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Insulin Binding and Insulin-dependent Phosphorylation of the Insulin Receptor Solubilized from Human Erythrocytes*

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Insulin receptors were extracted from human erythrocytes contained in 100 ml of blood with the nonionic detergent Triton X-100 with almost 100% yield. The solubilized receptor had binding characteristics similar to those of the intact cell. Using ^{125}I-monoiodoinsulin as tracer and a computer-assisted statistical curve-fitting program, a cooperative model gave values of 1.7×10^9 M^{-1} for the $\overline{K}_e$ (affinity of the empty receptor) and of 1.6×10^8 M^{-1} for $\overline{K}_f$ (affinity of the filled receptor). Bovine desalanine-desasparagine insulin inhibited tracer binding with 3–6% the potency of porcine insulin. Serum (B-8) containing anti-insulin receptor antibodies inhibited binding by 70% at a dilution of 1:100.

Receptor autophosphorylation reaction was studied by incubation of the Triton extract with [γ-^{32}P]ATP and Mn^{2+} in the presence or absence of insulin, and the receptor was identified by immunoprecipitation with anti-receptor antibodies and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Porcine insulin stimulated 4- to 5-fold the incorporation of ^{32}P in a protein of M_r = 95,000, corresponding to the β-subunit of the insulin receptor. Phosphoamino acid analysis revealed phosphorylation of the tyrosyl residues exclusively. The dose-response curve for insulin stimulation was sigmoidal; some effect of insulin was observed at 1 ng/ml but maximal effect was observed at 10 μg/ml. Bovine desalanine-desasparagine insulin, a noncooperative analogue of insulin, was able to fully stimulate the phosphate incorporation, but the dose-response curve was shifted to the right and steeper, consistent with the intrinsic affinity of this analogue for the insulin receptor. When insulin binding was performed under the same conditions as the phosphorylation, half-maximal stimulation of phosphate incorporation occurred with 6–29% of the fractional occupancy of the receptor. These data suggest that the insulin receptor of the human erythrocyte, as in other cells, is a tyrosine-specific protein kinase. Coupling between the receptor occupancy and kinase activation is complex. Furthermore, sufficient quantities of receptor can be easily obtained from a single individual to study the binding and kinase properties of the receptor opening the opportunity to a wide field of applications in human pathology.

Tyrosine protein kinases are rare in normal mammalian cells but recently have been recognized as a novel class of kinases which may have important regulatory functions (1–3). In addition to the tyrosine protein kinases associated with transforming proteins of certain retroviruses (1), receptors for epidermal growth factor (4), platelet-derived growth factor (5) and insulin (6–18) have shown to be included in this class.

Human erythrocytes are an extremely useful and easily accessible cellular model for the study of a variety of proteins. Red cells contain several protein kinases which act on serine and threonine residues. These have been well characterized in terms of dependence of cyclic AMP, calcium or calmodulin (19–23), and in several cases the cellular protein acceptors have been defined (for review see Ref. 24). More recently, a tyrosine-specific protein kinase has been suggested to be associated with the red cell membrane since the band 3,[1] the anion transporter protein of erythrocytes, has been shown to undergo a tyrosine phosphorylation (25). Human erythrocytes also contain specific insulin receptors (23) which have binding characteristics similar to those found in other classical insulin target cells (27–29). The erythrocyte insulin receptor, however, has received little biochemical characterization, primarily due to the relatively small number of receptors on these cells (30, 31). In the present study we have shown that the insulin receptors can be quantitatively extracted from human erythrocytes and that these receptors retain normal binding properties and undergo an insulin-stimulated tyrosine phosphorylation. In addition, we have characterized this reaction and correlated the phosphorylation with the fractional occupancy of the receptor.

EXPERIMENTAL PROCEDURES

Materials—Purified pork insulin was obtained from Elanco Co. (Indianapolis); bovine DAA[2] insulin was kindly supplied by Ronald Chance (Eli Lilly, Indianapolis, IN). [γ-^{32}P]ATP (5000 μCi/mmol), Na^{125}I (17.4 Ci/mg) and Triton X-100 were from New England Nuclear; protein A (Pansorbin) was from Calbiochem; tosylphenylalanyl chloromethyl ketone-treated trypsin was from Worthington (182 units/mg); 5'-ATP, phenylmethylsulfonyl fluoride and bovine γ-globulin (Fraction II) were from Sigma. Hypaque sodium (50%) was purchased from Winthrop Laboratories. Polyethylene glycol (Carbowax 6000) was from Fisher. All materials for gel electrophoresis were from Bio-Rad and other reagents were high performance liquid chromatography or analytic grade.

Preparation of Human Erythrocyte Membrane and Solubilized Receptor—100 ml of blood was freshly drawn from healthy young men

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[1] Nomenclature for erythrocyte membrane proteins is according to Steck (54).

[2] The abbreviations used are: DAA, desalanine-desasparagine; ED_{50}, half-effective dose; Hepes, *N*-(2-hydroxyethyl)-1-piperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ^{125}I-[Tyr^{A14}]-monoiodoinsulin, the isomer labeled with ^{125}I on tyrosyl residue in position 14 on the A chain of the insulin molecule.

into heparinized syringes. After centrifugation for 10 min at 400 × *g* at 20 °C, the plasma was discarded and the pellet mixed with an equal volume of a 9% NaCl solution and applied on a 50-ml Ficoll (0.9%)-Hypaque (33.9%) gradient and centrifuged for 20 min at 400 × *g* at 20 °C. This procedure was repeated twice resulting in erythrocytes essentially free of contamination with other cell types (32).

Inverted vesicles (inside-out ghosts) of erythrocytes were prepared as described by Steck *et al.* (33) with slight modifications (34). Hypotonic lysis was conducted in 30 volumes of 5 mM Tris-phosphate buffer, pH 8.0. This and all subsequent procedures were carried out at 4 °C and in the presence of the protease inhibitors phenylmethylsulfonyl fluoride (dissolved at 30 mg/ml in 95% ethanol) and aprotinin (1000 trypsin inhibitor units/ml). Membranes were pelleted by centrifugation for 30 min at 20,000 × *g* in Sorvall GSA rotor and washed 4–5 times in 10 volumes of 0.5 mM Tris phosphate buffer, pH 8.0. The final wash was in 50 mM Hepes, pH 7.6. The final membrane pellet was resuspended in 10 ml of 50 mM Hepes, 1% Triton X-100 (v/v), pH 7.6, and stirred for 1 h at 4 °C. The undissolved material was separated by ultracentrifugation at 200,000 × *g* for 90 min. The supernatant contained about 1.4 mg/ml of protein which represents 32% of the total membrane protein.

