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Internalization and recycling of insulin receptors in hepatoma cells

Absence of regulation by receptor occupancy

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Insulin receptors of Fao hepatoma cells were labelled with a ^{125}I-labelled photoreactive insulin analogue or by surface iodination catalysed by lactoperoxidase. Cells were then incubated at 37°C, and the cellular localization of the labelled receptors was assessed by limited exposure of intact cells to trypsin. The results show that: (1) photolabelled insulin–receptor complexes are internalized and recycled in Fao hepatoma cells; (2) the dynamics of photolabelled insulin receptors (internalization and recycling) is similar before and after down-regulation; (3) the unoccupied receptors labelled by surface iodination are internalized and recycled similarly to covalent insulin–receptor complexes; (4) insulin does not induce internalization of surface-iodinated insulin receptors. We conclude that internalization and recycling of insulin receptors are independent of receptor occupancy by insulin in Fao hepatoma cells.

After its binding to cell-surface receptors, insulin is internalized and partly degraded in target cells (Gorden *et al.*, 1980). Studies in freshly isolated hepatocytes have shown that covalent binding of insulin to plasma-membrane receptors is also followed by rapid internalization and progressive recycling of the insulin–receptor complexes (Fehlmann *et al.*, 1982*a*). The study of the biological implications of insulin (and receptor) internalization is, however, hampered in rat hepatocytes, owing to their limited viability in suspension that precludes long-term incubations necessary to demonstrate a receptor regulation. In the present study we have investigated the dynamics of insulin receptors in Fao cells, a rat hepatoma cell line that maintains many differentiated hepatic functions (Deschatrette & Weiss, 1969). This cell line has been shown to display a high content of insulin receptors (Fehlmann *et al.*, 1983) and to respond to the hormone for both short-term and long-term effects (Kasuga *et al.*, 1982*b*; Crettaz & Kahn, 1983; Fehlmann *et al.*, 1983).

Experimental

Materials

The photoreactive insulin analogue [(2-nitro-4-azidophenylacetyl)-B2]-des-Phe^{B1}-insulin was prepared by Dr. P. Thamm, Dr. D. Saunders and Dr. D. Brandenburg at the Wollforschungsinstitut, Aachen, Germany, as previously described (Thamm *et al.*, 1980). The photoreactive insulin was iodinated, in the dark, to a specific radioactivity of 200–250 μCi/μg by using the same method as that described for native insulin (referred to as 'the second modification' in Freychet, 1976). Pig monocomponent insulin was generously supplied by the Novo Research Institute, Copenhagen, Denmark. Na^{125}I was purchased from the Commissariat à l'Energie Atomique, Saclay, France. Triton X-100, phenylmethanesulphonyl fluoride, bovine serum albumin (fraction V), bacitracin and proteolytic enzymes were from Sigma. Reagents for SDS/polyacrylamide-gel electrophoresis were purchased from Bio-Rad. All other reagents were of the best grade commercially available. Serum from patient B9 with autoantibodies to insulin receptor was kindly given by Dr. C. R. Kahn (Joslin Research Laboratory, Boston, MA, U.S.A.) (Kahn *et al.*, 1976). Ham's F12 culture medium and foetal-calf serum were purchased from Flow Laboratories.

The Fao hepatoma cells are a well-differentiated clonal line derived from the H4-II-EC$_3$ line (Pitot *et al.*, 1964) established from a Reuber (1961) H-35 minimal-deviation hepatoma. This cell line was kindly provided to us by Dr. R. Bertolotti and Dr. M. Weiss (Gif-sur-Yvette, France) and exhibits several liver-specific properties, including an inducible tyrosine aminotransferase activity, the

Abbreviations used: SDS, sodium dodecyl sulphate; PBS, phosphate-buffered saline (137mM-NaCl/2.6mM-KCl / 8.1mM-Na_2HPO_4 / 1.5mM-KH_2PO_4 / 0.5mM-$MgCl_2$/0.7mM-$CaCl_2$, pH7,4).

