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Internalized insulin receptors are recycled to the cell surface in rat hepatocytes

(photoaffinity labeling/electron microscopic autoradiography/receptor subunits)

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ABSTRACT **We have followed the fate of cell surface insulin receptors in isolated rat hepatocytes by both a biochemical and a morphological approach. Hepatocytes were labeled with the photoreactive and biologically active ^{125}I-labeled insulin analogue, [2-nitro-4-azidophenylacetyl^{B2}]des-Phe^{B1}-insulin, under conditions that allow for minimal internalization (2 hr at 15°C). Analysis of the cell-associated radioactivity by NaDodSO$_4$/polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography revealed the specific labeling of a major insulin receptor subunit with M_r 130,000 and a minor degradation product with M_r 125,000. When the cells were exposed at 15°C to trypsin at the end of the association period, these two bands were no longer observed, indicating that the labeled receptors were at the cell surface. This trypsin sensitivity of the receptor disappeared within 30–60 min of incubation of the cells at 37°C, reflecting the internalization of the hormone–receptor complexes. Over the subsequent 4 hr of incubation, this was followed by a progressive reappearance of the receptor complexes at the cell surface, as indicated by the recovery of trypsin sensitivity of the labeled insulin receptors. An identical (both chronologically and quantitatively) journey of the insulin receptors was observed when the labeled material was studied by quantitative electron microscopic autoradiography. Thus, when the cells were incubated at 37°C there was a rapid decrease (30–60 min) in the percentage of autoradiographic grains associated with the plasma membrane, followed by a progressive increase in this percentage over the subsequent 4 hr of incubation. In conclusion, using a biochemical and morphological approach to trace the photoaffinity-labeled insulin receptor, we have shown that the internalized hormone–receptor complex is recycled back to the cell surface.**

The occurrence of receptor recycling that follows receptor-mediated endocytosis has been described for various ligands, including low density lipoproteins (LDL) (1, 2), asialoglycoproteins (3), mannose glycoconjugates (4), lysosomal enzymes (5), and α_2-macroglobulin (6, 7). In all of these cases, the major role of the receptors is to mediate ligand internalization, either by supplying the cells with cholesterol (LDL) or by allowing the removal of injurious agents from extracellular fluids (lysosomal enzymes, mannose, or galactose terminal glycoproteins) (8). However, little information is available concerning the fate of membrane receptors that bind hormones or neurotransmitters and whose major function is to convey a signal to the target cell. Recent studies suggest that receptors for epidermal growth factor (9, 10) and for acetylcholine (11) are internalized but not recycled in the presence of the ligand.

In the present study we have specifically labeled the insulin receptor *in situ* in isolated rat hepatocytes using a photoreactive and biologically active insulin analogue. The fate of the labeled receptor was followed by both a biochemical and a morphological approach. Our results demonstrate that insulin–receptor complexes are rapidly internalized and that the hormone–receptor complexes are recycled back to the cell surface.

MATERIALS AND METHODS

Photoreactive Insulin. The photoreactive insulin analogue [2-nitro-4-azidophenylacetyl^{B2}]des-Phe^{B1}-insulin was prepared as described (12). This analogue retains about 70% of the receptor-binding affinity and biological potency of native insulin (13). The photoreactive insulin was iodinated to a specific activity of 200–250 μCi/μg (1 Ci = 3.7×10^{10} becquerels) by using the same method as that described for native insulin (referred to as "the second modification" in ref. 14).

Isolation and Incubation of Hepatocytes. Hepatocytes were isolated from male Wistar rats (120–150 g) as described (15). Cells were incubated in Krebs–Ringer bicarbonate (KR bicarbonate) buffer (pH 7.4) containing dialyzed bovine serum albumin (fraction V) at 10 mg/ml, gentamycin at 50 μg/ml, bacitracin at 0.8 mg/ml, and, unless otherwise stated, 2 mM phenylmethylsulfonyl fluoride (PhMeSO$_2$F); the incubations were gassed with 5% CO_2/95% O_2. Immediately after isolation, hepatocytes (0.5×10^6 cells per ml) were incubated in the dark with photoreactive ^{125}I-labeled insulin (^{125}I-insulin) for 2 hr at 15°C, conditions in which steady-state binding with minimal insulin internalization is attained (13).

