Subunit Structure of the Insulin Receptor of the Human Lymphocyte†

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ABSTRACT: For a study of the subunit structure of the insulin receptor, cultured human lymphocytes (IM-9) were iodinated with ^{125}I by using a lactoperoxidase-catalyzed iodination technique which preferentially labeled cell surface proteins. A plasma membrane fraction was then prepared by differential centrifugation and solubilized with Triton X-100. The iodination procedure did not significantly alter the ability of the solubilized membranes to bind [^{131}I]insulin. When ^{125}I-labeled solubilized membranes were preincubated with [^{131}I]insulin and immunoprecipitated with highly specific antireceptor antibodies derived from patients with insulin-resistant diabetes, we observed a quantitative and parallel dose-dependent precipitation of [^{131}I]insulin bound to the receptor and ^{125}I-labeled membrane proteins. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the immunoprecipitated ^{125}I-labeled proteins revealed four peaks with apparent molecular weights of 90 000, 67 000, 56 000, and 34 000. Immunoprecipitation with two different antireceptor antibodies resulted in the same electrophoretic pattern. All peaks disappeared when the membranes were preincubated with trypsin at a concentration known to destroy insulin binding. Pretreatment of the solubilized membranes with insulin prior to immunoprecipitation caused a concentration- and time-dependent suppression of all four components, most marked with the three highest molecular weight components; the 34 000 molecular weight component was never completely suppressed. Treatment of the immunoprecipitate using more potent reducing conditions caused the 67 000 molecular weight component to disappear, whereas the 34 000 molecular weight peak increased. These findings suggest that the insulin receptor has a complex structure consisting of four different subunits with molecular weights of 34 000–90 000. The 67 000 molecular weight subunit may be a disulfide dimer of the 34 000 molecular weight subunit. Inhibition of antibody precipitation of the 90 000, 67 000, and 56 000 molecular weight components by insulin suggests that these three components may be insulin binding subunits or closely related to the insulin binding site.

The initial action of insulin consists of its binding to a specific (glyco)protein on the plasma membrane of the target cell known as the receptor (Ginsberg, 1977). This reaction has been studied in several laboratories and with a number of different receptor preparations including solubilized membranes. Receptors solubilized in Triton X-100 maintain their binding activity and may be studied by gel filtration or gel electrophoresis (Gavin et al., 1971; Cuatrecasas, 1972; Harrison et al., 1978; Ginsberg et al., 1976; Maturo & Hollenberg, 1978; Krupp & Livingston, 1978; Lang et al., 1979). By use of these techniques, the insulin receptor behaves as a high molecular weight species with estimates ranging from 300 000 to 1 000 000. In the presence of insulin this high molecular weight species has been shown to dissociate to a smaller insulin binding component (Ginsberg et al., 1976). Characterization of the insulin receptor after denaturation with sodium dodecyl sulfate ($NaDodSO_4$)[1] has been limited due to the difficulty in directly identifying the receptor. Notwithstanding, the molecular weight of the partially purified receptor analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis has been estimated to be 135 000 (Jacobs et al., 1977) or 74 000–90 000 with lower molecular weight components (Harrison et al., 1979a). Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis after affinity labeling the receptor in situ reveals two components of molecular weight 125 000–135 000 and 90 000 (Yip et al., 1978, 1979; Pilch & Czech, 1979).

In the present study we have attempted to define the structure of the insulin receptor by taking advantage of cell surface labeling and a highly specific technique for immunoprecipitating the insulin receptor (Harrison et al., 1979b) using naturally occurring antibodies to the receptor (Flier et al., 1975). When the immunoprecipitated ^{125}I-labeled insulin receptor of the cultured human lymphocyte was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, we found four components with molecular weights of 34 000, 56 000, 67 000, and 90 000. Further, insulin blocked the immunoprecipitation of the three higher molecular weight components, suggesting that these are related to the insulin binding site.

Materials and Methods

Materials. Porcine insulin (lot no. ODY44C) was purchased from Eli Lilly Co., bovine serum albumin was from Pentex Corp., $Na^{125}I$ (carrier free) was purchased from Amersham/Searle, and acrylamide and dithiothreitol (DTT) were from Sigma. Haptoglobin and transferrin were gifts from Dr. G. Ashwell (National Institutes of Health). Human IgG (normal pool) was purchased from Miles Laboratories and lactoperoxidase from Boehringer. The IgG fraction of goat antihuman IgG antiserum was purchased from Miles-Yeda Laboratories. All other chemicals were reagent grade.

Surface Labeling of IM-9 Lymphocytes. Cultured lymphocytes of the IM line were grown at 37 °C in continuous culture (Fahey et al., 1971; De Meyts, 1976). Prior to iodination the cells were washed 3 times in phosphate–saline buffer, pH 7.5, to remove serum proteins. Iodination of the cells was performed by the method of Marchalonis et al. (1971) with some modification. The reaction was carried out in 100 mL of phosphate–saline buffer, pH 7.5, which contained $(1–5) \times 10^6$ cells/mL, 5×10^{-7} M NaI, 1 mCi of $Na^{125}I$, and 2 mg of lactoperoxidase. One hundred microliters of 10^{-3} M H_2O_2 was added every minute for 15 min, and the suspension

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[1] Abbreviations used: DTT, dithiothreitol; PEG, poly(ethylene glycol); R_f, electrophoretic mobility relative to front moving boundary; $NaDodSO_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide.

