*Biochimica et Biophysica Acta*, 648 (1981) 137–144
Elsevier/North-Holland Biomedical Press



BBA 79403

# IDENTIFICATION OF LIVER CELL MEMBRANE GALACTOGLYCOPROTEINS INVOLVED IN THE PROCESS OF INSULIN BINDING

MARTINE CARON and JACQUES PICARD *

*Laboratoire de Biochimie-I.N.S.E.R.M. U 181, Faculté de Médecine Saint-Antoine, 27, Rue Chaligny, 75571 Paris Cedex 12 (France)*

(Received October 28th, 1980)
(Revised manuscript received March 17th, 1981)

*Key words: Insulin receptor; Galactoglycoprotein; Soluble receptor; Lectin; Glycosidase; (Liver)*

**The glycoproteinic nature of the insulin receptor was indicated using two different approaches: 1. [$^{125}$I]insulin binding to soluble receptors from mouse liver was inhibited by digestion with β-galactosidase or pretreatment with *Ricinus communis* I or concanavalin A. An other enzyme (neuraminidase) and lectins (wheat germ agglutinin, *Dolichos biflorus)* did not affect the binding reaction. These data confirmed that insulin directly interacts with the galactoglycoproteins of liver membranes. 2. The galactose oxidase-sodium boro[$^{3}$H]hydride technique, previously used for labeling accessible membrane galactoglycoproteins, was again utilized to discern the components that interact with insulin. When liver membranes were equilibrated with $10^{-7}$ M insulin prior to labeling, the SDS gel radioactive profiles were specifically modified within two galactoglycoproteins of apparent molecular sizes 195 000 and 145 000, compatible with their participation in the insulin binding interaction. Membrane pretreatment with β-galactosidase or *Sophora japonica* lectin reduced the labeling in most peaks, thus supporting the argument for labeling sensitivity. Preincubation of membranes with $10^{-7}$ M proinsulin slightly hindered labeling, while pretreatment with $10^{-7}$ M glucagon was ineffective, suggesting a specificity of the insulin effect. These data indicate the glycoprotein nature of the insulin receptor for two reasons: alteration of insulin binding after modification of the galactoglycoproteins, and alteration of galactoglycoprotein labeling after insulin binding. Two galactoglycoproteins, with apparent molecular weights 145 000 and 195 000, respectively, were identified and they are suggested to have insulin binding properties.**

## Introduction

Various arguments have suggested that the insulin receptor is an integral plasma membrane glycoprotein [1–7]: cell or membrane pretreatment with glycosidases [2–5] or lectins [4–6] prevents insulin binding; Triton X-100-soluble receptors are retained on Sepharose-immobilized lectins [5,7]; tunicamycin inhibits the synthesis of active insulin receptors [8]; purified receptor subunits are glycosylated [9,10].

We had previously demonstrated [4] that liver membrane galactosyl groups were required for the insulin binding process. Both their inactivation by β-galactosidase and their being masked by the galactose-specific lectins prevented hormone binding.

This report initially describes the influence of the enzymes and lectins on the insulin binding process to soluble receptors. Then, we attempted to test the reciprocal proposal, i.e. if the specific labeling of galactoglycoproteins was modified after insulin binding.

The externally oriented glycoproteins of intact and insulin presaturated plasma membranes ($10^{-7}$ M insulin) were labeled with galactose oxidase-sodium

* To whom correspondence should be addressed.
Abbreviation: SDS, sodium dodecyl sulfate.

0005-2736/81/0000–0000/$02.50 

borohydride [11,12] and characterized in SDS polyacrylamide gels [13]. The specificity and sensitivity of the labeling procedure was assessed by enzyme or lectin pretreatments. The specificity of the insulin effect was studied by means of proinsulin or glucagon.

In the present report, we confirm the galactoglycoprotein nature of the insulin receptor and observed that two components, of apparent molecular sizes 145 000 and 195 000, are involved in the insulin binding process.

