Comparison of Solubilized and Purified Plasma Membrane and Nuclear Insulin Receptors†

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ABSTRACT: Prior studies have detected biochemical and immunological differences between insulin receptors in plasma membranes and isolated nuclei. To further investigate these receptors, they were solubilized in Triton X-100 and partially purified by wheat germ agglutinin–agarose chromatography. In these preparations, the nuclear and plasma membrane receptors had very similar pH optima (pH 8.0) and reactivities to a group of polyclonal antireceptor antibodies. Further, both membrane preparations had identical binding activities when labeled insulin was competed for by unlabeled insulin (50% inhibition at 800 pM). Next, nuclear and plasma membranes were solubilized and purified to homogeneity by wheat germ agglutinin–agarose and insulin–agarose chromatography. In both receptors, labeled insulin was covalently cross-linked to a protein of 130 kilodaltons representing the insulin receptor α subunit. When preparations of both receptors were incubated with insulin and then adenosine 5′-[γ-^{32}P]triphosphate, a protein of 95 kilodaltons representing the insulin receptor β subunit was phosphorylated in a dose-dependent manner. These studies indicate, therefore, that solubilized plasma membrane and nuclear insulin receptors have similar structures and biochemical properties, and they suggest that they are the same (or very similar) proteins.

The initial interaction of insulin with target cells is via a protein receptor located on the plasma membrane (Cuatracasas, 1971; Freychet et al., 1971). This receptor serves both to concentrate insulin onto target cells and to initiate the cellular responses to the hormone (Goldfine, 1981). Various studies have recently revealed the major features of this receptor (Pilch & Czech, 1979; Jacobs & Cuatracasas, 1981). It is first synthesized as a 155-kilodalton (kDa)[1] precursor (Ullrich et al., 1985; Ebina et al., 1985) and then undergoes glycosylation in both the endoplasmic reticulum and the Golgi (Hedo et al., 1983; Forsayeth et al., 1986; Ronnett et al., 1984). In the Golgi, the precursor is split into one α and one β subunit. The mature subunits are 130 kDa (α) and 95 kDa (β). On the cell surface, the receptors are tetramers ($\alpha_2\beta_2$) linked by sulfide bonds. The β subunits are transmembrane and the α subunits are totally extracellular (Ullrich et al., 1985; Ebina et al., 1985; Hedo & Simpson, 1984).

In addition to the plasma membrane, insulin receptors have been identified in intracellular membranes, including the nuclear envelope (Horvat, 1978; Vigneri et al., 1978a), endoplasmic reticulum (Vigneri et al., 1978b; Horvat et al., 1975; Kahn, 1976), and Golgi apparatus (Bergeron et al., 1973). Prior studies from this and other laboratories have indicated that when insulin receptors are studied in purified membrane preparations, significant differences are found in the characteristics of insulin binding to plasma membrane and nuclear receptors. These differences include pH optima and binding affinity (Horvat, 1978; Vigneri et al., 1978a). In addition, the ability of an antireceptor antibody to inhibit insulin binding in the two preparations was different (Goldfine et al., 1977). These data suggested two possibilities. One was that the nuclear and the plasma membrane receptors were different proteins. The other was that they were the same protein but the different environments of the two membranes altered their hormone binding and immunological characteristics. In order to further investigate these possibilities, we studied insulin receptors from plasma membranes and nuclei after solubilization and purification in order to avoid interference by other membrane components.

Materials and Methods

^{125}I-Insulin (100–130 μCi/μg) was prepared as previously described (Goldfine & Smith, 1976).