Irradiation Procedure. At the end of the association of photoreactive ^{125}I-insulin, the cells were collected by centrifugation and resuspended in the same volume of insulin-free buffer. The irradiation was conducted at 4°C by using a water-cooled high pressure mercury lamp (Philips HPK 125 W). The light was filtered through a "black glass" filter (U.V.W. 55, Hanau, Federal Republic of Germany). Suspensions (3 mm depth) of hepatocytes were irradiated for 3 min at 9 cm from the lamp. Under these conditions, ≈10–15% of the cell-associated radioactivity was found to be covalently bound to the cells. By using this irradiation procedure the hepatocytes retained both morphological integrity and biological responsiveness to insulin (13).

Analysis of Cell-Associated Radioactivity. After UV irradiation the cell suspension was diluted 1:20 with insulin-free KR bicarbonate buffer and incubated for varying periods of time at 37°C. At each time point two aliquots (25 ml each) of the cell suspension (0.6×10^6 cells per sample) were centrifuged and the cells were resuspended in 450 μl of KR bicarbonate buffer

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Abbreviations: PhMeSO$_2$F, phenylmethylsulfonyl fluoride; KR bicarbonate, Krebs–Ringer bicarbonate; LDL, low density lipoproteins.

and transferred into Microfuge tubes. One of the tubes was centrifuged again and the cell pellet was fixed for 3–4 hr at room temperature with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for electron microscopic analysis. The fixed sample was processed for electron microscopic autoradiography as described (16, 17). In the other tube, hepatocytes were exposed to trypsin (50 μg/ml) for 10 min at 30°C, centrifuged, and solubilized in boiling 3% (wt/vol) $NaDodSO_4$ containing 10% (vol/vol) glycerol, 2% (vol/vol) 2-mercaptoethanol, and 0.01% (wt/vol) bromophenol blue. Cell lysates were then boiled for 4 min and analyzed by one-dimensional slab-gel polyacrylamide electrophoresis in the presence of $NaDodSO_4$ by using a 5–15% linear gradient of acrylamide as resolving gel (18).

The gels were stained with Coomassie blue, fluorographed, and vacuum-dried. Autoradiography was performed by exposing the gel to Kodak X-Omat film. Quantitative analysis of the labeled bands was carried out by scanning the film by microdensitometry. The standards used were: myosin, M_r 200,000; β-galactosidase, M_r 116,000; phosphorylase B, M_r 94,000; bovine serum albumin, M_r 67,000; ovalbumin, M_r 43,000; carbonic anhydrase, M_r 30,000; soybean trypsin inhibitor, M_r 20,000; and lysozyme, M_r 14,400.

RESULTS

Identification of Liver Insulin Receptors by Photoaffinity Labeling. When photoreactive ^{125}I-insulin was covalently bound to its receptor on isolated hepatocytes and the cell extracts were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions, three radioactive bands were revealed: a major band with M_r 130,000 and two minor bands with M_r 125,000 and 23,000 (Fig. 1, lane A). The three bands were not observed after binding of photoreactive ^{125}I-insulin in the presence of an excess of unlabeled insulin (5 μM), indicating that the bands represent binding proteins or their degradation product(s) to which insulin is specifically bound (Fig. 1, lane B). The M_r 130,000 band labeled with our photoreactive ^{125}I-insulin corresponds to the structure identified by us and other investigators as the α-subunit of the insulin receptor (19–22).

