Internalization of insulin receptors and HLA antigens in human hepatoma cells

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Human HepG2 hepatoma cells express a high number of insulin receptors. Growing cells exhibit 70% of their insulin receptors on the plasma membrane. Moreover, cell-surface insulin receptors form molecular complexes with class I major histocompatibility antigens, as determined by co-immunoprecipitation of the receptors by anti-class I monoclonal antibodies. On exposure to saturating concentrations of insulin, the hormone is rapidly internalized into a Pronase-resistant compartment. Internalization of insulin is accompanied by a rapid ($t_{1/2} = 2-3$ min) redistribution of insulin receptors from the cell surface to an intracellular compartment. On removal of insulin from the medium, functional receptors recycle back to the plasma membrane, where they can bind insulin again. With chronic exposure of HepG2 cells to insulin, the initial redistribution of receptors is followed by a slow ($t_{1/2} = 9$ h) down-regulation of the receptors. Finally, notwithstanding their interaction at the cell surface, insulin receptors and class I major histocompatibility antigens are internalized at different rates and with independent regulation.

INTRODUCTION

Chronic exposure of target cells to insulin results in a decrease of receptor number on the cell surface. This phenomenon, known as ligand-induced down-regulation, has been demonstrated for various tissue types *in vivo* [1] and *in vitro* [2–11]. Reversal of this effect is observed on removal of insulin [6,12]. It is thought that binding to the receptor could trigger the translocation of the complex (via endocytosis) into an intracellular compartment where receptor inactivation could occur. Internalization of insulin receptor has been demonstrated in rat liver *in vivo* [13], in isolated rat hepatocytes [14], in isolated rat adipocytes [15,16] and in cultured mouse fibroblasts [17]. Internalized receptors can be degraded [9,18] or recycled back to the plasma membrane [17,19,20].

Whether or not occupancy by insulin of its receptor can modulate the rate of receptor internalization and externalization is controversial. Insulin-dependent internalization has been shown in 3T3-L1 fat-cells [17], whereas in Fao rat hepatoma cells the presence of insulin has no effect on the rate of internalization and recycling of insulin receptors [21].

Moreover, insulin receptors have been shown to form non-covalent molecular complexes with major histocompatibility complex (MHC) antigens at the cell surface of different cells [22,23]. Such associations have been evoked to explain non-immunological functions of MHC antigens, including their interaction with cytoskeleton components (reviewed in [24]). However, whether these interactions are strong enough to allow their co-internalization with insulin receptors is not known.

In the present study we show that chronic exposure of

HepG2 hepatoma cells to insulin results in the loss of a large fraction of insulin receptors. By contrast, acute exposure of these cells to insulin induces a rapid redistribution of the receptor between the cell surface and a Pronase-resistant compartment. On removal of insulin, functional receptors recycle back to the plasma membrane.

Nevertheless, even if insulin receptors and class I MHC antigens also form molecular complexes in HepG2 cells, endocytosis of these two molecules proceeds independently.

EXPERIMENTAL

Materials

Pig monocomponent insulin, generously supplied by Dr. R. Chance (Eli Lilly Co.), was iodinated to a specific radioactivity of 140–200 $\mu\text{Ci}/\mu\text{g}$ with Na^{125}I (New England Nuclear Corp.) by an immobilized glucose oxidase/lactoperoxidase enzymic method (Enzymobeads; Bio-Rad Laboratories). The photoreactive insulin analogue [(2-nitro-4-azidophenyl-acetyl)-lysine^{B29}]-insulin was prepared as previously described [25] and radioiodinated by the chloramine-T method [26]. Anti-(insulin receptor) antibodies were from sera of patients with type B insulin resistance associated with acanthosis nigricans [27] and generously supplied to us by Dr R. Kahn (Joslin Diabetes Center, Boston). B-1-1-G-6 and B-9-12-1, two monoclonal antibodies directed against class-I antigen light and heavy chains, respectively, were obtained from Dr D. C. Mawas (INSERM U 119, Marseille, France). OKT9 is a monoclonal antibody reacting with the human

Abbreviations used: MEM, modified Eagle's medium; PBS, phosphate-buffered saline (134 mM-NaCl, 2.6 mM-KCl, 6.4 mM- Na_2HPO_4 , 1.46 mM- KH_2PO_4 , pH 7.4); DSP, dithiobis(succinimidyl propionate); RAM, rabbit anti-mouse IgG; MHC, major histocompatibility complex.