The following were purchased: Triton X-100 and [γ-^{32}P]ATP (3000 Ci/mmol) from New England Nuclear; Tris [tris(hydroxymethyl)aminomethane], Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], human γ-globulin, ATP, wheat germ agglutinin–agarose, N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone (TLCK), Nonidet NP-40, phenylmethanesulfonyl fluoride (PMSF), leupeptin, and bacitracin from Sigma Chemical Co., St. Louis, MO; bovine serum albumin (CRG-7) from Armour Pharmaceutical Co., Tarrytown, NY; Affi-Gel 15 from Bio-Rad Laboratories, Richmond, CA; poly(ethylene glycol) 6000 from Gall and Schlesinger, Carle Place, NY; disuccinimidyl suberate from Pierce Chemical Co., Rockford, IL; sodium dodecyl sulfate (SDS) from BDH Chemicals, Poole, England; protein A–Sepharose CL-4B from Pharmacia, Uppsala, Sweden; pork insulin (27.2 units/mg) from Elanco Products Co., Indianapolis, IN; and fixed, killed *Staphylococcus aureus* powder from Boehringer

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[1] Abbreviations: kDa, kilodalton(s); ATP, adenosine 5′-triphosphate; PMSF, phenylmethanesulfonyl fluoride; DSS, disuccinimidyl suberate; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TLCK, N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)-aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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Mannheim Biochemicals, Indianapolis, IN.

Rabbit polyclonal antibodies to purified human placental insulin receptors (R_1, R_2) were prepared as previously described (Forsayeth et al., 1986). Human polyclonal antibody B_7 was a gift of Dr. Simeon Taylor (NIH, Bethesda, MD).

Preparation of Insulin–Agarose. Twenty-five milliliters of Affi-Gel 15 was washed extensively by decantation, first with distilled water and then with 0.1 M sodium phosphate, pH 7.4, in 6 M urea. The washed and compressed gel was then suspended in 30 mL of phosphate–urea buffer, and 30 mg of insulin was added (with 1.6 μCi of ^{125}I-insulin as a tracer). The gel suspension was gently agitated for 16 h at 4 °C. Next, the resin was packed in a column and washed with 300 mL of 100 mM Tris, pH 7.4, to recover unbound insulin and to remove unreacted groups on the Affi-Gel. Total bound insulin was 0.8 mg of insulin/mL of packed resin.

Preparation of Receptors. Female Sprague-Dawley rats, 180–200 g, were used at 8:00–10:00 a.m. Purified liver plasma membranes were prepared as described (Ray, 1970). Purified nuclei were prepared by a modification of the method of Blobel and Potter (Vigneri et al., 1978a). Electron micrographs were routinely obtained to ensure that there was no contamination by other cellular organelles. Measurements of 5′-nucleotidase activity (Vigneri et al., 1978a), a plasma membrane marker, revealed that the nuclei had less than 0.1% of the 5′-nucleotidase activity of plasma membranes.

To solubilize both plasma membrane and nuclear receptors, 2 mg of protein/mL samples of either plasma membrane or nuclei were incubated at 24 °C in 0.25 M sucrose, 2% Triton X-100, 0.2 mM PMSF, and 50 mM Tris, pH 7.4, for 30 min. Then, 20 mL of solubilized receptors were applied to a 6.0-mL column of wheat germ agglutinin–agarose. Partially purified receptors were eluted with 0.3 M *N*-acetylglucosamine, 150 mM NaCl, 0.1% Triton X-100, 1.0 mM PMSF, 2 mg/mL bacitracin, and 50 mM Hepes, pH 7.6. To obtain highly purified receptors, the eluates from the wheat germ–agarose column were then applied to a 15-mL column of insulin–agarose and washed in buffer containing 1 M NaCl, 0.1 mM PMSF, 0.1% Triton X-100, and 50 mM Tris, pH 7.4. Purified receptors were then eluted with 1 mM NaCl, 0.1 mM PMSF, 0.1% Triton X-100, and 50 mM sodium acetate, pH 5.0. The receptor preparation was then neutralized with Tris to pH 7.0.

Nuclear envelopes were obtained from the aforementioned nuclei as described previously (Vigneri et al., 1978a).

Binding and Cross-Linking to Solubilized Receptors. To measure ^{125}I-insulin binding, 5 μL of solubilized receptor preparation was incubated with 100 pM ^{125}I-insulin for 2 h at 24 °C in 0.175 mL of buffer containing 0.1 M Hepes, 0.12 M NaCl, 1.2 mM $MgCl_2$, 2.5 mM KCl, 0.5 mM sodium acetate, 1 mM EDTA, 2 mM $MnCl_2$, 1 mg/mL bacitracin, and 10 mg/mL BSA, pH 7.9. Next, bound and free hormones were separated with poly(ethylene glycol) (Desbuquois & Aurbach, 1971).

