Functional Labeling of Insulin Receptor Subunits in Live Cells

αβ2 SPECIES IS THE MAJOR AUTOPHOSPHORYLATED FORM*

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Both receptor subunits were functionally labeled in order to provide methods allowing, in live cells and in broken cell systems, concomitant evaluation of the insulin receptor dual function, hormone binding, and kinase activity. In cell-free systems, insulin receptors were labeled on their α-subunit with 125I-photoactive insulin, and on their β-subunit by autophosphorylation. Thereafter, phosphorylated receptors were separated from the complete set of receptors by means of anti-phosphotyrosine antibodies. Using this approach, a subpopulation of receptors was found which had bound insulin, but which were not phosphorylated. Under nonreducing conditions, receptors appeared in three oligomeric species identified as αβ2, αβ, and α. Mainly the αβ2 receptor species was found to be phosphorylated, while insulin was bound to αβ2, αβ, and α forms.

In live cells, biosynthetic labeling of insulin receptors was used. Receptors were first labeled with [35S]methionine. Subsequently, the addition of insulin led to receptor autophosphorylation by virtue of the endogenous ATP pool. The total amount of [35S]methionine-labeled receptors was precipitated with antireceptor antibodies, whereas with anti-phosphotyrosine antibodies, only the phosphorylated receptors were isolated. Using this approach we made the two following key findings: (1) Both receptor species, αβ2 and αβ, are present in live cells and in comparable amounts. This indicates that the αβ form is not a degradation product of the αβ2 form artificially generated during receptor preparation. (2) The αβ2 species is the prevalently autophosphorylated form.

Insulin receptors are composed of α- and β-subunits. Both subunits possess a distinct function: the α-subunit contains the insulin binding site, a feature which has been evidenced by chemical cross-linking of insulin to its receptor (1) and by photoaffinity labeling (2, 3). The β-subunit displays an insulin-stimulatable tyrosine kinase activity, which is thought to play a key role in hormonal signaling (4–6). This activity was shown to be intrinsic to the receptor (7–9), as confirmed by the subsequent identification of an ATP binding site consensus sequence in the receptor β-subunit (10, 11). Receptor kinase activity appears to be essential for transmission of the insulin signal. Thus, in various physiopathological states associated with modifications of insulin action, the receptor tyrosine kinase is altered in parallel (12–14). More recently, conclusive evidence for the important role of the receptor kinase was presented by the demonstration that cells transfected with receptors mutated in the ATP binding site completely lose the ability to transmit an insulin response both for metabolic and mitogenic effects (15, 16).

When analyzed under nonreducing conditions which preserve disulfide bonds between the receptor subunits, or non-denaturing conditions which maintain native protein conformations, insulin receptors appear as multiple species (17–21). Some of those species were identified as being partially degraded proteolytic products, devoid of autophosphorylating activity (20, 21). Since most of those studies were performed with purified receptor preparations, the proteolytic forms could have been generated during the receptor isolation procedure; their “natural” occurrence in intact cells has not been addressed by the authors. In various pathophysiological conditions, receptor kinase activity has been related to insulin binding activity and parallel alterations in hormone action and kinase functioning have been taken as indications for a role of the receptor kinase in hormone signaling. The validity of these studies relies entirely on the assumption that insulin receptor forms with malfunctioning kinase are not artificially generated during the receptor extraction procedure.

In an attempt to provide a better approach to study the insulin receptor role, we have used functional labeling of receptor subunits combined with discriminating receptor immunoprecipitation. Thus, receptor subunits were labeled first by α-subunit tagging with iodinated hormone and then by β-subunit autophosphorylation; subsequently, phosphoreceptors were extracted with phosphotyrosine antibodies. Using this approach we found that both in live cells and cell-free systems, the αβ2 insulin receptor species was the prevalently autophosphorylated form and likely represents the signaling receptor.