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transferrin receptor (Ortho-mune, Aubervilliers, France). Rabbit anti-mouse IgG (RAM) was obtained from Dako (Glostrup, Denmark) and radioiodinated by the chloramine-T method. Culture medium, trypsin/EDTA and fetal bovine serum were from Gibco Laboratories. Pronase (type XIV), bovine serum albumin, cycloheximide and lactoperoxidase (EC 1.11.1.7) were from Sigma Chemical Co. Glucose oxidase (EC 1.1.3.4) was from Boehringer, Pansorbin from Calbiochem-Behring, and the cleavable bifunctional reagent DSP [dithiobis(succinimidyl propionate)] from Pierce Chemical Co.

Cell culture

The human hepatoma line HepG2 was kindly provided to us by Dr. A. Schwartz (Dana Farber Cancer Institute, Boston, MA, U.S.A.). The cells were grown in MEM containing 10% (v/v) fetal bovine serum in a humidified incubator at 37 °C under air/CO₂ (19:1). Cells were subcultured at ratios of 1:3 generally 4 days before assay.

Binding assay

Cells grown in 35 mm culture dishes were washed twice with PBS. Unless otherwise stated, binding was carried out in triplicate dishes at 4 °C for 2 h in binding medium (MEM with 0.1% bovine serum albumin and 20 mM-Hepes, pH 7.4) containing ¹²⁵I-insulin. After three washes by sequential immersion of the dishes in 1 litre of cold PBS, cells were solubilized in 1 ml of 1 M-NaOH and the radioactivity was determined by γ -radiation counting. Total binding was determined in the presence of 0.2 nM-¹²⁵I-insulin. Non-specific binding was determined in the presence of 3.5 μ M unlabelled insulin. Specific binding was total minus non-specific binding.

Uptake of ¹²⁵I-insulin

Cells growing in tissue culture dishes were washed with PBS, 0.5 ml of prewarmed (37 °C) binding medium was added to the cells and the dishes were immediately placed in a water bath at 37 °C. To initiate uptake, 0.1 ml of MEM containing 50 nM-¹²⁵I-insulin and 100 nM unlabelled insulin was added to each dish (final concn. of insulin 25 nM). At appropriate times, dishes were removed from the water bath, chilled immediately to 4 °C by immersion in cold PBS and treated as described for the binding assay. Non-specific uptake was determined in the presence of 35 μ M unlabelled insulin.

Insulin internalization was determined as follows: after incubation in the presence of ¹²⁵I-insulin, cells were chilled to 4 °C in cold PBS, then rinsed twice with PBS and thereafter incubated for 90 min at 4 °C with 1 ml of 1% Pronase in PBS. At the end of the incubation period, the cells were completely detached from the dishes by pipetting and centrifuged for 2 min in an Eppendorf Microfuge. The radioactivity of the pellet was then counted.

Recycling experiments

Cells were grown in 175 cm² flasks. Protein synthesis was inhibited by incubating the monolayers in culture medium containing 10 μ g of cycloheximide/ml for 2 h at 37 °C [28]. Medium was then removed and 10 ml of prewarmed (37 °C) insulin-free binding medium or medium containing 0.1 μ M-insulin was added to the flasks, which were then kept at 37 °C for 15 min. Media

