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Receptor-mediated phosphorylation of the hepatic insulin receptor: Evidence that the M_r 95,000 receptor subunit is its own kinase

(autoantibodies against insulin receptors/covalent affinity labeling/ATP)

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ABSTRACT **Insulin stimulates the phosphorylation of its own receptor. In the work reported here, the kinase activity responsible for the insulin-stimulated phosphorylation of the insulin receptor was localized. In a first approach, partially purified insulin receptors derived from normal rat hepatocytes were immunoprecipitated with antibodies specific for the insulin receptor; thereafter, the immunoprecipitates were incubated with [γ-^{32}P]-ATP in the absence or presence of insulin (1 μM). NaDodSO$_4$/polyacrylamide gel electrophoretic analysis of the immunoprecipitates under reducing conditions revealed autophosphorylation of the β subunit (M_r 95,000) of the insulin receptor; the α subunit (M_r 130,000) was not phosphorylated. Further, insulin specifically increased 3- to 4-fold the labeling of its own receptor β subunit, indicating that anti-receptor antibodies precipitate a functional and insulin-stimulable protein kinase that appears to be independent of cyclic AMP and calcium. To localize more precisely the insulin receptor-related kinase activity, we searched for an ATP-binding site on solubilized insulin receptors. By using covalent labeling with oxidized [α-^{32}P]ATP, a labeled polypeptide with precisely the same electrophoretic mobility as that of the β subunit of the insulin receptor (M_r 95,000) was specifically immunoprecipitated with anti-receptor antibodies. Further, its appearance was prevented when the immunoprecipitation was preceded by incubation with unlabeled insulin. In conclusion, we have shown that an insulin-stimulated phosphorylation site and an ATP-binding site coexist on the β subunit of the insulin receptor. The simultaneous presence of these two sites on the same receptor subunit indicates that the insulin receptor acts as its own protein kinase.**

Insulin binds to its specific membrane receptors located on target cells and initiates a complex array of cellular responses. Although the initial step in insulin's action (i.e., binding to its receptor) is well characterized, very little is known concerning the ensuing signal transduction (1–4).

It now is widely accepted that hormone-modulated protein phosphorylation–dephosphorylation reactions play a crucial role in the regulation of cellular events, including those mediated by insulin (3–5). More recently, evidence has been presented that receptors themselves are the subject of phosphorylation reactions (6–12) and might in some cases be the mediator of the kinase reaction (12). Recent observations have added the insulin receptor to the list of receptors that participate in receptor-mediated phosphorylation reactions. Thus, the β subunit of the insulin receptor is found to be phosphorylated, and its level of phosphorylation, to be increased by insulin in rat hepatoma cells, cultured human lymphocytes (13), and isolated rat hepatocytes (14). This ligand-stimulated receptor phosphorylation is observed not only in intact cells (13, 14) but also in partially purified insulin receptors (14, 15).

In this report we address the question of whether the insulin receptor possesses intrinsic kinase activity. We show that (*i*) the immunoprecipitated β subunit of the insulin receptor is associated with a functional insulin-stimulated kinase activity and (*ii*) an ATP binding site can be identified on the β subunit of the insulin receptor by covalent affinity labeling.

EXPERIMENTAL PROCEDURES

Materials. Porcine insulin (monocomponent) and glucagon were purchased from Novo (Copenhagen, Denmark); epidermal growth factor was from Collaborative Research (Waltham, MA); collagenase (type II) was from Worthington; Nonidet P-40, adenosine 5′-triphosphate, *N*-acetyl-D-glucosamine, wheat germ agglutinin-agarose, bovine serum albumin (fraction V), and bacitracin were from Sigma. Adenosine 5′-[γ-^{32}P]triphosphate, triethylammonium salt (aqueous solution, 5,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq), and adenosine 5′-[α-^{32}P]triphosphate (460 Ci/mmol) were from the Radiochemical Centre (Amersham, U.K.). $NaBH_3CN$ was from Ega Chemie (Albuch, Federal Republic of Germany); Na metaperiodate, from Merck; *Staphylococcus aureus* cells (Pansorbin), from Calbiochem–Behring; and protein A-Ultrogel, from I.B.F. (Paris, France). All reagents for NaDodSO$_4$/polyacrylamide gel electrophoresis were purchased from Bio-Rad. Serum from patient B_2 with autoantibodies to the insulin receptor was a kind gift from C. R. Kahn (Joslin Research Laboratory, Boston, MA) (16, 17). Purified catalytic subunit of cyclic AMP-dependent protein kinase (type II), isolated from beef heart, was a kind gift from J. Demaille (Montpellier, France).

