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Insulin Receptor: Insulin-Modulated Interconversion between Distinct Molecular Forms Involving Disulfide–Sulfhydryl Exchange†

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ABSTRACT: When insulin receptor was isolated from human placenta membranes by a sequential chromatographic procedure (method I) that did not expose the receptor to insulin, the purified receptor (about 2000-fold purification) was eluted on columns of Sepharose 6B in a volume corresponding to a K_{av} of 0.31 (R_I form of the receptor, with an apparent Stokes radius of about 7.2 nm). In contrast, the placenta receptor isolated by insulin–agarose affinity chromatography (method II) was eluted on Sepharose 6B columns with a K_{av} of 0.53 (R_{II} form of the receptor, with an apparent Stokes radius of about 3.8 nm). When receptor prepared by method I was exposed to insulin, the R_I form of the receptor was converted to the R_{II} form; this insulin-mediated change in the receptor elution behavior was mimicked by dithiothreitol (0.1 mM) treatment. In concert with the insulin-mediated conversion of the R_I to the R_{II} form of the receptor, we observed insulin-stimulated incorporation of ^{3}H-labeled *N*-ethylmaleimide (^{3}H-NEM) into the receptor preparation. Both the insulin-mediated interconversion of the receptor from the R_I to the R_{II} form and the insulin-stimulated incorporation of ^{3}H-NEM were dependent on insulin concentration; the concentration dependence indicated that a half-maximal effect occurred at about 1 nM insulin. Both ^{125}I-labeled receptor, converted to the R_{II} form in the presence of insulin, and the R_{II} form of the receptor labeled with ^{3}H-NEM in the presence of insulin were bound by anti-insulin receptor immunoglobulin, obtained from an individual with severe insulin resistance. Exposure of the R_I form of the receptor to insulin–agarose also led concurrently to the conversion of the receptor from the R_I to the R_{II} form and to the incorporation of ^{3}H-NEM. The R_{II} form of the receptor obtained by method II from insulin–agarose could be converted back to the R_I form by treatment with oxidized glutathione. The experiments with purified receptor preparations were complemented by studies with particulate membrane preparations cross-link labeled with ^{125}I-labeled insulin both at low (0.5 ng/mL) and comparatively high (25 ng/mL) insulin concentrations. Solubilization and immunoaffinity purification of receptor, cross-link labeled in the membranes with 0.5 ng/mL ^{125}I-labeled insulin, yielded material that on Sepharose 6B columns behaved like the R_I form of the receptor. In contrast, immunoaffinity-isolated receptor, cross-link labeled in the membranes with 25 ng/mL ^{125}I-labeled insulin, behaved on Sepharose 6B columns like the R_{II} form of the receptor; receptor cross-link labeled at the high (25 ng/mL) insulin concentration (but not at 0.5 ng/mL insulin) simultaneously incorporated ^{3}H-NEM. Our results indicate that both in membranes and in purified soluble receptor preparations, insulin causes an interconversion of its receptor from one hydrodynamic form to another by a process involving a disulfide–sulfhydryl exchange.

There has recently been considerable progress in understanding the molecular structure of the receptor for insulin [current views summarized in volume edited by Andreani et al. (1981)]. One salient feature of the heterodimeric structure proposed for the insulin receptor (Jacobs et al., 1980; Massagué et al., 1980) relates to the presence of a number of disulfide bonds that stabilize the proposed oligomeric $(\alpha\beta)_2$ structure. It is further evident that the entire receptor structure, as it exists in the cell membrane and in crude detergent extracts of cell membranes, may comprise not only the $(\alpha\beta)_2$ recognition oligomer that has been the species of intensive investigation but may also include other closely associated polypeptide chains. Evidence for the presence of such receptor-

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associated species has come not only from direct cross-link-labeling experiments using specific insulin receptor photoprobes (Yip et al., 1980, 1982) but also from indirect studies that have indicated the presence of nonrecognition moieties that modulate the affinity of the receptor for insulin (Maturo & Hollenberg, 1978; Harmon et al., 1980). It is possible that these nonrecognition substituents relate to the several molecular forms of the insulin receptor that have been detected in electrophoretic (Krupp & Livingston, 1978; Berhanu et al., 1982; Goren et al., 1982; Yip et al., 1982), chromatographic (Ginsberg et al., 1976; Maturo & Hollenberg, 1978; Baron et al., 1981), and sucrose gradient (Baron et al., 1981) systems.

In some of our previous work (Maturo & Hollenberg, 1978, 1979; Maturo et al., 1978), we have observed that the molecular form of the insulin receptor obtained subsequent to insulin–Sepharose affinity chromatography, from a number of tissue sources (liver, placenta, fat cells, fibroblasts), differs from the form of the receptor present in crude membrane extracts. Further, data obtained by us (Maturo & Hollenberg, 1978; Maturo et al., 1978; Hollenberg et al., 1981) and by others (Ginsberg et al., 1976; Baron et al., 1981) suggested that insulin per se could alter the hydrodynamic form of the receptor. In essence, it appears that exposure of the receptor to insulin increases the receptor's elution volume on columns of Sepharose 6B; this increase in elution volume has previously been taken to suggest an insulin-mediated decrease in receptor size. Preliminary work by us (Hollenberg et al., 1981), as well as data obtained by others (Jacobs & Cuatrecasas, 1980; Schweitzer et al., 1980; Massaguē & Czech, 1982), suggests that the oxidation state of the receptor may play an important role in terms of receptor structure and function. Therefore, in the present study we have explored further the insulin-mediated change in the chromatographic form of the insulin receptor and we have used sulfhydryl/disulfide-specific reagents (*N*-ethylmaleimide, oxidized glutathione, dithiothreitol) to evaluate the role of disulfide–sulfhydryl interchange in the insulin-mediated receptor interconversion process.

Materials and Methods

Chemicals. *N*-Ethylmaleimide (NEM) and Triton X-100 were purchased from Fluka Chemical Corp. Radiolabeled NEM [*N*-ethyl[2-^{3}H]maleimide (^{3}H-NEM); 692 mCi/mmol] was from New England Nuclear. The chemical reactivity of both unlabeled NEM and tritiated NEM was verified whenever utilized by measuring the inactivation and labeling of isocitrate dehydrogenase. Triton X-100 was purified and stored as suggested by Chang & Bock (1980).