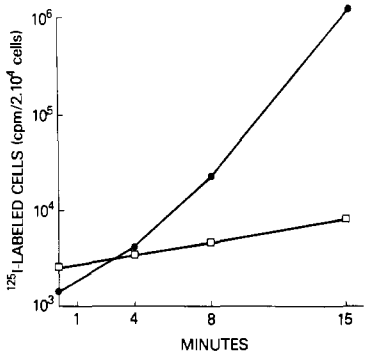

FIGURE 1: Time course of surface labeling of IM-9 lymphocytes with ^{125}I. 100 mL of cells [(1–5) × 10^6 cells/mL] was incubated with 1 mCi of Na^{125}I, 5 × 10^{-7} M NaI, and 2 mg of lactoperoxidase at room temperature in phosphate-buffered saline, pH 7.5. 100 μL of 10^{-3} M H_2O_2 was added every minute for 15 min. Duplicate 1-mL aliquots were removed at the times shown and immediately washed, and ^{125}I radioactivity was measured. This figure shows the time course of labeling in two separate experiments: (●) 95% viable cells by trypan blue exclusion; (□) 70–75% viable cells.

was gently mixed at room temperature. The cells were then washed 4 times with phosphate–saline buffer, pH 7.5. Preliminary experiments showed that a concentration of 2 × 10^{-6} M H_2O_2 achieved the best incorporation. Higher concentrations caused an inhibition of the iodination reaction. We also observed that the presence of 5 × 10^{-7} M NaI was necessary for optimal ^{125}I incorporation. In order to obtain a high level of incorporation of ^{125}I, it was very important to use only cell populations which contained 90–100% viable cells. Populations with only 70–75% viable cells had a much lower incorporation of ^{125}I (Figure 1). By use of cell populations with 90–95% viable cells, iodination did not significantly alter the insulin binding activity of cells (see Results).

Preparation of Solubilized Receptors. The cells were homogenized by a Potter–Elvehjem homogenizer in 40% sucrose at 4 °C in phosphate–saline buffer, pH 7.5. The homogenate was centrifuged at 600*g* for 10 min, and the supernatant was recentrifuged at 20000*g* to yield a pellet representing the crude membrane preparation. The crude membrane preparation was solubilized in 1% Triton X-100 at room temperature for 1 h with intermittent mild agitation. The preparation was then centrifuged at 100000*g* for 90 min at 3 °C, and the insoluble pellet was discarded.

Determination of Insulin Binding Activity. [^{131}I]Insulin was prepared by a modification of the Chloramine-T method to a specific activity of 120–150 μCi/μg (Roth, 1975). [^{131}I]-Insulin binding to membranes was assayed by the procedure previously described for liver (Kahn et al., 1974). In brief, [^{125}I]insulin was incubated with IM-9 membranes in phosphate–saline buffer, pH 7.5, containing 100 units/mL bacitracin to prevent degradation of the labeled hormone. The membrane-bound hormone was separated by centrifugation of duplicate samples through 200 μL of chilled buffer.

Insulin binding activity in the solubilized preparation was determined by the poly(ethylene glycol) precipitation method (Desbuquois & Aurbach, 1971). Solubilized IM-9 membranes (~200 μg/mL) were added to [^{131}I]insulin (0.1 ng/mL) in phosphate–saline buffer, pH 7.5, and 1 mg/mL bovine serum albumin to give a total volume of 0.5 mL and a final concentration of Triton X-100 of 0.1%. The mixture was incubated for 15 h at 4 °C, and the bound hormone was precipitated with 12% poly(ethylene glycol) in the presence of 0.05% γ-globulin. The [^{131}I]insulin bound in the presence of 10 μg/mL insulin was considered to represent nonspecific binding. Insulin degrading activity was determined by measuring the increase in [^{131}I]insulin that was soluble in 5% trichloroacetic acid (Cl_3AcOH). Protein concentration was measured by the fluorescamine method of Udenfriend et al. (1972).

