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The insulin receptor glycosidic moiety : its characterization and role

Martine CARON, Gisèle CHERQUI, Jacqueline CAPEAU, O. LASCOLS, J. PICARD (*)

Laboratoire de Biochimie, I.N.S.E.R.M., Unité 181,
Faculté de Médecine Saint-Antoine, 27, rue Chaligny, 75571 Paris Cedex 12, France.

Summary. 1. Using various exoglycosidases and/or lectins as specific glycosidic probes, we characterized the insulin receptor carbohydrate components in normal or transformed cells and investigated their importance in the insulin-receptor interaction. The insulin receptor structure was shown to contain sialylated oligosaccharide side chains of the complex N-linked type which participated in the insulin-receptor interaction through D-galactose, D-mannose and N-acetyl-D-glucosamine units. The treatment of rat fat cells with β-galactosidase plus neuraminidase resulted in a significant decrease of insulin binding which was not modified by either enzyme individually ; lectins specific for D-galactose (*Ricinus communis* I agglutinin : RCA I), D-mannose (concanavalin A : con A) or N-acetyl-D-glucosamine (wheat germ agglutinin : WGA) units markedly inhibited insulin binding and/or dissociation processes in fat cells, Zajdela hepatoma cells and liver membranes.

2. The receptor glycosidic moiety, involved in the « high affinity » binding process, seemed to be responsible for receptor affinity modulation. The almost linear Scatchard plots of insulin binding indicated that the inhibitory effect of lectins caused the disappearance of the « high affinity » insulin binding process without modifying receptor capacity. Sequential chromatographic purification of insulin receptors on immobilized lectins resulted in a progressive increase of receptor affinity for the hormone ; this increase was concomitant with a clear augmentation of insulin receptor sensitivity to exoglycosidases and lectins.

3. Studying the effect of tunicamycin on cultured hepatoma cells, we evaluated the importance of N-linked glycoproteins in the cellular biological response to insulin. Tunicamycin-treated cells (24-hour incubation) showed a dramatic decrease in insulin receptor number. Remaining receptors became resistant to the insulin-induced down-regulation process.

These data show that carbohydrate chains of the complex N-linked type play a key role at both insulin receptor and post-receptor levels.

Introduction.

Over the past ten years evidence has accumulated indicating that the insulin receptor is an integral membrane glycoprotein, and some questions have arisen as to the nature and importance of its carbohydrate components ; these

(*) To whom all correspondence should be addressed.

components have been shown to be involved in both the receptor (Cuatrecasas, 1971 ; Capeau, Picard and Caron, 1978 ; Caron, Picard and Kern, 1978 ; Maturo and Hollenberg, 1978 ; Capeau and Picard, 1980 ; Herzberg *et al.,* 1980 ; Caron and Picard, 1981 ; Cherqui *et al.,* 1981 ; Hedo *et al.,* 1981 ; Sorge and Hilf, 1981) and post-receptor (Cuatrecasas and Illiano, 1971 ; Tsuda, Taketomic and Iwatsuka, 1980) events concerning insulin.

The present paper describes the different methodological approaches developed in our laboratory for the identification of insulin receptor glycosidic units and elucidates their role in the initial interaction of insulin with its receptor and in its biological effects.

1. On normal or transformed cells we studied the modifications of insulin binding or dissociation processes when blocking or removing cell surface carbohydrate components by lectins or exoglycosidases, respectively.

2. We attempted to gain further insight into the role of the glycosidic moiety in the insulin-receptor interaction by studying (i) receptor affinity changes after preincubation of fat cells or liver membranes with lectins and (ii) the insulin-binding properties of lectin-purified receptors and the modifications of their sensitivity to agents that were found to alter receptor function.

3. Finally, we investigated the effect of tunicamycin (an agent that blocks the N-glycosylation of proteins) on the capacity of insulin to down-regulate the number of its own receptors in cultured Zajdela hepatoma cells.

Our data indicate that a sialylated glycosidic moiety of the complex N-linked type participates in the insulin-receptor interaction through D-galactose, D-mannose and N-acetyl-D-glucosamine units. This glycosidic core was shown to be involved in the control of receptor affinity for insulin. Furthermore, N-linked glycoproteins were important for the insulin-induced regulation of insulin binding sites.