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secretion of rat serum albumin and the synthesis of liver-specific isomers of alcohol dehydrogenase and aldolase (Deschatrette & Weiss, 1969). The cells were cultivated in Ham's F12 medium supplemented with 5% (v/v) foetal-calf serum.

Incubations with photoreactive insulin and irradiation procedure

Fao cells were grown in six-well (35mm-diameter) Corning plastic dishes. At confluency the culture medium was aspirated, and, after two washes of the cells with PBS, 1ml of serum-free medium containing 1% albumin and ^{125}I-labelled photoreactive insulin was added in each well. Cells were incubated in the dark for 90min at 15°C, conditions that permitted steady-state binding. At the end of this association step, the medium was aspirated, cells were washed with 2 × 4ml of PBS at 4°C and replaced in 1ml of fresh serum-free culture medium containing 1% albumin at 4°C. Irradiations were conducted under standardized conditions, in a cold-room (4°C), with a water-cooled high-pressure mercury lamp (Philips HPK 125 W/L). The light was filtered through a 'black glass' filter (U.V.W. 55, Glaswerk Wertheim, Wertheim, Germany), which decreases the short-wavelength u.v. emissions intensities to very low values (Fehlmann *et al.*, 1982*b*). Cells were irradiated in the culture dishes, with cover removed, at 330nm for 4min at a distance of 9cm from the lamp.

Surface iodination and immunoprecipitation

Fao cells were grown in 100mm-diameter Petri dishes. At confluency the culture medium was aspirated and cells were washed twice with PBS at room temperature. The reaction was initiated by adding 3ml of PBS containing 2 units of lactoperoxidase/ml and 20mM-glucose, 100μl of glucose oxidase in PBS (3 units/ml final concn.) and 500μCi of Na^{125}I. After 10min of incubation at 15°C, the reaction was boosted with 60μl of 1M-glucose and after 20min with 60μl of 1M-glucose and 10μl of 0.15mM-NaI. The reaction was stopped 10min later by aspirating the medium, and cells were washed extensively with PBS at 4°C. Iodinated cells were detached in PBS with a rubber policeman at 4°C, and collected by centrifugation (1500*g*, 5min). The pellet was resuspended in 150μl of 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (pH7.4)/150mM-NaCl containing 1% Triton X-100, 1mM-bacitracin, aprotinin (100 trypsin-inhibitor units/ml) and phenylmethanesulphonyl fluoride (2mM). Solubilization was performed by continuous stirring at 4°C for 90min. This preparation was centrifuged at 130000*g* for 15min (Beckman Airfuge) to remove insoluble material. Immunoprecipitations were performed by incubation with anti-receptor serum (Kahn *et al.*, 1976) at a 1:300 dilution for 16h at 4°C, followed by an additional 4h at 4°C with *Staphylococcus aureus* cells (Pansorbin). The immunoprecipitates were collected by centrifugation (1min at 2000*g* at 4°C) and the pellets were washed three times in 50mM-Hepes/150mM-NaCl.

Gel electrophoresis and autoradiography

Photolabelled cells were detached from the dishes with a rubber policeman and collected by centrifugation. The cell pellets and the immunoprecipitates were solubilized and boiled for 5min in a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, 10mM-sodium phosphate, 2% (v/v) 2-mercaptoethanol and 0.01% Bromophenol Blue. Samples were analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970) with a 7.5%-acrylamide gel as the resolving gel. The M_r values of the standards used were: myosin, 200000; β-galactosidase, 116000; phosphorylase *b*, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; soya-bean trypsin inhibitor, 20000; lysozyme, 14400. The gels were stained, destained, dried and autoradiographed by exposing the gels to Kodak X-Omat film as described by Fehlmann *et al.* (1982*b*). The autoradiograms were scanned in a microdensitometer (Gelman) for quantitative analysis.