## Materials and Methods

*Chemicals.* β-Galactosidase (*Charonias lampas,* EC 3.2.1.23, 1.1 U/mg), trypsin (beef pancreas, EC 3.4.21.4, 3000 U/mg), soybean trypsin inhibitor (120 000 BAEE units/mg) were purchased from Miles. Galactose oxidase (*Doctylium dendroides*, EC 1.1.3.9, 60 U/mg), β-galactosidase (EC 3.2.1.23, 70 U/mg, *Escherichia coli*), concanavalin A lectin (twice cristallized, 7 mg/ml) were purchased from Worthington. Galactose oxidase was preincubated at 50°C for 30 min to eliminate any proteolytic activity [11]. *Ricinus communis* I lectin was purchased from I.B.F. (France), *Sophora japonica* lectin was purified in our laboratory [4]. All enzyme and lectin specificities were tested as previously described [4] with or without 0.05% Triton X-100. [$^{125}$I]insulin (100–200 mCi/mg) and sodium boro[$^{3}$H]hydride (5–7 Ci/mM) were obtained from the Amersham Radiochemical Center.

*Insulin receptor solubilization.* Liver plasma membranes [4,14] were solubilized according to Cuatrecasas [15] in 0.05 M Tris-HCl/1% (v/v) Triton X-100 (pH 7.6) for 60 min at 4°C. After high speed centrifugation (105 000 × *g*, 90 min, 4°C), the supernatant was dialyzed in 0.05% Triton X-100 (16 h, 4°C). Protein determination [16] was performed with bovine serum albumin as standard, in the presence of Triton X-100. The white precipitate was pelleted after color development, or dissolved in 20 μl 10% SDS.

*Insulin binding studies.* 1 ml of assay volume contained 40–100 μg of soluble protein, $10^{-11}$–$10^{-6}$ M [$^{125}$I]insulin, with or without 20 μg unlabeled insulin in Krebs-Ringer phosphate buffer with 0.1% bovine serum albumin, pH 7.6. After incubation for 60 min at 22°C, the bound insulin was precipitated using 100 μl γ-globulin (1 mg/ml) and 900 μl 25% (w/v) poly(ethylene) glycol. After 10 min in ice, the samples were filtered through EHWP acetate filters (Millipore), and washed eight times with 1 ml 9% (w/v) poly(ethylene) glycol. The time and pH of incubation were determined and the insulin binding was linear and proportional to the soluble protein concentration within the range used in all experiments. Nonspecific binding in the presence of excess unlabelled insulin accounted for 25–30% of the total binding and was substracted. The degradation of [$^{125}$I]-insulin, measured using 4% final trichloroacetic acid, was minimal. All data were determined in triplicate for control tubes and in duplicate for 'nonspecific' tubes.

*Effect of enzymes or lectins.* Soluble receptors (1 mg/ml) were preincubated for 15 min at 37°C with various concentrations of enzyme, or for 50 min at 22°C with lectins in Krebs-Ringer phosphate/0.1% albumin, pH 7.6. The final Triton X-100 concentration did not exceed 0.05%. Trypsin digestion was stopped with soybean trypsin inhibitor (1 μg/unit trypsin). Binding experiments were performed with $1.6 \cdot 10^{-11}$ M insulin: it was shown (Scatchard analysis) that with this concentration, 40% of the total bound insulin was attributable to the high affinity sites. All data were corrected for nonspecific binding which was found to be unmodified upon enzyme or lectin treatment.

*Tritium labeling of galactoglycoconjugates.* The liver membrane galactoglycoconjugates were labelled according to Gahmberg [11,12]. Membranes (1 mg/ml) were treated with galactose oxidase (5 units/mg) for 40 min at 22°C in Krebs-Ringer phosphate, pH 7.6. Phenylmethylsulfonyl fluoride (0.2 mM) was added to avoid proteolysis of the membrane. The oxidized membranes were reduced with 100 μmol/mg protein sodium boro[$^{3}$H]hydride largely washed with Krebs-Ringer phosphate, pH 7.6, and solubilized in 1% Triton X-100 as described above. Nonspecific reduction, measured in the absence of enzymic oxidation, accounted for 20% of total tritium label.

*SDS-polyacrylamide gel electrophoresis.* SDS-polyacrylamide gel electrophoresis was performed in a discontinuous buffer system as described by Neville and Glossmann [13]. Labeled proteins were solubilized

in 0.05 M Tris/0.5 mM EDTA/1% (w/v) SDS with 1% (v/v) β-mercaptoethanol. When using Triton X-100-soluble material, the buffer was enriched in β-mercaptoethanol plus SDS. Samples were electrophoresed in 11 or 8% slab gels. Gels were in part stained for proteins with Coomassie brillant blue and in part cut into 1.5-mm slices. After depolymerization with $H_2O_2$, each slice was counted in Biofluor scintillation cocktail (New England Nuclear). Results are expressed as dpm/slice after appropriate quenching correction.