Material and methods.

Cell and plasma membrane preparation. — The preparation of isolated adipocytes, Zajdela hepatoma cells and liver plasma membranes has been described previously (Cherqui *et al.,* 1981 ; Capeau, Picard and Caron, 1978 ; Caron, Picard and Kern, 1978).

(^{125}I)-labelled insulin binding and dissociation studies. — (^{125}I)-labelled insulin binding and dissociation assays have been described elsewhere (Cherqui *et al.,* 1981 ; Capeau, Picard and Caron, 1978 ; Caron, Picard and Kern, 1978). For determining the insulin-binding parameters at steady state equilibrium, inhibition-competition assays were performed. Briefly, fat cells, liver membranes or lectin-purified receptors were incubated at 22 °C for 20 to 60 min with 50 to 100 pM (^{125}I)-labelled insulin in the presence of 0 to 1 $\times$ 10^5 ng/ml unlabelled insulin. The insulin receptor complex was isolated as in previous binding assays (Cherqui *et al.,* 1981 ; Capeau, Picard and Caron, 1978 ; Caron, Picard and Kern, 1978 ; Caron and Picard, 1981).

Lectin purification of insulin receptors. — Soluble liver membranes were sequentially chromatographed on con A- and ricin I-lectin columns at 4 °C in 50 mM Tris buffer 0.05 % Triton, in the presence of 1μ M Mg SO_4, 1μ M $CaCl_2$ (con A-lectin column). The elution buffers contained 0.2 M methyl α-mannoside (con A-lectin column) or 0.2 M D-galactose (ricin I-lectin column). Insulin binding studies were performed after dialysis in both the unbound fractions and eluates to determine receptor purification and recovery.

Enzymatic digestion and lectin preincubation. — The treatment of fat cells, Zajdela hepatoma cells and particulate or soluble liver membranes with either exoglycosidases or lectins has been described elsewhere (Cherqui *et al.*, 1981 ; Capeau and Picard, 1980 ; Caron, Picard and Kern. 1978 ; Caron and Picard, 1981). Cell viability was controled by the Trypan blue exclusion test. Agglutination assays have been described previously (Cherqui *et al.*, 1981).

Effect of tunicamycin on cultured hepatoma cells. — Cultured Zajdela hepatoma cells (ZHC) were used for these studies ; they were propagated as monolayer cultures in Dulbecco's modification of Eagle's medium (Gibco H 16) containing 8 mg/ml of D-glucose. The effect of tunicamycin (Calbiochem) or insulin (pig insulin MC, Novo) was tested in the absence of calf serum at 37 °C for various periods. The cells were then washed and scraped ; the binding assay was performed at 2 °C for 18 h in Hepes buffer (100 mM Hepes, 120 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 10 mM glucose, 15 mM sodium acetate) with 10 mg/ml of bovine serum albumin pH 7.6, in the presence of (^{125}I)-iodoinsulin (New England Nuclear). Non-specific binding was assessed with 10 μg/ml of native insulin (Novo).

Results and discussion.

Characterization of insulin receptor carbohydrate components. — The results presented in table 1 and figure 1 indicate that sialylated oligosaccharide side chains of the complex N-linked type participate in the insulin-receptor interaction through D-galactose, D-mannose and N-acetyl-D-glucosamine units.

The removal of terminal sialic acid residues by neuraminidase (from *Clostridium perfringens* or *Arthrobacter ureafaciens*) did not modify the insulin binding process whatever the biological material studied.

β-galactosidase pretreatment of fat cells, Zajdela hepatoma cells or liver plasma membranes, in either the absence or the presence of neuraminidase, decreased the insulin binding process by approximately 40 %. Similarly, treatment of intact adipocytes, liver membranes or desialylated Zajdela hepatoma cells with *Ricinus communis* agglutinin I (RCA I), specific for D-galactose units, resulted in a 30 to 50 % decrease of insulin binding.

Sialic acid units did not seem to be required for insulin-receptor interaction in any of the tissues examined, while terminal or penultimate D-galactose units were involved in this process.

