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Biochemistry

Insulin receptor: Covalent labeling and identification of subunits

(photoaffinity label/receptor purification/disulfide bonds/liver and placenta membranes/affinity chromatography)

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ABSTRACT **Two methods were used to label insulin receptors covalently with ^{125}I. In the first, an aryl azide derivative of insulin, ^{125}I-labeled 4-azido-2-nitrophenyl-insulin, was synthesized and used to photolabel the binding region of the insulin receptor in rat liver membranes and human placenta membranes. In the second, insulin receptors were purified from rat liver membranes and labeled with ^{125}I by use of chloramine-T; this method presumably has no specificity for the binding region of the receptor. The proteins labeled by both methods were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis after or without reduction by dithiothreitol. The photoaffinity label specifically labeled a single band in both liver and placenta that had an apparent molecular weight of 135,000 after reduction. A band with similar mobility was present in the chloramine-T-labeled preparation, which also contained a second major band with an apparent molecular weight of 45,000. Without reduction, both methods resulted in a single labeled band with an apparent molecular weight of about 310,000. These results indicate that the insulin receptor of both liver and placenta has a subunit of molecular weight 135,000 that binds insulin and that the receptor may be composed of at least two different subunits that are linked together or greatly stabilized by disulfide bonds.**

The molecular size of insulin receptors solubilized from several tissues, including membranes from rat liver (1), rat adipocytes (1), human placenta (2, 3), and turkey erythrocytes (4), has been determined by gel filtration. In each tissue the molecular weight has been estimated as being approximately 300,000. This high molecular weight species of the receptor has been reported to dissociate in the presence of insulin into forms of lower molecular weight (3–7). Consistent with this, the major Coomassie blue-staining band seen in sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels of a highly purified preparation of insulin receptors from liver membranes has an apparent molecular weight of 135,000 (8). A band of similar molecular weight has been specifically labeled in fat cells by using a photoaffinity label (9) or by covalently crosslinking ^{125}I-labeled insulin to its receptor (10).

In the present studies the photoaffinity label, 4-azido-2-nitrophenyl-insulin (ANP-insulin), was synthesized and used to label insulin receptors in membranes from rat liver and human placenta. The photoaffinity-labeled bands were compared with the bands present in the purified insulin receptor preparation by $NaDodSO_4$/polyacrylamide gel electrophoresis.

METHODS

Purification of Insulin Receptor from Rat Liver. Liver membranes were prepared by differential centrifugation as described (11). Insulin receptors were solubilized with Triton X-100 and purified by sequential chromatography on DEAE-cellulose, insulin-agarose, and concanavalin A-agarose by methods described previously (8).

Iodination of Purified Insulin Receptor. Fifty microliters of 100 mM sodium phosphate buffer (pH 7.4), containing 0.1% Triton X-100 and approximately 1 μg of purified insulin receptor protein, was added to 50 μl of 0.5 M sodium phosphate buffer (pH 7.4). To this, 1 mCi of $Na^{125}I$ in approximately 5 μl was added, followed by 20 μl of chloramine-T (1 mg/ml). After 15 sec, 50 μl of sodium metabisulfite (2 mg/ml) and 0.5 ml of 100 mM sodium phosphate buffer, pH 7.4/0.1% Triton X-100 were added. Free ^{125}I was separated from the protein-bound ^{125}I either by Sephadex G-75 chromatography or by a small concanavalin A affinity column.

Preparation of ANP-Insulin. ANP-insulin was prepared by a modification of the method described by Levy (12). Ten milligrams of porcine insulin was dissolved in 3 ml of dimethyl formamide. To the stirred solution, 2 μl of triethylamine was added, followed by 4.5 mg of 4-fluoro-3-nitrophenylazide. The reaction was allowed to proceed at room temperature for 24 hr, protected from light. The product, which was precipitated by the addition of 20 ml of petroleum ether and washed three times with 10 ml of ethyl acetate in order to remove unreacted 4-fluoro-3-nitrophenylazide, was used without further purification. ANP-insulin was iodinated by the chloramine-T method, and free iodide was removed by Sephadex chromatography.