Immunoprecipitation. Immunoprecipitation of the solubilized receptor was performed as previously described (Harrison et al., 1979b) by using sera B-2 and B-5 (Kahn et al., 1976; Flier et al., 1977) which had been partially purified for immunoglobulins by chromatography on protein A–Sepharose (Goding, 1978). The immunoprecipitation assay consisted of two steps: (a) the solubilized receptor was incubated with serum or a serum fraction containing antireceptor antibodies for 1 h at 22 °C; (b) the incubation mixture was then cooled to 4 °C, and immunoprecipitation was effected by addition of a slight excess of a second antibody (goat antihuman IgG, 1 μL of which precipitated 2.5 μg of normal IgG). After 4 h at 4 °C, the suspension was centrifuged for 5 min at 500*g* and the pellet was washed once with phosphate–saline buffer, pH 7.5, containing 0.1% Triton X-100 and counted in an Autogamma spectrometer. The binding of [^{131}I]insulin to the ^{125}I-labeled solubilized membranes was performed by incubating under steady-state conditions (15 h, 4 °C) before the addition of antireceptor antibody. Two controls were included in each experiment: (a) normal human γ-globulin to measure nonspecifically precipitated ("trapped") [^{131}I]insulin; (b) [^{131}I]insulin in Triton buffer without receptor ("receptor blank"). For some experiments, the ^{125}I-labeled receptor was purified by affinity chromatography using antireceptor IgG from serum B-2 which had been coupled to CNBr-activated Sepharose 4B (Pharmacia). ^{125}I-Labeled proteins bound to the affinity column were eluted with 2.5 M $MgCl_2$, pH 6.5, and immediately dialyzed against 0.1 M sodium borate buffer and 0.1% Triton, pH 7.4 (Harrison et al., 1979a).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed in a discontinuous buffer system as described by Neville (Neville & Glossman, 1972). The immunoprecipitates containing labeled membrane proteins were solubilized in a borate buffer, pH 9.6, containing 2% $NaDodSO_4$ and 1 mM DTT and heated at 100 °C for 5 min. The samples were then diluted to a final concentration of 0.1–0.3% $NaDodSO_4$, bromphenol blue tracking dye was added, and the solubilized proteins were electrophoresed in 11% polyacrylamide gels. Gels containing ^{125}I-labeled proteins were sliced into 2-mm slices (Peterson et al., 1974) and counted in an Autogamma spectrometer. Molecular weight markers used were bovine serum albumin (68 000), transferrin (77 000), haptoglobin (100 000, subunits 42 000 and 9000), and IgG (150 000, subunits 55 000 and 20 000).

Results

Distribution of Incorporated ^{125}I and Insulin Binding and Insulin Degrading Activity of ^{125}I-Labeled IM-9 Lymphocytes. By use of cell populations with 90–95% viable cells (see Materials and Methods), the lactoperoxidase-catalyzed iodination was linear for 15 min. At each time point a crude membrane fraction was prepared and the distribution of ^{125}I and insulin binding and insulin degrading activity were assessed (Table I). The specific activity of the radioactive iodine in the 20000*g* pellet, corresponding to the crude membrane fraction, was twice that in the other fractions. The insulin binding activity of the crude membrane fraction was also higher than in the other cell fractions, whereas the insulin degrading activity was slightly decreased. Relatively large amounts of radioactivity, however, were also present in the 600*g* and 100000*g* pellets. This was probalby due to incomplete homogenization of cells

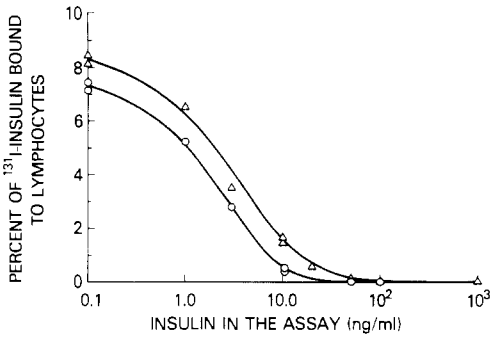

FIGURE 2: Binding of [^{131}I]insulin to solubilized membranes of ^{125}I-labeled IM-9 lymphocytes. [^{131}I]Insulin (0.1 ng/mL) was incubated at 15 °C in 0.5-mL volume with solubilized membranes (180 μg/mL) from ^{125}I-labeled (○) and unlabeled (△) cells. Unlabeled insulin was added in the indicated amounts. After 90 min the receptor-bound [^{131}I]insulin was precipitated with PEG as described under Materials and Methods. The [^{131}I]insulin bound is plotted as a function of the log of the concentration of the unlabeled insulin in the assay. Nonspecific binding has been subtracted from each point.

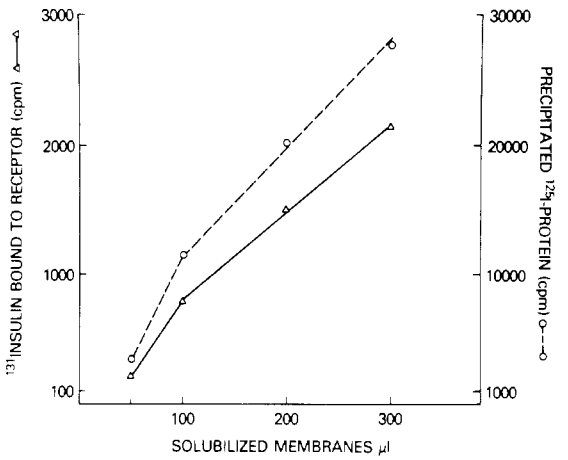

FIGURE 3: Determination of [^{131}I]insulin binding and precipitation of ^{125}I-labeled membrane proteins by poly(ethylene glycol). Triton-solubilized membrane proteins at the concentrations shown were incubated for 16 h at 4 °C with [^{131}I]insulin (0.1 ng/mL) in phosphate-buffered saline, pH 7.5. [^{131}I]Insulin bound to receptors was then determined by precipitation with PEG as described under Materials and Methods. Incubation mixtures containing unlabeled insulin (10 μg/mL) were used to determine nonspecific binding which was subtracted from the total binding to give the specific [^{131}I]insulin bound (△). Also shown (○) is the amount of ^{125}I-labeled membrane protein precipitated at each membrane concentration.

by the relatively gentle procedure used and to the inadequacy of the simple fractionation procedure in separating plasma membranes from other cell fractions. Similar findings were obtained when each of the cell fractions were solubilized (Table I). Thus, there was an enrichment of ^{125}I-labeled protein and insulin binding in the solubilized 20000*g* fraction, as compared to the solubilized 600*g* or 100000*g* pellet. In all cases, 95–98% of the ^{125}I was Cl_3AcOH precipitable, indicating that most of the ^{125}I was incorporated into proteins or polypeptides.