Preparation of Membranes and Isolation of Receptor. Fresh full-term human placentas were used as a source of the insulin receptor for these studies. The homogenization of tissue and the preparation of the crude membrane fraction was performed essentially as previously described (Maturo & Hollenberg, 1978; Hock & Hollenberg, 1980) with the exception that an inhibitor of proteolysis [phenylmethanesulfonyl fluoride (PMSF)] was, in some instances, included in the homogenization buffer. Results were the same for membranes prepared either with or without PMSF. Freshly prepared membranes were extracted for 1 h at 24 °C in 5 mM Tris[1]–1.0% v/v Triton X-100, pH 7.5. The resulting suspension was clarified by centrifugation at 200000*g* for 90 min at 4 °C.

The detergent-solubilized insulin binding material was purified by two different methods. Purification method I was as outlined in brief previously (Hollenberg et al., 1981), involving a series of sequential chromatographic steps: DEAE-Sephadex ion-exchange chromatography at pH 7.5, hydroxylapatite adsorption chromatography, concanavalin A (Con A)–agarose affinity chromatography, *Ricinus communis* 120–agarose affinity chromatography, and gel exclusion chromatography on Sepharose 6B.

For the sequential chromatographic procedure, the clarified soluble receptor preparation was applied at 4 °C at a flow rate of 50 mL/h to a DEAE-Sephadex column (5 × 20 cm) equilibrated with 10 mM Tris-HCl–40 mM NaCl buffer, pH 7.5, containing 0.1% v/v Triton X-100. The column was washed with 2 column volumes of buffer and was eluted with a 2-L linear gradient of NaCl (40–400 mM) in the same Tris–Triton buffer. The insulin-binding material, detected by the poly(ethylene glycol) (PEG) method (Cuatrecasas, 1972; Cuatrecasas & Hollenberg, 1976), was eluted when the NaCl concentration reached approximately 100 mM. The pooled insulin-binding fractions from the DEAE column (about 300 mL) were applied at a flow rate of 20 mL/h at 4 °C to a hydroxylapatite (Fast Flow, Calbiochem) column (1.6 × 10 cm) equilibrated with 10 mM Tris-HCl–0.1% Triton X-100. After sample application, the column was washed with 2 column volumes of buffer, and a 500-mL linear gradient of sodium phosphate (30–100 mM, pH 7.5) in the Tris–Triton buffer was begun. Insulin-binding fractions (eluted at about 60–70 mM phosphate) were pooled (about 30 mL) and were adsorbed at a flow rate of about 20 mL/h to a column (0.6 × 9 cm) of Con A–agarose (12 mg of lectin/mL of packed gel) that was preequilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM $CaCl_2$, 0.1 mM $MnCl_2$, and 0.1% v/v Triton X-100. The lectin column was washed with 100 mL of buffer, and insulin-binding material was eluted with 25 mL of Tris–Triton buffer, containing 0.5 M methyl α-D-mannopyranoside (Sigma); 1-mL fractions were collected and assayed for insulin binding. Receptor-containing fractions were pooled (12–15 mL), dialyzed against sugar-free Tris–Triton buffer, and applied to a column (0.6 × 9 cm) of *Ricinus communis* 120–agarose (Vector Laboratories; 6 mg of lectin/mL of packed gel). The lectin column was washed with sugar-free buffer and was eluted with buffer containing 0.5 M D-galactose; 1-mL fractions were collected and assayed for insulin binding. Receptor-containing fractions were pooled (12–15 mL), and 5-mL aliquots were applied to a column (2.6 × 93 cm) of Sepharose 6B (non-cross-linked) that was preequilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 0.1% Triton X-100. The column was eluted at 4 °C with buffer at a flow rate of 27 mL/h, and 3-mL fractions were collected. Fractions (no. 74–84) containing insulin-binding activity ($K_{av} \simeq 0.31$) were pooled and concentrated with a YM-30 filter (Amicon).

Purification method II was essentially that of Siegel et al. (1981), which omitted lectin–agarose chromatography and involved DEAE-cellulose ion-exchange chromatography at pH 6.3 and insulin–succinyldiaminopropylamino-Sepharose (insulin–Sepharose) affinity chromatography, utilizing an acid (pH 6.3)–4 M urea buffer for elution. The material eluted from the insulin affinity column was immediately added to 0.1 M phosphate–0.1% Triton–40% sucrose, pH 7.4, buffer, to accomplish a one-tenth dilution. The insulin-binding material from both purification procedures was concentrated with an Amicon YM-30 filter membrane. Only insulin-binding material that was eluted in a volume corresponding to a K_{av} of 0.31 (R_1 form of receptor) was used from purification method I. Thus method I avoided exposure of the receptor

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

both to insulin and to the harsh conditions of the acid–urea buffer used to elute the insulin affinity column. Method I achieved about a 2000-fold purification of receptor, a value that compares favorably with that of the purification achieved by the insulin affinity column (about 3000-fold).

Analytical Procedures. The preparation of radiolabeled insulin (Cuatrecasas & Hollenberg, 1976) and of ^{125}I-labeled receptor preparations (Hollenberg et al., 1981) was as previously described, using the chloramine T–sodium metabisulfite method. In some experiments, radiolabeled insulin was separated from reactants by chromatography on Sephadex G-25 rather than by adsorption to talc. For receptor labeling, reactants (~1 mCi of carrier-free ^{125}I, 10 mM chloramine T–25 mM sodium metabisulfite) were separated from protein by chromatography on Sephadex G-50, immediately following the addition of metabisulfite. The protein content of receptor preparations was estimated with the fluorescamine reagent, subsequent to alkaline hydrolysis [2 h at 100 °C in 0.5 M $Ba(OH)_2$] of the sample; bovine albumin was used for calibration of the assay. Gel exclusion chromatography was routinely performed at 4 °C on columns (1.5 × 87 cm) of Sepharose 6B (non-cross-linked preparation) preequilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 0.1% v/v Triton X-100. Before use, buffers were passed through Millipore Y-EGWP filters (0.1-μm pore size) to remove particulate aggregates. Columns were calibrated with proteins of known Stokes radii: thyroglobulin (8.5 nm), apoferritin (6.1 nm), γ-globulin (5.3 nm), and bovine albumin (3.6 nm). The binding of radiolabeled insulin by soluble receptor preparations was measured by the poly(ethylene glycol) method (Cuatrecasas, 1972; Cuatrecasas & Hollenberg, 1976).

