Characterization of Purified Insulin Receptor Subunits*

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Three insulin receptor subunits prepared from the purified receptor were isolated and characterized. Peptide mapping of the isolated subunits revealed that the $M_r = 125,000$ subunit ($\alpha$) is distinct from the $M_r = 90,000$ subunit ($\beta$) whereas the $M_r = 50,000$ subunit ($\beta_1$) shows considerable structural homology to $\beta$, indicating that the $\alpha$ and $\beta$ subunits are components of the intact insulin receptor.

From two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence and presence of dithiothreitol, the purified insulin receptor was shown to be composed of heterogeneous disulfide-linked complexes of $(\alpha_2, 2\beta)$, $(\alpha_2, \beta, \beta_1)$, $(\alpha_2, 2\beta_1)$, $(\alpha_2)$, $(\alpha\beta)$, and $(\alpha\beta_1)$. The largest disulfide-linked complex $(\alpha_2, 2\beta)$ appears to be the minimum unit of the intact insulin receptor whereas the other complexes appear to be generated from $(\alpha_2, 2\beta)$ by proteolytic degradation and/or reduction.

These studies provide conclusive evidence that the $\alpha_2\beta_2$ complex is the basic structural unit of insulin receptor, as previously proposed from affinity cross-linking experiments using crude membranes by Czech’s group (Czech, M. P., Massague, J., and Pilch, P. F. (1981) Trends Biochem. Sci. 6, 222–225). The biochemical approach described here should allow us to further elucidate the mechanism of insulin action.

The structure of the insulin receptor has been studied by a variety of indirect techniques (1–5). For instance, Czech and his co-workers affinity cross-linked $^{125}$I-insulin to the receptors on plasma membranes prepared from various tissues and analyzed by SDS-PAGE in the absence and presence of dithiothreitol (3, 6). They observed three major disulfide-linked complexes with $M_r = 350,000$, $320,000$, and $290,000$ under nonreducing conditions. These complexes were shown to have a combination of subunits with $M_r = 125,000–135,000$ ($\alpha$), $M_r = 90,000–95,000$ ($\beta$), and $M_r = 40,000–45,000$ ($\beta_1$). The composition of the nonreduced complexes was thus proposed to be $\alpha\beta_1$, $\alpha\beta_1\beta_1$, and $\alpha\beta_1\beta_1$, respectively (6), where the $\beta_1$ subunit was thought to be derived from the $\beta$ subunit as a result of proteolytic degradation (7).

Although affinity-labeling techniques have made significant contributions toward defining the insulin receptor subunits, the information obtained by these methods is inconclusive, because only specifically labeled proteins can be detected. For example, essential protein components which may lie far from the insulin binding site may not be labeled with $^{125}$I-insulin or membrane components which may exist near the insulin binding site but are not involved in insulin action may be artificially labeled. In fact, with the affinity cross-linking method, the $\beta$ subunit is very poorly labeled with insulin. Thus, to achieve a stoichiometric measurement of each subunit requires complete purification of the intact receptor.

Previously, we purified the insulin receptor from human placenta to apparent homogeneity. The purified receptor retained full insulin binding activity (8) as well as tyrosine-specific protein kinase activity (9), indicating that our purified receptor retains basic functions of the native insulin receptor. In addition, the purified receptor contained a significant amount of the $\beta$ subunit which had not been clearly observed by others (10–13), suggesting that this receptor preparation would be useful for studies on the subunit structure of native insulin receptor. In this report, the subunits of the purified receptor are isolated and characterized by various methods including isoelectric focusing, peptide mapping, two-dimensional SDS-PAGE in the absence and presence of dithiothreitol, and amino acid analysis.

EXPERIMENTAL PROCEDURES AND RESULTS$^2$

Subunit Composition of the Purified Receptor—Among nearly 50 preparations of purified insulin receptor, the most typical SDS-PAGE results of the receptor under reducing conditions are shown in Fig. 1. Both Coomassie blue and silver staining of the gel revealed almost the same densitometry patterns for three protein components with $M_r = 125,000 (\alpha)$, $90,000 (\beta)$, and $50,000 (\beta_1)$, as seen in Fig. 1, A2 and B1. The three components derived from disulfide-linked complexes with $\sim M_r = 300,000$ were previously shown to be

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* Portions of this paper (including "Materials and Methods," portions of "Results," and Figs. 2 and 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-1340, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

$^2$ Molecular weight of each subunit was previously estimated to be $135,000 (\alpha)$, $90,000 (\beta)$, and $60,000 from SDS-PAGE under the conditions described by Weber and Osborn (19). In this report, the values estimated from SDS-PAGE under Laemmli’s conditions are used. It should be noted that these molecular weights estimated from SDS-PAGE are tentative, mainly because the subunits are glycoproteins which are known to give ambiguity on molecular weight estimation.

