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Biochemistry

Insulin receptor: Interaction with nonreceptor glycoprotein from liver cell membranes

(affinity chromatography/receptor cooperativity/mobile receptor hypothesis)

JOSEPH M. MATURO III* AND MORLEY D. HOLLENBERG†‡

* Department of Biology, C. W. Post College, P.O. Greenvale, New York 11548; and † The Alan Bernstein Laboratories of the Division of Clinical Pharmacology, Department of Medicine and Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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ABSTRACT **In crude receptor preparations (either particulate or soluble) of rat liver membranes, the insulin receptor exhibits complicated binding kinetics (two binding plateaus, half-saturated at approximately 60 pM and 700 pM insulin) and an apparent chromatographic heterogeneity, suggested by the presence of two detectable, soluble insulin-binding components with apparent Stokes radii of 72 Å and 38 Å. In contrast, the insulin receptor isolated by affinity chromatography exhibits a simple binding isotherm (half-maximal saturation of binding at 700 pM insulin) without evidence for negative cooperativity and behaves as a single component (apparent Stokes radius of 38 Å) upon chromatography on Sepharose 6B. The apparent discrepancies between the properties of the unpurified insulin receptor and the affinity-purified receptor can be attributed to the presence in crude preparations of a nonreceptor constituent(s) having properties consistent with those of a membrane glycoprotein. A glycoprotein fraction from such crude soluble membrane preparations, freed from insulin receptor and subsequently partially purified using concanavalin-A-agarose, when combined with affinity-purified insulin receptor, causes both a reappearance of the complicated binding kinetics and an increase in the receptor's apparent Stokes radius from 38 Å to 72 Å. Similar results are observed for a glycoprotein fraction obtained from rat adipocyte membranes but are not observed for an identical fraction isolated from human erythrocyte membranes. We conclude that the insulin receptor in rat liver membranes can interact with another nonreceptor membrane glycoprotein that may represent either a nonrecognition moiety of the receptor oligomer or an effector molecule related to the biological action of insulin.**

There have been a number of studies of the binding of ^{125}I-labeled insulin to putative membrane receptors[§] in various tissues (summarized in ref. 1). When binding is measured over a sufficiently broad range of insulin concentrations, the data suggest that the interaction of insulin with a membrane-localized receptor may not be a simple bimolecular reaction. Mathematical analyses of the binding data [e.g., by the method of Scatchard (2)] have been interpreted either in terms of multiple classes of binding sites, as discussed by Kahn, *et al.* (3) or in terms of negative cooperativity between insulin-binding sites (4, 5). In the present study, we have focused on the binding properties of the solubilized insulin receptor from rat liver cell membranes over a broader range of insulin concentrations than have been previously studied, both before and after purification of the receptor by affinity chromatography (6–8). We observe that the apparently complicated insulin binding isotherm observed in crude, but not purified, insulin receptor preparations can be attributed to the presence of a nonreceptor constituent(s) having properties consistent with those of a membrane glycoprotein.