Cross-Linking of Insulin Receptor to ^{125}I-Labeled Insulin. Freshly prepared membranes were cross-linked with freshly prepared ^{125}I-labeled insulin using either the noncleavable disuccinimidyl suberate (at a final concentration of 1.0 mM) or the cleavable bis[2-[[(succinimidooxy)carbonyl]oxy]ethyl] sulfone (1.0 mM) according to the method of Pilch & Czech (1980). Both the cleavable and noncleavable cross-linking reagents were from Pierce Chemicals. The membrane concentration was 2.4 mg/mL, and the final insulin concentration used was 0.5–50 ng/mL.

Immunoaffinity Chromatography. Isolation of either affinity cross-link-labeled or radiolabeled (^{125}I or ^{3}H-NEM) receptor was achieved either with anti-insulin antibody, as outlined by Heinrich et al. (1980), or with anti-receptor immunoglobulin, obtained from a patient with severe insulin resistance, by employment of the immunoglobulin agarose affinity column approach described by Harrison & Itin (1980). The specificity of the patient's immunoglobulin for the insulin receptor was evaluated by double-antibody immunoprecipitation experiments, demonstrating the removal of insulin-binding activity from soluble receptor preparations, and by immunoglobulin–agarose adsorption experiments, in which 70–80% of the insulin-binding activity [poly(ethylene glycol) assay] of a detergent extract was recovered, upon elution of the column with 2.5 M $MgCl_2$ in 40 mM sodium acetate–0.1% Triton, pH 6.5 (Harrison & Itin, 1980). Control immunoglobulin–agarose columns did not bind the receptor. The receptor recovered from the antireceptor immunoaffinity column behaved on columns of Sepharose 6B like the R_I form of the receptor, isolated by method I (to be described below; see Figure 3B).

Results

In keeping with our previous observations with the insulin receptor from rat liver (Maturo & Hollenberg, 1978; Hollenberg et al., 1981), there was a marked difference in the chromatographic profile (Figure 1A) of the placental insulin receptor purified by insulin–agarose affinity chromatography (R_{II} form of receptor: method II, $K_{av} \sim 0.53$) compared with the receptor isolated by the sequential chromatographic procedure that does not expose the receptor to insulin (R_I form of receptor: method I, $K_{av} \sim 0.31$). Although the elution volumes corresponded to apparent Stokes radii of 7.2 (K_{av} = 0.31) and 3.8 (K_{av} = 0.53) nm, as calculated previously (Maturo & Hollenberg, 1978), our inability to separate these species on columns of cross-linked Sepharose 6B (data not shown) suggested that the elution volumes may not be indicative of true molecular hydrodynamic radii. Simply exposing the receptor prepared by method I to the acid–urea conditions used for elution in method II did not change the elution profile of the receptor (Hollenberg et al., 1981). Further, either upon rechromatography or upon storage of the receptor for as long as 5 months at 4 °C in 40% sucrose, the properties of the receptor prepared by method I (R_I form of receptor, $K_{av} \simeq$ 0.31) were stable, and there was no spontaneous appearance of receptor eluting with a K_{av} of 0.53, corresponding to the elution volume of receptor prepared by method II. Similarly, the R_{II} form of the receptor, obtained with insulin–Sepharose (method II, $K_{av} \simeq 0.53$), was stable upon storage for up to 1 month at 4 °C in 40% sucrose. Strikingly, when receptor material purified by method I (R_I form, $K_{av} \simeq 0.31$) was recovered from the Sepharose column (fraction 39, Figure 1A) and was exposed to comparatively high concentrations of ^{125}I-labeled insulin (25 ng/mL radiolabel, either in the absence or presence of 50 μg/mL unlabeled insulin), the elution volume of the specific insulin-binding component increased markedly, so as to correspond to a K_{av} of about 0.53 (R_{II} form of receptor, Figure 1A). The chromatographic properties of the receptor material purified by the two procedures could also be monitored with preparations that had been radiolabeled with carrier-free ^{125}I (Figure 1B); the iodination procedure did not affect the chromatographic profiles of the receptor material isolated by either of the two procedures (Figure 1B). Using the radiolabeled receptor preparations, it was possible to demonstrate that the effect of insulin on the chromatographic behavior of the R_I form of the receptor (prepared by method I) could be mimicked by exposing the receptor to low concentrations (0.1 mM) of dithiothreitol (Figure 1B). The experiments with radiolabeled receptor, prepared by method I, were amplified with columns of anti-receptor immunoglobulin–Sepharose for receptor isolation, prior to chromatography on Sepharose 6B (Figure 1C). When radiolabeling of the receptor was performed in the presence of insulin, the insulin-free radiolabeled receptor, isolated by immunoaffinity chromatography with anti-receptor immunoglobulin, migrated on Sepharose 6B as the R_{II} form of the receptor (open circles, Figure 1C). In contrast, the receptor, radiolabeled in the absence of insulin and isolated with the anti-receptor immunoglobulin column, migrated as the R_I form of the receptor (closed circles, Figure 1C). When immunoaffinity-purified radiolabeled receptor that was recovered from the Sepharose 6B column in the R_I form (fraction 39, Figure 1C) was exposed to insulin (25 ng/mL) and was rechromatographed (open squares, Figure 1C), the receptor eluted predominantly as the R_{II} form of the receptor. The inclusion of dithiothreitol (DTT), rather than insulin in the radiolabeling procedure, led to the formation of labeled receptor that, after immunoaffinity isolation, behaved as the R_{II} form of the receptor on Sepharose 6B (crosses, Figure 1C).

