Insulin Receptor Regulation and Desensitization in Rat Hepatoma Cells

THE LOSS OF THE OLIGOMERIC FORMS OF THE RECEPTOR CORRELATES WITH THE CHANGE IN RECEPTOR AFFINITY*

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We have previously reported that prolonged incubations of Fao cells, a cell line derived from the well-differentiated Reuber H35 rat hepatoma, with 10^{-6} M insulin, induced a decrease in receptor number (down-regulation), an increase in receptor affinity for insulin, and a loss of insulin's biological effect (desensitization). In the present study, we have investigated the relationship between these changes in insulin binding and action and changes in the structure of the insulin receptor. Intact cells were surface labeled with Na^{125}I and lactoperoxidase, and the {125}I-labeled insulin receptor was immunoprecipitated using specific antibodies and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiography of gels done under reducing conditions demonstrated the decrease in the number of receptors and an increase in receptor affinity. In these cells using immunoprecipitation of labeled receptors and gel electrophoresis under nonreduced conditions. We find that down-regulation and desensitization in hepatoma cells results in a preferential loss of the higher-molecular forms of the receptor. A similar increase in affinity and loss of higher-oligomeric forms is observed after treatment with reducing agents. These data suggest that there are differences in binding affinity and biological function of these different forms of receptor.

Over the past few years, studies using a number of different techniques have suggested that the insulin receptor is composed of two major subunits: an \( \alpha \) subunit (\( M_r = 135,000 \)) and a \( \beta \) subunit (\( M_r = 95,000 \)) (1-13). These subunits are linked by disulfide bonds to form oligomeric complexes of different molecular weights. By using covalent cross-linking of {125}I-insulin to its receptor in adipocytes, Massague et al. (14) have observed one predominant disulfide-linked oligomeric complex of \( \alpha \) and \( \beta \) subunits with an \( M_r = 350,000 \); in addition, some partially degraded forms with \( M_r = 320,000 \) and 290,000 were observed if proteolysis was allowed to occur. Using immunoprecipitation with antibodies to the insulin receptor and either biosynthetic or surface labeling of cultured human lymphocytes, we have observed two high-molecular-weight forms of the native insulin receptor (\( M_r = 520,000 \) and \( 350,000 \)) (15), as well as lower-molecular-weight oligomeric forms and free subunits. The possible roles of these different forms of the insulin receptor in the binding and the action of the hormone are unknown.

We have recently reported that insulin-induced receptor down-regulation in hepatoma cells in culture is characterized by a decrease in the number of receptors and an increase in insulin binding affinity (16). At the same time, there is a desensitization of biological response (16, 17). In the present study, we have studied the structure of insulin receptors in these cells using immunoprecipitation of labeled receptors and gel electrophoresis under nonreduced conditions. We find that down-regulation and desensitization in hepatoma cells results in a preferential loss of the higher-molecular forms of the receptor. A similar increase in affinity and loss of higher-oligomeric forms is observed after treatment with reducing agents. These data suggest that there are differences in binding affinity and biological function of these different forms of receptor.
Regulation of Insulin Receptor Structure in Hepatoma Cells

MATERIALS AND METHODS

Chemicals—Na\textsuperscript{251}I and Triton X-100 were purchased from New England Nuclear. Dithiothreitol, N-ethylmaleimide, aprotinin, and phenylmethylsulfonyl fluoride were obtained from Sigma; butyrylcholinesterase from Boehringer Mannheim; and staphylococcal protein A (Pansorbin) from Calbiochem. Reagents for gel electrophoresis were from Bio-Rad. Conn's modified Ham's F12 culture medium and fetal calf serum were obtained from Grand Island Biological Company (Grand Island, NY). All other chemicals were of the best grade commercially available. \textsuperscript{125}I-Insulin was prepared according to the modified chloramine-T method of Freychet (18).

