Biochemistry

 Volume 26, Number 11 June 2, 1987

Articles

Antibody-Induced Down-Regulation of a Mutated Insulin Receptor Lacking an Intact Cytoplasmic Domain†

David O. Morgan,‡ Leland Ellis,§,∥ William J. Rutter,§ and Richard A. Roth*,‡

Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305, and Hormone Research Institute, University of California, San Francisco, California 94143

Received July 14, 1986; Revised Manuscript Received January 15, 1987

ABSTRACT: Insulin receptor down-regulation was studied in various Chinese hamster ovary (CHO) cell lines expressing transfected human insulin receptor cDNAs. In addition to a cell line expressing the normal receptor (CHO.T line), three lines expressing mutated receptors were studied: the CHO.T-t line, which expresses a receptor with a degraded cytoplasmic domain due to the removal of the C-terminal 112 amino acids, and the CHO.YF1 and CHO.YF3 lines, in which important autophosphorylation sites of the receptor kinase (tyrosines-1162 and -1163) have been replaced by phenylalanine. A monoclonal anti-receptor antibody, but not insulin itself, was found to down-regulate cell surface receptor levels in all four cell lines by 60–80% after 18-h treatment at 37 °C. Down-regulation of the CHO.T and CHO.T-t receptors occurred at similar antibody concentrations and with a similar time course, although the maximum level of CHO.T-t down-regulation (60%) was generally lower than the level of CHO.T down-regulation (80%). Pulse–chase labeling of these two cell types with [^{35}S]methionine revealed that antibody treatment of both CHO.T and CHO.T-t cells resulted in a similar increase in the rate of degradation of mature receptor subunits. These results indicate that antibody-induced down-regulation of the insulin receptor in these cells can occur in the absence of various autophosphorylation sites of the receptor and that the mechanism of antibody-induced down-regulation is different from that for insulin.

Exposure of cells to insulin leads to a reversible decrease, or down-regulation, of insulin receptor levels on the cell surface (Gavin et al., 1974). This process involves the internalization of the hormone–receptor complex (Knutson et al., 1983; Standaert & Pollet, 1984; Hedo & Simpson, 1984) and, in some cells, an increase in the rate of receptor degradation (Kasuga et al., 1981). A similar loss of cell surface receptors is observed in cells exposed to various polyclonal (Grunfeld, 1984; Taylor & Marcus-Samuels, 1984; Maron et al., 1984) and monoclonal (Maron et al., 1984; Roth et al., 1983) antibody preparations; these antibodies also appear to act by accelerating receptor degradation (Taylor & Marcus-Samuels, 1984; Roth et al., 1983). Both insulin- and antibody-induced down-regulation may be important in several insulin-resistant disease states. For example, in obesity and type II diabetes mellitus, hyperinsulinemia is associated with decreased receptor number and insulin resistance (Roth & Grunfeld, 1981). In addition, insulin resistance in patients with high titers of anti-receptor autoantibodies may in part be due to receptor down-regulation (Kahn, 1979).

The mechanism by which insulin and anti-receptor antibodies enhance receptor internalization and degradation is unclear. There may be a role for insulin-stimulated receptor phosphorylation, both by an intrinsic tyrosine kinase activity in the receptor β-subunit and by other as yet uncharacterized serine/threonine kinases [for reviews, see Kahn (1985) and Carter-Su and Pratt (1984)]. Alternatively, another portion of the receptor cytoplasmic domain may interact with other cytoplasmic components.

† This work was supported by NIH Research Grants AM 34926 and 21344, Research Career Development Award AM 01393 (to R.A.R.), and an Alberta Heritage Foundation award (to D.O.M.).

* Correspondence should be addressed to this author.

‡ Stanford University School of Medicine.

§ University of California.

∥ Present address: Howard Hughes Medical Institute and Department of Biochemistry, The University of Texas Health Sciences Center, Dallas, TX 75235-9050.