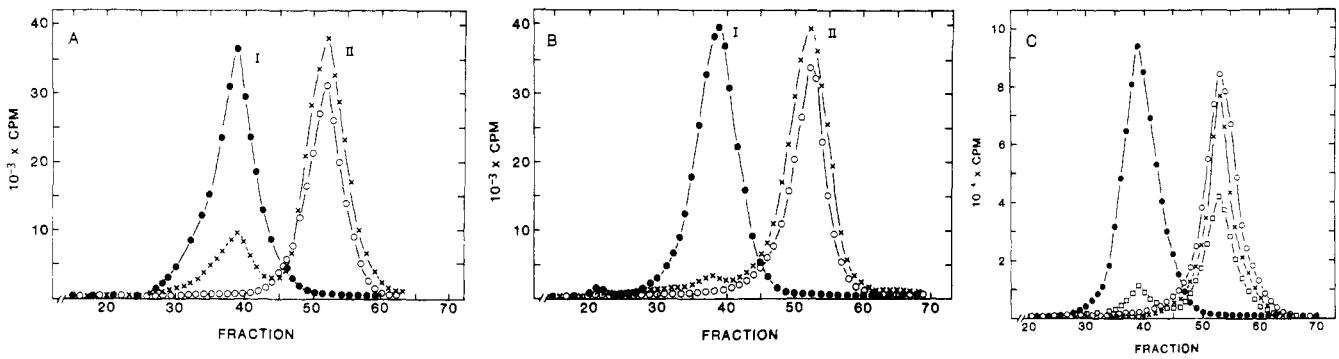

FIGURE 1: Chromatography of receptor on Sepharose 6B. (A) Comparative analysis of receptor isolated by methods I and II and effect of insulin on R_I form of the receptor. Soluble receptor (1–5 ng of protein) isolated either by method I (●) or by method II (○) was chromatographed (10 mL/h) on a column (1.5 × 87 cm) of Sepharose 6B, preequilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 0.1% v/v Triton X-100. Aliquots (50 μL) of the eluted fractions (1.8 mL) were used to measure the specific binding (cpm) of ^{125}I-labeled insulin by the PEG procedure. Receptor material isolated by method I that eluted in fraction 39 ($K_{av} \simeq 0.31$) was subsequently equilibrated with 25 ng/mL ^{125}I-labeled insulin in either the presence or absence of 50 μg/mL unlabeled insulin; both samples were rechromatographed, and the radioactivity in effluent fractions was measured. A difference plot (×) is recorded, representing the difference in radioactivity (cpm) in aliquots from identical fractions from samples preequilibrated either without or with unlabeled insulin prior to chromatography. (●) Insulin binding (cpm) by receptor prepared by method I; (○) binding (10^{-3} × cpm) by receptor prepared by method II; (×) radioactivity difference plot (cpm) for receptor (method I) incubated with 25 ng/mL ^{125}I-labeled insulin prior to rechromatography. (B) Chromatography of radiolabeled receptor and effect of DTT treatment. Receptor material isolated either by method I (●) or by method II (○) was radiolabeled (0.2 mCi of ^{125}I/5–10 ng of protein), freed from reactants on a Sephadex G-50 column, and then chromatographed on Sepharose 6B as outlined above. An aliquot of receptor material isolated by method I was treated first with 0.1 mM DTT and then with 10 mM NEM. After exhaustive dialysis, the DTT/NEM-treated receptor aliquot was radiolabeled and analyzed on the Sepharose 6B column (×). (Peak I) R_I form of receptor ($K_{av} \simeq 0.31$); (peak II) R_{II} form of receptor ($K_{av} \simeq 0.53$). (C) Effect of insulin and DTT on the chromatographic form of the radiolabeled receptor isolated by anti-receptor immunoglobulin–agarose. Three aliquots of receptor material isolated by method I were incubated: (1) (●) without addition, (2) (○) with insulin (50 ng/mL; 40 min, 24 °C), or (3) (×) with DTT (25 mM; 15 min, 24 °C; reaction stopped by the addition of NEM). The iodination procedure was then initiated by the addition of ^{125}I followed by other reactants. The reaction mixture was combined with sodium acetate buffer, pH 6, containing 4 M urea, and all reactants were immediately separated from radiolabeled receptor by gel filtration on Sephadex G-50. The three samples were applied in sequence to (1) a control immunoglobulin–Sepharose column and then (unadsorbed material) to (2) a column of anti-receptor immunoglobulin–Sepharose. Samples eluted with 2.5 M $MgCl_2$ from the anti-receptor column were analyzed by chromatography on a column of Sepharose 6B. Radioactivity in the effluent fractions was measured. Upon elution from the Sepharose 6B column, an aliquot of the untreated (i.e., without insulin or DTT) affinity column purified iodinated receptor (fraction 39) was incubated with insulin (25 ng/mL; 40 min, 24 °C) and rechromatographed on the column. (●) Affinity-purified receptor radiolabeled in the absence of insulin or DTT; (○) affinity-purified receptor radiolabeled in the presence of insulin; (×) affinity-purified receptor radiolabeled after treatment with DTT; (□) affinity-purified receptor recovered from fraction 39 (no previous treatment with insulin or DTT), exposed to insulin and rechromatographed.

Since both insulin and mild dithiothreitol treatment could convert the receptor from the R_I form to the R_{II} form, we were interested to evaluate further the participation of sulfhydryl groups in the process of insulin-mediated receptor interconversion. Thus, radioactive NEM was used as a probe to monitor the appearance of sulfhydryl groups during the process of insulin binding. Receptor material was first isolated by method I and pretreated with unlabeled NEM (10 mM) prior to insulin treatment, followed by removal of unreacted NEM by dialysis. Control experiments showed that the R_I form of the receptor was not changed by this procedure (Figure 2A) and that the procedure abolished the incorporation of radioactivity, upon subsequent addition of ^{3}H-labeled NEM to pretreated receptor (Figure 2A). In parallel experiments, it was observed that without pretreatment with unlabeled NEM, the R_I form of the receptor, prior to insulin exposure, could incorporate a small amount of radioactivity, upon addition of ^{3}H-labeled NEM (about 3% of the amount shown in Figure 2B); the ^{3}H-NEM-labeled R_I form of the receptor still migrated on Sepharose 6B with a K_{av} of about 0.31 (data not shown).

The NEM-pretreated R_I form of the receptor was applied to a column of insulin–agarose, was washed, and was then eluted with acid–urea buffer into the phosphate–Triton–sucrose buffer, containing ^{3}H-labeled NEM; the purified receptor was concentrated as well as freed from ^{3}H-NEM via diafiltration on an Amicon YM-30 filter membrane. In Figure 2A, it can be seen that the insulin–agarose procedure had not only converted the receptor from the R_I to the R_{II} form but had also exposed reactive sites, such that the R_{II} form of the receptor was labeled with ^{3}H-NEM. Importantly, the ^{3}H-NEM-labeled receptor was still capable of binding insulin.

The effect of the insulin–agarose column was mirrored by experiments in which the NEM-pretreated R_I form of the receptor was treated with increasing amounts of unlabeled insulin, after prior addition of ^{3}H-NEM. As shown in Figure 2B, treatment with insulin at concentrations of 2 ng/mL and higher (up to 50 ng/mL) led to the appearance of increasing amounts of the ^{3}H-labeled R_{II} form of the receptor. The concentration-effect curve for the insulin-mediated incorporation of ^{3}H-NEM into the R_{II} form of the receptor is shown in the inset of Figure 2B. In the absence of insulin, as in the previous series of experiments with insulin–agarose, no appreciable radioactive NEM was incorporated into the R_I form of the receptor that had been pretreated with unlabeled NEM.