To cross-link ^{125}I-insulin, highly purified receptors were incubated in the above buffer with 1 nM ^{125}I-insulin in a volume of 0.22 mL. After precipitation with poly(ethylene glycol), the precipitate was resuspended in 140 mM NaCl, 4 mM KCl, and 15 mM sodium phosphate, pH 7.4. Next, DSS in dimethyl sulfoxide was added to bring the final DSS concentration to 0.4 mM. The reaction was stopped by the addition of 10 μL of 1 M Tris, pH 7.4, and precipitated with poly(ethylene glycol). The precipitate was resuspended in sample buffer. The incubation mixture was then electrophoresed in a 7.5% polyacrylamide gel and the location of the radioactivity determined by fluorography.

Specific ^{125}I-insulin binding to intact purified plasma membranes and nuclear envelopes were carried out by a previously described microcentrifuge technique (Vigneri et al., 1978a).

Receptor Kinase Assay. Solubilized, wheat germ agglutinin purified receptors were incubated with increasing concentrations of insulin in buffer containing 0.3 M *N*-acetylglucosamine, 150 mM NaCl, 2.5 mM $MnCl_2$, 0.1% Triton X-100, 1.0 mM PMSF, 2 mg/mL bacitracin, and 50 mM Hepes, pH 7.6, in a final volume of 90 μL for 1 h at 24 °C. Next, 5 μL of [γ-^{32}P]ATP (100 μM) was added for 1 h at 24 °C. The reaction was stopped by the addition of 5 μL of 10 mM ATP, 20 mM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate, pH 7.4. The reaction mixture was then diluted to 1 mL in immunoprecipitation buffer containing 0.5 M NaCl, 2.0 mM EDTA, 0.5% Nonidet NP-40, 0.1% SDS, 100 μg/mL leupeptin, 0.25 mM TLCK, 0.1 mM PMSF, and 1.0 mg/mL bacitracin.

Immunoprecipitation of Labeled Receptors. The aforementioned reaction was first cleared twice by adding 80 μL of 10% (w/v) fixed, killed *Staphylococcus aureus*. Then, 10 μL of either antireceptor antiserum or nonimmune rabbit serum was added and the mixture incubated overnight at 4 °C. Tubes containing the mixtures were then centrifuged 3 min at 4 °C and the supernatants transferred to tubes containing 60 μL of protein A–Sepharose beads (70 mg/mL). Tubes were then rotated for 2 h at 4 °C and centrifuged 10 s, and the supernatants were discarded. The pellets were washed 5 times with 1.0 mL of immunoprecipitation buffer and once with 10 mM PO_4, pH 7.2, containing 0.1% SDS and 2.0 mM EDTA. The supernatants were discarded and pellets allowed to dry slightly. Thereafter, 60 μL of buffer containing 50 mM DTT was added, the tubes were boiled 3 min and centrifuged, and the supernatants were electrophoresed and fluorographed as described above.

Antireceptor Antibody Studies. The ability of the three antisera to precipitate the insulin receptors was quantitated as follows. Solubilized wheat germ agglutinin purified receptors (5 μL) in 50 mM Tris, pH 7.7, with 0.1% BSA and 0.1% Triton X-100 were incubated with ^{125}I-insulin (1 nM in a volume of 60 μL) at 24 °C for 2 h, after which time 10 μL of either immune or nonimmune serum was added. This mixture was incubated at 24 °C for 2 h. Next, a 100-μL slurry containing 10% (w/v) fixed, killed *Staphylococcus aureus* was added. After an additional 1 h at 24 °C, the insoluble material was collected by centrifugation and washed with 1 mL of 140 mM NaCl, 4 mM KCl, 16 mM Na_2HPO_4, pH 8.6, 0.1% BSA, 0.02% sodium azide, 0.5% Nonidet NP-40, and 0.1% SDS. The washed precipitate was collected by low-speed centrifugation, and the antibody-bound insulin–receptor complex was measured by scintillation counting.