The iodination of the cells prior to membrane fractionation and solubilization produced only a minimal decrease in the subsequent binding of [^{131}I]insulin to the preparation (Figure 2). Competition for the labeled insulin binding was also normal, suggesting that the iodination procedure produced little denaturation of the insulin receptor.

Relationship between Insulin Binding and ^{125}I-Labeled Proteins. To determine whether the ^{125}I label was distributed in proportion to the number of insulin receptors, the binding of [^{131}I]insulin was studied as a function of the amount of ^{125}I-labeled proteins. With increasing concentrations of

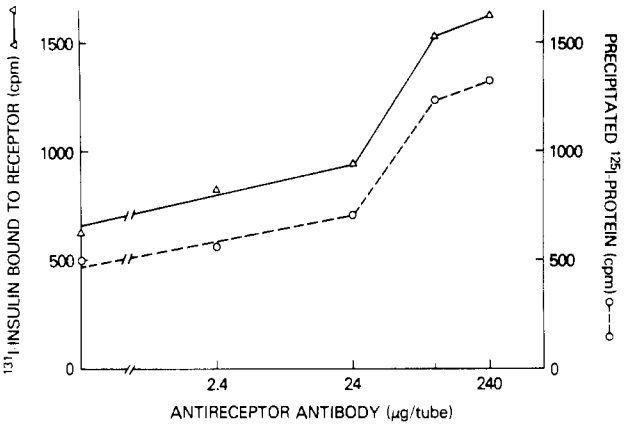

FIGURE 4: Dependence of specific [^{131}I]insulin binding and precipitation of ^{125}I-labeled insulin receptor on antireceptor antibody concentration. The solubilized labeled receptor was incubated with [^{131}I]insulin at 4 °C in phosphate–saline buffer, pH 7.5. After 16 h the antireceptor antibody was added at the concentrations shown and the mixture was incubated for 1 h at 20 °C. Antihuman IgG was then added and the mixture incubated for a further 4 h at 4 °C. The immune complexes were precipitated in a Beckman microfuge and washed once with 0.1% Triton buffer, and the radioactivity in the pellet was counted. (△) Precipitation of [^{131}I]insulin bound to receptor; (○) precipitation of ^{125}I-labeled membrane protein.

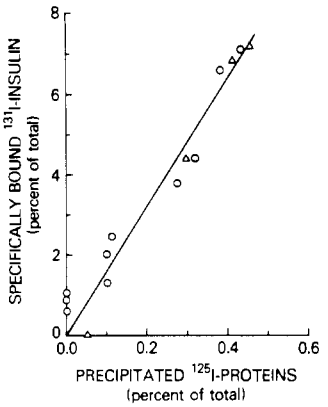

FIGURE 5: Relationship between [^{131}I]insulin bound to receptors and ^{125}I-labeled protein immunoprecipitated. [^{131}I]Insulin was bound to receptors, and ^{125}I-labeled proteins were precipitated with antireceptor antibody and goat antihuman IgG, as described in the legend to Figure 4. Total [^{131}I]insulin binding and total precipitated ^{125}I-labeled receptor proteins were corrected for nonspecific values by subtracting the radioactivity (^{131}I and ^{125}I) precipitated in the presence of normal IgG. (△) Data obtained with solubilized membranes; (○) data obtained with solubilized cells.

membrane protein, poly(ethylene glycol) (PEG) produced a parallel precipitation of ^{125}I-labeled proteins and [^{131}I]insulin bound to the receptor (Figure 3). Similar results were obtained when ^{125}I-labeled membranes and [^{131}I]insulin bound to the receptor were precipitated with the antireceptor antibody (Figure 4). With increasing antibody concentration there was a parallel increase of ^{125}I-labeled proteins and [^{131}I]insulin; however, in this case saturation was reached at a high antibody concentration since the solubilized insulin receptor had been completely immunoprecipitated.

