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THE INSULIN RECEPTOR: ITS STRUCTURE AND FUNCTION

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During the last 60 years much effort has been spent in trying to unravel the mechanism of action of insulin. Many of the final effects of this peptide hormone on carbohydrate, lipid, and protein metabolism have been well studied [1], but a conclusive explanation at the molecular level of how insulin elicits its complex array of cellular responses in target tissues is still lacking [2, 3]. However, it is now widely accepted that the first step in insulin action is binding to specific receptors in target cell surface membranes [4, 5]. Recently, a uniform picture of the insulin receptor has emerged from studies that employed a variety of methods [6–16]. Further, in the last two years a promising advance has been made to contribute to our understanding of the transfer of information from the hormone receptor to the cell by the demonstration that insulin stimulates the phosphorylation of its own receptor, both in intact cells and in cell-free systems [17–21]. In this review, we will briefly recount our data on the structural features of the insulin receptor, and on its phosphorylation.

STRUCTURE OF THE INSULIN RECEPTOR

1. *Covalent binding of* ^{125}I-*insulin*

To bind ^{125}I-insulin covalently to its receptor we used the technique originally described by Pilch and Czech [7] and adapted by us to the human cultured lymphocyte of the IM-9 line [10]. When ^{125}I-insulin was covalently cross-linked to human lymphocyte membranes by disuccinimidyl suberate, SDS-PAGE analysis under reducing conditions revealed a major ^{125}I-labeled band of M_r 130,000 and minor bands of 300,000 and 95,000 (Fig. 1). To assess the specificity of these bands, ^{125}I-insulin was crosslinked to lymphocyte membranes in the presence of the following unlabeled hormones: insulin, human growth hormone, and ovine placental lactogen (Fig. 1). The two latter hormones were chosen since it is known that the IM-9 lymphocyte has receptors for them. Insulin abolished both the M_r 130,000 and 95,000 bands in a dose-dependent manner, but neither human growth hormone nor ovine placental lactogen had any effect on their labeling. When ^{125}I-insulin was crosslinked in the presence of anti-receptor IgG from the sera of four patients with the syndrome of severe insulin resistance and acanthosis nicrigans, the two bands disappeared in accord with the titer of antibodies [2, 22–24]. In contrast, IgG from normal control sera had no effect on the labeling of the two bands (Fig. 1). Labeling of the M_r 300,000 band was specifically inhibited by insulin and anti-receptor antibody, suggesting that it may represent a high M_r aggregate containing the M_r 130,000 subunit or an incompletely reduced oligomeric receptor.

To address the question of whether the insulin-receptor complex can be immunoprecipitated, ^{125}I-insulin was first cross-linked to lymphocyte membranes, the membranes were solubilized, and thereafter exposed to antireceptor IgG. Resolution of the immunoprecipitate in SDS-PAGE again revealed a major band of M_r 130,000 and a minor band of 95,000. No radioactivity was precipitated by normal IgG. Most importantly, preincubation of solubilized ^{125}I-insulin receptor complexes with an excess of insulin inhibited subsequent immunoprecipitation of the two bands, whereas preincubation with human growth hormone was without effect.

To examine directly which membrane proteins interact with the antibody, ^{125}I-labeled monovalent fragments of anti-receptor IgG (^{125}I-Fab) were crosslinked to lymphocyte membranes. SDS-PAGE of these preparations revealed a major band of M_r 180,000 and a minor band of 125,000, in addition to a dense area of radioactivity at 50,000 corresponding to Fab fragments. Neither band was observed when ^{125}I-Fab fragments were crosslinked to membranes in the presence of excess unlabeled insulin or unlabeled anti-receptor IgG. We believe that the M_r 180,000 band represents a complex between the Fab-fragment (M_r 50,000) and the insulin binding site of the M_r 130,000 receptor subunit and the minor band 125,000 may represent a complex between Fab and the minor M_r 95,000 band documented above.