*Treatment of membranes prior to $^3H$ labeling.* Plasma membranes (1 mg/ml) were preincubated for 45 min at 22°C with *S. japonica* lectin (200 μg/ml), *R. communis* I lectin (100 μg/ml) or concanavalin A lectin (50 μg/ml); and for 15 min at 37°C with β-galactosidase (0.5 units/mg) or neuraminidase (1 unit/mg). Insulin receptor presaturation was effected with $10^{-8}$–$10^{-5}$ M native insulin for 30 min at 22°C. Preincubation of liver membranes with glucagon ($10^{-7}$ M) or proinsulin ($10^{-7}$ M) were performed in the same way for 30 min at 22°C.

Following these treatments, liver membranes were treated for 40 min at 22°C with galactose oxidase, for 5 min at 4°C with sodium boro[$^3$H]hydride, and largely washed to eliminate the excess of sodium boro[$^3$H]hydride and the various agents (enzyme, lectin or hormone). SDS gel analysis was performed after solubilization.

## Results

### *Soluble receptor studies*

*Binding characteristics.* A steady state of insulin binding was achieved within 1 h at 22°C. Nonspecific binding accounted for 25–30% of the total binding. After 60 min at 22°C, 95–98% of the [$^{125}$I]insulin remained precipitable in 4% final trichloroacetic acid, indicating no degradation of the free or bound insulin. Scatchard plot of insulin binding was curvilinear (Fig. 1). This may be explained by two orders of binding sites and/or negative cooperativity [17]. To facilitate comparison to the data obtained with plasma membranes, our calculations (±S.E.) followed the Scatchard method corrected by Kahn [18], high affinity: $K_d = (4.35 \pm 0.32) \cdot 10^{-11}$ M; low affinity: $K_d = (2.67 \pm 0.24) \cdot 10^{-9}$ M. When studying particulate liver membranes, the equilibrium data were found to be of the same order of magnitude [4,14].

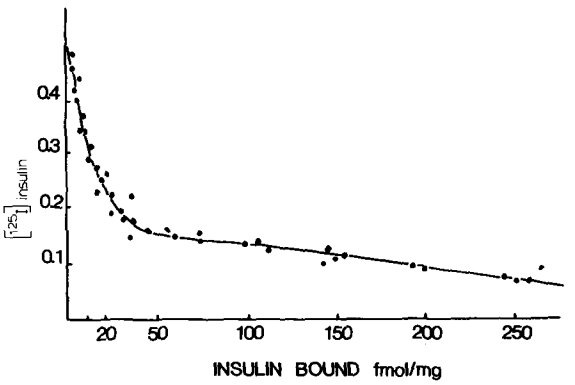


Fig. 1. Scatchard plot of insulin binding to soluble receptors of mouse liver. Soluble receptors (40 μg/ml) were incubated for 60 min at 24°C with [$^{125}$I]insulin, $10^{-11}$ mol/l–$10^{-6}$ mol/l, in the presence or absence of 20 μg/ml native insulin. All data have been corrected for nonspecific binding. Values were taken from ten separate experiments.

*Glycosylation properties (Table I).* Trypsin digestion of soluble membranes altered the binding of [$^{125}$I]insulin (16.6 pM). As compared to particulate membranes [4], higher concentrations of trypsin were required to obtain the same effect, probably due to an incorporation of the receptor or the enzyme into detergent micelles. Neuraminidase (up to 100 mU/mg protein) did not modify insulin binding. β-Galactosidase (500 mU/mg protein) reduced the binding of 16.6 pM [$^{125}$I]insulin by 30%. This effect is not attributable to contaminating proteases, since the same results were observed in the presence of phenylmethylsulfonyl fluoride (0.2 mM), an anti-proteolytic agent. As previously shown in particulate membranes [4], β-galactosidase pretreatment (500 mU/mg) almost linearized the Scatchard plot of insulin binding with a $K_d$ in the range of $10^{-9}$ M (data not shown).

Preincubation with galactose-specific lectins (*S. japonica* or *R. communis* I) reduced insulin binding (16.6 pM) by about 35%. Higher concentrations of lectins, i.e. up to 300 μg/mg protein, did not increase inhibition. When soluble receptors were incubated with concanavalin A lectin (mannose-specific), 50 μg/mg protein, insulin binding was inhibited by 40%. The hexosamine-specific lectins (*D. biflorus* or wheat germ agglutinin) did not appreciably affect the insulin binding reaction.