MATERIALS AND METHODS

Preparation of Soluble Insulin Receptor. Membranes were prepared from male CD-1 albino rats (100–140 g) as described (9) and were extracted (5 mg of membrane protein per ml of buffer) for 1 hr at 4° with 2% (vol/vol) Triton X-100 in a calcium-free phosphate buffer, pH 7.5, containing: 130 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4 \cdot 7\ H_2O$, and 8.6 mM Na_2HPO_4. After dialysis for 18 hr at 4° against the same buffer containing 0.1% (vol/vol) Triton X-100 (PT buffer), extracts were centrifuged at 150,000 × *g* for 60 min and the resulting supernatant was used as the source of the soluble insulin receptor. Subsequent experiments with soluble receptor were routinely done in PT buffer. Further purification of the insulin receptor was accomplished by affinity chromatography on insulin/succinyldiaminodipropylamino-Sepharose 4B (0.28 mg of insulin per ml of packed gel) prepared as described (derivative C in ref. 10) and washed with eluting buffer before regeneration with PT buffer. Receptor was eluted with an eluting buffer (0.05 M sodium acetate, pH 6.0/4 M urea/0.1% Triton X-100 in 1 ml fractions which were usually diluted immediately with 1 ml of 0.1 M sodium phosphate, pH 7.0. Insulin-binding fractions were pooled and immediately dialyzed against PT buffer for 18 hrs at 4°. The measurement of the specific binding by both soluble and particulate material of ^{125}I-labeled insulin (100–200 μCi/μg) as well as the preparation of ^{125}I-insulin have been described in detail (1, 11). Soluble receptor was measured by using polyethylene glycol [carbowax 6000; final conc. 10.4% (wt/vol)] to precipitate the hormone–receptor complex, as described (1). Calcium-free buffer (containing 0.1% Triton X-100 for experiments with soluble receptor) was used for the assay of insulin binding. Routinely, ^{125}I-labeled insulin isolated by the talc procedure (1) was used; equivalent data were obtained using monoiodo-insulin isolated by ion-exchange chromatography (12). Nonspecific binding, determined in the presence of 40–50 μg of unlabeled insulin per ml, usually amounted to about 25% of the total binding, both at low (0.5 ng/ml) and high (20 ng/ml) ^{125}I-labeled insulin concentrations. On occasion, with the polyethylene glycol assay, nonspecific binding of up to 75% of the total binding was observed. Importantly, even when nonspecific binding was high (e.g., 75%),

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Abbreviations: PT buffer, calcium-free phosphate buffer, pH 7.5, containing 0.1% (vol/vol) Triton X-100; Con A, concanavalin A.

‡ To whom correspondence should be addressed.

§ In this paper, the term "receptor" is used to designate only the recognition macromolecule for insulin.

the "specific binding" isotherm yielded results identical to those obtained in the presence of relatively low (i.e., 25%) nonspecific binding.

Preparation of Nonreceptor Glycoprotein Fraction. The solubilized membrane preparation was depleted of insulin receptor by repeated recycling (usually 3 or 4 times) of the sample (5 ml of soluble receptor extract) on a 1-ml insulin-Sepharose affinity column until insulin-binding activity in the effluent fractions could no longer be detected by the polyethylene glycol assay. The receptor-depleted fraction was then adsorbed on a 1-ml concanavalin A (Con A)-agarose affinity column (12 mg of Con A/ml of packed gel, prewashed with 5 ml of eluting buffer and preequilibrated with a minimum of 50 ml of PT buffer) which was then washed with 50 column-volumes of the PT buffer before eluting the adsorbed glycoproteins (approx. 2% of the protein applied) with 0.05 M sodium acetate (pH 6.0) containing 4 M urea, 0.05 M α-methylmannoside, 0.1% Triton X-100. The second through fifth eluted 1-ml fractions were pooled, dialyzed against PT buffer for 18 hrs, stored at 4°, and used within 3 days of preparation.

Insulin-Sepharose affinity columns were determined to be free from the "leakage" of interfering insulin by the measurement of the displacement of ^{125}I-labeled insulin from crude soluble receptor in a competitive binding assay. Routinely, 1-ml fractions that were collected immediately prior to the application of sample and prior to the elution of sample from the affinity columns were assayed. Similarly, effluent fractions from the Con A agarose affinity columns were determined to be free from interference in the insulin-binding assay.

Chromatography on Sepharose 6B. A 1.5 × 80 cm Sepharose 6B column was equilibrated at 4° with PT buffer for at least one week before use. In all experiments, 2-ml fractions were collected at a flow rate of 8 ml/hr. Radioactivity of the eluted fractions was measured by crystal scintillation counting (85% efficiency). Protein concentrations were estimated with the Folin–Ciocalteau reagent (13); bovine serum albumin was used as a standard.