The blockade of D-mannose units by concanavalin A (con A) decreased insulin binding by approximately 45 to 60 %. The lectin effects were specific

since they were almost entirely abolished in the presence of α-methyl-D-mannopyranoside (50 mM).

The blockade of N-acetyl-D-glucosamine by wheat germ lectin (WGA) markedly decreased insulin binding in adipocytes and Zajdela hepatoma cells by about 70 %. The lectin effect was no longer observed in the presence of N-acetyl-D-glucosamine (50 mM), thus attesting to its specificity. The failure of WGA to induce a decrease in insulin binding in particulate liver membranes suggested that N-acetyl-D-glucosamine units were not accessible to this lectin, but the possibility that these residues were present in the receptor structure cannot be excluded (see below).

TABLE 1

Effects of exoglycosidases and lectins on specific [^{125}I]-labelled insulin binding to various cells

Treatments	Rat fat cells	Zajdela hepatoma cells	Liver plasma membranes
	[^{125}I]-labelled insulin binding (% of control)		
None	100	100	100
Neuraminidase (2 mU/ml)	104	101	98
β-galactosidase (2 U/ml)	102	103	62
Neuraminidase (2 mU/ml) + β-galactosidase (2 U/ml)	66	68	61
α-L-fucosidase (50 mU/ml)	86	100	100
Ricin I (40 μg/ml)	59	69*	67
Concanavalin A (50 μg/ml)	40	51	54
Wheat Germ Agglutinin (50 μg/ml)	29	25	103

Rat fat cells, Zajdela hepatoma cells and liver plasma membranes were pretreated for 15-30 min at 37 °C with exoglycosidases or for 45-60 min at 4 or 22 °C with lectins. [^{125}I]-labelled insulin binding (30-75 pM) was then assayed at either 4 °C for 18 h (Zajdela hepatoma cells) or 22 °C for 20-60 min (rat fat cells, liver plasma membranes). All data were corrected for non-specific [^{125}I]-labelled insulin binding.

* Desialylated Zajdela hepatoma cells were used in this assay.

These results, indicating the involvement of D-galactose, D-mannose and N-acetyl-D-glucosamine units in the insulin-receptor interaction, were further confirmed when we investigated RCA I, con A and WGA effects on the dissociation process of bound (^{125}I)-labelled insulin (fig. 1).

In fat cells, the dissociation process of bound (^{125}I)-labelled insulin, measured 60 min after the addition of unlabelled insulin (time 0 of dissociation), was inhibited by 45, 78 and 84 % in the presence of RCA I, con A and WGA, respectively. Similar results were observed in liver membranes with RCA I and con A.