Cell Culture and Insulin Binding and Down-regulation—Fao (19) is a well-differentiated subclone derived from the Rb35 rat hepatoma (20). These cells were grown attached to Falcon plastic culture dishes in modified Ham's F12 medium (21), supplemented with 5% fetal calf serum, and equilibrated with 5% CO\textsubscript{2} in air in a humidified incubator at 37 °C. For these experiments, cells were detached with 0.05% trypsin and 0.02% EDTA in isotonic PBS, and cell pellets were washed with 5% fetal calf serum, and equilibrated with 5% CO\textsubscript{2} in PBS containing 0.1% bovine serum albumin (12). Two-dimensional electrophoreses were performed as described previously (15). Following one-dimensional electrophoresis in 5% polyacrylamide gel under nonreducing conditions, an entire lane was cut and rinsed in 0.125 M Tris-\(\text{HCl} \) (pH 6.8), 0.1% Na\textsubscript{2}SO\textsubscript{4}, for 40 min at room temperature. The lane was placed atop a second Na\textsubscript{2}SO\textsubscript{4}, gel, fixed in place with 1% agarsose, in 0.125 M Tris-\(\text{HCl} \) (pH 6.8), and overlaid with 0.1 M DTT. Electrophoresis was then performed into a resolving gel with an acrylamide concentration of 7.5%.

RESULTS

Confluent monolayers of rat hepatoma (Fao) cells were surface labeled with Na\textsuperscript{251}I and lactoperoxidase and the insulin receptor was immunoprecipitated by sera containing autoantibodies against the insulin receptor. Under reduced conditions, Na\textsubscript{2}SO\textsubscript{4}, polyacrylamide gel electrophoresis of immunoprecipitates revealed two specific bands of \(M_1 = 135,000 \) and 95,000 (Fig. 1, top). These bands correspond to the \( \alpha \) and \( \beta \) subunits of the receptor, as we and others have previously reported (1-6, 8). When electrophoresis was performed under nonreduced conditions, six major bands were specifically immunoprecipitated (Fig. 1, top). They correspond to the free \( \alpha (M_1 = 120,000) \) and \( \beta (M_1 = 95,000) \) subunits and four oligomeric forms of \( M_1 = 210,000, 270,000, 350,000, \) and 520,000.

In order to know the molecular composition of the oligomeric forms of the receptor, immunoprecipitates of \( \text{Na}^{251}I \)-labeled receptor were electrophoresed on Na\textsubscript{2}SO\textsubscript{4},polyacrylamide gel under nonreduced conditions, and the lane of interest was cut and placed horizontally atop a second and electrophoresed in the presence of 0.1 M DTT to reduce the disulfide bonds. Under these conditions, the proteins of \( M_1 = 210,000, 350,000, \) and 520,000 were separated into two bands with \( M_1 = 95,000 \) and 135,000, corresponding to the \( \alpha \) and \( \beta \) subunits (Ref. (11) and Fig. 1, bottom). The \( \beta \) subunit (Fig. 1, bottom) produced only one band of \( M_1 = 135,000 \) suggesting that this is an \( \alpha-\alpha \) dimer. The free \( \alpha \) and \( \beta \) subunits migrated as the \( M_1 = 135,000 \) and 95,000 proteins, respectively. The difference in mobility and apparent molecular weight of the \( \alpha \) subunit in the reduced gel compared to that in the nonreduced gel is presumably due to the existence of intramolecular disulfide bonds with unfolding of the molecule upon reduction (5, 15).