RESULTS

Similar binding isotherms for ^{125}I-labeled insulin were observed for both the particulate and the crude solubilized receptor preparations (Fig. 1). Interestingly, the concentration range over which the first plateau of insulin binding was observed (*Insets* for Fig. 1 *A* & *B*) corresponds to the range of concentrations over which insulin acts *in vivo* and is coincident with the dose-response curve for the enhancement of glucose transport in isolated adipocytes by the preparations of ^{125}I-labeled insulin used in these studies (data not shown). Only two principal plateaus of insulin binding were observed upon repeated analysis of the binding isotherms at closely spaced concentrations of insulin. The half-maximal concentration for the saturation of the two plateaus occurs reproducibly at approximately 6×10^{-11} M and 7×10^{-10} M. Identical isotherms were observed using either ^{125}I-labeled insulin isolated by talc adsorption (1) or mono-^{125}I-labeled insulin isolated by ion-exchange chromatography (12).

The possible degradation of ^{125}I-labeled insulin under the conditions of the binding assay was also evaluated. After 40 min at 24° in the presence of intact membranes, ^{125}I-labeled insulin (2.8 nM) exhibited the same membrane-binding properties as unincubated ^{125}I-labeled insulin; the insulin bound by identical membranes under these conditions, upon elution from the membranes, was also able to bind to a second aliquot of membranes with the same affinity as unincubated ^{125}I-labeled insulin. In other experiments, the precipitability with 3.3%

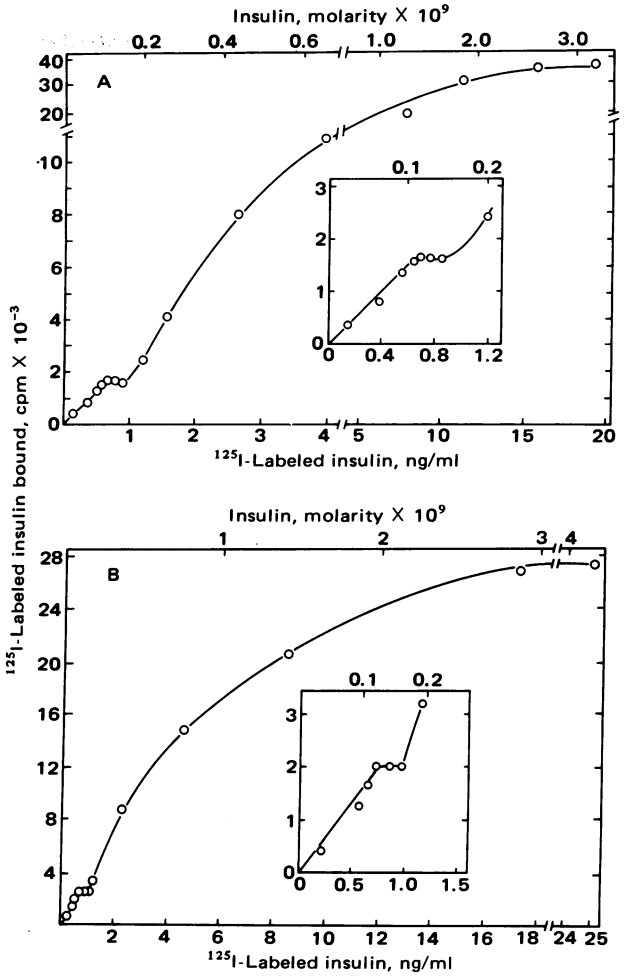

FIG. 1. Binding of ^{125}I-labeled insulin to receptor from rat liver membranes. Binding isotherms, measured as described (1), were determined either (*A*) for particulate membrane preparations (50 μg membrane protein per assay tube) or (*B*) for crude solubilized receptor (48 μg protein per assay tube). Nonspecific binding accounted for approximately 25% of the total amount of ^{125}I-labeled insulin bound, at both low (e.g., 0.5 ng/ml) and high (e.g., 20 ng/ml) ligand concentrations. The *Insets* show detail of the binding data at low concentrations of ^{125}I-labeled insulin.

