Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 154-160

PEPTIDE MAPPING OF THE INSULIN-BINDING SITE OF THE 130-kDa SUBUNIT OF THE INSULIN RECEPTOR BY MEANS OF A NOVEL CLEAVABLE RADIOACTIVE PHOTOPROBE

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Received October 14, 1985

A radioactive photoaffinity probe for the insulin receptor was prepared by derivatizing insulin at its B_{29} lysine with a novel crosslinking reagent having a cleavable azo linkage. Insulin receptors purified from human placental membranes were photoaffinity labeled with this probe. The photolabeled receptor was treated with dithionite to cleave the azo linkage, thereby removing the insulin ligand and transferring the radioactivity to the receptor protein. The radioactive labeled subunit was isolated and digested with elastase for peptide mapping and separation by high performance liquid chromatography. Results obtained indicated that it will be feasible to use this new photoaffinity probe to obtain radioactive peptides representing the insulin-binding site(s) on the receptor subunit.

The techniques of chemical crosslinking and photoaffinity labeling have been used successfully to identify specific hormone receptor by covalently crosslinking the hormone to its receptor. In the case of the insulin receptor, insulin is thus crosslinked to its receptor subunits of 125-130 kDa and 90-95 kDa (1-8). However, after crosslinking by these conventional reagents, the hormone cannot be cleaved from the receptor proteins. Therefore, studies of the hormone binding sites of the receptor is greatly complicated by the crosslinked ligand. In this study, we have prepared a novel radioactive and photoreactive insulin derivative that is cleavable through an azo linkage resulting in the transfer of radioactivity to the insulin

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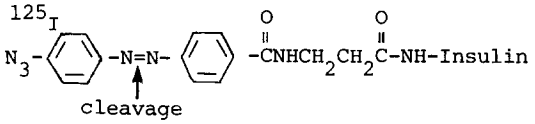
Abbreviations: HPLC, high-performance liquid chromatography; TEA, triethylamine; TFA, trifuoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

0006-291X/85 $1.50

receptor binding subunit. This photoprobe, illustrated below, was prepared by derivatization of bovine insulin with N-[4-(4'-azido-3'-[^{125}I]iodo-phenylazo)-benzoyl]-3-aminopropyl-N-oxy-succinimide ester (Denny-Jaffe reagent) (9).



We have used this radioactive photoprobe to label purified placental insulin receptors. After removal of the ligand by azo cleavage, the radioactive 130-kDa binding subunit was isolated and characterized by peptide mapping and HPLC analysis of its elastase digest.

MATERIALS AND METHODS

Materials: The following materials were obtained from the sources indicated: bovine insulin (Zn crystals) from Connaught Medical Laboratory, Canada; Denny-Jaffe reagent (2200 Ci/mmole) from New England Nuclear; AffiGel 10 and reagents for SDS-PAGE from Bio-Rad; elastase (EC 3.4.21.11), phenylmethylsulfonyl flouride, Triton X-100 from Sigma Chemicals.

Preparation of the Radioactive Photoaffinity Probe: The carrier benzene in the Denny-Jaffe reagent (1 mCi) was removed by a gentle stream of nitrogen. Bovine insulin (250 µg), dissolved in 100 ul of redistilled dimethylformamide and 2 µl of redistilled TEA, was added to the reagent. Under these conditions, insulin is derivatized at the ε-amino group of B-29 lysine. After 30 min at room temperature, the reaction was quenched with 50 µl of 0.1 M glycine. The reaction mixture was then precipitated by the addition of ice-cold absolute ethanol (300 µl) and diethyl ether (900 µl). The radioactive photoprobe was purified by reversed phase HPLC on a uBondapak C_{18} column (3.9 x 300 mm). The column was developed at a flow rate of 1 ml/min for 45 min with a linear gradient of acetonitrile generated from Solvent A composed of water/acetonitrile (80/20, v/v), 0.05 M TEA, pH 5, and Solvent B composed of water/acetonitrile (50/50, v/v), 0.05 M TEA, pH 5. Fractions of 0.5 ml were collected into tubes containing 0.1 ml of 0.2% BSA in 0.4 M phosphate buffer, pH 7.4. Figure 1 shows the elution profile and radioactive purity of the isolated photoprobe as analyzed by PAGE in urea-acetic acid (10). The photoprobe was lyophilized and stored in aliquots at -20 C.