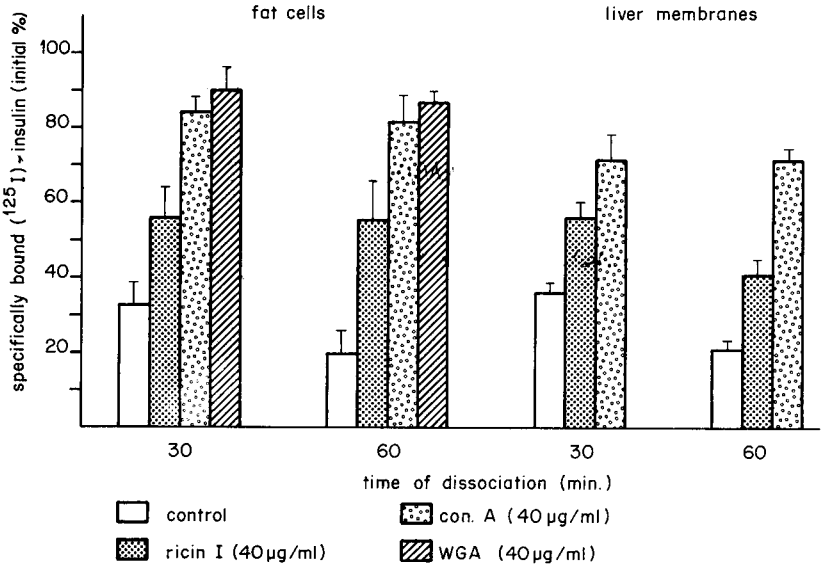

FIG. 1. — *Effects of lectins on the [^{125}I]-labelled insulin dissociation process on fat cells and liver plasma membranes.* Adipocytes (10^5 cells/ml) or liver plasma membranes (0.5 mg/ml) were incubated for 20 min at 22 °C with [^{125}I]-insulin (50-175 pM) and for a further 25 min with lectins. Dissociation of bound [^{125}I]-insulin was induced by the addition of unlabelled insulin (10 µg/ml, time 0).

Role of the glycosidic moiety in the insulin-receptor interaction. — As shown in figure 2, Scatchard analysis of the insulin binding data on lectin-preincubated fat cells (A, B) or liver plasma membranes (C) resulted in a profound loss of the high affinity portion of the typically curvilinear plot (control), while total binding capacity was presumably not altered. These data indicate that the integrity of the receptor glycosidic moiety is essential to the high affinity insulin binding process.

TABLE 2

Sequential purification of liver plasma membrane receptors on lectin columns

Samples	Total protein mg	Insulin binding capacity Ro (*) (ng/mg protein)	Purification fold	Receptor recovery %	[Ins] 50 % (**) ng/ml
Solubilized liver membranes	15 (100 %)	23	1	100	4
Concanavalin A eluate __	1.30 (8.6 %)	160	7	60	1.6
Ricin I eluate	0.57 (3.8 %)	340	15	53	0.9

(*) Determining from Scatchard analysis of inhibition-competition binding curves.
(**) Unlabelled-insulin concentration required for half-maximal inhibition of insulin binding in competitive binding assays.

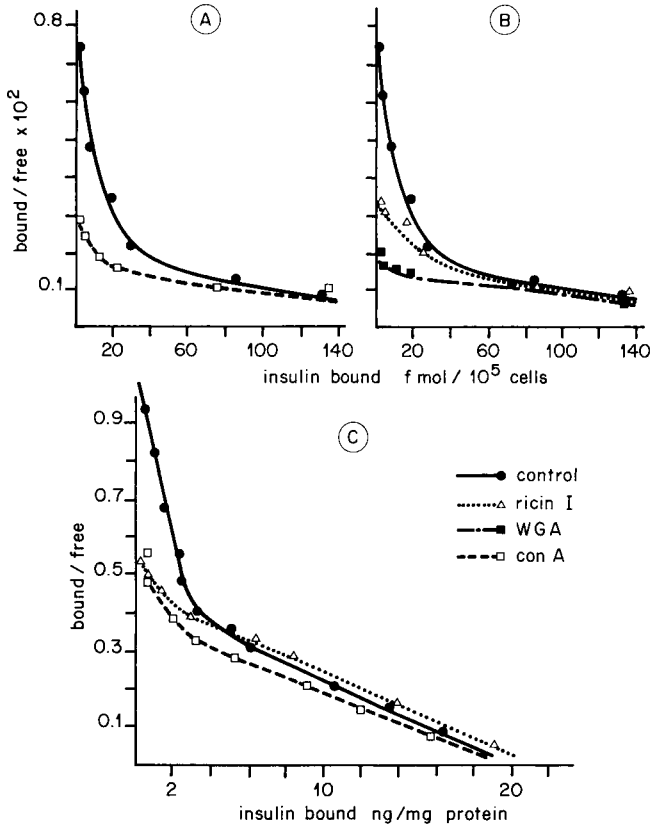

FIG. 2. — *Scatchard plots of [^{125}I]-labelled insulin binding to adipocytes* (A, B) *or liver plasma membranes* (C) *in the presence of various lectins.* Adipocytes (A, B) or liver plasma membranes (C) were preincubated at 22 °C for 30-45 min with 30-50 µg of the various lectins. Binding assays of 100 pM (A, B) or 0.15 ng/ml (C) of [^{125}I]-labelled insulin were performed in the presence of 0-1.10^5 ng/ml of unlabelled insulin. Data were corrected for non-specific binding.