Preparation of Partially Purified Insulin Receptors. Hepatocytes were isolated from male Wistar rats (150–200 g) by collagenase dissociation of the liver as described by Le Cam *et al.* (18). Immediately after isolation, hepatocytes were washed twice with cold buffer containing Hepes (50 mM; pH 7.4), NaCl (150 mM), bacitracin (1 mM), aprotinin (1,000 trypsin inhibitor units/ml), and soybean trypsin inhibitor (10,000 trypsin inhibitor units/ml). Cells were collected by centrifugation, resuspended in the wash buffer containing Nonidet P-40 (0.5%) and deoxycholate (0.5%), and homogenized in a Dounce homogenizer (140 strokes). Thereafter, the broken cells were solubilized for 90 min at 4°C by continuous stirring. This preparation was centrifuged at 100,000 × *g* for 90 min at 4°C, and the insoluble fraction was discarded. The supernatant was applied to a wheat germ agglutinin-agarose column, which was extensively washed at 4°C; bound glycoproteins were desorbed with *N*-acetylglucosamine (0.3 M). This chromatography allows a 20-fold purification with nearly 100% recovery of the insulin receptor

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as determined by ^{125}I-labeled insulin binding. The two fractions, which are known to contain insulin receptors (19–22), were pooled.

Immunoprecipitation of Insulin Receptors. Insulin receptors were specifically immunoprecipitated with antiserum containing autoantibodies to the insulin receptor as described (21–23). In brief, solubilized insulin receptors (300 μl) were incubated for 6 hr at 4°C with anti-receptor antiserum at a 1:800 or 1:300 dilution, corresponding to an IgG concentration of 25 and 65 μg/ml, respectively. In control experiments, pooled normal (i.e., nonimmune) sera were used at corresponding final IgG concentrations. Immunoprecipitation with protein A was achieved by addition of *S. aureus* cells (Pansorbin) or protein A-Ultrogel. After 2 hr at 4°C, the immunoprecipitates were collected by centrifugation (5 min at 500 × *g* and 4°C), and the pellets were washed three times in 50 mM Hepes/150 mM NaCl.

Covalent Affinity Labeling of the Insulin Receptor by Oxidized [α-^{32}P]ATP. Oxidized [α-^{32}P]ATP was prepared as described by Randerath and Randerath (24). To bind oxidized [α-^{32}P]ATP to insulin receptors, partially purified receptors were incubated at 0°C with oxidized [α-^{32}P]ATP (≈0.1 μM) in the absence or presence of an excess of unlabeled ATP (100-fold). The reaction was allowed to proceed overnight at 0°C in the presence of $NaBH_3CN$ (10 mM). The reaction was stopped by the addition of an excess of unlabeled ATP. Thereafter, the insulin receptors were incubated with nonimmune serum or with serum containing antibodies to the insulin receptor at a 1:800 dilution as described above. In one experiment incubation with anti-receptor antiserum was preceded by an incubation of the ATP-labeled receptors with 1 μM unlabeled insulin for 4 hr at 4°C. The immunoprecipitates were collected, washed, and finally analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by autoradiography.