TABLE I

EFFECT OF ENZYME OR LECTIN ON THE BINDING OF INSULIN TO SOLUBLE MEMBRANE RECEPTORS

Triton X-100-soluble receptors (1 mg/ml) were preincubated for 15 min at 37°C with enzymes or for 50 min at 22°C with lectins. Trypsin digestion was stopped by addition of 10 μg soybean trypsin inhibitor. Binding assays were performed using aliquots of the treated fractions (40 μg/ml) with 16.6 pM [$^{125}$I]insulin for 60 min at 22°C. Results are the mean ± S.E. of 12 determinations (control) and five determinations (treated).

| | | Specifically bound [$^{125}$I]insulin (fmol/mg protein) | Percent of control |
|---|---|---|---|
| Control | | 6.50 ± 0.14 | 100 |
| Trypsin | (10 U/ml) | 1.62 ± 0.15 | 25 |
| Neuraminidase | (100 mU/ml) | 6.65 ± 0.08 | 102 |
| β-Galactosidase | (500 mU/ml) | 4.55 ± 0.12 | 70 |
| *S. japonica* | (100 μg/ml) | 4.09 ± 0.07 | 63 |
| *R. communis* I | (100 μg/ml) | 4.48 ± 0.04 | 69 |
| Concanavalin A | (50 μg/ml) | 3.90 ± 0.03 | 60 |
| Wheat germ agglutinin | (100 μg/ml) | 6.63 ± 0.12 | 102 |
| *D. biflorus* | (100 μg/ml) | 6.55 ± 0.05 | 100 |

These results indicated that the soluble receptor for insulin, is or is closely associated with, a glycoprotein that contains mannose and galactose.

*Identification of glycosylated receptor components*

*Labeling of liver membrane galactoglycoproteins.* As shown in Table II, the liver membrane glycoproteins that possess accessible galactose were labeled with a highly specific activity (0.5 ± 0.15 μCi/mg membrane protein ± S.E.). The data were reproducible using the same batch of sodium borohydride, but could vary from batch to batch depending on the quality of the reductive agent. Nonspecific labeling (20% of total labeling) was probably due to tritium incorporation into cholesterol and phospholipids [11, 12]. If 50 μM galactose was added during the labeling assay, the specific tritium incorporation was inhibited by 98% (Table II).

As shown in Fig. 2A (full line), about 14 components were resolved in SDS polyacrylamide slab gels.

TABLE II

EFFECT OF VARIOUS AGENTS ON THE LABELING OF MEMBRANE GLYCOCONJUGATES USING THE GALACTOSE OXIDASE-SODIUM BORO[$^{3}$H]HYDRIDE ASSAY

Liver membranes (1 mg/ml) were pretreated with enzymes for 15 min at 37°C, or with lectins for 45 min at 22°C. Labeling was performed for 40 min at 22°C with 5 U galactose-oxidase, and for 5 min at 4°C with 100 μmol sodium boro[$^{3}$H]hydride. Nonspecific label by tritiated sodium borohydride alone, accounted for 28 164 dpm and was deduced from the total label. Data were the mean of four comparative experiments employing similar batches of galactose oxidase, tritium borohydride and liver membranes.

| Agent | | Specific tritium label/100 μg protein (dpm) | Percent of control |
|---|---|---|---|
| Control membranes | | 112 640 | 100 |
| D-Galactose | (50 μM) | 640 | 1 |
| β-Galactosidase | (500 mU/ml) | 21 440 | 19 |
| Neuraminidase | (1 U/ml) | 41 850 | 37 |
| *R. communis* | (100 μg/ml) | 48 835 | 43 |
| *S. japonica* | (200 μg/ml) | 83 842 | 74 |
| Concanavalin A | (50 μg/ml) | 132 848 | 118 |