$^3$ The $M_r = 50,000$ subunit is termed $\beta_1$ in this report. Although this subunit appears to be similar to the $\beta_1$ subunit described by Massague et al. (6), there is no direct evidence that they are the same molecule. The terminology ($\beta_1$) used in this report was adopted at the suggestion of the editor.
Characterization of Purified Insulin Receptor Subunits

**Fig. 1. SDS-polyacrylamide gel electrophoresis of the purified insulin receptor under reducing conditions.** Purified insulin receptor was boiled for 5 min in 50 mM Tris-HCl buffer, pH 6.8, containing 2% SDS and 20 mM dithiothreitol, and electrophoresed in a 7.5% gel according to Laemmli's methods. The gel was stained with silver (A) or Coomassie Blue (B). The stained peaks were monitored by densitometric scanning at 630 nm. A-1, standards: myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin. A-2, insulin receptor, 2.5 μg. B-1, insulin receptor, 4.4 μg.

**Amino acid composition of insulin receptor subunits**

Amino acid analysis of each isolated subunit was performed in duplicate after a 48-h hydrolysis at 110°C.

| Amino acid | Mα = 125,000 (α) | Mβ = 90,000 (β) | Mγ = 50,000 (γ) |
|------------|------------------|------------------|------------------|
|            | mol%             | mol%             | mol%             |
| Asx        | 11.4             | 9.4              | 9.1              |
| Thr        | 5.3              | 4.8              | 4.9              |
| Ser        | 7.6              | 7.8              | 7.9              |
| Glx        | 11.5             | 11.9             | 11.6             |
| Pro        | 5.2              | 5.9              | 5.6              |
| Gly        | 9.3              | 14.4             | 12.4             |
| Ala        | 4.6              | 5.8              | 6.6              |
| Cys        | 3.9p             | 2.4              | 1.5              |
| Val        | 4.4              | 5.3              | 6.5              |
| Met        | 1.3              | 1.4              | 1.1              |
| lle        | 5.7              | 5.2              | 4.9              |
| Leu        | 9.6              | 8.4              | 8.4              |
| Tyr        | 3.1              | 3.5              | 3.6              |
| Phe        | 4.4              | 4.1              | 4.3              |
| His        | 2.8              | 2.5              | 2.6              |
| Trp        | NDp              | ND               | ND               |
| Lys        | 4.1              | 2.8              | 3.3              |
| Arg        | 5.3              | 4.8              | 5.7              |

(NDp, not determined; p, amino sugar detected by amino acid analysis was also listed.)

**Table II**

Summary of two-dimensional SDS-PAGE analysis of the purified insulin receptor in the absence and presence of dithiothreitol

| Molecular species observed under nonreducing conditions | Subunit composition | Subunit structure |
|---------------------------------------------------------|---------------------|-------------------|
| a (320,000) α, β (2α, 2β)                               |                     |                   |
| b (300,000) α, β, δ, ε (2α, 2β, 2δ, 2ε)                 |                     |                   |
| c (275,000) α, δ, ε (2α, 2δ, 2ε)                         |                     |                   |
| d (250,000) α                                           |                     |                   |
| e (195,000) α, β (αβ)                                  |                     |                   |
| f (150,000) α, δ (αδ)                                  |                     |                   |
| g (110,000) α                                          |                     |                   |

*Disulfide-linked complexes detected under nonreducing conditions are named as shown in Fig. 5B.


d Molecular weight of the complexes was estimated by SDS-PAGE on a 5% polyacrylamide gel.

Subunit composition of each complex was estimated by SDS-PAGE, the β subunit sometimes showed slightly different mobility. The peptide map of such an anomalous Mγ = 50,000 subunit was very similar to that of the β subunit (data not shown).

**Amino Acid Analysis of the Insulin Receptor Subunits**—Approximately 18% of the original protein was recovered as isolated subunits from the SDS-polyacrylamide gel. The amino acid composition of each subunit was assayed in duplicate after desalting on Sephadex G-25. The results are summarized in Table I. Since the data obtained are based on 48-h hydrolysis, the values for Thr, Ser, Met, Val, Leu, Ile, and Tyr could be underestimated relative to the rest of amino acids, whereas Gly appears to be overestimated due to its contamination from the buffers.