The relative amount of the different forms of the surface-labeled insulin receptor was determined by either counting the radioactivity associated with bands on the gel or by scanning the density of the autoradiogram. Under our conditions, the species corresponding to \( \alpha \beta \), \( \alpha \), and \( \beta \) were approximately equal in amount, each representing about 15% of the total radioactivity (Table I). The reduced \( \alpha \) and \( \beta \) subunits account for close to 50% of the radioactivity. The difference in mobility with the different forms of the receptor is, however, an overestimation since, as shown by the two-dimensional gel, under these conditions the free \( \beta \) subunit is more extensively labeled than the covalently linked \( \beta \) subunit. The \( M_1 = 520,000 \) form of the receptor was the most variable, accounting for 0 (undetectable) to 13% of the total receptor forms.
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Relative proportion of the different forms of the surface-iodinated insulin receptor in Fa0 hepatoma cells

| Apparent Mr | Subunit composition | Per cent |
|-------------|---------------------|----------|
| 95,000      | \( \beta \)         | 19.1 ± 1.5 |
| 135,000     | \( \alpha \)         | 30.0 ± 2.2 |
| 210,000     | \( \alpha \beta \)    | 13.5 ± 0.9 |
| 270,000     | \( \alpha \)         | 16.0 ± 1.2 |
| 350,000     | \( \alpha \beta \)    | 15.7 ± 1.5 |
| 520,000     | \( \alpha \beta \delta \) | 5.5 ± 1.9 |

\footnote{a Determined by NaDodSO4-polyacrylamide gel electrophoresis.}
\footnote{b Deduced from two-dimensional gel analysis; the stoichiometry of the Mr, 350,000 and 520,000 forms is hypothetical.}
\footnote{c Per cent of total receptor forms determined by scanning densitometry of the autoradiogram and counting the radioactivity of specific labeled bands of the gels. Mean values of nine different experiments ± SE.}

FIG. 2. Immunoprecipitated insulin receptor surface labeled at 4 and 22 °C in control hepatoma Fa0 cells. Intact monolayers of control cells were surface labeled with Na\(^{125}\)I and lactoperoxidase at either 4 or 22 °C for 45 min. Cells were washed and solubilized at 4 °C. Immunoprecipitation was conducted at 4 °C for 18 h. The immunoprecipitates were analyzed on NaDodSO4-polyacrylamide (5%) gel electrophoresis in the absence of DTT.

Previous studies using affinity labeling, particularly in adipocytes (14), have suggested only one major oligomer form of receptor under nonreducing conditions and this has a Mr = 350,000. In order to know which forms of the insulin receptor present in the hepatoma cells could bind the hormone, \(^{125}\)I-insulin was bound to equilibrium to the cells and chemically cross-linked to the receptors using different concentrations of DSS (23). The autoradiogram of the gel run under nonreducing conditions after cross-linking with 0.2 mM DSS revealed a major band (Mr = 125,000) (Fig. 3). With increasing concentrations of the cross-linker, three additional bands appeared of Mr = 210,000, 250,000, and 320,000 and possibly of higher molecular weight. The labeling of these bands was specifically inhibited by the addition of unlabeled insulin. When the same cell extracts were analyzed on NaDodSO4-polyacrylamide (7.5%) gel in the presence of DTT, a single band of Mr = 135,000 was visible which corresponded to the

FIG. 1. Insulin receptor structure in hepatoma Fa0 cells. Top, autoradiogram showing \(^{125}\)I-labeled insulin receptor without (left) and with (right) reduction of disulfide bonds. Hepatoma cells were surface labeled with Na\(^{125}\)I and lactoperoxidase, solubilized with 1% Triton X-100, and immunoprecipitated by pooled control or antireceptor B-9 sera. The immunoprecipitates were analyzed on NaDodSO4-polyacrylamide (5%) gel electrophoresis before (left) and after (right) reduction with 0.1 M DTT. Bottom, autoradiogram of two-dimensional NaDodSO4-polyacrylamide gel electrophoresis of \(^{125}\)I-labeled insulin receptor. Electrophoresis in the first dimension was performed without reduction as shown on the top left. Following one-dimensional electrophoresis, the lane was cut out, rinsed in 0.125 M Tris-HCl, pH 6.8, 0.5% NaDodSO4, and placed atop a second NaDodSO4 gel, sealed with 1% agarose, and overlaid with 0.1 M DTT. Reduction of disulfide bonds was carried out during electrophoresis. The resolving gel on the second dimension had an acrylamide concentration of 7.5%.

also observed that the relative proportion of the oligomeric forms of the insulin receptor increased significantly with time when the solubilized receptor was stored at −70 °C; thus, in all subsequent experiments, the immunoprecipitates were immediately analyzed by gel electrophoresis.