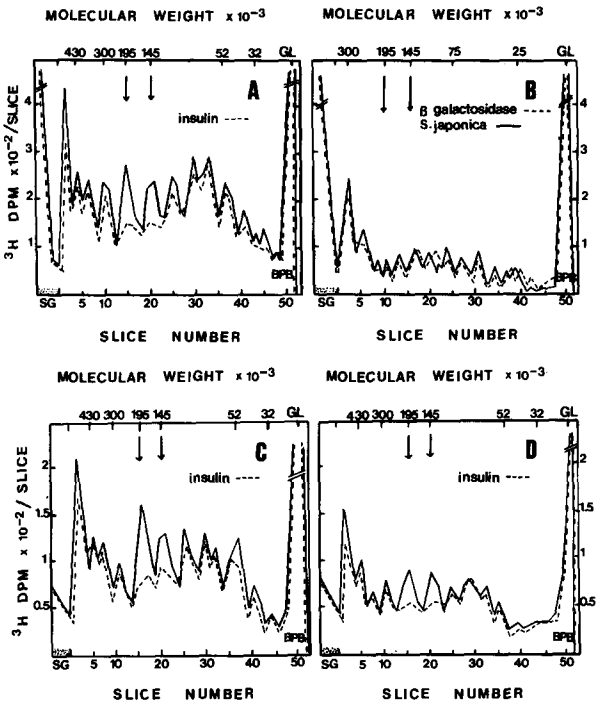


Fig. 2. SDS gel radioactivity profiles of liver membranes labeled with galactose oxidase-sodium boro[$^3$H]hydride. (A) Effect of insulin on the labeling of membrane galactoglycoproteins: 200 $\mu$g of control (full line) and insulin-presaturated (dashed line) membranes, were solubilized in SDS/mercaptoethanol and simultaneously electrophoresed in 8% slab gels ($0.28 \cdot 10^6$ dpm per sample). (B) Effect of $\beta$-galactosidase or *S. japonica* on specific labeling: Liver membranes (1 mg/ml) were digested with $\beta$-galactosidase (500 mU/ml) (dashed line) or treated with *S. japonica* lectin (200 $\mu$g/ml) (full line) before tritium labeling and 11% SDS gel analysis. (C) Studies of the Triton X-100-soluble galactoglycoproteins issued by $10^{-7}$ M insulin-prequilibrated membranes; 4 mg of control (full line) and insulin-presaturated (dashed line), membranes were labeled and solubilized in Triton X-100. Supernatants were enriched in 1% $\beta$-mercaptoethanol/1% SDS, and analyzed in 8% SDS gels (150 $\mu$g protein, $0.63 \cdot 10^6$ dpm for control, $0.5 \cdot 10^6$ dpm for insulin-pretreated). (D) Studies of the poly(ethylene glycol)-precipitable material issued from Triton X-100-soluble labeled membranes: 1 mg of labeled membranes was solubilized and precipitated with 9% final poly(ethylene glycol). The pellet was washed, enriched in $\beta$-mercaptoethanol/SDS and analyzed in 8% SDS gels ($0.2 \cdot 10^6$ dpm). Dashed line: material obtained from insulin-presaturated membranes. Labeling profiles are representative of one experiment performed eight times concerning A and C, and four times concerning B and D.

Labeling profiles were reproducible in ten experiments. Protein patterns, as described by Coomassie blue staining, were not appreciably modified after galactose oxidase-sodium boro[$^3$H]hydride labeling, indicating that no cross-linking or aggregation of proteins occurred. The absence of proteolysis was verified using azoalbumin [12]. The peak running just behind the dye front (GL) could be caused by plasma membrane galactoglycolipids [11–13].

*Specificity of the labeling assay.* As shown in Table II and Fig. 2, the labeling of liver membrane galactoglycoproteins was markedly modified by the various agents that either hydrolyze, bind or uncover the galactosyl groups: these data are evidence of the sensitivity and specificity of the labeling assay. Pretreatments of membranes with $\beta$-galactosidase (500 mU/mg protein), *R. communis* I lectin (100 $\mu$g/mg protein) or *S. japonica* lectin (200 $\mu$g/mg protein) reduced labeling by 81, 63 and 57%, respectively (Table II). When studying the radioactive profiles of the SDS gels (Fig. 2B, membranes treated with $\beta$-galactosidase or *S. japonica* lectin), we observed that most peaks disappeared. When the membranes were pretreated with neuraminidase (1 U/mg protein), the specific labeling was increased by 18%. Pretreatment with concanavalin A lectin (50 $\mu$g/mg protein) produced a 26% decrease.

*Effect of insulin receptor presaturation.* Preincubation of membranes with $10^{-7}$ M insulin for 30 min at 22°C, saturated 90–100% of receptors, as determined by displacement curves of 10 pM [$^{125}$I]insulin in presence of increasing concentrations of unlabeled insulin, $10^{-10}$–$10^{-5}$ M. As shown in Table III, a 20% decrease in specific labeling of galactoglycoproteins is observed. The labeling of insulin-pretreated membranes (Fig. 2A, dashed line) was markedly reduced in two glycoproteins with apparent molecular weights 195 000 and 145 000. The relative extent of inhibition was reproducible and other minor modifications were present, but not quantitatively interpretable.