were aspirated, and the monolayers were rinsed three times with cold (4 °C) PBS and incubated at 4 °C for 90 min in the presence of 1% Pronase in PBS. The cells were then gently pipetted free of the culture flasks, 2 ml of fetal bovine serum was added and the suspensions were centrifuged (1000 g for 10 min). The pellets were rinsed twice with binding medium containing 10% fetal bovine serum and twice with binding medium without fetal bovine serum. The cells were then resuspended in binding medium, divided into batches in Eppendorf Microfuge tubes and warmed up to 37 °C for various time periods with frequent agitation. At the end of each incubation time, tubes were chilled on ice for 5 min. Until this step cycloheximide was present in all solutions. Recycled receptors were quantified by incubating the cell suspension at 4 °C for 2 h in the presence of 0.2 nM-¹²⁵I-insulin. Non-specific binding was determined in the presence of an excess of unlabelled insulin. Cell suspensions were then centrifuged for 1 min in an Eppendorf Microfuge. The supernatants were aspirated and the radioactivity of the pellets was counted. Total binding was determined in triplicate and non-specific binding in duplicate.

Down-regulation experiment

To induce down-regulation, sterile insulin was added to the cell monolayers in culture media. The cells were further incubated at 37 °C for 24 h. At timed intervals after the addition of insulin-containing or insulin-free media, cell monolayers were subjected to a ligand-unbinding procedure to remove insulin from the cells. They were rinsed with PBS and incubated at 37 °C for 20 min in the presence of binding medium adjusted to pH 6.0. Medium was removed and the incubation was repeated under the same conditions. Cell monolayers were then rinsed twice with PBS. By this protocol, insulin is dissociated from the cell surface and, in addition, internalized insulin and the degradation products are released into the culture medium (results not shown).

Internalization of class I antigens

Cell monolayers were incubated for 1 h on ice in the presence of anti-class I monoclonal antibody at 25 μ g/ml. After two washes the cells were incubated for 1 h on ice in the presence of ¹²⁵I-labelled rabbit anti-mouse IgG (¹²⁵I-RAM). After three washes they were incubated at 37 °C for various periods of time in the absence or presence of 1 μ M-insulin. Cells were then washed with either ice-cold binding medium or ice-cold acid buffer, containing 85 mM-NaCl, 50 mM-glycine, 5 mM-KCl, 7.5 mM-NaN₃ and 0.1% albumin, pH 2.0. Cells were then solubilized as indicated above and radioactivity was determined by γ -radiation counting.

Other procedures

Cell-surface labelling and cross-linking were performed as previously described [23] with some modifications. Briefly, HepG2 cells were first labelled with ¹²⁵I-labelled photoreactive insulin at 4 °C. Cells were then incubated for 30 min with 5 mCi of Na¹²⁵I, 2.5 units of lactoperoxidase/ml, 8 units of glucose oxidase/ml and 160 mM-glucose. After three washes the cells were treated with DSP, washed and harvested. Cells were then lysed with PBS containing 1% Nonidet P40, 100 units of apro-tinin/ml and 1 mg of bacitracin/ml. Cell extracts were

Fig. 1. Insulin binding to HepG2 cells at 4 °C

HepG2 cells were incubated with 0.2 nM- 125 I-insulin in the absence or presence of various concentrations of unlabelled insulin. After 2 h at 4 °C, cell-bound insulin was determined by γ -radiation counting, and the data were plotted by the Scatchard method. Non-specific binding, defined as binding in the presence of 3.5 μ M unlabelled insulin, was subtracted from all data points. Each point is the mean of triplicate determinations.

pre-cleared by incubation with Pansorbin and incubated with antibodies for 4 h at 4 °C and a further 1 h at 4 °C with Pansorbin. The pellet was recovered by centrifugation (12000 *g* for 30 s), washed and solubilized in SDS buffer (10 mM- NaH_2PO_4 /10% glycerol/3% SDS, pH 6.8). The chemical cross-linker was cleaved by boiling the samples for 5 min in the presence of 2% β -mercaptoethanol. Labelled proteins were separated by polyacrylamide-gel electrophoresis as described by Laemmli [29], with 7.5–17.5% linear gradients of acrylamide as resolving gel, and revealed by autoradiography.

Protein was determined by the method of Lowry *et al.* [30]. Cell viability at the end of the experiments was above 95%, as judged by exclusion of Trypan Blue.