The relationship between receptor content and ^{125}I radioactivity was better seen when [^{131}I]insulin bound to insulin receptors was plotted as a function of immunoprecipitated ^{125}I-labeled membrane proteins (Figure 5). Note that a simple linear relationship between the two parameters was observed. It is also interesting to observe that solubilized crude membranes and solubilized whole cells showed the same correlation between precipitated ^{125}I-labeled proteins and bound [^{131}I]-

Table I: Distribution of ^{125}I Incorporation and Insulin Binding and Degrading Activity in Surface-Labeled IM-9 Lymphocytes[a]

fraction	^{125}I incorpd (cpm/200 μg of protein)	% Cl_3AcOH-precipitable ^{125}I	[^{131}I]insulin bound/200 μg of protein (% of total)	[^{131}I]insulin degraded/200 μg of protein (%/90 min at 15 °C)
nonsolubilized				
cells	17 919	86	5.7	0.9
600*g* pellet	14 542	96	5.0	8.7
20000*g* pellet	31 457	95	8.4	5.8
100000*g* pellet	16 833	93	3.2	6.5
100000*g* supernatant	870	98	0	7.0
solubilized				
cells	16 349	84	5.1	6.2
600*g* pellet	13 078	92	5.2	9.1
20000*g* pellet	29 884	98	7.5	6.5
100000*g* pellet	14 836	94	2.8	6.9
100000*g* supernatant	870	98	0	7.0

[a] IM-9 lymphocytes were surface labeled with Na^{125}I by using lactoperoxidase. The ^{125}I-labeled cells were washed 5 times and fractionated by differential centrifugation. The radioactivity of each cell fraction was measured, and its Cl_3AcOH precipitability was determined. At the same time, insulin binding activity and insulin degrading activity were measured, as described under Materials and Methods.

insulin, indicating that in both cases the insulin receptors represent a similar fraction of the ^{125}I-labeled proteins.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis of Immunoprecipitated ^{125}I-Labeled Proteins. The immunoprecipitates of ^{125}I-labeled and solubilized IM-9 cell membranes were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Precipitation with the antireceptor antibody (B-5) resulted in three or four distinct peaks of radioactivity which were not present when the membranes were precipitated with normal immunoglobulins (Figure 6, top). The major peak (I) was broad and usually had a shoulder, suggesting that it was composed of two peaks, Ia ($R_f \simeq 0.25$) and Ib ($R_f \simeq 0.34$). Two other major peaks were designated II ($R_f \simeq 0.39$) and III ($R_f \simeq 0.57$). A small peak of radioactivity with an R_f of ~ 0.8 was also observed in some gels. In an attempt to see if any of these components were related structurally, more complete reduction of the immunoprecipitated proteins was attempted by incubating the immunoprecipitate in 20 mM dithiothreitol (DTT) for 30 min at pH 9.2. Three percent $NaDodSO_4$ was then added, and the reaction mixture was boiled for 15 min. This treatment caused peak Ib to disappear while peak III showed a proportional increase (Figure 6, bottom). The loss of peak Ib also produced a narrowing of peak Ia and allowed peak III to be more clearly resolved. These observations suggest that the receptor may be composed of three subunits (Ia, II, and III) and that the fourth subunit which was observed under milder reducing conditions (Ib) is a dimer of peak III. The average molecular weights for peaks Ia, Ib, II, and III determined from seven separate experiments were 90 000, 67 000, 56 000, and 34 000, respectively.

Several lines of evidence suggest that all four labeled membrane components are parts of the insulin receptor rather than some other proteins which are precipitated by this antiserum. First, when the receptor was exposed to trypsin under conditions known to destroy the insulin binding site almost completely (1 mg/mL trypsin for 20 min at 37 °C), all the peaks which were observed after antireceptor antibody precipitation disappeared completely and the electrophoretic pattern was almost identical with that obtained after precipitation with normal immunoglobulins (Figure 7, top).[2] Second, a remarkably similar gel pattern of radioactivity was

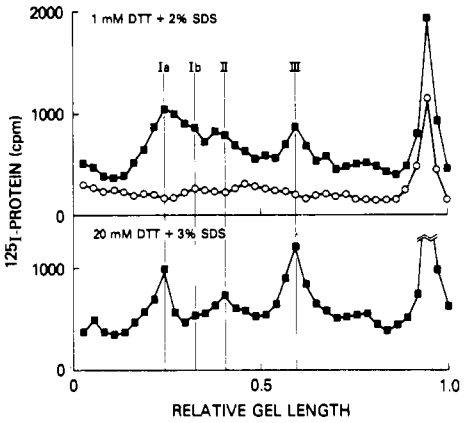

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of immunoprecipitates from solubilized ^{125}I-labeled membranes. Top: precipitates obtained with normal IgG (○) and antireceptor IgG (B-5) (■) and solubilized in 2% $NaDodSO_4$ and 1 mM dithiothreitol. Bottom: precipitate obtained with antireceptor IgG and solubilized in 3% $NaDodSO_4$ and 20 mM DTT. The gels were sliced into 2-mm segments, and the ^{125}I was counted in a γ spectrometer. The top of the resolving gel is indicated by a relative gel length of 0 and the position of the tracking dye by 1.0. Ia, Ib, II, and III refer to the subunits discussed in the text. The peak near the tracking dye probably represents iodinated small peptides or iodotyrosine trapped in the immunoprecipitate.