(wt/vol) trichloroacetic acid of ^{125}I-labeled insulin incubated in the presence of triton-solubilized membranes was observed to be indistinguishable from the precipitability (>98%) of unincubated ^{125}I-labeled insulin. We conclude that, under the conditions of the binding assay, insulin degradation does not occur to an appreciable extent and cannot account for the observed plateaus in the binding isotherms.

When soluble receptor was preequilibrated with ^{125}I-labeled insulin (5 ng/ml) prior to chromatography on Sepharose 6B in the presence and absence of unlabeled insulin, two peaks of insulin-binding activity (peaks a and b) were detected with distribution coefficients (K_{AV}) of 0.31 and 0.53, respectively, corresponding to stokes radii of about 72Å and 38Å, respectively (Fig. 2). Peak a contained the majority of the specific insulin-binding activity, whereas peak b contained both a small amount of specific binding activity and a substantial amount of nonspecific binding—i.e., the amount of radioactivity in peak b of Fig. 2 was only slightly decreased in the presence of a high concentration of unlabeled insulin. The distribution coefficient for the material in peak a is the same as that reported for solubilized insulin receptor (6). The insulin binding properties of the material in peaks a and b of Fig. 2 were further studied by

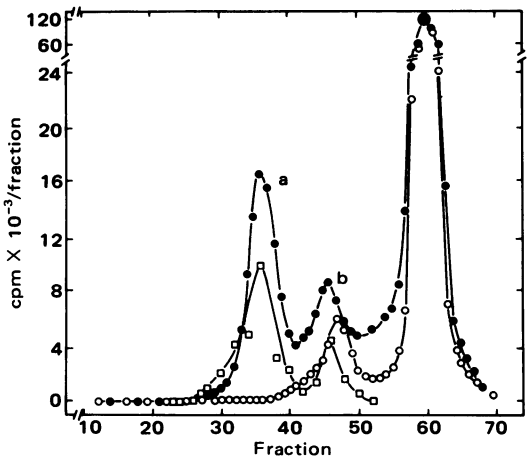

FIG. 2. Chromatography on Sepharose 6B of soluble insulin-binding material from rat liver membranes. Aliquots (0.5 ml) of the soluble extract from liver membranes (740 μg protein) were subjected to chromatography on a column (1.5 × 80 cm) of Sepharose 6B equilibrated with PT buffer. Two identical samples were analyzed subsequent to equilibration with ^{125}I-labeled insulin (7.6×10^{-10} M) in the absence (●) or presence (○) of unlabeled insulin (1.3×10^{-5} M); a third sample was chromatographed in the absence of insulin and aliquots (50 μl) of the effluent fractions (2 ml) were assayed for specific insulin binding (□) by the polyethylene glycol method (1).

the polyethylene glycol assay of insulin binding for fractions obtained subsequent to chromatography without prior exposure to insulin. This method substantiated the presence of specific insulin binding in both chromatographic peaks and quantitatively accounted for all of the insulin-binding activity applied to the column.

The binding isotherm of material eluted in peak a of Fig. 2, as shown in Fig. 3A, was closely similar to the isotherm either for liver membranes or for the unfractionated soluble receptor (Fig. 1). Half-maximal saturation of the two plateaus of binding was observed at 6×10^{-11} M and 8×10^{-10} M, respectively. Furthermore, when material isolated from peak a of Fig. 2 was rechromatographed, insulin-binding material was found both in the region of peak a and that of peak b. Strikingly, the binding isotherm of the material in peak b of Fig. 2, as shown in Fig. 3B, suggests the presence of only a single insulin-binding site with an apparent affinity of 7×10^{-10} M. It was therefore of interest to determine which of the two peaks corresponded to the elution volume of the soluble insulin receptor isolated by insulin-Sepharose affinity chromatography.