Results

Photolabelling of insulin receptors

^{125}I-labelled photoreactive insulin was first allowed to bind to Fao hepatoma cells for 90min at 15°C. The cells were then u.v.-irradiated or not, and covered by a large volume of insulin- and serum-free medium. The release of cell-associated radioactivity was followed as a function of incubation time at 37°C. Fig. 1 shows that, at all time points, the amount of radioactivity bound to u.v.-irradiated cells was higher than in controls (cells not u.v.-irradiated). It can be estimated from these results that 10–15% of the radioactivity originally bound at the steady state was covalently linked to the cells by u.v.-irradiation.

To determine the nature of cellular proteins to which ^{125}I-labelled photoreactive insulin was covalently bound, cells were directly solubilized in SDS immediately after u.v. irradiation, and proteins analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions followed by autoradiography. Fig. 2 (lane *b*) shows that more than 90% of the radioactivity was located in a band of M_r 130000. Two additional components, of M_r 95000 and 210000, were also labelled. Labelling of all species was inhibited by an excess of unlabelled insulin (5μM) during the association period of the

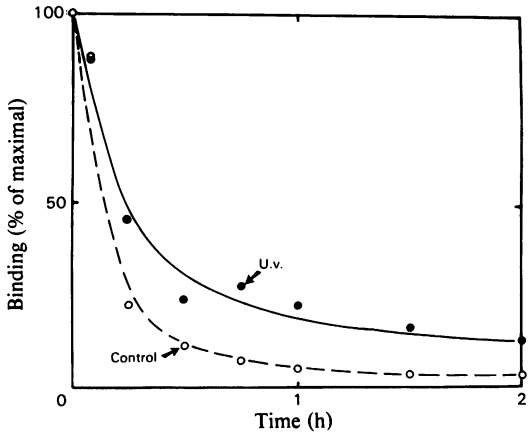

Fig. 1. *Time course of dissociation of ^{125}I-labelled photoreactive insulin from Fao hepatoma cells: effect of u.v.-irradiation*

Fao cells were incubated in the dark with 3nM ^{125}I-labelled photoreactive insulin for 90min at 15°C. Cells were then irradiated (●; u.v.) or kept in the dark (○; control), washed, covered with a large (10 times that during the association step) volume of insulin-free medium, and incubated at 37°C. After the time periods indicated the medium was aspirated, and the ^{125}I radioactivity associated with the cells was determined.

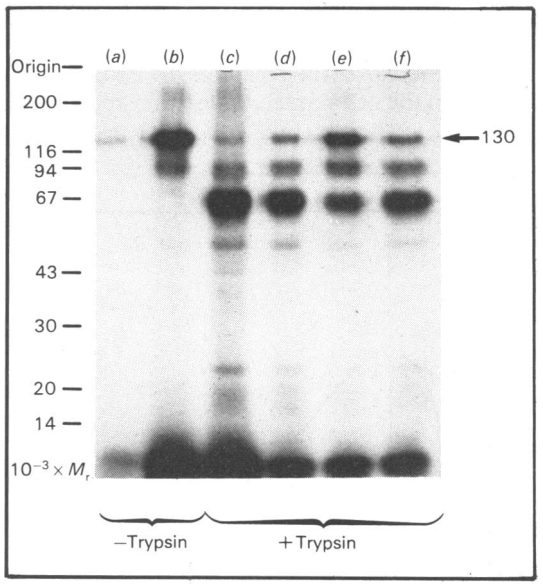

Fig. 2. *Trypsin-sensitivity of photolabelled insulin receptors of Fao hepatoma cells incubated at 37°C*

Fao cells were incubated in the dark with 3nM ^{125}I-labelled photoreactive insulin for 90min at 15°C in the absence (lanes *b–f*) or presence (lane *a*) of 2μM unlabelled insulin. Cells were then u.v.-irradiated, washed, replaced in fresh medium and incubated for 0min (lanes *a–c*), 10min (lane *d*), 60min (lane *e*) or 360min (lane *f*) at 37°C. At the end of the incubation period, cells were briefly exposed to 50μg of trypsin/ml (lanes *c–f*) or buffer (lanes *a* and *b*) and solubilized in boiling SDS. Cell extracts were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by autoradiography.