0006-2960/87/0426-2959$01.50/0

Recently, the cloning, sequencing, and expression of a human insulin receptor cDNA have provided a novel approach to the study of the mechanisms underlying receptor down-regulation (Ullrich et al., 1985; Ebina et al., 1985a,b). Truncations and site-directed mutagenesis were used to construct mutant insulin receptor cDNAs which were expressed in Chinese hamster ovary (CHO)[1] cells (Ellis et al., 1986). In addition to a cell line expressing the normal full-length receptor (CHO.T line), three lines expressing mutant receptors were developed. The CHO.T-t line expresses a truncated receptor lacking the C-terminal 112 amino acids of the β-subunit (Ellis et al., 1986). Antigenic mapping and phosphorylation studies in vivo and in vitro indicate that this receptor possesses a proteolyzed β-subunit cytoplasmic domain which completely lacks kinase activity and phosphorylation acceptor sites (Ellis et al., 1986). The CHO.YF1 and CHO.YF3 lines express receptors in which tyrosine-1162 or both tyrosines-1162 and -1163, respectively, have been replaced by phenylalanines. Replacement of these tyrosines was found to greatly reduce receptor autophosphorylation and kinase activity (Ellis et al., 1986). In the present study, these four cell lines were used to clarify the role of receptor kinase activity and the β-subunit cytoplasmic domain in down-regulation by a monoclonal anti-receptor antibody.

Experimental Procedures

Cell Lines. The development and characterization of the four stably transformed CHO cell lines are described elsewhere (Ellis et al., 1986).

Measurements of Insulin Receptor after Down-Regulation. Semiconfluent 16-mm wells of the various CHO cell lines were incubated in normal growth medium (Ham's F-12 plus 10% fetal calf serum) containing either no additions or the desired concentrations of insulin or monoclonal antibody MC51. After the desired incubation time at 37 °C, cells were washed twice with buffer B (100 mM Hepes, pH 7.8, 120 mM NaCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 15 mM sodium acetate, 10 mM glucose, and 1% bovine serum albumin) and then incubated 4 h at 4 °C or 1 h at 15 °C with 0.5 mL of buffer B containing 50000 cpm of ^{125}I-labeled monoclonal antibody 3D7 (iodinated by the Chloramine-T method to a specific activity of 15 Ci/g). Cells were then washed twice with ice-cold TBS (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl), solubilized 30 min at 37 °C with 0.5 mL of 0.03% SDS, and counted in a γ counter. Nonspecific binding of ^{125}I-3D7, determined by incubating normal CHO cells with ^{125}I-3D7 as described above, has been subtracted. Nonspecific binding was always less than 10% of total binding.

Measurements of Insulin Receptor Turnover. Semiconfluent 60-mm dishes of CHO.T or CHO.T-t cells were washed twice with methionine-free DME H16 medium and then incubated 30 min at 37 °C in 3.5 mL of this medium containing 10% dialyzed fetal calf serum and 0.15 mCi of [^{35}S]methionine (1330 Ci/mmol; Amersham). Cells were washed with 20 mM sodium phosphate, pH 7.4, and 150 mM NaCl and incubated for 0, 2, 6, or 24 h with 3 mL of complete medium containing 14 mM L-methionine plus either 1 μM insulin and 100 nM antibody MC51 or no additions. Cells were then washed twice with cold TBS and solubilized 15 min at 4 °C with 1.2 mL of 50 mM Hepes, pH 7.6, 1% Triton X-100, 1 mg/mL bacitracin, and 1 mM phenylmethanesulfonyl fluoride. Lysates were clarified by centrifugation (10000g, 10 min) and immunoprecipitated by a 16-h incubation at 4 °C with *Staphylococcus aureus* coated with rabbit anti-mouse IgG and a pool of monoclonal antibodies 5D9 and 3D7 (Ellis et al., 1986). After being extensively washed, the immunoprecipitated proteins were eluted from the *S. aureus*, reduced, and electrophoresed on a 7.5% polyacrylamide gel. Gels were soaked in Amplify (Amersham) before drying and autoradiography. Insulin receptor bands were excised and counted by liquid scintillation counting.

[1] Abbreviations: CHO, Chinese hamster ovary; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton(s).