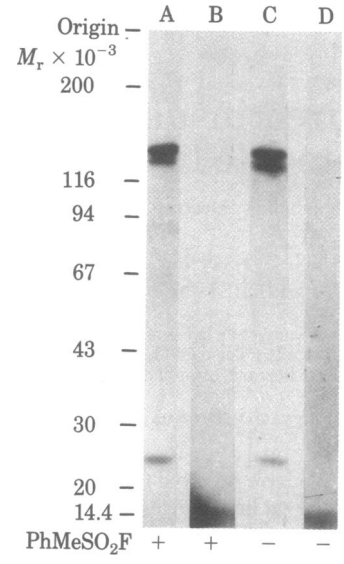

FIG. 1. Photoaffinity labeling of insulin receptors in isolated hepatocytes. Cells (0.5×10^6 per ml) were incubated in the dark for 2 hr at 15°C with photoreactive ^{125}I-insulin at 45 ng/ml (i.e., 7.5 nM) in the absence or presence of 2 mM $PhMeSO_2F$ and without (lanes A and C) or with (lanes B and D) 5 μM unlabeled insulin. After UV irradiation and centrifugation, the cells were directly solubilized in boiling $NaDodSO_4$. Extracts were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions; this was followed by autoradiography.

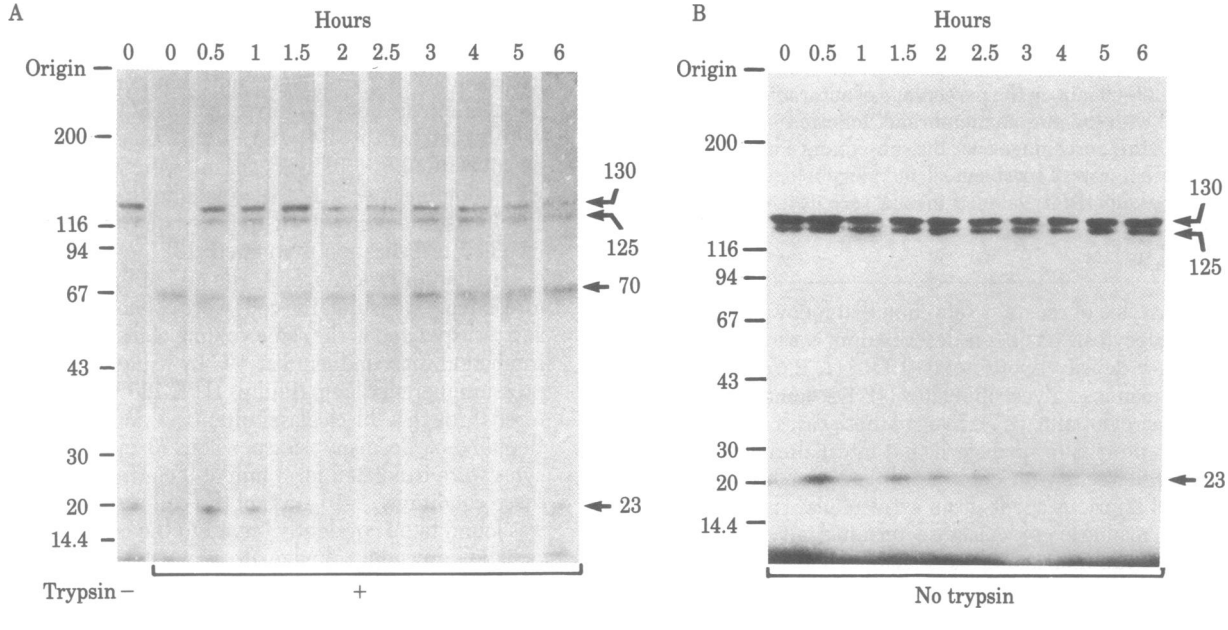

FIG. 2. (*A*) Trypsin sensitivity of the photolabeled insulin receptors of hepatocytes incubated at 37°C. Cells (0.5×10^6 per ml) first were incubated in the dark with photoreactive ^{125}I-insulin at 45 ng/ml for 2 hr at 15°C. Hepatocytes were then UV-irradiated, centrifuged, resuspended in insulin-free KR bicarbonate buffer, and incubated at 37°C. After the indicated periods of time (time at 37°C is given in hours; zero time refers to the onset of incubation at 37°C), hepatocytes were exposed to trypsin as described and solubilized in boiling $NaDodSO_4$, and cell extracts were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions. The same amount of protein (200 μg) was applied on each lane; this corresponded to ≈10,000 cpm for all time points. Molecular weights are given as $M_r \times 10^{-3}$. A typical autoradiogram is shown. (*B*) Subunit structure of the insulin receptor of hepatocytes incubated at 37°C. The experimental procedure of this experiment is identical to the one described in *A* except that the hepatocytes were not exposed to trypsin. Quantitative analysis of the relative amounts of radioactivity present in the bands did not reveal any statistically significant difference at the incubation periods studied (data not shown).

The two minor bands with M_r 125,000 and 23,000—observed irrespective of the presence or absence of the protease inhibitor $PhMeSO_2F$ during the binding step (Fig. 1, lanes A and C)—are likely to be degradation products of the M_r 130,000 subunit generated during the isolation of the hepatocytes with collagenase. Indeed, these bands were not observed when photoreactive ^{125}I-insulin was bound in the presence of $PhMeSO_2F$ to hepatic plasma membranes purified from rat livers (data not shown).

