We have studied the binding unit of the high affinity insulin receptor from the plasma membrane of the cultured human lymphoblastoid cell IM-9, solubilized with the nonionic detergent Triton X-100. We have previously shown this receptor to be an M, 310,000 protein associated with approximately 0.3 g of detergent per g of protein. High energy irradiation of the receptor in the frozen state decreased the receptor number without changing the binding affinity for insulin. The monoequational decrease in the number of active receptors with increasing radiation dose indicated a single-sized binding unit of M, 170,000 for this receptor.

The insulin receptor was covalently cross-linked to bound [*]H-insulin with disuccinimidyl suberate and solubilized with Triton X-100. Under reducing conditions, sodium dodecyl sulfate-polyacrylamide gel electrophoresis yielded a major subunit of M, 120,000 and a smaller amount of M, 50,000 material. To avoid ambiguities concerning sodium dodecyl sulfate binding and other critical factors, the size of the major subunit after reduction and alkylation of disulfide bonds was independently confirmed as M, 110,000 by gel filtration chromatography on Sepharose CL-4B in 6 M guanidine hydrochloride.

These results suggest that this insulin receptor, a multimeric globular glycoprotein of molecular weight 310,000, contains one or two insulin-binding units of molecular weight 170,000, each of which yields subunits of molecular weight 110,000-120,000 and 50,000 after denaturation and reduction of disulfide bonds. Furthermore, the results indicate that each of these two subunits of the receptor is required to maintain the integrity of the hormone-binding site. A working model for the insulin receptor based upon the above results is presented.

Insulin receptors from several sources have been characterized as membrane glycoproteins of molecular weight 300,000-350,000 (1-3). Subunit studies of highly purified insulin receptors or less pure preparations covalently bound to radiolabeled insulin using photoaffinity methods or cross-linking agents have demonstrated a major subunit with a molecular weight of 120,000-130,000 (2-4). In addition, several studies using these methods have identified additional smaller subunits with approximate molecular weights of 50,000 (2, 3) and 90,000 (3, 5, 6). Furthermore, examination of membrane proteins which are immunoprecipitated with antireceptor antibodies has suggested the presence of component peptides ranging from 30,000-90,000 (7).

In this report, we examine the relationship of these subunits to the insulin-binding process for the membrane receptor of the cultured lymphoblastoid cell IM-9 solubilized in Triton X-100. We have previously shown that this receptor is a multimeric globular glycoprotein of molecular weight 310,000 (8). We present here the results of radiation inactivation analysis of the insulin-binding unit. In addition, we have determined the molecular weight of the components of this binding unit which become covalently cross-linked to bound radiolabeled insulin with disuccinimidyl suberate, employing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We have independently confirmed the molecular weight of the major receptor subunit in these preparations by analytical gel filtration in 6 M guanidine hydrochloride. The results suggest a working model for this insulin receptor consisting of one or two binding units of molecular weight 170,000, each of which dissociates into subunits of molecular weight 110,000-120,000 and 50,000 following denaturation and reduction of disulfide bonds. These findings indicate that each of these two subunits is required to maintain the integrity of the insulin-binding site.

MATERIALS AND METHODS

Preparation of Detergent-solubilized Insulin Receptors—IM-9 lymphocytes were cultured and cell membranes prepared and stored at -70 °C as previously described (8). After thawing, the membranes were centrifuged for 15 min at 4 °C at 10,000 × g, resuspended at 20 mg of protein per ml in RRA buffer (100 mM HEPES, pH 7.2, 120 mM NaCl, 1.2 mM MgSO₄, 2.5 mM KCl, 15 mM Na acetate, 1 mM glucose, 1 mM EDTA, pH 7.6) containing 1% Triton X-100, and solubilized for 30 min at 4 °C. The solubilized receptor preparation was diluted 10-fold with cold RRA buffer and centrifuged for 30 min at 4 °C at 40,000 g to remove insoluble material. Alkaline phosphatase (2 mg/ml) was added as an internal standard for radiation inactivation analysis. 275-μl aliquots of the frozen receptor preparation were sealed in 2-ml glass ampoules and stored at -70 °C.