RESULTS

125 I-insulin binding to HepG2 cells

The number of insulin receptors on HepG2 cell membranes and their affinity for 125 I-insulin were determined by steady-state binding analysis at different insulin concentrations at 4 °C. At this temperature insulin binds only to the cell-surface receptors in a time-dependent manner, and steady state was reached within 2 h. Scatchard [31] plots of the binding data are shown in Fig. 1. The curvilinear pattern may reflect heterogeneity of sites [32] or negative co-operativity [33]. According to a two-population model, we estimated 4×10^4 high-affinity binding sites per cell with an apparent dissociation constant of 3×10^{-9} M, and 8×10^4 low-affinity binding sites per cell with an apparent

Fig. 2. Effect of protein cross-linking on the immunoprecipitation of insulin receptors by anti-class I antibodies

HepG2 cells were double-labelled with 125 I-labelled photoreactive insulin and by surface iodination and treated or not with DSP. After solubilization cell extracts were incubated with normal mouse IgGs (lanes a,f), anti-insulin receptor antibodies (lanes b,g), anti- β_2 -microglobulin antibodies B1.1.G.6 (lanes d,i), anti-class I heavy-chain antibodies B9.12.1 (lanes c,h) and anti-transferrin receptor OKT9 (lanes e,j). After SDS/polyacrylamide-gel electrophoresis, labelled immunoprecipitated proteins were revealed by autoradiography. Insulin-receptor α -subunit (INS-R), class I heavy chain (CI I) and β_2 -microglobulin (β_2 m) are identified by arrows on the gel: OR, origin. Positions of M_r markers are also indicated.

dissociation constant of 8×10^{-8} M. These values are in excellent agreement with those reported by Ciechanover *et al.* [34].

Molecular interaction between insulin receptors and class I MHC antigens

MHC antigens and insulin receptors have been found to form selective molecular complexes in mouse liver membranes [22] and human B lymphocytes [23]. To investigate possible similar interactions in HepG2 cells, surface proteins were double-labelled with the 125 I-labelled photoreactive insulin analogue and by lactoperoxidase-catalysed iodination. Intact cells were then exposed to buffer or to the bifunctional cross-linker DSP for 10 min at 4 °C. Under the latter conditions all the intimately associated proteins in which free amino groups are 1.2 nm apart can be covalently bound together. This cross-linking reagent has a cleavable disulphide bond which allows, under reducing conditions, isolation of individual chains that have been cross-linked. Fig. 2 shows that, in the absence of cross-linking (lanes a-e), anti-class I heavy chain (lane c) precipitated a component at 45 kDa and an additional component at

130 kDa, corresponding to the α subunit of the insulin receptor. The amount of insulin receptors precipitated by anti-class I antibody represents a small fraction of the amount precipitated by anti-(insulin receptor) antibody. When DSP treatment was introduced before cell solubilization and immunoprecipitation, the amount of insulin receptors being precipitated by anti-class I heavy-chain antibody increased markedly (lane *h*). Another unidentified component at 35 kDa was also precipitated (lane *h*). Similarly, when anti- β_2 -microglobulin antibody was used, an appreciable amount of insulin receptors was precipitated only after protein cross-linking (lanes *d* and *i*). No labelled material was precipitated by an equivalent amount of normal mouse IgG (lanes *a* and *f*). When the cell extracts were exposed to anti-transferrin-receptor antibody, there was no co-precipitation of any other protein with the transferrin receptor at 94 kDa (lanes *e* and *j*). Thus, in the absence of cross-linking, a small amount of insulin receptors were precipitated with class I MHC antigens and none with transferrin receptors. After membrane cross-linking, a high proportion of insulin receptors were co-precipitated with class I antigens, demonstrating their close vicinity in the plasma membrane. After cross-linking, anti-class I antibody precipitated a large fraction of insulin receptors, but anti-receptor antibodies did not precipitate class I molecules. Similar observations have been made in two human B-cell lines and extensively discussed [23].