Trypsin Sensitivity of Photolabeled Insulin Receptors. When hepatocytes were UV irradiated and exposed immediately thereafter to trypsin, analysis of cell extracts by Na-DodSO$_4$/polyacrylamide gel electrophoresis revealed that the M_r 130,000 and 125,000 bands were completely degraded (Fig. 2*A*, second lane from the left). The radioactivity was now found on a band of M_r 70,000–75,000. In contrast, the M_r 23,000 band was not sensitive to trypsin. These results confirm that the insulin receptor is highly susceptible to trypsin (23, 24) and clearly indicate that the M_r 130,000 receptor subunit (and the M_r 125,000 fragment) labeled after a 2-hr incubation at 15°C is fully accessible to the enzyme. Further, these data are in agreement with our previous observation that the insulin–receptor complex at 15°C remains essentially located on the cell surface (13).

When hepatocytes were UV irradiated after 2 hr of incubation at 15°C and were allowed to incubate further for 30 min at 37°C before trypsin treatment, the M_r 130,000 and 125,000 bands remained heavily labeled (Fig. 2*A*, lane 0.5). However, after 30 min at 37°C, ≈50% of the photolabeled receptor was no longer accessible to trypsin. As we have previously reported (13), these results provide strong evidence that a substantial amount of the ^{125}I-insulin–receptor complexes is internalized in hepatocytes within 30–60 min at 37°C.

With increasing incubation time of the hepatocytes at 37°C prior to trypsin treatment, the labeling of the M_r 130,000 band progressively decreased while the labeling of the M_r 70,000 band concomitantly increased (Fig. 2*A*, lanes 2–6; Fig. 3). This strongly suggests that the insulin receptor, which is internalized within 1 hr of incubation at 37°C, is progressively recycled back to the plasma membrane and again becomes sensitive to the enzyme. Quantitative analysis reveals that about 80% of the M_r 130,000 subunit that is initially internalized is recycled to the cell surface (Fig. 3). When hepatocytes were not exposed to trypsin after the incubations at 37°C, the M_r 130,000 band remained unchanged (Fig. 2*B*). This indicates that the insulin–receptor complex is not degraded within the cell and that the changes in the M_r 130,000 band observed after trypsin treatment are entirely due to changes in accessibility to the enzyme.

In contrast to the M_r 130,000 band, the M_r 125,000 component, which becomes protected from trypsin degradation (i.e., internalized) together with the M_r 130,000 subunit, remains essentially trypsin insensitive over the whole 6-hr incubation at 37°C (Fig. 3 *Lower*). This suggests that an apparently small (presumably degradation-linked) modification of the insulin receptor, which is reflected by the appearance of the M_r 125,000 band, does not interfere with the internalization of the receptor, but does hamper the recycling of this modified subunit. Finally, the M_r 23,000 fragment of the insulin receptor does not appear to be sensitive to trypsin from the onset of the incubation onward, suggesting that this proteolytic fragment is already too small to be further degraded by trypsin.