Photoaffinity Labeling and Isolation of the Labeled Receptor Subunit: Human placental membranes were prepared as described by Harrison and Itin (11). Solubilized receptors were obtained by Triton X-100 extraction of membranes from two placenta and purified on insulin-AffiGel 10 according to the method described by Fujita-Yamaguchi et al (12). The purified receptors were incubated overnight at 4 C with the radioactive photoprobe (0.7 nM) in 50 mM Tris-HCl, pH 7.4, containing 0.1% BSA and 0.1% Triton. The mixture was photolyzed for 30 sec (13). The labeled receptors were precipitated with 10% polyethylene glycol 6000 using bovine gamma-globulin as a carrier. The precipitate was solubilized by boiling for 20 min in 0.06 M Tris-HCl, pH 6.8, containing 3% SDS, 10% glycerol and 0.1 M DTT. The solubilized sample was electrophoresed according to the method of Laemmli (14). The 130-kDa subunit band was located by autoradiography and excised from the gel, electroeluted and lyophilized. The subunit protein was digested with elastase (100 µg in 50 mM NH_4HCO_3, pH 8) for 48 hr at 37 C. The digest was lyophilized and separated by HPLC on a Zorbax-PEP-RP-1

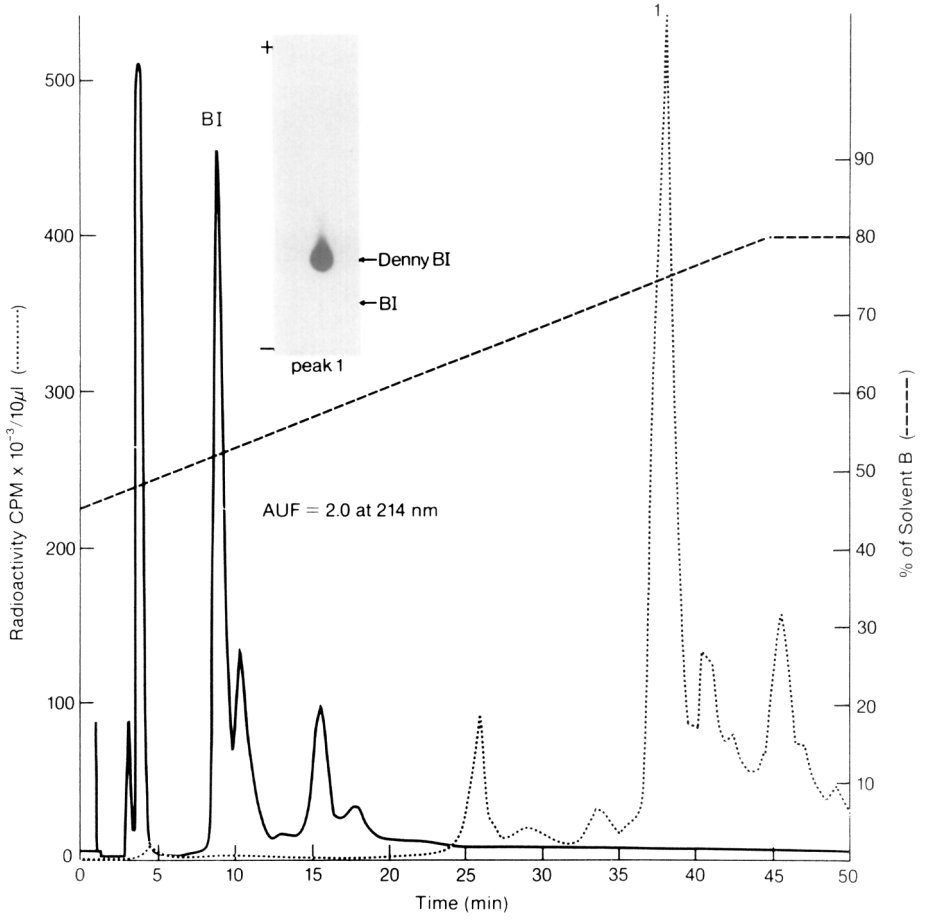


Figure 1: HPLC purification of the radioactive photoprobe. Fractions under Peak 1 containing the probe were pooled. The inset shows the radioactive purity of the pooled material as analyzed by PAGE in urea and acetic acid (10). BI, bovine insulin; AUF, absorbance (——) unit full-scale.

column (6.2 x 80 mm). The column was developed at a flow rate of 1 ml/min over a period of 60 min with a linear gradient of acetonitrile generated from Solvent A composed of 0.1 % TFA and 0.1 % morpholine in water, pH 3, and Solvent B composed of 0.1% TFA and 0.1 % morpholine in acetonitrile.

Peptide Mapping: Peptide mapping of the elastase digest was performed according to the method described by Elder et al (15).