Phosphorylation of the Isolated Insulin Receptors. Partially purified insulin receptors were immunoprecipitated with either a 1:300 or a 1:800 dilution of serum containing antibodies to the insulin receptor. In control experiments insulin receptors were incubated with pooled normal sera. Immunoprecipitation was achieved by addition of protein A. The incubation tubes were centrifuged; in some experiments the supernatants were saved and subjected to immunoprecipitation with antibodies to the insulin receptor as described earlier. The immunoprecipitates were washed three times with 50 mM Hepes/150 mM NaCl, pH 7.6 and then resuspended in the phosphorylation buffer. For the phosphorylation of the immunoprecipitates, a typical reaction mixture (final volume, 325 μl) contained resuspended immunoprecipitate (300 μl), Hepes (20 mM; pH 7.6), $MnCl_2$ (2 mM), bovine serum albumin (0.125%), and, if present, insulin (1 μM). To allow for insulin binding, the tubes were incubated for 2 hr at 20°C. Thereafter, the phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP (2 μM). The reaction tubes were incubated for 5 min at 20°C. The reaction was terminated by addition of 65 μl of ice-cold stopping solution containing NaF and EDTA at final concentrations of 100 mM and 10 mM, respectively. The immunoprecipitates were collected by centrifugation and washed three times with ice-cold stopping solution. Aliquots of the immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Gel Electrophoresis and Autoradiography. The immunoprecipitates were boiled for 5 min in a solution containing 3% (wt/vol) $NaDodSO_4$, 10% glycerol (vol/vol), 10 mM sodium phosphate, 2% (vol/vol) 2-mercaptoethanol, and 0.01% bromophenol blue. Aliquots of the precipitates were analyzed by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Laemmli (25), with either a 7.5% acrylamide gel or a 5–15% linear gradient of acrylamide as the resolving gel. The M_rs of the standards used were: myosin, 200,000; β-galactosidase, 116,000; phosphorylase B, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000; and lysozyme, 14,400. The gels were stained, dried, and autoradiographed by exposing the gels to Kodak X-Omat film as described (14, 21). The autoradiograms were scanned in a microdensitometer (Gelman) for quantitative analysis.

RESULTS

Immunoprecipitation of the Functional Insulin Receptor Kinase Activity. We first investigated whether the insulin receptor, isolated by specific immunoprecipitation with anti-receptor antibodies, was a substrate for phosphorylation. Fig. 1 shows that this was indeed the case. Thus, when partially purified insulin receptors were exposed to serum from normal individuals and the immunoprecipitates were incubated with [γ-^{32}P]ATP, $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography showed a few minor bands with M_rs ranging from 30,000 to 116,000 (Fig. 1, lane A). Only one additional band of M_r 95,000 was found when the immunoprecipitate obtained with specific anti-receptor antibodies was phosphorylated (Fig. 1, lane B). This polypeptide

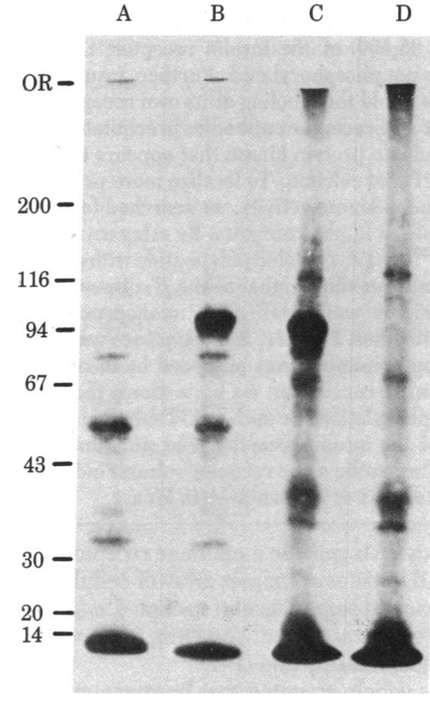

FIG. 1. Phosphorylation of immunoprecipitated insulin receptors. Partially purified insulin receptors were incubated with nonimmune serum (lane A) or serum containing antibodies to the insulin receptor (lane B) at a 1:300 dilution (i.e., IgG at 65 μg/ml). Thereafter, the IgGs were precipitated with protein A as described. Both supernatants were saved and subjected to immunoprecipitation with serum containing antibodies to the insulin receptor, and the immunoprecipitates were phosphorylated in the presence of [γ-^{32}P]ATP. The samples were then processed for $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions with a 7.5% acrylamide gel as the resolving gel. An autoradiograph of the gel shows the phosphorylated components of the immunoprecipitates. Lanes: A, precipitate obtained with nonimmune serum; B, precipitate obtained with anti-receptor antiserum; C, supernatant fluid obtained with nonimmune serum and subjected to immunoprecipitation with anti-receptor antiserum; D, supernatant fluid obtained with anti-receptor serum and subjected to a second immunoprecipitation with anti-receptor antiserum. M_rs are shown × 10^{-3}. OR, origin.

has precisely the same electrophoretic mobility as the β subunit of the insulin receptor identified by numerous techniques in various tissues, including hepatocytes (20–22, 26–31). As expected from previous demonstrations that these anti-receptor antibodies quantitatively precipitate the insulin receptor (21–23), the supernatant obtained with anti-receptor antiserum was totally devoid of insulin receptors that could be phosphorylated (Fig. 1, lane D). Conversely, in the supernatant obtained with nonimmune sera, a distinct capacity to phosphorylate insulin receptors was present (Fig. 1, lane C).