In summary, covalent crosslinking of ^{125}I-insulin to cell membranes reveals in SDS-PAGE under reducing conditions two species specific for the insulin receptor: a major band with a M_r of 130,000 and a minor band with a M_r of approximately 95,000. Anti-receptor IgG inhibits the formation of the M_r 130,000 and 95,000 insulin-receptor complexes identified by covalent crosslinking of ^{125}I-insulin, and will precipitate preformed covalent ^{125}I-insulin-receptor complexes. Crosslinking of ^{125}I-anti-receptor Fab to membranes results in complexes that are consistent with the binding of a M_r 50,000 Fab to both receptor subunits. Taken together, these data indicate that a major fraction of the autoantibodies to the insulin receptor are directed against determinants within the insulin binding sites of an oligomeric receptor.

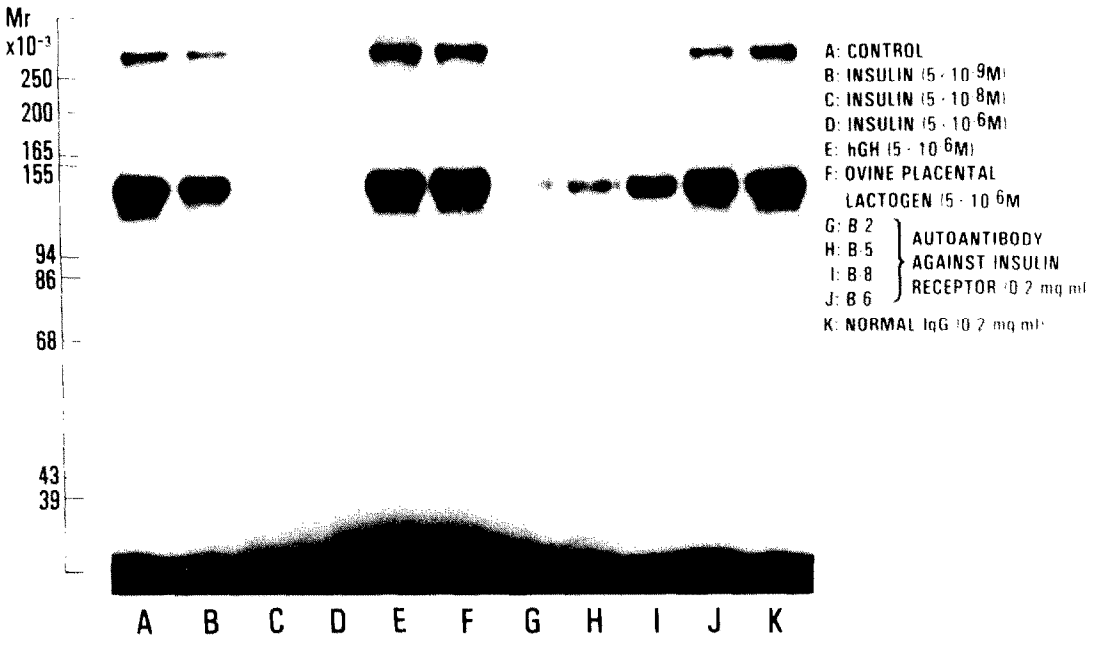

Fig. 1. Specificity of ^{125}I-insulin-receptor complex crosslinked to human lymphocyte membranes. The lymphocyte membranes were incubated for 90 min at 15° with 5 nM ^{125}I-insulin in the absence (A) and the presence of insulin (B) 5×10^{-9} M, (C) 5×10^{-8} M, (D) 5×10^{-6} M; human growth hormone (E) 5×10^{-6} M; ovine placental lactogen (F) 5×10^{-6} M; 0.2 ng/ml anti-receptor IgG (G) patient B-2, (H) patient B-5, (I) patient B-8, (J) patient B-6 or 0.2 ng/ml normal control IgG (K), crosslinked by disuccinimidyl suberate, and electrophoresed under reducing conditions (100 mM DTT) followed by staining, drying and autoradiography.