Insulin-binding Assay—Porcine insulin was labeled by the lactoperoxidase method (35) and the four monoiodoisomers were separated as described by Markussen and Larsen (36) using high performance liquid chromatography on a reverse phase column (μBondapak C18 column, 3.9 × 300 mm; Waters) with an isocratic elution buffer containing 0.1 M Tris phosphate, 0.05 M H_3PO_4, 1 mM EDTA, 39% ethanol. ^{125}I-[Tyr^{A14}]-monoiodoinsulin (specific activity of 360 μCi/μg) was used as tracer in all binding studies.

For the characterization of insulin binding, the solubilized insulin receptor was diluted 10 times to give a final concentration of Triton X-100 of 0.08%, containing approximately 0.13–0.15 mg/ml of protein. The standard assay was performed by incubation of the detergent extract (56 μg of protein), 50 pg/ml of monoiodoinsulin and increasing concentrations of unlabeled pork insulin or insulin analogues for 15 h at 4 °C in a final volume of 500 μl. The incubation buffer contained 50 mM Hepes, 150 mM NaCl, 0.08% Triton X-100, and 0.1% bovine serum albumin, pH 7.6. Receptor-bound hormone was separated from the free hormone by precipitation with polyethylene glycol at a final concentration of 12.5% using 0.1 mg/ml of bovine γ-globulin as carrier (37). After an incubation for 15 min at 4 °C, the samples were centrifuged for 5 min in a Beckman microfuge, washed with 12.5% polyethylene glycol and the pellet counted for radioactivity (Tracor Analytical 1290). Protein determination was carried out by dye-binding method (Bio-Rad) using bovine serum albumin as standard.

Protein Kinase Assay—The crude detergent extract of erythrocytes membranes (~1.4 mg/ml) was preincubated with different concentrations of pork insulin or DAA insulin for 15 h at 4 °C in the same buffer described for binding assay in the presence of protease inhibitors, in a final volume of 500 μl. The phosphorylating reaction was initiated by the addition of $MnCl_2$ (4 mM final concentration), 100 μCi/tube of [γ-^{32}P]ATP and cold ATP (10 μM final concentration) in conditions described in the figure legends. The reaction was stopped by addition of cold "stopping solution" containing 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA and 5 mM ATP. The insulin receptor was identified by specific immunoprecipitation as previously described (38) by incubation 6 h at 4 °C with serum B-8-containing antireceptor antibodies (dilution 1:50) followed by addition of 300 μl of protein A. Immunoprecipitates were solubilized by boiling for 3 min in Laemmli sample buffer (39) which contains 2% $NaDodSO_4$, 100 mM dithiothreitol, 0.01% bromphenol blue, 10% glycerol and 10 mM sodium phosphate (pH 7.0). The proteins were separated on 7.5% polyacrylamide gel electrophoresis according to Laemmli (39). The gels were stained with Coomassie blue in 50% trichloroacetic acid, destained overnight in 7% acetic acid and dried *in vacuo* at 80 °C for 1 h. The dry gel was submitted to autoradiography on Kodak X-Omat film with intensifying screen. The incorporation of ^{32}P into the β-subunit of the insulin receptor was quantitated by scanning densitometry of the films or by counting the corresponding bands of the gel in a β-scintillation counter. The background was estimated by counting a portion of the corresponding lane of the gel with the same surface as the band. The overall phosphorylation was expressed as per cent of the maximal stimulation in each experiment. The basal activity was subtracted from each experimental value.

Identification of the Phosphoamino Acids—Phosphoamino acids were identified by a modified version of the method of Hunter and Sefton (2). After the tryptic digestion of peptides as described in the legend of Fig. 7, the supernatant was lyophilized and dissolved in 100 μl of 6 N HCl (Pierce Chemical Co.) and hydrolyzed for 2 h at 110 °C. The phosphoamino acids were separated by high voltage electrophoresis on thin layer plates (Avicil, Analtech, 250 μ) using a solution of H_2O:acetic acid:pyridine (89:10:1) at pH 3.5. Phosphoserine, phosphothreonine and phosphotyrosine (Sigma) were added to all samples immediately before electrophoresis. These standards were identified by reaction with ninhydrin and the radioactivity was located by autoradiography.

Analysis of Data—The insulin-binding data were analyzed by a computer-assisted statistical curve fitting program contained in an original subroutine of MLAB program (40). The mathematical development and the validation of the method will be described in detail elsewhere.[3] Briefly, it consists of curve fitting using a nonlinear least square method of different mathematical models. The models tested in this study were the Hill-Sips model, a four-parameter stoichiometric cooperative model, and a model for two independent binding sites. The model functions were fitted to the raw data of the individual competition curves (expressed as counts per min) and include the nonspecific binding as a parameter. The method allows the calculation of stoichiometric binding parameters without the use of graphical methods. However, for purposes of clarity, simulated curves using a cooperative model were created using the Scatchard plot, since this is the graphic form with which most of the investigators are accustomed. The goodness of the fit was evaluated by the analysis of residuals. The values of root mean squares errors and final sum of squares were also used to evaluate the goodness of the fit within serial fittings of each model. All these steps of the interactive program were visualized on a Tectronix 4006-1 terminal.

The dose-response curves for the phosphorylation (expressed in counts per min of ^{32}P incorporated in the M_r = 95,000 band) were analyzed by curve fitting using a four-parameter logistic equation (41) in the form,

$$Y = \frac{a - d}{1 + \left(\frac{H}{c}\right)^b} + d \qquad (1)$$

where Y refers to the overall effect on phosphorylation, H is the arithmetic dose of insulin or insulin analogue, a is the basal activity when $H = 0$, d is the maximal effect when H is maximum, c is the ED_{50} and b is the "slope factor" (the steepness of the curve). The coupling plot (42) was obtained by calculation of the fractional occupancy of the receptor ($\bar{Y}$) as described in Ref. 43.

RESULTS

Characteristics of Insulin Binding to Solubilized Erythrocyte Receptors—Insulin receptors were extracted from inverted red cell ghosts using Triton X-100 and ^{125}I-insulin binding measured under equilibrium conditions (4 °C for 15 h). Previous studies (44) have shown that at high concentrations, Triton lowers insulin receptor binding by lowering receptor affinity, and thus, for the initial characterization of the receptor binding, the receptor solution was diluted to give a final Triton concentration of 0.08%. Under these conditions, the bound to free ratio with a tracer concentration of insulin was 0.7 per mg of protein. Unlabeled porcine insulin inhibited ^{125}I-insulin binding with an ED_{50} of 1–2 ng/ml (Fig. 1*A*).