We next addressed the question of the interrelationship between the insulin receptor and the kinase phosphorylating the insulin receptor. To approach this problem, we investigated whether the highly specific anti-receptor antibodies precipitate an insulin-stimulable kinase. Partially purified insulin receptors were first specifically precipitated with a 1:800 dilution of anti-receptor antiserum, then incubated in the absence or presence of insulin (1 μM), and finally exposed to [γ-^{32}P]ATP. Insulin significantly increased the phosphorylation level of the β subunit of the insulin receptor (Fig. 2, lanes A and B). Quantitative scanning of the autoradiograph revealed a 3- to 4-fold insulin-induced increase in the labeling of the M_r 95,000 receptor subunit. This effect of insulin on its own receptor subunit was selective because the few other labeled bands were not at all affected by insulin. Furthermore, insulin's effects on its own receptor subunit was specific because epidermal growth factor and glucagon were without effect (data not shown).

In an attempt to characterize the kinase responsible for the phosphorylation of the insulin receptor, the effect of the catalytic subunit of the cyclic AMP-dependent protein kinase was investigated. Partially purified insulin receptors were immunoprecipitated with anti-receptor antiserum after addition of protein A. Thereafter, the insulin receptors were incubated with [γ-^{32}P]ATP in the absence or presence of the catalytic subunit at a 0.1 μM concentration. The phosphorylation of the M_r 95,000 receptor subunit was not increased by the catalytic subunit when compared to its basal phosphorylation (Fig. 2, lanes A and C). These data would indicate that insulin's effect on its own receptor is not mediated by cyclic AMP-modulated protein kinases. We next investigated the calcium dependency of insulin's stimulating action on its receptor phosphorylation; insulin's effect was unaltered in the absence or presence of calcium ions (Fig. 2, lanes D–G). Note that basal phosphorylation also was unaltered in the absence or presence of calcium ions (data not shown).

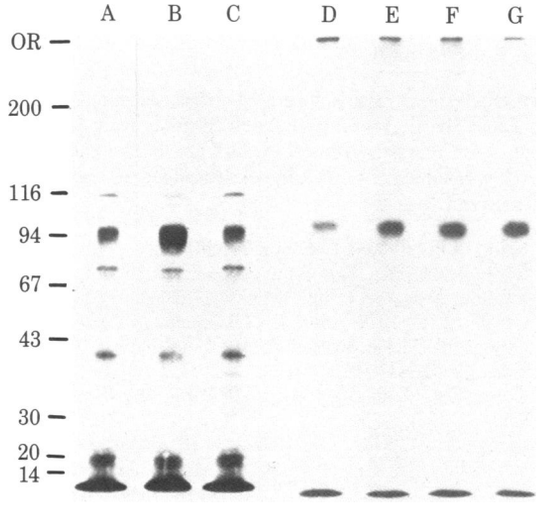

FIG. 2. Immunoprecipitation of an insulin-stimulated activity phosphorylating the insulin receptor. Partially purified insulin receptors were immunoprecipitated with antibodies to the insulin receptor as described in the legend to Fig. 1, except that a 1:800 serum dilution (IgG at 25 μg/ml) was used. Immunoprecipitation was achieved by addition of protein A; thereafter, the immunoprecipitates were resuspended as described in the phosphorylation buffer and the following additions. Lanes: A, buffer; B, insulin (1 μM); C, catalytic subunit of cyclic AMP-dependent protein kinase (0.1 μM); D, buffer; E, insulin (1 μM) and $CaCl_2$ (1 μM); F, insulin (1 μM) and $CaCl_2$ (200 μM); G, insulin (1 μM), EGTA (1 mM), and $CaCl_2$ (200 μM). The tubes were incubated for 2 hr at 20°C, followed by an incubation with [γ-^{32}P]ATP as described. Finally, aliquots of immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions. An autoradiograph of a 7.5% acrylamide gel is shown. M_rs are shown $\times 10^{-3}$. OR, origin.