The experiments with purified receptor material were substantiated by work with isolated membrane preparations. The receptor present in particulate placental membranes that had been pretreated with unlabeled NEM was cross-link labeled with the cleavable reagent bis[2-[[(succinimidooxy)-carbonyl]oxy]ethyl] sulfone and a comparatively low ^{125}I-labeled insulin concentration (0.5 ng/mL). Upon solubilization and immunoaffinity purification, the ^{125}I-radiolabeled receptor cross-linked at this low insulin concentration was observed on columns of Sepharose 6B to behave like the R_I form of the receptor (Figure 3). In contrast, when chemical cross-linking was performed at a high concentration of ^{125}I-labeled insulin (25 ng/mL), the radiolabeled receptor, after immunoaffinity purification, behaved like the R_{II} form of the receptor on columns of Sepharose 6B (Figure 3); in some preparations of the membranes, the insulin-mediated interconversion of the R_I to the R_{II} form of the receptor under these conditions was

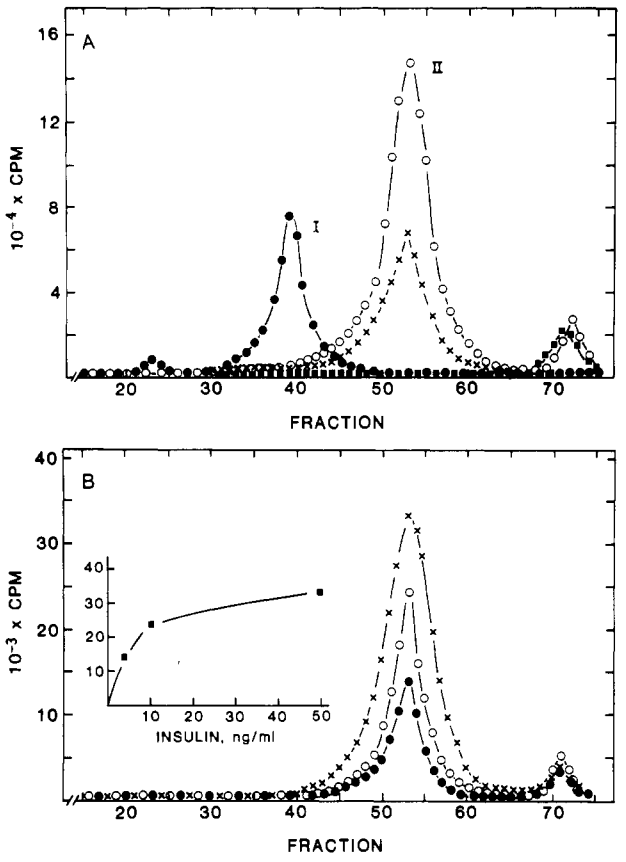

FIGURE 2: Effect of insulin on chromatographic behavior and on incorporation of ^{3}H-NEM. (A) Chromatography and ^{3}H-NEM incorporation before and after insulin exposure. Receptor isolated by method I (R_I form, approximately 10 ng of protein) was incubated with 10 nM unlabeled NEM (15 min at 0 °C); unreacted NEM was removed either by diafiltration or by chromatography on Sephadex G-50. An aliquot of the NEM-treated receptor preparation was incubated further with ^{3}H-NEM (50 nmol/mL), dialyzed, and analyzed by chromatography on Sepharose 6B. Eluted fractions were assayed for insulin binding (●) and for content of ^{3}H-NEM (■). A second aliquot of the NEM-treated receptor was then applied to an insulin–agarose affinity column and eluted into buffer containing ^{3}H-NEM (5 nmol/mL). The recovered receptor was both freed from unreacted ^{3}H-NEM and concentrated by dialysis and was then applied to the Sepharose 6B column. Eluted fractions were assayed for specific insulin binding (○) and for content of ^{3}H-NEM (×). (●, ■) Receptor prior to insulin exposure; (○, ×) receptor exposed to insulin. (B) Concentration dependence of insulin-mediated incorporation of ^{3}H-NEM. Aliquots of NEM-treated receptor isolated by method I (as above) were incubated in the presence of ^{3}H-NEM (5 nmol/mL) and were then treated with increasing concentrations of unlabeled insulin [2 ng/mL (●); 10 ng/mL (○); 50 ng/mL (×)]. Unreacted ^{3}H-NEM was removed by chromatography on Sephadex G-50, and receptor-containing samples were then applied to the Sepharose 6B column. Fractions eluted from the Sepharose 6B column were monitored for ^{3}H-NEM content by scintillation counting. No radioactivity was recovered from samples that had not been exposed to insulin. The inset shows the dependence of ^{3}H-NEM incorporation on insulin concentration.

not always as complete as is depicted in Figure 3 (data not shown). When the cross-linking reaction was performed at the high insulin concentration in the simultaneous presence of ^{3}H-labeled NEM, the immunoaffinity-purified receptor (R_{II} form) was observed to contain both ^{125}I-labeled insulin and ^{3}H-NEM (Figure 3). In contrast, at the low concentration of ^{125}I-labeled insulin (0.5 ng/mL), cross-link labeling in the simultaneous presence of ^{3}H-NEM resulted in the incorporation only of ^{125}I-labeled insulin and not ^{3}H-NEM into an R_I form of the receptor that could be isolated by the immunoaffinity method. In experiments wherein ^{125}I-labeled insulin was coupled to the receptor with the noncleavable cross-linking reagent (disuccinimidyl suberate), it was possible to show that the mild alkali treatment (1 h at 37 °C, pH 8), necessary to liberate ^{125}I-labeled insulin from the cleavable cross-linking reagent, did not affect the chromatographic beahvior of the receptor.

When the anti-receptor immunoglobulin–agarose column was used to isolate the ^{3}H-NEM-labeled receptor, subsequent to the insulin-mediated incorporation of radioactivity in membrane preparations, the receptor migrated as the R_{II} form on Sepharose 6B (Figure 3B). A receptor preparation treated in the same manner, but without the prior addition of insulin to the membranes, upon elution from the antireceptor immunoaffinity column, migrated as the R_I form of the receptor on Sepharose 6B. This result indicated that the acid–urea conditions used to dissociate insulin from the receptor prior to anti-receptor column isolation did not affect the chromatographic properties of the receptor.