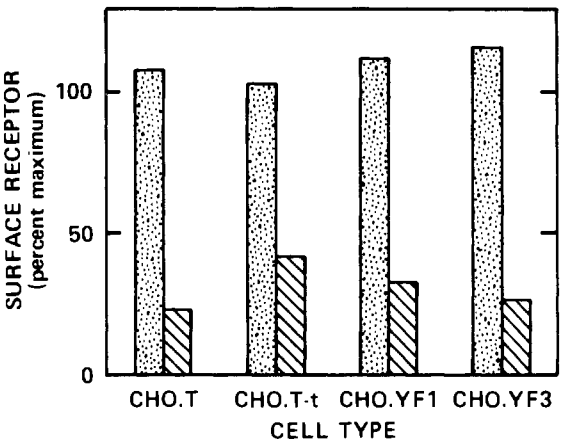

FIGURE 1: Effect of insulin and anti-receptor antibody treatment on cell surface insulin receptor levels in various CHO cell lines. Duplicate 16-mm wells of the various transformed CHO cell lines were incubated 18 h at 37 °C in the absence or presence of 1 μM insulin (stippled bars) or 100 nM antibody MC51 (hatched bars), and the amount of insulin receptor remaining on the cell surface was determined by the binding of ^{125}I-labeled antibody 3D7. 100% binding of ^{125}I-3D7, determined with untreated cells, was 3289, 4972, 2600, and 7466 cpm in CHO.T, CHO.T-t, CHO.YF1, and CHO.YF3 cells, respectively.

Results

The four transfected CHO cell lines were treated for 18 h at 37 °C with either 1 μM insulin or 100 nM monoclonal antibody MC51, which recognizes the insulin binding site of the human insulin receptor (Roth et al., 1982). The amount of insulin receptor remaining on the cell surface after hormone or antibody treatment was measured by the binding of ^{125}I-labeled monoclonal antibody 3D7. This antibody specifically recognizes the extracellular surface of the human insulin receptor at a site distinct from the insulin binding site, and therefore, the presence of insulin or antibody MC51 will not interfere with receptor quantitation by this method (Morgan et al., 1986; Morgan & Roth, 1986). Furthermore, antibody 3D7 does not bind to the endogenous hamster insulin receptor which is present at low levels on these cells.

Insulin treatment had no effect on surface receptor levels in any of the four cell lines (Figure 1). Treatment with antibody MC51, however, resulted in a major decrease (60–80%) in receptor levels in all four cell lines. To further confirm that insulin had no effect, similar experiments were performed in which surface receptor levels were measured by ^{125}I-labeled insulin binding. CHO.T cells were treated with insulin or MC51 as before and then washed extensively (Taylor & Marcus-Samuels, 1984) to remove the majority of the bound ligand. The binding of ^{125}I-insulin was reduced slightly (12%) in cells treated for 18 h with 1 μM insulin. Treatment with MC51 led to a major decrease (50%) in ^{125}I-insulin binding (not shown).

Subsequent experiments were then performed to characterize the ability of antibody MC51 to down-regulate the various receptor types. Since this antibody down-regulated

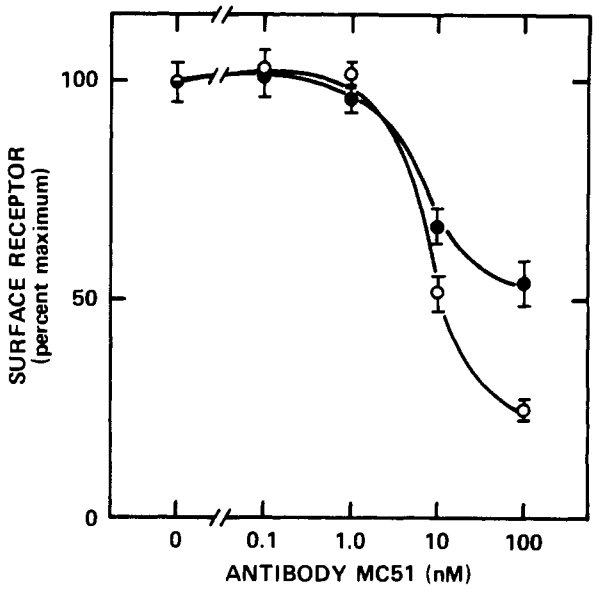

FIGURE 2: Insulin receptor down-regulation in CHO.T and CHO.T-t cells by various amounts of anti-receptor antibody. CHO.T (○) and CHO.T-t (●) cells were incubated 18 h at 37 °C in the presence of various concentrations of monoclonal antibody MC51, after which the amount of cell surface insulin receptor was determined by ^{125}I-3D7 binding. 100% values were 1051 cpm (○) and 1738 cpm (●). Values are means of triplicates ±SD.