Binding parameters for the insulin-receptor interaction under standard conditions were obtained by curve fitting for five individual competition experiments and are given in Table I. Based on statistical criteria, all three mathematical models used can adequately describe the data, *i.e.* there is a minimum of convergence, the residuals have a random distribution about the zero level, and the parameters have meaningful values, that is the product of the affinity constant and binding capacity were proportional to the initial bound/free. The Hill coefficient (n_H) for binding was 0.8 indicating either heterogeneity of binding sites or cooperative interactions of a neg-

[3] F. Grigorescu, R. C. Rodgers, and P. DeMeyts, manuscript in preparation.

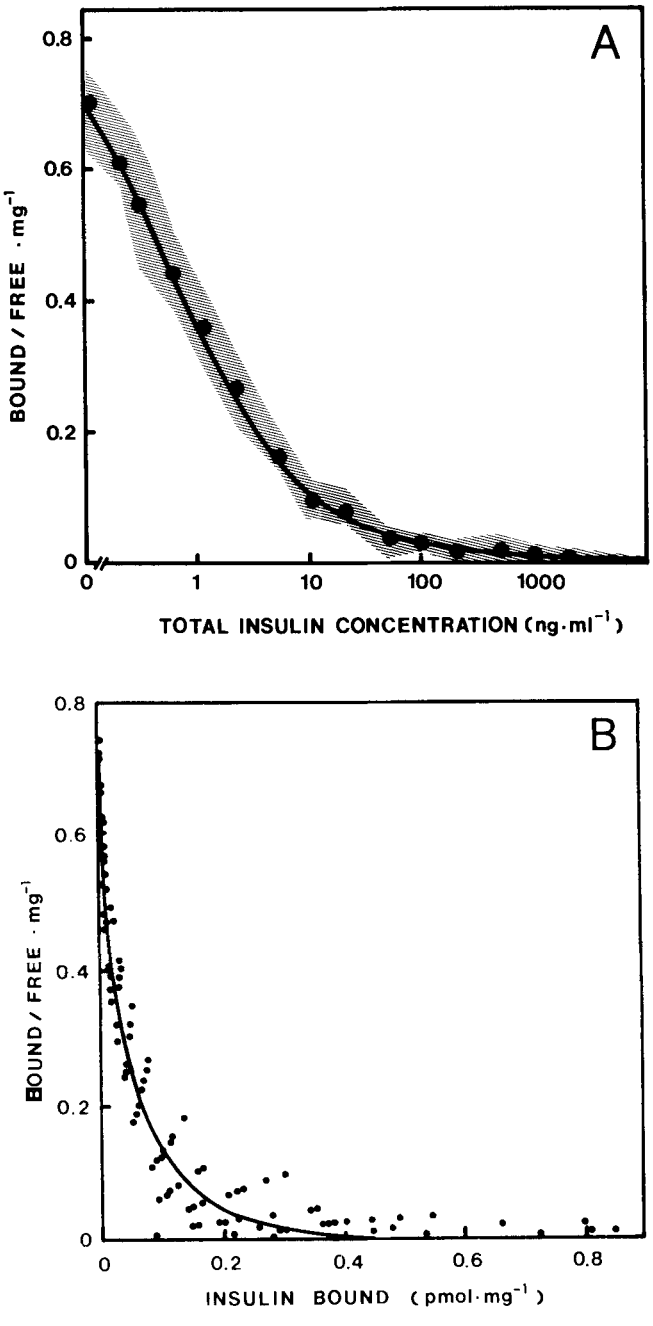


FIG. 1. **Insulin-binding characteristics of solubilized insulin receptor from human erythrocytes.** *A*, competition-inhibition curve. Detergent extract (about 0.14 mg/ml of protein) was incubated for 15 h at 4 °C in the presence of 50 pg/ml of ^{125}I-[Tyr^{A14}]-monoiodoinsulin and increasing concentration of unlabeled hormone (from 0 to 10,000 ng/ml of insulin), and the receptor-hormone complexes were precipitated with polyethylene glycol (12.5%) using γ-globulin as carrier (0.1 mg/ml). The values of bound/free were corrected by subtraction of the nonspecific binding (obtained by statistical curve fitting) and were corrected for the protein concentration. The *line* represents the mean of five experiments. *Shaded areas* represent the range of data and the *curve* is the "best fit" of a four-parameters logistic equation. *B*, simulated curve in Scatchard plot using an equation (DeMeyts) for a cooperative model. All values from five experiments, corrected for protein concentration, were plotted in the same graph. Note the range of data which have a similar shape to the 95% confidence interval in Scatchard plot. The simulated curve among experimental points is obtained with values from Table I for the cooperative model ($\bar{K}_e = 1.73 \times 10^9$ M^{-1}, $\alpha = 0.09$ and $R_0 = 0.44$ pmol·mg^{-1}).

TABLE I

Stoichiometric binding parameters of solubilized insulin receptor of human erythrocytes

Insulin-binding assay was performed under standard conditions (left column) as described under "Experimental Procedures" and in conditions used for the phosphorylation (right column): high Triton and protein concentrations, addition of $MnCl_2$ and ATP. Binding parameters were obtained by computer-assisted statistical curve-fitting (MLAB). The model functions were fitted directly on the competition curves and include a parameter for the nonspecific binding.[a] The best fitted values for five similar determinations were averaged and expressed in mean and range. $\bar{K}$ refers to the affinity constant and is express in M^{-1}; R refers to binding capacity and is expressed as pmol/mg of protein.

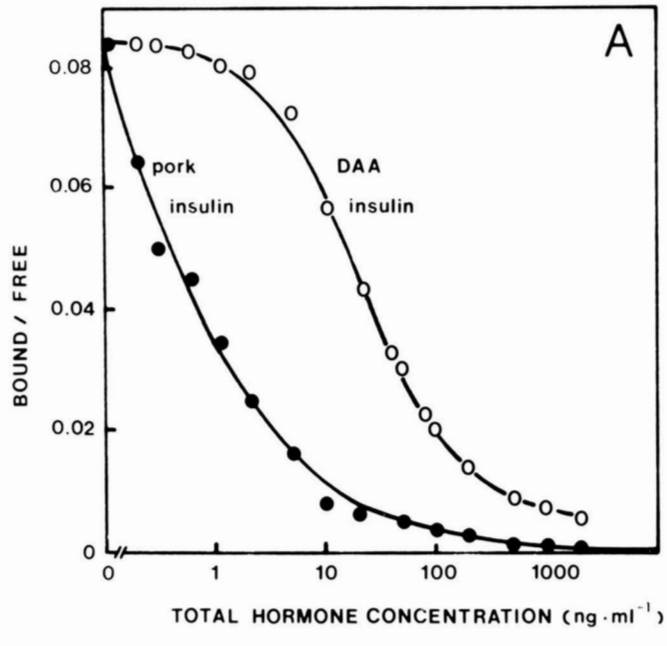
Model	Parameter[b,c,d,e,f]	Binding	
		Standard conditions	Phosphorylation conditions
Hill-Sips model	$\bar{K}_H \times 10^9$	1.55 (0.8–3.2)	0.41 (0.25–1.1)
	n_H	0.80 (0.77–0.88)	0.84 (0.74–0.95)
	R_H	0.22 (1.67–2.57)	0.24 (0.05–0.67)
Cooperative model	$\bar{K}_e \times 10^9$	1.73 (1.44–1.99)	0.13 (0.08–0.15)
	$\bar{K}_f \times 10^8$	1.58 (0.7–2.9)	0.03 (0.02–0.05)
	R_0	0.44 (0.37–0.49)	0.87 (0.59–1.1)
Two independent binding sites model	$\bar{K}_1 \times 10^{10}$	1.47 (1.02–1.9)	0.37 (0.21–0.77)
	$\bar{K}_2 \times 10^8$	5.34 (2.8–11.7)	0.17 (0.11–0.31)
	R_1	0.03 (0.02–0.04)	0.02 (0.01–0.034)
	R_2	0.31 (0.22–0.39)	0.62 (0.27–0.82)