EXPERIMENTAL PROCEDURES

Materials—Na125I was from CEA (France). [γ32P]ATP (trityl-ammonium salt; 3000 Ci/mmol) was from the Radiochemical Centre (Amersham Bucks, United Kingdom). Wheat germ agglutinin-agarose was from ICN (Bucks, U.K.). Antibodies to insulin receptor (serum from patient B5 or B7) was kindly provided by Dr. P. Gorden (National Institutes of Health, Bethesda, MD). Rat fibroblast cell line transfected with human insulin receptor cDNA and expressing 106 receptors/cell was a gift of Dr. A. Ullrich (Genentech, South San Francisco, CA). Antibodies to insulin were from Miles (Paris, France). Anti-phosphotyrosine antibodies were obtained from a rabbit injected with phosphotyrosine coupled to human IgG, and the serum was affinity purified on a phosphotyrosine-agarose column (22). All re-
agents for SDS-5-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad or from Serva (Heidelberg, Federal Republic of Germany).

Preparation of Partially Purified Insulin Receptors—Insulin receptors were prepared as described previously (13, 23) from skeletal muscle, hepatocytes, or from cells transfected with human insulin receptor cDNA by transfection (24). Briefly, tissues or cells were homogenized and solubilized in Hepes buffer (50 mM, pH 7.6), NaCl (150 mM), 1% Triton X-100, and protease inhibitors for 90 min at 4 °C by continuous stirring and centrifuging at 150,000 × g at 4 °C for 90 min. The supernatants were applied to a wheat germ agglutinin-agarose column and washed and equilibrated with 0.1 M DTT, bound glycoproteins were desorbed with 0.3 M N-acetylglucosamine in Hepes buffer (50 mM, pH 7.6), NaCl (30 mM), 0.1% Triton X-100 and stored at −80 °C until use. This preparation will be referred to as partially purified insulin receptors.

Photof ormative Labeling and Auto phosphorylation of Insulin Receptors—The photoreactive insulin analog, B2-(2-nitro-4-azidophenylacetyl)-desPhe6-insulin, was prepared as described previously (25) following an improved procedure2 and iodinated in the dark to a specific activity of 200–250 μCi/μg using the chloramine-T method (26). This photoreactive insulin is a full agonist with a 30% decrease in affinity for the insulin receptor compared to native hormone (3). Partially purified insulin receptors were incubated for 5 h at 15 °C in the dark with photoreactive [125I]-labeled insulin (5 × 10−4 M), conditions that permitted steady state binding. Samples were then put on ice and irradiated for 5 min under a mercury lamp (Philips HFP 125 W/L) at a 10 cm distance. The light was passed through a glass filter (WG 345, thickness 3 mm, Schott Glawerke, Mayence, P.R.G.) which suppresses the short UV emissions. This procedure was used in order to preserve the integrity of the kinase activity while insulin covalent labeling efficacy was maximal (data not shown). Following irradiation, phosphorylation was initiated with [γ-32P]ATP (15 pM), MnCl2 (4 mM), and MgCl2 (0.1 M) boiling SDS solution containing 10% glycerol (ν/v) and 0.01% bromphenol blue (w/v) without (nonreducing conditions) or with (reducing conditions) 2% β-mercaptoethanol (ν/v). The efficiency of the photoreactive insulin was a full agonist with a 30% decrease in affinity for the insulin receptor compared to native hormone (3). The photoreactive insulin is a full agonist with a 30% decrease in affinity for the insulin receptor compared to native hormone (3).