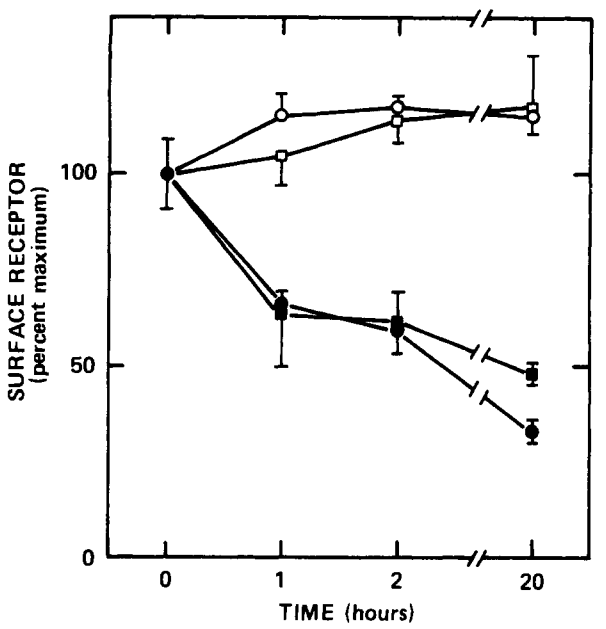

FIGURE 3: Time course of insulin receptor down-regulation by anti-receptor antibody. CHO.T (squares) and CHO.T-t (circles) cells were incubated for various times at 37 °C with 100 nM antibody MC51 (closed symbols) or normal mouse IgG (open symbols), and the amount of cell surface insulin receptor was determined by ^{125}I-3D7 binding. 100% values were 835 cpm (CHO.T) and 915 cpm (CHO.T-t). Values are means of triplicates ±SD.

all three mutant receptors (CHO.T-t, CHO.YF1, and CHO.YF3), it was clear that antibody-induced down-regulation not only occurred in the absence of the major receptor autophosphorylation sites (tyrosines-1162 and -1163) but also occurred in receptors lacking an intact cytoplasmic domain (CHO.T-t). Further studies were therefore confined to the CHO.T-t receptor, which possesses the most extreme alteration in the structure of the kinase domain of the receptor.

The effect of antibody MC51 on receptor levels was dose dependent, and half-maximal down-regulation of both receptor types occurred at similar concentrations of antibody (approximately 5 nM) (Figure 2). The CHO.T-t receptor was maximally down-regulated to a lesser extent (45%) than the full-length receptor (65%).

The time course of antibody-induced down-regulation revealed that an initially rapid phase was followed by a gradual decline (Figure 3). Once again, the patterns of down-regulation in the CHO.T and CHO.T-t cells were similar, although the extent of down-regulation in CHO.T-t was not as great as that seen in CHO.T cells. Normal mouse IgG had no significant effect on receptor levels (Figure 3).

The effect of MC51 on insulin receptor levels was also found to be specific: MC51 treatment for 18 h at 37 °C had no effect on the levels of another cell surface molecule, the receptor for insulin-like growth factor II (data not shown).

Previous studies have demonstrated that down-regulation is sometimes associated with an increased rate of receptor degradation (Kasuga et al., 1981; Taylor & Marcus-Samuels, 1984; Roth et al., 1983). To determine if similar changes in receptor degradation were occurring in down-regulated CHO.T and CHO.T-t cells, studies of receptor turnover were performed. Cells were pulse-labeled with [^{35}S]methionine for 30 min and then chased with unlabeled methionine for up to 24 h in the absence or presence of either 1 μM insulin or 100 nM antibody MC51. Cells were then solubilized, immunoprecipitated with anti-receptor antibodies, and analyzed on SDS–polyacrylamide gels (Figure 4). As seen in previous studies (Ellis et al., 1986), the CHO.T receptor was visible as three bands: the 200-kDa biosynthetic precursor, the 135-kDa α-subunit, and the 95-kDa β-subunit. The CHO.T-t receptor was visible as a 180-kDa precursor, a 150-kDa precursor (a proteolytic fragment of the 180-kDa species), and the 135-kDa α-subunit. The truncated β-subunit of the CHO.T-t receptor is known to be sensitive to proteolysis and is present in negligible amounts in these experiments [see Figure 4 and Ellis et al. (1986)]. None of these bands are present in immunoprecipitates with normal mouse IgG, as demonstrated previously (Ellis et al., 1986).