To evaluate the incidence of nonspecific insulin binding, we determined that with $10^{-7}$ M insulin, about 30–35% of the total bound insulin is attributable to nonspecific sites, in agreement with Kahn [1]. To ascertain that bound insulin was not displaced during the labeling assay, we verified that the same amount of insulin was bound before and after galactose oxidase treatment. To evaluate the specificity of

TABLE III

EFFECT OF INSULIN, PROINSULIN OR GLUCAGON ON THE LABELING OF LIVER MEMBRANE GALACTOGLYCOCONJUGATES

Membranes (1 mg/ml) were preincubated for 30 min at 22°C. Labeling was then performed as indicated in Table II. Nonspecific label by tritiated sodium borohydride alone, accounted for 28 164 dpm and was deduced from the total label. Results are the mean of three determinations for all hormones tested except for $10^{-7}$ M insulin where six experiments were analyzed.

| | Specific tritium label/100 μg protein (dpm) | Percent of control |
|---|---|---|
| Control membranes | 112 640 | 100 |
| Insulin ($10^{-8}$ M) | 93 480 | 83 |
| Insulin ($10^{-7}$ M) | 90 080 | 80 |
| Insulin ($10^{-5}$ M) | 70 960 | 63 |
| Proinsulin ($10^{-7}$ M) | 103 600 | 92 |
| Glucagon ($10^{-7}$ M) | 112 200 | 99 |

the insulin effect, we tested the effect of $10^{-7}$ M glucagon or $10^{-7}$ M proinsulin. The peptidic hormone glucagon was chosen due to the unglycosylated feature of its liver membrane receptor [1,19]. As shown in Table III, no modification in labeling was shown after preincubation with glucagon, while a 9% decrease was obtained with proinsulin, attesting to its poor affinity for the insulin receptor [20].

These data, in addition to the minimal nonspecific binding of $10^{-7}$ M insulin as well as the reduction of labeling in only two peaks, point out the specificity of the insulin effect.

Using $10^{-8}$ M insulin, receptor sites were partially saturated (35–50%) and the extent of inhibition in total labeling was less marked (Table III) than with $10^{-7}$ M insulin treatment. The inhibition observed in the two peaks (195 000 and 145 000) was almost similar (data not shown). These results confirm that insulin interacts with two galactoglycoproteins.

Using $10^{-5}$ M insulin, all sites were fully saturated, while the ratio of nonspecific/specific insulin binding was considerably higher. A further decrease in labeling (20%) was observed as compared to $10^{-8}$ M or $10^{-7}$ M insulin. Labeling alterations, randomly distributed throughout most membrane components, may be attributed to nonspecific insulin binding.

*Studies of Triton X-100-soluble or poly(ethylene glycol)-precipitable galactoglycoproteins.* When labeled membranes were solubilized in Triton X-100 (Fig. 2C) or precipitated with poly(ethylene glycol) (Fig. 2D), qualitatively the same galactoglycoproteins were retained, including the two components of $M_r$ 195 000 and 145 000, believed to be involved in the insulin receptor interaction. On the other hand, when we identified the labeled glycoproteins issued from insulin-presaturated membranes, the SDS gel radioactive patterns of soluble membranes (Fig. 2C, dashed line) or of poly(ethylene glycol)-precipitable material (Fig. 2D, dashed lined) lacked components 195 000 and 145 000, coinciding with the loss of their accessibility in the insulin presaturated membranes.

## Discussion

The kinetic properties of insulin receptors were retained after their release from the plasma membrane (Fig. 1), as reported elsewhere [15,21–23]. The concordant effects of β-galactosidase, *S. japonica* lectin and *R. communis* I lectin (Table I) upon insulin binding to soluble membranes demonstrate that soluble galactoglycoproteins are involved at the insulin receptor site.

In contrast with other studies [3,6,24], the effects of β-galactosidase or galactose-specific lectins did not require the desialylation of the soluble membranes. Since receptor subunits were recently shown to be sensitive to neuraminidase [9,10,25], one possibility is that mouse insulin receptors contained multibranched glycoproteins, the 'antenna' of which, implicated in binding, lacks sialic acid.