RESULTS

Discriminating Immunoprecipitation of Insulin Receptors following Functional Labeling—Taking advantage of the distinct biological properties of the insulin receptor α- and β-subunits, a selective labeling of each subunit was obtained. The receptor α-subunit, which contains the hormone binding site, was tagged with photolabeled [125I]-insulin, while the β-subunit was labeled by means of its autophosphorylation. To know whether all receptors were labeled on both subunits, samples were immunoprecipitated with the following antibodies: 1) antibodies to insulin receptors, which precipitate all insulin receptor species; 2) antibodies to phosphorylative residues, which recognize receptors having a tyrosine residue phosphorylated; and 3) antibodies to insulin, which immunoprecipitate hormone-receptor complexes, where insulin is covalently bound. Both the immunoprecipitates and the supernatants were analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 1, antireceptor antibodies precipitated two bands, a 130,000-Da protein corresponding to the α-subunit (labeled with [125I]-insulin) and a 95,000-Da subunit identified as the receptor β-subunit (labeled with [32P]). All the insulin receptor present in the samples (lane B or E) were immunoprecipitated, since no radioactivity remained in the corresponding supernatants (lanes A and D). The opposite situation was obtained when immunoprecipitation was performed with control serum (lanes A and D), all the receptors remaining in the supernatants (lanes A’ and D’). When samples were exposed to antibodies against phosphorylative residues, all the phosphorylated forms of the receptor were precipitated since the labeling of the β-subunit was similar to that obtained with antireceptor antibodies (lane C compared to lane B) and no labeled 95,000-Da band remained in the supernatant (lane C’). By contrast, a significant amount of the labeled 130,000-Da band was still present in this supernatant, indicating that some receptor population was covalently bound insulin not phosphorylated. When antibodies to insulin were used, nearly all the radioactivity present in the α-subunit was recovered in the precipitate (lane F), while most of the labeled β-subunits were recovered in the supernatant. This is expected, since the efficiency of the covalent labeling was found to be approximately 30% (results obtained with three different insulin receptor preparations).
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**FIG. 1.** Discriminating immunoprecipitation of insulin receptors labeled with \(^{125}\text{I}\)-photoreactive insulin and \([\gamma-^{32}\text{P}]\) ATP. Partially purified insulin receptors were incubated in the dark for 3 h at 15 °C with \(^{125}\text{I}\)-photoreactive insulin (5 × 10\(^{-8}\) M) and were irradiated with UV light for 5 min at 0 °C through a WG 345 filter. Phosphorylation was conducted for 15 min at 20 °C with 15 \(\mu\text{M} [\gamma-^{32}\text{P}]\text{ATP}, 4\text{ mM MnCl}_2, 8\text{ mM MgCl}_2\). After addition of stopping solution, samples were exposed to normal serum (Control, 1/150), antibodies to insulin receptor (Anti R, serum from patient B5, 1/150), antibodies to phosphotyrosine (Anti P Tyr, 1/20), or antibodies to insulin (Anti Ins, 1/20) for 12 h at 4 °C. Protein A was added and the immunoprecipitates were washed and analyzed under reducing conditions (left panel). Supernatants were subjected to a second immunoprecipitation with antibodies to insulin receptor (right panel). Lanes A–C and D–F correspond to two separate experiments. OR, origin.

**FIG. 2.** The different oligomeric insulin receptor forms are not equally phosphorylated. Insulin receptors were labeled either with \(^{125}\text{I}\)-photoreactive insulin alone (lanes A and E), with \([\gamma-^{32}\text{P}]\) ATP alone (lanes C, D, G and H), or together with \(^{125}\text{I}\)-photoreactive insulin and \([\gamma-^{32}\text{P}]\text{ATP} (lanes B and F). Samples were precipitated with antibodies to insulin receptor (lanes A, B, D, E, F, and H) or antibodies to phosphotyrosine (lanes C and G) as described in Fig. 1, and analyzed by SDS-PAGE in reducing (lanes A–D) or nonreducing (lanes E–H) conditions. OR, origin.