The subunit is relatively rich in Cys and Lys, while the β subunit is poor in Cys. Although the amount of GlcNAc estimated by the amino acid analyzer is not quantitative, this may be an indication of glycoprotein, since this sugar is a major component of the carbohydrate moieties which are usually found in glycoproteins. The results show that all three subunits seem to be glycoproteins and that the α subunit contains more carbohydrate residues than the β or δ subunits.

**Two-dimensional SDS-PAGE in the Absence and Presence of Dithiothreitol**—The purified receptor was electrophoresed in a 5% gel under nonreducing conditions for analysis in the first dimension. The gel slice was then placed on top of a 7.5% slab gel and the disulfide-linked complexes were analyzed in the second dimension under reducing conditions. The results are summarized in Fig. 5. The five major bands (a, c, e, f, and o) observed under nonreducing conditions were shown to be composed of α + β, α + β + δ + γ, α + γ, and α + δ, respectively. Since the molecular weights of the two smaller components (c and f) were 195,000 and 150,000, the subunit structure of the complexes was determined to be (αδ) and (αβ), respectively. The color intensity of the α and β subunits measured by densitometric scanning of the complex “e” allowed the calculation of a relative color factor of 0.32 for the β subunit.
Characterization of Purified Insulin Receptor Subunits

FIG. 3. Peptide mapping of insulin receptor subunits. The three insulin receptor subunits (approximately 3 µg for α, 2.5 µg for β, and 1 µg for β₁) were separated by SDS-PAGE, iodinated, and digested with TPCK-trypsin. The ¹²⁵I-labeled tryptic peptides were spotted on the origin (♦) on cellulose-coated TLC plates. Electrophoresis was performed in the first dimension, followed by chromatography (♦). The maps of the ¹²⁵I-labeled peptides were visualized after the plates were exposed to Kodak XAR-5 films for 3-5 h at -70 °C with intensifying screens. A, α subunit (Mᵦ, = 125,000); B, β subunit (Mᵦ, = 90,000); C, bovine serum albumin (BSA) as a control; D, β₁ subunit (Mᵦ, = 50,000).

against that of the α subunit. The subunit composition of the disulfide-linked complex “a” with Mᵦ, = 320,000 can therefore be deduced to be 2α and 2β subunits by quantitating the α and β subunits in the complex using the color factor. The other complex “c” appears to be composed of 2α and 2β₁ subunits while “d” appears to be 2α. Complex “b” (2α, β, β₁) may also exist in this preparation. However, resolution in this experiment was not good enough to identify the complex.

As summarized in Table II, two-dimensional SDS-PAGE revealed that the purified receptor is composed of the heterogeneous disulfide-linked complexes: (2α, 2β), (2α, β, β₁), and (2α, 2β₁) as major complexes and (α,β), (αβ), and (αβ₁) as minor complexes. Since (2α, 2β) is the largest disulfide-linked complex found in the preparation after SDS treatment, it is likely that it is an intact disulfide-linked complex and that the others are derived from it by either proteolytic degradation and/or reduction. It should be noted that the α-dimer was clearly detected in our purified receptor preparation as seen in Fig. 5A(2). This indicates that the two α subunits are linked together with disulfide bridges.

Insulin binding activity appeared as a single peak on Sepharose 6B chromatography, which gave a Stokes radius of 79 Å for the native purified insulin receptor-Triton X-100 complex (Data not shown). Since all disulfide-linked complexes were affinity-labeled with ¹²⁵I-insulin (Fig. 4B, 3 and 4), the small complexes (α,α), (αβ), and (αβ₁) must exist as larger forms.

DISCUSSION

We have previously reported that our purified insulin receptor retains the basic functions of the native insulin receptor such as a curvilinear Scatchard plot and autophosphorylation of the β₂ subunit (8, 9). In addition, a specific activity of 28 µg of insulin bound per mg of protein for our purified receptor is the highest value so far reported. These results indicate that the analysis of our purified receptor should be useful for directly determining the subunit structure of native insulin receptor.