obtained by using a second antireceptor antibody (B-2) to immunoprecipitate or affinity purify the ^{125}I-labeled proteins (Figure 7, bottom). This is significant because while both of these antisera (B-5 and B-2) contain other antibodies which might react with cellular components, the only activity which we have found in high titer in both is that directed against the insulin receptor (Harrison et al., 1979b). Finally, the most convincing piece of evidence is the effect of insulin on the antibody reaction. Increasing concentrations of unlabeled insulin have previously been shown to inhibit the precipitation of solubilized placental insulin receptors by serum (Harrison et al., 1979b) and the binding of purified antireceptor antibody to intact lymphocytes (Jarrett et al., 1976). Likewise, in the present experiments preincubation of the labeled, solubilized membranes with insulin resulted in inhibition of immunoprecipitation (Figure 7, top) and affinity purification (Figure 7, bottom) of the labeled components. This effect of insulin was both time and concentration dependent. Thus, insulin at 10^{-6} M for 16 h was more effective than 10^{-8} M for 16 h (Figure 7, top), and 16 h of incubation was more effective than 1.5 h (Figure 7, bottom). In both types of experiments (time

[2] The immunoprecipitate after trypsin treatment still shows the low, broad bands observed upon precipitation by normal IgG. This suggests that the radioactivity in these bands does not arise from active immunoprecipitation of any of the labeled membrane proteins.

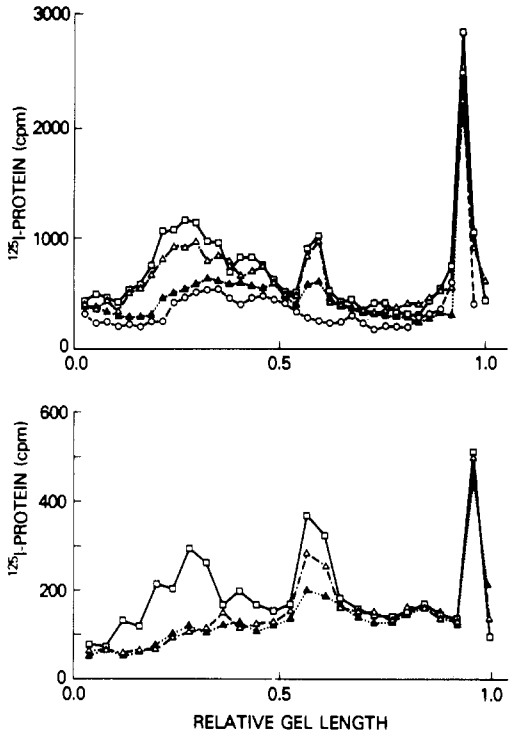

FIGURE 7: Top: $NaDodSO_4$–polyacrylamide gel electrophoresis (1 mM DTT) analysis of solubilized ^{125}I-labeled IM-9 lymphocyte membranes immunoprecipitated with antireceptor serum B-5. (□) Control; (○) solubilized labeled membranes pretreated with trypsin (1 mg/mL) for 20 min at 37 °C; (△) solubilized labeled membranes pretreated with 10^{-8} M insulin for 16 h at 4 °C; (▲) solubilized labeled membranes pretreated with 10^{-6} M insulin for 16 h at 4 °C. Bottom: $NaDodSO_4$–polyacrylamide gel electrophoresis (1 mM DTT) analysis of solubilized ^{125}I-labeled IM-9 lymphocyte membranes affinity purified on antireceptor IgG (B-2) coupled to Sepharose. The experimental conditions are described under Materials and Methods. (□) Control; (△) solubilized labeled membranes pretreated with 10^{-6} M insulin for 1.5 h at 4 °C; (▲) solubilized labeled membranes pretreated with 10^{-6} M insulin for 16 h at 4 °C.

and concentration), the ^{125}I-labeled proteins in peaks I and II were more sensitive to the effect of unlabeled insulin. Preincubation for 1.5 h with 10^{-6} M insulin caused only a slight decrease of peak III, and preincubation even for 16 h with the same concentration of insulin did not result in complete inhibition of this peak.

Discussion

Despite the fact that direct studies of insulin receptors have been possible for nearly a decade, the structure of the insulin receptor, like that of other receptors for peptide hormones, remains obscure. The receptor retains activity when solubilized in neutral detergents such as Triton X-100 and in this form has been characterized by gel filtration (Gavin et al., 1971; Cuatrecasas, 1972; Harrison et al., 1978; Ginsberg et al., 1976; Maturo & Hollenberg, 1978) and more recently by gel electrophoresis (Krupp & Livingston, 1978; Lang et al., 1979). The estimated molecular radius of the insulin receptor in Triton is 68–72 Å, and depending on the assumptions made regarding shape and detergent binding this corresponds to an estimated molecular weight of 300 000–1 000 000. At least three investigators (Ginsberg et al., 1976; Krupp & Livingston, 1978; Lang et al., 1979) have found that under some conditions an active receptor of smaller size (38–42 Å) can be detected, suggesting that the high molecular weight receptor is a complex composed of more than one component. Unfortunately, detergents such as $NaDodSO_4$, which are likely to reduce the receptor to its fundamental subunits, denature the receptor with respect to insulin binding, thereby making direct identification difficult.[3]

In the present study, we have attempted to elucidate the subunit structure of the receptor by taking advantage of a highly specific antibody to the insulin receptor which is found in the serum of certain insulin-resistant diabetics (Kahn et al., 1976; Flier et al., 1977). When cultured human lymphocytes were surface labeled with ^{125}I and solubilized membranes were prepared and immunoprecipitated with antireceptor antibody, $NaDodSO_4$–polyacrylamide gel electrophoresis revealed four peaks which we designated Ia, Ib, II, and III. These correspond to components with molecular weights of 90 000, 67 000, 56 000, and 34 000. Under more potent reducing conditions, the second peak (Ib) could be converted to a smaller component identical in size with peak III, leaving much sharper peaks at 90 000 and 56 000 daltons.