To determine whether some of these different forms of the receptor could be the consequence of receptor internalization or receptor processing occurring during the period of iodination (30 min at 22 °C), hepatoma cells were labeled, washed, and solubilized at 4 °C. At this temperature, receptor mobility and internalization have been shown to be significantly impaired (25). All six forms of the receptor were labeled at 4 °C (Fig. 2), suggesting that all are located at the plasma membranes and do not occur as a result of processing. As expected, the labeling was less effective at 4 °C than at 22 °C (Fig. 2). This resulted in a decreased intensity of all the bands except that of the Mr = 350,000 form of the receptor. The reasons for this discrepancy was not known.
 Regulation of Insulin Receptor Structure in Hepatoma Cells

**Regulation of Insulin Receptor Structure in Hepatoma Cells**

**Fig. 3.** Insulin receptor cross-linked with $^{125}$I-insulin using various concentrations of DSS in hepatoma Fao cells. Monolayers of hepatoma cells were incubated with $2-5 \times 10^{-10}$ M $^{125}$I-insulin in the presence and absence of $10^{-8}$ M unlabeled hormone for 4 h at 15°C. After washing to remove unbound insulin, various concentrations of DSS were added as indicated under “Materials and Methods.” Monolayers were solubilized by boiling 2% NaDodSO$_4$ and analyzed on NaDodS04-polyacrylamide (5%) gel electrophoresis in the absence of DTT.

**Fig. 4.** Scatchard plots of insulin binding in control, down-regulated, and DTT-treated hepatoma Fao cells. For down-regulation, six-well dishes with confluent monolayers of cells were incubated with $10^{-6}$ M insulin for 24 h at 37°C and extensively washed to remove insulin. In a parallel experiment, cells were incubated with PBS containing 0.1% bovine serum albumin and 7 mM DTT for 10 min at 22°C. The reaction was stopped by adding 12.5 mM NEM (final concentration), and the cells were washed another 10 min in PBS containing 0.1% bovine serum albumin. Insulin binding was measured in the control, down-regulated, and DTT-treated cells by incubating the monolayers with 1 ml of buffered medium containing $1 \times 10^{-11}$ M $^{125}$I-insulin and various concentrations of unlabeled hormone. When DTT and NEM were simultaneously added to the cells, no effect on insulin binding was observed. Each point is the mean of triplicate experiments.

**Fig. 5.** $^{125}$I-labeled insulin receptor immunoprecipitated by human antiserum (B-9) in control and down-regulated hepatoma Fao cells. Intact monolayers of control and down-regulated (incubated with $10^{-6}$ M insulin for 24 h) cells were labeled with NaI$^{251}$ and lactoperoxidase, solubilized, and immunoprecipitated as indicated in Fig. 1. Immunoprecipitates were analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis before (right) or after (left) reduction with 0.1 M DTT. The lanes of the gels of down-regulated cells contained twice the amount of extract as those of control cells, which showed changes in both receptor number and affinity; 2) in down-regulated IM-9 lymphocytes, which displayed a decrease in receptor number; and 3) in DTT-treated hepatoma cells, which bind insulin with different affinity.

**Structure of Insulin Receptor in Control and Down-regulated Hepatoma Cells**—As we have previously reported, incubations of hepatoma cells with insulin ($10^{-6}$ M) for 24 h induces a down-regulation of the receptor characterized by a decrease in receptor number but an increase in receptor affinity for insulin (Fig. 4). Both changes in the binding properties are dependent on time, temperature, and insulin concentration (16). Both changes are blocked by inhibitors of protein synthesis and are retained in isolated plasma membranes prepared from insulin-treated cells (16). These observations, in addition to receptor loss, suggested that a stable modification of the structure of the insulin receptor occurred during the process of down-regulation in hepatoma cells. To study this possibility, intact cells were labeled with NaI$^{251}$ and lactoperoxidase and insulin receptor was immunoprecipitated by specific autoantibodies and analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis before and after reduction of the disulfide bonds.