In the purified receptor, the β₂ subunit was clearly detected after Coomassie blue or silver staining of SDS-polyacrylamide gels. However, densitometric scanning of stained gels showed that the amount of the β₂ subunit was only about one-fourth of the α subunit. Densitometric scanning of a complex which contained only the α and β₂ subunits allowed the calculation of a relative color factor for the staining of β₂ subunit relative to α. This factor was only 0.32 which could explain why the amount of the β₂ subunit detected on stained gels always appeared less than that of the α subunit in the purified receptor. In addition, limited proteolysis could lead to a fur-
Characterization of Purified Insulin Receptor Subunits

Fig. 5. Two-dimensional SDS-PAGE of the purified insulin receptor. Purified insulin receptor (5.4 µg) was electrophoresed in the first dimension under nonreducing conditions and then in the second dimension under reducing conditions. The gels were stained with silver. A, the gel on top of the slab gel (1) shows the result of the first dimension (1.8 µg of the receptor was applied). The results of two independent experiments (1, 2) are shown. B, summary of two-dimensional SDS-PAGE. α- and β subunits indicate the disulfide-linked complexes observed under nonreducing conditions. Visible protein spots are circled by solid line (——) while expected spots are marked by dotted line (·····).

Other reduction of β subunit in the receptor preparations (see below). The reason that previous investigators have failed to show a clear picture of the β subunit in their preparations (8–11) could thus be due to a combination of low staining intensity and partial proteolysis of the β subunit.

The M, = 50,000 subunit (β1) was often detected in our purified receptor, although the amount varied from preparation to preparation. The peptide mapping experiments of each subunit revealed that the β1 subunit is related to the β subunit. It seems likely that the β1 subunit is derived by proteolytic degradation of the β subunit. Since the molecular weight of β1 is fairly consistent in different purified receptor preparations, the β subunit may have a region resistant to proteolytic digestion while the rest of its sequence is sensitive to proteases. This result supports the previous reports by Massaque et al. (7) using affinity cross-linking techniques, where they suggested that the M, = 49,000 subunit is generated as a result of limited proteolytic cleavage of the β subunit by lysosomal protease.

The α subunit was found in all disulfide-linked complexes detected in the purified receptor by SDS-PAGE under nonreducing conditions, suggesting that α is selectively concentrated by purifying insulin receptor with insulin-Sepharose affinity chromatography. In addition, affinity cross-linking of the purified receptor with 125I-insulin resulted in labeling of all disulfide-linked complexes including (αβ) and (αβ1). These results indicate that the α subunit is necessary for insulin binding as previously predicted (21) and that both (αβ1) and (αβ1) seem to have insulin binding activity.

The present study has revealed that insulin receptor purified with insulin-Sepharose affinity chromatography is not homogeneous in terms of subunit structure. SDS-PAGE under nonreducing conditions followed by silver staining showed the presence of three protein components with M, = 320,000, 300,000, and 270,000, two minor components with M, = 195,000 and 145,000, and a trace of the α subunit. These disulfide-linked complexes were identified as (2α, 2β) (2α, β), (2α, 2β1), (αβ), and (αβ1), respectively, as described under "Results." The largest disulfide-linked complex with M, = 320,000 has the subunit structure of (2α, 2β) which appears to be a minimum unit of the intact insulin receptor whereas the other complexes can be derived from it by proteolytic degradation and/or reduction as described in Fig. 6. The presence of α2 was clearly shown in the purified preparations.
as had previously been observed in crude preparations (4), suggesting that the two α subunits are linked together with disulfide bridges. These results provide conclusive evidence that \((α_2, 2β)\) is the basic structural unit of insulin receptor, which is consistent with the models proposed by Czech et al. (22, 23) and Jacobs et al. (1, 24). Recently, Yip et al. (25) and Baron and Sönksen (26) have reported the presence of another component with \(M_r = 40,000\) or 65,000, respectively, which does not seem to be related to the \(β\) subunit. The \(β_1\) subunit described in this paper is obviously different from these two components, which may be associated with the receptor in the membrane but cannot be purified by affinity chromatography.

Since more \(β_1\) subunit has been observed in receptor preparations purified from placental membranes stored at \(-20^°C\) for 4-6 months than from freshly prepared membranes,\(^6\) it is likely that during storage, the \(β\) subunit becomes more accessible to proteases and/or is more easily degraded by proteases present in the crude membrane preparations during purification. It is also possible, however, that heterogeneous receptors exist on intact cell membranes and are all purified by our methods. It is not clear at this moment whether \((α_2, 2β)\) is a biologically active molecule. The active form could be \((α_2, 2β)\) or even a higher molecular weight form such as a dimer \((α_2, 2β)\).

In a previous paper, we reported that our purified receptor has the highest specific activity so far obtained (8). The value, 28 ng of insulin bound per mg of protein, indicates that 1.4 mol of insulin binds to 1 mol of insulin receptor. This value is not conclusive since the present study revealed the presence of heterogeneity in terms of subunit structure in the receptor preparations. If one of the components such as \((α_2, 2β)\) represents native receptor and shows a curvilinear Scatchard

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\(^{a}\) Y. Fujita-Yamaguchi, unpublished.