Results

Binding of Insulin to Solubilized Receptors from Nuclei and Plasma Membranes and Immunological Studies. Solubilized and wheat germ agglutinin purified receptor preparations were incubated with ^{125}I-insulin and increasing concentrations of unlabeled insulin. Bound and free hormones were then separated with poly(ethylene glycol). With both plasma membrane and nuclear receptors, unlabeled hormone caused a dose-dependent decrease in the binding of ^{125}I-insulin. For both preparations, half-maximal inhibition of binding occurred at 800 pM (Figure 1). In contrast, as previously described, lower concentrations of insulin inhibited ^{125}I-insulin "binding" to intact plasma membranes when compared to intact nuclear envelopes (Figure 1) (Vigneri et al., 1978a).

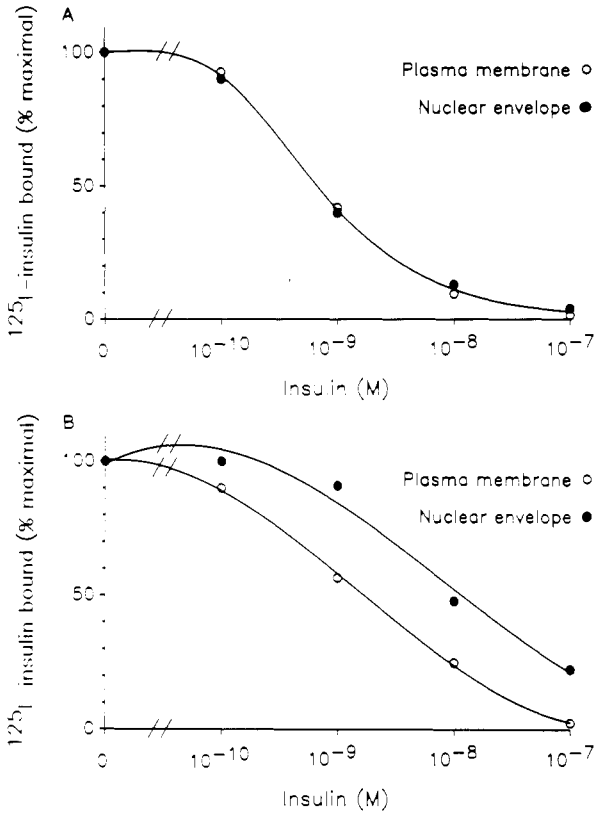

FIGURE 1: (A) Effect of increasing insulin concentrations on specific ^{125}I-insulin binding to solubilized and partially purified insulin receptors from plasma membranes (○) and nuclei (●). Receptors were incubated with 100 pM ^{125}I-insulin plus increasing concentrations of unlabeled insulin for 2 h at 24 °C. (B) Similar studies carried out with intact plasma membranes and intact nuclear envelopes. Each value is the mean of triplicate determinations.

Next, the pH optimum for binding was studied. In both preparations, the pH optimum was alkaline at 8.0 (Figure 2). The pH optimum for ^{125}I-insulin binding to intact plasma membranes was also alkaline, whereas, as previously described, the pH optimum for ^{125}I-insulin binding to intact nuclear envelopes was more acidic (Figure 2) (Vigneri et al., 1978a).

The ability of several antireceptor antisera to immunoprecipitate the plasma membrane and nuclear receptor preparations was compared. One antiserum from humans (B_7) and two antisera from rabbits (R_1, R_2) were studied. All three antisera immunoprecipitated both receptors with very similar dose–response curves. For both receptors, the order of potency of the antisera was $R_2 > B_7 > R_1$ (Figure 3).

Identification of the α Subunit by Cross-Linking Studies. Highly purified receptors were first incubated with ^{125}I-insulin, and then the insulin was covalently cross-linked to its receptor with the use of the bifunctional cross-linking reagent DSS. Next, the preparations were subjected to reducing conditions followed by polyacrylamide gel electrophoresis. After fluorography, one major band was seen in both preparations, having a molecular radius of 130 kDa (Figure 4). This protein has been previously identified as the α subunit of the insulin receptor (Pilch & Czech, 1979). In both preparations, a small amount of high molecular weight material (>300K) was also cross-linked. Presumably, this material represents the nonreduced receptor (Pilch & Czech, 1979).