When the receptor structure was studied under reduced conditions, the intensity of both the $\alpha$ and $\beta$ subunits were clearly decreased after down-regulation. This is apparent in Fig. 5, even though the gel of down-regulated cells was performed using the immunoprecipitate of twice as many cells as in the control. If the immunoprecipitated insulin receptors from the down-regulated cells were subjected to electrophoresis under nonreducing conditions, again several bands were observed (Fig. 5). The changes occurring in receptor structure during down-regulation was evaluated by measuring the intensity of the different bands (scanning densitometry) and calculating the ratio of the total intensity of the oligomeric forms to the total intensity of the free forms. This ratio gives a measurement of the degree of oligomerization of the insulin

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$^{2}$The value of 1 for this ratio indicates that 50% of the receptor subunits are disulfide linked (oligomeric forms). However, in the case of $^{125}$I-labeled receptors, due to differential labeling of the $\beta$ subunit, this ratio underestimates the actual degree of oligomerization.
receptor and is useful in comparing gels made of different amounts of receptors. In control receptors, this ratio was 1.05 ± 0.10 (mean ± of the nine experiments reported in Table I). In Fig. 5 (right), this ratio was 1.2 and 0.7 for control and down-regulated receptors, respectively. This indicated that the proportion of the oligomeric forms of the receptor in down-regulated cells was clearly decreased when compared to that of controls.

Although we have previously shown that the receptor after down-regulation is still recognized by the human antireceptor antisera B-9 (16), to exclude the possibility that these differences were due to an altered recognition of the receptor for immunoprecipitation, the 125I-labeled receptor was immunoprecipitated using a rabbit antisera to purified insulin receptor (kindly provided by S. Jacobs, Burroughs Wellcome). After surface labeling, NaDodSO4-gel electrophoresis of receptors from control cells under nonreduced conditions again revealed six bands. Likewise, after down-regulation, the relative intensities of the bands corresponding to the highest-molecular-weight oligomeric forms of the receptor were clearly decreased when compared to those of free subunits. In this experiment, the ratio of the oligomeric forms to the free subunits was 1.1 and 0.4 for control and down-regulated receptors, respectively (Fig. 6).

**Structure of Insulin Receptor in Control and Down-regulated IM-9 Lymphocytes**—In order to know whether the change in receptor structure occurring during down-regulation in hepatoma cells could be related in some way to the change in affinity measured in these cells, we studied the structure of insulin receptor in IM-9 lymphocytes before and after down-regulation, since insulin-induced receptor down-regulation in these cells is characterized by a decrease in receptor number without any change in receptor affinity (26). Control and down-regulated IM-9 lymphocytes were 125I-surface labeled and insulin receptor was immunoprecipitated by serum B-9 and analyzed under nonreduced conditions on NaDodSO4-polyacrylamide gel electrophoresis. The results of such an experiment are shown in Fig. 7. In control IM-9 lymphocytes, six specific bands, similar to those observed in Fao cells, were obtained. In down-regulated IM-9 lymphocytes, with the exception of the Mr = 520,000 form which was slightly more reduced, all the other bands were reduced by a similar extent (again the extract from twice the number of cells was applied to the gel for clarity). Thus, in IM-9 lymphocytes there was no change in receptor affinity and no preferential loss of the higher-molecular-weight oligomers of receptor.