Radiation Inactivation—The solubilized membranes were irradiated at the Armed Forces Radiobiology Research Institute (Bethesda, Maryland) employing a linear accelerator generating electrons with an energy of 13 MeV at a dose rate of approximately 0.5 Mrad/min. The logarithm of the fraction of insulin receptors remaining after increasing radiation doses was a linear function of the radiation dose.
Binding Unit of the Insulin Receptor

(Continued)

in (rads), and the slope of this function, K, was determined by linear regression analysis constrained to pass through 1.0 at zero dose. The molecular weight of the binding unit was calculated from the relation 9, 10 M = 6.4 \times 10^4 S/K, where S is a temperature factor. Each point represents the average of values performed in triplicate. The molecular weight determination is representative of three separate irradiations utilizing several membrane preparations. The temperature was maintained during irradiation at -130 °C ± 20 °C with a stream of cold nitrogen gas, and at this temperature S has been experimentally determined to have the value of 2.8 (11). Using this value, the average target size for the internal standard protein alkaline phosphatase (EC 3.1.3.1 calf intestine) was determined to be 72,000, in agreement with previous radiation inactivation analysis of its function (12) and with its known subunit structure (13). Additionally, the temperature factor for a given irradiation was determined from radiation inactivation of several internal standard proteins, including alkaline phosphatase described above.

Equilibrium Binding—Steady state insulin-binding activity following irradiation was assessed as described previously (8). Briefly, soluble receptors (100 µg of protein per ml) were incubated with 10^{-10} M ^{125}I-insulin for 90 min at 15 °C in RRA buffer containing 0.1% Triton X-100, and the insulin-receptor complex was precipitated with polyethylene glycol (14). Assays were corrected for nonspecific binding with 0.02 M phosphate (pH 7.4) in 0.15 M NaCl containing 0.19% Triton X-100 was previously shown to be linear (8), and Fig. 1 (inset) confirms the presence of a homogenous class of high affinity receptors with an affinity constant of 2.5 \times 10^9 M^{-1}.

These linear binding data permit an accurate determination of the binding site number. In order to characterize the binding unit of this insulin receptor, the preparation was subjected to radiation inactivation analysis (9, 21). This method determines the sensitivity of a given biochemical function to high energy radiation by measuring the number of functional units remaining after increasing radiation doses. This technique is based on the condition that the energy deposition resulting from the interaction of the ionizing radiation with the functional unit is sufficiently large to completely destroy the activity of the unit. Therefore, the fraction of activity remaining (A/Ao) after a given radiation dose (D) is given by A/Ao = e^{-\gamma D}, where \gamma is a constant. Target theory and consideration of empirical data has shown that \gamma = (5.59 \times 10^{-12}) M, for frozen samples irradiated at -130 °C, where M is the molecular weight of the functional unit (9-11).

The results of radiation inactivation at -130 °C of the frozen, detergent-solubilized insulin receptor preparation is shown in Fig. 1. As can be seen in the inset, Scatchard analysis of insulin binding after increasing radiation doses demonstrated a decrease in the number of insulin receptors as determined by the abscissa intercepts, with no detectable change in the binding affinity as measured by the slope of the binding data (22). These results satisfied the above condition for radiation inactivation analysis of the binding unit as a single target. The data in Fig. 1 show that the logarithm of the fraction of remaining receptors is a linear function of the radiation dose, as theoretically predicted for a single-sized target. The slope of this line yields a molecular weight of 167,000 for the functional unit required for insulin binding to this receptor. Three molecular weight determinations at -130 °C resulted in a value of 167,000 ± 15,000. Alternatively, the use of internal standard proteins of known target size to determine the temperature factor (S) for the molecular weight calculations likewise yielded 168,000 for the binding unit of the insulin receptor.