Since the experiments described above suggested that the insulin-mediated conversion of the receptor from the R_I to the R_{II} form involved the exposure of sulfhydryl groups, we reasoned that the R_{II} form of the receptor might be converted to the R_I form by reoxidation. Therefore, the R_I form of the receptor, prepared by method I, was converted to the R_{II} form by adsorption to a column of insulin–agarose, as described above. The acid–urea elution step was modified by the inclusion of freshly prepared 10 mM oxidized glutathione in the phosphate–Triton–sucrose buffer used for neutralizing and diluting the eluted, affinity-purified receptor. The eluted receptor material was rapidly dialyzed free of urea and concentrated on an Amicon YM-30 filter membrane. The concentrated, glutathione-treated receptor was then analyzed by chromatography on Sepharose 6B (Figure 4). It was evident that a large proportion of the receptor, which in the absence of glutathione treatment was in the R_{II} form (Figure 4), was converted by oxidized glutathione to the R_I form of the receptor (Figure 4).

Discussion

The major finding of this study is that insulin, upon binding to the human placental receptor either in intact membranes or in purified receptor preparations can cause an interconversion of the receptor from one hydrodynamic form (R_I) to a second form (R_{II}) that can be distinguished by chromatography on columns of Sepharose 6B. Our results substantially extend our preliminary observations (Hollenberg et al., 1981) and are in accord with the previous work by others (Ginsberg et al., 1976; Baron et al., 1981). Our data indicate that the interconversion caused by insulin results in the exposure of reactive receptor groups that can be radiolabeled with NEM; further, the interconversion from the R_I to the R_{II} form of the receptor caused by insulin can be reversed by treatment with oxidized glutathione. These data, coupled with the observation that dithiothreitol can mimic the insulin effect, suggest that disulfide–sulfhydryl exchange reactions may be involved in the insulin-mediated interconversion of the receptor from one form to another.

The two chromatographic forms of the receptor that we have observed (R_I, $K_{av} \simeq 0.31$; R_{II}, $K_{av} \simeq 0.53$) merit comment. By use of Sepharose 6B chromatography, analogous receptor forms labeled either by a photoprobe (Baron et al., 1981) or by chemical cross-link labeling (Heinrich et al., 1980) have been observed in work with the receptor in rat liver (Baron et al., 1981) and in adipocyte (Heinrich et al., 1980) membranes. The nomenclature we have used for the two receptor forms is in keeping with the designation by Baron et al. (1981), who observed that for rat liver preparations, the two chro-

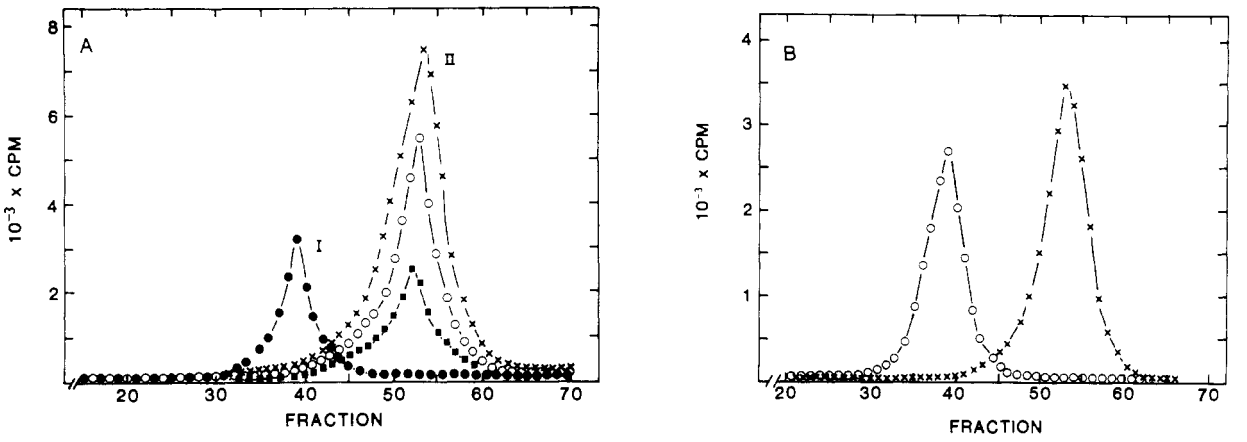

FIGURE 3: Chromatography of receptor isolated by either anti-insulin or anti-receptor antibody affinity chromatography. (A) Chromatographic profile of immunoaffinity isolated receptor obtained from placenta membranes cross-link labeled with either high or low ^{125}I-labeled insulin concentrations. Placenta membranes were pretreated with 10 mM unlabeled NEM and were washed free of unreacted NEM by centrifugation (five cycles). Washed membrane pellets were resuspended in pH 7.4 buffer (either Tris-HCl or Krebs–Ringer bicarbonate) containing ^{3}H-NEM (5 nmol/mL) and were incubated with either 0.5 or 25 ng/mL ^{125}I-labeled insulin. After 40 min at room temperature, the reaction was terminated by dilution with ice-cold buffer, and membranes were washed 5 times by centrifugation. Bound ^{125}I-labeled insulin was cross-linked to the receptor with the cleavable cross-linking reagent, as outlined under Materials and Methods. Nonspecific cross-linking of ^{125}I-labeled insulin was routinely 10% and always less than 25% of the total cross-linked radioactivity. Receptor was solubilized and purified by immunoaffinity chromatography with anti-insulin antibody, essentially as described by Heinrich et al. (1980). Samples eluted from the immunoaffinity adsorbent were chromatographed on Sepharose 6B either before (●, ○, ×) or after (■) release of the cross-linked ^{125}I-labeled insulin by incubation at pH 8.0 for 1 h at 37 °C. (●) ^{125}I cpm for cross-link-labeled receptor from membranes incubated with 0.5 ng/mL ^{125}I-labeled insulin; (○) ^{125}I cpm for cross-link-labeled receptor obtained from receptor incubated with 25 ng/mL ^{125}I-labeled insulin; (×) total radioactivity (^{125}I + ^{3}H) for receptor labeled at 25 ng/mL ^{125}I-labeled insulin; (■) ^{3}H-NEM radioactivity for immunoaffinity-purified receptor freed from cross-linked ^{125}I-labeled insulin by cleavage of the bifunctional reagent. (B) Chromatographic behavior of ^{3}H-NEM-labeled receptor recovered from membranes on anti-receptor immunoglobulin–Sepharose. Placenta membranes (4 mg/mL) were treated with 25 mM unlabeled NEM and were washed free of unreacted reagent by centrifugation (five cycles). Washed membrane pellets were resuspended in Krebs–Ringer bicarbonate, pH 7.4 buffer, containing ^{3}H-NEM (5 nmol/mL) to which unlabeled insulin was immediately added (25 ng/mL final concentration). After 40 min at room temperature, the reaction was terminated by dilution with ice-cold buffer, and membranes were washed 5 times by centrifugation. The pelleted membranes were resuspended in 50 mM Tris-HCl buffer, pH 7.5, containing 1% Triton X-100. After 90 min at room temperature, a clarified supernatant was obtained by high-speed centrifugation. A sample of the detergent extract was treated for 5 min with a 4 M urea pH 6.0 solution to dissociate bound insulin, which was then removed by chromatography of the extract on Sephadex G-50. The extract was then passed over a 1-mL column of anti-receptor immunoglobulin–Sepharose. Samples eluted from the receptor antibody affinity column were then chromatographed on Sepharose 6B. A sample of the unlabeled NEM-treated non-insulin-treated detergent extract, which was also subjected to 4 M urea–pH 6 treatment prior to affinity column isolation, was also chromatographed on Sepharose 6B, and fractions were assayed for specific insulin binding (○). This experiment was performed to eliminate the possibility that the K_{av} = 0.53 peak might have been generated by the acid–urea treatment procedure. (○) Insulin binding by immunoaffinity-isolated receptor; (×) ^{3}H-NEM-labeled receptor (insulin induced) isolated by immunoaffinity chromatography.