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Characterization of Purified Insulin Receptor Subunits

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EXPERIMENTAL PROCEDURES

Materials

Purified bovine insulin was kindly supplied by Lilly. [125I]-Labelled insulin, 
[127I]-labeled insulin, [3H]-labeled insulin, diiodotyrosine and NADP were purchased from New England Nuclear. Bovine serum albumin, myoglobin, gelatin, ovalbumin, phosphorylcholine, and phosphatase were purchased from Behring, bovine y-globulin and bovine serum albumin were from Sigma, and polybuffer 94 and polybuffer 14 were from Pharmacia. All other chemicals were used as reagent grade.

Methods

Purification of insulin receptor.

The insulin receptor was purified 2600-fold with a yield of 4% from human placental membranes by sequential affinity chromatography on wheat germ agglutinin and insulin-Sepharose columns as described previously. Approximately 50% of the purified receptor with a specific activity of 200u of insulin bound per mg of protein was obtained from two chromatographies. Chromatofocusing and gel filtration analyses indicated that the receptor preparation was apparently pure.

Polyacrylamide gel electrophoresis

Electrophoresis was performed using the discontinuous buffer system according to the method of Laemmli (14) in 4% stacking and 7% resolving gels in precast gels. Each gel slice from a control gel was incubated in 0.5 ml of 0.1M KCl, and then the pH was measured.

The first-dimension gel was incubated with 35S-labeled insulin (2.5 x 10-6 M, 10000 cpm/mg), dissolved in the stacking solution was directly applied to the chromatofocusing gel at 4°C after diluting with the same volume of the stacking solution. After electrophoresis, the gel was stained with 0.25% Coomassie blue and silver-stained protein bands were quantitated by scanning with an ISCO-AT automatic densitometer (Linear Dynamics). Following the SDS-PAGE analysis, the gels were stained with 0.25% Coomassie blue and silver-stained, and dried. The gel was used as standards to measure molecular weight.

Chromatofocusing

A column of polybuffer exchanger PE30 (0.6 x 20 cm, Pharmacia) was equilibrated with 20 mM polybuffer 94, pH 7.4, containing 0.1M NaCl, 0.1M DTT, and gel filtration buffer was applied to the top of the chromatofocusing gel. The eluate from an insulin-Sepharose column was directly applied to the chromatofocusing gel at 4°C after diluting with the same volume of the stacking solution. The gels were developed with 20% polybuffer 94, pH 7.4, containing 0.1M KCl, and then the protein bands were quantitated by scanning with a Bio-Rad densitometer (Linear Dynamics). The gels were stained with 0.25% Coomassie blue and silver-stained protein bands were quantitated by scanning with an ISCO-AT automatic densitometer (Linear Dynamics). Following the SDS-PAGE analysis, the gels were stained with 0.25% Coomassie blue and silver-stained, and dried. The gel was used as standards to measure molecular weight.

Peptide mapping

Peptide mapping was performed by following the method of Elder et al. (17), affixed homologous blue-stained protein band was cut out from the SDS-polyacrylamide gel, and subjected to gelatinase digestion. The gelatinase was digested with 20% polybuffer 94, pH 7.4, containing 0.1M KCl, and then the gels were stained with 0.25% Coomassie blue and silver-stained protein bands were quantitated by scanning with a Bio-Rad densitometer (Linear Dynamics). Following the SDS-PAGE analysis, the gels were stained with 0.25% Coomassie blue and silver-stained, and dried. The gel was used as standards to measure molecular weight.

RESULTS

Isoelectric Point of the Insulin Receptor and its Subunits

The insulin binding activity appeared as a single peak from the chromatofocusing column, indicating that the intact receptor has an isoelectric point of 4.0. The purified subunits were separated by isoelectric focusing gel electrophoresis. Figure 2.

Affinity Cross-linking of the Purified Insulin Receptor

Purified insulin receptor (-IgG) was cross-linked with [125I]-insulin in the absence (-IgG) and presence (+IgG) of excess cold insulin, and subjected to SDS-PAGE under reducing conditions (-IgG) or non-reducing conditions (+IgG). The gels were stained with 0.25% Coomassie blue and silver-stained, and dried. The gel was used as standards to measure molecular weight.
Characterization of purified insulin receptor subunits.

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