The subunit structure of the detergent-solubilized insulin receptor covalently cross-linked to ^{125}I-insulin with disuccinimidyl suberate was examined by SDS-PAGE (20). As shown in Fig. 2, following denaturation 1% sodium dodecylsulfate the insulin receptor demonstrated a small degree of migration consistent with a molecular weight of greater than 250,000. After denaturation and reduction of disulfide bonds with 1% β-mercaptoethanol, the preparation yielded a major receptor subunit with apparent molecular weight 120,000 and a smaller amount of M, 50,000 material. Densitometry showed that the radiolabel associated with the large subunit was approximately three times greater than that associated with the smaller subunit. Each of these bands was completely eliminated by the addition of 2 \times 10^{-7} M unlabeled insulin during the binding reaction, demonstrating the specificity of the insulin binding to these components. Consistent with the work of others (23), these results and the appearance of the 50,000-dalton subunit were not altered by the simultaneous presence of 4 broad spectrum of protease inhibitors, including phenylmethylsulfonyl fluoride (0.2 mM), aprotinin (20 units/ml), and with an applied current of 80 mA, following the method of Laemmli (20).
antipain (1 μg/ml), and ovomucoid (0.2 mg/ml), during membrane preparation, receptor solubilization, and subsequent steps.

Since molecular weight estimations by SDS-PAGE are critically dependent on the degree of SDS binding and the assumption of a rod-like conformation and yield anomalous results for glycoproteins, we more rigorously determined the molecular weight of the major subunit by analytical gel chromatography in 6 M guanidine hydrochloride (16, 17). The results for the detergent-solubilized insulin receptor under these totally denaturing conditions following reduction and alkylation of disulfide bonds are shown in Fig. 3. Under these conditions, proteins assume a random coil conformation, and for gel filtration chromatography the inverse error function complement of the distribution coefficient, \(erfc^{-1}K_a\), is a linear function of \(M_r^{0.50}\) (18) (inset). As shown in Fig. 3, the major subunit covalently cross-linked to \(^{125}\)I-insulin behaves as a protein of molecular weight 110,000. Thus, after denaturation and reduction of disulfide bonds, the detergent-solubilized insulin receptor covalently linked to \(^{125}\)I-insulin yields a major subunit of \(M_r 110,000-120,000\) as determined by two independent methods, and a smaller subunit of \(M_r 50,000\).

**DISCUSSION**

The hydrodynamic properties (1, 24, 25) and electrophoretic mobilities (2, 3) of detergent-solubilized insulin receptors from multiple sources have suggested molecular weights of 300,000-350,000. In addition, our previous studies of the hydrodynamic and sedimentation-equilibrium properties of the Triton X-100-solubilized insulin receptor of the cultured human lymphoblastoid cell IM-9 show it to be a multimeric globular glycoprotein of molecular weight 310,000 which binds approximately 0.5 g of detergent per g of protein (8).

The results of the present study of this insulin receptor preparation demonstrate by SDS-PAGE, and more rigorously by analytical gel chromatography in 6 M guanidine hydrochloride, that \(^{125}\)I-insulin, chemically cross-linked to the insulin receptor after the hormone binding event, becomes associated with a major subunit of \(M_r 110,000-120,000\). These findings are consistent with other SDS-PAGE studies which have identified a major subunit of \(M_r 120,000-130,000\) in highly purified insulin receptor preparations (2, 26, 27), and in less pure preparations after covalent linkage of bound \(^{125}\)I-insulin with cross-linking agents (3, 15) or photoreactive \(^{125}\)I-insulin derivatives (2, 4, 5), or after immunoprecipitation of \(^{[125]}\)I-methionine-labeled cellular proteins with antireceptor antibodies (6). Furthermore, our results also show a smaller amount of \(^{125}\)I-insulin cross-linked to \(M_r 50,000\) material, which is consistent with the findings of Jacobs et al. (2, 27) and Massague et al. (3), and with the study of Lang et al. (7) demonstrating peptides of \(M_r 34,000-90,000\) after denaturation, reduction, and SDS-PAGE analysis of those IM-9 lymphocyte membrane proteins which were immunoprecipitable with antireceptor antibodies. Although several studies have also indicated the presence of an \(M_r 90,000\) subunit (3, 5, 6), neither this subunit nor receptor heterogeneity (3) was apparent in our Triton X-100-solubilized insulin receptor preparation.