Surprisingly, the purified insulin receptor was eluted solely in the position of peak b (Fig. 4A) and, like the material from the crude preparation that eluted in the same volume, yielded a binding isotherm that suggests a single insulin-binding site with an apparent K_D of 7×10^{-10} M. The Hill coefficient for insulin binding to the purified receptor was 1.16 (Fig. 5B). From the above results it was hypothesized that, in the unfractionated soluble membrane extract, the insulin receptor might be associated with other components that could cause a portion of the receptor protein to migrate as a complex with a larger stokes radius and that might confer on the receptor the complicated insulin-binding properties illustrated in Figs. 1 and 3A. Thus, the complex of insulin receptor and another component or components might be expected to elute in the position of peak a of Fig. 2 and might be expected to dissociate, upon rechromatography, to yield uncomplexed receptor in the position of peak b.

To test the above hypothesis, a glycoprotein fraction free from insulin receptor was isolated. The unfractionated mem-

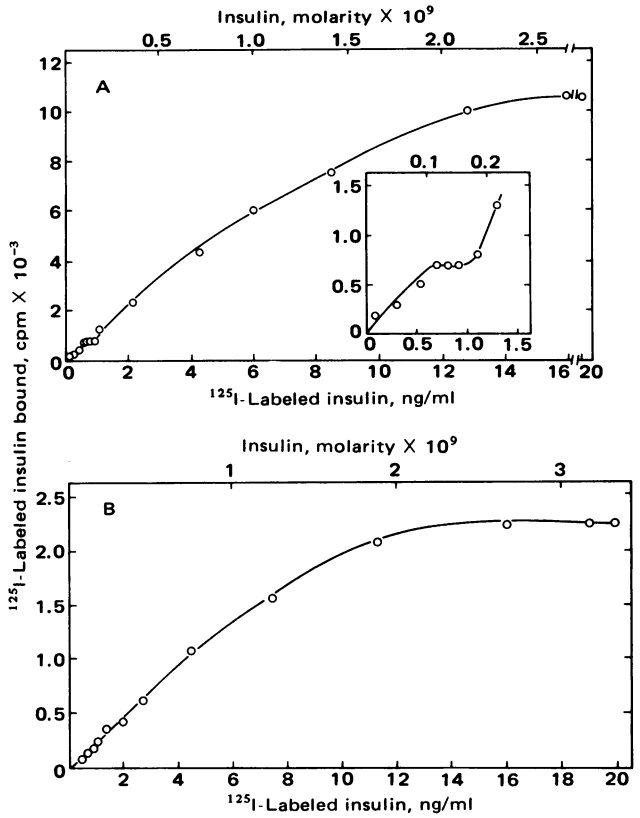

FIG. 3. Binding of ^{125}I-labeled insulin to soluble components isolated by chromatography on Sepharose 6B. Aliquots from the peaks shown in Fig. 2 were assayed for insulin binding as in Fig. 1. (*A*) Analysis of aliquots from peak a, Fig. 2 (fractions 35 & 36 were pooled). The *Inset* shows detail of data obtained at low concentrations of ^{125}I-labeled insulin. (*B*) Aliquots from peak b, Fig. 2 (fractions 47 & 48 were pooled).

brane extract was depleted of insulin-binding activity by recycling on an insulin-Sepharose column, and the resulting receptor-depleted extract was applied to a concanavalin A-Sepharose column. The glycoprotein fraction, containing 2% of the protein applied to the column and obtained as outlined in *Materials and Methods*, was devoid of specific insulin-binding activity. When added back to affinity-purified insulin receptor, the glycoprotein fraction caused the specific insulin-binding activity to elute as a higher molecular weight species (peak a, Fig. 4B), analogous to the result depicted in Fig. 2 (peak a). The appearance of insulin binding in peak a of Fig. 4B corresponded to a diminution in specific binding in peak b (Fig. 4B). Chromatography of the glycoprotein fraction alone (Fig. 4C) demonstrated the ability of the material to adsorb insulin only in a nonspecific manner, so as to contribute to the "nonspecific" insulin binding detected in peak b of Fig. 2; no reappearance of specific insulin binding was detected in this fraction.