[a] The nonspecific binding as output of the curve fitting is expressed in cpm and as per cent of total radioactivity and had values of 2% in standard conditions and 5% in phosphorylation conditions.
[b] n_H, Hill coefficient for binding.
[c] $\bar{K}_e$, affinity constant for "empty receptors."
[d] $\bar{K}_f$, affinity constant for filled receptors. The ratio $\bar{K}_f/\bar{K}_e$ is the "interaction factor" (DeMeyts) and has a value of $\alpha = 0.09$ at low Triton concentration and $\alpha = 0.024$ at high Triton concentration.
[e] $\bar{K}_1$, affinity constant of high affinity receptors.
[f] $\bar{K}_2$, affinity constant of low affinity receptors.

ative type among receptors. Using the DeMeyts model for negative cooperativity, the value of the "interaction factor" ($\bar{K}_f/\bar{K}_e$) was 0.09 consistent with a negative cooperative model and a curvilinear Scatchard plot as shown in Fig. 1*B*. The affinity constant of the empty receptor sites ($\bar{K}_e$) was 1.73×10^9 M^{-1} and was 50% higher than the value obtained in intact cells ($\bar{K}_e = 9.7 \times 10^8$ M^{-1}) using the same method of estimation.[3] The binding capacity (R_0) calculated for the cooperative model was 0.44 pmol/mg of protein, close to the predicted value calculated from the total number of sites contained in 100 ml of blood, indicating a yield of detergent extraction near 100%.

The specificity of binding was confirmed using insulin analogues and a serum-containing antibody to the insulin receptor. DAA insulin, a chemically modified analogue of insulin, inhibited insulin binding with about 3–6% the potency of porcine insulin (Fig. 2*A*). A serum (B-8) containing anti-receptor antibody inhibited binding by 70% at 1:100 dilution (Fig. 2*B*). Insulin competition curves performed after antibody treatment revealed that the effect of the serum was greatest at low insulin concentration and less at high insulin concentration, suggesting a change in apparent receptor affinity, as previously described (45). These data indicate that the detergent extract of human erythrocyte membrane contains a specific insulin receptor which is recognized immunologically, has conserved insulin analogue specificity and has binding properties similar to the intact cells.

Effect of Insulin on Receptor Phosphorylation—Recent studies have indicated that the insulin receptor has a protein kinase activity and undergoes an autophosphorylation reac-



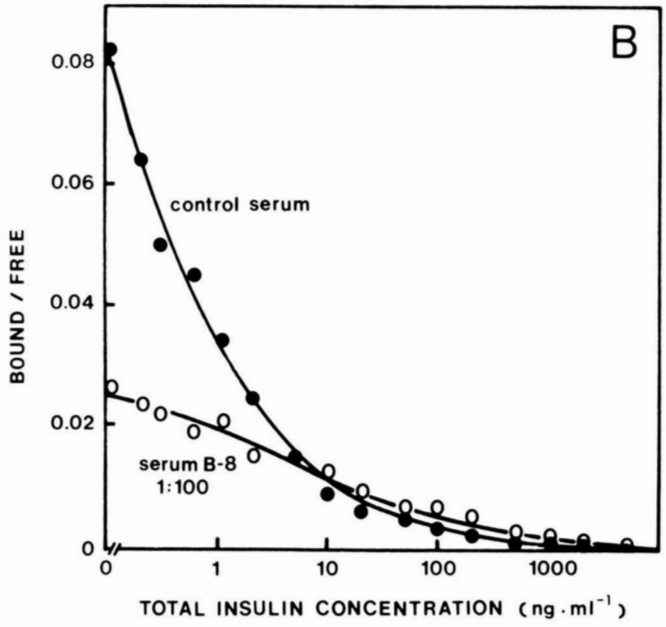


FIG. 2. **Specificity of insulin binding of solubilized erythrocyte insulin receptor.** *A*, competition curves of pork insulin (●) and DAA insulin (○) with native porcine ^{125}I-[Tyr^{A14}]-monoiodoinsulin. The solubilized receptor (0.14 mg/ml) was incubated for 15 h at 4 °C with tracer porcine and unlabeled porcine insulin or DAA insulin and the experiment proceeded as described in Fig. 1. *B*, competition curves of solubilized erythrocyte insulin receptor. The extract containing 0.14 mg/ml was preincubated for 6 h at 4 °C with control nonimmune serum (●) or serum B-8 (dilution 1:100) (○) containing anti-insulin receptor antibodies, and then a standard insulin binding was performed as described under "Experimental Procedures."

tion both *in vivo* and *in vitro* (6–15). The detergent extract of human erythrocyte membrane (mean 1.5 mg/ml of protein and 0.8% Triton X-100) was incubated with 100 μCi/tube of [γ-^{32}P]ATP, 10 μM cold ATP and 4 mM Mn^{2+} for 15 min at 20 °C. In the absence of immunoprecipitation, the gel electrophoresis and autoradiography reveal an incorporation of ^{32}P in a broad band with M_r = 88,000 to 110,000 and in several minor bands including those of M_r = 210,000, 50,000, and 65,000 (Fig. 3). The major band corresponds to band 3 of the erythrocyte membrane and the minor band of M_r = 210,000 most likely represents the erythrocyte cytoskeletal protein, spectrin (Band 2). When the phosphorylation was conducted in the presence of 10,000 ng/ml of insulin, no change in the pattern of phosphorylation was observed.