^{125}I-labelled photoreactive insulin with Fao cells (Fig. 2, lane *a*). Therefore the M_r-130000 component is the major molecular species implicated in the specific binding of insulin to Fao cells. A small amount of a M_r-95000 component can also be photolabelled, which confirms previous report that the β-subunit of the insulin receptor (M_r 95000) can be weakly labelled by affinity methods (Yip *et al.*, 1982). The M_r-210000 component is likely to represent the receptor precursor (Kasuga *et al.*, 1982*a*; Hedo *et al.*, 1983), which contains the binding site of the receptor.

The cellular localization of the insulin receptor can be demonstrated by a limited exposure of intact cells to trypsin (Fehlmann *et al.*, 1982*a*,*b*). In these conditions cell-surface receptors are cleaved by the enzyme, which results in the appearance of proteolytic fragments on SDS/polyacrylamide gels (Fehlmann *et al.*, 1982*a*,*b*) or in the loss of insulin-binding activity tested in intact or solubilized cells (Kono & Barham, 1971; Green & Olefsky, 1982). Conversely, internalized receptors are protected from trypsin degradation and thus retain their intact molecular structure and binding activity (Fehlmann *et al.*, 1982*a*,*b*). Fig. 2 (lane *c*) shows that a 10min exposure of Fao cells, photolabelled at 15°C, to 0.1mg of trypsin/ml before cell solubilization and analysis by SDS/polyacrylamide-gel electrophoresis results in the cleavage of more than 95% of the M_r-130000 subunit, giving rise to a M_r-70000 degradation fragment. This demonstrates that u.v. irradiation of Fao cells after incubation with a ^{125}I-labelled photoreactive insulin analogue at 15°C labels predominantly cell-surface insulin receptors.

Fate of the labelled insulin–receptor covalent complexes

When photolabelled Fao cells were first incubated for various periods at 37°C before trypsin treatment and analysis by SDS/polyacrylamide-gel electrophoresis, the M_r-130000 receptor subunit was progressively protected from trypsin degradation (Fig. 2, lanes *d* and *e*). However, after longer periods of incubation at 37°C it becomes again sensitive to the enzyme (Fig. 2, lane *f*). Quantification of the radioactivity associated with the M_r-130000 subunit by densitometric analysis of the autoradiograms reveals that the receptor is maximally protected from extracellular trypsin after 30min of

cell incubation at 37°C (Fig. 3). After this time, approx. 7.5% of all labelled receptors are internalized. Thereafter the receptors are progressively recycled to the cell surface, where they regain their sensitivity to trypsin (Fig. 3). Similar results were obtained when insulin receptors were labelled at 37°C for 5min, and then further incubated at the same temperature (results not shown).

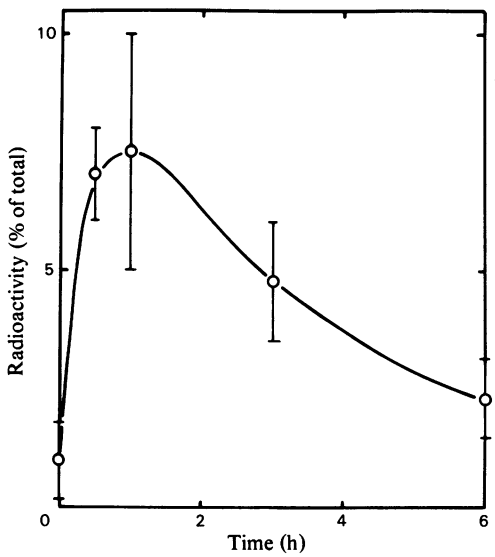

Fig. 3. *Time-dependence of the trypsin-sensitivity of photolabelled insulin receptors of Fao hepatoma cells incubated at 37°C*

The effect of trypsin on photolabelled insulin receptors was analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Autoradiograms were scanned, and for each indicated time point (0, 0.5, 1, 3 and 6h) the surface area corresponding to the band with M_r 130000 was measured and expressed as a percentage of the total surface area. Values represent means ± S.E.M. for three different experiments.