2. *Labeling of the protein moiety of the insulin receptor*

2.1. *Biosynthetic labeling of the insulin receptor.* To label the receptor biosynthetically IM-9 lymphocytes are cultured in the presence of ^{35}S-methionine; the cells are solubilized, and the labeled glycoproteins are partially purified on a wheat germ agglutinin agarose column. This lectin allows a 20-fold purification of the insulin receptor with nearly 100% recovery of the receptor as determined by ^{125}I-insulin binding [11, 13, 14]. The eluted glycoproteins are precipitated by anti-receptor antibodies. Finally, the immunoprecipitate is subjected to SDS-PAGE under reducing conditions and the labeled protein bands are identified by autoradiography. When the solubilized labeled glycoproteins were immunoprecipitated with serum from a normal individual, a few minor bands were observed ranging in M_r from 25,000 to 150,000 (Fig. 2, lane A). Immunoprecipitation with serum (B-2) containing autoantibodies against the insulin receptor revealed two major additional bands of M_r 130,000 and 90,000, and a minor band of M_r 200,000 (Fig. 2, lane B). Evidence that these labeled polypeptides are indeed subunits of the insulin receptor includes the fact that their immunoprecipitation is blocked by unlabeled insulin and that they disappear after "down-regulation" of cells, which markedly decreases the number of insulin receptors [11, 14]. Further, sera obtained from four different patients with anti-receptor antibodies all precipitate the M_r 130,000 and 90,000 bands [11].

2.2. *Cell surface labeling.* To label cell surface proteins, IM-9 lymphocytes were exposed to Na^{125}I and lactoperoxidase [14, 15]. The cells were solubilized and insulin receptors were partially purified by wheat germ lectin chromatography. Finally, they were quantitatively immunoprecipitated using autoantibodies against insulin receptors. Analysis of the immunoprecipitates by SDS-PAGE under reducing conditions followed by autoradiography revealed specific precipitation of two major bands with M_r 130,000 and 90,000. This is identical to the subunit composition of the insulin receptor found with biosynthetic labeling using ^{35}S-methionine.

3. *Labeling of the carbohydrate moiety of the insulin receptor*

3.1. *Biosynthetic labeling of the insulin receptor.* The technique used to label biosynthetically the carbohydrate moiety of the insulin receptor is identical to the one applied to label the proteins except that tritiated monosaccharides replace the ^{35}S-methionine [16]. In brief, cells grown in medium containing D-(^{3}H)glucosamine, L-(^{3}H)fucose, D-(^{3}H)galactose, and D-(^{3}H)mannose were solubilized; the extracts were subjected to lectin chromatography and immunoprecipitation. Analysis of the biosynthetically labeled insulin receptor by SDS-PAGE of the anti-receptor serum precipitate showed the presence of two major labeled subunits of M_r 134,000 ± 2000 (S.D., N = 3) and 98,000 ± 1500 (S.D., N = 3), and a minor component of molecular weight