RESULTS AND DISCUSSION

Figure 2 shows the labeling of the insulin receptor subunits using the radioactive photoprobe. Protein bands of 130 kDa, 90 kDa and to a lesser extent 40-45 kDa were specifically labeled by the photoprobe (Lanes 1,3 and 5). The 130-kDa and 90-kDa bands correspond to the alpha and beta receptor subunits, respectively. The 130-kDa band was labeled most intensely; about 17% of the radioactive photoprobe used was

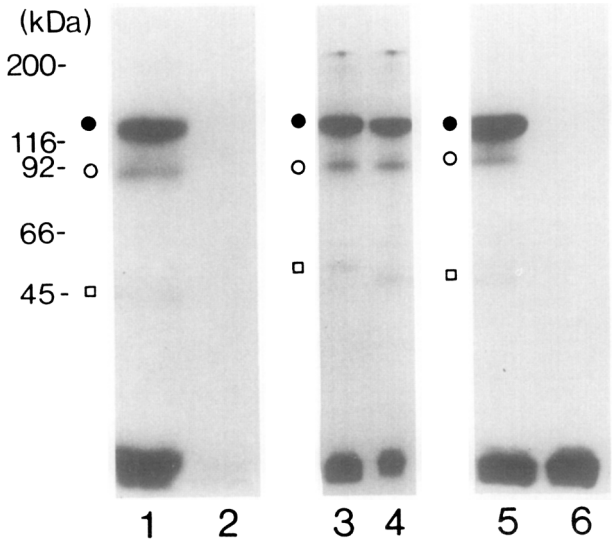


Figure 2: Autoradiograms of 3 gels showing the photoaffinity labeling of purified human placental insulin receptors with the radioactive cleavable photoprobe. SDS-PAGE was carried out as described in the text. Lane 1 shows the specific labeling of the receptor subunits which was displaced by an excess of insulin added to the incubation mixture before photolysis (Lane 2). The effect of azo cleavage on the mobility of the photolabeled receptor subunits is demonstrated in Lane 3 (before) and Lane 4 (after). The effect of azo cleavage of the photoprobe before photoaffinity labeling on the labeling of the receptor subunits is shown in Lane 6 (cleaved) and Lane 5 (not cleaved). 130-kDa (●), 90-kDa (O) and 40-45-kDa (□) subunit bands. Positions of the standard molecular weight markers are shown for Lanes 1 and 2.

crosslinked to this band. This is a high efficiency of photolabeling. Since the Denny-Jaffe reagent is cleavable through its azo linkage so that the radioactivity is transferred from the photoprobe to the crosslinked protein (9), we therefore used 0.2 M sodium dithionite to cleave the azo linkage after photolysis. We then analyzed the receptor subunits after reduction with DTT by SDS-PAGE. Figure 2 shows that the radioactive subunits (Lane 3) exhibited an increase in electrophoretic mobility after cleavage of the azo linkage (Lane 4). This was particularly noticeable in the case of the 40-45-kDa band. The increase in electrophoretic mobility would be expected from a decrease in molecular weight resulting from the release of the insulin B chain. No receptor subunit was radiolabeled when the photoprobe was treated with dithionite before photoaffinity labeling (Figure 2, Lane 6).

In order to characterize the insulin-binding sites of the subunit, the azo-cleaved radiolabeled 130-kDa band was electroeluted from several gels and digested with elastase, trypsin, chymotrypsin or V8 protease either separately or in

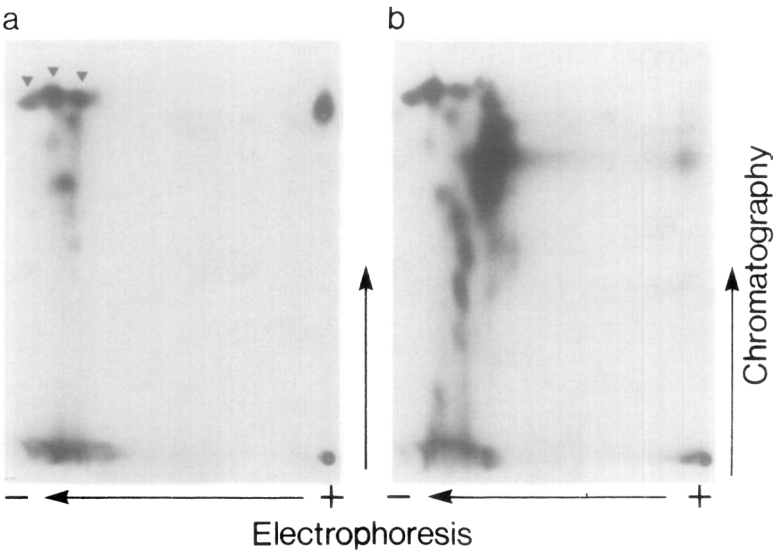


Figure 3: Peptide mapping of the elastase digest of the radiolabeled 130-kDa receptor subunit: (a) after cleavage of the azo linkage, (b) before cleavage. Thin-layer electrophoresis was carried out in acetic acid/formic acid/water (15/5/80, v/v). Chromatography was performed in n-butanol/pyridine/acetic acid/water (32.5/25/5/20, v/v).