Measurement of radioactivity in excised receptor bands demonstrated that insulin and antibody MC51 had the following effects. In both cell types, the amount of receptor precursors rose after 2 h and then declined to about 5% of their original level by the end of the 24-h chase period. There was no effect of insulin or antibody MC51 treatment on the rate of precursor processing in either CHO.T or CHO.T-t cells. Similarly, as seen in the studies of cell surface receptor (Figure 1), insulin had no effect on the levels of mature receptor subunits in both cell types. In both untreated and insulin-treated cells, the amounts of α- and β-subunits rose to a peak at 6 h and fell to about 50% of this level after 24 h. In contrast, antibody MC51 treatment caused a dramatic reduction in the amount of mature receptor subunits in both cell types at the 6- and 24-h time points. After 24 h, the amounts of ^{35}S-labeled CHO.T α-, CHO.T β-, and CHO.T-t α-subunits were decreased by 75%, 79%, and 74%, respectively, compared to their levels in untreated cells. This degree of receptor loss is similar to the 60–80% reduction in cell surface receptor observed after 18 h of antibody treatment (Figures 1–3).

Discussion

The present work demonstrates that a monoclonal antibody to the insulin receptor binding site is capable of down-regulating the levels of human receptors expressed from transfected cDNAs in CHO cells. Down-regulation by the antibody was dose dependent (Figure 2) and involved an initial rapid decrease in cell surface receptor levels, followed by a more gradual decline (Figure 3). Immunofluorescence studies (not

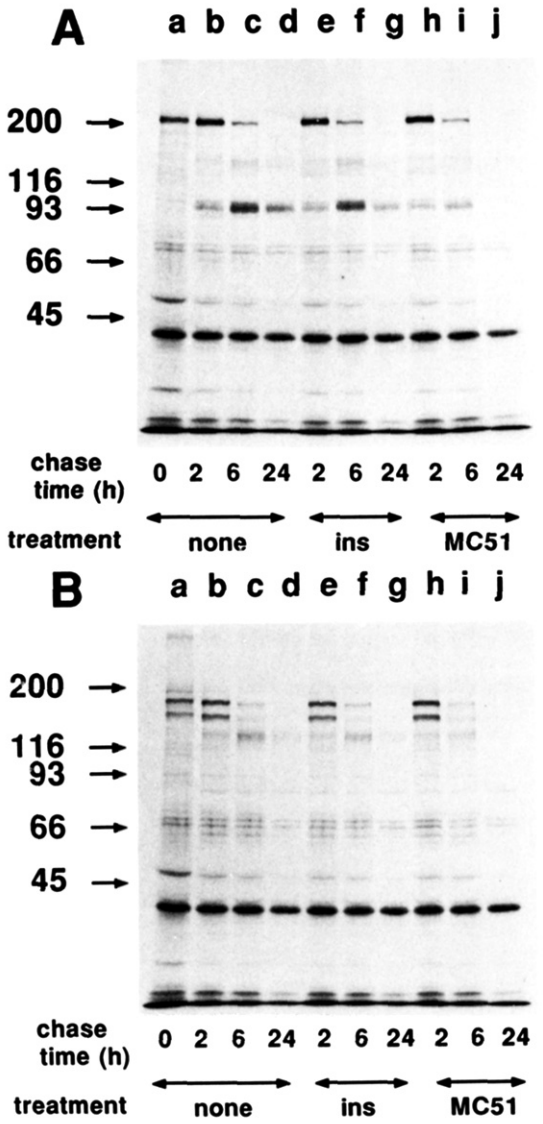

FIGURE 4: Effect of insulin and anti-receptor antibody on insulin receptor turnover in CHO.T and CHO.T-t cells. CHO.T (panel A) and CHO.T-t (panel B) cells were pulse-labeled with [^{35}S]methionine for 30 min at 37 °C and then chased with unlabeled methionine for 0 (lane a), 2 (b, e, and h), 6 (c, f, and i), or 24 h (d, g, and j) in the absence (b–d) or presence of 1 μM insulin (e–g) or 100 nM antibody MC51 (h–j). Cells were then solubilized, immunoprecipitated with monoclonal anti-receptor antibodies, and analyzed by polyacrylamide gel electrophoresis and autoradiography. Molecular weights of protein standards are shown.