Prolonged exposure of Fao cells to insulin results in a marked decrease in the number of insulin receptors expressed at the cell surface (Crettaz & Kahn, 1983), a process called 'down-regulation'. We have photolabelled insulin receptors in down-regulated Fao cells in order to evaluate the rates of internalization and recycling of the receptors that remain at the cell surface after down-regulation. Fig. 4 shows that, as expected, the amount of photolabelled receptors is decreased by approx. 60% in down-regulated cells compared with controls. However, the sensitivity of the photolabelled receptors to trypsin as a function of cell incubation time at 37°C is similar in control and down-regulated cells (Fig. 4). This demonstrates that insulin receptors that are expressed at the cell surface in the presence of a high concentration of insulin in the medium are internalized and recycled in similar proportions and at similar rates to those for total surface receptors that have not been exposed to insulin.

Surface iodination and immunoprecipitation of insulin receptors

Membrane proteins of intact Fao cells were labelled by using lactoperoxidase to catalyse surface iodination. Cells were solubilized and insulin receptors were immunoprecipitated with an antiserum containing autoantibodies to the insulin receptor (Kahn *et al.*, 1976, 1981), and analysed by SDS/polyacrylamide-gel electrophoresis. The autoradiogram (Fig. 5, lane *b*) reveals that three bands (M_r 130000, 95000 and 45000) are specifically immunoprecipitated. These bands are not present when serum from normal individuals is used for

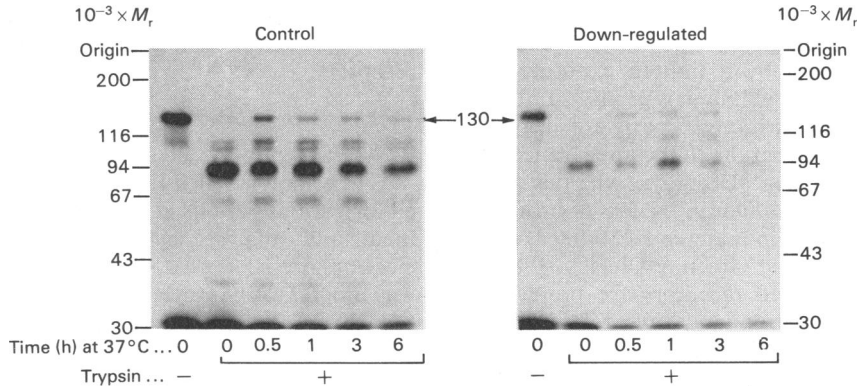

Fig. 4. *Trypsin-sensitivity of photolabelled insulin receptors in control and down-regulated Fao hepatoma cells incubated at 37°C*

Fao cells were exposed (down-regulated) or not (control) to 0.1 μM-insulin for 24h before photolabelling the receptor and investigating the trypsin-sensitivity of the photolabelled receptor as indicated in the legend to Fig. 2.