combination. We found that digestion with elastase alone gave the best result. Analysis of the elastase digest by SDS-PAGE established that the subunit was hydrolyzed to peptide fragments smaller than 14 kDa (data not shown). The digest was analyzed by peptide mapping and HPLC. Figure 3a shows a autoradiogram of the peptide map, revealing the presence of several major radioactive spots. It is worth noting that three of these (indicated by arrows in Figure 3a) had very similar electrophoretic and chromatographic properties in that they all migrated close to the solvent front. The digest was also partially resolved by HPLC into several major peaks eluting at 5, 13, 17 and 22 min respectively (Figure 4). An elastase digest of the uncleaved radioactive 130-kDa band produced a different and much more complicated peptide map with many more major spots (Figure 3b). It is likely that these spots represent peptide fragment(s) still crosslinked to the insulin B chain or its digested fragments.

It is generally accepted that the 130-kDa subunit interacts with one molecule of insulin. Our present finding of several radioactive peptide fragments after a long period (48 hr) of enzyme digestion might be interpreted incorrectly to indicate the

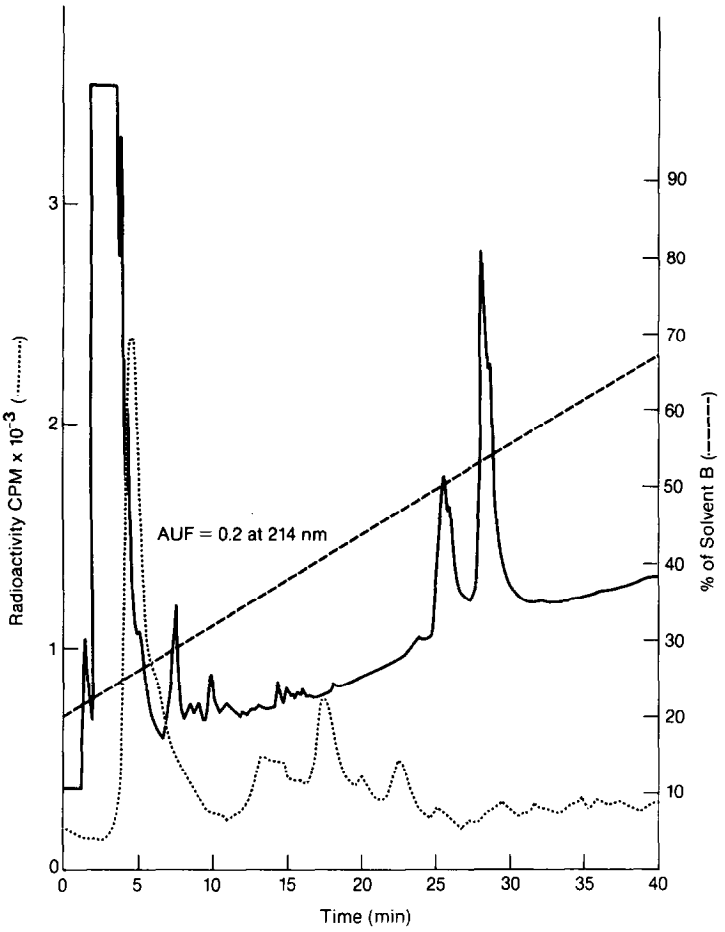


Figure 4: Reversed phase HPLC separation of the elastase digest of the radiolabeled 130-kDa subunit after cleavage of the azo linkage. AUF, absorbance (——) unit full-scale.

presence of more than one insulin-binding site on the subunit. However, the side chain of the B-29 lysine residue is sufficiently flexible to permit insertion in and crosslinking to more than one point on the same domain of the subunit. The same peptide fragment if crosslinked at different points could give rise to different spots on peptide mapping or different peaks in HPLC separation. The observed similarity in electrophoretic and chromatographic mobility of the three major radiolabeled spots supports this suggestion.

Recently Ullrich et al (16) reported the complete amino acid sequence of the insulin receptor precursor as deduced from human placental cDNA clones. However, the insulin-binding site(s) on the receptor remains to be determined. In our present study, we have used the approach of photoaffinity labeling with a novel cleavable

radioactive photoprobe and have labeled the receptor subunit peptide fragment(s) which interacts directly with insulin. It will now be possible to elucidate the binding site(s) on the insulin receptor by comparing the amino acid sequence, or even the amino acid composition, of the photolabeled peptide fragments obtained with this new photoprobe to that of the receptor. This approach is applicable to the study of receptor binding sites of other hormones.

ACKNOWLEDGMENTS: We thank Helga Hsu for her technical assistance. This study was supported by a grant from the Juvenile Diabetes Foundation International.

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