Internalization of surface-bound insulin

To determine the extent of insulin internalization by HepG2 cells, we used the procedure of Ciechanover *et al.* [34]. To discriminate between surface-bound and internalized ligand, cells were incubated with ^{125}I -insulin (25 nM) for various times at either 37 °C or 4 °C. At appropriate times, cells were chilled to 4 °C and treated without or with Pronase at 4 °C for 90 min. Only surface-bound ligand was accessible to the proteolytic enzyme and was released into the medium, whereas the internalized ligand was protected from proteolysis and recovered in the pellet. Fig. 3 shows that cell-associated ^{125}I -insulin reached an apparent steady state by 10–12 min. The curve had two components: a rapid phase, which resulted in 70% occupation of the binding sites within the first 1 min, and a slower component, which led to the occupation of the remaining 30% with an apparent half-time of 4–5 min. Fig. 3 also shows that Pronase-resistant binding increased with time. An increasing fraction of cell-associated ^{125}I -insulin became Pronase-resistant with time, and reached a maximum of 73%, with a half-time of approx. 2 min. These data indicate that insulin is rapidly internalized by HepG2 cells at 37 °C. The effects of temperature and metabolic inhibitors on insulin internalization were investigated. Cells were incubated with ^{125}I -insulin at 37 °C for 12 min in the absence or the presence of 10 mM-NaCN. The monolayers were chilled to 4 °C, and cell-associated and Pronase-resistant ^{125}I -insulin binding were determined. Table 1 shows that, in the absence of cyanide, 70% of labelled insulin was internalized after 12 min. In contrast, when cells were incubated in the presence of cyanide, only 4% of ^{125}I -insulin was Pronase-resistant, indicating that cyanide inhibits internalization.

When the experiment was performed at 4 °C for 3 h, only 6% of cell-associated ^{125}I -insulin was Pronase-resistant. This indicated that at 4 °C no internalization

Fig. 3. Binding and internalization of ^{125}I -insulin in HepG2 cells

Cell monolayers were washed and incubated with 25 nM- ^{125}I -insulin for various times at 37 °C. At appropriate times, dishes were chilled to 4 °C and rinsed free of unbound ^{125}I -insulin and treated (○) or not (●) with 1% Pronase for 90 min at 4 °C. The radioactivity remaining associated with the cells was determined. Data have been corrected for non-specific binding. The bars represent the range of duplicate or triplicate determinations. Inset: data replotted as the cell-surface-associated ^{125}I -insulin (Pronase sensitive) versus incubation time.

occurred. Therefore cell-associated ^{125}I -insulin, observed when receptor translocation was inhibited by a metabolic inhibitor or by low temperature, corresponded to ^{125}I -insulin bound exclusively to the cell surface.

Table 1 also shows that ^{125}I -insulin associated with the cells after 12 min in the absence of cyanide was 30% higher than in the presence of cyanide. This difference could be due to the inhibition of insulin binding by cyanide. This is not the case, since the same extent of insulin binding was observed when receptor translocation was inhibited by either cyanide or low temperature (Table 1). Further, the metabolic inhibitor did not inhibit insulin binding at 4 °C (results not shown). Thus only 70% of ^{125}I -insulin associated with cells after 12 min at 37 °C (in the absence of inhibitor) could be accounted for by insulin binding to receptors originally located at the cell surface. Additional evidence comes from the kinetics of ^{125}I -insulin (25 nM) binding to HepG2 cells in the presence of cyanide at 37 °C: in these conditions, only one phase was observed, and steady state was reached by 60–90 s. This result suggests that the rapid phase seen in Fig. 3, leading to 70% site occupation within 60 s, corresponds to ^{125}I -insulin binding to sites located on the cell surface. Later, the ^{125}I -insulin–receptor complexes are progressively internalized. This also suggests that the remaining 30% of the sites correspond to an intracellular pool of binding sites which are progressively externalized to the cell surface, where they were quickly occupied by hormone (since saturation of binding sites on the cell surface is rapid). The half-time for this exocytosis process was approx. 4–5 min.

The difference between rates of internalization and externalization in the presence of hormone resulted in a decrease of surface-associated ^{125}I -insulin, as shown in

Table 1. Effects of temperature and cyanide on insulin uptake by HepG2 cells

Cell monolayers were washed and incubated with 25 nM- 125 I-insulin in the presence or absence of 10 mM-NaCN for 3 h at 4 °C or for 12 min at 37 °C. The cells were then chilled to 4 °C and treated or not with 1% Pronase at 4 °C for 90 min. The cells were harvested, pelleted, and radioactivity associated with the pellet was determined. Each value represents the mean of triplicate determinations; data were corrected for non-specific uptake.