In contrast, if the erythrocyte extract was incubated with [γ-^{32}P]ATP under the same conditions, but subjected to specific immunoprecipitation using a serum containing antibodies to the insulin receptor and submitted to gel electrophoresis, the autoradiograms revealed the incorporation of ^{32}P into only one major band with M_r = 95,000 (Fig. 4). The incorporation of ^{32}P into this protein was stimulated by insulin in a dose-related fashion (*lanes B–L*) with a maximal stimulation of 4- to 5-fold. No specific ^{32}P-labeled band was observed when samples were immunoprecipitated with control nonimmune serum (Fig. 4, *lane A*). This M_r = 95,000 band was tentatively identified as the β-subunit of the insulin receptor, since its position corresponds exactly with the β-subunit identified by various techniques in other cellular systems, it is immunoprecipitated by anti-receptor antibody and its phosphorylation is stimulated by insulin. In the supernatant after immunoprecipitation, the gel electrophoresis of proteins and autoradiog-

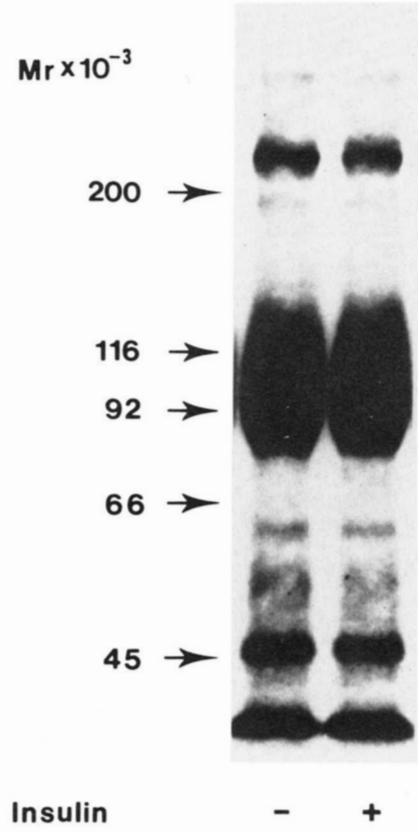


FIG. 3. **Autoradiogram showing the phosphorylation of crude membrane extract in the absence of immunoprecipitation of insulin receptor.** The crude detergent extract of erythrocyte membranes (750 μg of protein/500 μl) was preincubated in the absence or presence of 10,000 ng/ml of insulin for 15 h at 4 °C. After the addition of $MnCl_2$ (4 mM) the phosphorylation reaction was initiated by addition of 100 μCi/tube of [γ-^{32}P]ATP and 10 μM cold ATP for 15 min at 20 °C. The reaction was stopped by boiling an aliquot of 100 μl for 3 min in Laemmli buffer in reducing conditions and then was submitted to $NaDodSO_4$/PAGE and autoradiography. The molecular weight standards used were myosin (200,000), β-galactosidase (116,250), phosphorylase *b* (92,000), bovine serum albumin (66,200), and ovalbumin (45,000).

raphy revealed the same pattern of phosphorylated proteins as described in the experiment without immunoprecipitation. Again, no stimulation by insulin was observed (data not shown).

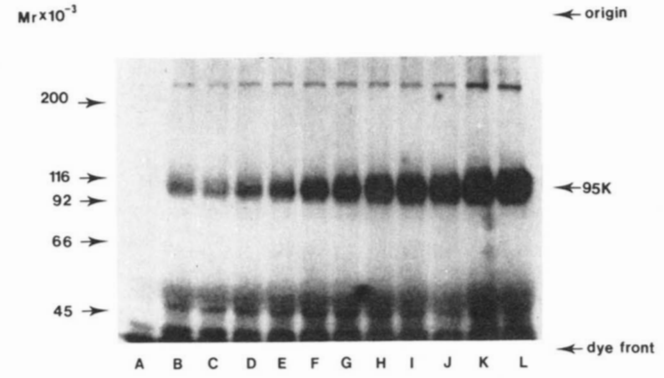


FIG. 4. **Autoradiogram showing the insulin-stimulated phosphorylation of 95,000-dalton subunit of the insulin receptor of human erythrocytes identified by specific immunoprecipitation.** The Triton X-100 extract (750 μg of protein/500 μl) was preincubated for 15 h at 4 °C with insulin from 0 to 10,000 ng/ml (*Lanes B* to *L*), and the protein kinase assay was performed as described in Fig. 3. The reaction was stopped by addition of cold "stopping solution," and the insulin receptor was identified by specific immunoprecipitation with serum-containing anti-receptor antibodies (serum B-8 at dilution 1:50) as described under "Experimental Procedures." The pellet was washed three times with a solution containing 1% Triton X-100, 0.5% SDS, and 25 mM Hepes, pH 7.6, and an aliquot of 80 μl was submitted to $NaDodSO_4$/PAGE under reducing conditions and autoradiography. Immunoprecipitation using control (nonimmune) serum is shown in *Lane A*.

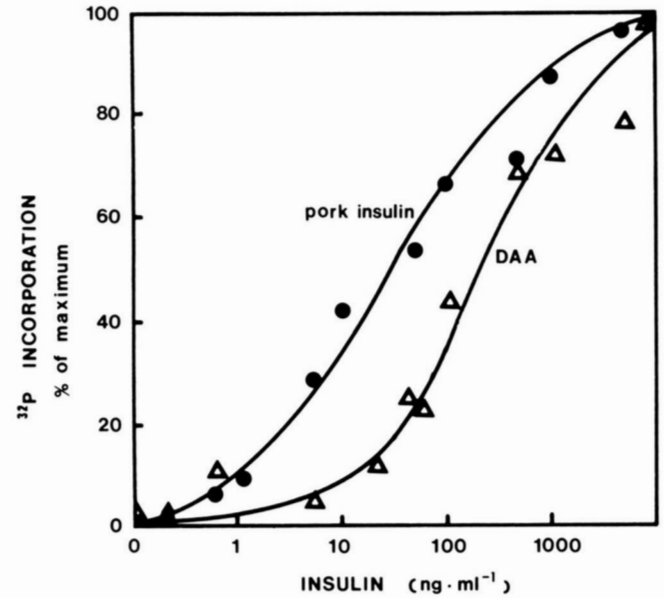


FIG. 5. **Dose-response curve for the phosphorylation of 95,000-dalton subunit of the insulin receptor of human erythrocytes.** The solubilized receptor (750 μg/500 μl) was preincubated for 15 h at 4 °C with increasing concentrations (from 0 to 10 μg/ml) of porcine insulin (●) or DAA insulin (△), and the protein kinase assay was performed as indicated in Fig. 4. The phosphorylated bands were located by autoradiography and were excised and counted in a β-scintillation counter. The basal activity (at point 0) was subtracted and the values were expressed in per cent of maximal stimulation (at 10 μg/ml of insulin). The *points* represent the means of six experiments. The curves are the "best fit" of a four-parameter logistic equation as described under "Experimental Procedures." Similar results were obtained if quantitation is made by scanning densitometry of the autoradiograms.