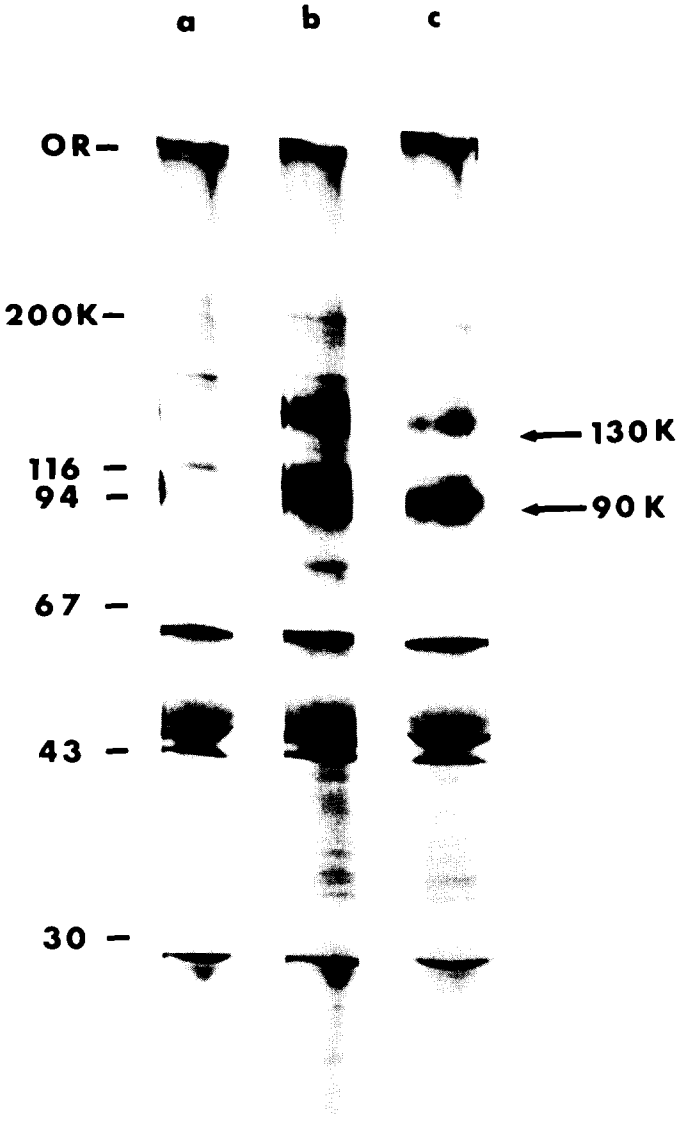

Fig. 2. Immunoprecipitation of ^{35}S-labeled subunits of the insulin receptor. Cells were labeled with ^{35}S-methionine, solubilized with Triton X-100, the insulin receptors were purified on wheat germ agglutinin–agarose and immunoprecipitation was performed. After solubilization the immunoprecipitates were electrophoresed in a 5–15% linear acrylamide gradient under reducing conditions. The gels were dried and subjected to autoradiography. The figure shows an autoradiograph of the gel. The different lanes correspond to the following immunoprecipitation conditions: a, normal serum (1/400 dilution or 25 μg/ml IgG); b, anti-receptor serum B-2 (1/800 dilution or 25 μg/ml IgG); c, preincubation with insulin (1.7×10^{-6} M) followed by immunoprecipitation with anti-receptor serum B-2 (1/800 dilution).

206,000 ± 2500 (S.D., N = 3) (Fig 3, lane a). The control serum precipitated only two minor bands of M_r 116,000, and 40,000 (lane b), which were also observed with the antireceptor serum. The ratio of activity in the M_r 134,000 band versus that in the M_r 98,000 band was 1.8:1 for (^{3}H)glucosamine, 1.9:1 for (^{3}H)fucose and 2:1 for (^{3}H)mannose. A somewhat lower ratio was observed with galactose (1.2:1). In contrast to the carbohydrate labeling, which always resulted in the M_r 134,000 subunit being more prominent, protein labeling with (^{35}S)methionine preferentially labels the M_r 98,000 band (M_r 134,000/M_r 98,000 = 0.3:1).

To further evaluate the specificity of the immunoprecipitation of the labeled bands, the samples were preincubated with insulin before immunoprecipitation. Insulin effectively inhibited immunoprecipitation of the receptor subunits. Two other findings indicate that the labeled glycoproteins are subunits of the insulin receptor. Firstly, sera from five different patients with antibodies to the insulin receptor precipitated the same labeled bands, and the extent