shown) indicated that the initial decrease in surface receptor levels was accompanied by receptor internalization. The long-term loss of surface receptors was associated with an increase in the rate of degradation of mature receptor subunits (Figure 4). Down-regulation was specific for the anti-receptor antibody, since normal mouse IgG had no effect on receptor levels (Figure 3). It was also specific for the insulin receptor, since MC51 had no effect on the levels of the receptor for insulin-like growth factor II. Thus, antibody-induced down-regulation in these cells is similar to that seen after treatment of other cell types with insulin or various antibody preparations (Gavin et al., 1974; Knutson et al., 1983; Standaert & Pollet, 1984; Hedo & Simpson, 1984; Kasuga et al., 1981; Grunfeld, 1984; Taylor & Marcus-Samuels, 1984; Maron et al., 1984; Roth et al., 1983).

Insulin treatment had no effect on insulin receptor levels, according to studies of the binding of ^{125}I-labeled antibody 3D7. This may indicate that insulin- and antibody-induced down-regulation occur by different mechanisms. The absence of a major insulin effect was also demonstrated in studies of ^{125}I-insulin binding, which decreased 12% after insulin treatment. Although this may indicate that insulin binding is a more sensitive method of receptor measurement, it is possible that this slight decrease is the result of incomplete removal of bound insulin after the 18-h treatment.

The lack of an insulin effect may have several explanations. For example, the human receptors expressed in these CHO cell lines may not be completely functional. This seems unlikely, since these receptors are known to (i) undergo insulin-stimulated phosphorylation in vivo and in vitro, (ii) transmit the signal for insulin-stimulated glucose uptake (Ebina et al., 1985b; Ellis et al., 1986), and (iii) mediate insulin internalization and degradation (J. Hari and R. A. Roth, unpublished results). Previous studies have also demonstrated that insulin-induced receptor down-regulation may not occur in several cell types which exhibit normal insulin responses and insulin-induced receptor internalization (Rennie & Gliemann, 1981; Bonser & Garcia-Webb, 1984). Second, the extremely large numbers of human receptors in these cell lines [about (0.5–1.0) $\times$ 10^6 receptors/cell] (Ellis et al., 1986) may be saturating the molecular mechanisms involved in insulin-stimulated down-regulation of the endogenous receptor in nontransfected CHO cells, which possess very few insulin receptors (about 3000 per cell) (Podskalny et al., 1984). However, we have recently found that insulin also does not down-regulate the transfected human insulin receptor in CHO cell lines expressing 15 000–20 000 receptors per cell. Third, preliminary evidence suggests that defective receptor glycosylation may interfere with insulin-stimulated down-regulation in CHO cells (Podskalny & Gorden, 1985); thus, the lack of an effect in the present work may be the result of differences in glycosylation between the endogenous CHO receptor and the transfected human insulin receptor. However, the transfected receptors appear to be expressed and processed normally (Figure 4). Finally, it is possible that this line of CHO cells, unlike those previously described (Podskalny & Gorden, 1985), cannot down-regulate its endogenous receptors. However, we have recently found that insulin treatment of these CHO cells does cause insulin receptor down-regulation (data not shown).

In any case, it is clear that none of these potential problems has interfered with antibody-induced down-regulation. Furthermore, in addition to down-regulating the normal receptor, antibody MC51 was found to down-regulate mutant receptors (CHO.YF1 and CHO.YF3) in which crucial autophosphorylation sites were replaced by phenylalanines. It is particularly remarkable that the antibody also down-regulated a highly defective mutant receptor (CHO.T-t) which possesses a β-subunit cytoplasmic domain from which the C-terminal 112 amino acids have been removed. This mutant receptor completely lacks kinase activity in vitro (Ellis et al., 1986). Moreover, no phosphorylation of this receptor was detected in immunoprecipitates of [^{32}P]orthophosphate-labeled whole cells, suggesting that no phosphorylation acceptor sites exist on this receptor (Ellis et al., 1986). Finally, immunoprecipitation of metabolically labeled CHO.T-t cells [see Figure 4 and Ellis et al. (1986)] as well as immunocytochemical studies with a panel of monoclonal antibodies to the β-subunit (unpublished studies), suggest that the entire cytoplasmic domain of this receptor has been extensively proteolyzed. Thus, we conclude that antibody-induced down-regulation and enhanced receptor degradation can occur in the absence of receptor kinase activity, receptor phosphorylation, and even

in the absence of an intact receptor cytoplasmic domain.