The average dose-response curve for stimulation of phosphorylation by pork insulin is depicted in Figure 5. The curve is sigmoidal in shape. The curve fitting using Equation 1 showed that some effect of insulin was observed at 1 ng/ml, the ED_{50} (parameter *c*) ranges between 10–15 ng/ml insulin and the slope factor (parameter *b*) ranges between 0.5–0.7. The maximal effect was obtained only at an insulin concentration between 5000–10,000 ng/ml. DAA insulin, an analogue which cannot induce negative cooperativity (46), also enhanced receptor phosphorylation, but the dose-response curve was shifted to the right and steeper (Fig. 5). The ED_{50} ranged between 110–176 ng/ml for DAA insulin and the slope factor was 0.86. High concentrations of this analogue were able to fully stimulate the phosphate incorporation into the receptor to the same extent as porcine insulin. The shape of the dose-response curve and the relative potency of the DAA insulin are consistent with the intrinsic affinity of this analogue for the insulin receptor.

The extent of phosphate incorporation in the β-subunit of the insulin receptor was proportional with the concentration of protein used in the phosphorylation assay from 0.07 to 1.12 mg/ml. In most experiments a final concentration of 1.1 mg/ml was utilized (Fig. 6).

Identification of Phosphoamino Acids in the Erythrocyte Insulin Receptor—To determine the phosphoamino acids in the insulin receptor, the detergent extract of the erythrocyte membrane was phosphorylated in the absence or presence of porcine insulin and the insulin receptor was identified as described in Fig. 4. The M_r = 95,000 band was cut from the gel, eluted by tryptic digestion, hydrolyzed and the phosphoamino acids in the M_r = 95,000 band were identified by high voltage electrophoresis on thin layer plates at pH 3.5 (Fig. 7). In the absence of insulin, the autoradiogram revealed only traces of phosphoamino acids, primarily phosphothreonine and phosphoserine. After insulin stimulation, there was a

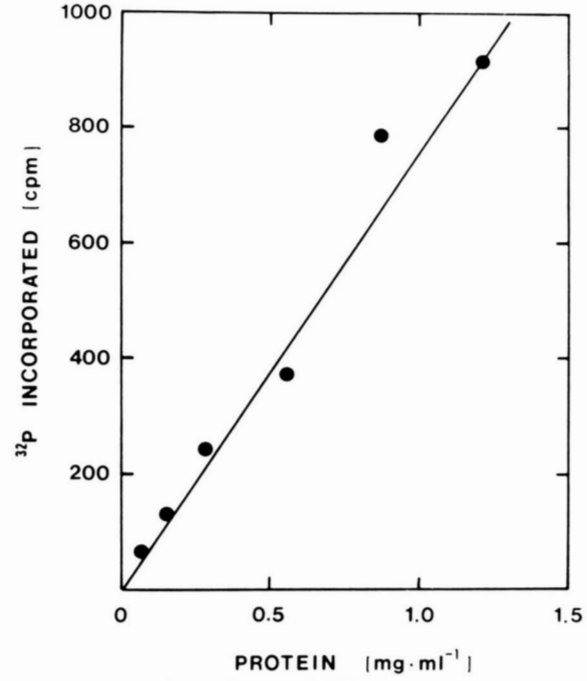


FIG. 6. **Protein dependence of ^{32}P incorporation in the β-subunit of the insulin receptor.** The solubilized receptor (1.4 mg/ml) was diluted when the concentrations of other components in the reaction mixture were unchanged. The protein kinase assay was performed as described in Fig. 4. The band corresponding to M_r = 95,000 was located by autoradiography, excited and the radioactivity was determined by β-scintillation counting.

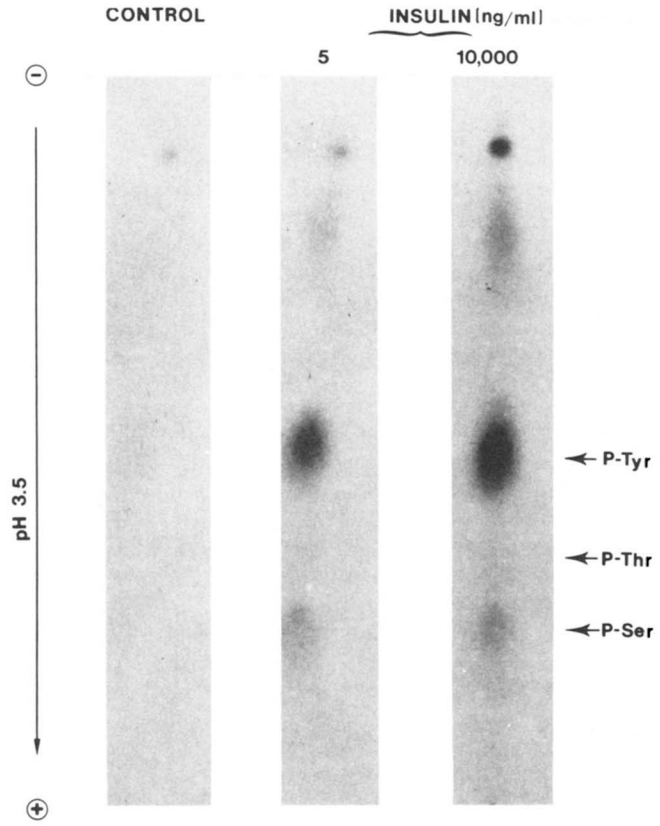
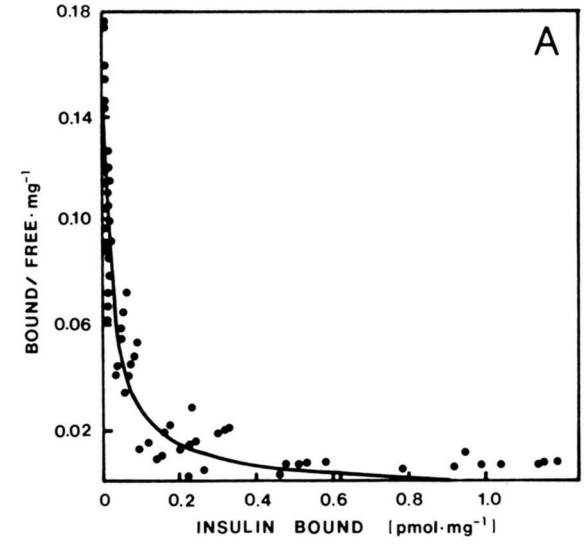
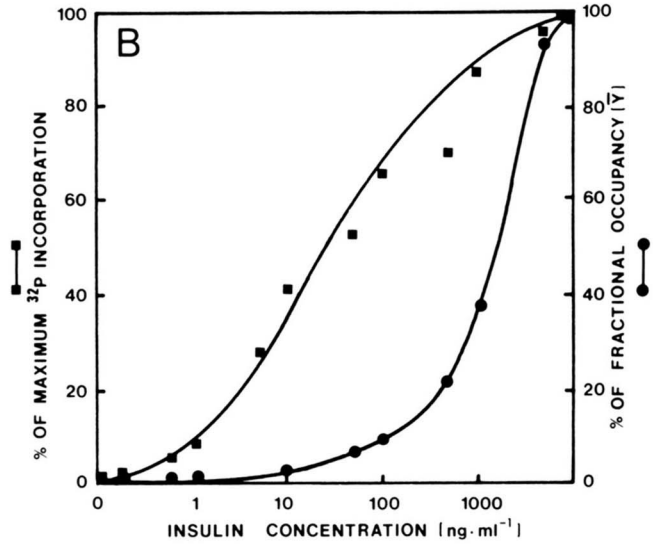