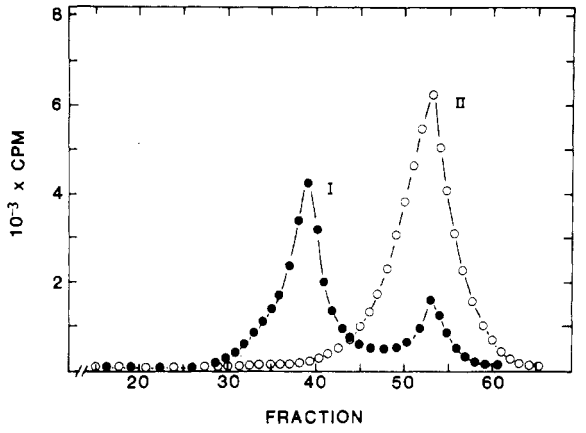

FIGURE 4: Conversion of receptor from R_{II} to R_I form. Receptor isolated by method I (R_I form) was applied to an insulin–agarose column that normally converts the receptor to the R_{II} form (Figure 1). Replicate receptor preparations were eluted into buffer either with (●) or without (○) 10 mM oxidized glutathione. Recovered receptor was dialyzed, concentrated, and chromatographed on Sepharose 6B as outlined in Figure 1. Eluted fractions were assayed for specific insulin binding (●, ○) by the PEG method. (●) Glutathione-treated receptor; (○) receptor recovered without glutathione treatment.

matographic forms observed upon Sepharose 6B chromatography (R_1, 7.0-nm Stokes radius; R_2, 3.6-nm Stokes radius) corresponded to two distinct species that could be detected in sucrose density gradients ($s_{20,w}$ of $R_1 \simeq 10.5$ S and $R_2 \simeq 6.5$ S). We have observed these same two chromatographic forms of the receptor in several previous studies with insulin receptors from a variety of tissues (Maturo & Hollenberg, 1978; Maturo et al., 1978; Maturo & Hollenberg, 1979); in our previous work, insulin–agarose chromatography has uniformly yielded a purified receptor that corresponds to the R_{II} form described in the present work and that exhibits a linear Scatchard plot for insulin binding. In contrast, Siegel and co-workers (Siegel et al., 1981), who used insulin–agarose affinity chromatography for purification of the human placental insulin receptor, observed only one chromatographic form on columns of Bio-Gel A1.5 M and only one principal component upon sedimentation in sucrose gradients ($s_{20,w}$ = 12 S). We have previously noticed that the two chromatographic receptor forms cannot be resolved on columns of cross-linked Sepharose 6B-CL (unpublished), and we now suspect that the two chromatographic forms of the receptor that we have observed need not necessarily represent species of markedly different molecular size; absorption of the R_{II} form of the receptor to the Sepharose 6B column cannot be ruled out. Given the linear Scatchard plot reported by Siegel et al. (1981), which we believe is characteristic of the R_{II} form of the receptor (Maturo & Hollenberg, 1978; Maturo et al., 1978; Maturo & Hollenberg, 1979), it appears quite possible that the material studied by Siegel et al. (1981) may represent the R_{II} form of the receptor that we describe in the present work. Very possibly, the two chromatographic forms of the receptor may not separate on Bio-Gel columns. Further work will be nec-

essary to explain what are apparent discrepancies between our present and previous (Maturo et al., 1978) work with the human placental insulin receptor and the results of Siegel et al. (1981).

Given the disulfide-stabilized heterodimeric (β–S–S–α–S–S–α–S–S–β) model proposed for the insulin receptor [Massagué et al. (1980) and contributions in Andreani et al. (1981)], it is perhaps not surprising that dithiothreitol converts the receptor from one hydrodynamic form to another. It is now recognized that reducing agents can markedly affect not only the electrophoretic forms (Massagué et al., 1980; Massagué & Czech, 1982) but also the functional properties (primarily, ligand binding; Jacobs & Cuatrecasas, 1980; Schweitzer et al., 1980; Massagué & Czech, 1982) of the receptor. On the basis of the proposed model, one might suspect that the R_{II} form of the receptor, resulting from dithiothreitol treatment, represents the β–S–S–α–SH species. What is remarkable, however, is that insulin itself can cause the interconversion of the receptor to the R_{II} form and that in the process of the interconversion, reactive groups (most probably, sulfhydryl) become available to combine with ^{3}H-NEM. Our results with insulin closely parallel observations made by Schweitzer and co-workers (Schweitzer et al., 1980), who demonstrated a reversible sulfhydryl-mediated change of the state of the insulin receptor in adipocyte membranes. The concentration-effect curve (Figure 2B) that we have observed for the interconversion clearly indicates that the process is saturable in a concentration range over which insulin has been observed to cause a biological response in vitro (Hollenberg, 1976). A receptor-related, rather than a nonspecific chemical, process is thereby implicated. It may be of significance that the insulin-mediated interconversion process occurs largely at insulin concentrations that are far in excess of the usual physiological range of insulin concentrations (up to 150 microunits/mL; 6 ng/mL or $\simeq 10^{-9}$ M). In view of the model suggested for the insulin receptor, it is possible that both putative insulin binding sites of the proposed heterodimeric structure must be occupied for the interconversion process to occur. Since this insulin-directed interconversion can occur under conditions wherein insulin is added to a receptor solution that already contains ^{3}H-NEM (Figure 2B), it is unlikely that the interconversion is mediated directly by a disulfide interchange involving a reduced insulin species (e.g., via insulin containing a reduced A-chain disulfide). As an alternative, the binding of insulin may indirectly perturb the receptor structure either to expose preexisting receptor sulfhydryl groups that are sterically masked from NEM in the absence of insulin or to promote the reduction of receptor disulfides by an as yet unknown process. This latter possibility is particularly intriguing to us in view of the nonrecognition regulatory constituents that we (Maturo & Hollenberg, 1978) and others (Harmon et al., 1980; Yip et al., 1980, 1982) believe may be closely associated with the ligand-recognition subunits of the receptor. In the context of our previous findings (Maturo & Hollenberg, 1978), we are tempted to propose that the nonreceptor glycoprotein fraction that we have previously described contains a constituent that in some manner can reoxidize the receptor subsequent to the insulin-mediated conversion of the receptor to the R_{II} state. In essence, we believe that the results with glutathione, documented in the present work, mimic the previously described (Maturo & Hollenberg, 1978) action of the nonreceptor glycoprotein affinity regulator (this material would be free of glutathione, given its method of preparation).