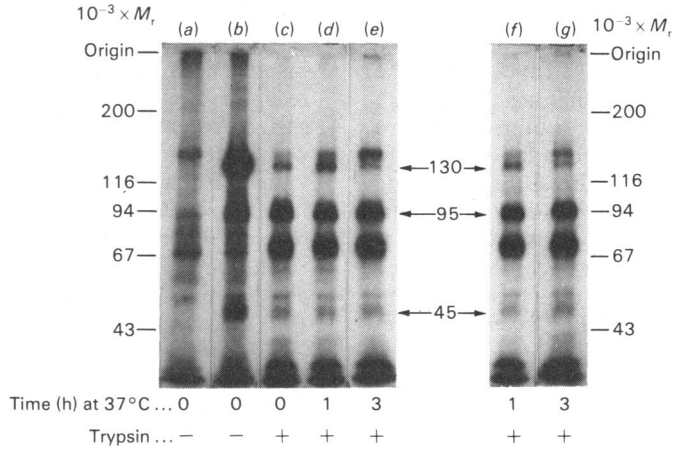

Fig. 5. *Trypsin-sensitivity of cell-surface-iodinated insulin receptors in Fao hepatoma cells: effect of insulin*
Cell-surface proteins of intact Fao cells were iodinated by using lactoperoxidase. Cells were washed, replaced in fresh medium and incubated at 37°C for the indicated periods in the absence (lanes *a–e*) or presence (lanes *f* and *g*) of 10nM-insulin. At the end of the incubation periods, cells were briefly exposed to 50μg of trypsin/ml (lanes *c–g*) or buffer (lanes *a* and *b*), solubilized in 1% Triton X-100 and exposed to anti-(insulin receptor) antibodies (lanes *b–g*) or to a control serum (lane *a*). The immunoprecipitates were solubilized in boiling SDS and analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by autoradiography.

immunoprecipitation (Fig. 5, lane *a*). This pattern is similar to that observed after surface iodination or biosynthetic labelling of insulin receptors in different cell types (Kahn *et al.*, 1981). In addition to the α (M_r-130000) and β (M_r-95000) subunits, a smaller component of M_r 45000 is also observed. When surface-iodinated Fao cells are briefly exposed to trypsin before solubilization and immunoprecipitation, more than 90% of the M_r-130000 and the M_r-45000 species are degraded by the enzyme. A fragment of M_r 70000 generated by trypsin is immunoprecipitated, which corresponds to the M_r-70000 fragment generated by trypsin in photolabelled cells. The β subunit (M_r 95000) is not affected by trypsin in intact cells. Similar results are found when other proteinases (chymotrypsin, elastase, subtilisin) are used instead of trypsin (results not shown). These results argue against the hypothesis that the M_r-45000 species (sensitive to trypsin) is a proteolytic fragment of the M_r-95000 subunit (insensitive to trypsin) and rather suggest that the M_r-45000 component is a naturally occurring constituent of the insulin receptor present at the cell surface.

The fate of plasma-membrane iodinated insulin receptors during cell incubation at physiological temperatures was followed by investigating their sensitivity to trypsin as a function of the incubation time of Fao cells at 37°C. Fig. 5 shows that the amount of iodinated receptors protected from trypsin degradation increases transitorily after 1h at 37°C (Fig. 5, lane *d*). Quantification of the radioactivity associated with intact M_r-130000 receptor subunits after cell exposure to trypsin reveals that 6% of all the iodinated receptors are internalized after 1h. This proportion is comparable with that found after photolabelling, thus indicating that the insulin receptors labelled by the photoprobe reflect a representative fraction of the total cell-surface receptor population. The effect of insulin on receptor internalization was then investigated by adding 10nM-insulin to the incubation medium. At this concentration all insulin receptors are saturated with insulin within a few minutes of incubation (results not shown). No detectable difference could be observed in the amount of receptor internalized after 1h (Fig. 5, lane *f*), nor in the amount recycled back to the cell surface (Fig. 5, lane *g*). It can be concluded that insulin receptors are internalized and recycled irrespective of the presence of insulin in the medium.

Discussion

Since the first report in 1978 that insulin enters its target cells after binding to cell-surface receptors (Gorden *et al.*, 1978*a*), the mechanism of insulin internalization has received a great deal of attention. The function of this receptor-mediated endocytosis of insulin is, however, not clear. It has been evoked as a mechanism responsible for phenomena such as degradation of insulin and thus termination of the hormonal signal, delivery of insulin to intracellular sites and regulation of

receptor number expressed at the cell surface (Gorden *et al.*, 1980).