Soluble liver membranes were sequentially chromatographed on con A- and ricin I-lectin columns (table 2). The receptors were purified 15-fold with a 53 % recovery.

When insulin binding parameters were determined with competition curves and Scatchard plots, the typically complex binding characteristics of the receptors were retained. The leftward shift in the competition curves — the points of half-maximal inhibition (INS 50 %) varied from 4 ng/ml to 1.6 and 0.9 ng/ml for the soluble membranes, con A-eluate and ricin I-eluate, respectively — and the steeper slopes of the Scatchard plots (data not shown) indicated that the apparent receptor affinity increased gradually in the course of purification.

As shown in table 3, specific galactose (β-galactosidase or ricin I), mannose (α-mannosidase or con A) and glucosamine (WGA) agents altered purified receptor binding activity.

As compared to soluble receptors, the effects of β-galactosidase and ricin I were clearly enhanced up to 90 % inhibition. WGA or LPA as well acquired the possibility to alter the insulin binding process. These data confirmed the reorganization of the insulin receptor carbohydrate units and the view that the receptor is an N-linked glycoprotein. Since enhancing receptor purity resulted in a concomitant increase in receptor binding affinity and sensitivity to glycosidases and lectins, we proposed that the affinity changes were controlled through perturbation of the receptor carbohydrate groups.

TABLE 3

Effects of various glycosidic probes on specific [^{125}I]-labelled insulin binding to intact soluble liver membranes as compared to lectin-purified receptors

Treatments	*[^{125}I] labelled insulin specifically bound % of control ± S.E.*	
	Soluble receptors	*Purified receptors*
None	100 ± 6	100 ± 8
β-galactosidase (2 U/ml)	53 ± 5	19 ± 8
Ricin I (50 µg/ml)	65 ± 6	8 ± 10
α-mannosidase (0.1 U/ml)	60 ± 4	42 ± 8
Concanavalin A (50 µg/ml)	60 ± 4	52 ± 13
Neuraminidase (0.1 U/ml)	100 ± 10	89 ± 15 (n.s.)
LPA (20 µg/ml)	100 ± 8	58 ± 6
WGA (25 µg/ml)	90 ± 8	20 ± 6

Soluble or purified liver membrane receptors were incubated either at 37 °C for 15 min with the enzymes or for 30 min at 22 °C with the lectins. [^{125}I-labelled insulin binding (0.145 ng/ml) was then assayed. Data were corrected for non-specific binding. The amounts of insulin bound for the controls were 0.32 ± 0.02 and 2.78 ± 0.21 ng/mg protein in soluble and purified receptors, respectively.

n.s. : not significant as compared to control.

Importance of glycosidic structures in insulin-induced receptor regulation. — When ZHC were incubated with tunicamycin, an inhibitor of N-linked glycoprotein synthesis, a dramatic decrease in insulin binding was observed. In this decline, related to tunicamycin concentration, the initial receptor level was decreased by about 80 % after the cells were treated with tunicamycin (0.5 µg/ml, 24 h) (fig. 3). Similar results have been described elsewhere (Keefer and De Meyts, 1981).

Insulin-induced receptor regulation was studied on control cells, 2-hour tunicamycin-treated cells and 24-hour tunicamycin-treated cells ; as indicated in

figure 3, a down-regulation phenomenon occurred on control and 2-hour treated cells after 2-hour incubation with 4.10^{-8} M insulin. The decrease in receptor number was 46 ± 4 % and 44 ± 5 %, as compared to controls incubated in the same conditions but in the absence of insulin.