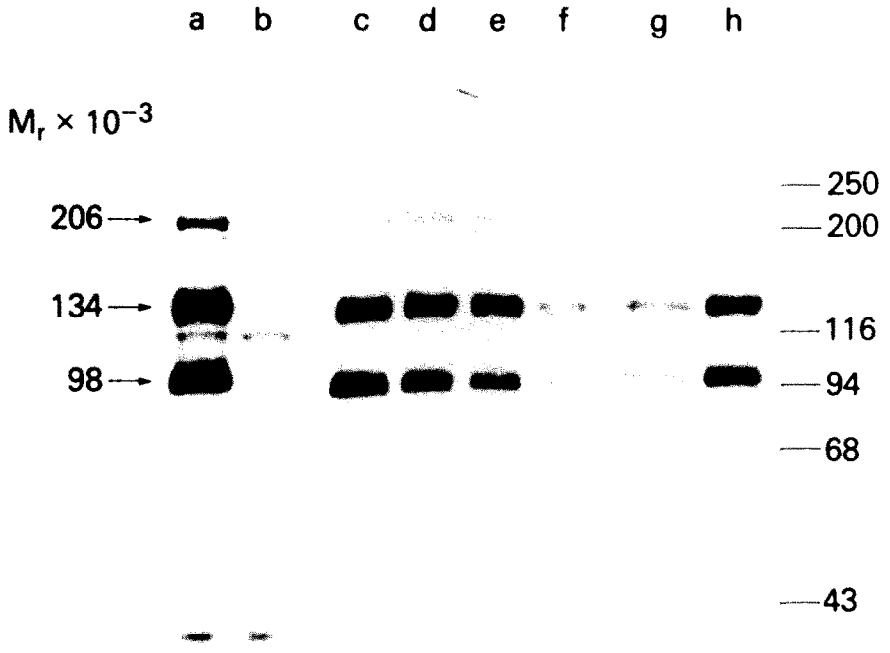

Fig. 3. SDS-PAGE and autoradiography of (^{3}H)glucosamine-labeled insulin receptors. IM-9 lymphocytes were cultured in the presence of (^{3}H)glucosamine and solubilized in Triton X-100; after partial purification on wheat germ agglutinin–agarose, the insulin receptors were immunoprecipitated with serum containing anti-receptor antibodies. The immunoprecipitates were solubilized with SDS/DTT/mercaptoethanol and subjected to electrophoresis in SDS/polyacrylamide gels. (Left) Immunoprecipitation with antireceptor serum from patient B-2 (lane a) and with nonimmune control serum (lane b). Both sera were diluted 1:400. (Middle) Effect of insulin on immunoprecipitation by anti-receptor antibodies. Anti-receptor serum B-2 was used at dilutions of 1:400 (lanes c and d) or 1:800 (lanes e and f). Prior to immunoprecipitation, some labeled samples were incubated with 1.7×10^{-6} M insulin for 12 hr at 4° (lanes d and f). Samples not incubated with insulin are shown for comparison (lanes c and e). (Right) Effect of down-regulation of insulin receptors on labeling of receptor subunits. IM-9 lymphocytes were incubated with (^{3}H)glucosamine in the absence (lane h) and the presence (lane g) of 0.1 μM insulin for 12 hr at 37°, which resulted in 70% decrease in receptor concentration as measured by ^{125}I-insulin binding. Subsequently, the cells were washed extensively to remove bound insulin, and processed as described above. Immunoprecipitation was performed with antireceptor serum B-2.

of precipitation correlated well with their titer of antireceptor antibodies. Secondly, the decrease in the number of insulin receptors observed in down-regulation of the insulin receptor was accompanied by a decrease in the labeling of these bands.

3.2. *External labeling of insulin receptors.* To study the external carbohydrate moiety of the insulin receptors the cell surface of intact lymphocytes was labeled with NaB^3H_4 using either the galactose oxidase (acts on nonreducing terminal galactose and *N*-acetylgalactosamine) technique or the periodate (oxidises sialic acid) technique [16]. Using the galactose oxidase method the incubation with galactose oxidase alone followed by borohydride reduction labeled only the 134,000 molecular weight subunit of the insulin receptor. Since sialic acid residues often are linked to penultimate galactosyl groups, increased labeling efficiency has been achieved for different cell surface glycoproteins by treating the cells with neuraminidase to remove sialic acids prior to labeling with galactose oxidase. Similar observations were found with the insulin receptor. Indeed, treatment with neuraminidase and galactose oxidase enhanced the labeling of the M_r 134,000 subunit, and revealed also the M_r 98,000 subunit, as well as the M_r 206,000 component.

To reveal the terminal sialic acid residues, IM-9 lymphocytes were labeled by using the periodate/NaB^3H_4 method. This technique labeled preferentially the M_r 98,000 subunit, although a faint M_r 134,000 band was also observed. Treatment of the cells with neuraminidase prior to labeling, leads to a decreased labeling of the M_r 98,000 band (a 35% decrease) and the disappearance of the M_r 134,000 component. Thus, as reported for other glycoproteins [25] sialic acid residues, that are radiolabeled by this method, may not all be accessible to neuraminidase.

Summary

Using both biosynthetic and external labeling techniques we have demonstrated that the insulin receptor consists of two major subunits with a molecular weight of approximately 135,000 and 95,000. These two major subunits are glycoproteins. Note that with the biosynthetic labeling using either ^{35}S-methionine [11, 14], or tritiated monosaccharides [16] we found routinely a minor component with a molecular weight of 210,000. Recent studies suggest that this M_r 210,000 component is a high-mannose type precursor of both the M_r 135,000 and 95,000 subunits of the insulin receptor [26]. Our data and observations from other laboratories have led to the suggestion that the insulin receptor is a heterodimer of the 135,000 and 95,000 subunit, and has in fact an immunoglobulin-like structure with heavy and light chains held together by disulfide bonds [10, 27, 28] (Fig. 4). Both subunits of the insulin receptor contain carbohydrate, whereas only the heavy chains of the immunoglobulin molecule are glycosylated. The incorporation of all four labeled monosaccharides (fucose, mannose, galactose, and glucosamine) into the two major subunits of the insulin receptor suggests that both are likely to contain carbohydrate chains of the complex, N-linked type [16]. The external labeling techniques demonstrate that both subunits possess a portion of their protein and carbohydrate moiety exposed at the external cell surface. Further, the labeling of the external oriented carbohydrates revealed remarkable differences in the nonreducing termini of the carbohydrate chains of both major subunits. Indeed, the galactose oxidase, NaB^3H_4 method preferentially labeled the M_r 134,000 subunit, whereas the periodate/NaB^3H_4 method preferentially labeled the M_r 95,000 subunit.