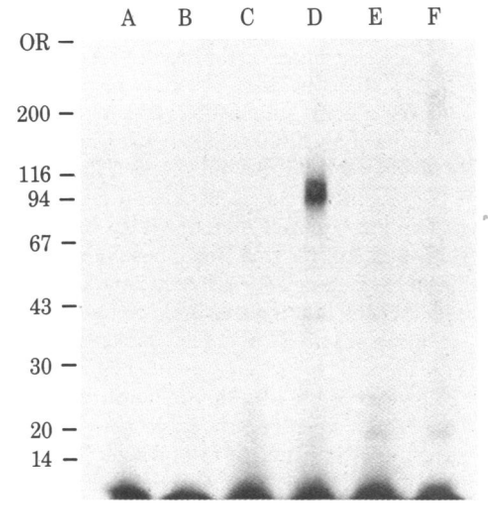

FIG. 3. Covalent affinity labeling of insulin receptors by oxidized [α-^{32}P]ATP. Partially purified insulin receptors were incubated with oxidized [α-^{32}P]ATP in the absence (lanes B, D, and F) or in the presence (lanes A, C, and E) of a 100-fold excess of unlabeled ATP. The reaction was allowed to proceed overnight at 0°C in the presence of $NaBH_3CN$ (10 mM) for the selective reduction of Schiff bases. At the end of the affinity labeling, buffer (lanes A, B, C, and D) or insulin (1 μM) (lanes E and F) was added for 4 hr at 4°C. Thereafter, the samples were incubated with nonimmune serum (lanes A and B) or anti-receptor antiserum (lanes C, D, E, and F) at a 1:800 dilution. Immunoprecipitation was achieved by addition of protein A-Ultrogel for 2 hr at 4°C. Finally, aliquots of the immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions. An autoradiograph of a gel with a 5–15% linear gradient of acrylamide as the resolving gel is shown. M_rs are shown $\times 10^{-3}$. OR, origin.

Covalent Affinity Labeling by Oxidized [α-^{32}P]ATP of the Insulin Receptor. To identify a potential ATP binding site on the insulin receptor, partially purified receptors were incubated with periodate-oxidized [α-^{32}P]ATP and $NaBH_3CN$ for the selective reduction of Schiff bases. A unique and major labeled polypeptide of M_r 95,000 was immunoprecipitated with antibodies to the insulin receptor (Fig. 3). This polypeptide was totally absent when an excess of unlabeled ATP (100-fold) was added to the incubation mixture, indicating the specific labeling of an adenosine-binding site. We have identified the M_r 95,000 polypeptide as the β subunit of the insulin receptor on the basis of its M_r and the two following observations. First, no radioactivity was found in this region when nonimmune serum was used instead of anti-receptor antiserum to precipitate the insulin receptors (Fig. 3, lanes A and B). Second, when the immunoprecipitation with anti-receptor antiserum was preceded by an incubation with insulin (1 μM), the M_r 95,000 labeled polypeptide was no longer precipitated (Fig. 3, lanes E and F).

DISCUSSION

Recently, a coherent picture of the insulin receptor structure has arisen from studies that used a variety of techniques (20–22, 26–31). To be specific, the insulin receptor consists of glycoprotein subunits of M_r 130,000 (α subunits) and M_r 95,000 (β subunits) linked together by disulfide bonds. The α subunit appears to be the subunit that binds insulin; the β subunit is likely to be the nonbinding subunit. Although it has been recognized for many years now that the first step in insulin's action is binding of the hormone to its cell-surface receptors, the signal transfer occurring after the binding step and ultimately resulting in the final actions of insulin is not clear. Recently, a promising advance has been made to further our understanding of insulin-regulated cellular processes by the demonstration that the β subunit of the insulin receptor is the subject of phosphorylation reactions, which are stimulated by insulin itself (13, 14). We also were able to show that this ligand-stimulated receptor phosphorylation occurs not only in intact cells but also, more important, in partially purified insulin receptors (14).