An important question is whether all of the observed bands are components of the receptor or some other coprecipitated proteins not related to the receptor. While it is impossible to totally exclude the latter, several lines of evidence indicate that all of the components are likely, in fact, to be parts of the receptor. First, the specificity of the antibodies used in this study has been extensively demonstrated. The antibodies block insulin binding to a wide variety of tissues from all vertebrate species (Flier et al., 1975; Muggeo et al., 1979). In tissues which respond to insulin, the antireceptor antibodies also mimic the acute biological effects of insulin (Kahn et al., 1977). The antibodies do not block insulin degradation or the binding of other hormones including glucagon, growth hormone, epidermal growth factor, and the insulin-like growth factors (Flier et al., 1977). Second, ^{125}I-labeled antireceptor antibodies bind to cells in proportion to the number of insulin receptors, and this binding is blocked by insulin and insulin analogues (Jarrett et al., 1976). Finally, the sera used in this study specifically and quantitatively immunoprecipitate the solubilized insulin receptor without depleting insulin degrading activity or receptors for other hormones (Harrison et al., 1979b) and do not precipitate any of the major proteins seen by Coomassie Blue staining in solubilized crude membranes.

In the present study, several additional findings support the notion that the antibodies precipitate primarily, if not exclusively, the insulin receptor. Preincubation of the labeled, solubilized membranes with trypsin suppressed the precipitation of all the peaks, in agreement with the evidence that this enzyme destroys insulin binding activity (Kahn et al., 1977). Further, two different antireceptor antibodies (B-2 and B-5) gave a very similar electrophoretic pattern. Three components (peaks Ia, Ib, and II) were also quantitatively suppressed by preincubating the solubilized membranes with 10^{-6} M insulin. These components may be more closely related to the insulin binding site or have a higher affinity for insulin. Alternatively, all four peaks could simply represent a covalently linked oligomer which loses affinity for insulin when broken down to smaller components. In favor of this latter hypothesis is the observation that the immunoprecipitation of peak Ib is blocked well by insulin while peak III is not, despite the fact that Ib appears to be converted to III by more potent reducing conditions. It is highly unlikely that any of the components represented F_c receptors, since the solubilized membranes were

[3] $NaDodSO_4$ gel electrophoresis has been performed on the partially purified receptor (Jacobs et al., 1977). Since this preparation is only partially pure and since $NaDodSO_4$ destroys all binding activity, it is impossible to be certain which, if any, of the bands observed in gel electrophoresis are in fact the receptor.

routinely pretreated with normal δ-globulin coupled to Sepharose 4B prior to purification of ^{125}I-labeled receptors on the receptor antibody affinity column.

Morphological studies by Gonatas et al. (1976) indicated that after lactoperoxidase-catalyzed iodination of lymphocytes significant radioactivity was associated with components other than plasma membrane. Similarly, Schmidt-Ulrich et al. (1974) have reported that with labeled thymocytes 10% of the radioactivity is in a nuclear fraction, 30% in soluble cytoplasmic proteins, and 52% in a microsomal fraction including the plasma membranes. Although insulin receptors have been identified on endomembranes such as golgi (Bergeron et al., 1973; Posner et al., 1978), endoplasmic reticulum (Kahn, 1976; Horvat et al., 1975), and even nuclei (Goldfine et al., 1977a), there is no evidence that the heterogeneity of subunits observed here is due to this phenomenon. A previous study by Goldfine et al. (1977b), using one of the antireceptor antibodies used in this study (B-2), suggested that the insulin binding sites present on endoplasmic reticulum and nuclei are immunologically distinct from those in the plasma membrane. Further, our studies with cultured IM-9 lymphocytes show that no soluble cytoplasmic proteins are iodinated, and it seems likely that the radioactivity associated with the other cell fractions is due to contamination by plasma membranes rather than to direct labeling of receptors on endomembranes. Thus, when solubilized cells or solubilized membranes were preincubated with [^{131}I]insulin and immunoprecipitated with different concentrations of antireceptor antibody, we observed a quantitative dose-dependent precipitation of ^{125}I-labeled proteins which was absolutely parallel to the precipitation of the bound [^{131}I]insulin.