PHOSPHORYLATION OF THE INSULIN RECEPTOR

The observation that the entire spectrum of metabolic effects (both acute and late) of insulin can be initiated by the interaction of ligands other than insulin (i.e. autoantibodies to insulin receptor) with the receptor has been taken as evidence that the receptor contains all the necessary information for insulin action [5, 29]. Further, information has been gathered suggesting that insulin action results in phosphorylation–dephosphorylation reactions in some cellular proteins [3].

Based on this series of observations an important question arose as to whether the receptor itself possesses an enzymatic activity leading to phosphorylation–dephosphorylation, or is a substrate for such enzymatic activity.

1. *Phosphorylation of insulin receptors in intact hepatocytes*

To investigate whether the insulin receptor can be phosphorylated in intact hepatocytes, isolated rat hepatocytes were first incubated with ^{32}P-orthophosphate and then without or with insulin. After solubilization and partial purification by lectin chromatography insulin receptors were specifically precipitated with anti-receptor antibodies and analyzed by SDS-PAGE under reducing conditions. When solubilized labeled glycoproteins obtained from cells incubated in the absence of insulin were precipitated with antireceptor antibodies a major labeled band was found with M_r 95,000 [20]. We recognize this molecular species as the β-subunit of the insulin receptor for the following reasons. First, nonimmune serum did not precipitate a labeled band with a similar electrophoretic mobility. Second, using biosynthetic and external labeling methods, we have previously identified the β-subunit of the insulin receptor with identical electrophoretic mobility. Insulin induced a small, but consistent increase in the labeling of its own receptor subunit. Thus, quantitative scanning reveals a 30–50% insulin-induced stimulation of the β-subunit phosphorylation. This effect of insulin is specific, since epidermal growth factor, which binds to and increases the phosphorylation of its own receptors in these cells, was without effect on insulin receptor phosphorylation. Phosphoamino acid analysis of the β-subunit of the insulin receptor revealed the presence of phosphothreonine and phosphoserine when the cells were labeled in the absence of insulin. Further, the stimulatory action of insulin on receptor phophorylation is accounted for by an increase in phosphoserine [30].

2. *Phosphorylation of insulin receptors in cell-free systems*

To further characterise the molecular basis of the insulin receptor phosphorylation we have used a cell-free system. In brief, partially purified insulin receptors were incubated with (γ-^{32}P)ATP in the absence or presence of insulin. Thereafter, the insulin receptors were specifically precipitated with antireceptor antibodies. SDS-PAGE analysis of the immune precipitates under reducing conditions revealed that a major band of M_r 95,000 was phosphorylated in the basal state [20] (Fig. 5). Again, this M_r 95,000 phosphoprotein was identified as the β-subunit of the insulin receptor by its specific precipitation by antireceptor antibodies and by its electrophoretic mobility, which was identical to that found for the β receptor subunit labeled by other techniques [11, 14–16]. Similar to its effect in intact cells, insulin increased 2–4-fold the phosphorylation of its M_r 95,000 receptor subunit [20]. Further, the dose-relationship of insulin's effect on phosphorylation of

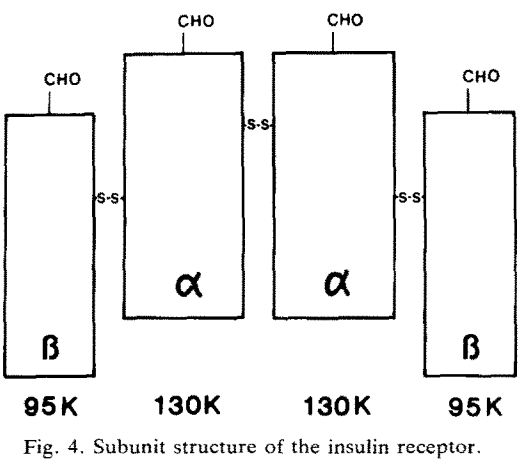

Fig. 4. Subunit structure of the insulin receptor.