FIG. 7. **Phosphoamino acids content of the 95,000-dalton protein submitted to phosphorylation reaction in the absence or presence of insulin (5 and 10,000 ng/ml).** The position of the phosphorylated proteins separated by polyacrylamide gel electrophoresis was determined by autoradiography. The corresponding bands were excised, washed for 12 h at 37 °C in 10% methanol, dried at 100 °C for 1 h, and rehydrated with 2 ml of 50 mM NH_4HCO_3 containing 50 μg/ml of trypsin, pH 8. The enzymatic digestion was allowed to proceed for 24 h at 37 °C, and the supernatant was submitted to hydrolysis and high voltage electrophoresis as described under "Experimental Procedures."

marked increase in phosphotyrosine with little or no change in other phosphoamino acids. Thus, the insulin receptor of the erythrocyte, like other insulin receptors, undergoes a specific tyrosine phosphorylation *in vitro*.

Relation between Insulin Receptor Phosphorylation and Occupancy of the Receptor by Insulin—In order to estimate the relationship between fractional receptor occupancy and phosphorylation, it was necessary to measure the insulin binding under conditions similar to those used for the phosphorylation reaction. This included higher Triton and protein concentrations and the addition of ATP and Mn^{2+}. Under these conditions, the specific binding of the extracted insulin receptor was reduced by 10-fold (bound/free = 0.07/mg of protein) and the ED_{50} for insulin competition-inhibition curve was about





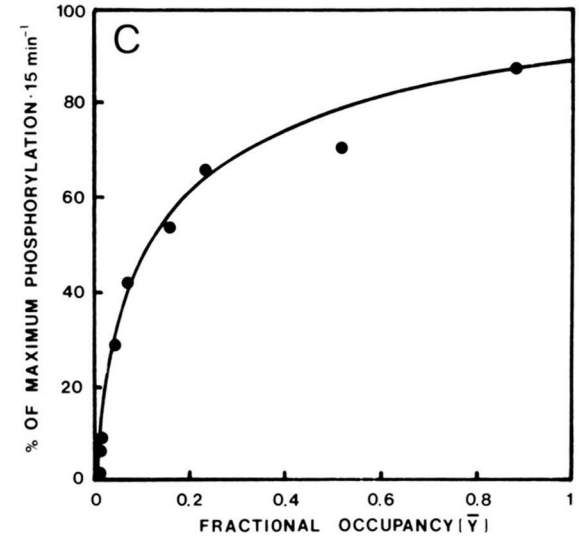


FIG. 8. **Relationship between the insulin binding and phosphorylation of the erythrocyte insulin receptor.** *A*, insulin-binding characteristics of the receptor determined in conditions similar to the kinase assay: 1.5 mg/ml of protein of detergent extract, 0.8% Triton X-100 and addition of $MnCl_2$ and ATP. The experimental points of five experiments were plotted in the same graph as described in Fig. 1. A simulated curve using a cooperative model was obtained in Scatchard plot giving the values from Table I ($\bar{K}_e$ = 1.3 × 10^8 M^{-1}, α = 0.024 and R_0 = 0.87 pmol·mg^{-1} insulin). *B*, the fractional occupancy of receptor ($\bar{Y}$) expressed in per cent and the per cent of maximum phosphorylation (at 15 min) of the β-subunit of the receptor is plotted against the log of insulin concentration. *C*, coupling plot of phosphate incorporated at 15 min.

5–10 ng/ml of insulin. The stoichiometric binding parameters obtained by statistical curve fitting of the binding data are shown in Table I and indicate that the decrease in binding was due primarily to a reduction in receptor affinity. A simulated curve in Scatchard plot using the binding parameters for a cooperative model is shown in Fig. 8*A*. Both affinity constants were decreased but the effect is marked more on the $\overline{K}_f$ (filled receptors).

If one directly compares the dose-response curve for phosphorylation and the fractional occupancy of the receptor measured under identical conditions, half-maximal stimulation of phosphorylation occurred at 6–29% of total occupancy of receptor, regardless of the model chosen for analysis of the data (Fig. 8*B*). Thus, the coupling (42) between phosphorylation measured at this time point and the occupancy was nonlinear and convex upward (Fig. 8*C*). Since insulin enhances both the rate and extent of phosphorylation of the receptor, further studies will be needed to analyze the coupling in terms of initial reaction rates.

DISCUSSION

Since our initial demonstration that the insulin receptor undergoes an insulin-stimulated phosphorylation reaction (6–10), an increasing body of evidence has suggested that the insulin receptor is a hormone-stimulated tyrosine-specific protein kinase (11–15). Both the insulin binding and kinase activity appear to be intrinsic to the receptor, and thus, both copurify with the receptor (13), both are retained in receptor preparations purified to near homogeneity (12), and both are present in the receptor after precipitation with anti-receptor antibodies (12). These two functions of the receptor appear to be contained in two distinct subunits. The α-subunit of the receptor which has a M_r = 135,000 (47) is labeled by insulin affinity labeling techniques (48), suggesting that it contains the insulin-binding site. The β-subunit, on the other hand, has a M_r = 95,000 and appears to contain the protein kinase activity. The subunit can be affinity labeled by ATP (15) and analogues of ATP (16, 17) and undergoes an insulin-stimulated phosphorylation. In the native receptor these two subunits are linked by disulfide bonds to form a complex or complexes with $M_r > 300{,}000$.