In the present report, we have extended our investigation to a study of the relationship of the observed structural subunits to the functional unit within the receptor which is required for insulin binding. The method of radiation...
vation is ideally suited to elucidate this relationship, since it examines the sensitivity of the binding function of the receptor to ionizing radiation independently of the spatial proximity and orientation of particular reactive groups on the receptor subunits and the bound radiolabeled hormone which may be subject to subsequent chemical cross-linking. The applicability of the radiation technique has been amply demonstrated for the determination of the size of the functional units within numerous enzyme preparations (9). For this purpose, the Triton X-100-solubilized receptor preparation of the IM-9 lymphoblastoid cell offers advantages over membrane insulin receptors and other detergent-solubilized insulin receptors, including (i) homogeneous high affinity binding which yields linear Scatchard plots and accurate determinations of binding site number; (ii) physical homogeneity, as demonstrated by previous characterization (8), without evidence of heterogeneity (3) or multiple oxidation-reduction forms (28) of the receptor; and (iii) absence of receptor-mediated hormone degradation (29). Furthermore, since this insulin receptor preparation appears to be free of a putative large inhibitor of high energy target size corresponds closely with that of the radiation technique has been amply demonstrated for the determination of the size of the functional units within membrane insulin receptor preparations (3), in approximate analogy to the immunoglobulin G molecule. Confirmation of a dimeric structure for the insulin receptor must await an independent determination of the binding valence presently in progress.

We therefore, propose a working structural model for the Triton X-100-solubilized insulin receptor of the lymphoblastoid cell, as shown schematically in Fig. 4. This M, 310,000 glycoprotein (8) contains one or two (dimer model) disulfide-linked insulin-binding units of molecular weight 170,000, each of which contains subunits of molecular weight 110,000-120,000 and 50,000. This lower molecular weight component is consistent with that observed for the purified insulin receptor (2) and with the \( \beta \) subunit proposed by Massague et al. (3). Although this model does not include structural heterogeneity (3) of the insulin receptor, the finding that two subunits of the receptor are required for insulin binding would also appear to be valid for other insulin receptor preparations containing the 50,000-dalton subunit (8), since recent evidence indicates that the \( \beta \) subunit may be derived from the \( \beta \) subunit (23).

While these findings and their internal self-consistency indicate that the binding unit of this receptor consists of two subunits, it is the larger (M, 110,000-120,000) subunit which is predominantly covalently cross-linked to radiolabeled hormone with disuccinimidyl suberate. We can speculate that the 50,000-dalton subunit participates in the hormone-binding site but does not contain amino acid residues with reactive amino groups in sufficient proximity to reactive groups on the radiolabeled hormone to permit an equal degree of chemical cross-linking. Alternatively, the smaller subunit may be required to maintain or regulate the structural integrity of the insulin-binding site on the major subunit with less direct participation in the binding interaction with the hormone. Further studies will be required to examine the relationship and interactions among these receptor subunits in order to elucidate the detailed mechanism by which they participate in the hormone-binding reaction and the subsequent transduction of this event into the multiplicity of hormone-induced biochemical effects.

REFERENCES
Binding Unit of the Insulin Receptor

Structure of the insulin receptor of the cultured human lymphoblastoid cell IM-9.
Evidence suggesting that two subunits are required for insulin binding.
R J Pollet, E S Kempner, M L Standaert and B A Haase


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