Other Procedures. Placenta membranes were prepared as described (13) with the minor exception that 10 μg of phenylmethylsulfonyl fluoride per ml and 2 mM EDTA were added to the sucrose homogenizing buffer to inhibit proteolysis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed according to Laemmli (14), with a 3% stacking gel and a 7.5% separating gel. Samples were boiled for 5 min in sample buffer containing 2% $NaDodSO_4$.

RESULTS

$NaDodSO_4$/polyacrylamide gels at various stages of purification of insulin receptors are shown in Fig. 1. The eluate from insulin-agarose (lane C) contained a major Coomassie blue-staining band with an apparent molecular weight of 135,000. Minor bands with molecular weights of 90,000, 75,000, and 45,000 were also present. It is significant that the 135,000 molecular weight band was not visible in the eluate from DEAE-cellulose (lane B), indicating that it must be a very minor component of the total protein at this stage of the purification. This is consistent with the very low concentration of insulin receptor, as determined by insulin binding, in the DEAE-cellulose eluate. By further purification on concanavalin A-agarose, it was possible to selectively remove the 75,000 molecular weight band (lane D). When the eluates from insulin-agarose and concanavalin A-agarose were iodinated with chloramine T and run on $NaDodSO_4$/polyacrylamide gels, autoradiograms

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Abbreviations: ANP-insulin, 4-azido-2-nitrophenyl-insulin; $NaDodSO_4$, sodium dodecyl sulfate.

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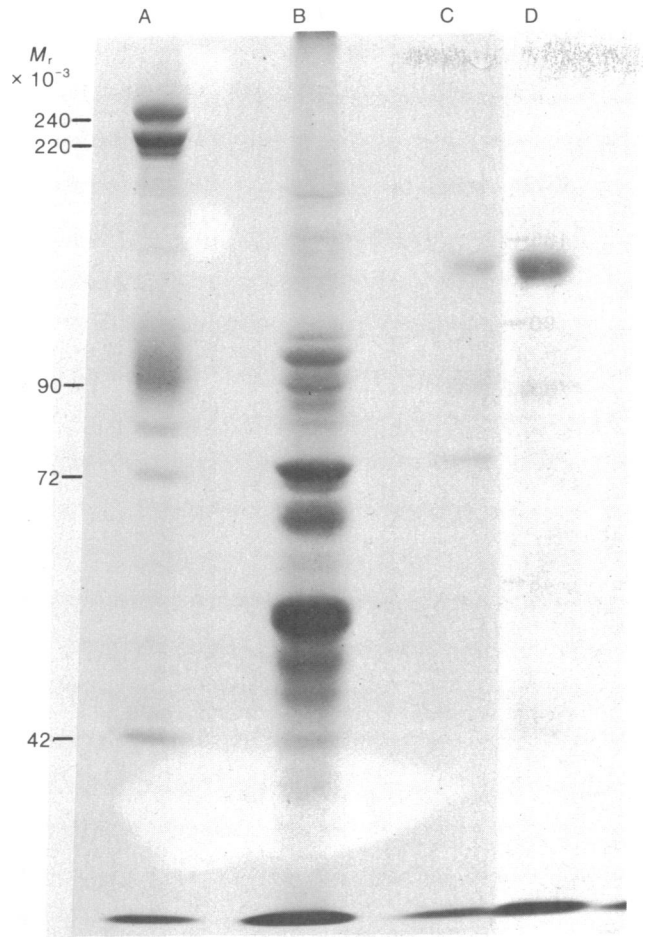

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of rat liver insulin receptors at various stages of purification. Lane A, human erythrocyte ghosts used as marker proteins; lane B, after DEAE-cellulose chromatography; lane C, after insulin-agarose affinity chromatography; lane D, after concanavalin A affinity chromatography. Electrophoresis was performed after reduction with 10 mM dithiothreitol. Gels were stained with Coomassie blue.