Importantly, a glycoprotein fraction from human erythrocytes, prepared by the same procedure used for liver membranes has no effect on the elution properties of the insulin receptor (Fig. 4 B and 4 C), whereas a similar fraction from fat-cell membranes does alter the receptor elution volume in the manner depicted in Fig. 4 for the fraction from liver membranes (data not shown). Whereas the receptor alone displays an apparently homogeneous insulin-binding site (Fig. 5B) with an apparent K_D of 7×10^{-10} M, adding back the partially purified glycoprotein fraction resulted in an isotherm similar to the one observed for liver membranes and exhibiting half-

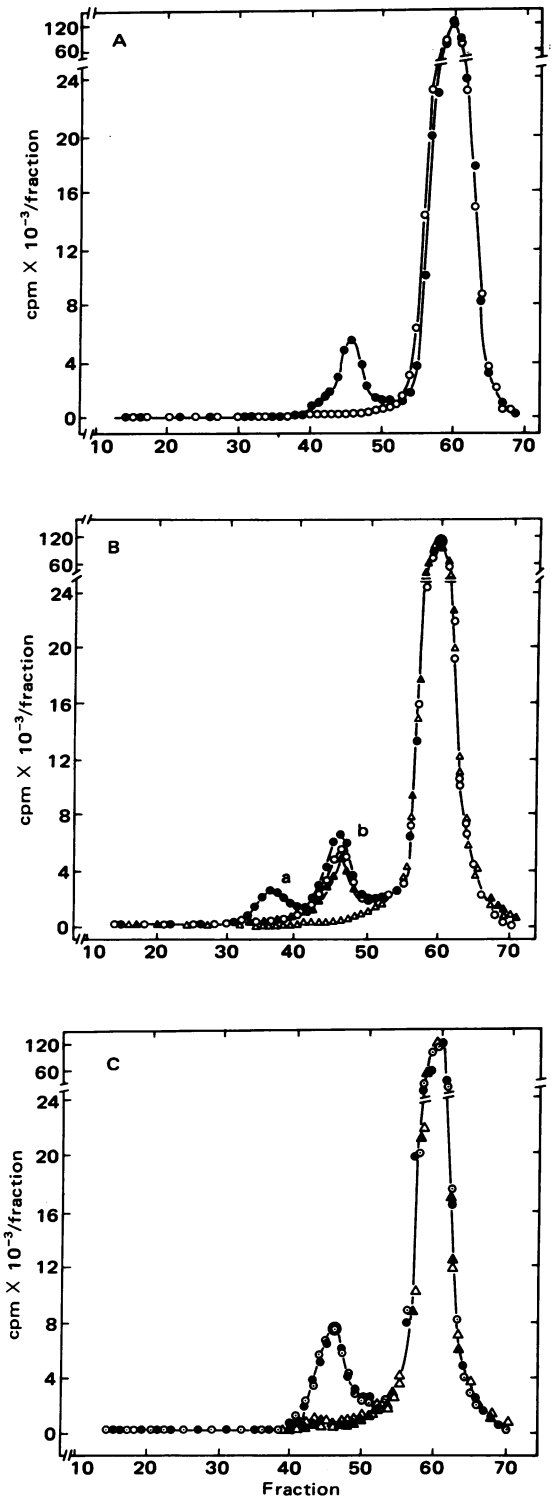

FIG. 4. Chromatography of affinity-purified insulin receptor and nonreceptor glycoproteins on Sepharose 6B. Affinity-purified insulin receptor and nonreceptor glycoprotein fractions were equilibrated (½ hr, 24°) with ^{125}I-labeled insulin in either the presence (○, △) or absence (●, ▲) of unlabeled insulin and subjected to chromatography as outlined in the legend to Fig. 2. (*A*) Chromatography of affinity-purified insulin receptor. (*B*) Chromatography of affinity-purified insulin receptor equilibrated (½ hr, 24°) with a glycoprotein fraction (50 μg protein) obtained either from liver membranes (●, ○) or from human erythrocytes (▲, △). (*C*) Chromatography of glycoprotein fractions either from liver membranes (●, ○) or from human erythrocyte membranes (▲, △). Specific insulin binding is indicated by a reduction of radioactivity in the peaks caused by the presence of high concentrations of unlabeled insulin.