In the present study we have investigated the effect of insulin on the dynamics of insulin receptors in Fao cells, a highly insulin-responsive cell line. By using two different labelling techniques, we were able to address the following questions: (*a*) does insulin induce the internalization of its receptor?; (*b*) does the nature of the bond between insulin and its receptor affect the rate of endocytosis of the insulin–receptor complex?; (*c*) does receptor recycling occur irrespective of the presence of insulin?

The observations that photoreactive insulin analogues covalently coupled to insulin receptors can enter into target cells (Berhanu *et al.*, 1982; Fehlmann *et al.*, 1982*a*,*b*) were the first direct demonstration of the internalization of a hormone receptor together with its ligand during receptor-mediated endocytosis. The photolabelling approach, however, raises the possibility that covalent insulin–receptor complexes are processed differently from naturally interacting molecules, although such covalent associations have been reported to occur *in vivo* (Clark & Harrison, 1982). This limitation applies even better for receptor recycling, since it is improbable that the insulin receptor is recycled *in vivo* with the ligand molecule still bound on it. Indeed there is a general agreement that, during receptor-mediated endocytosis, ligands are dissociated from receptors in acidic prelysosomal vesicles (Helenius *et al.*, 1983). The present study confirms that photolabelled insulin receptors are internalized and recycled in insulin-responsive cells and further demonstrates that these processes also occur for unoccupied receptors labelled by surface iodination.

Down-regulation of insulin receptors after chronic cell exposure to insulin, usually monitored by insulin binding to intact cells, corresponds to a decrease in the number of functional cell-surface insulin receptors. Ultimately it has been shown that the new steady-state receptor number obtained after down-regulation reflects not a receptor redistribution between the cell surface and intracellular pools, but rather a total receptor loss. Indeed, using a biosynthetic pulse label, Kasuga *et al.* (1981) have demonstrated that the down-regulation phenomenon is associated with an accelerated receptor degradation. The mechanism that leads to this receptor decay has not been elucidated. Insulin either could induce internalization of its receptors, which would be degraded intracellularly, or alternatively could control the degradation of the receptor irrespective of any induced redistribution. The present results demonstrate that endocytosis of insulin receptors in hepatoma cells need not to be initiated by insulin binding. Insulin-receptor internalization is thus a constitutive endocytotic process. Consequently, insulin does not induce internalization of its receptor, but simply shares, after binding to surface receptors, a continuously operating pathway. The biological implication of these observations is that insulin-induced down-regulation of insulin receptors in Fao cells is independent from receptor internalization or recycling.

These results contrast with those obtained in rat adipocytes (Green & Olefsky, 1982; Wang *et al.*, 1983) and cultured fibroblasts (Knutson *et al.*, 1983), suggesting that insulin internalization and down-regulation are linked processes. It is at present unknown whether the hepatoma cells used in the present study behave differently from adipocytes or fibroblasts in the control of insulin-receptor expression. Nevertheless, in Fao cells, as in adipocytes, insulin rapidly induces a marked down-regulation of its receptor (Crettaz & Kahn, 1983), and we found that receptors remaining at the cell surface after down-regulation display similar dynamic properties to receptors sensitive to down-regulation. In addition, insulin at concentrations that induce a marked decrease in surface receptors within 4h (Crettaz & Kahn, 1983) does not alter the dynamics of the receptor during this period.

Constitutive endocytosis has previously been described for a variety of ligands (Renston *et al.*, 1980; Abrahamson & Rodewald, 1981; Kaplan & Keogh, 1981), and data suggest that low-density-lipoprotein receptors (Anderson *et al.*, 1976) and epidermal-growth-factor receptors (Gorden *et al.*, 1978*b*) may localize in coated pits even in the absence of ligand, and presumably become internalized as such. Whether a similar mechanism applies for insulin in its major target cells requires investigation.

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