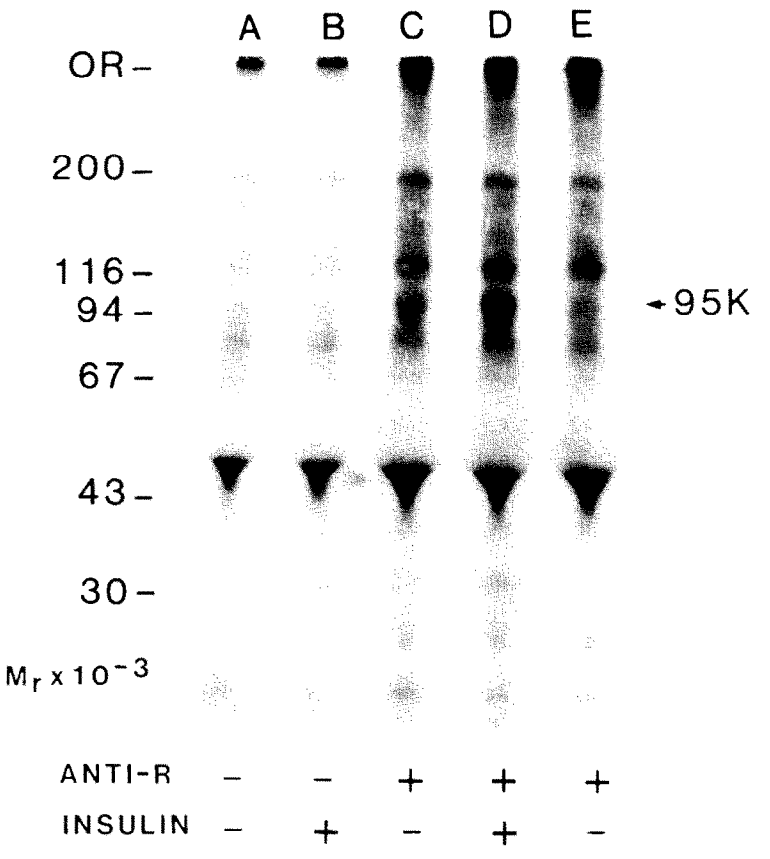

Fig. 5. Phosphorylation of M_r 95,000 insulin receptor subunit in a cell-free system: purified insulin receptors were incubated with (γ-^{32}P)ATP in the absence (a, c) or presence of insulin (10^{-7}M) (b, d), EGF (10^{-7}M) (e). Labeled insulin receptors were then exposed to anti-receptor serum (c–e) or normal serum (a, b). The precipitates were analyzed by SDS-PAGE under reducing conditions followed by autoradiography.

its receptor subunit was remarkably similar to that previously described in our laboratory for insulin binding to the high affinity component in these hepatocytes. This stimulatory action of insulin is specific since EGF and glucagon had no effect. The stimulatory action of insulin on its receptor phosphorylation has therefore the typical affinity and specificity of a receptor-mediated insulin effect.

We next addressed the question of the physical interrelationship between the insulin receptor and the kinase phosphorylating the receptor. We first investigated whether the insulin receptor isolated by specific immunoprecipitation with anti-receptor antibodies is a substrate for phosphorylation [21]. Figure 6 shows that this is indeed the case. More important, insulin increased 2-fold the phosphorylation of the isolated insulin receptor indicating that antireceptor antibodies precipitate a functional insulin-stimulable kinase activity, which is contained in, or closely associated with the receptor. Insulin's effect on its own receptor phosphorylation appears to be independent of cyclic AMP and calcium [21]. To localize more precisely the kinase activity phosphorylating the insulin receptor we searched for a potential ATP binding site on the insulin receptor using covalent affinity labeling [21]. When partially purified insulin receptors were first incubated with oxidized (α-^{32}P)ATP and $NaBH_3CN$, and then subjected to immunoprecipitation, a unique and major labeled polypeptide of M_r 95,000 was precipitated with anti-receptor antibodies We recognize this polypeptide as the β-subunit of the insulin receptor based on its electrophoretic mobility and the established specificity of the antireceptor antibodies.