Identification of the β Subunit by Receptor Kinase Studies. The ability of insulin to stimulate the autophosphorylation of its own receptor β subunit was studied in solubilized and purified plasma membrane and nuclear receptors (Figure 5). In the absence of insulin, very little phosphorylation was seen.

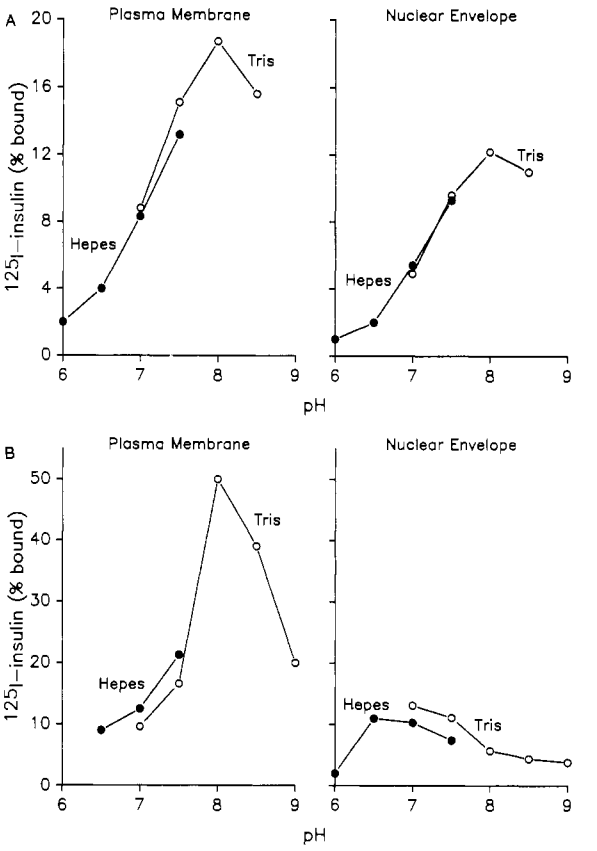

FIGURE 2: (A) Effect of pH on specific ^{125}I-insulin binding to solubilized and partially purified plasma membrane and nuclear insulin receptors. Receptors were incubated with 100 pM ^{125}I-insulin for 2 h at 24 °C. Each value is the mean of triplicate determinations; open circles represent Tris buffers, and closed circles represent Hepes buffers. (B) Similar studies were carried out with intact plasma membranes and intact nuclear envelopes. Each value is the mean of duplicate determinations.

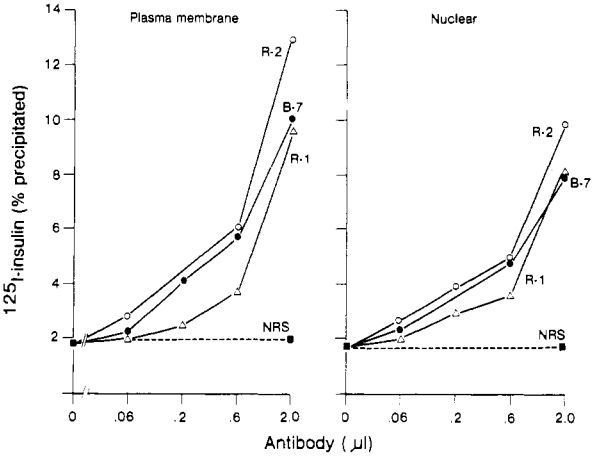

FIGURE 3: Effect of increasing concentrations of three different antireceptor antisera on the precipitation of ^{125}I-insulin prebound to solubilized and partially purified plasma membrane and nuclear insulin receptors. Data shown are the percent of ^{125}I-insulin added that was precipitated. Each point on the graph is the result of a single determination.

In a dose-dependent manner, insulin stimulated the phosphorylation of a 95-kDa protein representing the β subunit of the receptors. In both preparations, this effect was maximal at 10 nM insulin.