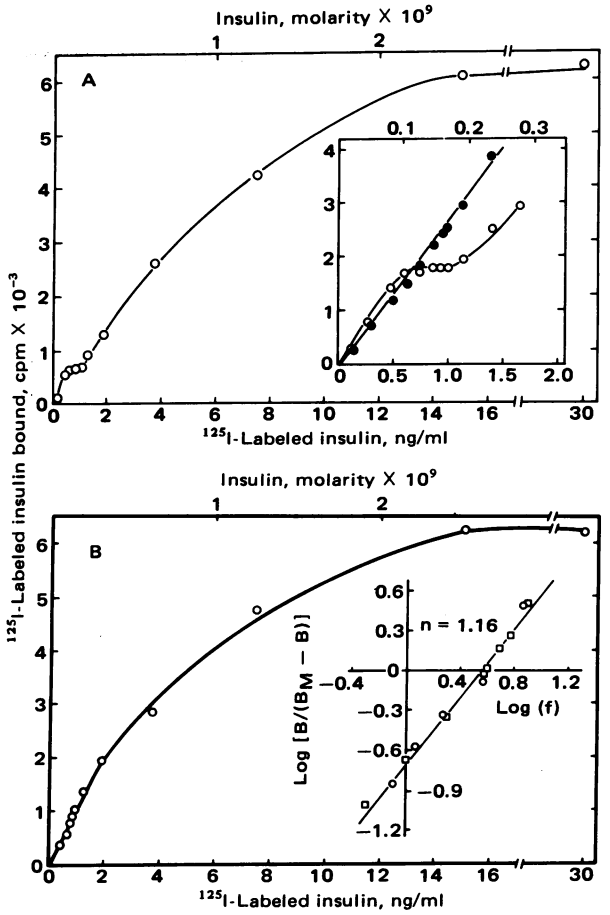

FIG. 5. Binding of ^{125}I-labeled insulin to affinity-purified receptor in the presence and absence of nonreceptor glycoprotein. (*A*) Soluble insulin receptor, isolated by affinity chromatography, was equilibrated with nonreceptor glycoprotein from liver membranes (48 μg) and the binding isotherm determined by the polyethylene glycol assay. The *Inset* shows detail of measurements at low insulin concentrations for twice the amount of receptor either in the presence (○) or absence (●) of glycoprotein. (*B*) Binding of insulin to the identical amount of receptor as in (*A*) in the absence of glycoprotein; the maximum binding capacity is unchanged. A Hill plot of the binding data (*Inset*) was calculated using both experimental (○) and interpolated (□) values from binding isotherm. f = free insulin concentration (ng/ml).

maximal saturation of two binding plateaus at 6×10^{-11} M and 7×10^{-10} M (*inset*, Fig. 5*A*).

DISCUSSION

It is evident from the ^{125}I-labeled insulin-binding isotherms either for liver membranes or for a crude, solubilized membrane preparation (Fig. 1) that, unless sufficient data points are obtained in the low concentration range (0–2 ng/ml), the first "high-affinity" plateau of binding may easily go undetected when present. Although it has been repeatedly pointed out that the shapes of biological dose-response curves for agonists need not necessarily bear any relationship to the agonist-receptor binding isotherms (discussed in ref. 1), it may be more than fortuitous that the saturation of the biological insulin dose-response curve for isolated adipocytes *in vitro* occurs exactly over this low concentration range (14) and is coincident with the first plateau of binding observed in the present study. Likewise, the reappearance of this "high-affinity" plateau of binding upon adding the nonreceptor glycoprotein fraction to the purified receptor may suggest a role for the nonreceptor constituent(s) in the biological action of insulin.