Cells	Specific binding of 125 I-insulin (% of control)		
	4 °C	37 °C	
		10 mM-CN ⁻	No CN ⁻
Control	100	102	130
Pronase-treated	7	5	86

the inset of Fig. 3. In other words, on addition of hormone, insulin receptors rapidly redistributed to a cellular compartment inaccessible to Pronase.

Taken together, our results suggest the following sequence. At zero time of the experiment depicted in Fig. 3, 70% of receptors were on the cell surface and 30% inside the cell. The addition of insulin (25 nM) induced a rapid endocytosis of hormone-receptor complexes, and, more slowly, an exocytosis of internal ligand-free receptors. After 12 min about 70–75% of the receptors were inside (Pronase-resistant) and 25–30% were on the cell surface.

Recycling of internalized receptors

The data reported above suggested that insulin receptors inside the cells can recycle back to the cell membrane. To obtain direct evidence for this, we performed the following experiment: cells were incubated in the presence of 0.1 μ M unlabelled insulin for 12 min at 37 °C to allow the receptors to redistribute inside the cells. The monolayers were then chilled to 4 °C, and receptors still at the cell surface were destroyed by treatment with Pronase at 4 °C, and the presence of functional receptors at the cell surface was assessed by measuring 125 I-insulin binding. Protein synthesis was inhibited by 10 μ g of cycloheximide/ml. A similar experiment was carried out without allowing the receptors to redistribute inside the cells. When the cells were treated with insulin before inactivation of the external receptors by Pronase, binding activity reappeared at the cell surface in a time-dependent manner ($t_{1/2}$ = 30–40 min) (Fig. 4). The possibility that these binding sites corresponded to newly synthesized receptors can be ruled out, since the experiment was carried out in the presence of a concentration of cycloheximide which fully inhibits protein synthesis [28]. Further, they could not have arisen as the result of a mobilization of receptors synthesized before addition of cycloheximide and progressively processed, since very little recycling was observed when the cells were incubated in the absence of insulin before the Pronase treatment. Thus

Fig. 4. Recycling of internalized insulin receptors in hepatoma cells

Cell monolayers were washed and incubated at 37 °C in the absence (○) or presence (●) of 0.1 μ M unlabelled insulin for 15 min. Dishes were chilled to 4 °C, rinsed of unbound insulin, treated with 1% Pronase at 4 °C for 90 min, and cells were harvested and extensively washed to remove Pronase, then warmed to 37 °C for various times. Cycloheximide (10 μ g/ml) was present in all the solutions. At appropriate times, cell samples were chilled again to 4 °C and assayed for 125 I-insulin-binding capacity. Details are described in the Experimental section.

these results further indicate that insulin, on binding to cells, induces a redistribution of its receptor to an intracellular compartment, and they demonstrate that internalized receptors recycle back to the cell surface. These processes are not inhibited by cycloheximide.

Insulin-induced down-regulation

The ability of HepG2 cells to exhibit insulin-induced down-regulation of cell-surface insulin receptors was investigated. Cell monolayers were exposed to either insulin-free medium or medium containing 1 μ M-insulin at 37 °C for various times. Cells were then subjected to the ligand-unbinding procedure (to remove insulin from the cells) as described in the Experimental section, and cell-surface 125 I-insulin binding capacity was determined at 4 °C. Fig. 5 shows that chronic exposure to insulin resulted in a time-dependent decrease of insulin-binding capacity.

During the first 3 h, the receptor internalized in the presence of insulin recycled back to the surface during the unbinding protocol, so that no decrease in insulin-binding capacity was observed during this period. After this lag time, insulin-binding capacity fell to approx. 40% of its original value, with a half-time of 7 h. Scatchard analysis of 125 I-insulin binding at the cell surface (4 °C) of control and down-regulated cells (16 h) indicated that cell-surface receptor number fell from 47000 to 19200 high-affinity sites/cell, but the apparent affinity of these receptors for insulin remained unchanged: 2.3 and 1.8 nM for control and down-regulated cells respectively.