**Dithiothreitol Effect on Insulin Binding and Insulin-receptor Structure in Fao Hepatoma Cells**—Since the down-regulation study suggested that the state of molecular aggregation of the receptor could play a role in the affinity of the receptor for insulin, intact hepatoma cells were treated with the reducing agent DTT to alter the redox state of the receptor subunits. The free sulphhydryl groups generated by DTT treatment were alkylated with NEM. In preliminary experiments, we observed that NEM alone had no effect on insulin binding as long as the concentration did not exceed 5 mM, and thus these experiments were designed to maintain the concentration of NEM below this concentration.

The effect of various DTT concentrations (followed by NEM) on 125I-insulin binding to hepatoma cells was studied. Treatment of cells with increasing DTT concentrations produced a progressive increase in 125I-insulin binding up to a maximal effect at 7 mM (data not shown). Further increases in DTT concentrations were associated with a reduction in insulin binding (data not shown). Assuming a negative cooperative model for the insulin-receptor interaction, Scatchard plots of complete competition curves indicated that the increase in insulin binding in DTT-treated cells was due to an increase in affinity with no change in total receptor number (Fig. 4).

![Fig. 6](image-url) 125I-labeled insulin receptor immunoprecipitated by rabbit antiserum in control and down-regulated hepatoma Fao cells. Cells were labeled with Na125I and lactoperoxidase and solubilized as indicated in Fig. 1, and immunoprecipitated with rabbit control serum or rabbit serum containing antireceptor antibodies. Immunoprecipitates were analyzed by NaDodSO4-polyacrylamide gel electrophoresis. The gels of down-regulated cells contained twice the amount of extract as those of control cells.

![Fig. 7](image-url) 125I-labeled insulin receptor immunoprecipitated by human antiserum (B-9) in control and down-regulated IM-9 lymphocytes. Intact control and down-regulated (incubated with 10−8 M insulin for 24 h) cells were labeled with Na125I and lactoperoxidase and immunoprecipitated as described in Fig. 1. The gels of down-regulated cells contained twice the amount of extract as those of control cells.
Fig. 1. DTT, to remove free Nalz5I, labeled cells were treated with either 7 mM serum in control and DTT-treated hepatoma Fao cells. Cells were then solubilized and immunoprecipitated as described in the protocol described above. The cells were then solubilized, and labeled and then treated with 7 mM DTT according to a protocol described above. The cells were then solubilized, and 125I-labeled receptor was immunoprecipitated and analyzed on gel electrophoresis under nonreduced conditions (Fig. 8). In cells treated with 7 mM DTT (followed by akylation with NEM), all four oligomeric forms of the insulin receptor decreased. The decrease was most marked in the Mr = 520,000, 350,000, and 270,000 forms. The oligomeric Mr = 210,000 form also decreased slightly and there was a concomitant increase in the free α and β subunits. Treatment with DTT alone gave qualitatively identical results, whereas NEM alone had no effect on the distribution of the different forms of the insulin receptor (Fig. 8). In addition to the change in distribution of receptors, after DTT treatment, the intensity of all bands of the immunoprecipitated receptor decreased. This probably reflects some effect of small amounts of residual DTT which could have interfered with the immunoprecipitation by partially reducing the IgG molecules.

The effect of DTT treatment on insulin receptor structure was also studied. To avoid any effect of DTT on cell labeling, confluent monolayer cultures of hepatoma cells were first 125I-labeled and then treated with 7 mM DTT according to a protocol described above. The cells were then solubilized, and 125I-labeled receptor was immunoprecipitated and analyzed on gel electrophoresis under nonreduced conditions (Fig. 8). In cells treated with 7 mM DTT (followed by akylation with NEM), all four oligomeric forms of the insulin receptor decreased. The decrease was most marked in the Mr = 520,000, 350,000, and 270,000 forms. The oligomeric Mr = 210,000 form also decreased slightly and there was a concomitant increase in the free α and β subunits. Treatment with DTT alone gave qualitatively identical results, whereas NEM alone had no effect on the distribution of the different forms of the insulin receptor (Fig. 8). In addition to the change in distribution of receptors, after DTT treatment, the intensity of all bands of the immunoprecipitated receptor decreased. This probably reflects some effect of small amounts of residual DTT which could have interfered with the immunoprecipitation by partially reducing the IgG molecules.