Discussion

In the present study, we examined solubilized and purified insulin receptors from rat liver plasma membranes and nuclei and found similar characteristics in the ability of insulin to

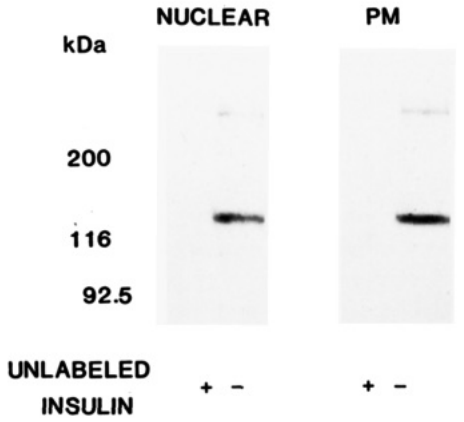

FIGURE 4: Fluorograms of highly purified nuclear and plasma membrane receptors after cross-linking with ^{125}I-insulin. 1 nM ^{125}I-insulin (in the absence and presence of 10 μM unlabeled insulin) was incubated with highly purified receptors for 2 h at 24 °C. Next, 0.4 mM DSS was added to covalently cross-link the labeled hormone. After exposure to reducing conditions, the incubation mixture was electrophoresed and fluorographed.

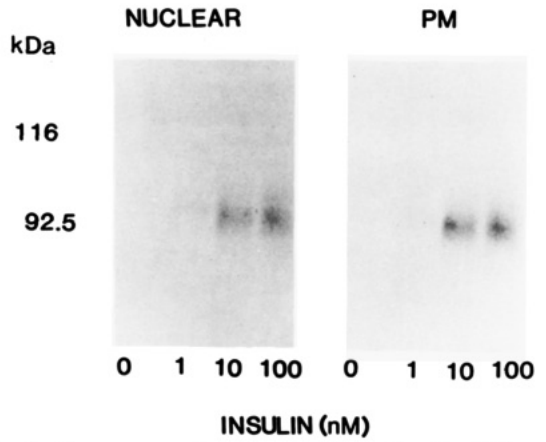

FIGURE 5: Fluorograms of insulin-stimulated phosphorylation of the β subunit of highly purified nuclear and plasma membrane insulin receptors. The autophosphorylation of solubilized receptors was determined in the presence of increasing insulin concentrations. The receptors were then immunoprecipitated with an excess of antireceptor antiserum, exposed to reducing conditions, electrophoresed, and fluorographed.

bind to both preparations. For both, the competition–inhibition curves and the pH optima were similar. In our previous studies (Vigneri et al., 1978a), in the present study, and also in studies of others (Horvat, 1978), a number of significant differences in the ability of insulin to bind to its receptors were observed between purified plasma membranes and nuclear envelopes. Since the solubilized and purified receptors (in contrast to receptors in membranes) bind to insulin in a similar manner, the most likely explanation for these differences is that insulin binding to receptors is influenced by the membrane environment.

Nuclear envelopes and plasma membranes differ in several ultrastructural and biochemical characteristics, including protein and lipid content (Harris & Agutter, 1976). For instance, in comparison to plasma membranes, the nuclear envelope has a low concentration of cholesterol and sphingomyelin. Since the lipid environment influences insulin receptor binding (Amatruda & Finch, 1979), one possible explanation for the difference in insulin binding to its receptor in membranes is the difference in membrane lipids.

Our previous study indicated that insulin receptor binding sites, when in intact intracellular organelles, are immunologically distinct in plasma membranes and nuclei (Goldfine et al., 1977). The present study however, employing solubilized and purified insulin receptors from nuclei and plasma membranes, indicated that these receptors were immunologically similar. The explanation for this apparent discrepancy may reside again in differences in membrane structure and composition. For instance, the same receptor could have different antigenic sites exposed to an antibody when the receptor is embedded in either plasma or nuclear membranes.

When we labeled insulin and cross-linked it to highly purified receptor preparations from plasma membranes and nuclei, the labeled insulin was associated with a protein of 130 kDa. Prior studies have identified this protein as the α subunit of the receptor. Goidl and colleagues have cross-linked ^{125}I-insulin to mouse plasma membrane and nuclear receptors and have identified a similar protein of approximately 120 kDa (Goidl, 1981). Our studies and these studies suggest, therefore, that the α subunits of both the nuclear and plasma membrane insulin receptors are very similar.

