limiting step in the degradation of the receptor. The pathway for receptor degradation

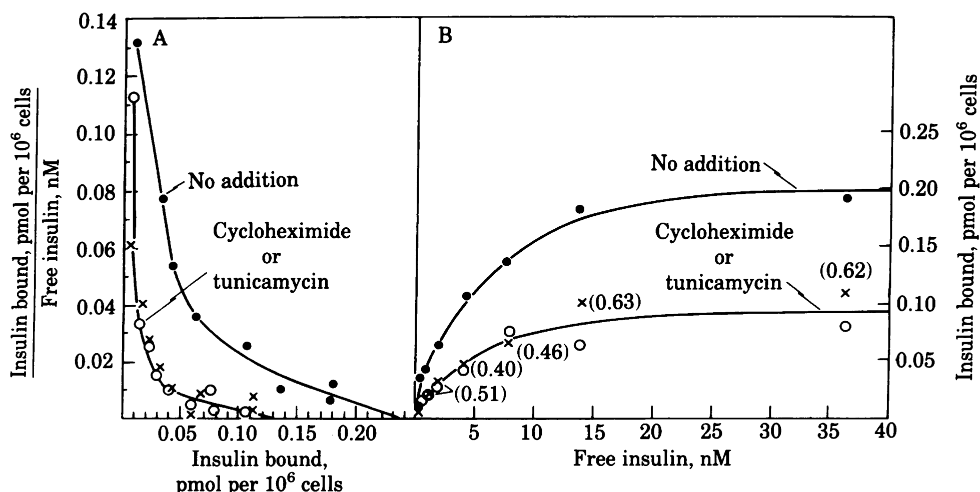

FIG. 5. Insulin binding isotherms for Triton X-100-extracted, isopycnically banded insulin receptor from untreated (●) and tunicamycin-treated (○) or cycloheximide treated (×) 3T3-L1 adipocytes. Treated cells were incubated with medium containing tunicamycin (1.3 μg/ml) or cycloheximide (5 μg/ml) for approximately one receptor half-life—i.e., 13 or 23 hr, respectively. The numbers in parentheses represent fractional values for insulin binding by receptor from cycloheximide-treated cells divided by insulin binding by receptor from untreated cells at identical insulin concentration.

is complex, apparently involving several steps, including receptor clustering on the plasma membrane, endocytosis, fusion of the endocytotic vesicles with lysosomes, and final breakdown in the lysosome (22–25). Hence, there are numerous sites at which such a short-lived protein could function. The fact that insulin receptors on the cell surface *per se* also exhibit a long half-life (11) in the presence of cycloheximide suggests that the site of action of this putative short-lived protein precedes internalization of the receptor into the intracellular compartment. This is supported by the observation that insulin-induced receptor down-regulation, which appears to involve the translocation of receptors into the cell (26), is inhibited by cycloheximide (27).

A recent report (11) indicated that the rate of loss of the insulin binding capacity of 3T3-L1 adipocytes was considerably faster ($t_{1/2}$ = 9 hr) in the presence of tunicamycin than in the presence of an inhibitor of protein synthesis ($t_{1/2} \approx$ 24 hr). While these results suggested that tunicamycin accelerated the depletion of cell-surface receptors, the approach employed could not distinguish between an effect on rate of receptor formation and an effect on rate of receptor degradation. The use of the density-shift technique in the present investigation permits the distinction between synthesis and degradation. The possibility that tunicamycin causes receptor depletion by accelerating the rate of old light receptor degradation was ruled out by the finding that the half-life for light receptor degradation was, in fact, not decreased by the inhibitor; rather, the half-life for receptor degradation was somewhat increased by tunicamycin (Fig. 4). Actually, tunicamycin totally blocked the formation of active dense insulin receptors in cells shifted to medium containing heavy amino acids (Fig. 2). This suggests that *N*-glycosylation of the receptor (or a receptor-associated) polypeptide(s) is required to produce functional receptor. It is possible that tunicamycin inhibits the glycosylation and activation of another protein which is required for the processing, activation, or both of the insulin receptor.