Although insulin receptors have been demonstrated on almost all mammalian cells, thus far most studies of the structure of the receptor and its kinase properties have been performed using receptors extracted from solid tissues such as human placenta, liver or adipose tissue, or from cultured cell lines. The human erythrocyte has been shown to possess insulin receptors (26), but these have received little chemical characterization. Such studies are important, however, for several reasons. First, erythrocytes are one of the few tissues readily and repeatedly accessible for studies of physiology and pathophysiology in humans. Secondly, the membrane of the red cell is well characterized in terms of protein structure and enzymatic activity, and thus the interaction of the insulin-receptor kinase with other membrane proteins and kinases can be studied in a simplified system. The preparation of solubilized receptors from human erythrocytes presents some difficulties due to the small number of insulin receptors on these cells (30). Hara *et al.* (31) have reported some characteristics of the insulin receptor solubilized from human red cells and also showed that the solubilized erythrocyte receptor has a $M_r \cong 350{,}000$ by gel filtration, suggesting that it is chemically similar to the receptor in other cellular systems (49).

In the present study we have prepared a solubilized insulin receptor from human erythrocyte membranes with a very high yield and which retained all binding properties of the native receptor. Insulin binding yielded curvilinear Scatchard plots. The Hill coefficient for binding was less than unity indicating heterogeneity in binding sites or cooperative interaction of negative type, similar to our studies in the intact cell. Further analysis using a cooperative model or a model for two independent binding sites indicated that based on statistical criteria, all three rival mathematical models adequately describe the data. The curvilinearity of Scatchard plot was not due to tracer heterogeneity since these studies were performed with a homogeneous tracer, the ^{125}I-[Tyr^{A14}]-monoiodoinsulin. As in other systems (44), increasing the Triton concentration resulted in a decrease of insulin binding due to a decrease in receptor affinity.

When the receptor preparation was incubated *in vitro* with [γ-^{32}P]ATP and Mn^{2+}, an insulin-dependent phosphorylation of the M_r = 95,000 subunit was observed. This protein has been identified as the β-subunit of the insulin receptor by three criteria. 1) It is immunoprecipitated by antibodies to the insulin receptor; 2) its migration in NaDodSO$_4$/PAGE is identical with that of the β-subunit identified by various techniques in other cellular systems; and 3) its phosphorylation is stimulated several-fold by addition of insulin. As in other systems studied, (7, 11, 13) the phosphorylation occurs primarily, if not exclusively, on tyrosyl residues.

The mechanism by which insulin activates its receptor kinase is unknown. It has been suggested that some conformational changes occur in the receptor upon insulin binding (11, 18), and this may also be responsible for the phenomenon of "negative cooperativity" and/or signal transmission by the receptor (50). In this sense, it was interesting to investigate the possibility of kinase activation using a noncooperative analogue of insulin, DAA insulin. DeMeyts (46) has previously shown the DAA insulin is unable to increase the dissociation of insulin from the receptor, *i.e.* it is "noncooperative." Our data show that the DAA insulin is able to fully stimulate the receptor phosphorylation suggesting that the mechanisms involved in negative cooperativity differ from those involved in activation of the receptor kinase or that the receptor is positive cooperative stimulated even in the presence of a negative cooperative binding of the ligand. Furthermore, when we attempted to correlate the phosphorylation with the fractional occupancy of the receptor, half-maximal phosphorylation was observed at a fractional occupancy of 6–29% suggesting that the coupling between binding and kinase activation is nonlinear and implicating some amplification steps in the transmission of the signal beyond the receptor. Since, in these particular experiments, the coupling was measured at one time point only, further studies will be necessary to establish how the coupling of the occupied receptor is related in the rates of activation of the kinase.

The relationship of the insulin receptor kinase to other kinases in the erythrocyte and the potential implications for insulin action in red cells must also be considered. Red cells are devoid of protein synthesis and respond to metabolic changes by either allosteric or covalent modifications (51). Indeed, human erythrocytes possess several protein kinases which act on red cell proteins as substrates. A cyclic AMP-dependent protein kinase has been identified in erythrocytes which is capable of phosphorylating several minor membrane polypeptides, such as band 2.1, 4.1, 4.5, and 4.8 (20, 23, 52). Red cells also possess a cyclic AMP-independent protein kinase which has been implicated in the phosphorylation of spectrin (the major component of erythrocyte cytoskeleton) and the proteins of band 2.1, 2.2 and band 3 (19, 52). Another kinase which has been studied in erythrocytes is a membrane-

bound protein kinase which is able to phosphorylate band 3. This kinase requires Mg-ATP for the activity and also acts on casein, but not histone, as exogenous substrates (52). These kinases have all acted primarily on serine or threonine residues. The present study also indicates that erythrocytes possess a tyrosine-specific insulin-dependent protein kinase which phosphorylates the insulin receptor. This kinase activity is likely present in the insulin receptor itself and is in a position to interact with the other kinases or proteins of the red cell.

One particular protein which deserves discussion in regard to the insulin receptor is the anion transport protein present in band 3 of the erythrocyte. The molecular weight of the β-subunit of the insulin receptor-kinase after reduction is 95,000, and accordingly, in gel electrophoresis, would be included in the band 3 region, which is a broad protein extending from M_r = 88,000 to 105,000 (53). Band 3 has been shown to undergo phosphorylation primarily at serine and threonine residues (24), but recently also has been found to possess phosphotyrosine (25). It seems unlikely that the phosphoprotein observed in our study is band 3 since it was specifically precipitated by antibody to the insulin receptor, since the phosphorylation was stimulated by insulin *in vitro* and since there is little or no phosphoserine in this protein on phosphoamino acid analysis. If the phosphoprotein was band 3 isolated by cross-reactivity with anti-receptor antibody, one would have expected phosphoserine and phosphothreonine as major phosphoamino acids in this protein. On the other hand, we cannot exclude the possibility that the phosphorylated insulin receptor might contaminate an analysis of band 3. As noted above, band 3 is broad and would include the region of the insulin receptor β-subunit. Tyrosine phosphorylation of band 3 has been reported to occur on a M_r = 23,000 fragment from the cytoplasmic domain near the NH_2-terminal region of the protein (25). Whether such a fragment could have been generated from the insulin receptor will need further study.

In summary, we have shown that the insulin receptor of the human erythrocyte can be solubilized and that both its binding and kinase properties can be studied *in vitro*. Since the proteins of the red cell are relatively few in number and have been well characterized, this provides the opportunity to study the interaction of the insulin receptor with other proteins in this cell. In addition, the method presented here opens the way for investigation of the insulin receptor kinase of erythrocytes since the red cells are an easily accessible tissue and full dose-response curves for both binding and phosphorylation can be performed from 100 ml of blood. Finally, by comparing the binding and kinase activity of the insulin receptor solubilized from red cells, a possible "functional marker" of insulin action becomes available using circulating blood cells.

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