The data we present are entirely consistent with *apparently* conflicting data previously reported in the literature that have focused either on the low-capacity, high-affinity portion of the binding isotherm or on the lower-affinity, high-capacity portion of the isotherm. When all of the insulin binding activity in a particular soluble preparation was adsorbed by recycling on insulin-Sepharose, the receptor material subsequently eluted displayed a simple binding isotherm without any detectable plateau in the low concentration range (Fig. 5*B*) and without any evidence for negative cooperativity. No specific insulin-binding activity could be detected in the unadsorbed material, either before or after isolation with Con A-agarose. The absence of specific insulin binding in the fraction eluted from the lectin affinity column is of particular significance. Insulin, which might have been present due to "leakage" from the insulin-agarose column so as to mask a small amount of "high-affinity" binding in the recycled fraction, would be removed prior to elution from the lectin columns. Insulin receptor protein which is known to bind to Con A-agarose columns would have been readily detected subsequent to elution. It can be concluded that the complicated binding kinetics for insulin observed in crude receptor preparations result from the presence of an apparently homogeneous insulin-binding species, rather than from multiple insulin-binding species. The reappearance of complicated binding kinetics upon adding back the glycoprotein fraction to an isolated receptor fraction substantiates this conclusion.

The interaction of the insulin receptor with the nonreceptor glycoprotein fraction is demonstrated both by the alteration in the receptor elution volume on Sepharose 6B caused by the presence of the nonreceptor fraction and by the perturbation of the insulin-binding isotherm. It has not yet proved possible to isolate sufficiently pure glycoprotein either to examine the stoichiometry of this interaction or to estimate the recovery of the factor(s) subsequent to chromatography. Nonetheless, one may speculate that the presence of the glycoprotein may be required for the complicated ligand-binding behavior—i.e., nonlinear Scatchard plots and insulin-mediated acceleration of the dissociation of bound ^{125}I-labeled insulin—that has previously been interpreted in terms of negative cooperativity between insulin receptors (4). It is to be noted that in cultured lymphocytes, kinetic binding data obtained at low insulin receptor occupancy (15) do not support the negative cooperativity model of insulin binding, as initially formulated (4). The absence of cooperative binding kinetics for the affinity-isolated receptor (Fig. 5B) further confirms the conclusions of Pollet *et al.* (15) and emphasizes the possible influence of the glycoprotein factor(s) on insulin binding.

The nature of the constituent(s) responsible for the alteration in the binding kinetics and elution properties of the insulin receptor is as yet clearly a matter for speculation. Because the fraction studied both adsorbs to Con A and contains a substantial amount of protein, it is most likely that the factor(s) is a glycoprotein. It should not be overlooked, however, that a complex glycolipid might conceivably account for the results we observe. One possibility is that the glycoprotein fraction may contain "effector" molecule(s) with which the insulin receptor may interact within the plane of the membrane, as postulated by the mobile receptor paradigm of hormone action (1, 16–19). The complicated binding kinetics generated by the interaction of such effector molecules with a unique recognition moiety can be predicted mathematically (19). A second alternative is that the glycoprotein(s) may form an integral part of the insulin-receptor oligomer that is separate from the recognition subunit but that confers special binding properties on the receptor complex. It is possible that the insulin-mediated reduction in the Stokes radius of the insulin-recognition site observed in a previous study (20) results from a dissociation of a glycoprotein from the recognition site rather than from negatively cooperative interactions *between* insulin-binding sites. The ability of the glycoprotein fraction to adsorb insulin in a "nonspecific" manner—i.e., high concentrations of unlabeled insulin do not compete for the binding of ^{125}I-labeled insulin; Fig. 4*C*—is difficult to interpret. It is as yet unclear whether or not the component(s) responsible for this "nonspecific" binding are the same as the one(s) responsible for the interaction with insulin receptors. It thus becomes of considerable interest to isolate the glycoprotein factor(s) responsible for the perturbation of the insulin-binding properties that we have observed, so as to examine the stoichiometry of the interaction and to test the above hypotheses.

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