There are four other studies which have attempted to analyze the structure of the insulin receptor from other tissues by using $NaDodSO_4$–polyacrylamide gel electrophoresis. Jacobs et al. (1977) analyzed the rat liver receptor, partially purified from an insulin–agarose column, and found a major band with a molecular weight of 135 000 and other minor bands of lower molecular weight. Yip et al. (1978, 1979), using a radiolabeled photoaffinity probe prepared from insulin, and Pilch & Czech (1979), using chemically cross-linked radiolabeled insulin, identified two major proteins of molecular weight 125 000 and 90 000. Recently, we have purified the insulin receptor of human placenta by sequential wheat germ and receptor antibody affinity chromatography (Harrison et al., 1979a). Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions revealed four bands of molecular weight 82 000 and 35 000 (major bands) and 67 000 and 55 000 (minor bands). The results obtained by different techniques and in different laboratories show reasonable agreement; in both of the current studies there is evidence for several subunits of the receptor. We do not observe a major component of higher molecular weight ($\simeq$125 000) but are in agreement with the finding of a major component of molecular weight $\simeq$90 000.[4]

In contrast, studies of the receptor solubilized in Triton X-100 under nondenaturing conditions have resulted in much higher estimates of molecular weight. Besides the fact that $NaDodSO_4$ and dithiothreitol together destroy most noncovalent and disulfide bonds and reduce the receptor to its fundamental subunits, receptors in Triton probably carry a significant amount of detergent and may behave as micellar aggregates. Harmon et al. (1979), in studying the receptor by radiation inactivation, found a much higher molecular weight for the receptor solubilized in Triton X-100 than for the nonsolubilized receptor. Significantly, however, with this technique the estimated molecular weight of the binding component of the receptor was ~90 000, remarkably close to the major peak(s) observed in our direct studies.

In summary, the present study indicates that the insulin receptor may consist of four subunits with molecular weights of 90 000, 67 000, 56 000, and 34 000. The 67 000 molecular weight subunit appears to be a disulfide dimer of the 34 000 molecular weight subunit. Immunoprecipitations with two different antireceptor antibodies resulted in the same electrophoretic pattern. All four components were destroyed when the labeled solubilized membranes were preincubated with trypsin, whereas preincubation with insulin suppressed completely the three peaks of higher molecular weight. Taken together these data suggest a complex structure for the receptor. Further studies will be required to determine the chemistry of these subunits and their interrelationship with respect to insulin binding.

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[4] It is important to note that determination of the molecular weight of the receptor in $NaDodSO_4$–polyacrylamide gel electrophoresis at only one concentration of acrylamide could be subject to considerable error, since the native receptor, being a glycoprotein, may behave anomalously.

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Photoaffinity Labeling of Insulin Receptor Proteins of Liver Plasma Membrane Preparations†

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ABSTRACT: The photoreactive insulin derivatives $N^{\epsilon B29}$-(azidobenzoyl)insulin (MAB-insulin) and $N^{\alpha A1}$,$N^{\epsilon B29}$-di(azidobenzoyl)insulin (DAB-insulin) were synthesized by reacting bovine insulin with the *N*-hydroxysuccinimide ester of 4-azidobenzoic acid. These derivatives were purified by ion-exchange chromatography on SP-Sephadex, and their identities were established by polyacrylamide gel electrophoresis, amino acid analysis, and end-group determination. Their biological activities were measured by receptor binding assay and fat cell assay. The photoreactivity of these two derivatives was demonstrated by spectral changes and by the formation of covalent polymers of high molecular weight when exposed to light. Radioactive MAB-insulin and DAB-insulin were prepared by iodination with [^{125}I]iodine. These radioactive derivatives were characterized for their photoreactivity, immunoreactivity, and receptor binding to liver plasma membrane. Liver plasma membrane preparations of rat, mouse, and guinea pig were incubated with these radioactive insulin derivatives and irradiated with light. Sodium dodecyl sulfate gel electrophoresis of these plasma membrane preparations after solubilization and reduction showed that two proteins were specifically labeled. The molecular weights of the two radioactive bands were estimated to be about 130 000 and 90 000 in all three species of animals.

The initial event in the action of insulin, like other polypeptide hormones, is its binding to specific receptor on the plasma membrane of the target tissues (Cuatrecasas, 1969; Roth, 1973). Studies on the characterization of the insulin receptor have been indirect in that either the receptor is identified and characterized after disruption of the plasma membrane by solubilization or its characterization is deduced from the effects of enzymic or chemical modifications of the membrane on the binding of insulin (Cuatrecasas, 1971, 1972; Ginsberg et al., 1976; Jacobs et al., 1977). In order to identify the insulin receptor in situ, we have applied the technique of photoaffinity labeling which has been used successfully to label specific functional sites on cell membranes (Haley & Hoffman, 1974; Cabantchik et al., 1976; Ji, 1977; Das et al., 1977; Rosenblit & Levy, 1977; Trosper & Levy, 1977; Bregman & Levy, 1977). Levy (1973) had reported on the preparation of photoreactive aryl azide derivatives of insulin, but the use of these derivatives to label insulin receptor proteins of plasma membrane was not described. In a preliminary communication (Yip et al., 1978), we reported the synthesis and use of a photoreactive insulin derivative, (4-azidobenzoyl)insulin, to label specifically a protein ($M_r \sim 125\,000$) in the plasma membrane of rat adipocytes. The photoreactive insulin preparation used in that preliminary study was a mixture of mono- and di(azidobenzoyl) derivatives of insulin obtained by reacting ^{125}I-labeled insulin with (4-azidobenzoyl)-*N*-hydroxysuccinimide. In the present study, we first synthesized, purified, and characterized two photoreactive insulin analogues: $N^{\epsilon B29}$-(azidobenzoyl)insulin and $N^{\alpha A1}$,$N^{\epsilon B29}$-di(azido-

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