Fig. 5. Down-regulation of insulin receptors in hepatoma cells

Dishes with 60–80% confluent monolayers of HepG2 cells were incubated in the absence (○) or presence (●) of 1 μ M-insulin at 37 °C for various times. At appropriate times dishes were washed and cells were subjected to the unbinding protocol as described in the Experimental section (i.e. two incubations for 20 min at pH 6.0 and 37 °C). Insulin binding was measured by incubating the cells with 0.2 nM- 125 I-insulin at 4 °C for 2 h. Data were corrected for non-specific binding. Each point is the mean of duplicate determinations.

Fig. 6. Internalization of class I antigens in HepG2 cells

Cells were successively preincubated at 4 °C with anti-class I antibodies and with 125 I-RAM to label cell-surface MHC antigens. Cells were then warmed to 37 °C for various periods of time. The proportion of class I antigens internalized during these periods was evaluated by measuring the proportion of 125 I-RAM that remained cell-associated after an acid wash of the cells (○). The effect of insulin was measured by adding 1 μ M-insulin during the incubation period at 37 °C (●).

Internalization of class I MHC antigens

Internalization of MHC antigens was quantified by measuring the variation at 37 °C of the acid-releasable portion of anti-class I antibodies prebound to intact cells at low temperature. In this protocol, antigen–antibody– 125 I-RAM complexes that have been internalized remain cell-associated when the cells are briefly incubated at pH 2.0. Fig. 6 shows that 98% of the 125 I-RAM antibodies bound to class I antigen–anti-class I antibody complexes could be released by an acid wash when the cells were incubated for 1 h at 4 °C. At 37 °C the fraction of 125 I-RAM remaining associated with the cells after the acid wash increased with time. Within 1 h HepG2 cells have internalized 20–25% of their cell-surface class I antigens. When insulin was present during the incubation at 37 °C, no effect on internalization of class I antigen could be detected. Reciprocally, treatment of the cells with anti-class I antibody and unlabelled RAM at 4 °C had no effect on insulin internalization (results not shown). In addition to this lack of transmodulation, internalization of class I antigens ($t_{1/2} \approx 2$ h) and insulin receptors ($t_{1/2} \approx 3$ min) takes place at very different rates. Taken together, these results thus indicate that class I MHC antigens and insulin receptors are internalized independently.

DISCUSSION

The human hepatoma cell line HepG2 provides a model [35] which presents a metabolically stable population of defined cells. This monolayer-forming cell line offers the possibility of performing experiments such as recycling, which need very severe treatments (many

temperature changes, proteolytic digestion, incubation for long periods). These experiments were not feasible with isolated hepatocytes or adipocytes.

Once bound to its receptor, insulin is rapidly internalized at 37 °C. This is shown by the rapid acquisition of resistance to extracellular Pronase. This cellular uptake process is temperature- and energy-dependent (Table 1), suggesting that internalization is mediated by receptor endocytosis. After 6 min about 70–75% of the specific cell-associated radioactivity is intracellular; the half-time required to reach this value is 2 min (calculated from Fig. 3).

HepG2 cells behave like isolated rat hepatocytes. Indeed, a similar large fraction of insulin receptors is internalized in the presence of insulin [14], and a comparable half-time has been measured for internalization of insulin–receptor complex [36].

It should be noted that no insulin degradation products could be detected during the first 30 min of incubation (results not shown), indicating that HepG2 cells internalize insulin in the intact form.