The High Molecular Weight Insulin Receptor Species Is the Major Phosphorylated Form in Partially Purified Receptor Preparations—We next wanted to know whether all the oligomeric receptor forms were equally phosphorylated. In Fig. 2, we compared insulin receptors labeled with \(^{125}\text{I}\)-photoreactive insulin or with \(^{32}\text{P}\) only or together with \(^{125}\text{I}\)-photoreactive insulin and \(^{32}\text{P}\), SDS-PAGE being performed both under nonreducing or reducing conditions. When receptors were labeled with \(^{125}\text{I}\)-insulin alone and samples analyzed under reducing conditions, one band was obtained with 130,000 Da, which we identified as the insulin receptor \(\alpha\)-subunit (lane A). Under nonreducing conditions (lane E), two major bands with \(M_r\) higher than 300,000 and a minor one with \(M_r\) approximately 230,000 were visualized, which correspond to oligomeric receptor forms. Those bands were specific, since no labeling could be found in the presence of an excess of native insulin or when samples were immunoprecipitated with control serum (data not shown). When receptors tagged with \(^{125}\text{I}\)-insulin were subjected to phosphorylation with \([\gamma-^{32}\text{P}]\) ATP before a similar analysis, a labeled band appeared at 95,000 Da under reducing conditions corresponding to the receptor \(\beta\)-subunit (lane B). Under nonreducing conditions, only the intensity of the highest molecular weight form increased significantly (compare lane F to lane E). When receptors were labeled with \([\gamma-^{32}\text{P}]\text{ATP} alone and samples analyzed under reducing conditions, one phosphoprotein, the receptor \(\beta\)-subunit, was obtained whether samples were precipitated with antibodies to phosphotyrosine (lane C) or to insulin receptor (lane D). Under nonreducing conditions, two phosphoproteins were observed, both with anti-phosphotyrosine antibodies (lane G) or with antireceptor antibodies (lane H), but only the highest one was heavily labeled. This experiment also shows that the different oligomeric phosphorylated forms of the receptor were similarly identified by anti-phosphotyrosine and antireceptor antibodies.

The High Molecular Weight Insulin Receptor Species Is the Major Phosphorylated Form in Live Cells—We next extended our study to live cells (HIR cells) using another “double labeling” of insulin receptors. Cells were first labeled biosynthetically with \(^{35}\text{S}\)methionine for 12 h, and then exposed to insulin for 10 min to allow autophosphorylation of insulin receptors by endogenous ATP. They were then solubilized and the cell extracts subjected to immunoprecipitation with antibodies to insulin receptor or to phosphotyrosine. As shown in Fig. 3, lanes A and C, in control (basal) or insulin-treated...
cells with antireceptor antibodies three bands were found under reducing conditions: the heavily labeled receptor α- and β-subunits and the less intensely labeled insulin receptor precursor with an apparent molecular mass of 200,000 Da (23). In cells not exposed to insulin, those bands were absent when samples were immunoprecipitated with antiphosphotyrosine antibodies (Fig. 3, lane B). By contrast, when cells have been exposed to insulin for 10 min, a large proportion of insulin receptors were phosphorylated on tyrosine residues as shown by the appearance of 95,000- and 130,000-Da receptor subunits following precipitation with antiphosphotyrosine antibodies (Fig. 3, lane D). More important, when the same samples were analyzed under nonreducing conditions, only the highest band was clearly visible when samples were precipitated with antiphosphotyrosine antibodies (Fig. 3, lane H), while all the oligomeric forms were present in samples precipitated with antireceptor antibodies (Fig. 3, lanes E and G).

The precise molecular composition of the oligomeric receptor forms is difficult to define, since in this range molecular weight determinations are not reliable for large hydrophobic glycoproteins. Indeed, the intermediary form seen under nonreducing conditions has been reported to be either αβ (17) or αββ′, where β′ would represent a proteolytically derived β-subunit fragment (21). To discriminate between those two possibilities, we performed the next analysis. Immunoprecipitates of [35S]methionine labeled receptor were subjected to SDS-PAGE under nonreducing conditions. The lane of interest was cut out and placed horizontally atop a second gel and subjected to electrophoresis in the presence of 0.1 M DTT to reduce disulfide bonds (Fig. 4). Under these conditions, the two highest bands were separated into two bands with M, 95,000 and 130,000 corresponding to α- and β-subunits, respectively. For the two bands, the relative amounts of radioactivity in the two subunits were different. Thus, from the highest band, we obtain under reducing conditions twice as much radioactivity in the β- as in the α-subunit. This was also observed in the gel shown in Fig. 3, lane A. Since the α- and the β-subunits contain, respectively, 9 and 20 methionine residues (10, 11), these results indicate that the composition of the highest form is αββ. By contrast, the intermediate form gave rise to two bands (the α- and β-subunits) containing the same amount of counts, indicating that this form corresponds to αβ. As expected, the lowest form migrated under reducing conditions as α-subunits only, and is therefore identified as α2. It should be noted that the amount of the two species, αββ and αβ, was similar. Some free β-subunits were also visible.