**DISCUSSION**

Over the past few years, a variety of different experimental approaches have suggested that the insulin receptor is an oligomeric protein composed of two subunits linked by disulfide bonds (1-13). Covalent cross-linking of 125I-insulin to its receptor either using photoreactive insulin analogues or bifunctional reagents (5, 6) has suggested that the α subunit (Mr = 135,000) contains the insulin-binding site. The β subunit (Mr = 95,000) appears to be the effector portion of the receptor, and possesses a tyrosine-specific protein kinase activity which is stimulated by insulin (27-29). The present study indicates that in cultured hepatoma cells, these two subunits are joined to form several forms of the insulin receptor expressed in the plasma membrane in the nonreduced (native) state. These are four oligomeric forms of the insulin receptor (α-β heterodimer, α-α dimer, and two higher oligomers of α and β), as well as free or reduced α and β subunits. When the hepatoma cells are down-regulated, the oligomeric forms of the receptor are preferentially lost and, in this situation, there is a decrease in receptor number with a concomitant increase in affinity of the receptor for insulin (16). Similar molecular organization of the receptor was found in IM-9 lymphocytes, although in the IM-9 cells, the Mr = 520,000 form also appears to be composed of both α-β oligomers and a proreceptor molecule of Mr = 210,000 (15). In contrast to hepatoma cells, in IM-9 lymphocytes there is no change in the distribution of receptor forms and no change in receptor affinity after down-regulation.

We believe that the heterogeneity observed by gel electrophoresis of immunoprecipitates of surface iodinated receptors under nonreducing conditions is not the result of a partial oxidation or reduction for several reasons. First, two-dimensional gels suggest that free α and β subunits have different iodination properties than those involved in oligomer formation; that is, in the oligomeric forms of the receptor, the α subunit is much more heavily labeled than the β subunit, whereas the free α and β subunits appear to be almost equally labeled. If the free subunits occurred by artifactual reduction of oligomeric forms, a significant difference in labeling between α and β should be observed. The reason for the low labeling of the β subunit in the oligomeric forms is unknown but this may suggest that the accessibility of the β subunit to lactoperoxidase is decreased in the oligomeric forms of the receptor. Second, when the cells were treated with NEM to prevent any possible oxidation, free α and β subunits still appear on the nonreduced gel. In addition, it is well established that the β-subunit of the insulin receptor is phosphorylated (30). We have observed that all the forms of the receptor were phosphorylated except the 270,000-Da and the 135,000-Da forms,3 which are the dimeric αα and the free α form, respectively. These observations strongly support the existence of reduced subunits of the insulin receptor, as well as the α-β heterodimer, the α-α homodimer, and two oligomers of α and β subunits of high molecular weight.

Labeling of surface proteins done at 4 °C, a condition under which very low internalization of plasma membrane proteins occurs (25), indicates that all the forms of the insulin receptor are located in the plasma membrane. In addition, at least four of these forms can actively bind insulin. NaDodSO₄-gel electrophoresis after cross-linking of labeled insulin to intact hepatoma cells revealed bands with apparent Mr, of 125,000, 210,000, 250,000, and 320,000. These are presumably identical to the different forms of the receptor identified by specific antibodies. Under our conditions, the free α subunit labels most easily, consistent with the notion that it may have the highest affinity for insulin. A similar observation has been reported using a photoreactive analog of insulin and liver membranes (31). This observation contrasts with some other studies (6, 14, 32), particularly those done with isolated adipocytes, which have not reported the existence of significant amounts of free subunit. According to our experience, spontaneous oxidation of the free subunits as well as the Mr = 210,000 and 270,000 forms of the receptor occurs upon storage at -70 °C (or freezing and thawing) of either wheat-germ-purified or NaDodSO₄-solubilized receptors. Whether the discrepancy between our observations and those of others is due to such technical differences, to differences among cell types, or has other explanations is not yet clear.