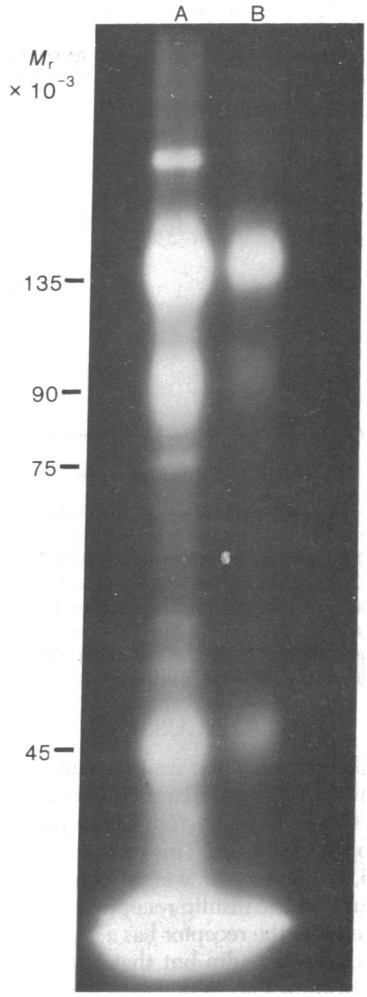

FIG. 2. Purified insulin receptor preparations labeled with ^{125}I by use of chloroamine-T. The eluates from insulin-agarose (lane A) and concanavalin A-agarose (lane B) were labeled with ^{125}I by use of chloramine T. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed after reduction with 10 mM dithiothreitol. Autoradiography was performed on the dried gels.

of the gels (Fig. 2) revealed the same bands that were present in the Coomassie blue-stained gels (Fig. 1). An important difference, however, is that in the autoradiograms the 45,000 molecular weight band appeared to constitute a considerably greater fraction of the total protein present than was suggested by Coomassie blue staining.

The photoaffinity labeling insulin derivative, ^{125}I-labeled ANP-insulin, binds specifically to liver membranes. Native insulin was equipotent in inhibiting the binding of ^{125}I-labeled ANP-insulin compared to ^{125}I-labeled insulin, indicating that ^{125}I-labeled ANP-insulin bound to the insulin receptor (Fig. 3).

In liver and placenta membranes, ^{125}I-labeled ANP-insulin photolabeled specifically a single protein band that had a mobility similar to that of the 135,000 molecular weight band present in the preparation of purified insulin receptor (Fig. 4). Under the conditions of photolysis used, approximately 3.0% of the radioactivity specifically bound to the membranes was incorporated into this band. Photolabeling of this band was inhibited by addition of native insulin (2 μg/ml) during the binding reaction. The 45,000 molecular weight band present in the preparation of purified insulin receptor was not labeled by ^{125}I-labeled ANP-insulin, nor were the other minor bands. In the photolysis studies, some radioactivity was also found at the interface between the stacking gel and the spacer gel. The significance of this is not clear.

To further evaluate the specificity of photolabeling of the 135,000 molecular weight band by ^{125}I-labeled ANP-insulin, we determined the ability of various concentrations of native insulin, proinsulin, or the unrelated polypeptide, epidermal growth factor, to inhibit photolabeling of this band (Table 1). The potency of native insulin to inhibit photolabeling of this band was similar to its potency in inhibiting the binding of ^{125}I-labeled insulin to liver membranes (Fig. 3). Proinsulin was less potent in inhibiting photolabeling, and epidermal growth factor, as expected, had virtually no inhibitory activity.

In the studies described above, samples were reduced with 10 mM dithiothreitol prior to electrophoresis. When this step of reduction was eliminated, the 135,000 and 45,000 molecular weight bands of the preparation of purified insulin receptor were no longer seen. Instead, there was a band with a molecular weight of about 310,000 (Fig. 5, lanes A and D). Similarly, when reduction was eliminated, the 135,000 molecular weight band seen when liver or placenta membranes were photolabeled with ^{125}I-labeled ANP-insulin disappeared and was replaced with a new band with a molecular weight greater than 300,000 (Fig. 5, lanes B, C, E, and F).