The possibility of an αββ′ composition for the intermediate unreduced form appears unlikely, since no labeled phosphoprotein could be observed at a molecular weight of 45,000 (even after a long exposure of the autoradiogram, data not shown). However, the methionine residues are mainly located in the cytoplasmic tail of the insulin receptor β-subunit (10, 11), and thus the β′ fragment would not be heavily labeled with methionine. Therefore, we performed cell surface iodination, which labels the insulin receptor at the level of the extracellular portion of the β-subunit (which would give rise to the β′ fragment) and the entire α-subunit. Using this labeling technique, we found under nonreducing conditions the three major molecular species, which we identified previously using biosynthetic labeling as αββ (the highest), αβ (intermediate), and α (the lowest) (Fig. 5). Some free β-subunits were also visible. The absence of a polypeptide with M, 45,000 under reducing conditions confirms that the intermediate form corresponds to αβ and not to αββ′.

To verify that the αβ and α subunit structures are not anomalies resulting from abnormal synthesis or degradation in the particular transfected cell line used in most of the experiments reported above, normal cells were studied in the experiments illustrated in Fig. 6. First, when hepatocytes were labeled with [125I]-photoreactive insulin, the αββ, αβ, α insulin receptor species were observed under nonreducing conditions (Fig. 6, lane B), this labeling being specific since it was not found when the labeling was performed in the presence of an excess of unlabeled insulin (lane A). Second, when insulin

![Fig. 4. Composition of the different insulin receptor species.](image-url)

![Fig. 5. Cell surface labeling of insulin receptors.](image-url)
Fig. 6. Different insulin receptor species in normal cells. Left panel, rat hepatocytes were incubated for 3 h at 15 °C in the dark with 125I-phoreactive insulin without (lane B) or with (lane A) unlabeled insulin (10^{-7} M). Cells were solubilized, extracted, and precipitated with antibodies to insulin receptor. Right panel, insulin receptors were partially purified from skeletal muscle and phosphorylated with [γ-32P]ATP as described in the legend to Fig. 1. Samples were exposed to normal serum (lane C) or to antibodies to insulin receptor (lane D). The immune pellets were analyzed by SDS-PAGE under nonreducing conditions.

DISCUSSION

In our experiments with partially purified preparations, the insulin receptor α-subunit was labeled with 125I-phoreactive hormone before autophosphorylation with [γ-32P]ATP. Subsequently, by the use of discriminating immunoprecipitations it was possible to extract, with an anti-insulin antibody, receptor species occupied by the hormone and, with antiphosphotyrosine antibodies, phosphorylated insulin receptors. Our experiments show that a population of insulin receptors carrying covalently bound insulin is not phosphorylated on tyrosine residues. These results suggest that either those insulin receptors were dephosphorylated or, as shown by O’Hare and Pilch (21, 28), that some receptor forms with intact hormone binding capacity have lost autophosphorylating ability. Analyses of samples in nonreducing conditions were in favor of the second hypothesis, since it was mainly the highest molecular weight species, identified as αβ2, that was autophosphorylated. By contrast, the intermediate species, recognized as αβ, was able to bind insulin but was not autophosphorylated.

The experiments discussed so far were performed using partially purified, solubilized insulin receptors, which were phosphorylated in vitro following exposure to photoreactive insulin and UV irradiation. To prevent artefactual generation of some oligomeric forms, we have used a short (5 min) solubilization procedure in SDS, since it has been shown that longer incubations give rise to an increased amount of intermediate oligomeric forms (29). For the same reason, freshly prepared insulin receptor preparations were routinely used, since storage at −70 °C increases the appearance of reduced insulin receptor forms (18).