The purified plasma membrane insulin receptor has intrinsic insulin-regulated tyrosine kinase activity in its β subunit (Kasuga et al., 1982; Cobb & Rosen, 1984). When insulin binds to the α subunit, the β subunit is activated in a dose-dependent manner, and it can either autophosphorylate or phosphorylate other proteins. We studied, therefore, insulin receptor autophosphorylation in solubilized receptor preparations from nuclei and plasma membranes and found that in both preparations insulin induced a dose-dependent phosphorylation of a 95-kDa protein. This observation suggests that in both preparations insulin induces autophosphorylation of its own receptor. In addition, preliminary studies by Brossette et al. have suggested that the nuclear insulin receptor can undergo insulin-induced receptor autophosphorylation of its β subunit (Monier et al., 1984). These studies indicate, therefore, that the nuclear and plasma membrane insulin receptors have α and β subunits which have the same molecular weights and are similar in their various characteristics.

Since the solubilized nuclear and plasma membrane receptors have subunits of the same size, can bind insulin in the same manner, and have similar immunological characteristics and receptor kinase activity, it is quite likely that only one type of insulin receptor molecule is synthesized in target cells. This receptor is then distributed to various cellular membranes, including the plasma membrane, the nuclear envelope, and the Golgi apparatus. At present, only one insulin receptor gene has been identified in target cells (Ullrich et al., 1985). The present studies suggesting the presence of only one form of insulin receptor in different organelles of target cells are in agreement, therefore, with this observation.

Registry No. Insulin, 9004-10-8; agarose, 9012-36-6; insulin receptor kinase, 88201-45-0.

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Characterization of Dodecylphosphocholine/Myelin Basic Protein Complexes†

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ABSTRACT: The stoichiometry of myelin basic protein (MBP)/dodecylphosphocholine (DPC) complexes and the location of protein segments in the micelle have been investigated by electron paramagnetic resonance (EPR), ultracentrifugation, photon correlation light scattering, ^{31}P, ^{13}C, and ^{1}H nuclear magnetic resonance (NMR), and electron microscopy. Ultracentrifugation measurements indicate that MBP forms stoichiometrically well-defined complexes consisting of 1 protein molecule and approximately 140 detergent molecules. The spin-labels 5-, 12-, and 16-doxylstearate have been incorporated into DPC/MBP aggregates. EPR spectral parameters and ^{13}C and ^{1}H NMR relaxation times indicate that the addition of MBP does not affect the environment and location of the labels or the organization of the micelles except for a slight increase in size. Previous results indicating that the protein lies primarily near the surface of the micelle have been confirmed by comparing ^{13}C NMR spectra of the detergent with and without protein with spectra of protein/detergent aggregates containing spin-labels. Electron micrographs of the complexes taken by using the freeze-fracture technique confirm the estimated size obtained by light-scattering measurements. Overall, these results indicate that mixtures of MBP and DPC can form highly porous particles with well-defined protein and lipid stoichiometry. The structural integrity of these particles appears to be based on protein–lipid interactions. In addition, electron micrographs of aqueous DPC/MBP suspensions show the formation of a small amount of material consisting of large arrays of detergent micelles, suggesting that MBP is capable of inducing large changes in the overall organization of the detergent.

Understanding the molecular architecture of the myelin sheath requires the characterization of the interactions between its various components, the lipids and proteins. The association of the myelin basic protein (MBP) with lipid components plays an important role in stabilizing the multilamellar structure of myelin. Hence, knowledge of the location, structure, and nature of the binding of the basic protein to lipid systems may provide insights into intermolecular interactions of functional significance in the organization of myelin.

For MBP to contribute to the stability of the myelin double bilayer it must interact with lipids in specific ways. A simple model would envisage the protein acquiring definite conformations in protein–lipid complexes, in the manner of intrinsic structural proteins. The conformational rigidity resulting from the protein–lipid interactions acts as a stabilizing factor on the membrane. Outside this milieu the protein is unable to undergo adequate folding. Another view would consider the

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