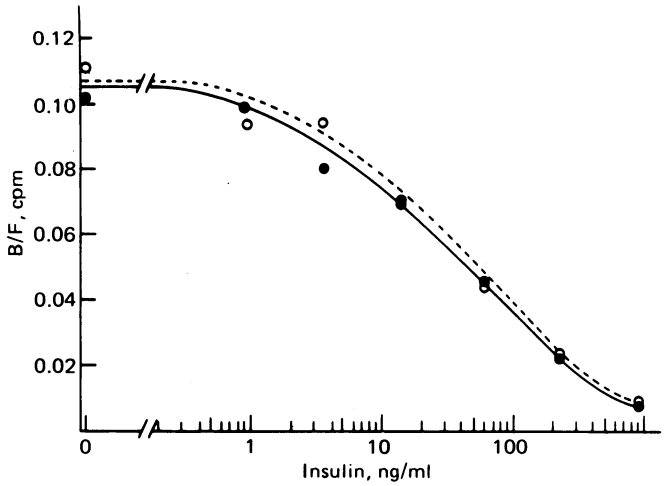

FIG. 3. Inhibition of binding of ^{125}I-labeled ANP-insulin or ^{125}I-labeled insulin to liver membranes by native insulin. Liver membranes (200 μg) were incubated with 10,000 cpm of ^{125}I-labeled ANP-insulin (3.4×10^8 cmp/μg) (O- - -O) or of ^{125}I-labeled insulin (2.1×10^8 cpm/μg) (●—●) and various concentrations of native porcine insulin in 0.2 ml of KRB buffer/0.2% albumin at 15°C for 90 min in the dark. Binding of the labeled peptides was measured, without photolysis, by EGWP Millipore filters as described (15).

DISCUSSION

The major 135,000 molecular weight component present in the purified insulin receptor preparation has virtually the identical mobility on the same gel system as the protein photolabeled specifically by ANP-insulin. This provides convincing evidence that the 135,000 molecular weight component is an insulin-binding subunit of the insulin receptor. Previous reports (3, 5) have suggested that the receptor has a glycoprotein subunit that itself does not bind insulin but that alters the affinity of the receptor for insulin. The 45,000 molecular weight component of the preparation of purified insulin receptor is present in various batches of receptor purified from liver by the methods described above. It is possible that this 45,000 molecular weight component, which is not labeled by ANP-insulin, is a subunit of the insulin receptor not directly involved in insulin binding. Consistent with this, we find that antibodies, prepared by immunizing with the 135,000 molecular weight band eluted from $NaDodSO_4$/polyacrylamide gels, which do not directly cross-react with the 45,000 molecular weight band, immunoprecipitate the 45,000 molecular weight band because of its association with the 135,000 molecular weight band (unpublished data). Heavily glycosylated proteins often stain poorly with Coomassie blue. This may explain why the 45,000 molecular weight component of purified receptor preparations is not readily visible in gels stained with Coomassie blue whereas it is clearly detectable if the same preparations are labeled with ^{125}I.

Table 1. Specificity of photolabeling of the 135,000 molecular weight band

Native peptide, ng/ml	cpm incorporated
None	1910
Insulin:	
10	1430
100	900
1,000	640
10,000	660
Proinsulin: 1,000	1170
Epidermal growth factor: 1,000	1820

Liver membranes were photolabeled as described in the legend to Fig. 4, except that various concentrations of native peptides were present during the incubation of the membranes with ^{125}I-labeled ANP-insulin. The membranes were then subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis after reduction with 10 mM dithiothreitol, and the amount of radioactivity incorporated into the 135,000 molecular weight band was determined.