In broken cell systems, the two functions of the insulin receptor (kinase activity and hormone binding) do not present the same sensitivity to proteolysis, kinase function being more labile than binding function (20, 21, 30). To verify that the different high molecular weight insulin receptor forms correspond to native receptor species and that they did not lose their autophosphorylating properties during preparation, we performed a second series of experiments in live cells expressing a high number of insulin receptors after transfection with human insulin receptor cDNA (HIR cells). In this case, insulin receptors were biosynthetically labeled with [35S]methionine, and phosphorylation occurred "naturally" by means of the endogenous unlabeled ATP pool. The phosphorylated insulin receptor species could subsequently be separated from the entire receptor pool using anti-phosphotyrosine antibodies. This approach permitted us to study receptor autophosphorylation in live cells, thus excluding the possibility that the receptor kinase activity could have been partly destroyed during purification. In these experiments, anti-phosphotyrosine antibodies precipitated only the αβ2 insulin receptor form, despite the fact that there was a nearly equal amount of αβ2 and αβ insulin receptor species as shown by methionine labeling. If the αβ form were a degraded product of the αβ2 species, it should also have been precipitated by the anti-phosphotyrosine antibody since the remaining β-subunit should be phosphorylated. Therefore, these observations indicate that αβ insulin receptors exist in live cells, and that they are not artificially induced degradation products of the αβ2 receptor, and that they were not autophosphorylated. It should be noted that although the αβ receptor does not appear to undergo autophosphorylation we cannot exclude that this species can phosphorylate cellular proteins or initiate intracellular signals. Our recent demonstration (31) that antibodies to intracellular receptor domains stimulate the receptor substrate phosphorylation capacity without modifying receptor autophosphorylation makes this certainly a reasonable possibility.

The precise molecular composition of the oligomeric receptor species appears to vary depending upon the tissue and the receptor extraction procedure. In some studies, intermediary receptor forms appear as either αβ or αββ′, in which β′ would be a truncated β-subunit (20, 21, 28, 30). In our preparations, detectable degradation of the β-subunit does not seem to occur, since we could not find a labeled band in the 45-kDa region either with methionine labeling or with cell surface iodination. The variation between our results and those reported by others (20, 21, 28, 30) could be explained by the different preparations used. In our study insulin receptors were solubilized directly from intact tissues without using an intermediary step of membrane preparation. In contrast, in the studies mentioned, insulin receptors were purified by a longer preparation procedure, which consisted of placental membrane preparation, solubilization, Sephacyr 400 chromatography, wheat germ agglutinin chromatography, and purification of the different forms on mono Q chromatography. Note that using cell surface labeling, we found a significant amount of a polypeptide which we identified as α2 based on its tagging with 125I-phoreactive insulin and its subunit composition. Knowing that the α-subunit is not a transmembrane glycoprotein (10, 11), the occurrence of a α2 species associated with the cell surface must imply that the α-subunits
are withheld by the transmembrane $\beta$-subunits through non-cova
tent interactions.

In most studies on possible alterations of the insulin recep
tor kinase, quantitation of kinase activity is normalized to
hormone binding capacity (12-14,32). In light of the different
sensitivity to degradation of the two insulin receptor func
tions, the validity of this mode of expression could be ques
tioned. The results reported here show clearly that a subpop
culation of insulin receptors is able to bind insulin without
being autophosphorylated. Furthermore, this does not seem
to be due to artefacts in insulin receptor preparations, since
we found an identical receptor labeling pattern using live
cells. The precise mechanism underlying the appearance of
pathological factors which are able to interfere with the rela
tive abundance of $\alpha\beta_2$ and $\alpha\beta$ receptor forms. Another pos
sibility which has also to be considered is that the $\alpha\beta$ receptor form
imparts the signaling of the $\alpha\beta_2$ receptor. This situation
would be reminiscent of the inhibition of normal receptor
function by kinase deficient insulin receptors (35).

Finally, the results reported in this paper, that in live cells
only the $\alpha\beta_2$ oligomeric form is capable of autophosphoryla
tion, add further support to the idea that the $\alpha\beta-\alpha\beta$ interaction
is critical for receptor activation and autophosphorylation. In
purified preparations, the dithiothreitol reduction of the te
trameric receptor into $\alpha\beta$ dimers is accompanied by a disa
pearence of insulin-dependent autophosphorylation, and
those dimers need to reassociate, but not necessarily cova
lently, to express insulin-activated kinase activity (34-36).
Our data are in accord with these findings and indicate that
despite an equal hormone binding to the receptor $\alpha$-subunits,
the presence of only one $\beta$-subunit in the $\alpha\beta$ receptor is not su
fficient to induce autophosphorylation.

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