The exact nature of the R_{II} hydrodynamic form of the receptor generated by insulin remains to be established. On the basis of a report that appeared upon completion of our study (Massagué & Czech, 1982), we suspect that insulin binding leads to the cleavage of the so-called "class I" receptor disulfides, which can be split by dithiothreitol added to intact cells or membranes; the so-called "class II" disulfides become available for dithiothreitol reduction only after denaturation of the receptor in sodium dodecyl sulfate (Massagué & Czech, 1982). Given the specific activity of the NEM that we used, it would appear that at least several reactive sites on the receptor must become available subsequent to the binding of insulin. The data of Massagué & Czech (1982) would add weight to the supposition that the R_{II} hydrodynamic form of the receptor may correspond to the dimeric form of the receptor (β–S–S–α). However, preliminary data obtained by Goren et al. (1982), on the basis of electrophoretic analysis in nonreducing gels, indicate that the R_{II} form of the ^{125}I-labeled insulin cross-link-labeled receptor, isolated by Sepharose 6B chromatography ($K_{av} \simeq 0.53$), exhibits a molecular weight less than 150 000. Thus, the assumption that the R_{II} form represents the species β–S–S–α–SH (this species would have a molecular weight of about 230 000) is open to question. In continuing work, we plan to establish the precise subunit structure of the insulin-mediated R_{II} form of the receptor and to identify the location of the NEM-reactive groups that are involved in the R_I to R_{II} interconversion process. We believe that this receptor-interconversion process may relate to the biological action of insulin in intact cell systems and to the different affinity states of the receptor that have been documented both in cultured rat hepatocytes and in membranes from hepatocytes, adipocytes, and placenta cells (Donner & Corin, 1980; Corin & Donner, 1982; Jacobs & Cuatrecasas, 1980; Schweitzer et al., 1980; Ozaki et al., 1982).

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Registry No. Insulin, 9004-10-8.

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Purification of the Receptor for 1α,25-Dihydroxyvitamin D_3 from Chicken Intestine†

Robert U. Simpson, Alan Hamstra, Nancy C. Kendrick, and Hector F. DeLuca*

ABSTRACT: Methods were investigated for use in the purification of the chicken intestinal receptor for 1α,25-dihydroxyvitamin D_3. The techniques investigated include column isoelectric focusing, gel exclusion, polyacrylamide gel electrophoresis, and DNA–cellulose, DEAE-cellulose, and hydroxylapatite chromatography. For the starting receptor preparation, a nuclear extract of chicken intestinal mucosa was found to be enriched above cytosol preparations and a plentiful source of receptor. A five-step purification scheme that resulted in the purification of the receptor protein by 5800-fold with 8% yield has been described. Analysis of the purified proteins on polyacrylamide gel electrophoresis containing sodium dodecyl sulfate suggests homogeneity. Analysis using two-dimensional polyacrylamide electrophoresis characterized the purified protein as having a molecular weight of approximately 63 000 and a p*I* of 6.0–6.2. Furthermore, assessment of protein purity by ^{125}I iodination followed by sucrose gradient analysis revealed that approximately 90% of the iodinated macromolecules have the same sedimentation coefficient as the titrated 1α,25-dihydroxyvitamin D_3 receptor complex. The final purified receptor that bound tritiated 1α,25-dihydroxyvitamin D_3 retained affinity for DNA–cellulose and possesses a 3.7S sedimentation coefficient. The receptor has an estimated Stokes radius of 37 Å.

Vitamin D_3 has been shown to exert its physiological actions involved in the control of serum calcium and phosphate homeostasis via metabolic activation. It is now accepted that 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$][1] is the hormonally active vitamin D_3 metabolite responsible for the actions of the vitamin (DeLuca & Schnoes, 1976).

Studies have shown that the intracellular mechanism of action of 1,25-$(OH)_2D_3$ for control of intestinal calcium absorption involves the modulation of gene transcription (Eisenstein & Passavoy, 1964; Zull et al., 1965; Norman, 1965). Autoradiographic and subcellular fractionation studies have further shown specific nuclear localization of 1,25-$(OH)_2$-[^3H]D_3 (Haussler et al., 1968; Chen & DeLuca, 1973) and revealed specific high-affinity 1,25-$(OH)_2D_3$ receptor-like protein in intestinal cytosol and nuclei (Brumbaugh & Haussler, 1974; Kream et al., 1976). Current findings suggest that 1,25-$(OH)_2D_3$ acts on the intestine in a manner similar to that proposed for other steroid hormones. Central to the intraceullular mechanism of action of 1,25-$(OH)_2D_3$ is the interaction of the hormone with specific receptor proteins.

Our laboratory along with others have characterized 1,25-$(OH)_2D_3$ receptor-like proteins. They exist in over 20 target organs [for review, see Norman et al. (1982)] including intestine (Brumbaugh & Haussler, 1974; Kream et al., 1976), bone (Kream et al., 1977; Mellon & DeLuca, 1980), kidney (Simpson et al., 1980; Chandler et al., 1979), pancreas (Christakos & Norman, 1979), skin (Simpson & DeLuca, 1980; Colston et al., 1980), and mammary tissue (Reinhardt

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[1] Abbreviations: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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