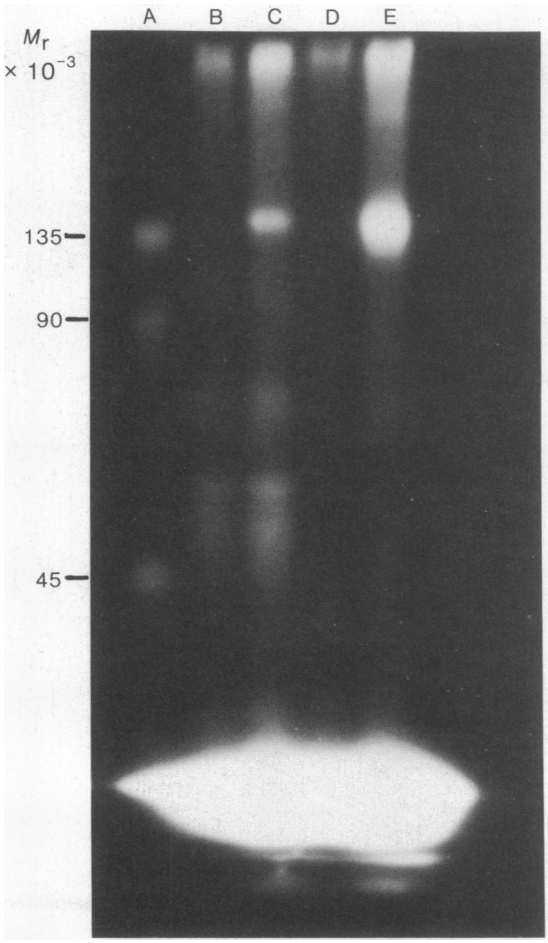

FIG. 4. Comparison of proteins present in the preparation of purified rat liver insulin receptor with proteins of rat liver and human placenta that were photolabeled by ^{125}I-labeled ANP-insulin. Lane A, purified insulin receptor iodinated with chloramine-T; lane B, rat liver membranes incubated with ^{125}I-labeled ANP-insulin in the presence of native insulin (2 μg/ml) and photolysed; lane C, rat liver membranes incubated with ^{125}I-labeled ANP-insulin in the absence of native insulin and photolysed; lane D, human placenta membranes incubated with ^{125}I-labeled ANP-insulin in the presence of native insulin (2 μg/ml) and photolysed; and lane E, human placenta membranes incubated with ^{125}I-labeled ANP-insulin in the absence of native insulin and photolysed. In the photoaffinity labeling studies, 400 μg of liver membranes or 400 μg of placenta membranes was incubated with 10^6 cpm of ^{125}I-labeled ANP-insulin (3.4×10^8 cpm/μg) in the presence or absence of native insulin in 0.4 ml of 100 mM sodium phosphate buffer (pH 7.4)/0.2% albumin at 15°C in the dark. After 90 min the membranes were diluted with 0.4 ml of buffer and photolysed at 4°C. Photolysis was carried out for 5 min, 20 cm from a Hanovia 450-W mercury arc lamp fitted with a Pyrex filter. The membranes were then diluted with 0.4 ml of buffer and centrifuged through buffer containing 10% sucrose. The supernatant was aspirated and the pellet was boiled in Laemmli sample buffer containing 2% $NaDodSO_4$. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed after reduction with 10 mM dithiothreitol. Autoradiograms were made from the dried gels.

It has been reported recently (16) that in liver membranes, azidobenzoyl-insulin derivatives specifically photolabel a band that is probably identical to the 135,000 molecular weight band