The coexistence of an ATP binding site and an insulin-stimulable phosphorylation site on the β-subunit of the insulin receptor suggests that the insulin receptor itself is a protein kinase capable of self-phosphorylation. Recently, we were able to show that the insulin receptor is not only a protein kinase toward itself, but is also capable of catalysing the phosphorylation of exogenous protein substrates. Further, in cell-free systems the insulin receptor is phosphorylated on tyrosine-residues, and insulin stimulatory action on phosphorylation of its own receptor and exogenous protein substrates is largely accounted for by an increase in phosphotyrosine [17, 30, 31].

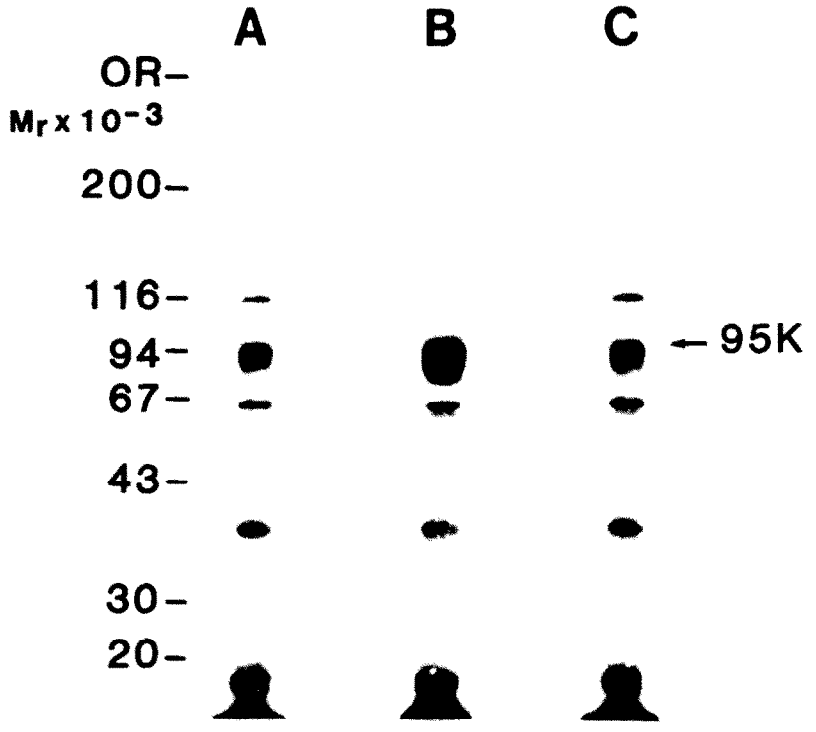

Fig. 6. Immunoprecipitation of an insulin-stimulated activity phosphorylating the insulin receptor. Partially purified insulin receptors were precipitated with anti-receptor antibodies at a 1:800 serum dilution. The precipitates were resuspended in the phosphorylation buffer supplemented with buffer (A), insulin (10^{-6}M) (B), catalytic subunit of cAMP dependent protein kinase (10^{-7}M) (C). After phosphorylation with (γ-^{32}P)ATP the precipitates were analyzed by SDS-PAGE under reducing conditions followed by autoradiography.

CONCLUSIONS

The insulin receptor is a high molecular weight integral membrane glycoprotein consisting of two major subunits with a M_r 130,000 (α-subunit), and M_r 95,000 (β-subunit). The two subunits seem to have distinct functions. Thus, the α-subunit contains the insulin binding site, whereas the β-subunit is an insulin-activated tyrosine-specific protein kinase. It is an appealing idea to envision that the insulin-receptor-kinase complex functions as an integrated system for transfer of hormonal information across the cell membrane. In this model the binding of insulin to its receptor stimulates the protein kinase activity of the receptor itself leading to autophosphorylation. The activated receptor protein kinase would then provoke the phosphorylation of cellular proteins, and through a cascade of phosphorylation–dephosphorylation reactions this would eventuate in the effects of insulin. Major challenges for the significance of this concept are the identification of native substrates of the insulin receptor kinase and the demonstration that receptor phosphorylation is a sufficient requisite for insulin to act.

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