The effect of β-galactosidase, galactose or mannose-specific lectins were maximal at low insulin receptor occupancy, indicating a correlation between the degree of receptor occupancy and galactoglycoprotein involvement. These data could be interpreted as follows: (a) insulin receptors comprise separate proteins [18] that are heterogeneous with respect to binding affinity and glycosylation; (b) insulin receptors may be the same glycoprotein species which can exist in multiple interconvertible states [17] and the increase of receptor occupancy may simultaneously minimize the binding affinity and glycoprotein involvement; (c) finally, an alternative possibility is that glycoproteins confer special binding properties

to the insulin receptor complex, while being structurally separate from the recognition moiety [17,26]. The present results did not provide further resolution of the matter, but recent data that indicate that all receptor subunits were glycosylated [8,25,29] might favor the second hypothesis.

To better discern the interaction between insulin and galactoglycoproteins, quite a different approach was put to use: a comparison was made of the galactoglycoprotein accessibility in intact and insulin-presaturated membranes using the galactose oxidase-sodium boro[$^3$H]hydride assay in such a manner as to allow the preservation of membrane functions. The sensitivity and specificity of the labeling was demonstrated using the agents that modify the galactose accessibility (Table II, Fig. 2): $\beta$-galactosidase or *S. japonica* lectin treatments largely reduced the extent of labeling in most peaks (Fig. 2B); mild neuraminidase digestion, exposing subterminal galactose residues, enhanced incorporation efficiency. Concanavalin A lectin (mannose-specific) unexpectedly reduced tritium labeling, probably due to either a sterical hindrance (that would affect adjacent galactose residues) or to a reorientation in a few accessible plasma membrane galactoglycoproteins [27]. Thus, the mild and specific alterations in galactoglycoprotein accessibility were clearly visualizable in SDS gels.

Presaturation of membranes with insulin ($10^{-7}$ M) prevented the labeling of two components of $M_r$ 195 000 and 145 000, with little modification in the other peaks. About the same results were obtained using $10^{-8}$ M insulin. Several lines of evidence bring out the specificity of the insulin effect; firstly, concerning the experimental conditions: an almost fully saturating dose of insulin was chosen in order to saturate specific receptors with minimal nonspecific absorption; membrane labeling was performed in brief and mild conditions, in the presence of an anti-proteolytic agent so as not to allow the dissociation of the bound insulin. Secondly, taken into account that the reduction was detected only on two peaks, that $10^{-8}$ M insulin produced an almost similar effect, and that glucagon (whose receptor sites were not glycosylated) was not potent enough to alter labeling, the specificity of the insulin effect was confirmed. With higher concentrations of insulin ($10^{-5}$ M), an increased proportion of nonspecific insulin binding was awaited: the further decrease in overall labeling could be due to nonspecific inhibitions.

The loss of accessibility of two galactoglycoproteins when insulin receptors are occupied could be explained in two main ways: a change at the receptor level or a change at the effector level. According to the first possibility, these components might participate in the receptor structure, as the recognition moiety; more likely, insulin binding could lead to rearrangements within the receptor: aggregation of subunits [17], lateral displacement [7,28], initial step of internalization [29]. In these cases, the identified components would be localized in close proximity to the insulin binding molecules, but their role would be secondary, probably as regulatory subunits.

According to the second possibility, as postulated by the mobile receptor paradigm [6,7,30], insulin binding would transmit a signal to adjacent glycoproteins (effector molecules), thus modifying their accessibility. However, regarding the marked effects of glycosidase or lectins on insulin receptor binding, it seems more probable that the identified components belong to the receptor structure, either as receptor subunits or as modulatory components involved in site-site interactions.

The estimated molecular weight of the insulin receptor in Triton X-100 is about 300 000 [21,22, 26,31,32]. The insulin receptor consists of two subunits of $M_r$ 135 000 and two subunits of $M_r$ 45 000 [9,10,33–36] that are associated by means of disulfide bonds and strong noncovalent interactions. Receptors could be observed in SDS gels under multiple redox forms depending on the degree of subunit association. Using low dithiothreitol concentrations, Jacobs et al. [10] recently identified in SDS gels, two components of $M_r$ 135 000 and 180 000, the latter being shown to contain the 135 000 plus 45 000 subunits.

These data converge with the present results if we except the 7% overestimation in molecular weights (195 000 and 145 000 as compared to 180 000 and 135 000) that is attributable to the range of error of the SDS gel electrophoresis technique [13].