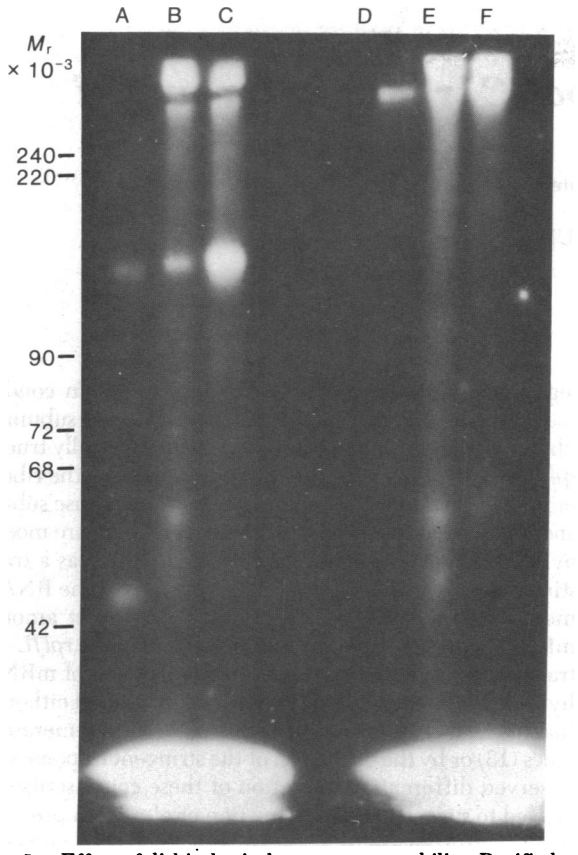

FIG. 5. Effect of dithiothreitol on receptor mobility. Purified rat liver insulin receptor iodinated with chloramine T (lanes A and D) or rat liver membranes photolabeled with ^{125}I-labeled ANP-insulin (lanes B and E) and human placenta membranes (lanes C and F) photolabeled with ^{125}I-labeled ANP-insulin as described in the legend to Fig. 4 were run on $NaDodSO_4$/polyacrylamide gels either after reduction with 10 mM dithiothreitol (lanes A–C) or without reduction (lanes D–F).

that we observe to be labeled by ANP-insulin. In addition, these azidobenzoyl-insulin derivatives label a 90,000 molecular weight band (16). We do not find that this 90,000 molecular weight band is labeled by ANP-insulin. However, a 90,000 molecular weight band is present as a minor component in the preparation of purified rat liver insulin receptor and it is possible that this 90,000 molecular weight band may be structurally and functionally related to the insulin receptor. Because the relative amount of this 90,000 molecular weight band varies considerably from preparation to preparation, it is unlikely that it is associated with the receptor in a fixed stoichiometric complex.

We find (8) that the undenatured insulin receptor purified from liver membranes by affinity chromatography has a molecular weight of 300,000, the same size as solubilized receptor prior to purification (1). A surprising finding of the current work is that even when the receptor is denatured by boiling in 2% $NaDodSO_4$, it still behaves as a large molecular weight complex (i.e., molecular weight 310,000) on $NaDodSO_4$/polyacrylamide gel electrophoresis. Only after reduction does it break into subunits, suggesting that the subunits are linked, or at least greatly stabilized, by disulfide bonds. This is similar to results obtained with the acetylcholine receptor from *Torpedo californica*, which has been shown to exist as disulfide-linked oligomers (17–19). It is difficult to reconcile this finding with reports that the insulin receptor breaks up into subunits simply upon binding insulin (3–5, 7). It is conceivable that insulin binding might catalyze the opening of disulfide bonds in the receptor. Alternatively, it is possible that some of the conditions encountered in this work during the purification or labeling of the receptor might have artifactually led to the formation of disulfide bonds, as has been reported to occur during the purification of HLA antigens (20) and acetylcholine receptors from *Electrophorus electricus* (21). To investigate the latter possibility further, we homogenized rat livers in 1 mM iodoacetamide and included 1 mM iodoacetamide in all buffers during the preparation of liver membranes and subsequent photolabeling with ^{125}I-labeled ANP-insulin. $NaDodSO_4$/polyacrylamide gels of liver membranes prepared and photolabeled under these conditions still contain the specifically labeled 135,000 molecular weight band after reduction by dithiothreitol but only the 310,000 molecular weight band without reduction (data not shown).

It is interesting that the insulin-binding component of rat liver and human placenta, as demonstrated in Figs. 4 and 5, and rat fat cells, as demonstrated by using a photoaffinity label (9) or by chemically crosslinking insulin to its receptor (10), are all of approximately the same molecular weight, 135,000. We have found that a 135,000 molecular weight band also is specifically labeled by ^{125}I-labeled ANP-insulin in differentiated 3T3-L1 cells (data not shown). This similarity is consistent with other similar physical, immunological, and binding properties of insulin receptors from different tissues and species.

Note Added in Proof. After submission of our manuscript, we learned of an independent study (22) in which it was also found that the insulin receptor is composed of disulfide-linked subunits.

We thank S. Cook for her expert assistance in carrying out these studies.

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