The loss of biochemical function of glycoproteins, either by cleavage of the oligosaccharide chain or by preventing its addition to the apoprotein, has been demonstrated for only a few glycoproteins (28, 29), including the insulin receptor. Because the insulin receptor mediates its biochemical effects from the cell surface, further study of the role of the oligosaccharide chain of this receptor system in signal transductions is warranted. Tunicamycin should prove useful in such studies because it could provide a means by which the aglyco-receptor can be generated and investigated.

We acknowledge helpful discussions with Dr. Gerald Hart during the course of this investigation. This investigation was supported by grants from the National Institutes of Health (AM-14574), the American Heart Association (78822), and the Kroc Foundation. B.C.R. was supported by a postdoctoral fellowship from the National Institutes of Health (AM-05506). G.V.R. was supported by Medical Scientist Training Program Grant GM-07309 from the National Institues of Health.

1. Kahn, C. R. (1980) *Metabolism* **29**, 455–466.
2. Harrison, L. C., Martin, F. I. R. & Melick, R. A. (1976) *J. Clin. Invest.* **58**, 1435–1441.
3. Crettaz, M. & Jeanrenaud, B. (1980) *Metabolism* **2**, 467–473.
4. Hoffman, S. S. & Kolodny, G. M. (1977) *Exp. Cell Res.* **107**, 293–299.
5. Reed, B. C., Kaufmann, S. H., Mackall, J. C., Student, A. K. & Lane, M. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4876–4880.
6. Rubin, C. S., Hirsch, A., Fung, C. & Rosen, O. M. (1978) *J. Biol. Chem.* **253**, 7570–7578.
7. Karlsson, F. A., Grunfeld, C., Kahn, C. R. & Roth, J. (1979) *Endocrinology* **104**, 1383–1391.
8. Jacobs, S., Hazum, E. & Cuatrecasas, P. (1980) *J. Biol. Chem.* **255**, 6937–6940.
9. Cuatrecasas, P. (1974) *Annu. Rev. Biochem.* **43**, 169–214.
10. Maturo, J. M. & Hollenberg, M. D. (1979) *Can. J. Biochem.* **57**, 497–506.
11. Rosen, O. M., Chia, G. H., Fung, C. & Rubin, C. S. (1979) *J. Cell. Physiol.* **99**, 37–42.
12. Takatsuki, A., Kohno, K. & Tamura, G. (1975) *Agric. Biol. Chem.* **39**, 2089–2091.
13. Tkacz, J. S. & Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun.* **65**, 248–257.
14. Struck, D. K. & Lennarz, W. J. (1977) *J. Biol. Chem.* **252**, 1007–1013.
15. Reed, B. C. & Lane, M. D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 285–289.
16. Reed, B. C. & Lane, M. D. (1980) *Adv. Enzyme Regul.* **18**, 97–117.
17. Mackall, J. C., Student, A. K., Polakis, S. E. & Lane, M. D. (1976) *J. Biol. Chem.* **251**, 6462–6464.
18. Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 318–322.
19. Krupp, M. N. & Livingston, J. N. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2593–2598.
20. Reed, B. C., Ronnett, G. V., Clements, P. R. & Lane, M. D. (1981) *J. Biol. Chem.* **256**, 3917–3925.
21. Lane, M. D., Reed, B. C. & Clements, P. R. (1980) in *Control of Cellular Division and Development*, ICN–UCLA Symposia on Molecular and Cell Biology, ed. Fox, C. F. (Liss, New York), Vol. 18, in press.
22. Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1979) *Nature (London)* **279**, 679–685.
23. Brown, M. S. & Goldstein, J. L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3330–3337.
24. Gorden, P., Carpentier, J.-L., Freychet, P. & Orci, L. (1980) *Diabetologia* **18**, 263–274.
25. Schlessinger, J. (1980) *Trends Biochem. Sci.* **5**, 210–214.
26. Krupp, M. & Lane, M. D. (1981) *J. Biol. Chem.* **256**, 1689–1694.
27. Kosamakos, F. C. & Roth, J. (1980) *J. Biol. Chem.* **255**, 9860–9869.
28. Weintraub, B. C., Stannard, B. S., Linnekin, D. & Marshall, M. (1980) *J. Biol. Chem.* **255**, 5715–5723.
29. Struck, D. K. & Lennarz, W. J. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans*, ed. Lennarz, W. J. (Plenum, New York), pp. 35–83.