In this work we have sought to localize a kinase activity on the insulin receptor itself in order to answer the question of whether the receptor phosphorylation is due to either a kinase intrinsic to the receptor or a kinase physically separated from the receptor. In the first series of experiments, we examined the phosphorylating activity of the immunoprecipitated insulin receptor. We showed not only that the β subunit of the receptor isolated by specific immunoprecipitation is a substrate for phosphorylation, but also that its level of phosphorylation is specifically stimulated by insulin. These data indicate that the anti-receptor antibodies precipitate a functional kinase activity that is contained in the insulin receptor or is closely associated with the receptor. Because the anti-receptor antibodies have been shown to precipitate both the α and β subunit of the insulin receptor derived from biosynthetically or surface-labeled cells (21, 22), the kinase activity could be closely associated with or localized in either one of the receptor subunits. Further, at present it is not clear whether the β-subunit phosphorylation seen in the absence of insulin reflects true basal phosphorylation. Indeed, because the anti-receptor antibodies used to precipitate the receptor prior to the phosphorylation reaction have been shown to mimic most of insulin's actions (16, 17), it is not impossible that at least part of the ^{32}P incorporation in the β subunit could be due to a stimulating effect induced by the antibodies themselves. Note also that because of the previous observation that these anti-receptor antibodies reduce the affinity of the receptor for insulin (16, 17), high concentrations of insulin were used to detect a hormone-stimulated effect on immunoprecipitated receptor phosphorylation.

The concept that the insulin receptor itself might have intrinsic kinase activity was further substantiated by our ATP affinity-labeling studies. To localize the kinase that phosphorylates the insulin receptor, we chose the technique of covalent affinity labeling of an ATP-binding site rather than the possible alternative approach, which would consist of demonstrating the absence or presence of phosphorylating activity in a highly purified receptor preparation. Compared to the latter, our approach offers a major advantage. Indeed, whereas copurification of an enzymatic activity is extremely difficult (not to say impossible) to rule out, the affinity-labeling approach permits one to obtain unambiguous results. Our ATP affinity probe, oxidized [α-^{32}P]ATP, has been used to identify an ATP-binding site on diphtheria toxin (32), the large T protein that has ATPase activity in polyoma virus and in simian virus 40 (33), and purified Na^+/K^+-ATPase derived from dog kidney cells. Using covalent affinity labeling with oxidized [α-^{32}P]ATP and partially purified insulin receptors, we were able to label a polypeptide of M_r 95,000. We identified this polypeptide as the β subunit of the insulin receptor on the basis of its electrophoretic mobility and its immunoprecipitation with highly specific anti-receptor antibodies, which is abolished by preincubation with unlabeled insulin. The observation that the M_r 95,000 insulin receptor subunit isolated by specific immunoprecipitation is a substrate for ligand-stimulated phosphorylation reactions, coupled to the identification of an ATP-binding site on this same receptor subunit, allows us to conclude that the M_r 95,000 receptor subunit possesses intrinsic kinase activity. This kinase activity appears to be independent of cyclic AMP and calcium.

A series of elegant studies by Stanley Cohen and coworkers indicate that, for the epidermal growth factor receptor, the three receptor domains (i.e., the binding-, kinase-, and substrate-domain) reside on one and the same molecule (6, 7, 12). The available data would suggest that such a centralized domain distribution is not found with the insulin receptor. Indeed, affinity labeling of the insulin receptor with labeled photoreactive insulin or with chemical crosslinking of labeled insulin preferentially labels the α subunit (M_r 130,000) of the receptor. Thus far, neither in intact hepatocytes nor in solubilized hepatic insulin receptors have we been able to detect routinely ^{32}P incorporation in the α subunit (14). Likewise, when both subunits are immunoprecipitated with anti-receptor antibodies and then used in a phosphorylation reaction, only the β subunit is found to be phosphorylated. Taken together, these data are consistent with a model in which the α subunit is the insulin-binding site, whereas the β subunit is the effector. With the observation in mind that insulin stimulates the phosphorylation of its β subunit in immunoprecipitated receptors, we envision a model in which insulin binds to the α subunit of its receptor, leading to a conformational change that is propagated to the β subunit. This ligand-induced change in conformation would stimulate the protein kinase activity, resulting in an increase in autophosphorylation of the β subunit.

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