Distribution of the Cell-Associated Radioactivity as a Function of Time. As previously observed (13, 16), at the end of the 2-hr association period at 15°C the autoradiographic grains were predominantly located at the cell periphery. When their distribution was analyzed quantitatively, >80% of the grains were found within a distance of ±250 nm from the plasma membrane

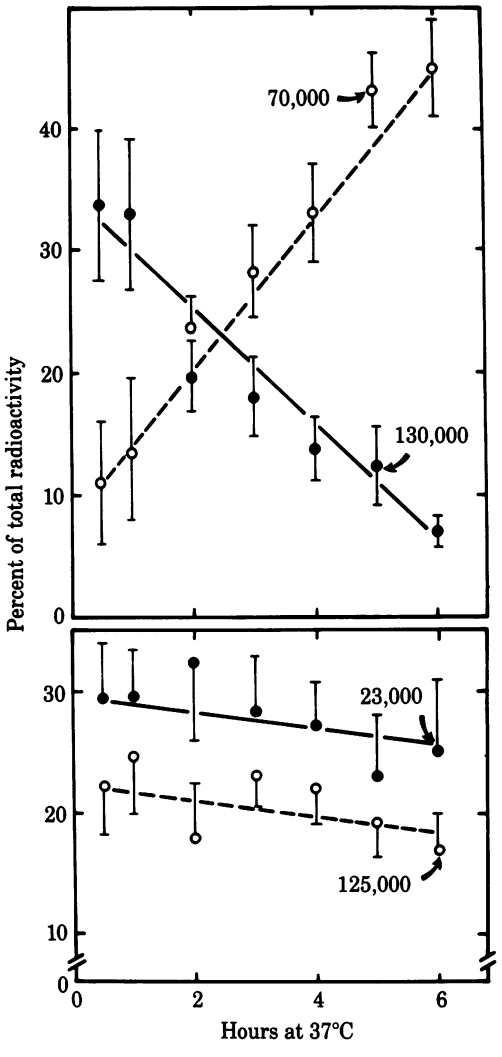

FIG. 3. Time dependence of the trypsin sensitivity of the photolabeled insulin receptor. The effect of trypsin on photolabeled insulin receptors was analyzed by electrophoresis and autoradiography as described in the legend to Fig. 2. Autoradiograms were scanned, and for each time point the surface area of the peaks corresponding to the bands with M_r 130,000, 125,000, 70,000, and 23,000 was taken as 100%; each band was expressed as a percentage of the total surface area. Each value represents the mean ± SEM of three separate experiments.

and therefore were considered to be associated with the plasma membrane (Fig. 4). When the cells were UV irradiated and further incubated for various periods of time at 37°C, the percentage of grains associated with the plasma membrane first dropped to a value close to 20% by 30 min of incubation, but later on (between 1 and 5 hr) their percentage progressively increased to reach a value close to 60% by 5 hr of incubation (Fig. 4). Note that after 6 hr at 37°C the morphological study showed an increased number of necrotic cells. Therefore, this 6 hr time point was not used in the autoradiographic analysis. Because the total cell-associated radioactivity from 2 to 5 hr of incubation is relatively constant (Fig. 4) and because the hormone–receptor complex is not degraded (Fig. 2*B*), these data indicate that the complex is shifting from one compartment to another rather than being preferentially lost from one compartment.