Recent data have indicated that antibodies to the epidermal growth factor receptor can also induce the internalization of this receptor without affecting its phosphorylation state (Sunada et al., 1986). Moreover, a truncated form of this receptor which lacks kinase activity has also been reported to be down-regulated by epidermal growth factor (Prywes et al., 1986). These studies raise the possibility that the cytoplasmic structures of the insulin and epidermal growth factor receptors are not necessary for receptor internalization. Other transmembrane proteins, associated with extracellular receptor regions, may therefore provide the necessary connections with the cell interior. Studies of receptors mutated at extracellular sites will be useful in identifying regions of the receptor involved in this process.

Registry No. Insulin, 9004-10-8; insulin receptor tyrosine kinase, 88201-45-0.

REFERENCES

Bonser, A. M., & Garcia-Webb, P. (1984) *Biochem. Biophys. Res. Commun. 119*, 706–712.

Carter-Su, C., & Pratt, W. B. (1984) in *The Receptors* (Conn, P. M., Ed.) Vol. 1, pp 541–585, Academic Press, Orlando, FL.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., & Rutter, W. J. (1985a) *Cell (Cambridge, Mass.) 40*, 747–758.

Ebina, Y., Edery, M., Ellis, L., Standring, D., Beaudoin, J., Roth, R. A., & Rutter, W. J. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8014–8018.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., & Rutter, W. J. (1986) *Cell (Cambridge, Mass.) 45*, 721–732.

Gavin, J. R., III, Roth, J., Neville, D. M., Jr., De Meyts, P., & Buell, D. N. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 84–88.

Grunfeld, C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2508–2511.

Hedo, J. A., & Simpson, I. A. (1984) *J. Biol. Chem. 259*, 11083–11089.

Kahn, C. R. (1979) *Proc. Soc. Exp. Biol. Med. 162*, 13–21.

Kahn, C. R. (1985) *Annu. Rev. Med. 36*, 429–451.

Kasuga, M., Kahn, C. R., Hedo, J. A., Van Obberghen, E., & Yamada, K. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6917–6921.

Knutson, V. P., Ronnett, G. V., & Lane, M. D. (1983) *J. Biol. Chem. 258*, 12139–12142.

Maron, R., Jackson, R. A., Jacobs, S., Eisenbarth, G., & Kahn, C. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7446–7450.

Morgan, D. O., & Roth, R. A. (1986) *Biochemistry 25*, 1364–1371.

Morgan, D. O., Ho, L., Korn, L. J., & Roth, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 328–332.

Podskalny, J. M., Gorden, P. (1985) *Endocrinology (Baltimore) (Suppl.) 116*, 4.

Podskalny, J. M., McElduff, A., & Gorden, P. (1984) *Biochem. Biophys. Res. Commun. 125*, 70–75.

Prywes, R., Livneh, E., Ullrich, A., & Schlessinger, J. (1986) *EMBO J. 5*, 2179–2190.

Rennie, P., & Gliemann, J. (1981) *Biochem. Biophys. Res. Commun. 102*, 824–831.

Roth, J., & Grunfeld, C. (1981) in *Textbook of Endocrinology* (Williams, R. H., Ed.) Sixth ed., pp 15–72, W. B. Saunders, Philadelphia, PA.

Roth, R. A., Cassell, D. J., Wong, K. Y., Maddux, B. A., & Goldfine, I. D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7312–7316.

Roth, R. A., Maddux, B. A., Cassell, D. J., & Goldfine, I. D. (1983) *J. Biol. Chem. 258*, 12094–12097.

Standaert, M. L., & Pollet, R. J. (1984) *J. Biol. Chem. 259*, 2346–2354.

Sunada, H., Magun, B. E., Mendelsohn, J., & MacLeod, C. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 833*, 3825–3829.

Taylor, S. I., & Marcus-Samuels, B. (1984) *J. Clin. Endocrinol. Metab. 58*, 182–186.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, J. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature (London) 313*, 756–761.