In a previous study (16), we had shown that down-regulation of insulin receptors in hepatoma cells was associated with

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3 M. Crettaz, C. R. Kahn, unpublished observations.
a decrease of receptor number and a marked increase in binding affinity. The increase in affinity was indicated by a change in the curvilinearity of the Scatchard plots of insulin binding in the down-regulated cells, as well as a decrease in dissociation rate. The changes in receptor organization after down-regulation led us to test the hypothesis that the degree of oligomerization of the insulin receptor could modulate its affinity for insulin. Previous studies have reported that DTT treatment of intact adipocytes (33) and liver or adipocytes plasma membranes (33, 34) resulted in an increase in receptor affinity. While similar treatment of human placenta membranes produced a decrease in affinity (33, 35). When we treated intact hepatoma cells with DTT in this study, there was a clear increase in receptor affinity. This treatment also produced a reduction of the oligomeric forms of the receptor and resulted in a structural organization similar to that observed in down-regulated cells. By contrast, down-regulation of receptor in IM-9 lymphocytes occurred without any change in receptor affinity (26), and without a major change in the proportion of the different forms of the receptor. These observations strongly suggest that the affinity of the α (binding) subunit of the insulin receptor for hormone might be different in its different oligomer states in the plasma membrane.

DTT treatment of hepatoma cells produces a significant decrease in the oligomeric forms of the receptor with a concomitant increase in the free α subunit. As DTT treatment increases the binding affinity without modifying the total number of binding sites, this suggests that the free α actually binds insulin with high affinity. As noted above, $^35$I-insulin was cross-linked to the free α subunit with much lower concentrations of DSS than those needed to label the oligomeric forms of the receptor. Thus, the formation of disulfide bonds between the receptor subunits may produce a modification in the molecular conformation of the α subunit that would lead to changes in both the affinity of the receptor for the hormone and the efficiency of discusniform subunits to cross-link insulin to its receptor. This is also consistent with studies of radiation inactivation of the receptor which reveal an increase in receptor affinity at low levels of radiation which might preferentially disrupt the disulfide-linked receptor oligomers (36, 37).

Thus, insulin binding to intact cells may be more complex than previously envisioned. Although there is a single insulin-binding subunit, the subunit exists in five different oligomeric forms and these appear to bind insulin with a different affinity. In addition, the oligomeric forms of $M_0 = 520,000, 350,000, and 270,000$ presumably contain at least two binding subunits, and may therefore display negative cooperativity by direct allosteric interaction. Another interesting consideration is the possibility that the different forms of the receptor are responsible for different effects of the hormone or are differentially important in insulin action. In this context, it is interesting to note that down-regulated cells, which have lost most of the high-molecular forms of the receptor, are totally insensitive to insulin (16). These forms are also the forms which are most intensely labeled when studies of receptor autophosphorylation are conducted (38). This suggests that the high-molecular weight forms of the receptor, which have lower affinity for insulin, may be preferentially involved in insulin action.

The exact mechanism by which insulin induces down-regulation of its receptor in the hepatoma cell is as yet unclear. However, in lymphocytes, insulin increases the rate of receptor degradation (39). Other potential mechanisms include internalization and sequestration of the receptors (40) and shedding of receptors into the incubation medium (41). Internalization is believed to occur through an aggregation of receptors into coated or noncoated pits and formation of endosomes and vesicles. One suggestion is that the insulin receptor could undergo a sequential aggregation leading to internalization and down-regulation. It is possible to imagine the following sequence of events: reduced α and β subunits of the receptor ↔ various oligomeric forms ↔ receptor clustering and higher covalent forms ↔ aggregation ↔ internalization ↔ degradation or recycling. Insulin could then act to increase degradation by acting on one or more of these steps. Obviously, many more studies are needed to clarify important questions.