DISCUSSION

The concept of membrane recycling was postulated to explain the high rate of membrane internalization in mouse peritoneal

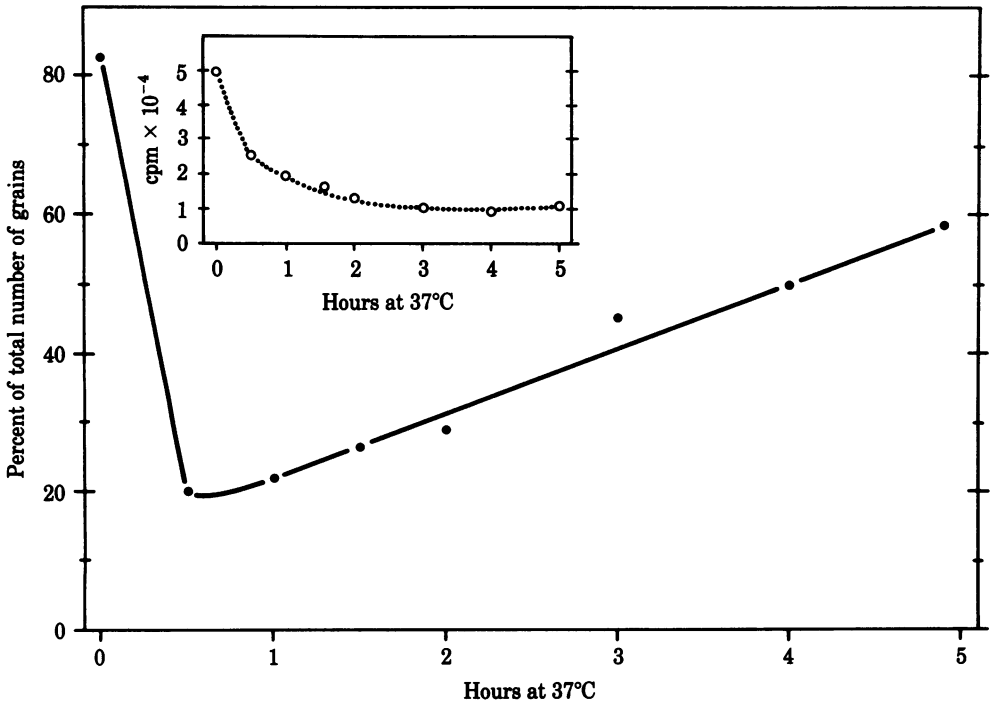

FIG. 4. Relationship of autoradiographic grains with the plasma membrane of isolated hepatocytes. Incubation conditions (incubation, UV irradiation, centrifugation, and resuspension) were as indicated in the legend to Fig. 2. Grains were considered to be associated with the plasma membrane if their center was ±250 nm from the plasma membrane. (*Inset*) Time course of total amount of cell associated radioactivity under the same experimental conditions.

macrophages (for review, see ref. 25). More recently, direct experimental evidence in support of membrane reutilization was presented by Schneider *et al.* in their study using anti-plasma membrane immunoglobulins (26). The occurrence of recycling of membrane receptors—which represent highly specialized structural units in the membranes—has been inferred from studies of various receptors. However, most, if not all, of the evidence in favor of receptor recycling is based on theoretical considerations derived from biochemical and morphological studies (8).

The present work affords both a biochemical and a morphological demonstration of recycling of a hormone receptor. Indeed, we show that when labeled insulin is covalently attached to its cell surface receptor, the hormone–receptor complex is first internalized into a cellular compartment, and thereafter this complex reappears at the cell surface. The rates of insulin receptor internalization and recycling appear to be widely different. Thus, maximal internalization is observed within 30–60 min, whereas even after 6 hr the entire amount of internalized receptors is not recycled back to the cell surface (Figs. 3 and 4). Even if both the measurement of the accessibility of labeled receptors to trypsin and the quantitative autoradiographic analysis are likely to represent a steady-state picture of the situation rather than an actual evaluation of the rates of recycling, the insulin receptor recycling in isolated hepatocytes appears to be much slower than the recycling of LDL receptors in human fibroblasts (2) and the recycling of the receptor for mannose *N*-acetylglucosamine in alveolar macrophages (4). These varying rates may be ligand- or cell-specific and may reflect various mechanisms, including differences in preexisting intracellular receptor pools (27–29), intracellular routes, and processing.