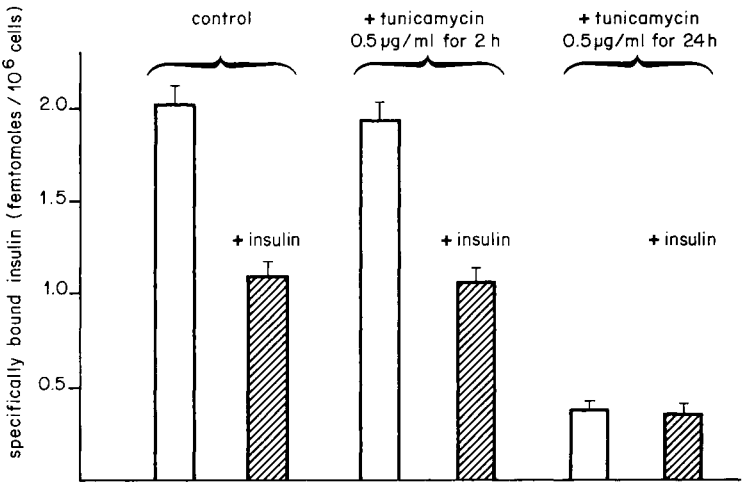

FIG. 3. — *The effect of tunicamycin on insulin-induced down-regulation of insulin receptors in hepatoma cells.* Cultured Zajdela hepatoma cells were employed. The effect of insulin was tested with 4.10^{-8} M insulin for 2 h at 37 °C.

In contrast, the insulin-induced down-regulation of cells treated for 24 h with tunicamycin was not significant (6 % decrease in insulin binding capacity). These cells presented a dramatic decrease in receptor number. Furthermore, although these binding sites retained their normal binding characteristics (unpublished data), the insulin biological effect studied — *i.e.* insulin-induced receptor regulation — no longer occurred, strongly suggesting the presence of a resistant post-receptor state related to the inhibition of N-glycoprotein synthesis. These results provide further evidence for the N-glycoproteinic nature of the insulin receptor and indicate that insulin-induced down-regulation occurs through N-linked glycoproteins of the post-receptor system.

Conclusion.

From the present study which developed methodological approaches to demonstrate the importance of glycoproteins in the binding and action of insulin, it is concluded that carbohydrate chains of the complex N-linked type play a key role at both insulin receptor and post-receptor levels.

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Résumé. *Caractérisation et rôle de la partie glucidique du récepteur de l'insuline.*

1. Nous avons utilisé diverses exoglycosidases et/ou lectines comme agents spécifiques des unités glucidiques pour caractériser les constituants saccharidiques du récepteur de l'insuline des cellules normales ou transformées, et avons évalué leur importance pour l'interaction insuline-récepteur. Le récepteur de l'insuline contient des chaînes oligosaccharidiques sialylées de type N-acetyllactosaminique qui participent à l'interaction insuline-récepteur par l'intermédiaire de résidus D-galactose, D-mannose et N-acetyl-D-glucosamine. Le traitement des adipocytes de rat avec la β-galactosidase en présence de neuraminidase diminue significativement la fixation de l'insuline ; chaque enzyme utilisé individuellement reste inefficace. Les lectines spécifiques des unités D-galactose (lectine de Ricin, RCAI), D-mannose (Concanavaline A, Con A) ou N-acetyl-D-glucosamine (lectine de germe de blé, WGA) inhibent les processus de fixation ou de dissociation de l'insuline dans les adipocytes, les cellules de l'hépatome de Zajdela ou les membranes plasmiques hépatiques.

2. L'effet des lectines dépend directement du degré d'occupation du récepteur puisque le processus de liaison de « haute affinité » de l'insuline est totalement inhibé sans que le nombre de sites ne soit significativement modifié. Ces données sont indiquées par la perte de curvilinéarité des courbes de Scatchard. La purification séquentielle des récepteurs de l'insuline par chromatographie sur colonnes de lectine entraîne une augmentation progressive de l'affinité du récepteur pour l'hormone qui est accompagnée d'une nette augmentation de leur sensibilité aux exoglycosidases et lectines.

3. Nous avons évalué l'importance des N-glycosyl-protéines pour la réponse biologique des cellules à l'insuline, en étudiant l'effet de la tunicamycine sur des cellules d'hépatome en culture. Après 24 h d'incubation, les cellules traitées à la tunicamycine montrent une très importante diminution du nombre de leurs récepteurs pour l'insuline, et de plus, les récepteurs résiduels sont devenus résistants au processus de « régulation en retour » induit par l'insuline.

Ces données nous permettent de conclure que des N-glycoprotéines du récepteur de l'insuline jouent un rôle clef à la fois aux niveaux récepteur et post-récepteur.

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