The time course of cell association of insulin exhibits two phases. The first represents the rapid occupation of the cell-surface binding sites, and the second very likely corresponds to the occupation of sites originally inside the cell which are progressively externalized. At a given time, the number of receptors present at the cell surface results from the balance between the rate of internalization and the rate of externalization of the receptors. Since binding of insulin to its receptors is faster than receptor translocation, the drastic and time-dependent decrease in radioactivity on the cell surface (Fig. 3 inset) indicates that externalization is slower than internalization. In other words, if the process of receptor internalization/externalization occurs continuously even in the absence

of ligand, the presence of insulin accelerates internalization or decreases externalization, thus causing depletion of surface receptors and accumulation of receptors inside the cell. More information will be necessary to distinguish further between the two possibilities. In HepG2 cells, 70% of the insulin receptors are on the cell surface and 30% in an intracellular pool. On addition of hormone, there is a rapid redistribution of receptors (about 70–75% inside, 25–30% at the surface). Insulin-induced accumulation of insulin receptors into an intracellular compartment is also demonstrated by the fact that very little recycling is observed if the receptors have not been previously internalized by acute exposure of the cells to insulin. In contrast with our finding, it has been reported that insulin does not induce internalization of its receptors in Fao rat hepatoma cells [21]. The reason for this discrepancy is not clear. However, it should be noted that at maximal internalization only 7.5% of the receptors are internalized by these cells, compared with 70% in isolated rat hepatocytes [14] and human hepatoma cells (Fig. 3 and Table 1). This raises the possibility that Fao rat hepatoma cells have lost the machinery for ligand-induced endocytosis and that only the constitutive endocytosis remains in these cells.

As mentioned above, the present work affords a demonstration of recycling of insulin receptors in HepG2 cells. Since these experiments were carried out in the presence of cycloheximide, we can conclude that: (i) the re-appearance of insulin receptors on the cell surface is not due to receptor synthesis *de novo*; (ii) cycloheximide does not prevent internalization or recycling of insulin receptors in HepG2 cells, as previously reported for 3T3-C2 fibroblasts [17], but in contrast with what is observed in isolated rat adipocytes [37].

The rates of insulin-receptor internalization and recycling appear to be very different, since maximal internalization is observed within a few minutes, whereas even after 2 h recycling of internalized receptors is not complete (Figs. 3 and 4). Similar results have been reported for 3T3 fibroblasts [17]. Even larger differences in the rates of internalization and recycling have been observed for the insulin-receptor complex (by using photo-reactive insulin) in isolated rat hepatocytes [19]. On the other hand, the rate of recycling is lower than that of externalization, as deduced from the uptake experiment depicted in Fig. 3. However the two measurements do not reflect the same process. Indeed, in the uptake experiment the receptors that are externalized during the first 12 min come from an intracellular pool of ligand-free receptors; once at the cell surface these receptors bind insulin and are re-internalized. Thus, after the initial rapid decrease of cell-surface receptors, internalization of receptors continues more slowly, probably even after 12 min. These internalized receptors with insulin bound to them follow a pathway in which ligand and receptor are probably dissociated in acidic vesicles before recycling.

It is well established that chronic exposure of cells to high concentrations of insulin induces a down-regulation of insulin receptors. It has been demonstrated in cultured lymphocytes [8] and cultured adipocytes [9] that receptor decay is due to an acceleration of receptor degradation, with no change in synthesis rate. It has also been proposed [19] that down-regulation could result from an internalization of the hormone-receptor complex, accompanied by a decrease in receptor recycling. Our results

support these views; thus insulin, by rapidly inducing redistribution of its receptors to an intracellular compartment (by increasing internalization or decreasing recycling), increases the rate of receptor degradation.

Molecular interactions between insulin receptors and class I MHC antigens have been demonstrated in mouse liver membranes [22] and human B-cell lines [23] by co-precipitation of photolabelled insulin receptors and MHC antigens with anti-class I monoclonal antibodies. By contrast with mouse liver membranes, very few insulin receptors could be co-precipitated with class I antigens in HepG2 cells. However, stabilization of the association between insulin receptors and class I antigens could be obtained *in situ* by treating the cells with the homobifunctional cross-linker DSP, indicating that insulin receptors selectively adjoin class I antigens in HepG2 cells.

It could be expected that the existence of non-covalent complexes between class I antigens and insulin receptors could influence the dynamic properties of the two partners. Our results show, however, that insulin receptors and class I antigens are internalized at different rates and that no transmodulation occurs.

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