**Acknowledgements**

This work has been supported by grants from the 'Institut National de la Santé et de la Recherche

Médicale' (CRAT 49.77.81 and CRL 77.1.191.7), and the 'Centre National de la Recherche Scientifique' (ERA 691). The authors thank Gisèle Cherqui for helpful discussions, Christiane Horn (Melbourne, Australia) for reviewing the manuscript, and Betty Jacquin for secretarial assistance.

## References

1 Kahn, C.R. (1976) J. Cell. Biol. 70, 261–286
2 Cuatrecasas, P. (1972) in Insulin Action (Fritz, I.B., ed.), pp. 137–169, Academic Press, London
3 Cuatrecasas, P. and Illiano, G. (1971) J. Biol. Chem. 246, 4938–4946
4 Caron, M., Picard, J. and Kern, P. (1978) Biochim. Biophys. Acta 512, 29–40
5 Cuatrecasas, P. (1973) J. Biol. Chem. 246, 3528–3534
6 Capeau, J., Picard, J. and Caron, M. (1978) Cancer Res. 38, 3930–3937
7 Jacobs, S. and Cuatrecasas, P. (1976) Biochim. Biophys. Acta, 433, 482–485
8 Rosen, O.M., Chia, G.H., Fung, C. and Rubin, C.S. (1979) J. Cell. Physiol. 99, 37–42
9 Jacobs, S., Hazum, E. and Cuatrecasas, P. (1980) Biochem. Biophys. Res. Commun. 94, 1066–1073
10 Jacobs, S., Hazum, E. and Cuatrecasas, P. (1980) J. Biol. Chem. 255, 6937–6940
11 Gahmberg, C.G. (1976) J. Biol. Chem. 251, 510–515
12 Gahmberg, C.G. and Hakomori, S.I. (1973) J. Biol. Chem. 248, 4311–4317
13 Neville, D.M. and Glossmann, H. (1971) J. Biol. Chem. 246, 6335–6338
14 Kern, P., Picard, J., Caron, M. and Veissiere, D. (1975) Biochim. Biophys. Acta 389, 281–289
15 Cuatrecasas, P. (1972) J. Biol. Chem. 247, 1980–1991
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 De Meyts, P., Bianco, A.R. and Roth, J. (1976) J. Biol. Chem. 251, 1877–1888
18 Kahn, C.R., Freychet, P., Roth, J. and Neville, D.M., Jr. (1974) J. Biol. Chem. 249, 2249–2257
19 Giorgio, N.A., Johnson, C.B. and Blecher, M. (1974) J. Biol. Chem. 249, 428–437
20 Freychet, P. (1974) J. Clin. Invest. 54, 1020–1031
21 Harrison, L.C., Billington, T., East, I.J., Nichols, R.J. and Clark, S. (1978) Endocrinology 102, 1485–1495
22 Maturo, J.M., Schackelford, W.H. and Hollenberg, M.D. (1978) Life Sci. 23, 2063–2072
23 Ginsberg, B.H., Cohen, R.M., Kahn, C.R. and Roth, J. (1978) Biochim. Biophys. Acta 542, 88–100
24 Tsuda, M., Taketomi, S. and Iwatsuka, H. (1980) Am. J. Physiol. 239, E186–E191
25 Yip, C.C., Moule, M.L. and Yeung, C.W.T. (1980) Biochem. Biophys. Res. Commun. 96, 1671–1678
26 Maturo, J.M. and Hollenberg, M.D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3070–3074
27 Hughes, C. (1976) Membrane Glycoproteins. A review of structure and functions, Butterworth, London
28 Schlessinger, J., Shechter, Y., Willingham, M.C. and Pastan, I. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5353–5357
29 Gordon, Ph., Carpentier, J.L., Freychet, P. and Orci, L. (1980) Diabetologia 18, 263–274
30 Hollenberg, M.D. (1979) Pharmacol. Rev. 30, 393–410
31 Cuatrecasas, P. (1972) J. Biol. Chem. 247, 1980–1991
32 Krupp, M.N. and Livingston, J.N. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2593–2597
33 Jacobs, S., Shechter, Y., Bissell, K. and Cuatrecasas, P. (1977) Biochem. Biophys. Res. Commun. 77, 981–988
34 Yip, C.C., Yeung, C.W.T. and Moule, M.D. (1978) J. Biol. Chem. 253, 1743–1745
35 Pilch, P.F. and Czech, M.P. (1979) J. Biol. Chem. 254, 3375–3381
36 Jacobs, S., Hazum, E., Shechter, Y. and Cuatrecasas, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4918–4921