Another striking difference between internalization and recycling of insulin receptors in hepatocytes relates to the receptor subunit integrity. The photoreactive insulin analogue that we have employed allows the labeling of a unique M_r 130,000 subunit in purified liver membranes (data not shown), whereas in freshly isolated hepatocytes an additional component with a slightly reduced molecular weight (M_r 125,000) was also labeled. This fragment is likely to be a proteolytic product generated during the cell isolation, with a collagenase preparation contaminated with proteases. Indeed, collagenase treatment of liver membranes prior to incubation with photoreactive ^{125}I-insulin and photolysis generated a fragment with the same molecular weight (data not shown). Interestingly, the totality of both the M_r 130,000 and M_r 125,000 components is internalized within 30–60 min (Fig. 2). In contrast, only the M_r 130,000 subunit appeared to be recycled (Fig. 3). This observation suggests that only receptors with intact subunits can be translocated to or reinserted in the plasma membrane (or both), whereas the partly fragmented receptors are probably further degraded and do not reappear at the cell surface.

The biological importance of insulin–receptor internalization is not fully understood. It has been proposed that this process plays an important role in the degradation of the hormone and possibly in the termination of the hormonal signal (reviewed in ref. 30). It has also been proposed that the internalization process is a means of delivering the hormone or a proteolytic fragment of the hormone (or both) to intracellular sites, where it (they) could directly mediate the biological effects of insulin (31). The direct demonstration that the insulin receptor is also internalized during the receptor-mediated uptake of insulin (13) and the present observation that receptors can be recycled back to the cell surface provide new insights into the biological implications of the hormone–receptor internalization process.

It is well established that insulin induces a decrease of its own cell surface receptors. This process termed "down regulation" occurs in various target cells (32–36) including hepatocytes (29, 33). The mechanism underlying this process is not established, but is likely to involve the combination of endocytosis and degradation of receptors. The possible role of internalization of insulin receptors in down regulation was originally suggested based on morphological evidence of an increased endocytotic activity in cultured human lymphocytes (IM-9 line) exposed to ambient insulin levels that reduce the number of cell surface insulin receptors (37). Using external and biosynthetic labeling of the insulin receptor, Kasuga *et al.* have shown that in these lymphocytes an accelerated degradation of insulin receptors accounts for the decrease in receptor concentration observed in down regulation (38). Furthermore, a recent study of hepatocytes has revealed that cell-surface insulin receptors are

translocated to an intracellular compartment during down regulation (29). It has also been suggested that down regulation of insulin receptors in isolated adipocytes occurs through a receptor-mediated endocytosis of the insulin–receptor complexes (34, 35).

On the basis of these data it has been proposed that a chronic exposure of cells to insulin enhances the rate of hormone–receptor internalization, thus decreasing the amount of receptors available at the cell surface (30). However, against this simple model is the existence of a poor correlation (or the absence of a correlation) between the extent of insulin (and receptor) internalization and the extent of the hormone's ability to decrease the number of its cell surface receptors. For example, in cultured human lymphocytes insulin can decrease the number of receptors by 80% (32, 36) and yet only a small amount of labeled insulin can be shown to be internalized (39). In contrast, in 3T3-L1 adipocytes, which apparently are unable to down-regulate insulin receptors when exposed to the hormone (40), a substantial quantity of labeled insulin is internalized (41).

Our present observation that insulin receptors are recycled back to the cell surface leads us to put forward an alternative and more satisfactory model for insulin-induced down regulation. We propose that the loss of cell-surface insulin receptors upon prolonged exposure of cells to the hormone results from an internalization of hormone–receptor complexes accompanied by a reduction in receptor recycling. The varying degrees of insulin-induced receptor loss found in different cell types could then be explained by a different contribution of both phenomena—internalization and recycling—in the down-regulation process. Further, the decreased receptor recycling could result from an inhibition of the recycling process itself or from an increased intracellular degradation of receptors or both. The experimental approach presented in this study, which involves the photoaffinity labeling of insulin receptors *in situ* and the determination of the cellular localization of the labeled receptors, should enable us to elucidate the precise mechanism of down regulation by assessing the respective contributions of receptor internalization and receptor recycling.

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